ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

PRODUCTION OF GLUCONIC ACID FROM CANE MOLASSES THROUGH SUBMERGED FERMENTATION USING *ASPERGILLUS CARNEUS*.

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Production of gluconic acid from cane molasses through submerged fermentation using *Aspergillus Carneus*.

A thesis presented to the research and graduate school of Addis Ababa university, Addis Ababa institute of technology, school of chemical and bio engineering in partial fulfillment of the requirements for the award of a master of science in chemical and bio engineering under process engineering stream.

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Declaration

I declare that this thesis work entitled “PRODUCTION OF GLUCONIC ACID FROM CANE MOLASSES THROUGH SUBMERGED FERMENTATION USING *ASPERGILLUS CARNEUs”* is my own work and has not been presented in any form for another degree, diploma or an award at any university or other institution of the tertiary education. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and discussions. Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given. This thesis work has been done under the guidance of Dr. ing ANURADHA.J (Associate Professor) instructor in Addis Ababa University, School of Chemical and Bio Engineering.

Name: FENTAHUN TAREKEGN

Signature: _____

Date of Submission: June, 2018

Dedication

When I started MSc I had one strength with me that Initiates and motivates to do more. But now I lost that memorable initiation, since I lost my father last year.

This work is dedicated to my father for all your motivation and your strength throughout your life. Please come back and see my success!!! I always say RIP!!! You are my best!!!

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Above all, I thank Almighty God for always being with me in all my endeavors and giving me a chance and endurance to complete my research.

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ABSTRACT

Gluconic acid is a multifunctional organic acid which has increasing interest in different application because of its non-corrosiveness, non-toxicity, readily biodegradability and environmentally friendly properties. This study involves gluconic acid production and investigation of different parameters on the yield of gluconic acid. Gluconic acid was produced from treated molasses by using *Aspergillus carneus* strain under submerged fermentation. To reduce the inhibition of heavy metals and inorganic salts in crude molasses, it requires being pretreated. Gluconic acid production was achieved by three subsequent steps such as pretreatment, fermentation and separation with characterization. To investigate the effect of variables, four factors with three levels Box -Bhenken Design expert 7.0.0 trial was used. Chemical characterization of gluconic acid was examined by Fourier transform infrared spectroscopy analysis. Results of Box -Behnken Design indicates that the optimum parameter were temperature 30℃, Carbon source concentration 250.00g/L, pH 7 and incubation time 6.86 days to produce maximum yield of gluconic acid 61.96%. Fourier transform infrared spectroscopy analysis identifies and confirms the presence of gluconic acid in the product. Results showed that use of treated molasses for production of maximum yield of gluconic acid through submerged fermentation by using *Aspergillus carneus* were valuable, feasible and waste minimal.

Keywords*: Molasses; Aspergillus Carneus; Submerged Fermentation; Glucose Oxidase; Gluconic Acid*

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ACRONYMS

ANOVA	Analysis Of Variance
BBD	Box -Bhenken Design
CIF	Cost Insurance and Freight
CM	Crude Molasses
DF	Degree of Freedom
EDTA	Ethylene Diamine Tetra Acetic acid
ES	Ethiopian Standard
ETB	Ethiopian Birr
FT-IR	Fourier Transform Infrared Spectroscopy
GA	Gluconic Acid
GdL	Glucono-delta Lactone
GOD	Glucose oxidase
ICUMSA	International Commission Uniform Methods of Sugar Analysis
IUPAC	International Union Pure And Applied Chemistry
NIST	National institute of standards and technology
RSM	Response Surface Methodology
SMF	Submerged Fermentation
SSF	Solid State Fermentation
TM	Treated Molasses
TRS	Total Reducing Sugar

CHAPTER ONE

INTRODUCTION

1.1. Background

Fungal biotechnology is one of the excellent commercial production processes of organic acids (Idnurm & Meyer, 2014). Fungal secondary metabolites are extremely important to our health and nutrition and have tremendous economic impact. However, unlike penicillin, the organic acids produced by other fungi species have had a little visible impact on human well-being. Indeed, organic acid fermentations are often not even identified as fungal bioprocesses, having been overshadowed by the successful placement of the chemical synthesis (Adrio & Demain, 2003). Yet, in terms of productivity, fungal organic acid processes may be the best examples of all (Magnuson & Lasure, 2004). Hence, fungal spores are known to be able to act as biocatalysts and can often perform the same reactions as the corresponding strain(Gbnie et.al, 1996).

Gluconic acid (also known as hexanoic acid, penta-hydroxy-caproic acid, with IUPAC designation is 2,3,4,5,6-penta hydroxyl hexanoic acid)is the simple oxidation product of glucose and a mild organic acid that has gained much interest in biotechnology(Abdul Qadir et.al, 2012), due to its varied and wide applications in industries such as in the pharmaceutical, food, animal feed, and leather and textile industries(Ahmed et.al, 2015). It is also applied for the solubilisation of mineral phosphates(Stella & Halimi, 2015), decontamination agent(Zepedal et al., 1994),scale removal in metal cleaning and metal finishing and as additive in cement to control the setting time and increase strength and water resistance(Walford & du Boil, 2006). Gluconic acid is a noncorrosive, nonvolatile, nontoxic mild organic acid so it imports as a refreshing sour taste in many food items such as wine, fruit juices. Since gluconic acid is listed as a generally permitted food additive. Gluconic acid abundantly available in fruit, honey, wine and other food staffs(Ramachandran et.al, 2006). As a food additive (E574), it is an acidity regulator. It is also used in cleaning products, where it dissolves mineral deposits, especially in alkaline solution(Jiménez-hornero et al., 2016).

There are many approaches for production of gluconic acid such as chemical, electrochemical, biochemical and bio-electrochemical. There are mainly three different methods for the commercial production of gluconic acid like; chemical oxidation of glucose

by hypochlorite solution; electrolytic oxidation of glucose solution in presence of bromide and fermentation process where specific micro-organisms are grown in medium containing glucose and other ingredients(Pal et.al, 2016). The microbial fermentation processes offer attractive techniques for the gluconic acid production to reduce the problems related to chemical production such as the inevitable side reactions and also to further economize the bioprocess(Zepedal et al., 1994).

Moulds are generally used as a source of the commercial preparations of microorganism for different application. *Aspergillus* and *penicillium* species are frequently used because of the high glucose oxidase activities exhibited by members of the genera. Strains of *aspergilli* are also important in the production of other industrial enzymes and other valuable chemicals product(Fawole & Odunfa, 2003).

Gluconic acid can be prepared by filamentous fungi microorganisms that have the ability for gluconic acid production(Pai , 2003). Industrial gluconic acid fermentation can be done in three different ways: by surface fermentation, submerged fermentation and solid state fermentation, each of these methods requires raw material and inoculum preparation(Ali, 2012). The production of gluconic acid is mainly in batch cultivation using *A. niger, A.terreus, Penicillium, Gliocadium, Scopulariopsis and Gonatabotrys* have been tested and reviewed (Purane, 2012).Among the different fungal genera, it has been reported that the accumulation of large amounts of the gluconic acid and its salts are restricted to certain species of *Aspergillus* especially *Aspergillus niger* which considered as the most industrially important gluconic acid producer in fermentation industry. But other *aspergillus* species has also similar potential to produce gluconic acid(Ahmed et al., 2015).

A variety of agro-industrial residues and by-products has also been used with submerged fermentation techniques for their excellent potential to be used as substrates for gluconic acid production(Jiménez-hornero et al., 2016). Cane molasses is preferably used as the source of sugar for microbial production of gluconic acid due to its relatively low cost and high sugar content (40–55 %). Since it is a by-product of sugar refining, the quality of molasses varies considerably. The cane molasses composition depends on various factors like the variety of cane, methods of cultivation, conditions of storage and handling (transport, temperature variations), etc. but there are considerable yield variations within each type. In the case of cane molasses, generally it contains some metals (iron, calcium, magnesium, manganese,

zinc) which retard gluconic acid synthesis and it requires some pretreatment for the reduction of them(Soccol et.al, 2006).

The present study will aim to produce and optimize the operating variables of gluconic acid production from molasses as the carbon source substrate by using submerged fermentation and investigate the potential production of *Aspergillus carneus*.

1.2. Statement of the problem

A large quantity of agro-food byproducts are generated during food processing technology. These byproducts may not useful without further processing which was considered as waste. Utilization of these waste materials can be one part of environmental impact control method, on the other hand production of value added product for commercial benefit. Molasses is one of an agro-industrial byproduct often used as carbon source for fermentative commercial products. Especially sugar cane molasses is an available agro-industrial raw material for gluconic acid production in Ethiopia. Due to its cheap cost, availability, and optimal carbon source for microorganism metabolism it is suitable for gluconic acid production. The waste accumulated around sugar factory is the main environmental issue. Bioconversion of these waste materials can be a part of environmental pollution control on one hand and their utility for production of cost effective products of commercial significance on the other, thus changing its grade from byproduct or waste to the potential provider one current issue of us. Utilization of such waste to valuable commercial products is the way how to overcome environmental impact has initiated the research. Most food, pharmaceutical, and textile industries use gluconic acid. To sustain the industries life and viable by economics, they must use renewable local resource. So the production of lab grade gluconic acid from local raw material molasses is encourages the research idea. Moreover, Governmental pressure and environmental regulation are forcefully promoted the search and development of new valuable commercial grade products from waste. Since gluconic acid is pollution free, nontoxic, nonvolatile, noncorrosive, less waste and economically feasible.

1.3. Objective

1.3.1. General objective

The general objective of this thesis is to produce gluconic acid from waste molasses through submerged fermentation by using *Aspergillus carneus*.

1.3.2. Specific objective

- Pretreatment and clarification of crude molasses.
- Study the fermentation of molasses by submerged fermentation.
- Investigate filtration and purification of gluconic acid technically.
- Study the main and interaction effect of the fermentation processing variables.
- Investigate the optimal processing variables for the production of gluconic acid.
- Evaluate the main characteristic of gluconic acid.

1.4. Significance of study

The importance of this research is to utilize a waste product of sugarcane factory to value added commercial products. Utilization of byproducts will also overcome the environmental impact positively through environmentally friendly product. In addition to these, uses of locally available raw material for gluconic acid production for different application are very viable. Since gluconic acid is imported, it can see the potential to produce from our resource and will save currency. Moreover it can serve as starting material for further research studies on the techno grade production, application, and kinetic study of gluconic acid.

1.5. Scope of study

In this research pretreatment and proximate analysis composition of sugarcane molasses is carried out. Selection and inoculation of microorganism, fermentation technique, separation and analysis of gluconic acid are carried out. Further the determinations of dry cell mass also carried out.

CHAPTER TWO

LITERATURE REVIEW

Organic acids are broadly distributed in nature, and humans have used them in their natural sources since early ages. Organic acids are the building blocks of numerous commercial chemical, pharmaceutical, food and beverage additive products. The main organic acids in industrial use are citric, acetic, tartaric, malic, lactic and gluconic acid(Vashishth et.al, 2014).

Gluconic acid fermentation is one of the largest biotechnological industries before dating back a century. Today most of the gluconic acid used in food, pharmaceutical and other industries obtained from fungal fermentation especially with filamentous fungus *Aspergillus niger*(Plassard et al., 2009).

2.1. Gluconic acid and its application

Gluconic acid production dates back to 1870 when Hlasiwetz and Habermann discovered gluconic acid. Gluconic acid (pentahydroxycaproic acid, $C_6H_{12}O_7$), a polyhydroxyl carboxylic ligand, has been investigated for many years, mainly due to its importance in a variety of industrial, pharmaceutical and biological processes(Perez, 1996).

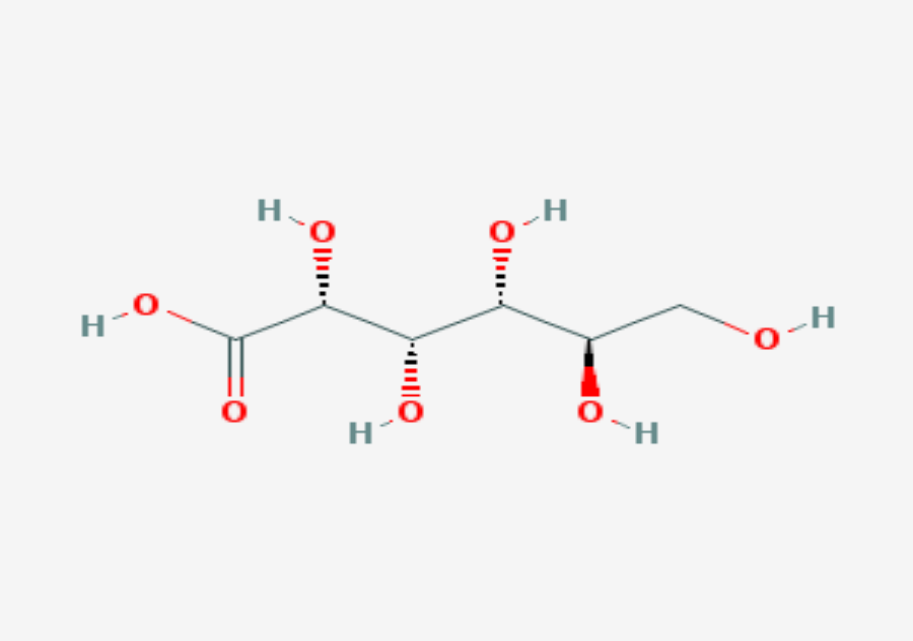


Figure 2.1: 2D structure of gluconic acid

Gluconic acid is the oxidized product (aldonic acid) of glucose. The aldehyde group of glucose is oxidised to the carboxyl group by the action-of the enzyme, glucose oxidase(Wardman, 1994). Gluconic acid and its derivative salts are abundantly available in natural plants, fruits and other foodstuffs such as rice, meat, dairy products, wine (up to 0.25 %), honey (up to 1 %),apple, grape and vinegar(Jiménez-hornero et al., 2016). The physiological D-form of gluconic acid is usually formed by the microbial oxidation of glucose(Gothoskar, 2014). Gluconic acid is stable at the boiling point even of concentrated alkaline solutions and is easily degraded in waste water treatment plants (98% after 2 days). Concentrated solutions of gluconic acid contain the neutral cyclic ester glucono D-lactone (GdL; $C_6H_{10}O_6$), which is less soluble in the cold and possesses no actual acid properties. About 5% of glucono D-lactone are present in the 50% gluconic acid solution at room temperature(Anastassiadis & Morgunov, 2007b).

Gluconic acid is also one of industrial organic acid and recognized as a generally permitted food additive (E574) in the European Parliament and the Council Directive No. 95/2/EC. Some specific properties of gluconic acid (pollution free, nontoxic, nonvolatile, noncorrosive, less waste and economically feasible) permit its wide and varied applications in preventing the deposition of milk stone or in removing it in dairy industry and in preventing cloudiness in beverages. Gluconic acid is also used as an additive in cement to control the setting and increase the strength and water resistance. This organic acid can produce and improve the taste of fruit juices and can form complexes with metal ions in various foods. It is also used as water conditioner by removing alkali and protein films. The non-corrosiveness nature of this acid may be used as gentle metal cleaning agent where cleaning and degreasing of ferrous and non-ferrous metal ions like Ca^{2+}, Fe^{2+}, Al^{3+} and other heavy metals is possible(Pal et al., 2016; Singh et.al, 2003).

Gluconic acid production by *Aspergillus* species is an aerobic fermentation with a high oxygen demand. The biotransformation of glucose to gluconic acid represents a simple dehydrogenation reaction without involvement of complex metabolic cell pathways and is realized with high selectivity, high rate and high yield of conversion(Crognale et.al ,2008). Gluconic acid is formed by the hydrolysis of the gluconolactone ($C_6H_{10}O_6$); the by-product of the reaction (H_2O_2) is decomposed to water and oxygen by enzyme catalysis, which is present in the living cells.

The overall reaction mechanism can be described as follows(Znad et.al , 2004).

