COMPARATIVE STUDY OF GLUTAMIC ACID PRODUCTION BY WILD-TYPE AND MUTANT STRAINS OF CORYNEBACTERIUM GLUTAMICUM

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BY

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DEPARTMENT OF MICROBIOLOGY,
FACULTY OF SCIENCE, AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

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DECLARATION

I declare that the work in this dissertation entitled "Comparative study on glutamic acid production by wild-type and mutant strains of *Corynebacterium glutamicum*" has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

_____ _____

Musa BISHIR Date

CERTIFICATION

This dissertation entitled "COMPARATIVE STUDY ON GLUTAMIC ACID PRODUCTION BY WILD-TYPE AND MUTANT STRAINS OF CORYNEBACTERIUM GLUTAMICUM" by Musa BISHIR meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This piece of work is dedicated to my parents, siblings and my wife.

ABSTRACT

Several different lignocellulosic biomass of agricultural origin hold remarkable potential for conversion into commodity products presenting dual advantage of sustainable resource supply and environmental quality. There is generally an increasing demand for amino acids especially L-glutamic acid as growth promoting factor, as well as flavour enhancer in foods. The present study was an investigation on comparative L-glutamic acid production by wild-type and a mutant strain of Corynebacterium glutamicum (CG^{NTA}) using rice husk pretreated with 1.0M H_2SO_4 and 1.0M KOH. The acid-treated and alkali-treated rice husk with high carbohydrate content of 64.25% and 76.37% respectively as determined, were used for the production of glutamic acid by submerged fermentation. The acid-treated and alkali-treated rice husk at concentration of 4% gave the highest glutamic acid yield of 27.84g/L and 15.72g/L respectively with the developed mutant strain (CG^{NTA}) under predetermined optimum fermentation conditions (30^oC, pH 7.0, 4% substrate concentration and 7% inoculum size). In contrast, lower yields of 10.40g/L and 9.08g/L respectively were obtained with the wild type strain under similar optimum culture conditions. Out of four parameters optimized, all were found to significantly ($p \leq 0.05$) influence glutamate production from both the acid and alkali-treated rice husk by the CG^{NTA}. Similarly, all parameters except variation in the concentrations of the acid and alkali-treated rice husk ($p \leq 0.05$)were found to be significant on the performance of the wild-type strain in glutamate production. Acid-treated rice husk hydrolysate was determined to be a better substrate for L-glutamate production by the CG^{NTA} mutant than the wild type strain of C. glutamicum. The mutant strain (CG^{NTA}) developed could, therefore, be useful in the industrial production of glutamic acid using rice husk as substrate pretreated with acid. This may perhaps form the basis of starting a microbial L-glutamate production industry from rice husk as substrate in this locality and Nigeria as a whole.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Corynebacterium glutamicum is a rod-shaped Gram-positive aerobic bacterium, which can be found in soil, sewages, vegetables, and fruits (Eggeling and Bott, 2005). This bacterium is capable of utilizing various sugars as well as organic acids (Blombach and Seibold, 2010). Among others, *C. glutamicum* has the ability to metabolize glucose, fructose, and sucrose as well as lactate, pyruvate, and acetate (Blombach and Seibold, 2010); additionally, *C. glutamicum* has the ability to grow on mixtures of different carbon sources with a monoauxic growth (Wendisch *et al*., 2000) as opposed to diauxic growth observed for many other microorganisms such as *Escherichia coli* and *Bacillus subtilis*. Only a few exceptions have been reported as in the case of glucose-ethanol or acetate-ethanol mixtures, where preferential substrate utilization was observed (Zahoor *et al*., 2012). Since its discovery, *C. glutamicum* has become an indispensable microorganism for the biotechnological industry (Wendisch, 2014). With the development of amino acid market, a new era for the production of these amino acids by many companies and academic associations have enthusiastically arisen with the start of research and development in this field to increase the rate of amino acid production. This technological race has expedited the expansion of amino acid production by various methods. Thus, almost all the amino acids can be produced by any of the four methods which include; chemical synthesis, protein hydrolysis, enzymatic synthesis and fermentation. However, industrially, the most advantageous and economical method used for amino acids manufacture is microbial

method, that is fermentation (Ikeda, 2003). For almost fifty years, amino acids have been produced through fermentation (Rastegari *et al*., 2013). Out of ten non-essential amino acids, glutamic acid is second to alanine in priority due to that it stands first in the list as it is commercially very important amino acid used as flavor enhancer in foods (Javaid *et al*., 2012).

Among all biochemical methods, fermentation is the most economical, practical and eco-friendly means of producing glutamic acid, with low temperature requirement and the possibility of using cheaper carbon sources such as agricultural residues (Ekwealor and Obeta, 2005).

Microorganisms have regulatory mechanisms to control the quantities and qualities of enzymes that are involved in the synthesis of amino acids. Therefore, it is necessary to use these regulatory mechanisms in order to get the mass production of the target amino acid. Moreover the titre of amino acid increases if the enzymes involved in the production of the required amino acid are found in large amounts under workable situations. For this purpose, strains of microorganisms are improved using several techniques to make this process possible (Kothari, 2009).

Microorganisms employed for amino acid production are categorized into four groups; including the wild-type, auxotrophic, regulatory and auxotrophic regulatory mutants. The species of *Corynebacterium* or *Brevibacterium* are widely used for glutamic acid production (Choi *et al*., 2004). Similarly, their mutant strains that are auxotrophic or resistant to certain chemicals result in enhanced production of glutamic acid (Anastassiadis, 2007).

In most developing countries including Nigeria, hundred thousand tons of agricultural residues are produced annually (Khan *et al*., 2006). These residues can be utilized as substrates for generation of different value-added products such as amino acids. Rice husk is one of the highly utilized residues which have a perceptible amount of reducing and non-reducing carbohydrates.

Rice husk is the outer covering of rice that is separated from the starchy endosperm during the first stage of milling. It is rich in vitamin B, minerals, fiber, high level of carbohydrates and proteins (Sramkova *et al.,* 2009). It is an agricultural waste that is produced as bulks in rice milling. It can also be used as cheapest source of energy for fermentation. Rice husk also contains about 10% of paddy and accessible in large amounts in major rice developing regions of the world (Ambreen *et al*., 2006).

Pre-treatment of rice husk, increases the availability of cellulose which can be hydrolyzed to glucose by microorganisms (Shafaghat *et al*., 2010).

L-Glutamate has a distinctive taste, known as "umami" that is not sweet, sour, salty, nor bitter (Nakamura *et al*., 2006) and is mainly used as a flavouring agent or enhancer. Globally, about 1.8 million tons of monosodium glutamate is produced annually by fermentation using coryneform bacteria (Nakamura *et al*., 2006). L-glutamate is a non-essential amino acid and is recently reported to act as neurotransmitter (Hawkins, 2009).

Corynebacterium glutamicum is a biotin auxotroph that secretes L-glutamic acid in response to biotin limitation; this process is employed in industrial L-glutamic acid production. Fatty acid ester surfactants such as Tween 40 and Tween 80 as well as

penicillin also induce L-glutamic acid secretion, even in the presence of biotin (Nottebrock *et al*., 2003). However, the mechanism of glutamic acid secretion remains unclear.

1.2. Statement of Research Problem

There is generally an increasing demand for amino acids especially L-glutamic acid as growth promoting factor, as well as flavour enhancer in foods. This is of great importance worldwide (Wendisch, 2014)

One of the major problems affecting large scale synthesis and utilization of L-glutamic acid is the cost of raw materials or chemicals used (Nampoothri *et al*., 2002). The process is also tedious, and non-economical probably due to process inefficiency as a result of undesirable product formation due to side reactions (Mahmood, 1996). The use of chemicals used exerts serious health risks due to their mutagenicity and carcinogenicity as opposed to the use of microorganisms (Ahmed *et al*., 2013). Chemical synthesis of amino acid also produces a racemic (DL-glutamate) mixture, which requires additional optical resolution, since the amino acid in the L-isomeric form is the active form (Wendisch, 2014). Similarly, when chemicals are used for glutamic acid production, certain special vessels/ containers may be needed to avoid damage of the fermentor due to chemical corrosiveness usually accompanied by great economic loss (Hermann, 2003). Another disadvantage of using chemicals for amino acids production is the need for neutralizing agent where acidic or alkaline solutions are used in order to bring the pH to near neutrality. Hence, the need for microbial synthesis of amino acids including L-glutamic acid.

Strikingly, utilization of expensive media for the microbial production of L- glutamic acid is often not cost effective. This in turn affects the market price of this particular amino acid worldwide (Mostafa and Ahmed, 2006).

The wild-type strains of *C. glutamicum* lack the ability to utilize the pentose fractions of lignocellulosic hydrolysates. Similarly, the intracellular accumulation of L-glutamic acid as opposed to the industrial need (secretion) is a common characteristic of the wild-type strains of *C. glutamicum*; which in turn affects the quantity of the L- glutamic acid produced by a wild strain of the *Corynebacterium glutamicum* (Nakamura *et al*., 2007)

Production of L-glutamic acid in Nigeria using the abundant agricultural residues as raw materials through fermentation processes will reduce the high importation cost and boost local industrial utilization which in turn has positive impact on the foreign exchange and economy of the country.

1.3 Justification of the Study

The search for new biological materials to be used as drugs and pharmaceuticals, flavours and food additives, resulted in a phenomenal growth of industrial Microbiology on one side, and fermentation engineering on the other hand. The increased industrial utilization of biological processes suggest that biotechnology will be the major growth industry in near future and this will affect the lives and welfare of people all over the world (Vijayalakshmi and Sarvamangala, 2011).

Apart from the role of microorganisms in the production of drugs and pharmaceuticals, it was observed that biotransformation could also be utilised for the production of food and feed materials including amino acids such as L-lysine and L-glutamic acid (Shagufta,

2014). In the field of food Microbiology, the role of microorganisms in the preservation of raw and cooked food materials and improvement of flavours and colours has been fully established. However, with the establishment of the role of growth promoting substances such as vitamins, amino acids and gibberellins, much attention is now focussed on the utilization of microorganisms and their enzymes to produce these valuable substances. A large number of microbial strains were developed for the industrial production of these useful organics and the help of fermentation process came to the advantage of mankind (Shafaghat, 2010).

The demand of L-glutamate is still increasing in the field of foods, animal feeds, pharmaceuticals and chemicals. To meet these increasing and diversified demands, there is still room for strain improvement based on the knowledge of microbial physiology (Rastegari *et al*., 2013). Furthermore, studies on process optimization, especially for lowering the expense of carbon and energy sources are also desirable (Adnan *et al*., 2011).

L-glutamate may be produced either by isolation from natural materials (originally from the hydrolysis of animal or plant proteins) or by chemical, microbial or enzymatic synthesis. Although, the chemical synthesis of amino acid also produces a racemic (DL-glutamate) product, the amino acid in the L-isomeric form is required in all its applications. This technical problem is overcome by the microbial synthesis of L-glutamate, which however, gives rise to optically pure L-glutamate. This therefore makes the process advantageous over the synthetic one (Shafaghat, 2010).

It is also known that the wild-type of *Corynebacterium glutamicum* lacks ability to utilize the pentose fractions of lignocellulosic hydrolysates, but on the contrary, its certain mutants

are able to grow with the pentoses such as xylose as well as with arabinose as sole sources of carbon and energy on media containing acidic rice straw (Damisa *et al.*, 2008) or wheat husk hydrolysates for the production of L-glutamate. This may reveal that acid hydrolysates of agricultural wastes materials may provide an alternative feedstock for large scale amino acid production.

The success in industrial production of glutamic acid stimulated further interest in finding strains capable of over-producing glutamic acid and other amino acids as well. For the extracellular production of a desired amino acid, changes in cellular metabolism and/or regulatory controls are required (Pasha *et al*., 2011).

Most amino acids are produced nowadays by the use of mutants that contain a combination of auxotrophic and regulatory mutants (Wendisch, 2007). Even more prolific amino acid-producing strains have been obtained by eliminating the ability of the organism to degrade the product and by providing cell permeability in favour of excretion of the end product (Nakamura *et al*., 2007).

Interestingly, certain local materials such as sugarcane baggase, cassava, wheat bran, rice husk and maize cobs have shown promising ability to substitute the expensive media used in the L-glutamate production as substrates (Jyothi *et al*., 2005). Thus, this helps to significantly reduce the level of environmental land pollution that usually results from dumping of such wastes, and also save a lot of costs.

This research is therefore aimed to explore the local bacterial flora (*Corynebacterium glutamicum*) and to develop and isolate regulatory mutants resistant to feedback inhibition for an increased yield of L-glutamate. In addition, special emphasis will also be given on

the exploration of the locally available raw material (rice husk) for the process design in the laboratory production of L-glutamate. Hence, this study was undertaken to utilize rice husk as carbon and energy sources, and 4-fluoroglutamate – a toxic analogue of L-glutamate to produce glutamic acid by liquid state fermentation.

1.4 Aim of the Study

The aim of this study was to isolate *Corynebacterium glutamicum* from soil and compare the level of L-glutamic acid produced by mutant and wild-type strains of the isolate using rice husk as substrate.

1.5 Objectives of the Study

The objectives of this study were to

1. Determine the proximate composition of the pre-treated rice husk substrate.
2. Isolate and Characterize *Corynebacterium glutamicum* from Different Soil Samples Using Conventional Cultural and Biochemical Methods.
3. Screen the *Corynebacterium glutamicum* isolates for L-glutamic acid production.
4. Produce Regulatory Mutant Strains of the *C. glutamicum* Isolate with the Best Potential for L-glutamic acid Production.
5. Determine the Effects of Various Optimization Parameters on the Production of L-glutamic acid.
6. Produce L-glutamic acid from the Pre-treated Rice Husk Using the Wild-type and the Regulatory Mutant of the *C. glutamicum*.
7. Determine the Qualitative Characteristics of the glutamic acid Produced using Industrial glutamic acid as Control.

1.6 Research Hypothesis

H_0 = 0: The level of L-glutamic acid production by the mutant and wild-type strains of *C. glutamicum* is the same for all treatments under all conditions.

H_A ≠ 0: The level of L-glutamic acid production by the mutant and wild-type strains of *C. glutamicum* is not the same for all treatments under all conditions.

CHAPTER TWO

2.0 Literature Review

2.1 Amino Acids

In 1956, a research program started at Kyowa Hakko Kogyo Co., Ltd., Tokyo that was aimed at obtaining a microorganism that could accumulate and secrete glutamic acid extracellularly. Several isolates were found and one that might be fit for the purpose was named as *Micrococcus glutamicus* No.534. Further studies revealed that this microorganism could accumulate glutamic acid at a limiting concentration of biotin present in the medium. This suggested that biotin must play a key role in the physiology of the cells and their glutamate forming ability. By way of microscopic observation of cultures at various stages, it was observed that the cellular morphology can change considerably and for this reason, coupled with further taxonomical studies, the bacterium was renamed. From several manipulations such as mutation carried out on this organism, together with discoveries regarding key regulatory features, it was found that other amino acids, such as lysine, arginine, threonine, etc., could be accumulated. Most of these amino acids are now produced commercially (Ikeda, 2003).

Amino acids produced by such a process are all in their natural (L) form and this gives microbial production a big advantage over chemical synthesis. Thus, a new industry called amino acid fermentation was established. The commercial production of amino acids until the discovery of *C. glutamicum* had relied on the decomposition of natural protein of plants and animal origin and consequently the isolation of its constituent amino acids. The new process, on the contrary, was a biosynthetic process using carbohydrate and ammonium

ions, therefore, fermentation process can contribute to the amino acid supply and also helps to increase the absolute amount of protein in the world. Since the world population continues to increase year by year, so will the demand for amino acids and protein. After World War II, two new fermentation industries were born in Japan. These are the amino acid and nucleotide fermentation industries (Ikeda, 2003).

2.2 Production of Amino Acids

The production of amino acids is a big industrial factor in both the chemical and biotechnological industries. There has always been a competing demand of amino acids between these two fields which necessitate their efforts to produce amino acids in a cheap and energy reducing mode. Amino acids have many special properties which make them very valuable, for example; their contribution to nutrition, the taste, the chemical features and their importance in physiological activities. The proteinogenic amino acids otherwise, referred to as the "magic 20" are the building blocks of proteins; they are important intermediates on the pathway from the genetic to the protein level through the synthesis of mRNA transcript by transcription to the synthesis of gene product (polypeptide) by way of translating the information coded on the mRNA transcript.

Fermentation technology has played a crucial role in the progress made, and currently the fermented amino acids represent important products of biotechnology in both volume and value. This area is highly competitive in the world market and process economics are of primary importance. For cost effective production, many technologies have been developed to establish high productive fermentation and recovery process (Asakura *et al*., 2007). Over production of glutamate by *C. glutamicum* is induced by biotin limitation (Nakamura *et al*., 2007) or by adding specific detergents (Eggeling *et al*., 2001; Amin and Al- Talhi,

2007) or by adding sub-lethal amounts of penicillin in early exponential growth phase or at last, by a temperature shift-up of the culture broth. Glucose and other carbon sources such as beet molasses (Shagufta, 2014) and cassava residues (Jyothi *et al*., 2005) are used as substrates. Vijayalakshmi and Sarvamangala (2011) reported the use of cheap agricultural wastes as carbon sources such as *Muntingia calabura L.* for production of L-glutamate.

In addition to exploitation of wild type strains for amino acid biosynthesis, some workers used advanced techniques for improvement of strains by mutagenesis, cloning and protoplasm fusion techniques (Atef *et al.,* 2007). Pasha *et al*. (2011) tested the UV and chemical mutagens on *C. glutamicum* for increasing glutamate productivity.

The varied use of amino acids is as supplements to human and animal food, medical infusions, cosmetics and intermediates in the chemical industry. On the bases of amino acid usage, amino acids have been divided into 38% for food, 54% for feed and 8% for other applications (Shagufta, 2014).

It has since been observed that the world population is increasing at alarming rate and so the demand of food in the form of protein. Animal scientists are now working day and night to develop some conventional and non- conventional methods to enhance the productivity of meat producing animals (Tryfona and Bustard, 2004). Moreover Nigeria being a developing country, its feed sector needs to develop such processes that are more economical and beneficial (Damisa *et al.,* 2008).

Glutamic acid is a non-essential amino acid for both human and animals. Majority of amino acids are commercially produced by fermentation process. *Corynebacterium* has been widely used for industrial production of glutamic acid (Ahmed *et al.,* 2013). Glutamic acid, which is 2-aminopentandioic acid has the molecular formula$C_5H_9NO_4$and molecular

mass 147.13g/mol. It is an acidic amino acid having two carboxylic groups, one of them, on α – position and other at the end of a 5- carbon aliphatic chain (Malothu *et al*., 2012). It is the second most important amino acid with annual production of more than 800,000 tons and its requirement has been increasing day by day (Anastassiadis, 2007).