Cell growth:

$$C_6H_{12}O_6 + \text{biomass} + O_2 \rightarrow \text{biomass}$$

Glucose oxidation:

$$C_6H_{12}O_6 + O_2 \xrightarrow{\text{GOD}} C_6H_{10}O_6 + H_2O_2$$

Gluconolactone hydrolysis:

$$C_6H_{10}O_6 + H_2O \rightarrow C_6H_{12}O_7\ (\text{GA})$$

H_2O_2 decomposition:

$$H_2O_2 \xrightarrow{\text{Catalysis}} 2H_2O + O_2$$

Gluconic acid and its salts are important materials used in different industries such as; pharmaceutical, food, textile, detergent, leather, photographic and other biological industries. Gluconic acid is a bulk chemical with many uses in the food, beverage and other industries as acidifying and flavor enhancing agents and is produced commercially by submerged fermentation using *aspergillus* species and sugar cane molasses as the carbon source (Dowdells et.al, 2010).

The most important property of gluconic acid bases on its excellent chelating power for calcium, iron, copper, aluminum and other heavy metals, especially in alkaline and concentrated alkaline solutions, surpassing all other known chelating agents, such as EDTA and related compounds. Gluconic acid is widely used as a chelating agent in metal cleaning operations in the dairy industry, in detergents for the cleaning of surfaces, as a component for detergents, in alkaline derusting operations in the metallurgic industry and iron deposition prevention in the textile industry, and as a chelating agent for the extraction of ions like calcium, copper and iron. Furthermore, the sodium salt of gluconic acid can be used as an additive in cement mixtures in the construction industry, enhancing the cement's resistance and stability under extreme climatic conditions, e.g. frost and water, and prolonging its duration(Anastassiadis & Morgunov, 2007b).

2.2. Current status of gluconic acid

2.2.1. Gluconic acid in the world

Today, gluconic and citric acids are the most dominant organic acid and their global production showed an upward trend over the last 25 years(Roukas, 2000). A total amount of 65,000 to 100,000 metric tons of GA and gluconates is produced each year, virtually exclusively via fermentation processes(Crognale et al., 2008). The production and uses of GA and its salts are described in detail elsewhere. Briefly, these substances are used as ingredients of personal care products, pharmaceuticals, chelating agents (e.g., for calcium in the building industry), industrial cleaners and metal surface treatments as well as stabilizing agents for textile bleachers. Additionally, because of their prebiotic properties, GA and its major derivatives are being used as additives in foods and drinks and are considered as key components in the development of new beverages. Despite their flexibility and the large amounts used, no potential hazards justifying further investigation of their safety have so far been identified(Singh & Singh, 2006).

The major driver for the market is its wide application in the food and pharmaceutical market, innovation in technology, economic biotechnological production process, the increased demand for biodegradable acid and environmental concerns(Ramachandran et al., 2006). Gluconic acid is a non-corrosive, easily biodegradable, nontoxic and non-volatile acid and due to these properties, its demand in the market is increasing. In addition to other production processes of gluconic acid, biotech fermentation process has proved to be a boon for manufacturers which has increased its market growth(Jiménez-hornero et al., 2016).

The global consumption of gluconic acid is increasing driven by its applications demand and technological production. The production of gluconic acid is very high in some regions while some regions depend on import of gluconic acid to meet the domestic demand. The market segmentation by geography includes the regions North America, Europe, Asia Pacific, South America and Africa. Europe and Asia pacific are the major market of gluconic acid. China dominates the global gluconic acid market by largest share while Japan and the Netherlands are the fastest growing countries.

2.2.2. The Status of gluconic acid in Ethiopia

Gluconic acid production in Ethiopia is not utilized up to date and linked with sugar factories byproduct. At present, the main supply line in the domestic market is dominated by imported product from European countries with the annual cost; insurance and freight (CIF) expense of more than 5 million ETB for only gluconic acid. The imported gluconic acid and its salt were reagent grade for esterification.

Table2.1: value and Net weight of annually imported gluconic acid and its salt by origin

Year	Country(origin)	Net weight (Kg)	CIF value (USD)	CIF value (ETB)
2006	Kenya	14,737.00	44,494.03	390,866.72
	Denmark	1,445.00	6,616.21	58,121.45
	Sweden	4.00	18.39	161.56
2007	Kenya	22,263.00	62,987.86	569,895.29
2008	India	1.00	10.87	105.32
2009	China	8,882.00	2,618.17	31,085.25
	United kingdom	3.00	65.38	776.20
2010	China	4,000.00	2,104.90	30,656.88
	France	58.00	40.47	589.39
	India	582.00	1,628.91	23,724.27
2011	Cyprus	13,416.00	277,827.71	4,741,046.57
2012	United States	200.00	972.36	17,371.71
2013	Germany	4,500.00	88,304.60	1,660,135.35
	China	150.00	512.32	9,631.67
2014	China	29,326	47.754.77	961,953.04
2015	China	2,008.00	2,333.48	48,494.38
	Switzerland	2,500.00	3,219.27	66,902.82
	United States	340.00	760.33	15,801.14
	India	249.93	1,632.47	33,926.00
	Germany	4.00	57.29	1,190.66
2016	China	181,054.00	173,160.41	3,799,693.29
	Sweden	1,600.00	4,694.64	103,015.42
2017	India	716.62	3908.30	94,646.87
	France	4,366.66	19,514.03	472,569.27
	United States	20.00	96.93	2,347.23
	China	141,200	119,905.55	2,903,740.77

Source: *Ethiopian Statistical Agency, 2018*

Ethiopia has a great potential to produce a valuable organic acid for many application. Since gluconic acid can be produce from sugarcane molasses and other starches, it has a very renewable resources and opportunities. Even though Ethiopia has many sugar factories that produce huge amount of molasses, we did not transform to valuable products rather than animal feed stock and ethanol. To change this potential into real work, the government developed a strategic plan in 2007 considering agro-processing byproducts for industrial usage, improving environmental sustainability and save energy to maintain green economy.

2.3. Raw materials for gluconic acid production

The gluconic acid is produced commercially by fermentation of hydrolyzed starch of cereal grains (corn, wheat and potato)(Elena et.al, 2009), grape and banana must(Ahmed et al., 2015), food processing residues, figs, cheese whey, refined glucose and sucrose, molasses or other materials with high starch and/or sugar contents(Purane, 2012). The fermentation process involves conversion of sugars to gluconic acid by the fungus *aspergillus carneus* aerobically outside the cell wall by the action of glucose oxidase. Among others, Molasses is commonly used as a feedstock for gluconic acid production. It supplies all the sugar that glucose oxidase needs for growth and energy along with part of the needed nitrogen (Crognale et al., 2008).

2.3.1. Aspergillus carneus

Aspergillus carneus can be described as filamentous fungus that can be found in soil, plant products and living plants. *A.carneus* has been reported as a high gluconic acid yielding strain among all gluconic acid producing microorganisms. Some UV and Gamma mutated strains have shown high gluconic acid production in batch fermentation and molasses as a best carbon source(Magnuson & Lasure, 2004). Filamentous fungi are preferred sources of glucose oxidase as they produce extracellular enzymes. The most productive species belong to the genera *Aspergillus , Rhizopus, Mucor, Penicillium and Geotrichum.* In biodiversity, our laboratory initiated a screen that identified *Aspergillus carneus* as the fungus producing gluconic acid(Saxena et.al, 2003).

During fermentation process, molasses that accumulates in the culture converts into gluconic acid through the action of glucose oxidase enzymes. The glucose oxidase enzymes, which are involved in the direct synthesis of gluconic acid from molasses, consist of D-glucono-δ-lactone. It is well known that molasses acts as a precursor in gluconic acid synthesis.

Gluconic acid production ceases when all the molasses in the culture depletes and the *aspergillus carneus* is died.

2.3.2. Molasses

Actually, great amounts of agro-industrial wastes are used for organic acids and enzymes production with industrial interest using fermentation. Molasses and sugarcane bagasse are some by-products generated from sugar industry and these can be harnessed and converted with high valorization. Molasses is a viscous and dark brown slurry liquid, final effluent obtained during the preparation of sugar by repeated crystallization and clarification(Veana, et.al 2014).

Molasses could cause environmental pollution through aesthetic degradation if spills are not properly cleaned. It can also cause water pollution if major spills or factory effluents enter river streams. It is therefore important to consider critically the handling and disposal of molasses particularly in situations where supply exceeds demand. This can arise especially where industrial use of molasses is not well diversified(Ndegwa, 2011). But know most industrial products were fabricated from sugar factory byproducts (molasses and bagasse) such as organic acids, bio plastics, pharmaceutical products and other detergent ingredients. The molasses produced in Ethiopia is mainly used in manufacture of ethanol, production of alcoholic drinks, and also used as animal feeds. Due to lack of some suitable technology for utilization of molasses were not well done. Ethiopia has three large sugar factories, which can produce molasses as by-product that is methehara, Fincha, and Wonji/Shoa sugar factories. Ethiopian Sugar Corporation recorded average annual molasses production from theses factories are 80,000-100,000 and above tons per year. The capacities of molasses production in three sugar factory for the last fifteen years are shown in the Table 2.2.

Molasses is one of a valuable by-product of sugar manufacture and exists in a range of grades: edible molasses, cane and beet molasses, and refinery molasses. It is used as carbon source for animal's feed additive, bio-fertilizer, and raw material in the fermentation industrial production of rum and other beverages(Sirianuntapiboon, et.al 2004). For industrial use, molasses is one of the low-cost and suitable raw materials for fermentation processes such as baker's yeast production, amino acid fermentation and alcohol fermentation and other organic acids such as citric acid, gluconic acid, lactic acid and other fermentation processes(Eggleston & Lima, 2015). Molasses, the non-crystallizable residue remaining after

sucrose purification, has some advantages: it is a relatively inexpensive raw material, readily available, it does not require starch hydrolysis and already used for gluconic acid production. The molasses obtained after sugar beet processing contains about 60% sucrose and 40% other components. The non-sucrose substances include inorganic salts, raffinose, organic acids and nitrogen containing compounds. Molasses is used in the baker's yeast production, in the fermentation technology for ethanol, citric, lactic and gluconic acids production, as well as glycerol, butanol and acetone production, as an ingredient of mixed feeds or in the production of amino acids. The fermentative glucose oxidase is largely used in gluconic acid production using such renewable biomass as sugar cane molasses as the main carbon source(Cazetta, 2007).

Table 2.2: Capacities of molasses production in three sugar factory for the last fifteen years

Years	Factories in (tones)			Total
	Wonji	Fincha	Metehara	
2002/03	19954.6	23815.5	39499.4	83269.5
2003/04	18753.7	24607.8	35748.5	79110
2004/05	18386.5	22224.6	37726.9	78338
2005/06	17901.0	21606.8	41343.4	80851.2
2006/07	17578.7	24375.6	41455.3	83409.6
2007/08	15356.1	25573.0	41082.2	82011.3
2008/09	17561.4	28479.0	42482.0	88522.4
2009/10	15981.0	31306.6	39329.0	86616.6
2010/11	20085.1	30277.5	32689.0	83051.6
2011/12	20947.0	31830.0	30967.0	83744
2012/13	18534.7	38410.0	26935.0	83879.7
2013/14	21034.2	33506.0	28323.5	82863.7
2014/15	20345.1	35638.9	33208.1	89192.1
2015/16	21205.1	37885.8	38272.6	97363.5

Source: Ethiopian Sugar Corporation

One major disadvantage in using molasses for gluconic acid production is its high ash content that contains some trace metals, which inhibits efficient gluconic acid production. To reduce or eliminate the inhibitory action of metal, appropriate pretreatment is given to the molasses solution. *A. carne*us needs a variety of divalent trace elements such as Fe^{++}, Cu^{++}, Zn^{++}, Mn^{++} & Mg^{++} etc. for growth and gluconic acid production. However, gluconic acid production is very sensitive to the concentration of these metals in the fermentation media. Therefore, the concentration of these heavy metals should be decreased well below that required for the

optimal fungal growth .Since the concentration of trace element affects gluconic acid production profoundly(Ashraf & Ali, 2015).

The chemical composition of cane molasses is very varied which depends on the climatic factors, variety and maturity of cane as well as the processing conditions. Consequently, considerable variations may be found in the nutrient contents, flavour, colour and viscosity. The cane molasses which is making a blackish homogeneous liquid with high viscosity contained the following composition(Shetty, 2015).

Table 2.3: Cane molasses composition from literature

Molasses type	Water content %	Sugar content%	Non-sugar content%	Ash content (inorganic salts) %	PH	Nitrogen content %
Cane	20	62	10	8	6.3	<3

2.3.3. Pretreatment of molasses for gluconic acid production

Crude cane molasses contains many organic and inorganic salts that inhibit/ impede the metabolism activity/growth of microorganism for specific application. Before fermentation process, cane molasses that used to be pretreated to decrease the amount of various inhibitors. Common pretreatment methods using acid could decrease the concentration excessive metallic ions and raise the quality of fermentation product(Ndegwa, 2011). Most commonly tested pretreatment techniques were EDTA, sulfuric acid, potassium ferrocyanide, tricalcium phosphate and tircalcium phosphate with hydrochloric acid treatment(Shetty, 2015).The potassium ferrocyanide treatment considerably reduced the concentration of inorganic salts and heavy metal ions as compared to other treatment techniques. The removal of heavy metals and inorganic salts from molasses by potassium ferrocyanide was more efficient than other techniques(Abdel-Rahman,et.al 2016).

The crude cane molasses pretreatment was subjected into two stages. The first stage was achieved by physical treatment that diluted molasses was subjected to centrifugation and mixing with activated carbon followed by filtration to remove colorful agents for

decolorization. In addition to decolorization, some metal complexes are absorbed by activated carbon and reduced the concentration of inhibitors. The second stage was achieved by the chemical treatment, i.e potassium ferrocyanide was used. The treated cane molasses from first stage mixed with potassium ferrocyanide and heated to 90℃ for 2h and placed until precipitate formations. The mixture was centrifuged and the clear liquid was treated molasses(.Nassar et.al. 2016).

The first process step in gluconic acid production is molasses treatment. This stage envisages a reduction in the level of impurities and unwanted trace elements, notably Calcium salts to facilitate the next operations, i.e., fermentation and separation. This guarantees better performance with regard to separation process where the reduction in scaling will be significant, thus permitting better yields and lower operating cost(Garhi, 2012). The need of some pre-treatment of raw materials may enhance the fermentation efficiency. In order to find a cheaper substitute to glucose for gluconic acid production molasses was tried by various workers. Bose (1947) successfully used molasses as a carbon source for gluconic acid production by *Aspergillus niger*. Chughtai and Shah (1966) reported that *Aspergillus niger* NRRL 372 (98) and *Aspergillus niger* NRRL - 3 produced gluconic acid 32 .37)& and 34.29%, respectively on the 6 days of fermentation when molasses was used as a carbon source. Molasses though used by the aforesaid workers for gluconic acid production the yield was lower in comparison to that obtained using glucose. This low yield of gluconic acid was attributed by Taha et al (1963), Chughtai and Shah (1966) to the trace elements present in high concentration in the molasses which somehow inhibited gluconic acid production. Although there is no published report about the effect of refined molasses on gluconic acid fermentation Nasim et al (1981) observed that if the molasses is pretreated with Bentonite and then added to the medium as carbon source citric acid production by *Aspergillus niger* was more than that obtained with untreated molasses.

2.4. Routes of gluconic acid production

Numerous methods have been extensively described for the production of gluconic acid, including chemical and electrochemical catalysis, enzymatic bio-catalysis in enzyme bioreactor, microbial production using free growing or immobilized cells(Anastassiadis & Rehm, 2006). There are several different oxidizing agents available, but still the process appears to be costlier and less efficient compared to the fermentation processes. At present the fermentation process is appreciably competitive with chemical techniques and the method

of choice by industry. However, the choice in turn depends on the economic viability of the processes which in turn depend upon the activity, selectivity, lifetime and cost of the catalyst as well as on the measures required for downstream purification and energy demands. Although the conversion is a simple one-step process, the chemical method is not favoured. Thus, fed-batch and batch fermentation have been one of the efficient and dominant techniques for manufacturing gluconic acid(Crognale et al., 2008). Among various microbial fermentation processes, the method utilizing the fungus *Aspergillus* species is one of the most widely used ones(Ramachandran et al., 2006).

2.4.1. Chemical methods

Chemical synthesis of gluconic acid is related to high energy costs and as in the most fermentative processes large amounts of following byproducts have to be separated from product. The development of chemical (electrolytic or catalytic) oxidation methods for glucose as alternatives to the microbiological synthesis of GA(Anastassiadis & Morgunov, 2007a). Chemical, electrochemical, biochemical and bio-electrochemical approaches are adopted in production of gluconic acid(Pal et al., 2016). Although glucose can be easily oxidized by heterogeneous catalysis in an aqueous solution, the efficiency of the process depends on the activity, selectivity and stability of the catalyst(Jiménez-hornero et al., 2016). Several studies have been reported on heterogeneous catalysis of glucose to produce gluconic acid by using Pt, Pd or Au nanoparticles as catalysts and oxygen from air as oxidant under mild conditions. Due to its high selectivity as well as steady activity Au has been considered the most competitive catalyst for glucose oxidation, however there is now a growing excitement regarding the potential of applying gold for catalyzing industrial reactions, including glucose oxidation in to gluconic acid. Recently the focus has shifted to new raw materials and more inexpensive and environmentally friendly reagents. For example, aqueous hydrogen peroxide has been used as an alternative reagent for chemical conversion of glucose of 99% at 25℃ using a $FeSO_4$ catalyst(Jiménez-hornero et al., 2016). But chemical synthesis of gluconic acid is less attractive due to high cost of the catalyst, high probability of getting deactivated, difficulty in recycling and recovery of the heterogeneous catalysts, difficulty of down streaming process, made it difficult for gluconic acid production(Pal et al., 2016).

2.4.2. Fermentation

Fermentation has been one of the dominant methods for producing gluconic acid at the present time(Liu et.al, 2002). It performs simple/complex transformation on organic material to produce a valuable desired product by using the metabolic activity of microorganisms. A biocatalyst either a cell or enzyme, especially glucose oxidase, converts the organic material to the desired product. The process steps required during production of gluconic acid through fermentation are fermentation, gluconic acid isolation and purification(Pal et al., 2016). The advantage of fermentation process over normal chemical synthesis includes product specificity, lower energy costs and higher yields. The current method of gluconic acid preparation is a bio process based on glucose syrup employing species (strain) converted to it extracellularlly(Walford & du Boil, 2006).

The most common biotechnological process for GA production is based on fungi and uses *Aspergillus carneus*. This microorganism yields the GA via D-glucono-δ-lactone as intermediate reaction product. Specifically, the enzyme glucose oxidase catalyses the conversion of glucose to the lactone, which subsequently gives GA (fast at either acidic or alkaline conditions and slow at neutral pH; also the hydrolysis of glucono-δ-lactone is enzymatically facilitated by lactonase, specially required at neutral pH). The process requires carefully adjusting the pH of the medium and ensuring an adequate supply of oxygen. In fact, the high oxygen demand of the biotransformation process, and the typical difficulties of homogenizing a highly viscous medium such as one with fungal mycelia, considerably raise operational costs and hinder homogeneous distribution of dissolved oxygen in the culture medium. Also, the pH of the medium should be controlled in the range 4.5-7.0 because a lower value triggers the tricarboxylic acid cycle and leads to citric rather than GA.

Every fermentation process requires specific medium but certain basic requirements components must be present within fermentation media. All microorganisms used in fermentation require water, various carbon sources, nitrogen sources, certain minerals, vitamins and oxygen if process is aerobic. Glucose and cane molasses is the best carbon sources for gluconic acid production. For gluconic acid production wide range of carbon and nitrogen sources have been used are described in following table(Chaudhary et.al, 2014):

Table 2.4: Composition of production media

Element	Source
Carbon	Glucose Xylose Starch Sucrose Lactose Cane molasses Beet molasses Cereal grains
Nitrogen	Corn steep liquor Ammonium salts Urea Nitrates Peptone
Minerals	$ZnSO_4$ KH_2PO_4 $MgSO_4.7H_2O$ $CuSO_4.5H_2O$
Oxygen	Natural sterile air (Through aeration)

Various techniques can be employed for the production of gluconic acid through enzyme glucose oxidase which includes solid state, surface and submerged fermentations. Submerged fermentation is found to be more effective in the production of gluconic acid and other industrial enzymes because it is easier to control the environmental factors in this technique as compared to solid state and surface fermentation(Macris., 1995).

2.4.2.1. Submerged fermentation

The submerged fermentation process has become the method of choice in the industrialized countries because it requires less labor for operating, gives higher production rate and uses less space. Under submerged fermentation method the media was subjected to vigorous controlled aeration and agitation in large bioreactor. Submerged culture method of gluconic

acid product ion is much superior to the other methods not only in respect to the time required for fermentation but also in the yield and ease of production. In gluconic acid fermentation it was observed that a medium of one composition is suitable for maintaining the growth of the culture ,a 2^{nd} medium for inducing sporulation, 3^{rd} medium for stimulating germination and another medium is for gluconic acid fermentation(Dubey et.al, 2017).

Mostly gluconic acid is produced by using submerged fermentation (SMF) method. It is highly reliable method for gluconic acid biosynthesis. The growth of aerobic microorganisms in a submerged fermentation (SMF) is controlled by the availability of substrates (sugars), energy and enzymes produced by microorganisms. Production cultures are always of a heterogeneous nature hence, the rates of biochemical reactions can't be limited by the rate of substrate or product transfer at a particular interface. Mahmoud et al (1977) studied gluconic acid production by *Aspergillus niger* using both fermentation methods by surface and submerged culture technique and obtained better production of gluconic acid in case of submerged culture fermentation technique.

2.4.2.2. Solid state fermentation

Solid-state fermentation (SSF) refers to the growth of microorganism on solid substrate to support the growth in the presence of moisture. It has been termed as an alternative method to produce gluconic acid from agro-industrial residues. Gluconic acid production by SSF was first developed in Japan and is as the simplest method for its production. SSF can be carried out using several raw materials. Generally, the substrate is moistened to about 70% moisture depending on the substrate absorption capacity. It provides the opportunity for economical production of fermented food, organic acids and flavour inducing compounds by initiating the natural growth on water- insoluble solid support in the absence of free liquid medium(Pal et al., 2016). The initial pH is normally adjusted to 4.5-6.0 and the temperature of incubation can vary from 28 to 30℃. The most commonly organism which is used for gluconic acid production was *A. niger*. One of the important advantages of SSF process is that the presence of trace elements may not affect gluconic acid production. Consequently, substrate pre-treatment is not required(Vandenberghe, et.al, 1983).Different types of fermenters such as conical flasks, glass incubators and trays, etc. have been used for gluconic acid fermentation in SSF.

2.4.2.3. Surface fermentation

Liquid surface culture is the classic gluconic acid production process characterized by low yield, lower energy consumption and less man power. Karklins et.al. (1996) studied the utilization of different microorganisms in gluconic acid production by surface fermentation using cane molasses as a substrate. Elnaghy and Megalla (1975) used this technique for gluconic acid production by eight different species of *Penicillium* using surface culture technique.

2.5. Parameters affecting gluconic acid production

There are several physical, chemical and biological variables that affect fermentation of gluconic acid production which includes use of high gluconic acid yielding microorganisms, incubation time, production media, type of fermentation operation, physiological conditions (temperature, pH and moisture), aeration & agitation and minerals(Chaudhary, et.al, 2014). Maximum gluconic acid production efficiency can be achieved when these factors are considered and the production system is operating under the optimal fungal growth condition(Fawole & Odunfa, 2003). Response surface methodology (RSM) is a global optimization method which can assess and optimize the interaction between independent variables in different chemical, bioenvironmental and biochemical processes(Rasoulnia & Mousavi, 2016).

Roukas(2000) studied the effect of fermentation parameter such as temperature, pH, moisture and methanol concentration on kinetic parameters of fig fermentation for gluconic acid production. From this investigation the optimum operating conditions were 9 day fermentation time, 75% moisture content, 7 initial pH, 30℃ temperature , 6% addition methanol concentration resulted in increased gluconic acid concentration(Roukas, 2000).

2.5.1. Initial pH

Most filamentous fungi are observed to grow well under slightly acidic conditions, ranging from 3 – 6, but some fungi are able to grow at a pH below 3. Many strains of *Aspergillus* can lower the pH of their environment by oxidizing glucose outside the cell wall, converting it to gluconic acid via the action of the enzyme glucose oxidase(Magnuson & Lasure, 2004). But as reported by sexan et.al (2003) for *aspergillus carneus* the optimum pH is 8 to lipase production(Saxena et al., 2003).

For the production of gluconic acid by *A. niger* there is no agreement in the literature about optimal initial pH which seem, to be strain dependent. However, the pH range of 3– 6 is commonly used in solid state and submerged fermentation. Roukas and Harvey (1988) reported that at low pH values, citric acid is produced whereas at high pH values gluconic acid is formed. The pH of culture media may change in response to microbial metabolic activities; the most obvious reason is the secretion of organic acids, which will cause the pH decrease. Change in pH kinetics depends also on the microorganism, in case of the *Aspergillus sp., Penicilium sp. and Rhizopus sp.,* pH can drop very quickly to less than 3.0. However, for other groups of fungi such as *Trichoderma, Sporotrichum, Pleurotus sp.,* the pH is usually stable between 4.0 and 5.0. The nature of the substrate and the production technique also influence pH kinetics. In this way initial pH must be very well defined and optimized depending on the microorganism, substrate and production technique.

2.5.2. Temperature

There are variations of optimum temperature for gluconic acid production from different species of *aspergillus* as reported by(Shindia & Sheriff, 2006) an optimum temperature of 30℃ for maximum gluconic acid production from *aspergillus niger.* Mostly, 30℃ was the optimum temperature for different species as reported by many authers(Znad et al., 2004;Anastassiadis and Morgunov, 2007;Stella & Halimi, 2015). The optimal temperature obtained from this study facilitates the strain growth and high quality gluconic acid preparation and production for food processing industry usage. The higher gluconic acid production found at optimal temperatures can be explained by an enhanced enzyme activity of glucose oxidase and enzyme secretion into the medium, because higher biomass concentrations are obtained at lower temperature(Anastassiadis & Morgunov, 2007a).

2.5.3. Incubation time

Duration of fermentation process for gluconic acid production depends on the type of fermentation (whether solid surface culture or submerged culture), percent of glucose present, and the vigour of inoculum added. Taha et al (1960) studied the effect of incubation period for gluconic acid production by *Aspergillus phoenicis* and found that 6 days incubation was enough to achieve maximum yield of acid after which the organism appreciably began to break down the gluconic acid accumulated. Chopra et al (1975) observed that gluconic acid fermentation from 30% glucose by *Aspergillus niger* in shake flask completed within 76 h.

Mahmoud et al (1976) found that for maximum production of gluconic acid by *Aspergillus niger NRRL 3* required 120 h in shake flask culture in submerged cultivation.

2.5.4. Carbon source concentration

Carbon source concentration and type of source can affect the amount of gluconic acid production qualitatively and quantitatively. For gluconic acid fermentation usually glucose is used as carbon source(Sirianuntapiboon et al., 2004). The use of carbon source other than glucose for gluconic acid production are sucrose, corn starch, whey, grape must, banana must, molasses and other sugar containing agro-byproducts. As reported by(Ahmed et al., 2015) sugar cane molasses can give highest result from other sources. So utilization of molasses is a sole carbon source for gluconic acid production through submerged fermentation by *aspergillus carneus*.

2.5.5. Nitrogen source concentration

Gluconic acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. urea, ammonium sulfate, ammonium cloture, peptone, malt extract, etc. Nitrogen consumption leads to pH decrease, which is very important point in gluconic acid fermentation. However, it is necessary to maintain pH values in the first day of fermentation prior to a certain quantity of biomass production(Shindia & Sheriff, 2006). The concentration of nitrogen source required for gluconic acid fermentation is 0.1 to 0.4N /liter. A high nitrogen concentration increases fungal growth and the consumption of sugars, but decreases the amount of gluconic acid produced(Shetty, 2015).

2.5.6. Aeration and agitation

In aerobic submerged fermentation supply enough oxygen is the basic rate limiting factor for bioconversion of glucose to gluconic acid(Pal et al., 2016). Aeration supplies the necessary oxygen to the microorganisms, and agitation maintains uniform conditions within the fermenter (incubator shaker). Altogether, the aeration and agitation are important factors in promoting effective mass transfer to liquid medium in the incubator shaker. The main function of a properly designed bioreactor is to provide a controlled environment in order to achieve the optimal growth and/or product formation in the particular cell system employed. In laboratory shake flasks, aeration and agitation are accomplished by the rotary or reciprocating action of the shaker apparatus. In pilot-scale and production-scale fermenters,

oxygen is generally supplied by compressed air, and mechanical devices are used to agitate the liquid broth. In aerobic fermentation processes, oxygen is a key substrate, and because of its low solubility in aqueous solutions, a number of studies to enhance the efficiency of oxygen mass transfer have been conducted. The concentration of dissolved oxygen in a suspension of respiring microorganisms generally depends on the rate of oxygen transfer from the gas phase to the liquid, on the rate at which oxygen is transported to the site of utilization, and on the rate of its consumption by the microorganism. In the conventional water soluble carbohydrate substrate processes, it has frequently been found that the rate of oxygen transfer from dispersed air bubbles can become the rate limiting factor by the rate of supply of oxygen(Shindia & Sheriff, 2006).

As studied by(Ambekar, et.al, 1965) the rate of aeration were increases, as the utilization of glucose increases which cause the yield of gluconic acid increases appreciably. Humphrey (1967) has reviewed the engineering problems associated with hydrocarbon fermentations. One such problem is that of supplying sufficient oxygen to meet the relatively-high microbial respiration rate demands in these fermentations. Darlington (1964) has shown that hydrocarbon substrate growth requires about 2.7 times the rate of oxygen transfer from air as does microbial growth on glucose.

2.6. Separation and Analysis of gluconic acid production yield

For the downstream purification process of gluconic acid from the fermentation broth, membranes separation like, microfiltration, ultrafiltration, nano-filtration, reverse osmosis and electro-dialysis membrane can play effective role. For the separation of organic acids which may be neutral or charged solutes nano-filtration (NF) membrane has been practiced well recently. Nano-filtration is relatively new class of the pressure-driven membrane processes for the downstream purification and recovery of gluconic acid. Application of NF membrane for such purposes is a viable option over traditional methods like extraction, ion-exchange, evaporation and distillation. It has been recently developed and adept very well for the separation of small neutral and charged solutes in aqueous solution (Pal et al., 2016). Measurement of the concentration of secreted organic acids (gluconic acid), was conducted using titration method, FTIR and statistical analysis of variance (ANOVA). Gluconic acid was the sole organic acid found in the cultivation broth. This feature agreed with the fact that sodium hydroxide consumption for pH control was closely related to gluconic acid synthesis(Gbnie et al., 1996).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

The major raw materials used in this work were sugarcane molasses, peptone, yeast extract, sodium hydroxide, sulfuric acid, potassium chloride, calcium carbonate, ethanol, mono potassium phosphate, hydrated magnesium phosphate, potato dextrose agar, potato dextrose broth, benedict solution, phenolphthalein solution, standard glucose hydrous and *Aspergillus carneus*. Sugar cane molasses could be obtained from Oromia region of Ethiopia in Wonji/shoa sugar factory. *Aspergillus carneus* was obtained from Ethiopian biodiversity institute or gene bank in Addis Ababa around Megenagna. All the other chemicals used for fungus development are analytical reagent grade and purchased from different chemical stores in Addis Ababa around chirkos market center.

3.2. Equipment

The equipment's used during the experiment were magnetic stirrer, pH meter, water bath, tongs, beakers, beam balance, measuring cylinders, cell loops, indicator, aluminum and silica dish, petri dish, aluminum foil, centrifuge, autoclave, oven, Muffle furnace, different size conical and Erlenmeyer flasks, heater, incubator shaker, refractometer, spectrometer, FTIR and inoculating chamber are will be used. All the experiments including proximate analysis of molasses, pretreatment of molasses, media preparation, inoculation, fermentation and separation of gluconic acid and dry cell mass have been carried out in school of chemical and bio engineering laboratory, AAiT. The functional group analysis FTIR was done at faculty of natural science department of chemistry, in Arat kilo chemistry laboratory. The overall structure of the experimental works is shown in Figure 3.1.