After the evolutionary changes that occur during 1970s, several successful mutation methods were discovered and were used to produce a valuable amount of this amino acid which led to the expansion of amino acid industry using fermentation with several microorganisms such as *C. glutamicum, B. flavum, B. lactofermentum* (Hassan *et al.*, 2008). The valuable and high cost compounds are manufactured through fermentation by using cheap industrial and agricultural wastes. Moreover, this method of amino acid fermentation is economical, environmentally friendly and more efficient. The fermentative method of amino acids production is more advantageous as it produced 100 % L-amino acids that are metabolized by the microorganism whereas by chemical method, 50% D-amino acids and 50% L- amino acids are synthesized and the D-enantiomer cannot be assimilated for protein synthesis by living organisms (Khan *et al*. 2006). It was reported by Anastassiadis (2007) that fermentation has more attraction for biotechnologist compared to synthetic methods of amino acid production due to stereo specificity. Furthermore after identification of more glutamic acid producing strains, mutant strains of *Brevibacterium* and *Corynebacterium* were developed and were used to produce glutamic acid and other amino acids such as lysine. Moreover, fermentation technology also replaced the classical methods such as acid hydrolysis that was costly and low product yielding method as compared to these emerging techniques of fermentation.

At industrial scale fermentation is carried out basically by different types of culturing *viz*; continuous, fed-batch and batch culture. Batch fermentation is however mostly practiced for pilot studies and laboratory scale production. The fed batch was found to be the best suitable culturing method with reference to product and biomass formation. Moreover, metabolism of microbes was greatly affected by environmental factors for over production of the desired product. It has also been reported that microbes are cooperated with facility to synthesize all the amino acid as essential components but their production is usually limited. For this purpose, to enhance its productivity, scientists have developed large variety of mutant strains by using various techniques. For fermentation, the selection of substrate is as important as microbe selection. But during the selection, it is normally preferable to use cheap and economical substrate such as molasses, cereal husk and starch hydrolysates (Shah and Khan, 2008).

It is possible to synthesize all amino acids in the traditional chemical way but for many of them it would be much more profitable to produce with other different methods. The advantage of the enzymatic synthesis and the direct fermentation is the modern enantio selective production of either the L-enantiomeric or D-enantiomeric form. There are examples for each of the production possibilities, for instance, Glycine is the only non-chiral amino acid; therefore the chemical process is without competition because there is no racemic product mixture to purify. L-asparagine, L-arginine, L-histidine and L-cysteine for example are produced by extraction from protein hydrolysates, L-tryptophan and L-aspartic acid are obtained using enzymes or immobilized cells.

The best activities of multi-enzyme systems are reached when the effectiveness of the microbial cell as enzyme-membrane reactor is much higher in spite of side reactions and

by-products. On this account, the direct fermentation is the preferable process in commercial production of L-glutamic acid. One of the drawbacks of microbial synthesis of glutamic acid is the strong regulated biosynthesis in wild type microorganisms. The produced amino acid itself restricts the formation of necessary enzymes (feedback repression) and/or reduces the activity of key enzymes for the metabolic building pathway (feedback inhibition). This therefore, calls for deactivation of the control mechanisms. In addition, side reactions and the degradation of end and intermediate products have to be blocked.

2.3 Raw Materials for the Production of Amino Acids

Selection of raw materials is essential for economic amino acid production. The carbon source especially represents a major part of variable production costs. This explains the sometimes tight connection between amino acid and sugar producers which developed over time. Some amino acid producers are located very close to sugar plants or form joint ventures in order to decrease transport costs f the raw materials. Depending on the geographical location of the plant carbon sources like cane molasses, beet molasses, or starch hydrolysates from corn, potato or cassava are used.

Lignocellulosic biomass holds remarkable potential for conversion into commodity products presenting dual advantage of sustainable resource supply and environmental quality. Though their utilization does not compete with food and feed demand, its bioconversion and utilization is facilitated by pretreatment.

Rice husk is a low cost source of cellulose (Narasimha *et al.,* 2011), abundantly available in northern parts of Nigeria and other parts of the world where rice is being cultivated as a

staple food crop. Rice husk is commonly pre-treated separately using sodium hydroxide and sulphuric acid and is used as substrate for fermentation to glutamic acid production.

While molasses are common in Europe, South America and China, starch hydrolysate is the most important carbon source in North America and West Africa including Nigeria. Tapioca hydrolysate, the starch hydrolysate from cassava is widespread in South-East Asia. Pure sugars are usually preferred compared to molasses because of unwanted side reactions and changing qualities of the complex media components. Some types of molasses for example contain higher concentrations of biotin which inhibits corynebacterial L-glutamic acid biosynthesis. Even though, some authors describe processes with ethanol, acetic acid or n-paraffins as carbon source, these substrates have significant economical disadvantages compared with sugar carbon sources. Methylotrophic bacteria are able to convert methanol into L-glutamic acid, L-lysine (*Bacillus* sp., *Methylobacillus glycogenes*) or L-threonine (Motoyama *et al.,* 2001). Similar processes with methanol are described for production of L-phenylalanine or L-methionine. Nitrogen sources vary from inorganic pure ammonia and ammonium salts like ammonium sulphate to complex organic components like peptone hydrolysates or corn steep liquor. These components might be either introduced into the initial media of the process or dosed into the bioreactor during the process like ammonia water or gas which is commonly used for pH regulation. Carbonate or hydrogen carbonate ions interact as counter-ions for produced basic (positively charged) amino acids like L-lysine or L-histidine (Itoyama *et al.,* 2001). This procedure reduces the consumption of ammonium sulphate in the bioprocess. The advantage of inorganic nitrogen sources is the stable quality obtainable on the market. Peptone hydrolysates from animal origin are considered problematic in bioprocess media recipes since the bovine spongy form

encephalopathy (BSE) incidents. More expensive peptone sources like yeast extract have more defined ingredients. Corn steep liquor is another cheap and often standardized nitrogen source rich in amino acids, oligopeptides, vitamins and nucleotides. However, as a by-product of a lactic acid bioprocess with corn as substrate, corn-steep liquor has changing qualities. These fluctuations originate in the source and age of the corn and the course of the steeping process. Fluctuation in raw material quality is well-known as source of bad bioprocess results. Cheap corn steep liquor qualities often have increased L-lysine contents leading to reduced yields in e.g. penicillin production (Banuelos *et al*., 2000). Changing concentrations of iron in corn steep liquor batches were shown to influence L-threonine production with *E. coli* significantly (Okamoto and Ikeda, 2000). It was shown that by addition of inorganic iron the process got independent from fluctuating iron concentrations in the system. Corn steep liquor contains significant amounts of inorganic and organic phosphate, another important ingredient of bioprocess medium recipes. Nevertheless, main phosphate sources are inorganic salts like potassium or sodium phosphate. Same is valid for magnesium, sulphur and other ions necessary for the growth of coryneform bacteria. Vitamins like biotin are usually added into the process in their pure form. Some organic components have a limited stability at higher temperatures which occur during sterilization or under these conditions react with others (i.e. Maillard reaction). Composition of the media must, therefore, be adapted to the conditions of sterilization. Furthermore, salts might form insoluble complexes which limit their availability for the organism. As mentioned for repeated fed batch and continuous bioprocesses, continuous sterilization is usually more favorable than batch-wise sterilization. However, quality check of new raw material lots by either instrumental analytical methods (i.e. mass spectrometry) or biotests is essential. Thus, several different

agricultural residues have proven to be promising as good substrates for amino acid production, particularly L-glutamic acid.

2.4 Discovery of L-Glutamic Acid

Glutamic acid was first discovered by Ritthausen in 1866. Some of the seed proteins, especially the prolamines, yielded 20-45 percent of glutamic acid on hydrolysis. The man who first noted in 1908 the commercial importance of glutamic acid was Kikunae Ikeda. He discovered that monosodium glutamate (MSG-sodium salt of glutamic acid strongly enriched the flavour). Glutamate was obtained by decomposing plant proteins such as soybean and wheat.

In 1955, Kinoshita aimed to find an appropriate organism, which could convert non proteinaceous raw material into an amino acid and excrete it out of cells abundantly. A screening programme for such microorganisms was started in1955, headed by Dr. Shigezo Udaka who found the novel bacterium (*Corynebacyerium glutamicum*), which can accumulate up to 10.3 g/l of glutamic acid in the medium. This left no doubt that glutamic acid produced was the result of direct bacterial fermentation process (Asakura, 2007).

The discovery of a potent glutamic acid-producing bacterium by Kinoshita *et al*. (1957) was the start of the subsequent development of amino acid production by regulation of biosynthetic metabolism. The glutamic acid producing bacterium first discovered was reported as *Micrococcus glutamicus* in 1958, and other glutamic acid-producing coryneform species, all potent strains, were subsequently isolated by many others. *C. glutamicum, C. lilium, C. herculis, Brevibacterium flavum, B. lactofermentum,B. divaricatum, B. ammoniagenes, B. thiogenetalis and Mycobacteriumammoniaphilum* are

among the potent glutamic acid producing strains. Some more of the glutamic acid secreting bacteria can be easily found in nature such as *Escherichia coli, Bacillus megaterium, Bacillus circulans, Bacillus cereus* and *Sarcina lutea*. All of these organisms can produce glutamic acid from carbohydrates (Nasab *et al.,* 2007). Representative strains producing glutamic acid by direct fermentation were classified into four genera; *Corynebacterium,Nicrobacterium, Arthrobacter* and *Brevibacterium.*

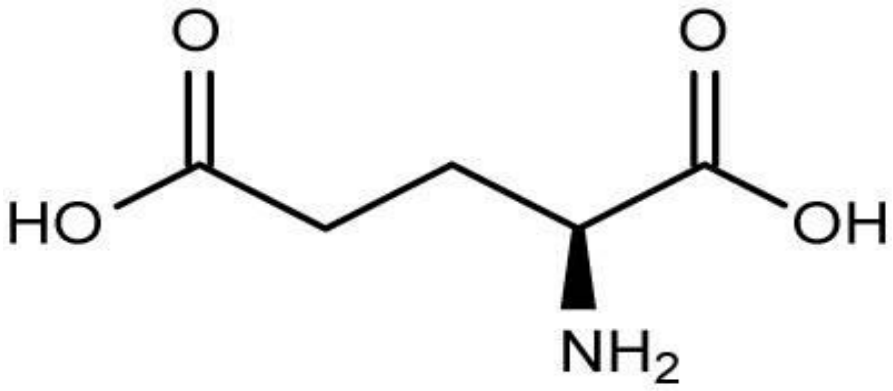


Figure 2.1: L-Glutamic Acid Chemical Structure (Chemical Formula: $C_5H_9NO_4$ and M.WT: 147.13)

2.5 Chemistry of L-Glutamic Acid

Glutamic acid, which is 2-aminopentaandioic acid has the molecular formula$C_5H_9NO_4$and molecular mass 147.13 g. It is an acidic amino acid having two carboxylic groups, one on the α – position and the other at the end on a 5- carbon aliphatic chain (Malothu *et al*., 2012). Glutamic acid has one additional methylene group in its side chain than do aspartic acid. The side chain carboxyl of aspartic acid is referred to as the β carboxyl group, while that of glutamic acid is referred to as the γ carboxyl group. The pK_a of the γ carboxyl group for glutamic acid in a polypeptide is about 4.3, significantly higher than that of aspartic acid. This is due to the inductive effect of the additional methylene group. In some proteins, due to a vitamin K-dependent carboxylase, glutamic acids may be regarded as di-carboxylic acids, referred to as γ- carboxyglutamic acid that forms tight binding sites for calcium ion.

There are two common forms of glutamic acid, L-glutamic acid and D-glutamic acid. L-glutamic acid found in protein is referred to as 'bound' or 'protein bound' glutamic acid. In reality, amino acids of natural foods are rarely free. Rather, they are linked or bound in long chains to other amino acids in peptides or proteins. D-glutamic acid 'outside of protein' or 'free glutamic acid' is artificially and chemically produced outside the body in form of sodium salt. This is what is known as monosodium glutamate or MSG. Because it has a carboxylic acid moiety on the side chain, glutamic acid is one of the only two amino acids (the other being aspartic acid) that have a net negative charge at physiological pH. This negative charge makes glutamic acid a very polar molecule and it is usually found on the outside of proteins and enzymes where it is free to interact with the aqueous intracellular surroundings. On a molar basis, glutamic acid is incorporated into proteins at a rate of 6.2 percent compared to other amino acids. Glutamic acid is also a precursor of GABA, an important neurotransmitter in the central nervous system.

2.6 Importance of L-Glutamic Acid

Glutamic Acid is sometimes referred to as glutamate. Glutamic acid is a nonessential amino acid that functions as an important metabolic intermediate. Glutamic acid can be synthesized from oxoglutaric acid (α-ketoglutarate), formed in the metabolism of carbohydrates as an intermediate of tricarboxylic acid cycle,, so it does not require direct dietary sources. Glutamic acid is biosynthesized from a number of amino acids including ornithine and arginine. Glutamic acid is a nonessential amino acid that the body uses to build proteins. (Wendisch, 2007).

2.6.1 Sources and uses of glutamic acid

Protein builds muscle, organ, and all body tissues. It aids in the digestion of foods. It provides structure to hair, fingernails, and ligaments. It produces stomach acid, aids the movement of muscles, helps in the production of antibodies, and delivers oxygen to blood. It is therefore very essential to life.

Proteins are amino acids linked together in chains. Of the twenty known amino acids, ten are classified as 'essential', signifying the body does not produce them so they must be obtained from dietary sources. The other ten amino acids are considered ‘non-essential’, signifying that the body is capable of making them so they are not diet-dependent.

Glutamic acid is a 'non-essential' classified amino acid that is very common in plants and animals. Besides being a building block of protein, glutamic acid is vital in the transmission of nerve impulses, and is even manufactured in the brain.

When food containing protein is consumed, the body breaks it down or hydrolyzes it in the stomach and lower intestines through the action of hydrochloric acid and digestive enzymes. In a healthy person, the body controls the amount of glutamic acid that it takes

from protein. Excess glutamic acid is not stored by the body, preventing toxicity. It is passed off as waste. Utilized this way, glutamic acid from eating protein is harmless.

In the chemical MSG manufacturing plant, however, the bound glutamic acid in foods is normally broken down or made 'free of protein' by various processes (hydrolyzed, autolyzed, modified or fermented with strong chemicals, acids as well as bacteria, or enzymes, which are often genetically modified) and refined to a white crystal powder that resembles salt or sugar. Chemical MSG contains 78% glutamate, 12.2% sodium, and 9.6% water.

When purely manufactured MSG is ingested, a rapid effect occurs from the glutamate. This 'free of protein' glutamic acid, unlike the naturally occurring 'protein bound' glutamate is not attached to other amino acids. The normal digestive disassembly process does not happen because there are no 'peptide' bonds to slow the process. The sudden increase in free glutamic acid is then rapidly absorbed and can raise blood levels of glutamate about eight to ten times, thereby causing toxicity.

Good sources of glutamic acid such as dairy products, meat, poultry and fish are considered to be natural brain food by improving mental capacities and the glutamic acid is used by the body to build proteins.

2.6.2 Biomedical uses of L-glutamic acid

Glutamic acid can be used as fuel in the brain, and can attach itself to nitrogen atoms in the process of forming glutamine, and this action also detoxifies the body of ammonia. This action is the only way in which the brain can be detoxified from ammonia. Glutamate is the most common excitatory (stimulating) neurotransmitter in the central nervous system and

is also important in the metabolism of sugars and fats. It helps with the transportation of potassium across the blood-brain barrier, although it does not pass this barrier so easily. It also shows promise in the future treatment of neurological conditions, ulcers, hypoglycemia, muscular dystrophy, epilepsy, Parkinson's disease, and mental retardation. The fluid produced by the prostate gland contains significant amounts of glutamic acid, and this amino acid may play a role in normal function of the prostate gland (Wendisch, 2007). Glutamic acid may have protective effects on the heart muscle in people with heart disease. Monosodium glutamate (MSG), the form of glutamic acid that is used as a flavor enhancer, has been reported in anecdotal studies to have a number of different adverse effects (including headache, fatigue, and depression) (Reeds *et al*., 2000).

Glutamic acid is classified as a non-essential amino acid. It is an important excitatory neurotransmitter and required for lipid and glucose metabolism. Dosage based on the results from clinical studies with positive results have shown that daily dosage of glutamic acid ranges from between 2 - 15 grams (high doses may produce symptoms like Headache and Neurological problems) required for lipid and glucose metabolism (Reeds *et al*., 2000).

Other therapeutic uses of glutamic acid include but are not limited to the following;

1. Treatment of childhood behavioral disorders.

2. Treatment of neurological conditions such as, epilepsy, mental retardation, muscular dystrophy, Parkinson's disease

3. Glutamic Acid injections (I.V.) have been shown to increase exercise tolerance and heart function in population with stable angina pectoris

4. Treatment of BPH (Benign Prostate Hyperplasia).

Fermentation technology has played crucial roles over a period of time and currently the amino acid produced by fermentation represent chief products of biotechnology in both volume and value (Eggeling *et al*., 2003).

2.6.3 Commercial benefits of glutamic acid

L-amino acids have a wide spectrum of commercial use as food additives, feed supplements, infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agrochemicals (Ahmed *et al.,* 2013). Monosodium glutamate (MSG), the sodium salt of glutamic acid is used commercially as a flavour enhancer, usually in combination with nucleotides inosinate to provide an expansion and extension of taste in processed food such as soups, biscuits, noodles, Chinese foods, meat and vegetable processing etc. Glutamic acid mother liquor in MSG production is being used in the manufacture of Sauce and as soil conditioner, fertilizer etc. Several strains of *Corynebacterium* and *Brevibacterium* are used as cost effective bio-converters, which have been exploited by the fermentation industry to provide various amino acids, including L-glutamic acid (Mostafa and Ahmed, 2006).

Owing to the importance of the particular industrial fermentation, much effort was still going on to improve the glutamic acid fermentation process especially from the standpoint of savings in production cost (Nasab, 2007; Narasimha *et al.*, 2011). Cassava *(Manihot esculenta* Crantz*)* popularly known as tapioca, is one of the major tuber crops of the world, being cultivated extensively in tropical countries and obviously provides a major source of calories to millions of the people in the world. On dry weight basis, tapioca contains about

80-82% starch (fresh roots have about 22-30% starch) of which 55-60% is recoverable as starch. It is an excellent substrate for the reducing sugars such as (maltose, glucose etc), especially because of its ease of liquefaction.