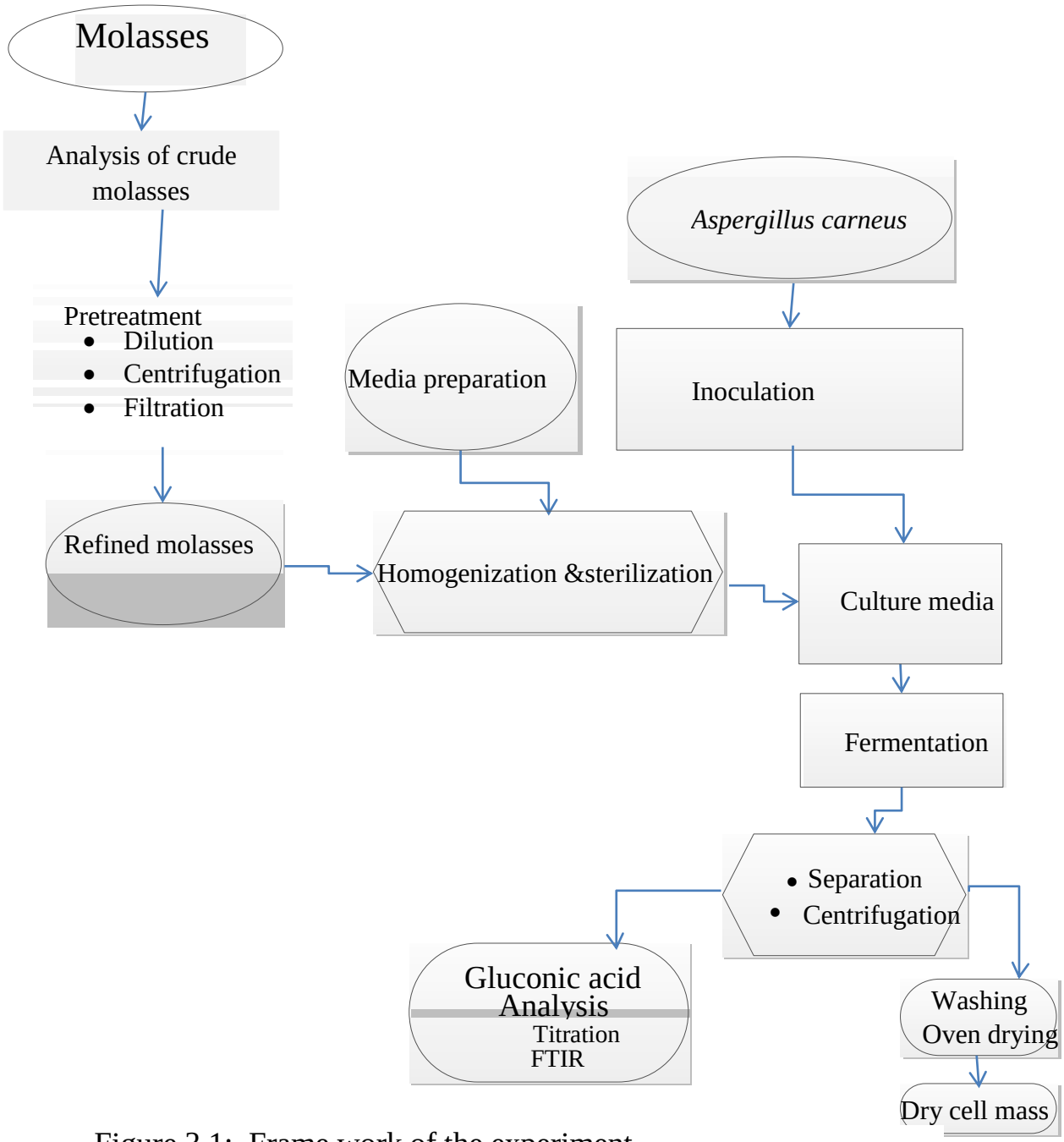


Figure 3.1: Frame work of the experiment

3.3. Experimental methods

3.3.1. Collection and pretreatment of cane molasses

Crude molasses (CM) was obtained to contain high concentrations of heavy metals and other compounds that inhibited gluconic acid fermentation, hence it was treated with potassium ferrocyanide prior to use. Cane molasses was mixed with an equal quantity of distilled water. The diluted mixture was kept at 40℃ for 5 hours (h) followed by centrifugation at 2000

revolution per minute (rpm) for 15 minutes (min). The clarified molasses liquid was used for the pretreatment methods. The clarified liquid was mixed with the activated charcoal 100 g/L for decolourization and filter by vacuum filtration. Potassium ferrocyanide (100ppm) was added with continuous stirring to the decolorized molasses at pH 5.0–5.5, followed by heating at 70–90℃ for 15 min. The precipitate formed containing metallic complex was removed by centrifugation, and the clear liquid was referred as treated cane molasses (TM). The pH of clarified molasses was adjusted to 5.5 before is used for gluconic acid fermentation.

3.3.1.1. Inorganic salts and Heavy Metals Analysis of treated molasses

The inorganic salt and heavy metals were determined according to the method described in(Abdel-Rahman et.al, 2016). The treated molasses sample was dried in an oven at 105℃. The dried sample was ash in muffle furnace at 500℃ until the sample was completely combusted (ash turned to white /gray or slightly colored). The obtained ash was reflected the amount of inorganic salts and heavy metals (cadmium, nickel, copper and lead) in general.

3.3.2. Chemical compositions and nutritional analysis of crude molasses

3.3.2.1. Total dissolved solid content

According the procedure of ICUMSA (1994) total dissolved substance composition commonly termed as brix for cane molasses sample were determined in triplicates(Favero et.al, 2014). About 100 gms of molasses sample was weighed on analytical balance with accuracy 0.01 and poured into Erlenmeyer flask at which about 200 g distilled water was add to bring to about 300 gm of total weight. The sample was kept under magnetic stirrer and stirred until homogenized solution is obtained. Using covered funnel with a watch glass to minimize evaporation the sample was filtered through a fluted filter paper. The first 20 mL of filtrate was rejected and sufficient filtrate was collected in a 150 mL beaker for brix analysis. The temperature of the filtered molasses solution was kept at 20℃ using water bath supported by inbuilt thermo circulator. Brix reading was taken using Anton paar digital automatic Referactometer.

3.3.2.2. Determination of Total Reducing Sugar content

The concentration of total reducing sugar content of cane molasses which was obtained from wonji sugar cane factory was determined using a spectrophotometer by measuring absorbance vs. sugar concentration. Benedict solution and standard glucose solution was used to plot the calibration curve to show the percentage of absorbance of red light by the standard glucose solutions. Benedict's solution is designed to detect the presence of reducing sugars. In hot alkaline solutions, reducing sugars reduce the blue copper (II) ions to brick red copper (I) oxide precipitate. As the reaction proceeds, the color of the reaction mixture changes progressively from blue to green, yellow, orange and red. When the conditions are carefully controlled, the coloration developed and the amount of precipitate formed depends upon the amount of reducing sugars present. Hence, in most conditions, a sufficiently good estimation of the concentration of glucose-equivalent reducing sugars present in a sample can be obtained.

Standard glucose dilution series solution was prepared at different concentration of 15, 12.5, 8, 4, 2, 1, 0.5 and 0(w/v%). 1ml of each of the standard glucose solution is added into labeled test tubes, each containing 5 ml of benedict's solution and mixed by shaking. The labeled test tubes were heated in 90℃ water bath for 5 minutes. The test tubes removed from the water bath and filtered the samples using filter paper to remove any red precipitate formed when reducing sugar in the samples reacted with benedict reagent. The absorbance of the samples was read using spectrophotometer. Finally, plot a calibration curve to show the percentage of absorbance of red light by the standard glucose solution.

$$Con.of\ unknown\ sample\ = \frac{(absorbance\ of\ unkwon\ sample)-(Y_{intercept})}{slope} \dots\dots\dots\dots\dots\dots\dots (3.1)$$

$$Yield\ of\ TRS\ (\%) = \frac{glucose\ conc}{gram\ of\ sample} *\ \ molasses\ volume * 100 \dots\dots\dots\dots\dots\dots\dots (3.2)$$

3.3.2.3. Determination of Moisture content

Accurately weigh about 2g of the sample homogenized and placed in a weighing bottle which has been previously dried to a constant weight. Dry it in a vacuum oven at a temperature of 105℃ for four hours, cool to room temperature in a desiccator and weigh. Repeat vacuum drying until the loss in weight does not exceed 2mg per hour in the drying

period(Anonymous, 2001). Calculate the moisture content in the sample from the following formula. Round off fractions to the first decimal place.

$$\%, moisture \text{ Content} = \frac{W0-W1}{W0} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.. (3.3)$$

Where:-W_0: Amount (g) of sample collected

W_1: Weight (g) of sample after drying

3.3.2.4. Determination of Ash content

As per the procedure of, the sample has been well mixed; samples solutions were prepared in triplicates in crucibles by taking the required value of pre-dried sample from moisture determination. The crucibles were heated using Bunsen burner (oven) in a fume cupboard until completely dried. The sample crucibles were placed in a muffle furnace at 550℃ for 24 hours. Removed, cooled, and added in the desiccators and weighed. Repeat the procedure until the loss in weight does not exceed 0.1% per hour in the burning period. The ash, percent by mass was evaluated form the following relations.

$$\% \text{ ash content} = \frac{M2-Mo}{M1-Mo} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.4)$$

Where: - Mo = Mass of empty crucible

M1 = Mass of the original sample and crucible

M2 = Mass of the residue and crucible

3.3.2.5. Determination of Nitrogen Content

The nitrogen content of sample was determined by using kjeldahl method described by ES (2004). In the method all nitrogen was converted to ammonia by digestion with a mixture of conc. suplphuric acid and conc.orthophosphoric acid containing potassium sulphate as boiling point raising agent and selenium as catalyst. The ammonia released after alkalization with sodium hydroxide was steam distilled into boric acid and titrated with sulphuric acid. Weighing out molasses sample transferred into a testator tube, Place it in the tecator rack. Adding 5mL of NH_4Cl solution in to each tecator tubes, and 6mL of the acid mixture that was mixed of 5 parts of conc Orthophosphoric acid with 100 parts of conc Sulphuric acid. It has mixed the molasses sample and acid carefully. 3.5 mL of hydrogen peroxide was added step-

by step. There was a violent reaction. By adding 3gm of the catalyst mixture for 30 min before digestion, Digestion was going on for more than 3 hour at 370℃. Distillation has been placed on a 250 ml conical flask contain 25 mL of the boric into the solution. Transfer the digested and diluted solution in to the sample compartment and added 25 mL of the 40% sodium hydroxide solution into the compartment; it has been rinsed down with a small amount of water, and switch on the steam. It has been distilled until 100 mL then continued until a total volume of a few mL of water before the receiver was removed. Then it was titrated with 0.1N sulphuric acid to a reddish color using the radio meter pH stat.

$$\text{Total percent of nitrogen in a sample} = \frac{\left((T-B)\times N\times 14\times 100\right)}{W} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.5)$$

Where:
T=Volume in mL of the standard sulphuric acid solution used in the titration for the test.
B=Volume in mL of the standard sulphuric acid solution used in the titration for the blank determination
N=Normality of standard sulphuric acid.
W=Weigh in grams of the test material.

3.3.3. Microorganism inoculation

After extensive screening of gluconic acid producers, *A. carneus* was selected from the laboratory culture collection. Pure cultures of *Aspergillus carneus* were collected form gene bank. The organism was maintained on potato dextrose agar slants and incubated aerobically at 25℃ for 3 days. The slants were stored at 4℃ and sub-cultured every month. The spore suspension was prepared by suspending the spores on the slant in 10 mL of sterilized saline solution and used as inoculum (2–3%, v/v) for batch fermentation.

3.3.4. Fermentation technique

Gluconic acid fermentation was carried out by submerged fermentation in 250 mL cotton wool plugged Erlenmeyer shake flasks with composition of fermentation media: Glucose varying gram; peptone 1.5g; KH_2PO_4 0.5g;KCl 0.025g; $MgSO_4$ $7H_2O$ 0.025g;Yeast extract 1.6g; distilled water 1000mL. The medium was modified by substituting glucose from pretreated molasses and adjusting the pH of each solution with H_2SO_4, sterilized at 121℃ for 15 minutes and cooled to room temperature.

In the submerged fermentation (SmF) process, $100cm^3$ of the fermentation medium was inoculated in Erlenmeyer flasks ($500cm^3$) with mutant *A. carneus* and incubated in an orbital

shaker with constant shaking (150rpm) for wanted days. After completion of fermentation, the biomass was separated by centrifugation under constant5000 rpm for 20 minute and the fermented media was analyzed for residual sugar and gluconic acid concentrations(Singh et al., 2003).

3.3.5. Estimation of dry cell mass

The dry cell mass was determined by centrifuging the culture media through a pre-weighed centrifuge tube. The separated mycelia were washed several times with deionized water and mycelia were then dried in an oven at 105℃ for 2hours to constant weight after repeated weighing. The supernatant was used for further analysis(Yigitoglu, 1992).

3.3.6. Determination of gluconic acid

The total acidity of the culture filtrates was estimated by titration method, by taking 10mL of fermented broth against 0.1N NaOH(standard alkaline solution) using phenolphthalein as indicator (Peppler, 1967; El-Ktatney, 1978) and strength of acid production was calculated in terms of molarity (M). The fermented broth showing greatest acid production were selected for further study(Shindia & Sheriff, 2006).

The quantity of this acid was determined by titration of the culture solution with 0.1 N sodium hydroxide at room temperature that adapted from(Herrick&May,1928). Excess alkali was added, and the solution was maintained at a temperature of 50-55℃. At this temperature it was found that any glucose present did not react with appreciable quantities of the sodium hydroxide and that the gluconic acid latone was completely hydrolyzed to the acid in from 1 to 2 minutes. After 2 minutes at this temperature, the solution was titrated with 0.1N sulfuric acid and the gluconic acid resulting from the hydrolysis of the lactone was added to the first titration. As a rule the lactone equaled from 5 to 10 per cent of the total quantity of gluconic acid. The total acid was then calculated in gm. of gluconic acid, since no citric or oxalic acids were found in appreciable quantities. To check these results several determinations of gluconic acid, as calcium gluconate, were made by neutralizing an aliquot portion of the culture liquor with calcium carbonate, heating to boiling, filtering, and precipitating the salt with 3 volumes of 95 per cent ethanol. The mixture was allowed to stand for 2 days to insure complete precipitation and was then filtered in a weighed crucible. The precipitate was washed with 60 percent ethanol, after which it was dried to constant weight at 90℃. The

agreement between the quantity of acid calculated from the titration and the quantity actually recovered was satisfactory(May et.al, 1927).

3.4. Fourier transform infrared spectroscopy analysis of gluconic acid

The infrared spectrum was recorded by passing a beam of infrared light through the sample. The functional group analysis of the gluconic acid product was carried using Fourier-Transformed Infrared (FTIR) spectroscopy. The FTIR spectra were recorded on spectrum 65 FT-IR (perkinElmer) equipped with KBr beam splitter. Diffuse reflectance system (DRS) was used for powder samples and NaCl plate for liquid samples by thin film deposition technique. A regular scanning range of 400-4000 cm^{-1} was used for 20 repeated scans at a spectral resolution of 4 cm^{-1}. All the spectra were recorded and processed using essential FTIR software.

3.5. Experimental design for gluconic acid production

In this thesis work the gluconic acid was produced using purified molasses, *Aspergillus carneus*, and mixture of media. Experimental data analysis was done by accounting the influential factors with different combinations of their test levels using software – Design – Expert ® version 7.0.0 trial. To demonstrate the influence of each factor on responses and to determine the optimum combination of operational level of factors, contour and 3D surface plots was generated using software – Design – Expert ® version 7.0.0 trial. Randomization of the experimental runs as well as appropriate analysis technique was the bases to conclude about the research through proper utilization of software – Design – Expert ® version 7.0.0 trial.

The experimental design selected for this study was response surface methodology. RSM is a collection of mathematical and statistical methods which determines operating conditions and regression model equations using quantitative data obtained from the appropriate experiments; four-factor with three-level Box-Behnken Design (BBD) and the response variables measured were the dry cell mass and gluconic acid yield(Rasoulnia & Mousavi, 2016). The four independent variables studied for the fermentation process were temperature, pH, molasses concentration and incubation time. The independent variables interaction effect was analyzed to obtain maximum product of gluconic acid and optimum dry cell mass. To achieve maximum conversion of molasses rotational speed was set at 150 rpm, amount of

inoculated strain 2% and the amount of mixture of media was fixed to the minimum amount for microorganism requirement.

Four-factor with three-level- BBD was used in the optimization study which requires 29 experiments to be conducted. The twenty-nine experiments were done and the data was statistically analyzed using software – Design – Expert ® version 7.0.0 trial to obtain a suitable model equation for the percentage production of gluconic acid as a function of the independent variables(Montgomery, 2002).Table 3.1shows that the range and levels of the four independent variables studied in which, the lower and higher levels are chosen by considering the operating limits of gluconic acid fermentation process conditions under submerged and batch fermentation.

Table 3.1: Experimental factors and levels used in the BBD for the gluconic acid production

Factors	Levels		
Temperature (℃)	25	32.5	40
Incubating time (day)	3	5	7
pH	3	5	7
Molasses concentration (g)	50	150	250

The experimental design including the selected factors and their levels with gluconic acid concentration as response variable have been presented in table 3.2. As the number of selected factors was 4, 16 (2^4) factorial points and 8(2 *4) axial points with 5 center points made the total number of experiments 29(Jia et al., 2015). For each factor the center point is the mean value of the lowest and highest level of the variable(Rasoulnia & Mousavi, 2016).

3.5.1. Experimental setup

The feed materials of the experiment prior to fermentation process (mixture of media, purified molasses and *aspergillus carneus*) were carried out on a Fast Flow v-Cabinet hood equipped with laminar flow, U.V light and light source. The fermentation process was carried out in an incubator shaker equipped with an rpm control, time controller and temperature controller to control the actual fermentation temperature. The incubator shaker can be adjusted to the desired temperature, fermentation time and rotational speed. For fermentation process, a 100 mL conical glass flask was used in all experiments. The batch fermentation process system was employed for gluconic acid production as shown in the schematic diagram figure3.2.

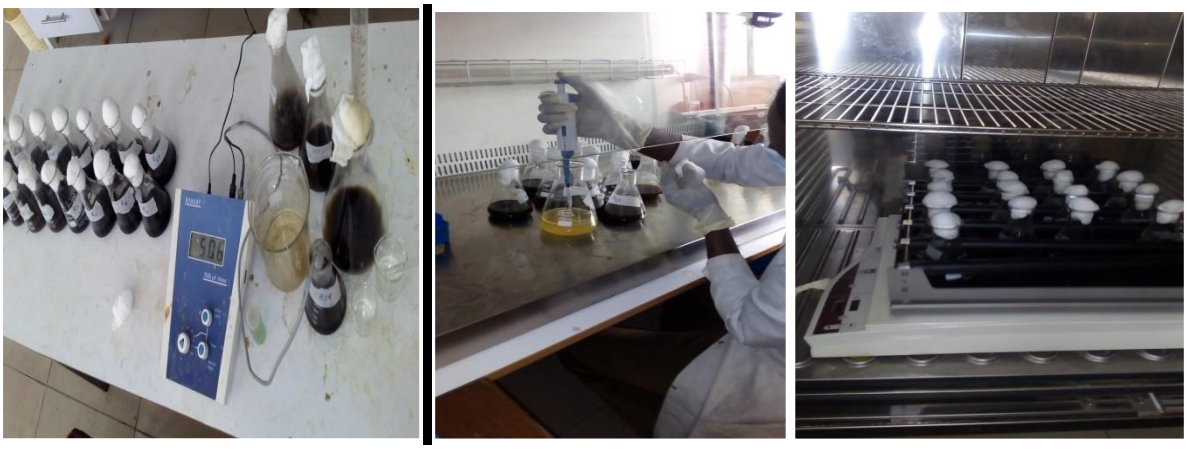


Figure 3.2: Experimental set-up for gluconic acid production through fermentation

Table 3.2: The complete experimental design expert matrix

Std	Run	Temperature(℃)	Carbon source concentration(g/l)	pH	Incubation time(day)
1	7	25	50	5	5
2	25	40	50	5	5
3	5	25	250	5	5
4	18	40	250	5	5
5	8	32.5	150	3	3
6	20	32.5	150	7	3
7	26	32.5	150	3	7
8	28	32.5	150	7	7
9	19	25	150	5	3
10	21	40	150	5	3
11	17	25	150	5	7
12	10	40	150	5	7
13	13	32.5	50	3	5
14	1	32.5	250	3	5
15	23	32.5	50	7	5
16	16	32.5	250	7	5
17	14	25	150	3	5
18	12	40	150	3	5
19	6	25	150	7	5
20	3	40	150	7	5
21	4	32.5	50	5	3
22	27	32.5	250	5	3
23	11	32.5	50	5	7
24	22	32.5	250	5	7
25	29	32.5	150	5	5
26	9	32.5	150	5	5
27	15	32.5	150	5	5
28	2	32.5	150	5	5
29	24	32.5	150	5	5

CHAPTER FOUR

RESULT AND DISCUSSION

4.1. Proximate analysis of the cane molasses

The composition of molasses is influenced by many factors such as soil type, season of production, moisture, temperature, variety, extraction technology, evaporation and clarification techniques can control the amount of components. Due to these factors the cane molasses collected from wonji/shoa sugar factory were analyzed for the determination of ash, moisture, pH, total dissolved solids, total sugar and total reducing sugar. As tabulated in Table 4.1, the percentages of each amount of samples were determined in triplicate samples and take the average value. The obtained result shows that cane molasses composition somehow in the Ethiopian standard range. The molasses contains enough sugar for fermentation production of organic acids by using some microorganism. Since it contains glucose, nitrogen, and other trace metal complex.

Table 4.1: Composition of crude cane molasses collected from wonji compared with Ethiopian standard

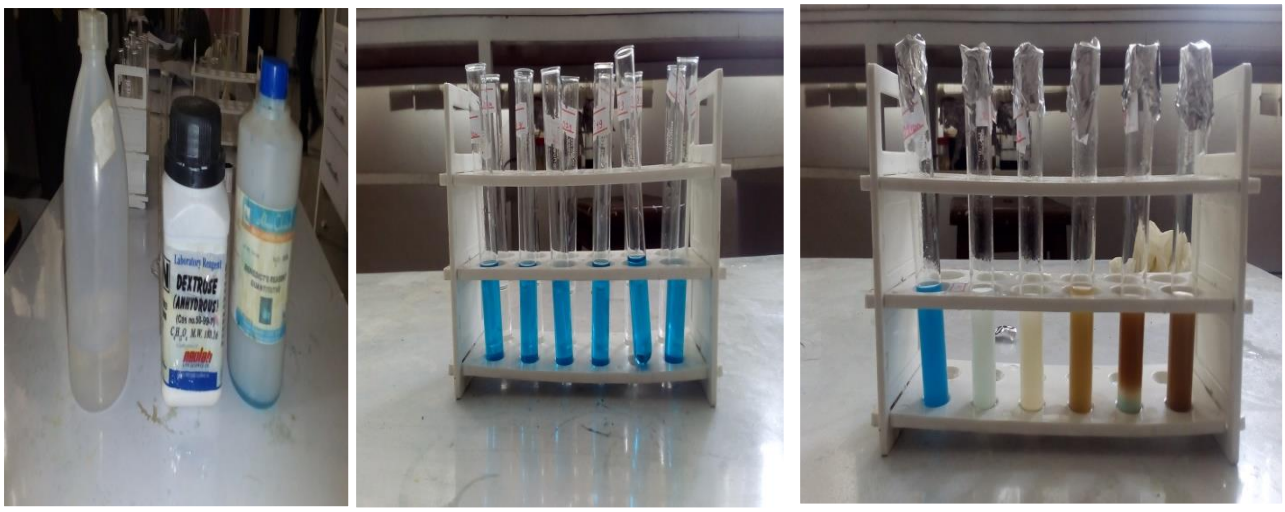
No	Characteristics	Ethiopian Standard	Laboratory result
1	Moisture content (%)	15	14.35
2	Ash content (%)	14	13.45
3	Total dissolved solid content (%)	85	85.65
4	Total sugar content (%)	50	51
5	Total reducing sugar content (%)	15	14.42
6	Total Nitrogen content (%)	<3	0.4
7	pH	…..	6.7

Table 4.1, shows that cane molasses was very rich in dissolved solid (85.65 %) but low in ash content (13.45%). The molasses has well-enough sugar content (51 %) for organic acid production especially for gluconic acid production. The amount of nitrogen present in molasses (0.4%) is not sufficient for fungus growth specifically for *Aspergillus carneus* growth. So it requires some source of nitrogen for optimum growth. The above finding was disagreement with the finding of(Bakhiet & Al-mokhtar, 2015) who reported as the percentage of moisture content was 65%, ash was 6.50% and the pH was 6.0±0.2. Even though it has low ash (inorganic salt) content its amount and type of salt should not be determined for microorganism growth. So inorganic salt would result inhibited the growth of

microorganisms for gluconic acid production .it should be treated and made comfortable for cell growth.

- **Total reducing sugar measurement**

In this study, the total reduced sugar content of cane molasses was determined. The crude molasses collected from sugar crystallization process was diluted 1:1 of distilled water. The amount of reduced sugar was investigated from standard calibration curve of glucose and its average absorbance as reported by(Anonymous, 2001). By preparing accurate weigh of standard glucose anhydrous in distilled water and common volume (5mL) of benedict solution. The prepared solution was heated in water bath and forms the following figure.



(a) (b) (c)

Figure4.1: Reduced sugar determination: (a) standard raw materials, (b) only benedict solution (c) Standard glucose solutions after being reacted with benedict's solution

Table 4.2: Absorbance of standard glucose solution reacted with benedict solution.

Standard glucose concentration (g/l)	av. Absorbance @ 540 nm
0	0
0.05	0.0005
0.1	0.0250
0.4	0.1550
0.8	0.2515
1	0.3355
1.25	0.4035
1.5	0.4510

By using spectrophotometer which measures the intensity of light, absorbance of standard glucose solution reacted with benedict solution was recorded in digital spectrophotometer at 540nm wavelength. Experimental results of average absorbance and calibration curve were shown in Table 4.2 and Figure 4.2 respectively.

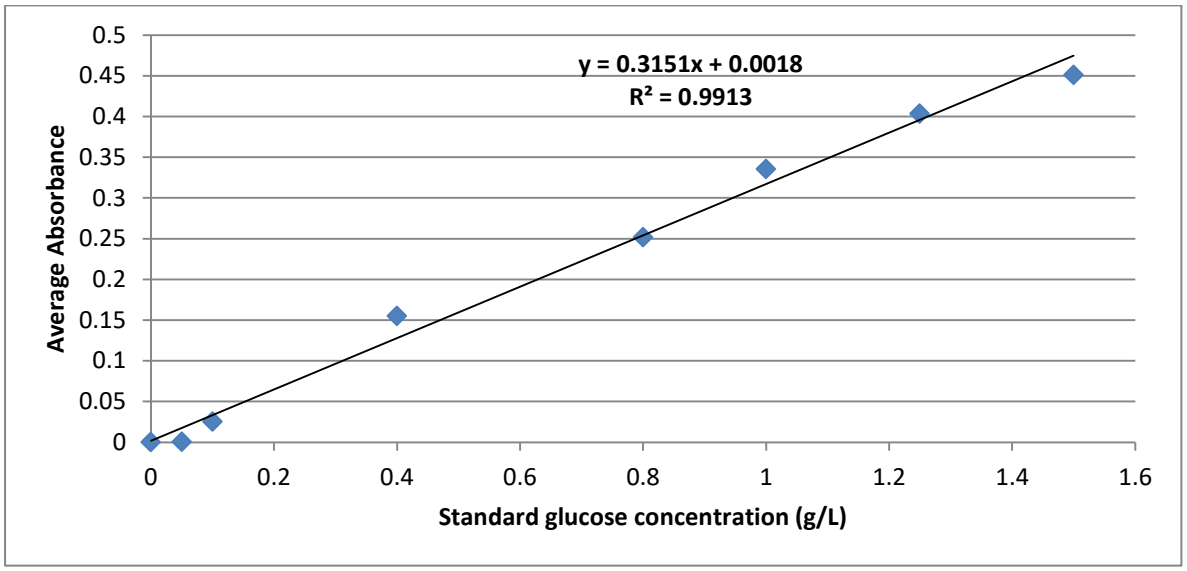


Figure 4.2: Calibration curve of standard glucose concentration with average absorbance

The concentrations of unknown sugar samples (molasses) were determined from a standard curve of glucose concentration ($Y = 0.3151X + 0.0018$) and the average absorbance of molasses sample was 0.8983. The amount of yield of total reduced sugar in molasses is 14.426%. This confirms the Ethiopian standard proclamation.

4.2. Treated cane molasses

For this study potassium ferrocyanide pretreatment technique were used and the concentration of inorganic salts and metallic ions could decrease and raise the quality and yield of fermentation.

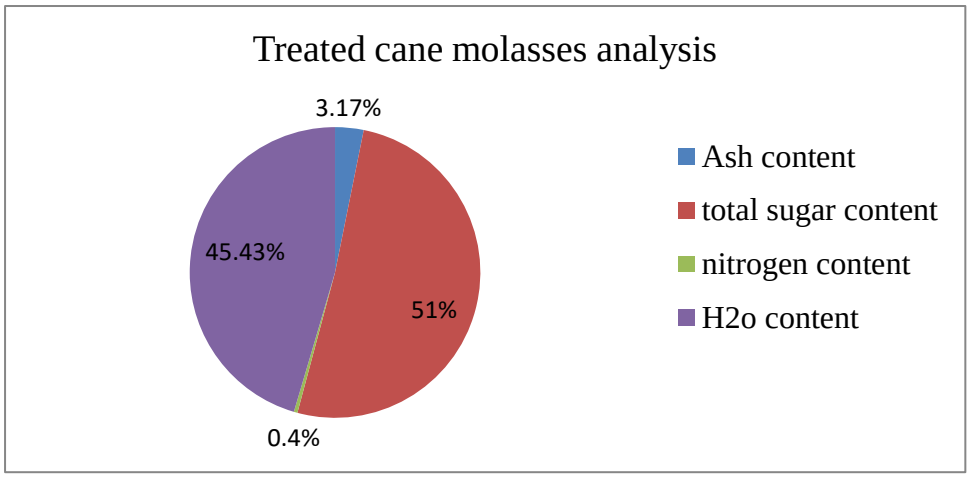


Figure 4.3: Treated cane molasses analysis

The level of metal ions and inorganic salts after pretreatment technique is summarized in figure4.3. As shown this figure ash content of treated molasses (3.17 %) is very low as compared to crude molasses ash content (13.45%). This reduction of ash shows low level of metal ions and inorganic salts in the treated cane molasses since all organic compounds are burnt and volatilized. So the effect of metal ions and inorganic salts on microorganism growth was reduced. The effect of inhibitors were decreased and made molasses comfortable to fermentation. It can be observed that potassium ferrocyanide treatment reduces the metal ions and inorganic salts considerably which was agreed to(Rao & Panda, 1994) that found the potassium ferrocyanide pretreatment technique is the promising one from other techniques. As reported by(Ashraf & Ali, 2015) potassium ferrocyanide was employed best metal complexing agent for molasses pre-treatment. Treatment of molasses increases the amount and quality of acid production. But at higher concentration of potassium ferrocyanide the acid production was decreased because at high level of potassium ferrocyanide the fungal growth were inhibited. This finding confirms the need for the reduction of heavy metals in molasses.

4.3. Dry cell mass

The dry cell mass was obtained after centrifugation of fermented broth sample through pre-weighted centrifuge tubes. The cell was washed with distilled water and dried to get constant weight. The cell growth was increases as the carbon source concentration was increases appreciably as shown from figure 4.4. It can be seen that the minimum amount of dry cell (1.3&1.5g/L) were recorded at a minimum carbon source concentration(50g/L)which shows that the growth of cell in low concentration is not facilitated(Singh & Singh, 2006). To get best result of gluconic acid the growth of cell makes spore and secrete glucose oxidase enzyme. The considerable cell growth took place in 150g/L carbon source concentration under 5 days. After this day the cell concentration was approximately increases with carbon source concentration. The maximum cell production rate was (11.3g/L per 5day $=$ $2.26 g/L\ day$) in temperature 32.5℃ and pH 7 which lead cell concentration had significant effect on gluconic acid accumulation. So the cell concentration with significant effect stimulates the production of gluconic acid at different conditions of variables as reported by(Znad *et. al.,* 2004).