2.7 Industrially Important Microorganisms for Glutamic Acid Production

Microorganisms are able to synthesize both essential and non-essential amino acids. While some tend to produce extracellular glutamic acid that can be easily recovered from the fermentation broth, others accumulate the product intracellularly and therefore efficient method of recovery is needed. Glutamic acid can be produced via the following methods; preparation of protein hydrolysates and then extraction of amino acids, enzymatic method of amino acid manufacture, chemical and fermentative processes that was used for amino acid manufacture (Faurie and Thommel, 2003). Initially, extraction from protein hydrolysate was the main process through which glutamic acid was produced, but then, gradually, chemical and enzymatic synthesis took over (Shah *et al*., 2002). Chemical synthesis produced only D and L form of amino acids and required additional step of optical resolution to render them biologically active L-isomers. Enzymatic synthesis, on the other hand, produced optically active L amino acids in high concentrations. It also produced lesser amounts of by-products making the downstream process simpler. This method employs the use of cheap and readily available substrates that are converted to amino acids by microorganisms (Faurie and Thommel, 2003).

Fermentative processes opened up a vast arena for the production of industrially important metabolites such as L-glutamic acid. *C. glutamicum* was discovered to produce glutamic acid through fermentation in 1958 at Kyowa Hakko's plant in Japan (Kelle *et al*., 2005).

Since then, there has been no turning back for fermentation industry. Recombinant DNA technology and strain improvement of microbes producing amino acids have enabled production of lysine and glutamic acid at industrial scale (Ali *et al*. 2009). Fermentation contributes 80% of the lysine and glutamic acid that is annually produced worldwide while the remaining 20% is contributed by other processes (Coello *et al*., 2000 and 2002) This method is preferred over all other methods because it employs low temperature, low pressure, low cost carbon sources and renders biological form of glutamic acid as the final product (Nasab *et al*., 2007).

Microorganisms play important roles in the production of various products that are needed in different aspects of life, such as energy, food, chemicals, diagnostics and pharmaceuticals. They are also used to produce enzymes, organic acids, amino acids, vitamins, antibiotics, single cell proteins and other useful chemicals that are of commercial value (Gupta *et al*., 2012).

Microorganisms that have been reported to produce glutamic acid include *Corynebacterium glutamicum* (Nelofer *et al.,* 2008), *Bacillus megaterium* (Ekwealor and Obeta, 2005).

Among these, *C. glutamicum* has been widely exploited industrially for glutamic acid production (Pfefferle *et al.,* 2003). *C. glutamicum* and its other subspecies are known to be well glutamic acid-producing stains including *B. flavum, B. lactofermentum* and *C. lilium*. They have their specific characteristic features being rod shaped, Gram positive and aerobic bacteria. These strains are industrially important and famous for amino acid fermentation. In order to investigate the control of sugar transportation mechanism and

glutamic acid production mechanism, these strains are under observation. Moreover, it was explored that *B. flavum*, *C. glutamicum* and *B. lactofermentum* are genetically closely related to each other (Ruklisha and Ionina, 2000).

2.8 Fermentation Media for Glutamic Acid Production

Fermentation media for glutamic acid production must have a good substrate, organic or inorganic nitrogen source, some growth factors, minerals and essential additives. Some complex media contain special components including; peptone, tryptone, yeast extract, corn steep liquor and molasses that serves not only as substrates but also mineral reservoirs. The vitamins and minerals that are used in the fermentation process include biotin, thiamine, iron and manganese salts and various types of inorganic ions are used in glutamic acid fermentation which include di-potassium hydrogen phosphate that is the source of phosphate and potassium, magnesium sulfate and magnesium chloride which provides the magnesium which is a macro nutrient, whereas ferrous chloride, ferrous sulfate and manganese sulfate serves as micro nutrient (Ahmed *et al.*, 2013). Adnan *et al*. (2011) reported glutamic acid yield of 23.57g/L with the organism *B. linen* where they utilized some agricultural wastes like rice husk, rice husk, wheat husk and soya bean meal for solid state fermentation and also optimized different physical and nutritional parameters like 13.0g soybean meal,4.0% inoculum size, 87.50% moisture level, and 0.65g ammonium sulphate. All over the world, carbohydrates are exclusively used as significant feedstock. Different researchers utilized various components for the production of their required product through fermentation, as Ahmed *et al.* (2010) performed the fermentation by sequential culturing of *Arachniotus* sp. followed by *Candida utilis* at 35^{o}C for 72 h of incubation and produced microbial biomass protein that is enriched with essential amino

acids. The fermentation medium used was 1% molasses, 6% corn steep liquor, 0.0075% $CaCl_2.2H_2O$, 0.01% KH_2PO_4, 0.005% $MgSO_4.7H_2O$ and (30:1) C: N ratio.

With the passage of time to accomplish the increasing demand of lysine and glutamic acid, manufacturers are building various project to optimize numerous levels of carbohydrates and other conditions (Ishikawa *et al*., 2008). Moreover, the coryneform bacteria have special metabolism to utilize beet pulp, molasses, sucrose and other sugars as major carbon sources which are important cost reducing factors of finally produced amino acid on industrial scale. Different researchers worked to optimize the fermentation media for enhanced production of glutamic acid. Among them are Sattar *et al.* (2008) who described their findings after a study on *B. flavum* that was found as one of the important coryneform bacteria used for glutamic acid production. Actually, they enhanced the glutamic acid contents in yeast sludge from 1.54% to 4.78%. They found molasses (1%), protein hydrolysate (25 mg/100mL) and corn steep liquor (4%) as optimum level for maximum glutamic acid production with yeast sludge as substrate at 30^oC and pH 7. The time course of 48 hours was maintained in their study using *B. flavum* as the fermenting agent.

Glutamic acid synthesis was also investigated by Nasab *et al.* (2007), they reported that a number of valuable products like single cell proteins, enzymes and amino acids can be produced by utilizing agricultural wastes as cheap sources of carbon and energy. Therefore, they used a variety of agricultural wastes as carbon sources in their studies for fermentation using a reported strain of *C. glutamicum*. By-products of agricultural origin like date pulp and molasses were used and the results were compared with those obtained when glucose was used as substrate. A maximum production of glutamic acid (48 g/L) after 96 hours of incubation with molasses as substrate was found. Different nitrogen sources for hyper

production of glutamic acid were optimized and observed that during the investigation process, ammonium sulphate was the best nitrogen source. Furthermore, after increasing the incubation time from the optimum time, glutamic acid titer was found to have decreased.

Ekwealor and Obeta (2005) optimized different ionic and minerals concentration and described their effect on glutamic acid production using *Bacillus megaterium* as fermenting agent. The use of complex substances like molasses, yeast extract, corn steep liquor necessitates the process of optimization that involves optimizing parameters such as components of the fermentation media like substrate concentration and inoculum size, temperature and pH It is a pre-requisite for scaling up of all biotechnological process and determining the feasibility of any process (Eggeling, 2005). Optimization of cultural conditions in fermentation aims to increase the titre of glutamic acid production and reduce the amount of undesirable by-products that are likely to be formed through side reactions under unfavorable conditions (Kircher and Pfeerle, 2001).

Overproduction and secretion carrier of glutamic acid are closely related to each other as reported by (Tryfona and Bustard, 2004). Although secretion carrier of glutamic acid is present in both wild and mutant strains, but overproduction is shown to be possessed only by the mutant strain. Therefore, wild strains of coryneform are subjected to mutation to enhance their potential for glutamic acid production and sometimes even the secretion.

2.9 Mutagenesis

There exist several reports on the very low potential for L-glutamic acid production by the wild strains of coryneform bacteria. To date, many studies on strain improvement have been carried out using various methods including conventional mutagenesis and screening, genetic engineering, and metabolic engineering (Malothu *et al*. 2012). Recent work on *C. glutamicum* was the development of complete genome sequencing, and the genetic information is very valuable for strain development for higher performance, in coordination with various "omics" analyses such as transcriptomics, proteomics as well as metabolomics. A large number of amino acid producing isolates were developed over the years through random mutation and selection (Nakamura *et al*., 2007). Many regulatory and auxotrophic mutants of this species have been developed and have been well characterized as hyper-producers of L-glutamic acid. Amongst the regulatory mutants, the commonest are the feedback resistant mutants which are developed by screening mutants for their ability to tolerate glutamic acid analogues so as to obtain mutants that resist feedback inhibition phenomena and overproduce L-glutamic acid. Many recombinant strains of *C. glutamicum* have been developed and are used to improve the production of lysine and glutamic acid (Shah *et al*., 2002;Blombach *et al*., 2009). The over production of desirable product by microbial metabolism requires different techniques. Mutagenesis is one of the processes which are applied to modify an organism. Chemical and physical agents are the two types of mutagens which may be used for genetic control of feedback inhibition in wild type strains, but the rate at which mutations are induced in an organism and the nature of the mutation are some of the important factors to be considered. After mutation, the "mutant strain" should not be revertible. Strong chemical mutagens such as

nitroso-compounds like N-methyl-N-nitro-N-nitroso-Gaunidine (MNNG) and alkylating agents like ethyl methane sulfonate (EMS) are usually employed. Whereas, physical mutagens such as heat, UV- radiations (254 nm), X-rays and ionizing radiations are used in mutagenesis. UV-radiation is the most recommended physical mutagen because both frame shift and base pair mutations can be induced. But it can be reversed by photo-reactivation after exposure to light or dark reactivation when the culture is incubated in darkness under normal growth conditions (Ali *et al*., 2009).

Development of regulatory mutant is another method in which mutant should be insensitive to feedback inhibition or repression. Typically such types of mutants are deficient in regulatory enzymes. *B. flavum* also showed such sort of performance in glutamic acid production (Shah and Khan, 2008). Similarly, *Escherichia coli* was isolated from irrigation water channel by Nadeem *et al.* (2001) and the strain was formerly accomplished of producing 1.8 g/L of glutamic acid but was later progressively upgraded through mutation for glutamic acid production. The wild bacterial cells were exposed to MNNG (100 µg/mL) as a mutagen for 90 minutes. The mutant showed resistance against (4-fluoroglutamic acid), a glutamic acid toxic analogue, and was able to produce 13-15 g/L of glutamic acid.

2.10 The Genus *Corynebacterium*

2.10.1 Taxonomy

The genus *Corynebacterium* was created by Lehmann and Neumann in 1896 as a taxonomic group to contain the bacterial rods responsible for causing diphtheria. The genus was defined based on morphological characteristics. Studies on 16S-rRNA were later used to groupthe genus into the subdivision of Gram-positive eubacteria with high G: C content, with close phylogenetic relationship to *Arthrobacter*, *Mycobacterium*, *Nocardia*, and *Streptomyces*. The term*Corynebacterium* comes from the Greek word, corönë ("knotted rod") and bacterion ("rod"). The term "diphtheroids" is used to represent corynebacteria that are nonpathogenic; for example,*C. diphtheriae* would be excluded.

2.10.2 Genomics

Comparative analysis of Corynebacterial genomes has led to the identification of several conserved signature indels which are unique to the genus. Two examples of these conserved signature indels are a two amino acid insertion in a conserved region of the enzyme phosphoribose diphosphate: decaprenyl-phosphate phosphoribosyltransferase and a three amino acid insertion in acetate kinase, both of which are found only in *Corynebacterium* species. Both of these indwells serve as molecular markers for species of the genus *Corynebacterium*. Additionally, 16 conserved signature proteins, which are uniquely found in *Corynebacterium* species, have been identified. Three of the conserved signature proteins have homologs found in *Dietzia* species, which is believed to be the closest related genus to *Corynebacterium*. In phylogenetic trees, based on concatenated protein sequences or 16S rRNA, the genus *Corynebacterium* forms distinct clade, within

which is a distinct subclade, cluster I. The cluster is made up of the species *C. diptheriae, C. pseudotuberculosis, C. ulcerans, C. aurimucosum, C. glutamicum and C. efficiens.* This cluster is distinguished by several conserved signature indels, such as a two amino acid insertion in LepA and a 7 or 8 amino acid insertions in RpoC. There are also 21 conserved signature proteins which are found only in members of cluster I. Another cluster has been proposed consisting of *C. jeikeium and C. urealyticum,* which is supported by the presence of 19 distinct conserved signature proteins which are unique to these two species (Gao, 2012).

2.10.3 Features, growth characteristics and uses of *Corynebacterium*

The principal features of the *Corynebacterium* genus were described by Collins and Cummins in 1986. They are Gram-positive, catalase positive, nonspore-forming, non-motile, rod-shaped bacteria that are straight or slightly curved (Yassin *et al.*, 2003). Metachromatic granules are usually present representing stored phosphate regions. Their size falls between 2-6 micrometers in length and 0.5 micrometers in diameter. The bacteria are grouped together in a characteristic way, which has been described as the form of a "V", "palisade", or "Chinese letters". They may also appear elliptical. They are aerobic or facultatively anaerobic, chemoorganotrophs, with 51–65% genomic G: C content. They are pleomorphic through their life cycles; they occur in various lengths and frequently have thickenings at either end, depending on the surrounding conditions. **(**Fawole and Oso, 2007**).**

The cell wall is distinctive, with a predominance of meso-diaminopimelic acid in the murein wall (Yassin *et al*., 2003) and many repetitions of arabinogalactan as well as corynemycolic acid (a mycolic acid with 22 to 26 carbon atoms), tied together by

disaccharide bonds called L-Rhap-(1 → 4)--D-GlcNAc-phosphate. These form a complex commonly seen in *Corynebacterium* species: the mycolyl-AG–peptidoglycan (mAGP)

Corynebacteria grow slowly, even on enriched media. In terms of nutritional requirements, all need biotin to grow. Some strains also need thiamine and para-aminobenzoic acid (PABA). Some of the *Corynebacterium* species with sequenced genomes have between 2.5 and 3 million base pairs. The bacteria grow on Loeffler's medium, blood agar, modified Hoyle's medium and trypticase soy agar (TSA). They form small greyish colonies with a granular appearance, mostly translucent, but with opaque centers, convex, with continuous borders (Yassin *et al.*, 2003). The colour tends to be yellowish-white on Loeffler's medium. On TSA, they can form grey colonies with black centers and dentated borders that look similar to flowers (*C. gravis*), or continuous borders (*C. mitis*), or a mix between the two forms (*C. intermedium*) while on modified Hoyle's medium, they form black, slightly mucoid colonies following the reduction of potassium tellurite (e.g. *C. glutamicum*).

Corynebacterium species occur commonly in nature in the soil, water, plants, and food products .The non-diphtheiroid *Corynebacterium* species can even be found in the mucosa and normal skin flora of humans and animals. Some species are known for their pathogenic effects in humans and other animals. Perhaps the most notable one is *C. diphtheriae*, which acquires the capacity to produce diphtheria toxin only after interacting with a bacteriophage . Other pathogenic species in humans include: *C. amicolatum, C. striatum, C. jeikeium, C. urealyticum,* and *C. xerosis*; all of these are important as pathogens in immunosuppressed patients. Pathogenic species in other animals include *C. bovis* and *C. renale.*

Non-pathogenic species of *Corynebacterium* are used for several important industrial applications, such as the production of amino acids like L- glutamic acid, nucleotides, and other nutritional factors (Hermann, 2003); bioconversion of steroids; degradation of hydrocarbons; cheese aging; and production of enzymes. Some species produce metabolites similar to antibiotics: bacteriocins of the corynecin-linocin type, antitumor agents etc. One of the most studied species is *C. glutamicum*, whose name refers to its capacity to produce glutamic acid under aerobic conditions. Glutamic acid is used in the foods industry as monosodium glutamate (MSG) in the production of soy sauce and yogurt. The most widely use of these bacteria utilizes the bacterial capacity to produce glutamic acid under aerobic conditions. Species of *Corynebacterium* have been used in the mass production of various amino acids including glutamic acid, a food additive that is made at a rate of over 1.5 million tons/year. The metabolic pathways of *Corynebacterium* have been further manipulated to overproduce glutamic acid and threonine.

2.11 *Corynebacterium glutamicum*

2.11.1 Strain information

In 1957, Kinoshita *et al*. isolated a bacterial strain which was able to overproduce L-glutamic acid in minimal media with glucose as carbon source and release the product in the extracellular environment. The isolated soil bacterium was named *Corynebacterium glutamicum*. In taxonomic terms it belongs to the family of Corynebacteriaceae. Its cell wall formation is very characteristic (gram positive), especially the existence of mycolic acids which surround the entire cell as a structured layer (Eggling *et al*., 2001). The wild type strains are mostly able to grow aerobically on basic minimal media containing a

carbon source like glucose and other minerals such as phosphate, sulphate, ammonia and additionally biotin due to the fact that this bacterial species is completely biotin dependent (Biotin is an essential growth factor to this organism). Since the isolation in 1957, high amounts of L-glutamic acid have been produced with newly developed or advanced strains of this species (Eggling *et al*., 2005).

Because of many experiences the scientists gained over the last decades about this organism and its metabolic fluxes in context of amino acid production, *Corynebacterium glutamicum* has become the most important bacterial strain for amino acid overproduction. It has been observed that the regulatory system is much simpler than that of *Escherichia coli* (Zahoor *et al*., 2012).

2.11.2 Regulation of L-glutamic acid production with *Corynebacterium glutamicum*

The process to gain L-glutamic acid with *Corynebacterium glutamicum* by direct fermentation (Asakura *et al*., 2007) is very well investigated. Key factors for the cultivation process in order to reach high amounts of L-glutamic acid are the optimal concentration of biotin to influence and support cell growth and the secretion of the product in the extracellular environment (Nakamura *et al.,* 2007). Another important factor to prevent side reactions and undesirable by-products is the oxygen supply. This is because, under partially anaerobic conditions, other/additional products like lactic acid could be produced (Ikeda, 2003). Several different enzymes are involved in the formation of glutamic acid from acetyl-coA through alpha-ketoglutarate via the TCA cycle. The enzymes given in Figure 2.2 are listed below;

1. Phosphoenolpyruvate carboxylase. 2. Pyruvate kinase. 3. Pyruvate carboxylase. 4. Pyruvate dehydrogenase. 5. Citrate synthetase. 6. Aconitase. 7. Isocitrate dehydrogenase. 8. L-glutamate dehydrogenase (GDH). 9. α-ketoglutarate dehydrogenase (KDH).10. Isocitrate lyase. 11. Malate synthetase.

The most important factor for L-glutamate overproduction is the activity of the enzymes GDH and KDH (Figure 2.2). In overproducers, the conversion velocity of α-ketoglutarate to L-glutamic acid with GDH is 150 times higher than the side reaction of the substrate with KDH which leads back to the citric acid cycle (Wendisch, 2007). In Figure 2.2 the versatile regulatory mechanisms in biological pathways for L-glutamic acid (feedback inhibition and repression) are shown, the problems in modifying these metabolic fluxes in desired directions are quite obvious due to the complexity and various connections in these metabolic cycles.