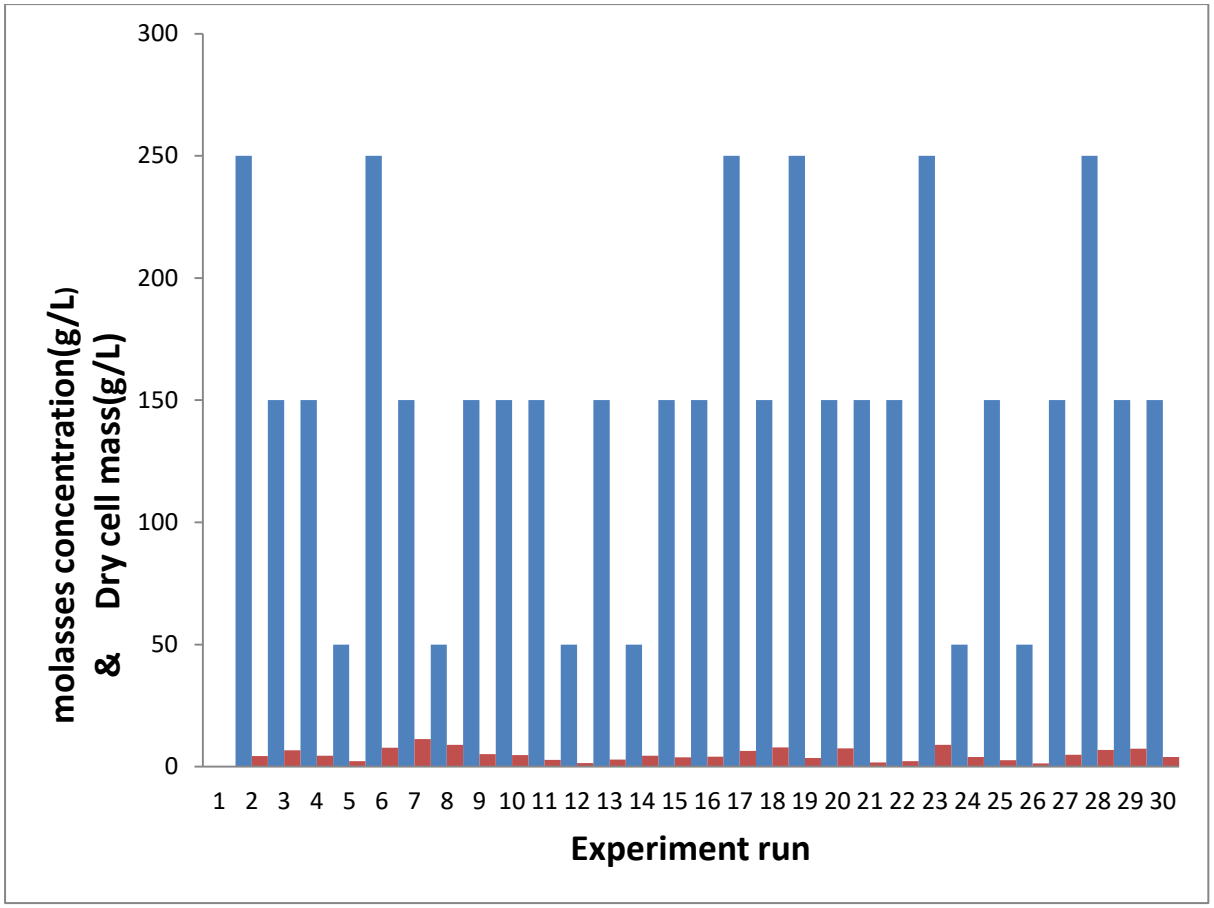


Figure 4.4: dry cell mass determination at each experimental run

4.4. Statistical analysis of gluconic acid production

The experimental design selected for this thesis work was the Box-Behnken Design (BBD) and the response variables measured were the dry cell mass and gluconic acid yield. Box – Behnken design is the commonly used experimental design model for four factors with three levels which minimizes the run of experiments(Montgomery, 2002). Box – Behnken design considers the optimum combination of factor levels to compensate effective evaluation of factor levels.

Table 4.3: Design summary

Study type	Response surface
Initial design	Box-Behnken
Design model	Cubic polynomial
Runs	29
Blocks	No blocks
Center points	5

Design-Expert Software 7.0.0 trial was used in the least squares regression analysis of variance (ANOVA). The statistical software program is used to generate the model equation, effect of individual variables, interaction effects of the independent variables, contour and surface plots using the fitted equation obtained from the regression analysis holding one or two of the independent variables are constant. The BBD conditions and their respective responses are given in Table 4-4.

Table 4.4: Box-Behnken design conditions with respective response

run	Temperature (℃)	Carbon source concentration (g/L)	pH	Incubation time (day)	Dry cell mass(g/L)	Gluconic acid(g/L medium)	%yield of gluconic acid
1	32.5	250	3	5	4.4	128.1	51.24
2	32.5	150	5	5	6.7	58.33	38.88
3	40	150	7	5	4.5	73.2	48.8
4	32.5	50	5	3	2.2	19.22	38.44
5	25	250	5	5	7.8	96.35	38.14
6	25	150	7	5	11.3	10.91	7.27
7	25	50	5	5	8.9	9.30	18.60
8	32.5	150	3	3	5.1	42.12	28.16
9	32.5	150	5	5	4.8	52.9	35.28
10	40	150	5	7	2.8	52.20	34.80
11	32.5	50	5	7	1.5	23.93	47.86
12	40	150	3	5	2.9	21.70	14.47
13	32.5	50	3	5	4.5	11.45	22.90
14	25	150	3	5	3.8	24.8	16.5
15	32.5	150	5	5	4.1	56.94	37.96
16	32.5	250	7	5	6.5	141.58	56.63
17	25	150	5	7	7.9	54.10	36.06
18	40	250	5	5	3.5	45.51	18.20
19	25	150	5	3	7.5	34.10	22.73
20	32.5	150	7	3	1.7	63.22	42.15
21	40	150	5	3	2.3	27.90	18.60
22	32.5	250	5	7	8.9	114.2	45.68
23	32.5	50	7	5	4	11.78	23.56
24	32.5	150	5	5	2.7	49.10	32.73
25	40	50	5	5	1.3	12.50	24.30
26	32.5	150	3	7	4.9	44.76	29.84
27	32.5	250	5	3	6.8	87.28	34.91
28	32.5	150	7	7	7.3	67.24	44.83
29	32.5	150	5	5	3.9	58.69	39.13

The results of the 29 conducted experiments were statistically analyzed to evaluate the effect and significance of the variables(Montgomery, 2002). The results of ANOVA for the predicted models by the design expert software are shown in Table 4.5.

Table 4.5: Analysis of variance (ANOVA) for Response Surface Cubic Model

Source	Sum of squares	Df	Mean square	F-value	P-value prob>F	Remark
Model	5502.64	22	250.12	8.89	0.006	Significant
A-temperature	15.76	1	15.76	0.56	0.4824	
B-carbon source concen	1102.24	1	1102.24	39.19	0.0008	
C-pH	578.64	1	578.64	20.56	0.0039	
D-incubation time	313.14	1	313.14	11.13	0.0157	
AB	0.20	1	0.20	7.04E-03	0.9359	
AC	480.05	1	480.05	17.07	0.0061	
AD	65.61	1	65.61	2.33	0.1775	
BC	580.09	1	580.09	20.65	0.0039	
BD	32.15	1	32.15	1.14	0.3261	
CD	20.66	1	20.66	0.73	0.4243	
A^2	3.77	1	3.77	0.13	0.7267	
B^2	29.73	1	29.73	1.06	0.3435	
C^2	157.87	1	157.87	5.61	0.0556	
D^2	295.95	1	295.95	10.52	0.0176	
A^2B	379.36	1	379.36	13.49	0.0104	
A^2C	5.76	1	5.76	0.20	0.6667	
A^2D	332.69	1	332.69	11.83	0.0138	
AB^2	0.83	1	0.83	0.029	0.8696	
AC^2	293.30	1	293.30	10.43	0.0179	
B^2C	0.20	1	0.20	7.06E-03	0.9358	
B^2D	229.94	1	229.94	8.18	0.0288	
BC^2	734.79	1	734.79	26.13	0.0022	
Residual	168.73	6	28.12			
Lack of fift	9.33	2	4.66	0.12	0.8925	Not significant
Pure error	159.41	4	39.85			
Cor total	5671.37	28				

The Model F-value of 8.89 implies the model is significant. There is only a 0.60% chance that a "Model F-Value" this large could occur due to disturbance or personal error. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Higher F-values show the increased significance of the factors or models and approaching of F-value to one indicates that the model is not significant and the factor doesn't have significant effects(Rasoulnia & Mousavi, 2016). On the other hand "Prob > F" value greater than 0.1000 shows the model terms are not much significant. In this case B, C, D, AC, BC, D^2, A^2B, A^2D, AC^2, B^2D, BC^2 are significant model terms. This shows that the individual parameters which are carbon source concentration, initial pH and incubation time affects significantly on the fermentation of molasses to gluconic acid. In addition to these the interaction effect of temperature and pH, carbon source concentration and pH, square of the incubation time, square of temperature and carbon source concentration, square of temperature and incubation time, temperature and square of pH, square of carbon source concentration and incubation time, carbon source concentration and square of pH, affects the percentage yield of gluconic acid in fermentation process significantly(Montgomery, 2002).

The "Lack of Fit F-value" of 0.12 implies that the Lack of Fit is not significant relative to the pure error. There is an 89.25% chance that a "Lack of Fit F-value" this large could occur due to noise or personal error. Non-significant lack of fit is good since we want the model to fit exactly. The data obtained from the statistical models versus experimental data have been shown in Figure.4.15. The points in the vicinity of the diagonal line imply presence of a satisfactory correlation between the predicted and experimental data, confirming the fitness of the models.

4.4.1. Effect of gluconic acid production variables

Based on the analysis of variance, fermentation process of gluconic acid was significantly affected by various process variables and interactions between the process variables. This result demonstrated that the advantage of using BBD surface response for experimental data analysis in capturing the interaction between variables that affects the fermentation process(Zhang, et.al, 2016). To investigate the effects of fermentation influencing factors of temperature, Carbon source concentration, pH and inoculation time on the production of gluconic acid was analyzed by point scattering and interaction effect of these factors, the two dimensional and three dimensional contour plots have been presented. Each plot in each section illustrates their effects on production of gluconic acid.

4.4.2. Effect of individual production variables

4.4.2.1. Effect of temperature

The effect of temperature on the fermentation process of gluconic acid production within the range of 25 to 40℃ has slight effect as shown in ANOVA (Table 4.5) at high concentration of carbon source. Since the microorganism can develop and excrete glucose oxidase enzyme for molasses oxidation. But at low concentration of carbon source, low pH and low incubation time have significant effect on the production of gluconic acid as shown in figure 4.5. From the figure it can be seen that with increasing temperature until it reaches around center value would result increasing in the percentage yield of gluconic acid, then after it starts to drop as the temperature tend to increase above the center limit. This reduction of yield might be due to many reasons such as denaturation of microorganism at high temperature and the conditions under which the fermentation took place. The highest gluconic acid was recorded at 32.5℃ with best agreement to published documents. As reported by (Mounir et al., 2016)at high temperature the process could produce acetic acid rather than gluconic acid under batch fermentation conditions. This indicates beyond 35℃ the growth of microorganism could be depleted. 30℃ was also found to be the optimum temperature for efficient fermentation for highest gluconic acid production in medium carbon source concentration (molasses concentration) as reported by(Ahmed et al., 2015). As Roukas studied that the maximum gluconic acid concentration and gluconic acid yield were obtained at 30℃ (Roukas, 2000).

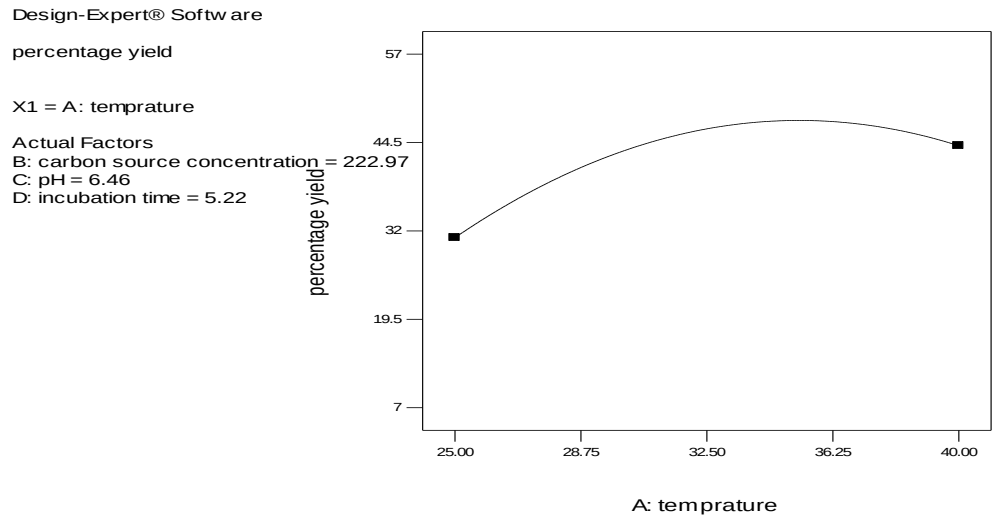


Figure 4.5: Effect of temperature on the yield of gluconic acid at high carbon source concentration and initial pH, mean value Incubation time.

4.4.2.2. Effect of carbon source concentration

Concentration of carbon source highly affects the yield of gluconic acid. As shown from figure4.6, the results illustrates that maximum gluconic acid was obtained at high concentration of molasses. So increases of molasses concentration would result increase of gluconic acid yield until it reach the optimum concentration requirement (250g/L) and then the yield decreases as further increases of the concentration of molasses since unreacted molasses concentration increases appreciably inhibitory response to microorganism as studied by(Singh et.al, 2001). Ahmed found that utilization of molasses for gluconic acid production under submerged fermentation by *aspergillus niger* was the efficient sole carbon source(Ahmed et al., 2015).

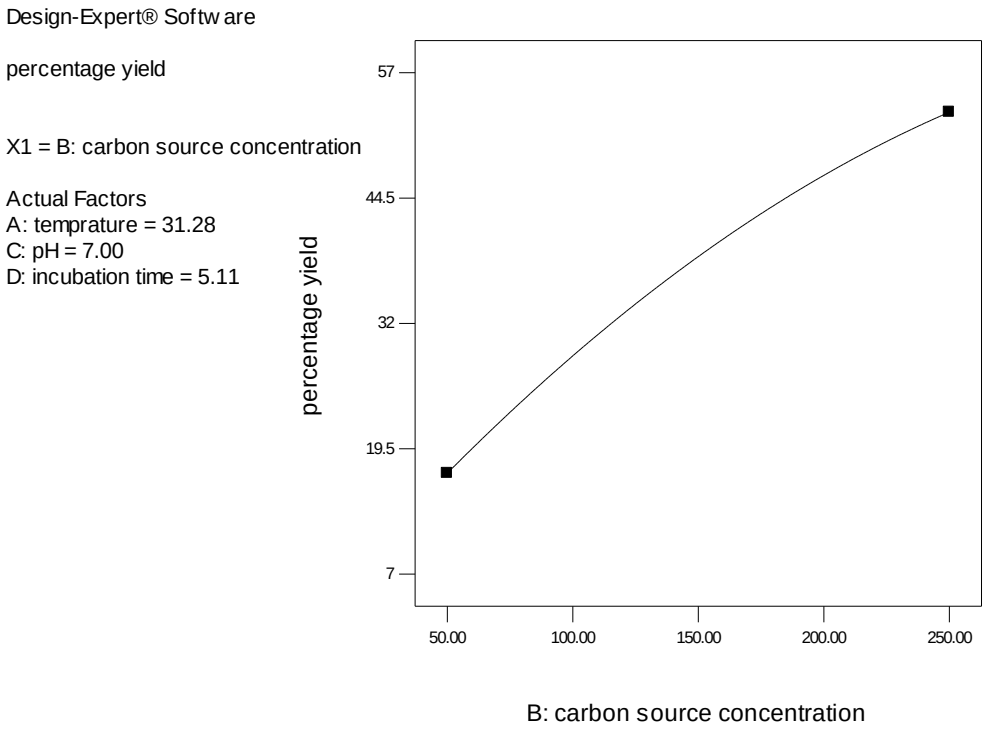


Figure 4.6: Effect of carbon source concentration on the yield of gluconic acid at high initial pH, mean value of temperature and Incubation time.