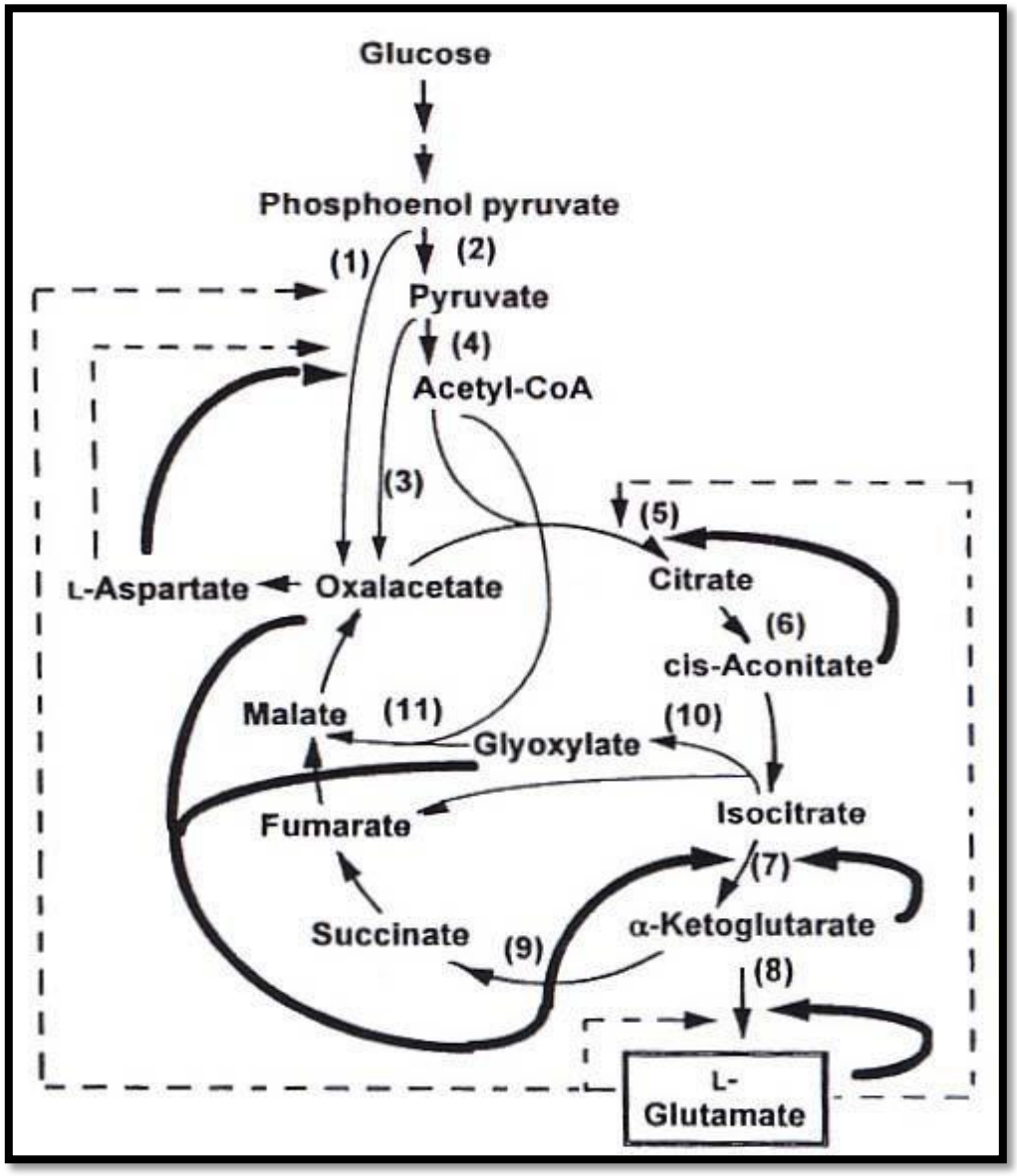


Figure 2.2: Pathways of regulation of L-glutamic acid biosynthesis in *Corynebacterium glutamicum* (Wendisch, 2007); thick lines represent feedback inhibition, dashed lines represent feedback repression.

2.12 Industrial Methods for Recovery and Purification of Glutamic acid

2.12.1 Filtration and centrifugation of fermented broth

Batches consisting of fermented broth (obtained from batch fermentation in fermenter) are normally filtered using a microfiltration unit (Millipore, USA), fitted with a 50 mm membrane diameter with a pore size of 0.45 µm under vacuum using a pump. The filtrate is usually centrifuged at 10,000 rpm for 10 minutes to get the supernatant, which is then used for the recovery of the product.

2.12.2 Preparation of resin

Spherical particles of cation exchange resin are normally used. Prior to use, the resins are usually pre-conditioned and then washed thoroughly two times with 4 N HCI. After two washes with distilled water, the resin is then washed with 2 N NaOH until the filtrate becomes alkaline. The resulting material (sodium salt of the resin) is usually suspended in 3-times its volume of 1 N NaOH and heated over a steam bath for 2 h with occasional mixing. The supernatant fluid is then decanted after 30 minutes of settling and replaced with fresh hot 1 N NaOH. The procedure is repeated two times. The resin is then filtered and washed with 2 L of distilled water to make it free of alkali. The resin is finally stored as the moist sodium salt.

2.12.3 Packing the column

Resin (as above) is placed in a column (3 cm × 50 cm) containing distilled water and filled up to 25 cm^3. The excess water is then removed using a siphon.

2.12.4 Separation process by ion-exchange column

The chromatographic conditions are selected to minimize the inhibitory effect of co-existing inorganic ions on the adsorption of amino acids by ion-exchange resins. Removal of impurities from the broth is done by filtering and centrifuging the broth. The pH of the broth normally drops and this is the most important factor affecting the adsorption of glutamic acid on the resin because the ionic forms vary with the pH.

The processes involved in the column are adsorption and elution. In the adsorption process, the broth used is adjusted to a suitable pH in order to charge the glutamic acid so that the ion exchange between the glutamic acid and the resin could occur. The broth is continuously recycled at a flow rate of 20 ml/minute (retention time 50 minutes) until glutamic acid is fully adsorbed in the column, leaving other ions. In the elution process, the pH is increased to 3.8-4.0 by treating the broth with urea and sodium hydroxide. This is done to release the glutamic acid bound on the resin by changing the glutamic acid charge.

2.12.5 Crystallization of L-glutamic acid

After adsorption and elution, the eluate containing the glutamic acid is acidified to pH 3.22, the isoelectric point of glutamic acid with 1 N HCl. Storage at 20°C for 48 h results in the formation of crystals of glutamic acid. After evaporation of the eluent, the dry solid crystals are obtained.

CHAPTER THREE

3.0 Materials and Method

3.1 Experimental Design

The experimental design consisted of two substrate's treatments (Acidic and Alkaline), and four replicates, two for each of the regulatory mutants and the wild-type strains of *Corynebacterium glutamicum*.

3.2 Collection of Samples

Four distinctive locations of Samaru village were chosen for soil sampling to isolate *Corynebacterium glutamicum*. A total of five soil samples were collected from different parts of flower bed around the Department of Microbiology, Ahmadu Bello University, Zaria, and two samples each from paddock, chicken-run and sheep-pen were collected from Samaru village at a depth of about 10cm. Each sample was packaged in a clean polythene bag, labelled appropriately and then transported to the Department of Microbiology, Ahmadu Bello University, Zaria for analyses.

About 1kg of fresh and fine-textured rice husk was collected from rice milling station at Samaru village, Sabon Gari Local Government Area of Kaduna state. The rice husk sample was packaged into polythene bag, labelled appropriately and then transported to the Department of Microbiology, A.B.U. Zaria for analyses.

3.3 Treatment of the Rice Husk (Substrate Pre-treatment)

3.3.1 Alkaline pre-treatment

A weighed amount of 25g of the fresh rice husk was placed in a 1000mL Erlenmeyer flask and 225 mL of 1.0M KOH solution was added. The flask was cotton- plugged and autoclaved at 121ºC for 30 min. The material obtained after treatment was then filtered through muslin cloth and washed several times under running distilled water until no color was visible in the wash water and the pH adjusted to physiological value (7.2). The neutralized residue was then pressed manually to remove excess water and used for the enzymatic hydrolysis (Narasimha *et al*., 2011). A small portion of the treated biomass was dried in an oven at 70ºC for 24 h and was ground to fine particle size in a laboratory mill for the proximate analysis (Rakesh *et al*., 2013) at the Department of Food Science, Institute of Agricultural Research, Ahmadu Bello University, Zaria.

3.3.2 Acidic pre-treatment

The method described by Rakesh *et al*. (2013) was adopted with modifications. About 25g of the rice husk sample was added to a 1000mL flask and about 225mL of 1.0M sulphuric acid was added to the sample. The mixture was autoclaved at 121°C for 30 min and the material obtained after treatment was then filtered through muslin cloth and washed several times under running distilled water until no color is visible in the wash water and the pH adjusted to physiological value (7.2) following calcium hydroxide over-liming. The neutralized residue was then pressed manually to remove excess water and used for the enzymatic hydrolysis. A small portion of the treated biomass was dried in an oven at 70ºC for 24 h and was ground to fine particle size in a laboratory mill for the proximate analysis (Rakesh *et al*., 2013) at the Department of Food Science, Institute of Agricultural Research, Ahmadu Bello University, Zaria.

3.4 Proximate Analyses of the Pre-treated Rice Husk

Eight grams of each of the acid-treated and alkali-treated dried unfermented rice husk samples were used to carry out proximate analyses at the Department of Food Science, Institute of Agricultural Research, Ahmadu Bello University, Zaria. Hence analyzed for the included carbohydrate, the crude protein, crude fat, crude fibre, ash content and moisture as the percentage compositions of the substrate according to the methods adopted by Ahmed *et al*. (2013) with modifications. The methods are described as follows:

3.4.1 Determination of moisture content:

Crucibles were washed and dried to a constant weight in an oven at 100^{o}C. They were later removed and cooled in a desiccator and weighed (**W1**). Two grams of the grounded (powder) each of acid-treated and alkali-treated samples were placed separately in the weighed moisture dish (**W2**). The dishes containing the samples were kept in an oven set at 100^{o}C for about 3hr, the samples were removed and cooled in the desiccator and weighed **W3** (AACC, 2000).

The % of moisture content was then calculated as: $\frac{\mathbf{W2-W3}}{\mathbf{W2-W1}} \times 100$

3.4.2 Determination of ash content (AC)

Crucibles were cleansed and dried in an oven, after which they were cooled in a desiccator and weighed (**W1**). Two grams of the grounded (powder) each of acid-treated and alkali-treated samples was placed separately in the crucibles and weighed (**W2**). They were then transferred into a muffled furnace (SXL Model) set at 550^{o}C for 2hrs. Thereafter, the crucibles were removed, cooled in the desiccator and weighed (**W3**) (AACC, 2000).

The % Ash of each sample was calculated as: $\frac{\mathbf{W3-W1}}{\mathbf{W2-W1}} \times 100$

3.4.3 Determination of crude fibre content (CFb)

Two grams of each sample was placed separately in a beaker containing 1.3ml of H_2SO_4 per 100ml of solution and boiled for about 30min, the residue was filtered and washed with hot water, it was later transferred to another beaker containing 1.2g of NaOH per 100ml of solution and boiled again for another 30min, the residue was washed with hot water and dried in an oven and weighed (**C2**), the weighed sample was then incinerated in a muffled furnace set at a temperature of about 550^{o}C for 2hrs, removed, allowed to cool and weighed as **C3.**

The % Crude Fibre was calculated as thus; $\frac{\mathbf{C2-C3}}{\mathbf{W}} \times 100$

3.4.4 Determination of crude fat (CF)

The crude fat content of each sample was estimated by following the standard AOAC (Association of Official Analytical Chemists) procedure as described by Shagufta *et al.* (2014) with modifications as follows:

Two clean boiling flasks of 250ml capacity were dried in an oven and transferred into desiccators and allowed to cool. Empty filter paper labelled **W1** was weighed and recorded. Two grams of each sample was weighed into the labelled thimbles (filter papers) and recorded as **W2.** Each of the boiling flasks was filled with n-hexane after which the soxhlet apparatus was assembled and allowed for refluxing for 8hr. The samples were then removed and transferred to an oven set at 100^{o}C to dry and then to a desiccator to cool and then weighed and recorded as **W3.**

The % Crude Fat of each sample was calculated as: $\frac{\mathbf{W2-W3}}{\mathbf{W2-W1}} \times 100$

3.4.5 Determination of crude protein (CP)

The crude protein was determined by Kjeldhal method as described by Fakhar-un-Nisa *et al*. (2015) with modifications as follows:

The nitrogen content in each sample was determined by using Kjeltech apparatus based on Kjeldhal's AACC Method 46-10 (AACC, 2000). The percentage protein was calculated by multiplying the nitrogen content with the standard protein factor for all forages and feeds including rice husk i.e. 6.25

a. Digestion

Two grams of the sample was placed into a Kjeldahl flask and a catalyst (Kjeltabs Cu/3.5 containing 3.5g of K_2SO_4 and 0.4g of $CuSO_4$. $5H_2O$) was added followed by 15ml of concentrated sulphuric acid. The tubes were assembled in a digestion box and the fume cupboard closed. The content of each tube was heated until the solution assumes a green colour and then cooled after which each digest was transferred with several washings into 100ml with distilled water.

b. Distillation

The digest was first steamed through the Markham distillation apparatus for about 15min. Under the condenser, a 100ml Erlenmeyer flask containing 10ml of 4% boric acid was placed and two drops of indicator was added. Ten millilitres of 40% NaOH was added to 10ml of the digest after which the NaOH-sample mixture was distilled and the distillate collected in the boric acid-indicator mixture until it turned green. This was then titrated with N/100 (0.01N) hydrochloric acid.

c. Titration

The solution in the receiving flask was titrated with 0.01N hydrochloric acid. The Nitrogen content and hence the protein content of the sample was calculated as follows:

The % protein content was then calculated as; *Final reading – Initial reading X standard number of nitrogen (1.4) divided by initial weight X standard protein factor (6.25)*

$$\% \text{ Protein} = \frac{(V_2 - V_1) \times 1.4}{W \times 6.25}$$

Where: V_1 = Initial reading, V_2 = Final reading, W = Initial weight of the rice husk sample

3.4.6 Determination of carbohydrate (CHO)

By difference, in this method, carbohydrate content was obtained by calculations having estimated all other fractions by proximate analyses i.e.

CHO = 100- (% moisture + %Ash + % Protein + %Fat)

3.5 Isolation and Characterization of *Corynebacterium glutamicum*

3.5.1 Media preparation for the Isolation of *Corynebacterium glutamicum*:

3.5.1.1 *Loeffler's Blood Serum Medium:*

Preparation of Medium: A weighed amount of 19.2g of the dehydrated powder of Loeffler's Blood Serum medium was added to 240mL of distilled water, mixed thoroughly and adjusted to a pH of 7.2. The mixture was gently heated and brought to boiling to dissolve, dispensed in bottles and tightly capped, then autoclaved as slants for 15 min at 15 psi pressure and a temperature of 121°C.

3.5.1.2 *Modified Hoyle's Medium:*

Preparation of Medium: A weighed amount of 8.64g of the dehydrated powder of Hoyle's medium base was added to 240mL of distilled water, mixed thoroughly and adjusted to a pH of 7.2. The mixture was gently heated and brought to boiling to dissolve, then autoclaved for 15 min at 15 psi pressure and at 121°C then allowed to cool before adding 5% (13mL) of sheep blood, 12ml of 1% potassium tellurite solution and 50µg of biotin. The mixture was then poured into sterile Petri- dishes and allowed to set.

3.5.1.3 *Sheep Blood Agar*:

Preparation of Medium: A weighed amount of 9.6g of the dehydrated powder of blood agar base was added to 240mL of distilled/deionised water, mixed thoroughly and adjusted to a pH of 7.2. The mixture was gently heated and brought to boiling to dissolve, then autoclaved for 15 min at 15 psi pressure and at 121°C and allowed to cool before adding 5% (13mL) of de-fibrinated sheep blood and 50µg of biotin. The mixture was then poured into sterile Petri dishes and allowed to set.

3.5.1.4 *Motility medium:*

A weighed amount of 5.22g of the dehydrated powder of motility medium was added to 120mL of distilled water and mixed thoroughly. The mixture was gently heated and brought to boiling to dissolve, 10mL each was then dispensed in cleaned test tubes, then plugged with cotton wool and autoclaved at 121^{o}C for 15 min at 15 psi pressure. The medium was allowed to cool and about 25µg of biotin was added to each test tube, allowed to set and then stored in refrigerator at 4^{o}C until required.

3.5.2 Isolation of *Corynebacterium glutamicum:*

A weighed amount of 25g of each of the five (5) soil samples was separately added to 225mL of sterile distilled water and a tenfold serial dilution was carried out to a dilution of 10^{-5} using sterile normal saline as diluent. A loopful (0.1mL) from each of the $1:10^2$ diluted soil suspensions was separately inoculated onto slants of Loeffler's medium which was the primary isolation medium by streaking. The inoculated slants were then incubated at 35^oC for 48 hr. Discrete; well isolated colonies were selected and identified. These were sub-cultured on the selective medium- modified Hoyle's medium and also incubated at 35^oC for 48 hr. The isolates were used as source of culture to be screened for the production of L-glutamic acid (Ahmed *et al.*, 2013). The isolates were sub-cultured onto nutrient agar slants for subsequent identification and use.

3.5.3 Identification of *Corynebacterium glutamicum*

Identification of *Corynebacterium glutamicum* was primarily based on the taxonomic comparison. The characteristic morphological, cultural and biochemical properties were observed (Bergey, 2004).

3.5.3.1 Cultural characterization

Loeffler's Blood Serum Medium:

The colonies on the slants were examined for size, pigmentation, form, margin and elevation. They form small yellowish-white colonies with a granular appearance, mostly translucent, but with opaque centres, convex, with continuous borders.

Modified Hoyle's Medium:

The plates of the Modified Hoyle's Medium were examined for size, pigmentation, form, margin and elevation of the colonies following incubation for 48h.

Sheep Blood Agar:

The already prepared sheep blood agar plates were inoculated with the isolates from the modified Hoyle's medium and incubated at 35^{o}C for 48 hr. The plates were then examined for size, pigmentation, form, margin, elevation and type of haemolysis.

Motility Test:

The presumptively identified isolates from the selective isolation medium were stab-inoculated using a straight wire to about two-thirds of the tube length (20cm). The tubes were then incubated at 35^{o}C for 48 hours. At the end of the incubation period, tubes were examined for pattern of growth (i.e. along the line of stabbing or staggered along the line into the medium).

3.5.3.2 ***Morphological characterization***

Such morphological characteristics as the cell shape, cell arrangement as well as the Gram's reaction of the organism were determined by Gram staining technique. Other staining techniques such as spore-staining were also carried out to morphologically characterize the isolates. (Mahmood, 1996).

Gram Staining:

A smear was prepared from the 48hr agar culture, where a pinch of well separated colony (pure culture) was placed directly into a drop of physiological saline on a clean, dried, grease-free slide, smeared and then allowed to air-dry. The smear was then heat-fixed by passing the slide over a Bunsen flame for three quick successions. The slide was then flooded with crystal violet solution and allowed to stand for one minute, then washed with slow-running tap water and then flooded with Gram's iodine (mordant) and allowed to act also for one minute. The slide was then washed with tap water and decolourized with 95% ethyl alcohol for 30sec. Thereafter, the slide was rinsed with tap water and then counter-

stained with safranin for 30sec. The slide was finally rinsed with slow-running tap water, allowed to air-dry and then examined microscopically under oil immersion objective lens (x100). Typical Gram positive, non-spore bearing rod shaped cells, arranged as palisades (V-shaped) appearing purple were considered presumptively as *Corynebacterium* spp. (Cowans and Steel, 2004).