4.4.2.3. Effect of initial pH

The initial pH value of culture media is one of the most critical factors affecting the fungal growth as well as the fermentation process of gluconic acid formation. The quantities of gluconic acid and dry cell mass with respect to initial pH of the fermentation media are presented in Table 4.4. Experimental results showed that the effect of the initial pH on the gluconic acid production and the growth of *aspergillus carneus* through submerged fermentation in batch cultivation ranged from 3 to 7 have significant effect. As shown from figure 4.7, the percentage yield of gluconic acid production was increases continuously as pH increases. The highest gluconic acid (141.58g/L medium) was produced at pH 7 for the combination of mean value temperature and carbon source concentration and high incubation time. Gluconic acid production by irradiated *A. niger* in pH 6 is the optimum value for the growth of this strain and its gluconic acid production as reported by(Ahmed et al., 2015). This result is the best in agreement to the current research work, that initial pH 7 is the optimum value for the growth of *aspergillus carneus* and its gluconic acid production as studied by(Roukas, 2000). The pH range of the fungi for the production of gluconic acid is around 4.5 to 7.0. pH 5.5 is generally considered as optimum for *Aspergillus* species (Ramachandran et al., 2006) was agreed with our result. At low pH value the fermentation process facilitates the citric acid formation rather than gluconic acid, since low pH triggers the citric acid cycle.

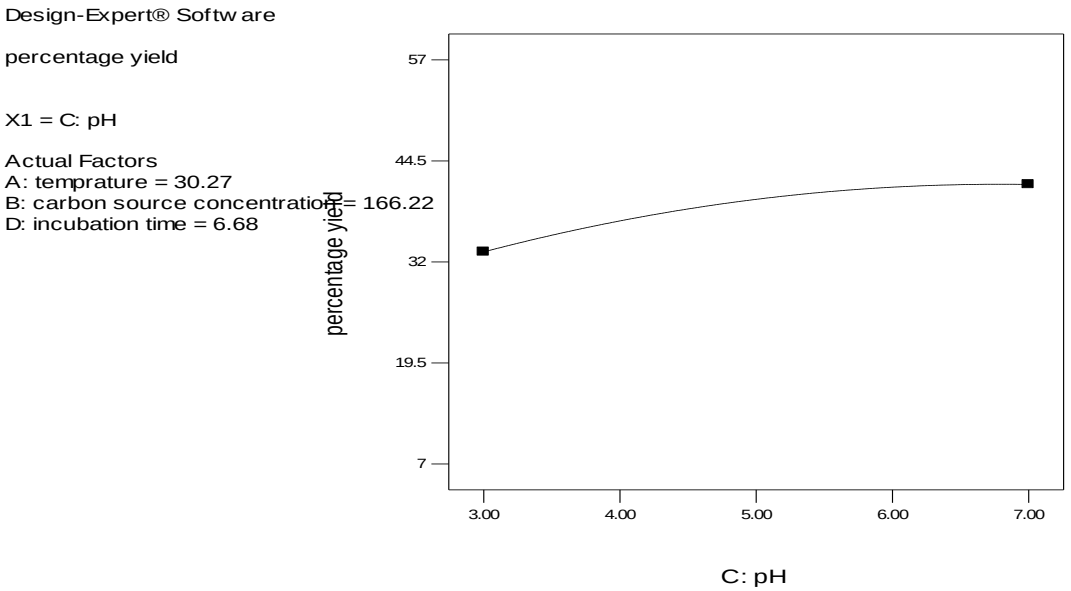


Figure 4.7: Effect of pH on the yield of gluconic acid at high Incubation time, mean value of temperature and carbon source concentration.

4.4.2.4. Effect of incubation time

The period of incubation also has an effect on the growth of *Aspergillus carneus* and the production of gluconic acid. Figure 4.8 showed that the maximum production of gluconic acid was gained at high incubation time (7days). The production of gluconic acid and the growth of fungi are affected with the change of the incubation period slightly, whereas gluconic acid production was decreased by increasing or decreasing the incubation period of 7 days. These result are similar to data showed by(Ahmed et al., 2015) Whereas tabulated data (Table4.4) showed that the maximum gluconic acid production was 141.57g/L produced by *A. carneus* at incubation time of 5 days.

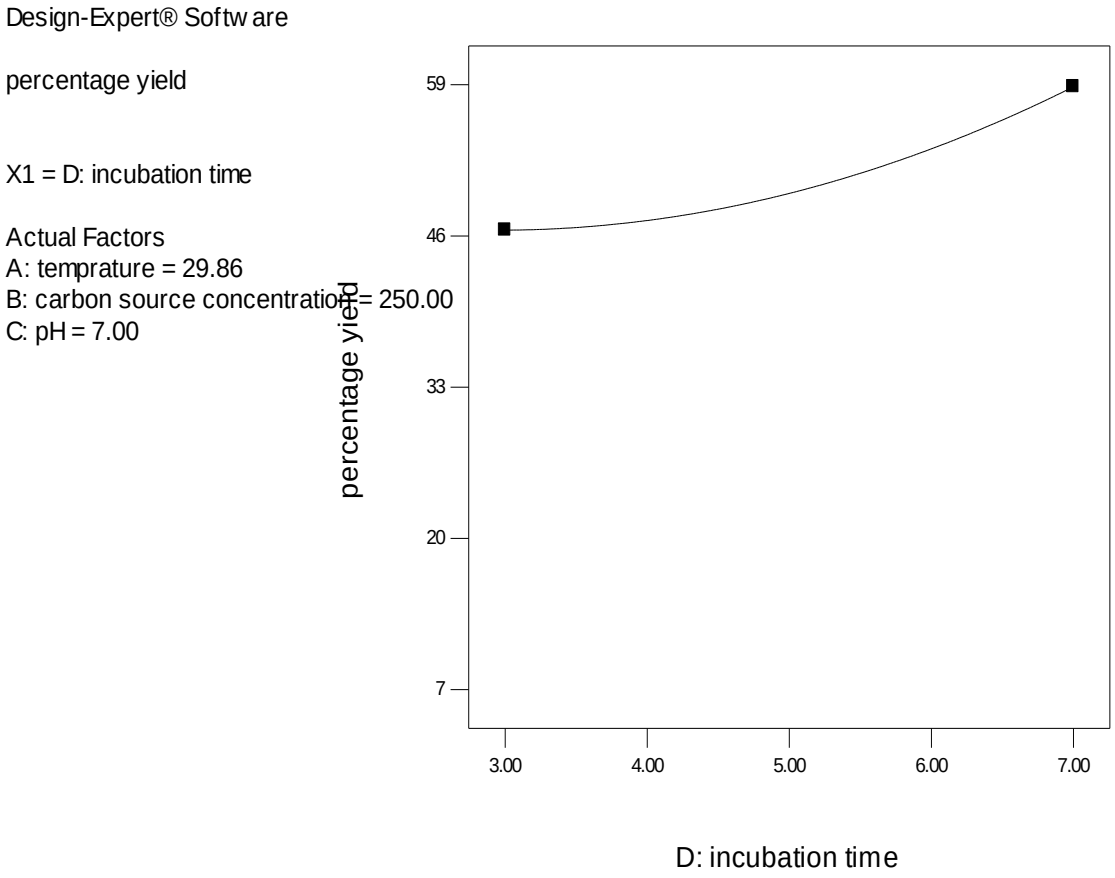


Figure 4.8: Effect of Incubation time on the yield of gluconic acid at high initial pH and carbon source concentration, mean value of temperature.

4.4.3. Effect of interaction b/n production variables

The most common way to summarize the results of a Box – Behnken design experiment is in the form of a response surface plot and response contour plot. As shown from Table4.5 the Interaction effect of temperature and pH, carbon source concentration and pH, square of the incubation time, square of temperature and carbon source concentration, square of temperature and incubation time, square of carbon source concentration and incubation time, carbon source concentration and square of pH, affects the percentage yield of gluconic acid in fermentation process significantly. As shown from figure 4.9 the interaction effect of temperature and pH on the yield of gulconic acid production at high carbon source concentration and mean value of incubation time. It can be seen that high yield of gluconic acid was obtained at high pH whereas at low pH the yield of gluconic acid was decreases as the temperature Increases continuously. To get high yield the experiment should proceed at high pH 7 and mean value of temperature32.5℃. This Interaction effect was agreed with (Ahmed et al., 2015). The results of (El-Sherbeny & Shindia, 2005)are in good agreement with our results that the optimum PH and temperature for glucose oxidase production were 5.6 and 37℃ respectively.

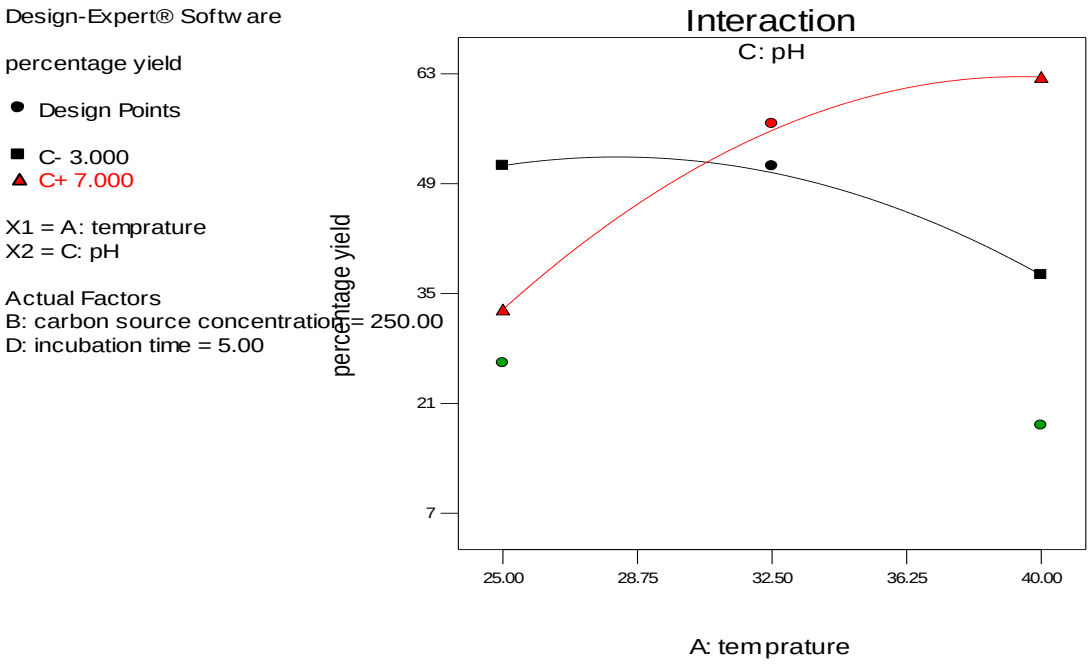


Figure 4.9: Interaction effect of temperature and pH on the yield of gluconic acid at high carbon source concentration and mean value of incubation time.

The contour plot of the interaction effect of temperature and incubation time on the yield of gluconic acid has slight effect as shown in figure 4.10. The percentage yield of gluconic acid at high incubation time and at low incubation time are 39.85%and 34.96% respectively. This result shows low difference between them which implies the interaction effect of optimum temperature and incubation time that much not significant at high carbon source concentration and mean value of pH. This finding was not agreed to the finding of (Macris., 1995)that shows the optimal glucose oxidase growth was at incubation temperature of 45℃ and fermentation time of 72 h in submerged fermentation.

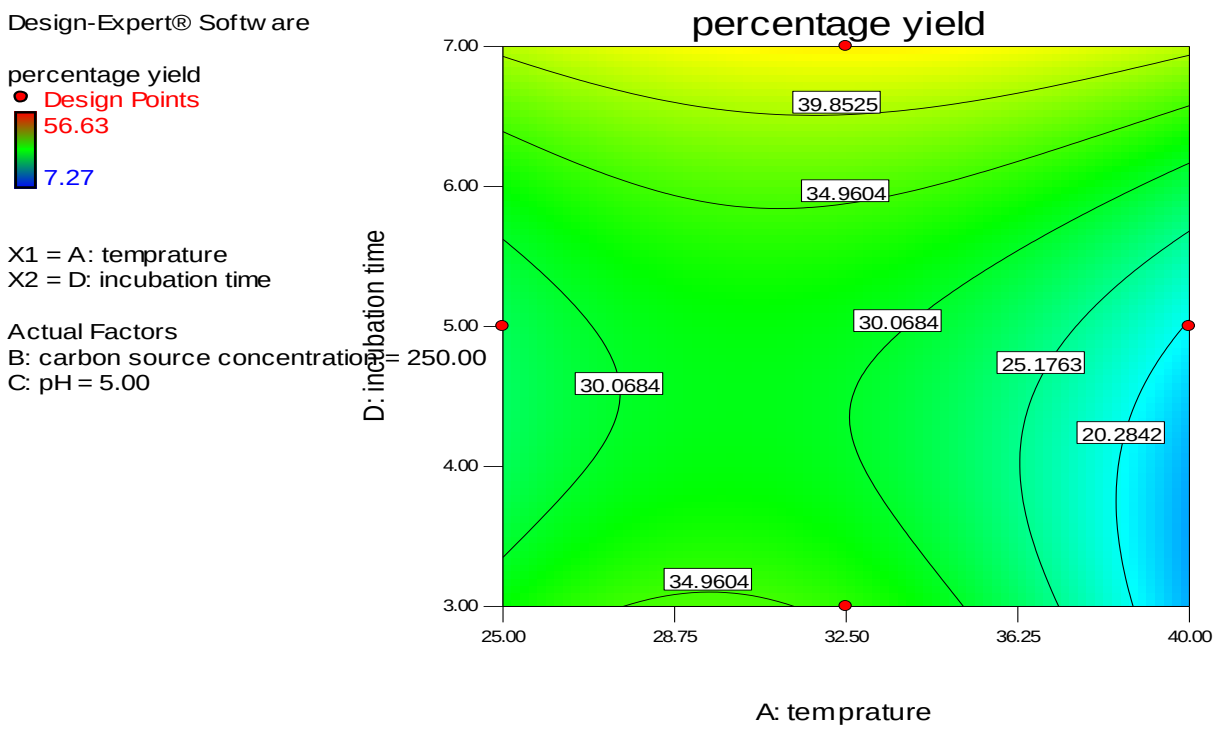


Figure 4.10: Contour plot of the interaction effect of temperature and incubation time on the yield of gluconic acid at high carbon source concentration and mean value of pH.

The Contour plot of the interaction effect of carbon source concentration and pH on the yield of gluconic acid has significant effect as shown below in figure4.11. The maximum production were obtained at high both carbon source concentration and pH value respectively. These finding was agreed with the finding of (Ramachandran et al., 2006)which shows that the optimal condition for gluconic acid production from Glucose at concentrations between 110–250 g/L and pH value of medium around 4.5 to 6.5. Also as reported by (Nagra, 2002), the concentration of carbon source and pH are play an important role on the conversion of glucose into gluconic acid and its calcium salt. In this study the maximum

glucose conversion into gluconic acid was found when the glucose concentration is 150g/L and the pH was at 6.5. Therefore, for ensuring efficient gluconic acid production with A*spergillus carneus* entails using relatively high initial concentrations of molasses and at high pH. This conclusion also agreed to(Jimenez-hornero et al., 2017) In gluconic acid production by acetic acid bacteria that found low initial concentrations of molasses and low pH were found to inhibit the formation of gluconic acid.