Spore staining:

A dried smear was prepared and covered with a piece of paper towel and then flooded with 5% Malachite green solution and steam for five minutes keeping the paper towel moist with the Malachite green. The paper was then removed and discarded and the slide was washed well with running tap water and then counter-stained with 5% aqueous carbol fuchsin for one minute. The slide was then washed under slow running tap water, dried with blotting paper and examined under oil immersion objective (x100). Non-spore forming rods appearing red were observed (Fawole and Oso, 2010). This distinguishes *Corynebacterium* spp. from other Gram positive but spore-forming soil bacteria.

3.5.3.3 *Biochemical characterization*

Media preparation: All media used for biochemical tests were of analytical grade (OXOID) and were prepared according to manufacturer's instructions.

Carbohydrate fermentation test:

A series of test tubes containing sterile nutrient broth with 1% each of membrane-filter-sterilized (0.45µm) single fermentable sugar and inverted Durham's tubes were prepared. The tubes were then inoculated with the test organisms stored on modified nutrient agar slant. The sugar fermentation tubes were incubated at 35^{o}C for 24 hours. At the end of the incubation period, all tubes were examined for acid following the addition of methyl red indicator and for gas production in the Durham's tubes and then compared with the control

for interpretation (Cowans and Steel, 2004). A total of seven (7) sugars were used for the fermentation tests, these included; Glucose, Arabinose, Fructose, Galactose, Lactose, Maltose and Sucrose.

Catalase test:

Using an inoculating needle, the 24 hours culture of each of the isolates (from the slant) was picked and placed separately on a clean, grease-free slide already containing three drops of 3% H_2O_2. The slides were immediately observed for bubbling (Effervescence) which indicated a positive test. (Cowans and Steel, 2004).

Nitrate Reduction Test:

Test tubes containing 5mL of nitrate broth each with inverted Durham tubes were inoculated with the suspected colony of *Corynebacterium glutamicum* using a straight wire. The tubes were incubated at 35^{o}C for 24hrs. After 24hrs of incubation, the Durham tubes were observed for gas collection. All tubes had no gas collection, 10 drops of reagent B (α-naphthylamine in 5N acetic acid) was added followed by 10 drops of reagent A (Sulphanilic acid in 5N acetic acid). An immediate red colour developed in tubes where nitrate has been reduced to nitrite. But in tubes where no colour developed within 1 minute, a pinch of zinc granules was added. Development of red colour around the zinc granules within two minutes and eventually colouring the broth means nitrate was not reduced and still present. However, in tubes where there was no development of colour within two minutes, then nitrate was no longer present in the broth, i.e. it has been reduced beyond the nitrite stage but not as far as the nitrogen gas stage since no gas was observed in the inverted Durham tubes. (Cowans and Steel, 2004).

Methyl Red test:

Test tubes containing 5mL of MR-VP broth each were inoculated with the suspected colony of *Corynebacterium glutamicum* using wire loop. The tubes were incubated at 35^{o}C for 48hrs. After incubation, about 3ml of the cultured broth was transferred to a small test tube to which three drops of methyl red indicator was added. Formation of red colour on addition of the indicator signifies a positive methyl red test and a yellow colour signifies a negative test (Cowans and Steel, 2004).

Voges-Proskauer test:

Test tubes containing 5mL of MR-VP broth each were inoculated with the suspected colony of *Corynebacterium glutamicum* using wire loop. The tubes were incubated at 35^{o}C for 48hrs. After incubation, about 3mL of the cultured broth was transferred to a small test tube to which five drops of 40% potassium hydroxide (KOH) was added followed by the addition of 15 drops of 5% α-naphthol in ethanol. The mixture was shaken and the tubes placed in a sloping position. Development of red colour starting from the liquid-air interface within one hour indicates a positive VP test and no colour change occurs in a VP negative test (Cowans and Steel, 2004).

Aesculin hydrolysis:

A pinch of the suspected colonies of *Corynebacterium glutamicum* was inoculated on bile aesculin agar and incubated at 37^{o}C for 24hrs. A positive test was indicated by a dark brown to black colouration of the whole medium after 24hr of incubation. A negative reaction was indicated by lack of colour change throughout the medium. (Cowans and Steel, 2004). This differentiates it from some *Bacillus* species that are also found in the soil.

Gelatine liquefaction:

The test organisms were inoculated in nutrient gelatine tubes by means of stab-inoculation. The tubes were then incubated at 35^{o}C for 24 hrs. At the end of the incubation period, the tubes were placed in refrigerator at 4^{o}C for 30 minutes and observed for the liquefaction of the medium and compared with the control (un-inoculated tubes of nutrient gelatine). Liquefaction of gelatine indicates a positive test (Cowans and Steel, 2004).

Urease test:

Urea agar slants in Bijou bottles were inoculated with the test isolates. The bottles were then incubated at 35^{o}C for 24hrs. The development of a bright-pink or red colour indicated a positive reaction (Cowans and Steel, 2004).

3.6 Screening of the *C. glutamicum* Isolates for Glutamic Acid Production

3.6.1 Screening Medium

The compositions of the screening medium used for L-glutamate production per 1000 ml of dH_2O is as follows; glucose, 5gm; calcium carbonate, 1gm; ammonium sulphate, 1gm; potassium di-hydrogen phosphate, 0.3gm; di-potassium hydrogen phosphate, 0.7gm; magnesium sulphate hepta-hydrate, 0.01gm; ferrous sulphate hepta-hydrate, 0.2mg; magnesium chloride tetra hydrate, 0.2mg; thymine hydro chloride, 20µg; and d-biotin, 10µg.

3.6.2 Preliminary screening of the isolates

3.6.2.1 *Culture procedure*

Screening medium (100 ml) was taken in each 250 ml sterilized flask and inoculated with 24 hours old bacterial broth culture. After inoculation, the flasks were incubated on a

shaker incubator at 37ºC for 96h at 180 rpm. Samples were taken after 96h and centrifuged at 5000rpm for 10 min. Supernatants were then examined for L- glutamic acid.

3.6.2.2 ***Qualitative estimation of L-glutamic acid***

Qualitative analysis for L-Glutamate was done by paper chromatographic technique (Hassan *et al.,* 2003). For paper chromatography, Whatman No. 1 filter paper having dimensions 19x10cm was used, spotted with standard glutamate solution and solution from the supernatant of the samples, 3cm above from the bottom by means of micro capillary tube. At room temperature, spots were air-dried. The paper was then dipped up to 1.5cm in solvent system having 30ml Butanol, 5ml acetic acid and 5ml dH_2O (6:1:1). By using ascending method, spots were developed by allowing the solvent to run for three hours, up to 12cm from the bottom. After air-drying, the paper was sprayed uniformly with 0.5% ninhydrin in (95%) acetone. The paper was then placed in an oven set at 65ºC for five minutes after air-drying. Coloured spots of amino acids were produced which indicated the positions of amino acids present in the sample(s). Through these well-defined coloured spots, the retention factor (RF) was also calculated and the glutamic acid present in the unknown samples was identified by comparing with the standard glutamic acid coloured spot based on their RF. The retention factor was calculated using the following formula;

$$RF = \frac{\text{Distance moved by the spot}}{\text{Distance moved by the solvent}} \quad \text{(No unit)}$$

3.6.2.3 ***Quantitative estimation of L-glutamic acid***

For quantitative estimation of the amino acid produced, two millilitres of the supernatant from each of the fermented screening medium was taken separately in test tubes and two millilitres of 5% ninhydrin in acetone was added and heated for 15 min in boiling water

bath. The tubes were then cooled to room temperature and glutamate was quantitatively estimated by taking readings at 570 nm using spectrophotometer with reference to the standard curve.

3.6.3 Standard curve of L-glutamic acid

To estimate glutamic acid quantitatively by spectrophometry method as described by Shagufta (2014), the standard curve was firstly plotted between various diluted concentrations (100mg/L-500mg/L) of standard glutamic acid and their respective optical densities (OD) at 570 nm. The trend in Figure3.1 represented the increasing OD with increasing standard glutamic acid concentrations. The maximum OD (0.9) at 570nm was corresponding with the highest standard glutamate concentration of 400mg/L.

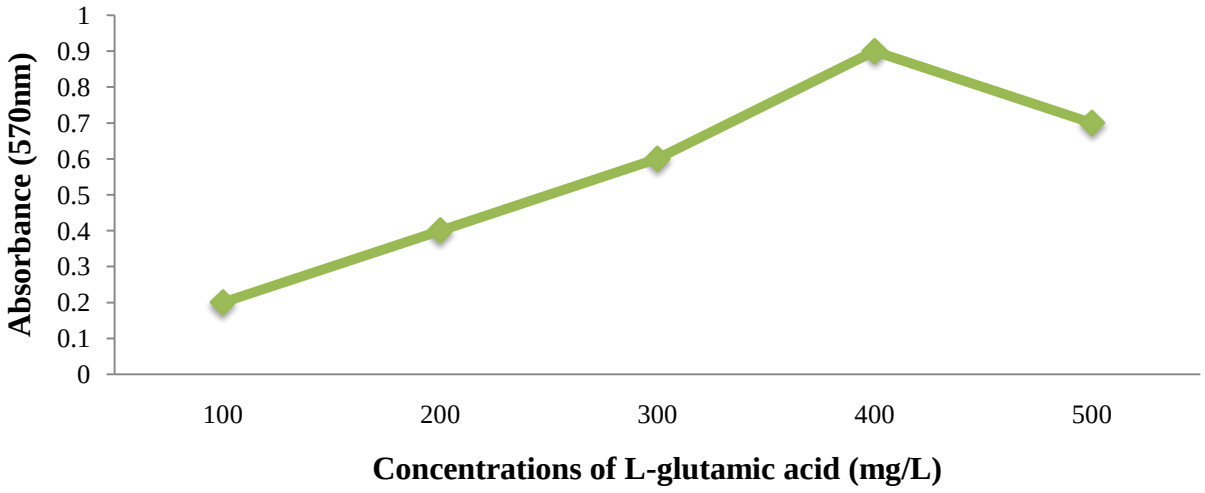


Figure 3.1: Standard curve of L-glutamic acid

3.7 Production of Regulatory Mutant of the Selected *C. glutamicum* Isolate

3.7.1 Mutation with nitrous acid

The isolates were grown in biotin-supplemented nutrient broth for 24 h. Acetate buffer (0.2 M, pH 4.4) was prepared in accordance with the procedure adopted by Ado (2004). To fifty milliliters of 50:50 organism: acetate buffer suspension in a 150mL flask, a 1.5mL of membrane filter (0.2μm pore size) - sterilized aqueous solution of 2.0M sodium nitrate was added. This was allowed to stand at room temperature (30 ± 2°C) for twenty minutes. The reaction was then terminated by serial dilution with Tris HCl prepared in accordance with the procedure of Ado (2004): 121 g of Tris base was dissolved in 800mL of distilled water. The pH value was adjusted to 7.4 by adding 84 mL of 0.1M HCl to 100 ml of 0.1M Tris base. The volume was made up to one litre with distilled water. The treated organisms were inoculated, using pour plate technique on biotin-supplemented nutrient agar and incubated at 37°C. Survivals were further subjected to mutant selection medium containing 4-Fluoroglutamic acid as toxic analogue of L-glutamic acid and screened out for their potential to produce L- glutamate.

3.7.2 Isolation of Regulatory Mutants Using a Toxic analogue (4-Fluoroglutamic Acid-Resistant Mutants)

Suspected mutant isolates from the nutrient agar supplemented with biotin were inoculated directly onto modified Hoyle's medium supplemented with 2.0mg/ml of 4-Fluoroglutamic acid. Colonies that appear on the surface of the agar plate within two to seven days of incubation were picked up as the regulatory mutants resistant to 4-Fluoroglutamic acid (Nakamura *et al*., 2007).

3.8 Glutamic Acid Production from Rice Husk by Submerged Fermentation

3.8.1 Inoculum preparation:

For the preparation of inoculum, loop full of the refreshed culture of *C. glutamicum* was aseptically transferred to sterile inoculum medium (25mL) in 250mL Erlenmeyer flask. The inoculum medium contained 0.8 % nutrient broth (pH= 7.0± 0.2) and was kept for 18 hours on an orbital shaker at 30^{o}C and 120 rpm (Nasab *et al*., 2007). The optical density of the culture was adjusted to 0.6 at 600 nm by diluting the culture with sterile distilled water (Athar *et al*., 2009).

3.8.2 Basal Medium

For L-glutamic acid production by *C. glutamicum* through submerged fermentation, optimization of such parameters like substrate-water ratio, temperature, pH and inoculum size, cells were cultured in basal salt (BS) medium per litre. The basal salt medium comprised the following per litre: 5 g, $(NH_4)_2SO_4$; 5 g, urea; 2 g, KH_2PO_4; 2 g, K_2HPO_4; 0.25 g , $MgSO_4 \cdot 7H_2O$; 0.01 g, $FeSO_4 \cdot 7H_2O$; 0.01 g, $MnSO_4 \cdot 5H_2O$; 0.01 g, $CaCl_2 \cdot 2H_2O$; 0.03 mg, $ZnSO_4 \cdot 7H_2O$; 0.1 mg, H_3BO_4; 0.07 mg, $CoCl_2 \cdot 6H_2O$; 0.03 mg, $CuCl_2 \cdot 2H_2O$; 0.01 mg, $NiCl_2$; 0.1 mg of $NaMo_2O_4 \cdot 2H_2O$; 200µg of biotin (pH 7.0) . The initial pH was adjusted at 7.2 with 1N NaOH.

3.8.3 Shake Flask Fermentation

Fifty millilitres of the basal medium was mixed separately with 13g each of the acid-pre-treated and alkali-pre-treated substrate in 250mL Erlenmeyer flasks and labelled appropriately. Four millilitres each of the 18hours-old culture of the wild-type and the regulatory mutants were added appropriately and incubated on a rotary flask shaker (180 rpm) at 37°C for 96hrs (Ahmed *et al*., 2013). The qualitative and quantitative analyses of the glutamic acid produced were carried out and results were recorded accordingly.

3.9 Parameters Optimized for L-glutamate Production

The following parameters were used in order to obtain the optimum conditions for L-glutamic acid production using the wild-type and mutant strains of *C. glutamicum*:

1. Substrate water ratio (1 to 5%) with incubation period of 96hrs.

2. Temperature (25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C).

3. Inoculum size (1%, 3%, 5% 7% and 9 %)

4. Initial pH (6.6, 6.8, 7.0, 7.2 and 7.4)

All the parameters of fermentation were used to determine the optimum conditions on micro-scale in 250 mL Erlenmeyer flasks having 63 mL of the total fermentation medium.

3.9.1 Effects of Substrate Concentration of Glutamic Acid Production

Acid and alkali pre-treated rice husk substrates were used to optimize different levels of substrate-water ratio with one to five percent (1-5%). The different concentrations were used to determine the best concentration that would produce the highest yield of L-glutamic acid. The fermentation medium contained all the basal nutrients as mentioned in basal medium. It was monitored after 96hrs for glutamic acid production.

3.9.2 Effects of Temperature on Glutamic Acid Production

To find the metabolically favorable temperature of *C. glutamicum* to optimally secrete glutamic acid, different degrees of temperature such as 25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C were studied. At the termination of the fermentation period, the produced glutamic acid was estimated to find the optimum temperature for maximum glutamic acid production.

3.9.3 Effects of pH on Glutamic Acid Production

Various ranges; 6.6, 6.8, 7.0, 7.2 and 7.4 of pH were used to determine the optimum pH for L-glutamate hyper production.

3.9.4 Effects of Inoculum Size on Glutamic Acid Production

The size of seed culture medium (inoculum) is a deciding parameter that utilize fermentation medium and confirm its conversion into required product completely. Different percentages of inoculum (1, 3, 5, 7 and 9%) were used to optimize the size of inoculum to get high titre of glutamic acid.

3.10 Analytical Methods for Glutamic Acid Detection

L-glutamate estimation

The fermented broth was autoclaved and cell mass was separated by centrifugation (10,000 rpm) for 10 minutes. Then qualitative and quantitative estimation of produced glutamate from the supernatant was done (Hassan *et al.*, 2003) by using the same protocols for qualitative and quantitative estimation of glutamic acid as in 3.6.2.2 and 3.6.2.3 respectively.

3.10.1 Identification of the Glutamic Acid Produced by Crystallization Technique

Fermented broth (10mL) collected from contents of shaking medium was centrifuged at 10,000 rpm for 10 min, and the supernatant was partially evaporated in a shaker water bath. The concentrated medium was then acidified to a pH of 3.22 which is the iso-electric point of glutamic acid using 1N HCl and allowed to stand still in a refrigerator until the glutamic acid crystals were precipitated there from (Ahmed *et al*., 2013).

3.10.2 Purity Determination

The Thin Layer Chromatographic (TLC) technique was used to detect the purity of the L-glutamate produced as described by Shagufta, (2014). Aliquot of 0.1g of the clear crystals obtained was dissolved in 10mL of distilled water and was used as the sample solution. Standard and test sample solutions were (5.0µl each) spotted on an aluminium TLC plate of 0.2mm thickness and developed with a mixture of n-propanol and strong ammonia: water (67:33) solution to the distance of about 10cm and dried in air. After spraying a solution of ninhydrin in acetone (0.5g of ninhydrin in 100mL acetone), the plate was dried at 65^{o}C for 5min to observe for the spots. Purity of the product was detected by the presence of only one purple spot of the same retention factor (RF) value of 0.3 as the standard L-glutamate (Hassan *et al*., 2003).

3.11 Statistical Analysis

Data processing was carried out and the results obtained from the optimization parameters were analyzed by comparison of means through paired t-test.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Compositions of the Acid Pre-treated and Alkali Pre-treated Rice Husk

Proximate analyses of both the acid and alkali-treated dried unfermented rice husk samples revealed higher ash content of 26.45% with the acid-treated rice husk while the alkali-treated rice husk had only 13.90%. However, higher carbohydrate content of 76.37% was observed with the alkali-treated rice husk and the acid-treated rice husk had 64.25%. Other compositions determined include moisture content, crude fat, crude protein content and crude fibre as shown in Table 4.1

4.2 Bacterial Strain Isolation and Identification

Eight isolates were identified; seven of the isolates were confirmed to be *C. glutamicum* based on cultural, microscopic as well as biochemical characterizations as shown in Table 4.2. The percentage occurrence of the *Corynebacterium glutamicum* isolates in various soil samples collected from Samaru Village, Zaria is presented in Table 4.3 with soil samples from flower bed having the least isolation rate of 20%, while soils from paddock, chicken pen and sheep pen had isolation rate of 100% each.

4.3 Screening for Glutamic Acid Production

The seven isolates were screened for L-glutamic acid production and were found to be capable of L-glutamic acid production which was identified qualitatively by paper chromatography, revealing the same retention factor (RF) of 0.3 as the standard L-glutamic acid calculated as follows;

$$RF = \frac{\text{Distance moved by the spots}}{\text{Distance moved by the solvent}} = \frac{4\text{cm}}{12\text{cm}} = 0.3$$

The *C. glutamicum* isolate from chiken-pen named as SFCD2 gave the maximum (0.25g/L) L-glutamic acid yield, with isolate from sheep-pen named as SFSD1 giving the least yield of 0.12g/L when determined spectrophotometrically at 570nm and using the standard curve (Table 4.4).

Table 4.1: Proximate Compositions of the Treated Rice Husk Substrates

Proximate Compositions (%)	**Sample Name**	
	Acid-treated rice husk	Alkali-treated rice husk
Moisture	3.55	4.80
Ash	26.45	13.90
CF	4.00	3.20
CP	1.75	1.73
CFb	46.55	51.65
CHO	64.25	76.37

KEY:

CF; Crude Fat, **CP**; Crude Protein, **CFb**; Crude Fibre, **CHO**; Carbohydrate

Table 4.2: Cultural, Microscopic and Biochemical Characteristics of the Isolates

Isolate's Code	Growth on LM	MHM	GRM	Biochemical Tests Mot.	Haem	Cat.	U	MR	VP	NR	GL	AH	Sugar Fermentation Glc	Gal	Fru	Suc	Ara	Mal	Lac	Inference
HLS1	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisades	-	γ	+	+	+	-	+	-	+	+	-	+	+	-	+	+	*C. glutamicum*
HLS2	Whitish-yellow colonies	Black non-mucoid colonies	Gram +ve cocci in clusters	-	β	+	-	+	-	+	+	±	+	-	+	+	-	+	+	*Staphylococcus* spp.
SFCD1	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisade	+	γ	+	-	+	-	-	-	+	+	-	+	-	-	±	±	*C. glutamicum*
SFCD2	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisade	-	γ	+	±	+		-	+	-	+	-	-	-	±	+	-	-	*C. glutamicum*
SFSD1	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisade	-	γ	+	+	+	-	-	-	+	+G	+	+	+	+	+	+	*C. glutamicum*
SFSD2	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisade	-	γ	+	+	+	-	-	-	+	+G	+	+	+	+	+	-	*C. glutamicum*
SFHD1	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisade	-	γ	+	+	+	-	-	-	+	+G	+	+	-	-	-	±	*C. glutamicum*
SFHD2	Whitish-yellow colonies	Black slightly mucoid colonies	Gram +ve palisade	-	γ	+	+	+	-	-	-	+	+G	+	-	±	-	-	±	*C. glutamicum*

KEY: LM; Loeffler's medium, **MHM**; Modified Hoyle's medium, **GRM**; Gram reaction and Morphology, **Mot;** Motility, **Haem;** Haemolysis, **Cat;** Catalase, **U;** Urease, **MR**; Methyl red, **VP**; Voges-Proskauer, **NR**; Nitrate Reduction, **GL**; Gelatin Liquefaction, **AH**; Aesculin Hydrolysis, **Glc**; Glucose, **Gal**; Galactose, **Fruc;** Fructose, Suc; Sucrose, **Ara**; Arabinose, **Mal**; Maltose, **Lac**; Lactose, **G**; Gas, **+**;Positive, **-**;Negative, **±**; Weakly positive, **β**; Beta, **γ**; Gamma

Table 4.3: Occurrence of *Corynebacterium glutamicum* in Various Soil Samples Collected from Samaru Village, Zaria

N= 11

Sampling Location	Number of Samples Collected	Number Positive (%)
Flower bed	5	1(20)
Paddock	2	2(100)
Chicken-pen	2	2(100)
Sheep-pen	2	2(100)

Key: N = Total number of samples collected

Table 4.4: Screening for L-Glutamic Acid Production by the *C. glutamicum*[a] Isolates

Soil Isolate's Code	Glutamic Acid Produced (g/L) [b]
HLS1	0.20
SFC D1	0.23
SFCD2[c]	**0.25**
SFSD1	0.12
SFSD2	0.21
SFHD1	0.19
SFHD2	0.23

Key:

[a]Shake flask fermentation at pH 7.0 , Temperature: 37^{o}C for 96h.

[b]Data are approximated to 2 decimal places

[c]Designation of the best L-Glutamic acid- producing isolate

4.4 Random Mutagenesis Using Nitrous Acid

Nitrous acid-induced mutant strain of the SFCD2 isolate was found to be phenotypically resistant to 4-fluoro-glutamic acid appearing whitish in colour as mucoid colonies as indicated on Plate I.

4.5 Determination of Optimum Conditions Affecting L-glutamic Acid Production by Wild- type Strain of *C. glutamicum*

4.5.1 Effect of Substrates Concentration on Glutamic Acid Production

Various concentrations (1 to 5%) of each of the pre-treated substrates were investigated and it was found that 4% (w/v) of the acid-treated rice husk gave the highest production of glutamic acid (6.37 g/L) after 96 hours of incubation. The alkali-treated rice husk, gave the highest glutamic acid yield of 4.30 g/L at 4% of its concentration after 96 hours of incubation as shown in Figure 4.1.

4.5.2 Effect of Temperature on Glutamic Acid Production

Different temperatures (25, 30, 35 & 40^{o}C) were used to determine the optimum temperature for L-glutamate production from wild *C. glutamicum* with 4% acid-treated and 4% alkali-treated rice husk using 5% inoculum. The maximum glutamate (10.1 g/L) was produced at 30^{o}C. With increasing temperature, a decrease of glutamate yield was found as shown in Figure 4.2

4.5.3 Effect of Inoculum Size on Glutamic Acid Production

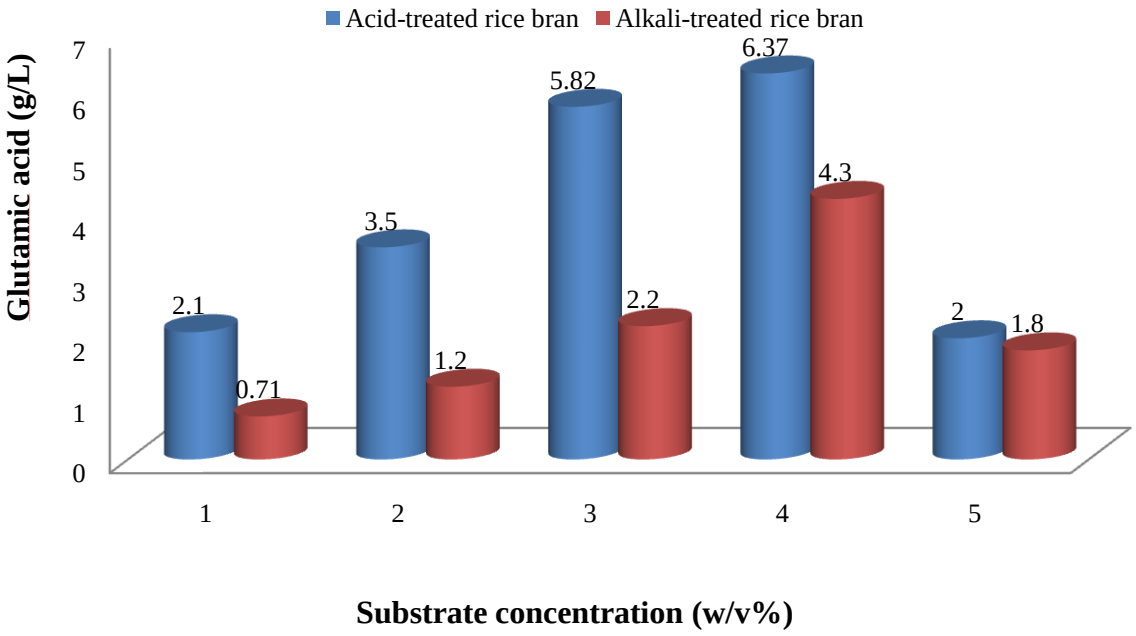
Different inoculum concentrations (1, 3, 5, 7 and 9%) were used to find the optimum level for maximum glutamate production using wild *C. glutamicum* with pre-optimized conditions. Inoculum size of 7% produced the maximum glutamic acid (10.4 g/L). Thereafter, increasing the concentration of the inoculum resulted in a decreased glutamic acid yield as shown in Figure 4.3

4.5.4 Effect of Initial pH on Glutamic Acid Production

The effects of various levels of initial pH (6.6, 6.8, 7.0, 7.2 & 7.4) were investigated by using 4% acid-treated, 4% of alkali-treated and 7% inoculum at 30^{o}C with wild *C. glutamicum* as fermenting agent. Glutamic acid production of 16.06 g/L and 9.64g/L were observed for acid-treated and alkali-treated rice husk respectively at pH 7.0. Further decrease in glutamic acid yield was found with increasing pH as shown in Figure 4.4



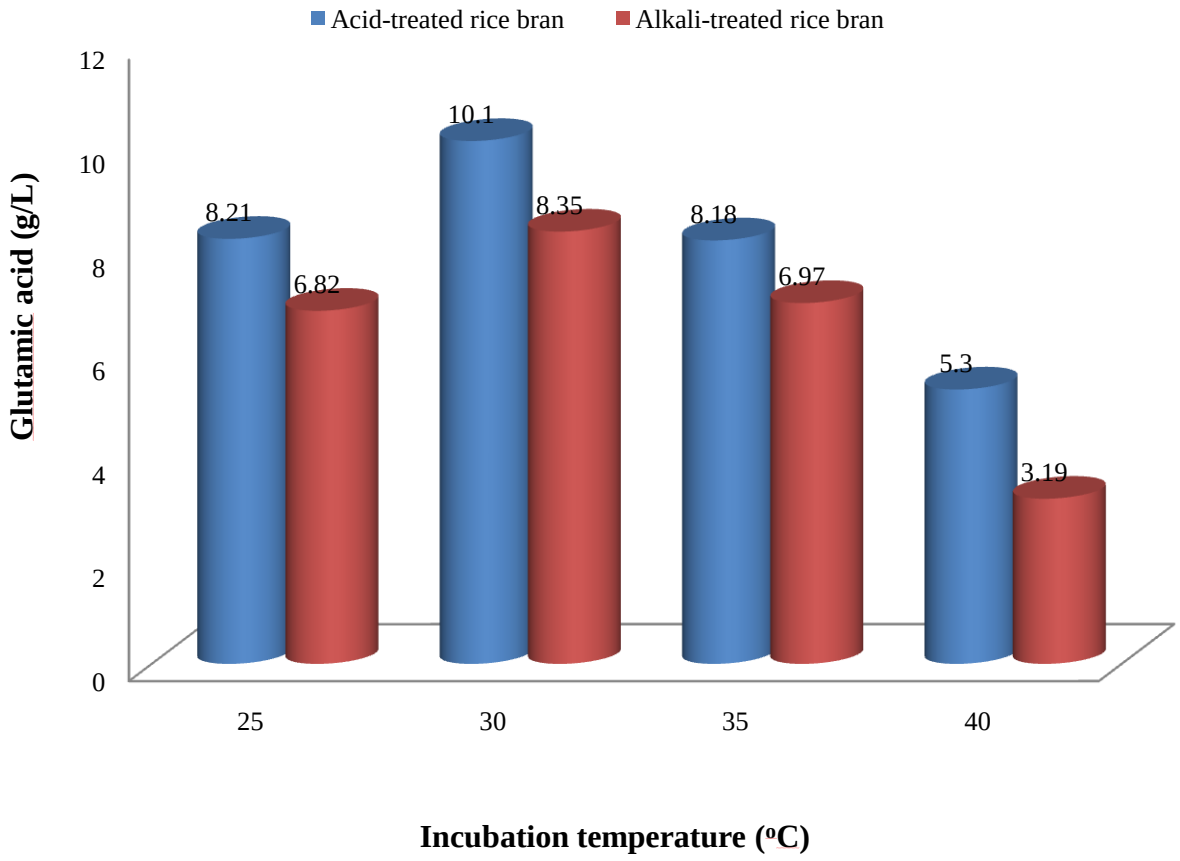
Plate I: Phenotypic Variations between the Wild-type (Left) and Regulatory mutant (Right) strains of *C. glutamicum*



Acid-treated rice husk: *p = 0.407* *df = 4* *t = -0.925*

Alkali-treated rice husk: *p = 0.183* *df = 4* *t = 1.607*

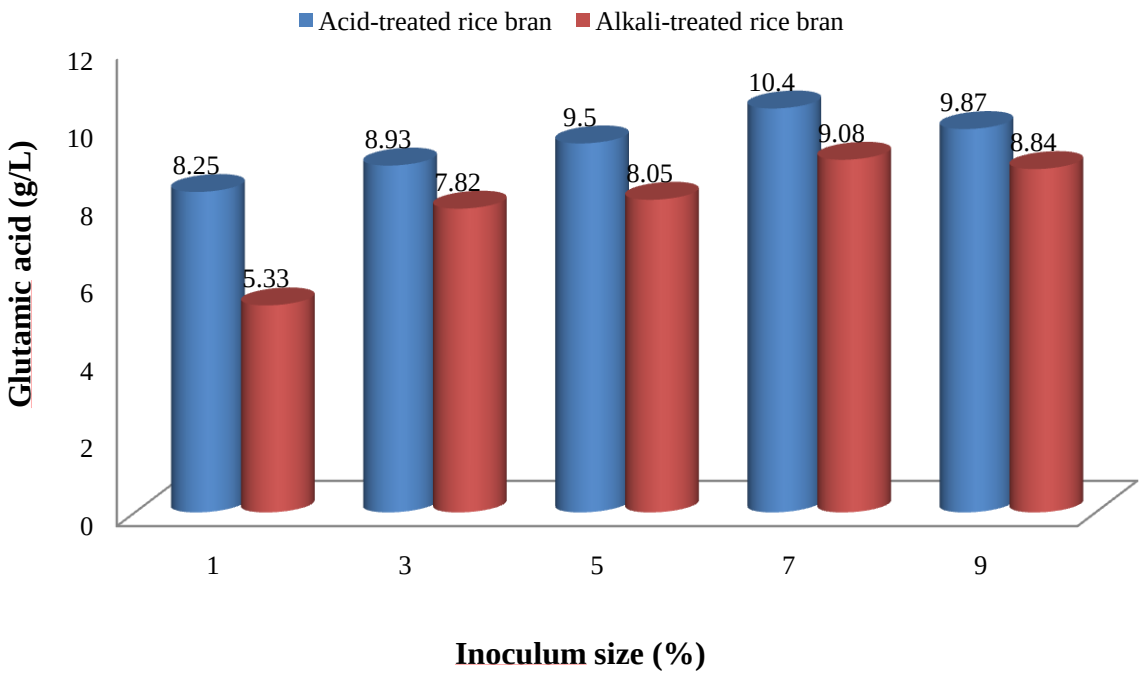
Figure 4.1: Influence of different substrates concentrations (w/v %) on glutamic acid production by wild-type *C. glutamicum*



Acid-treated rice husk: *p = 0.009* *df = 3* *t = 6.170*

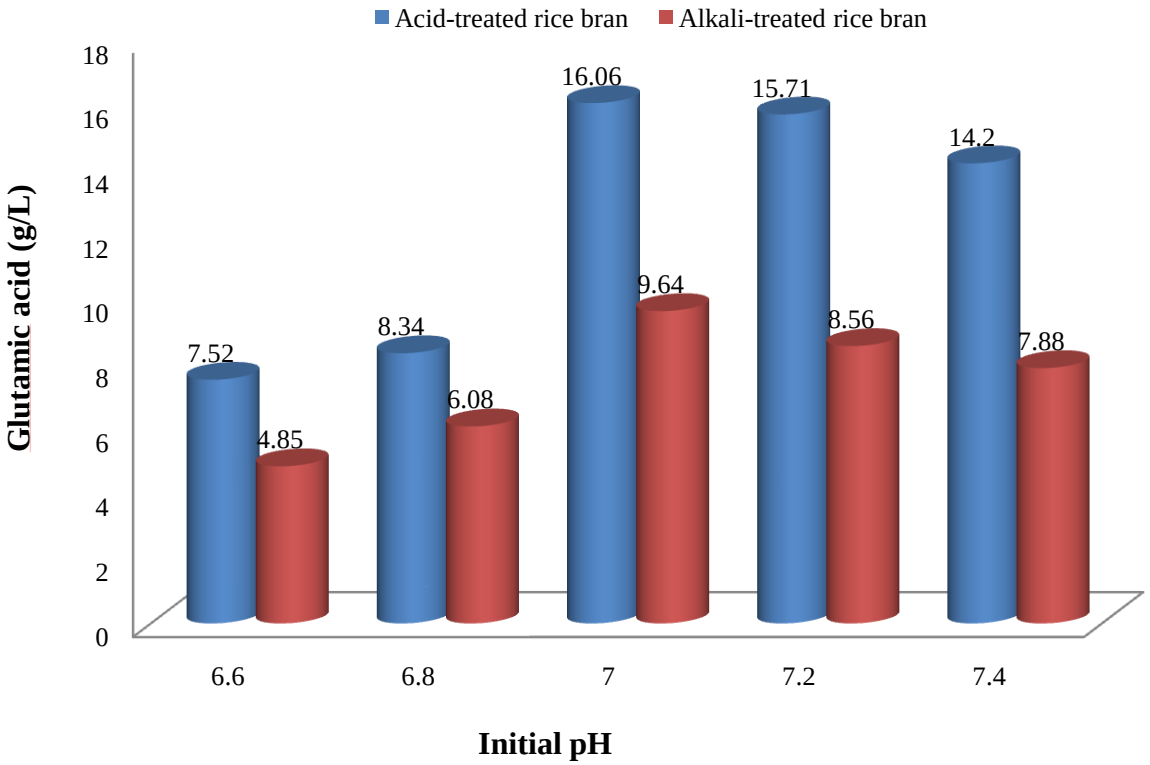
Alkali-treated rice husk: *p = 0.008* *df = 3* *t = 6.395*

Figure 4.2: Effect of incubation temperature on glutamic acid production by wild-type *C. glutamicum*



Acid-treated rice husk:	***p = 0.016***	***df = 4***	***t= -4.013***
Alkali-treated rice husk:	***p = 0.033***	***df = 4***	***t= -3.181***

Figure 4.3: Effect of inoculum size on glutamic acid production by wild-type *C. glutamicum*



Acid-treated rice husk:	***p = 0.036***	***df = 4***	***t = -3.098***
Alkali-treated rice husk:	***p = 0.629***	***df = 4***	***t = -0.523***

Figure 4.4: Effect of Initial pH on glutamic acid production by wild-type *C. glutamicum*

4.6 Optimization of Parameters for L-glutamic Acid Production by CG^{NTA} Mutant

4.6.1 Growth curve of mutant

The optical density (OD) at zero hour was 0.066 and there was rapid increase after 24h and the highest optical density of 2.225 was obtained after 48h. By increasing time from 48 to 56 hours, the OD of the growing organism started decreasing with an OD of 1.985. A similar decline was also observed as time increases but remains almost constant after 72 to 80 hours of incubation as presented in Figure 4.5

4.6.2 Effect of different concentrations of treated rice husk substrate for glutamic acid production by the mutant (CG^{NTA}).

Of all the various concentrations (1, 2, 3, 4, and 5%) of treated rice husk substrates tested, the highest yield of glutamic acid (25.35 g/L) was observed with 4% acid-treated rice husk, whereas, a lower yield (14.15g/L) was obtained with the same 4% of the alkali-treated rice husk. A decline of the glutamic acid titre was observed with increased substrate concentration as depicted in Figure 4.6

4.6.3 Influence of temperature on glutamic acid production by the mutant (CG^{NTA}).

For the various degrees of temperature (25, 30, 35 & $40^{o}C$) used for the fermentation of 4% treated rice husk by the mutant (CG^{NTA}) , maximum glutamic acid yield of 26.43g/L was obtained at $30^{o}C$ after 96 hours of incubation. A decrease in glutamic acid titre was observed with increasing temperature as shown in Figure 4.7

4.6.4: Effect of pH on glutamic acid production by the mutant (CG^{NTA}).

The highest glutamic acid yield of 20.18 g/L and 12.39g/L was observed respectively for acid-treated and alkali-treated rice husk at pH 7.0. A decrease of glutamic acid was seen with increasing pH value as shown in Figure 4.8 below;

4.6.5: Effect of inoculum size on glutamic acid production by the mutant (CG^{NTA}).

Out of all the inoculum concentrations (1, 3, 5, 7 and 9%) used for optimum glutamate production by CG^{NTA} under pre-optimized conditions, the highest glutamic acid concentration of 27.84 g/L was observed with 7% inoculum from 18 hours culture broth and a decrease of glutamic acid titre was noticed as the inoculum size increased from 7 to 9% as represented in Figure 4.9

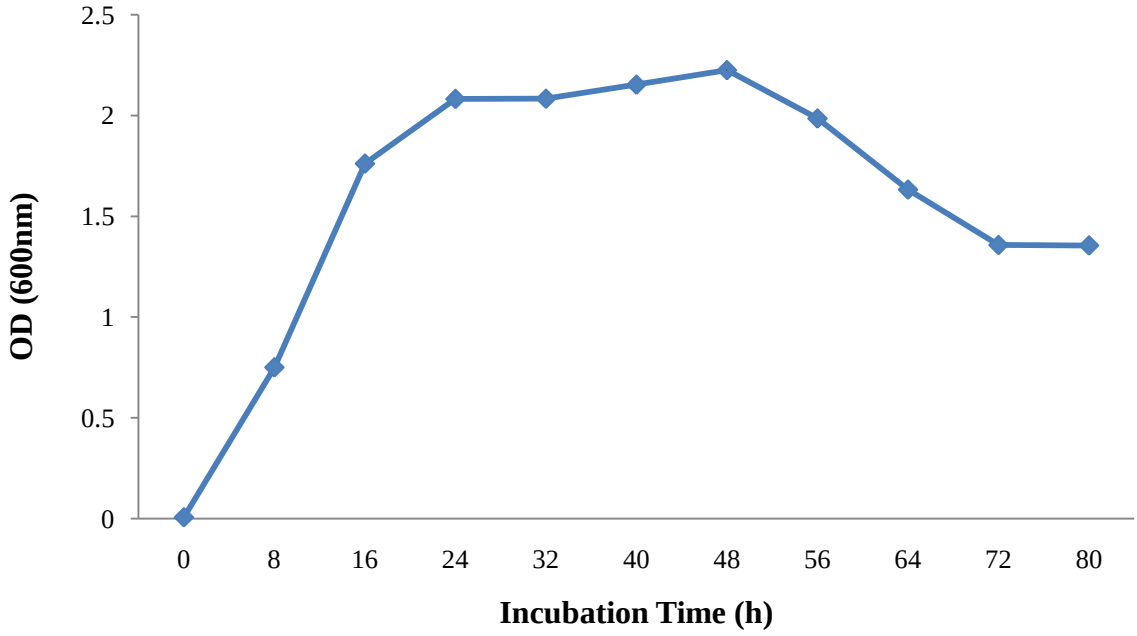
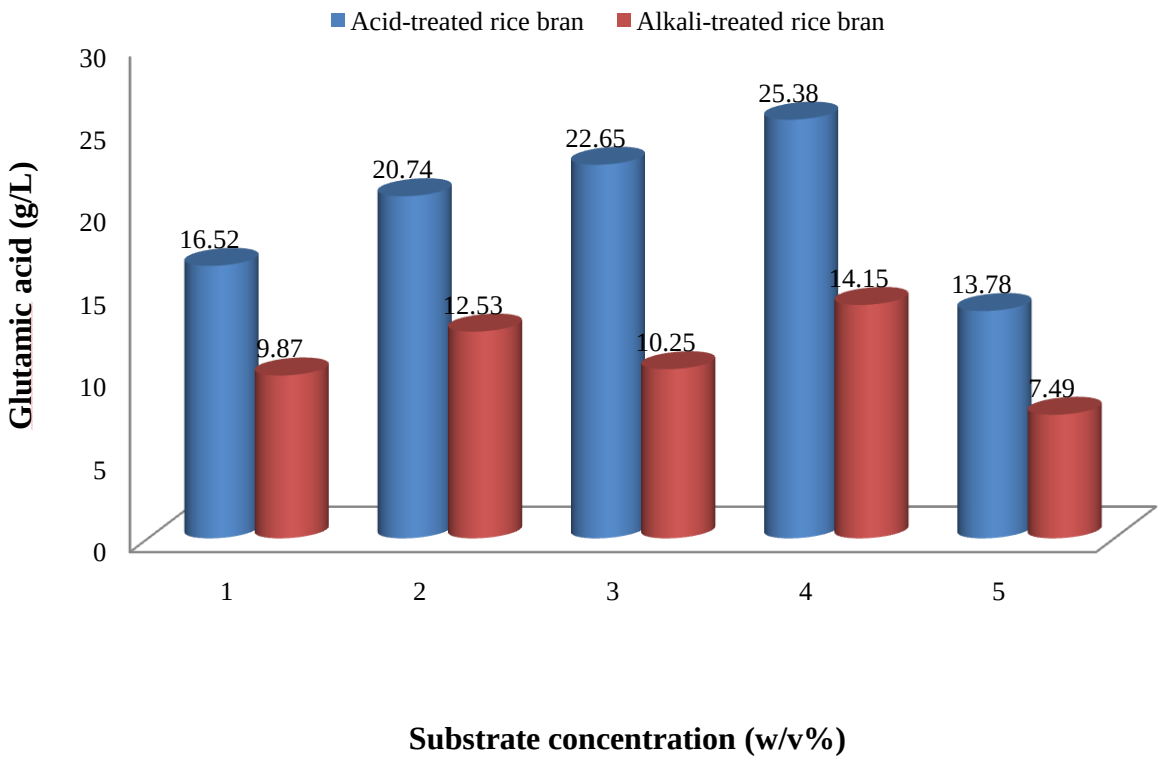


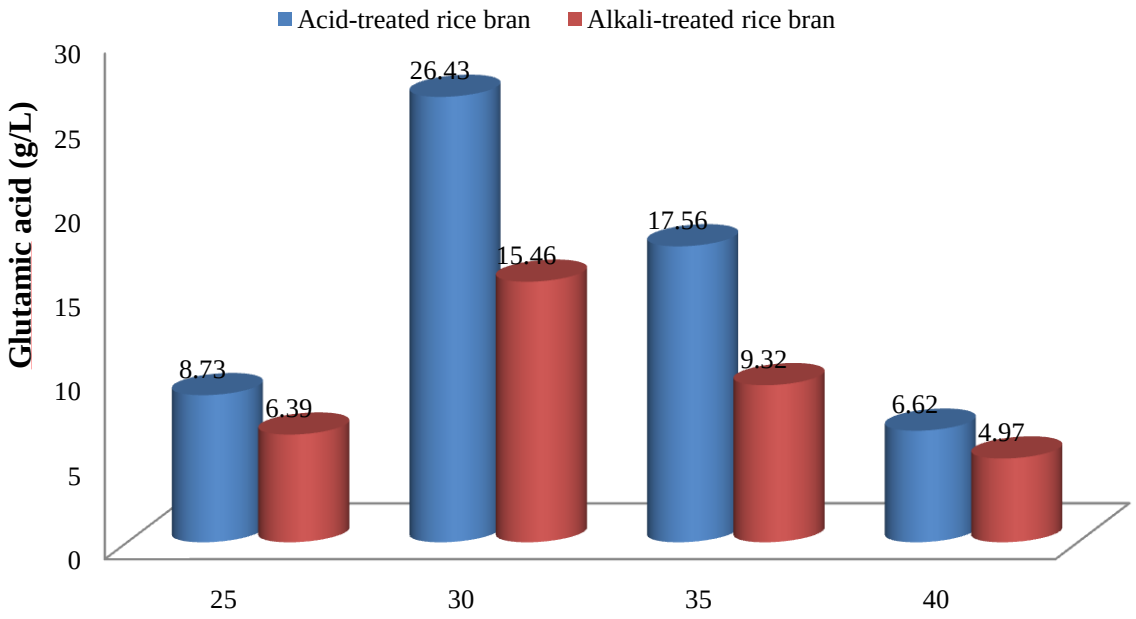
Figure 4.5: Trend of mutant (CG^{NTA}) growth observed at 600 nm after every 8hours



Acid-treated rice husk: *p = 0.002* *df = 4* *t = -7.564*

Alkali-treated rice husk: *p = 0.008* *df = 4* *t = -4.950*

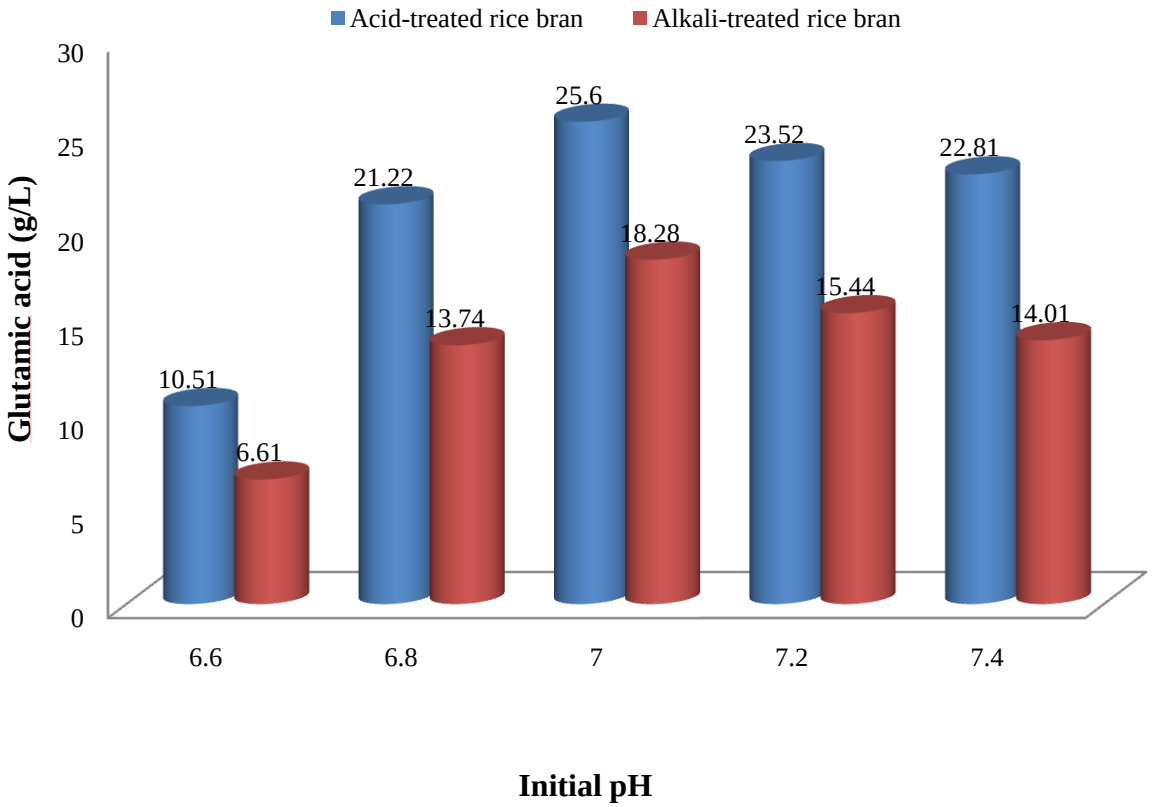
Figure 4.6: Effect of different concentrations of treated rice husk for the production of glutamic acid by the mutant (CG^{NTA})at 30^{o}C and 96 hours of incubation time



Acid-treated rice husk: *p = 0.063* *df = 3* *t = 2.892*

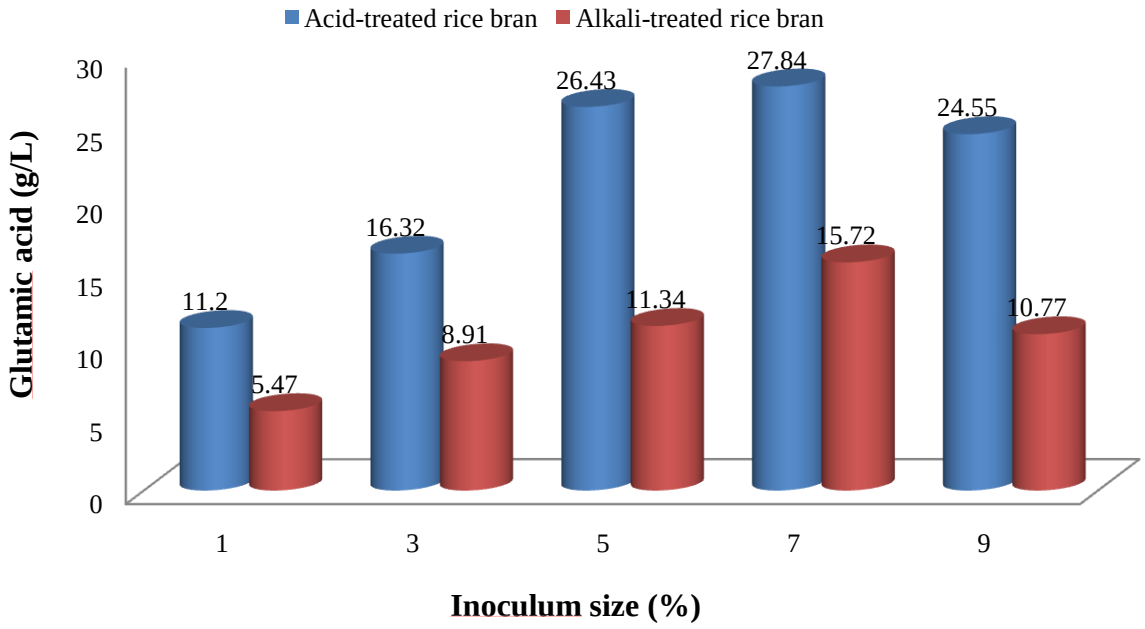
Alkali-treated rice husk: *p = 0.014* *df = 3* *t = 5.226*

Figure 4.7: Effect of different temperatures on the production of glutamic acid with 4% substrate and 5%inoculum by the mutant (CG^{NTA}) for 96 hours.



Acid-treated rice husk:	*p = 0.006*	*df = 4*	*t = -5.383*
Alkali-treated rice husk:	*p = 0.023*	*df = 4*	*t = -3.584*

Figure 4.8: Effect of initial pH for the production of glutamic acid with 4% substrate by the mutant (CG^{NTA}) at 30°C, 5% inoculum and 96 hours of incubation time.



Acid-treated rice husk:	*p = 0.002*	*df = 4*	*t = - 7.521*
Alkali-treated rice husk:	*p = 0.009*	*df = 4*	*t = - 4.755*

Figure 4.9: Influence of various concentrations of inoculum for enhanced glutamic acid production by the mutant (CG^{NTA}) with 4% substrate for 96 hours of fermentation at 30°C and pH 7.

4.7. Comparative Production of Glutamic Acid by the Mutant and Wild-Type Strains of *C. glutamicum* Under Optimum Parameters

Table 4.4 shows the yields of L-Glutamic acid (g/L) obtained from the acid-treated rice husk by the mutant and wild type strains of *C. glutamicum* under optimum conditions. The mutant strain showed an improved glutamic acid production under all the conditions as compared to the wild type.

Table 4.5 shows the yields of L-Glutamic acid (g/L) obtained from the alkali-treated rice husk by the mutant and wild type strains of *C. glutamicum* under optimum conditions. The mutant strain showed an improved glutamic acid production under all the conditions as compared to the wild type.

4.8. Purity Determination of the Produced L-Glutamic Acid

The spots developed on TLC plate for purity determination of glutamic acid produced are shown on Plate II. The produced glutamic acid labelled S1 had a retention factor of 0.27, a value that is only slightly different from the standard L-glutamic acid having an RF value of 0.3.

Table 4.5: Comparative Glutamic Acid Production (g/L) by Mutant and Wild Strains of *C. gutamicum* from Acid-treated Rice Husk Hydrolysates under Optimum Fermentation Conditions

Parameter (Optimum value)	**Wild Type A**	**Mutant (CG^{NTA}) B**	***Fold-increment[a]**
Substrate concentration (4%)	6.37	25.38	3.98
Incubation temperature ($30^{o}C$)	10.10	26.43	2.62
Initial pH (7.0)	16.06	25.60	1.59
Inoculum size (7%)	**10.40**	**27.84**	**2.68**

[a]Data are approximated to 2 decimal places

***Fold-increment = B/A**

Table 4.6: Comparative Glutamic Acid Production (g/L) by Mutant and Wild Strains of *C. gutamicum* from Alkali-treated Rice Husk Hydrolysates under Optimum Fermentation Conditions

Parameter (Optimum value)	**Wild-Type A**	**Mutant (CG^{NTA}) B**	***Fold-increment[a]**
Substrate concentration (4%)	4.30	14.15	3.29
Incubation temperature (30^{o}C)	8.35	15.46	1.85
Initial pH (7.0)	9.64	18.28	1.89
Inoculum size (7%)	**9.08**	**15.72**	**1.73**

[a]Data are approximated to 2 decimal places

***Fold-increment = B/A**

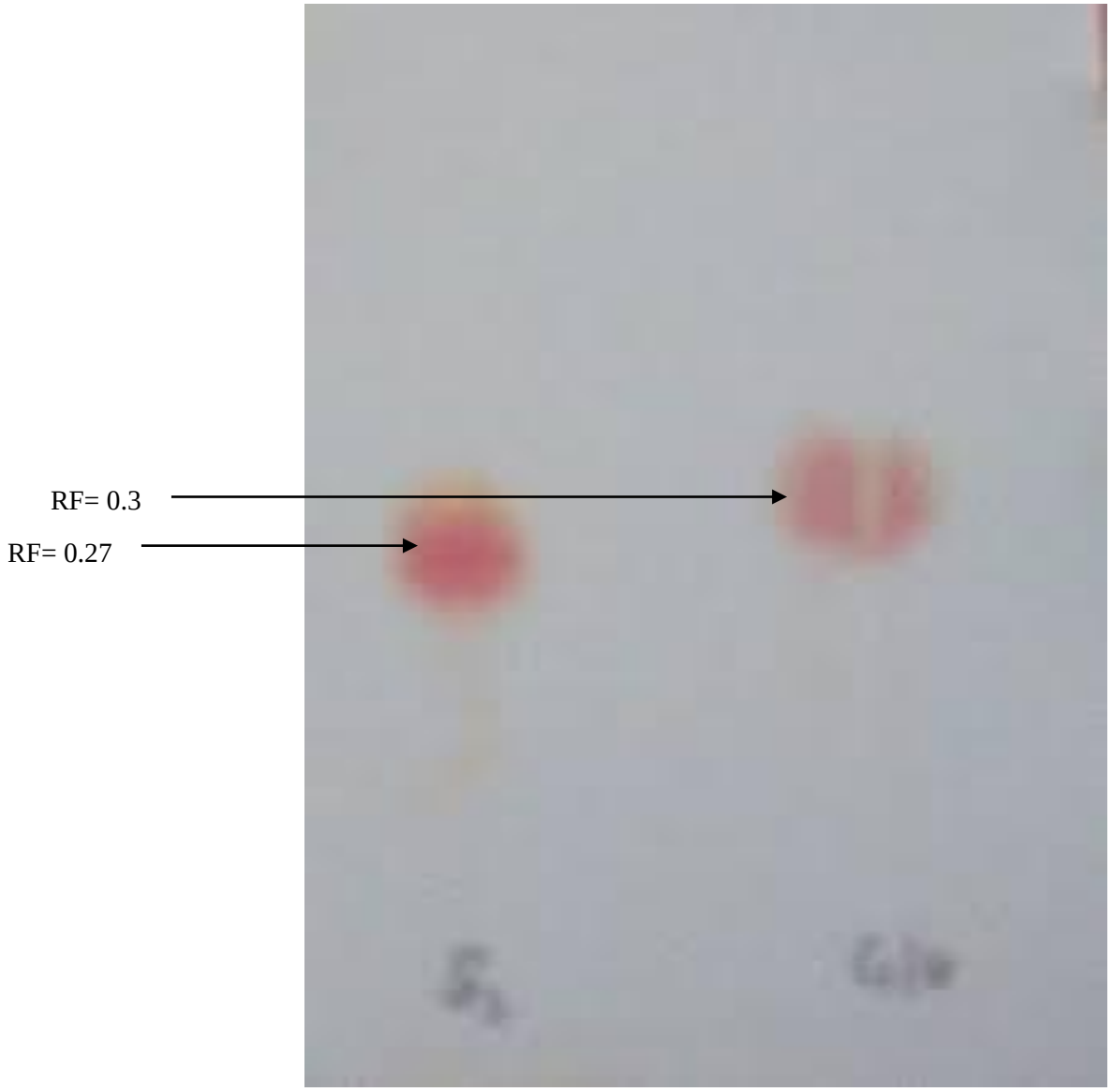


Plate II: Spots of Glutamic acids on TLC plate for purity determination

Key: Glu = Standard Glutamic Acid

S1 = Test Fermentation Product

CHAPTER FIVE

5.0 DISCUSSION

Pre-treatment of the substrate was carried out in this research and glutamic acid produced from this substrate by both the wild type and mutant strains of *C. glutamicum* was found to be higher with the acid-treated substrate (10.40g/L and 27.84g/L respectively) than with the alkali-treated substrate (9.08g/L and 15.72g/L respectively). This might be due to the higher percentages of ash (26.4%) and protein (1.75%) contents observed in the acid-treated hydrolysate after proximate analysis. This corroborates with the findings of Damisa *et al*. (2008) who reported the highest ash content (7.02%) from acid-treated corn straw when he tested different lignocellulosic materials for cellulase production. Although, a lower carbohydrate (CHO) content (64.25%) was recorded for the acid-treated hydrolysate than with the alkali-treated hydrolysate (76.37%), but the former presented to be a better candidate for glutamate production by virtue of its high ash (mineral salts) and protein (nitrogen source) contents that are required by *C. glutamicum* for growth and glutamate production. The higher amount of sugar observed in alkali-treated hydrolysate might be as a result of higher delignification potential possess by alkali, the mechanism of which is believed to be the saponification of intermolecular ester bonds cross-linking lignin and hemicelluloses as well as opening of the crystalline structure of cellulose more efficiently than acid so that an increased number of reactive sites available for enzyme activity is achieved. This may be accountable based on the fact that acid pretreatment of residues usually does not remove lignin from the substrate, but only modifies the lignin-CHO linkage as reported by Damisa *et al*. (2008). This also is in agreement with the work of Houghton *et al*. (2006) who stated that acidic treatment of substrate may even permit cellulose to re-anneal, leading to horrification of cellulose in to microfibrils

instead. Similar results on the lower sugar contents of acid-treated hydrolysate were reported by Narasimha *et al*. (2011) who reported a higher (4.973mg/g) content of sugar from KOH-treated hydrolysate than that obtained from H_2SO_4-treated rice husk hydrolysate (3.410mg/g) with respective 73.26% and 54.50% removal of lignin. Rakesh *et al*. (2013) also reported that there was about 63.6% delignification by KOH.

Overall, the recalcitrant nature of the lignocellulosic substrate in fact becomes impediment to its industrial exploitation, thus, pretreatment are needed to increase the surface area of cellulose by removing the lignin seal, solubilizing the hemicelluloses, disrupting crystallinity and increasing the pore volume and then making the substrate amenable for enzymatic hydrolysis or microbial biodegradation for improved glutamic acid production.

In this study, a total of eleven (11) soil samples from four different locations were used for the isolation of *C. glutamicum*, of which a prevalence rate of (100%) was obtained with soil from paddock, chicken-pen and sheep-pen whereas soil from flower bed had the least prevalence of 20%. This might be due to the richness in the nutritional composition of the humic soil from the animal houses, whereas, the flower bed might have little organic matter as nutrient to the organism. This agrees with the findings of Zahoor *et al*. (2012) who reported a higher isolation rate (15.6%) from organically-rich soil than from nutrient poor soil with 5.74%. Out of seven (7) isolates confirmed to be *C. glutamicum* and screened for L-glutamic acid production, the isolate from chicken-pen (SFCD2) produced the highest quantity of glutamic acid (0.25g/L) whereas, the lowest glutamic acid production (0.12g/L) was observed with isolate named SFSD2 from sheep-pen. This might be due to the variability and adaptability in terms of the nutritional diversity of *C. glutamicum* as it is not unconnected with the nature of the environment from which they were isolated. Glutamic acid concentration obtained with

SFCD2 in this study, is much lower than that obtained by Hadia *et al*. (2012) who reported a concentration of 1.5g/L after screening. This difference may be accounted for by the higher biotin concentration (200µg) used in this study as opposed to the lower concentration of 50µg used by Hadia *et al*. (2012). This is likely because the activity of 2-oxoglutarate dehydrogenase complex (ODHC) reportedly decreases during L-glutamate production in response to biotin limitation as reported by Nakamura *et al*. (2007). Since ODHC links the tricarboxylic acid (TCA) cycle and L-glutamate biosynthesis, a decrease in ODHC activity could switch the metabolic flow from the TCA cycle to L-glutamate synthesis.

The best glutamic acid producer (SFCD2) was observed to possess some novel characteristics such as white colonies characterized by increased viscosity and resistance to 4-Fluoroglutamic acid (4FGA) - a toxic analogue of L-glutamic acid, all of which were not observed with the wild type strain. These differences may be due to disruption of the likely present pigment-producing gene in the mutant strain which may account for the variation in coloration and increased secretion of complex polysaccharides which may be connected with the increased viscosity observed. While the phenotypic resistance to 4FGA might be due to mutation of the NCgl1221 gene which codes for an L-glutamate exporter, reducing the entry of 4FGA into the mutant cell and hence become resistant to the 4FGA and ultimately to feedback inhibition. Thus these characteristics result in hypersecretion of glutamic acid as explained by Nakamura *et al*. (2007). This corroborates with the findings of Paisrisan *et al*. (2013) who reported a remarkable phenotypic variation between the mutant and the wild type strain of *C. glutamicum*.

Substrate is the most important and basic requirement for microbial biosynthesis. A cheap substrate with multiple nutrients is ideal for industrial biotechnological processes.

Acid-treated rice husk hydrolysate proved to be a better substrate used in present study as a good carbon source. Out of the various concentrations of both acid-treated and alkali-treated hydrolysates used in this study, 4% was found to be the optimum for the production of glutamic acid using the wild type and mutant strains of *C. glutamicum*. At 4%, the yield of glutamate produced by CG^{NTA} from acid and alkali-treated hydrolysate (25.38g/L and14.15g/L respectively) was found to be significantly higher ($P< 0.05$) than that produced from other concentrations. Whereas, the difference between the yield obtained at 4% and other concentrations with the wild-type from acid-treated hydrolysate (6.37g/L) and alkali treated hydrolysate (4.30g/L) was not statistically significant (p□ 0.05). The observed variations in the amount of glutamate produced with substrate concentrations could be explained by the fact that at lower concentration (higher water to substrate ratio), the medium might be too diluted and therefore contained relatively very low nutrients where the organism had to employ energy from the hydrolysis of ATP in order to take up the nutrients by active transport mechanism. Similarly, at higher concentrations of the substrate (higher substrate to water ratio), characterized by low water activity (a_w), the fermentation medium might become hypertonic to the fermenting organism, thereby causing the cell to shrink leading to cell death and eventually reduces the inoculum size that ultimately leads to low glutamic acid production. Hence an average concentration is needed for optimum glutamate production. The results of these findings are therefore arguing in favour of the works carried out by Chen *et al*. (2008) and Shagufta *et al*. (2014).

Metabolic activity of an organism is greatly affected by change in temperature. Therefore to optimize suitable temperature, fermentation was carried out with 4% treated hydrolysates as substrates for 96 hours of incubation with various ranges of temperature. The results showed that glutamate production was highest at 30^oC

compared to all other temperatures. Further increase in temperature resulted in decreased glutamate production. At 30^{o}C, the yield of glutamate produced by CG^{NTA} from acid and alkali-treated hydrolysate (26.43g/L and15.46g/L respectively) and the yields obtained with the wild-type from acid-treated hydrolysate (10.10g/L) and alkali-treated hydrolysate (8.35g/L) at 30^{o}C were all found to be significantly higher ($P< 0.05$) than those obtained at other temperatures. This is because cardinality of temperature plays a very important role in the growth and metabolism of an organism. Thus, growth and metabolic functions at extreme temperatures are greatly inhibited and sometimes become almost impossible. This is simply because; at low temperatures, enzymes get inactivated while membrane lipoproteins which are important in glutamate excretion become hardened. Whereas, at extreme temperatures, enzymes and membrane proteins get denatured and therefore loss their activities. The results of present study are in line with the work of Sthiannopkao *et al*. (2001), who observed a maximum glutamate production at 30^{o}C during the study with three strains of coryneform bacteria as fermenting agents. The results of Shah *et al*. (2002); Nelofer *et al*. (2008) and Rao *et al*. (2011), showed that at 30^{o} C, the metabolism of coryneform bacteria are highly active for the production of enzymes and amino acids. Temperature used by Javaid *et al*. (2012) was 32^{o}C with mutated strain of *B. flavum* using glucose (3%), 8% inoculum size and pH of 7.5 and reported glutamate production of about 17.8g/L in shake flask. Naz *et al.* (2001) reported a maximum glutamate (21.48 g/L) production by *C. glutamicum* at 37^{o}C, in contrast to present findings. This is also similar to the work of Pasha *et al*. (2011) who reported that the effect of temperature shift-up from 30 to 39°C through incubation, the mutant strain and wild strain show that the specific production rate of glutamic acid at 30^{o}C increased apparently by 2 and 1.5 fold respectively on average from other temperatures. Contrary results were obtained by Choi *et al*. (2004)

who reported enhanced glutamic acid production by *Brevibacterium* sp. with temperature shift-up from 30 to 38°C. The mutant produced higher glutamate than their wild type bacterial strains at a temperature of 30^{o}C. This disagrees with the work of Ahmed *et al.* (2013) who reported that at temperature above 30^{o}C, the glutamate was actively produced, meanwhile the activities of ODHC and pyruvate dehydrogenase (PDH) were found to have 35% reduced activity.

The growth of bacteria is sensitive to changes in pH which also influences membrane potential for secretion of glutamic acid (Shagufta *et al*., 2014). Therefore, to get the highest growth with good secretion of glutamate, various ranges (6.6, 6.8, 7.0, 7.2 & 7.4) of initial pH of fermentation medium were investigated to get optimum titre of glutamic acid. At pH 7.0 , the yield of glutamate produced by CG^{NTA} from acid and alkali-treated hydrolysate (25.60g/L and 18.28g/L respectively) and the yields obtained with the wild-type from acid-treated hydrolysate (16.06g/L) at pH 7.0 were all found to be significantly higher ($P< 0.05$) than those obtained at other pH, whereas, the yield obtained at the same pH of 7.0 with alkali-treated hydrolysate (9.64g/L) was not statistically significant ($P = 0.629$) as shown in Figure 4.4 and 4.8 respectively. The reason might be due to the physiological nature of the pH 7.0 at which best metabolic functions are carried out. Extreme pH of the fermentation media might have negatively affected the membrane stability (membrane fluidity), enzymatic activities as well as transport of nutrients for growth and development, hence low yield of glutamate recorded. The results of present studies are in line with that of Javed *et al.* (2011) who reported a maximum glutamate production of 38.5 g/L at pH 7 in growth medium as compared to 28.3, 25.7 and 14.6 g/L glutamate at pH 6.5, 7.5 and 8.0 respectively. Similar results were reported by Sattar *et al.* (2008) who obtained high yield of glutamate (27mg/ 100mL) at pH 7 after 48 hours of incubation. The results of Naz *et al.*

(2001) were also in agreement with the present findings. While Rehman *et al.* (2012) obtained 12.5 g/L of glutamate at optimum value of pH 7.6 after 60 hours of incubation.

Vegetative growth of bacterium may be promoted at the cost of glutamate production due to competition for available nutrient (Reddy *et al*., 2008). Therefore, a number of inoculum concentrations (1, 3, 5, 7 and 9%) were used to find the optimum inoculum size for hyper production of glutamate from mutant and wild type *C. glutamicum* with 4% acid-treated and alkali-treated rice husk hydrolysates as substrate for 96hours of fermentation period at 30^{o}C and pH 7.0. The trend of glutamic acid production by the CG^{NTA} showed that significantly ($P< 0.05$) higher titres (27.84 g/L and 15.72g/L) of glutamic acid and by the wild-type (10.10g/L and 8.35g/L) were obtained with 7% of inoculum from acid and alkali-treated substrate respectively as shown in Figure 4.3 and 4.9. This might not be unconnected with the density-dependent bacterial communication (Quorum sensing) based on which metabolic functions of a microbial entity are determined. This result is in agreement with that of Adnan *et al.* (2011). Javaid *et al.* (2012) and Rehman *et al.* (2012) reported maximum glutamic acid production at 8% and 10% inoculum size respectively.

In the present study, the total amount of glutamic acid produced at optimum conditions from acid-treated and alkali-treated rice husk hydrolysates by CN^{NTA} were 27.84 g/L and 15.72g/L respectively. Whereas, 10.40g/L and 9.08g/L were respectively produced by the wild type strain of *C. glutamicum* from acid-treated and alkali-treated rice husk hydrolysates. This shows that agricultural wastes such as rice husk are a good substrate for the production of glutamic acid. The results agrees with the findings of Asakura *et al*. (2007) who reported that 17.8g/L of glutamate was produced from 15g/L of barley through heterogenous expression of *Corynebacterium thermocellum endoglucanase* in *C. glutamicum* suggesting that direct utilization of cellulosic materials for glutamate

production is possible. Yugandhar *et al*. (2007) also revealed that a maximum yield of L-glutamic acid was obtained with *C. glutamicum* free cells under optimum parameters. However, Amin and Al-tahi.(2007) reported that only 73g/L of glutamate was produced by *C. glutamicum* ATCC 13022 from 75.7% of rice husk hydrolysate. Maximum of 39.32mg/ml glutamate was reported to be produced by *C. glutamicum* CECT 690 strain from date waste juice as reported by Tavakkoli (2009).

The purified glutamic acid produced was found to appear as a spot with a retention factor (RF) of 0.27 which is nearly the same as 0.3 of the standard L-Glutamic acid. The slight difference observed in the RF values might be due to the difference in the efficiency of the purification methods used.

This research therefore presents the potentials of acid-treated rice husk in the production of L-glutamate under optimized conditions from which some economic and environmental benefits may be derived.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The overall conclusions that could be drawn from the present study are;

That the proximate compositions of the acid and alkali-treated rice husk were found to be different as determined in the study.

That *C. glutamicum* was isolated from all the sampling locations but with different rates of occurrence.

All the *C. glutamicum* isolates were found to have potential for L-glutamate production after screening.

That a strain of *C. glutamicum* (CG^{NTA}), a regulatory mutant resistant to 4-fluoroglutamic acid was developed which produced L-glutamic acid in significant quantity.

That a high yield of L-glutamic acid was obtained through submerged fermentation.

That the yield of L-glutamic acid could be controlled by various optimization conditions such as the temperature, substrate concentration, pH and inoculum size.

That a highly efficient method of rice husk pre-treatment for efficient and reliable glutamic acid production was achieved.

That the use of locally available and nutrient-rich raw material (like rice husk) as organic substrate for glutamic acid production is possible and would help in clearing the environment of the waste.

6.2 Recommendations

1. Molecular analyses such as genome sequencing should be carried out on the mutants and wild type strains of *C. glutamicum* as this will be of great industrial biotechnological importance.
2. Other organisms as well as other wastes of animal or agricultural origin should also be tried for their potentials to serve respectively as fermenting agents and substrates for glutamic acid production.
3. Rapid and inexpensive recovery and purification methods for glutamic acid so produced should be established to save time, increase recovery rate and purity.
4. Nigerian government should encourage the establishment of local industries for glutamic acid production from this substrate so as to reduce importation as well as helping to reduce unemployment.

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