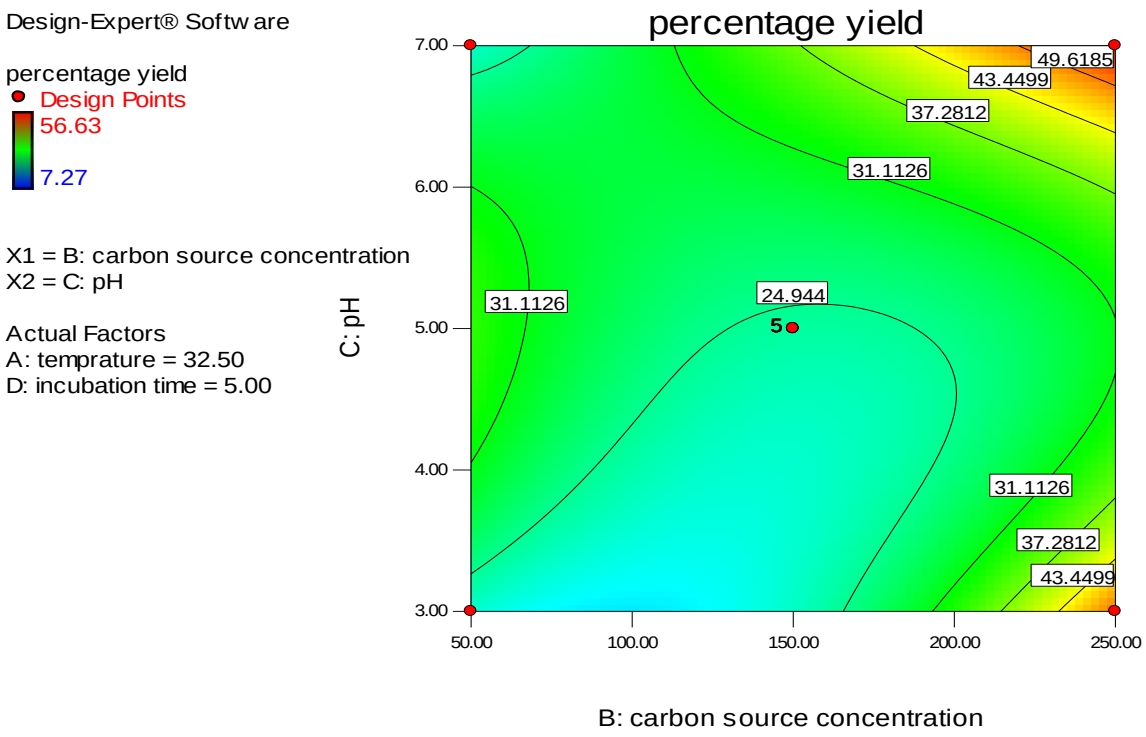


Figure 4.11: Contour plot of the Interaction effect of carbon source concentration and pH on the yield of gluconic acid at mean value of temperature and incubation time.

The surface plot of the interaction effect of incubation time and pH on the yield of gluconic acid was indicated from in figure 4.12. It can be seen that the percentage yield of gluconic acid at mean value of pH is very low from low to high incubation time. Whereas the percentage yields of gluconic acid at high pH is increases from low to high incubation time. Thus, high percentage yield of gluconic acid was obtained at high pH (7) and high incubation time (7days) with optimal additional factors of carbon source concentration (250g/L) and mean value of temperature (32.5℃).

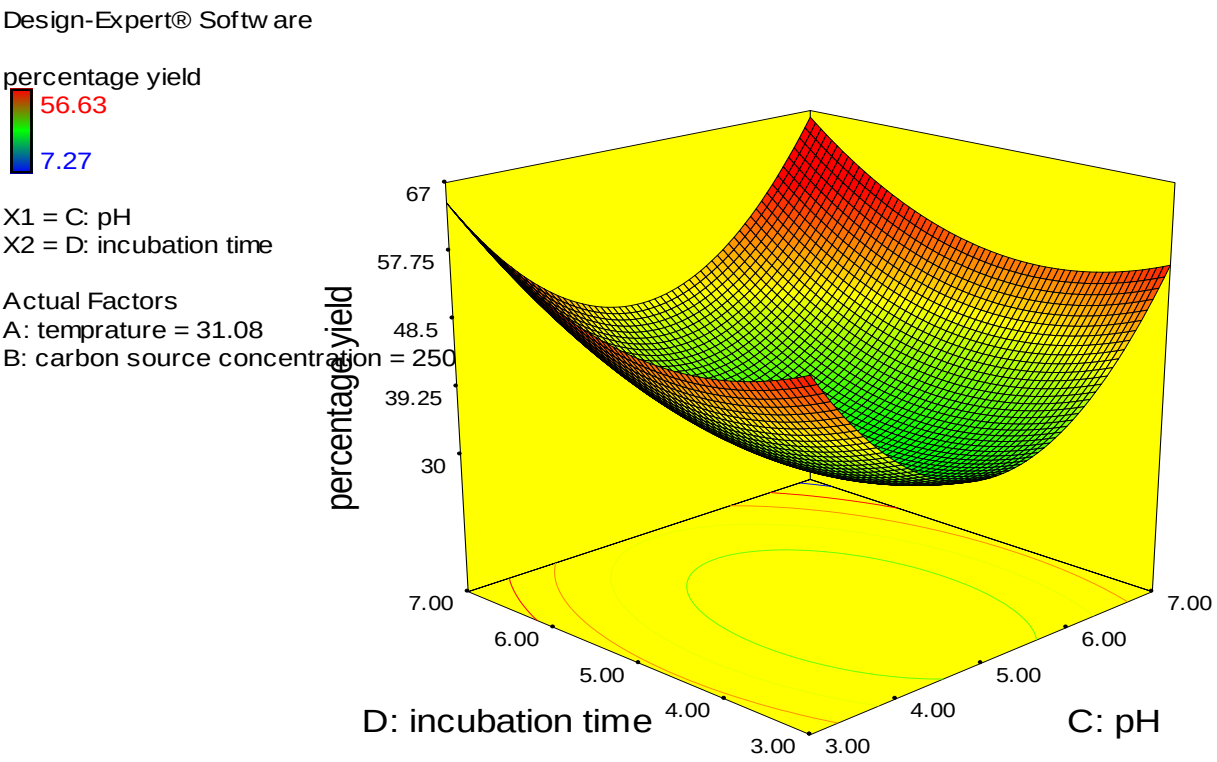


Figure 4.12: Surface plot of the Interaction effect of incubation time and pH on the yield of gluconic acid at high carbon source concentration and mean value of temperature.

The surface plot of the Interaction effect of carbon source concentration and incubation time on the yield of gluconic acid was indicated as below in figure4.13. As shown from this figure the percentage yield of gluconic acid was increases as both factors are increases simultaneously. However at low carbon source concentration and incubation time produces other organic acids rather than gluconic acid since low concentration of carbon source favors citric acid formation. If the amount of carbon source concentration was increases above high limit (250g/L), the formation of gluconic acid was decreases and the residual sugar after fermentation was increases abundantly at high incubation time. Furthermore, further increase of incubation time(>7days) decreases the formation of gluconic acid due to inactivation of microorganism to oxidize the molasses at high pH(7) and mean value of temperature (32.5℃). As reported by(Dowdells et.al, 2010) the maximum gluconic acid production by *A.terreus* was after 6days and at high glucose concentration above 100g/L. Before this day and at low glucose concentration the accumulation of gluconic acid is not sufficient.

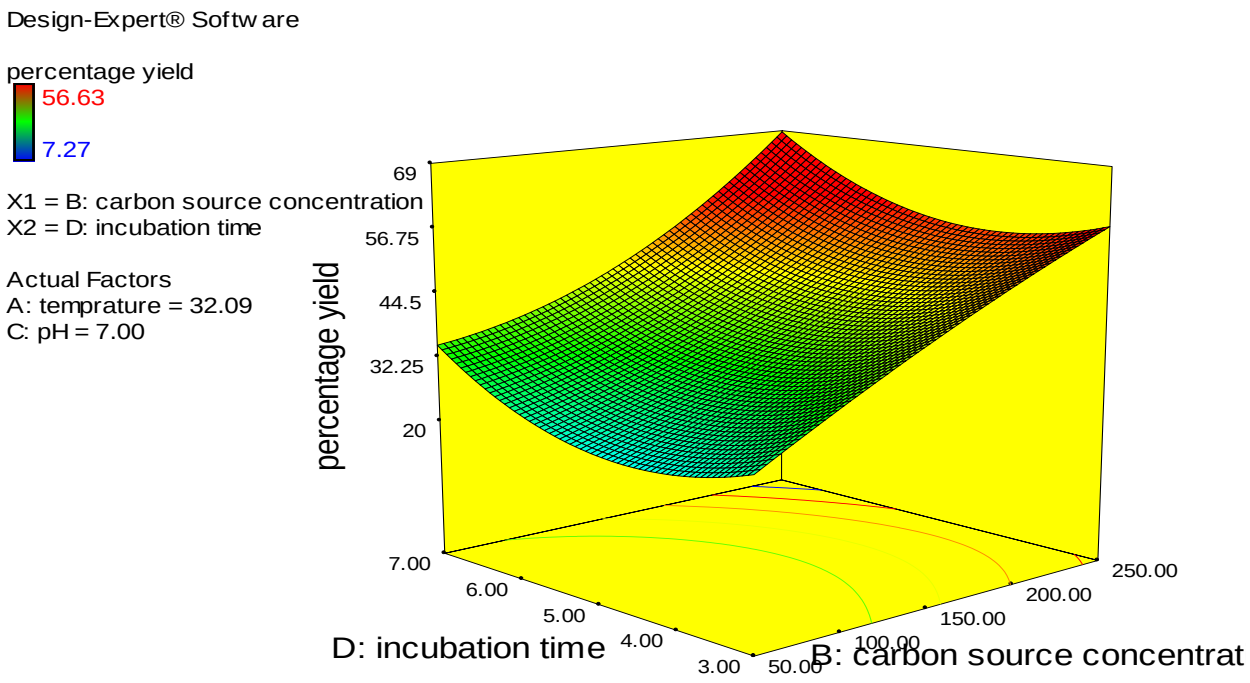


Figure 4.13: Surface plot of thee Interaction effect of carbon source concentration and incubation time on the yield of gluconic acid at high pH and mean value of temperature.

The cube plot of the Interaction effect of pH, carbon source concentration and incubation time on the yield of gluconic acid was indicated in figure4.14. As shown from the figure the percentage yield of gluconic acid was plotted as corner value of cube. Cube plot has only considers low and high value of influential factors. It doesn't consider mean values of variables. Even though, it has less application to conclude the interaction effect, it shows the interaction of three influential factors at a time with respect to the response. It can be seen the minimum yield of gluconic acid (25.97%) was obtained at low values of pH (3), carbon source concentration (50g/L) and incubation time (3days). Whereas the maximum yield of gluconic acid (69.80%) was recorded at hih values of influential factors of pH, carbon source concentration and incubation time (7, 250g/L and 7days) respectively at mean value of temperature (32.5^{o}C). Another maximum option of yield of gluconic acid (63.91%) was obtained at of pH(3), carbon source concentration(250g/L) and incubation time(7days).

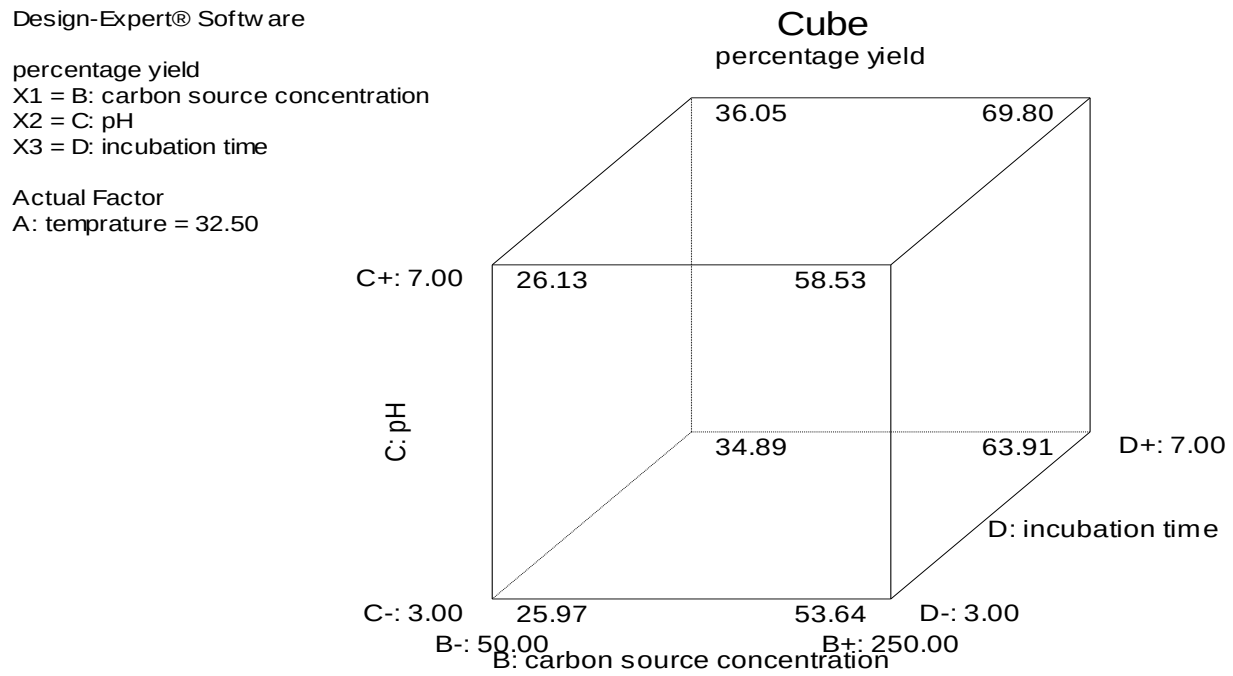


Figure4.14: cube plot of the Interaction effect of pH, carbon source concentration and incubation time on the yield of gluconic acid at mean value of temperature.

4.4.4. Optimization of process variables

Actually, Optimization process by itself is not easy process, since it considers other constraints other than process variables such as cost, market study and environmental regulation etc. But know it considers only the process variables. This optimization was aimed to get the highest percentage yield of gluconic acid by using the model regression developed from design expert. To validate the optimization process it follows optimization criteria. The optimization of fermentation process criteria for gluconic acid production from molasses by using *Aspergillus carneus* microorganism was summarized in Table4.6.

Table 4.6: Optimization criteria for optimum yield of gluconic acid

Name	Goal	Lower limit	Upper limit
Temperature(℃)	in range	25	40
Carbon source conc(g/L)	Maximum	50	250
pH	maximum	3	7
Incubation time(day)	in range	3	7
Dry cell mass(g/L)	Maximum	1.3	11.3
Gluconic acid(g/L)	Maximum	9.3	141.58
Percentage yield (%)	Maximum	7.27	56.63

After making the criteria the software displays optimum possible solutions with desirability. This desirability lies between 0 and 1 to represent the closeness of response to ideal value. If the desirability is close to 0, a response falls within the unacceptable intervals and the optimization isn't satisfactory. If the desirability is close to 1 and perfect 1, a response falls within the ideal intervals or the response reaches its ideal value and the optimization is definitely efficient.

Based on the desirability analysis the first optimum possible solution is selected which gives the maximum percentage yield of gluconic acid 61.96% was found at temperature 30.13℃,carbon source concentration 250g/L, pH 7.00 and incubation time 6.86 days and the value of desirability obtained was 1.

Table4.7: Optimum possible solutions

Soln num	Temp	Carbon concen	pH	Incubation time	Dry cell mass	Gluconic acid	%yield	Desirability
1	**30.13**	**250.00**	**7.00**	**6.86**	**11.402**	**144.869**	**61.96**	**1.00 (selected)**
2	29.26	249.99	7.00	6.57	11.299	136.952	57.180	0.979
3	28.29	250.00	7.00	7.00	12.532	134.462	57.684	0.966
4	33.60	250.00	7.40	7.00	10.105	160.57	72.426	0.963
5	30.58	249.12	6.57	7.00	10.548	135.792	56.632	0.963
6	26.74	247.60	7.00	7.00	13.322	118.54	51.435	0.944
7	33.42	250.00	7.00	5.60	8.178	141.959	60.104	0.928
8	32.79	248.67	6.33	7.00	9.279	135.267	56.629	0.911
9	32.72	250.00	7.00	4.94	7.535	135.147	56.058	0.899
10	27.84	245.55	6.37	7.00	11.299	113.086	46.982	0.877

4.4.5. Model equation development

The model equation developed that correlates the response (percentage yield of gluconic acid) to the fermentation process variables in terms of actual value after excluding the insignificant terms was determined well. Since some terms are aliased with another one. The predicted model for percentage yield of gluconic acid in terms of the coded factors is given in equation (4.1).

$$Percentage\ yield\ of\ gluconic\ acid = +17.87 + 1.98A - 16.60B - 12.03C - 8.85D - 0.22AB + 10.95AC + 4.05AD - 12.04BC - 2.83BD + 2.27CD - 0.76A^2 + 2.14B^2 + 4.93C^2 + 6.75D^2 + 13.77A^2B - 1.70\ A^2C + 12.90A^2D + 0.64AB^2 - 12.11AC^2 + 0.32B^2C + 10.72B^2D + 19.17BC^2 \quad \ldots\ldots (4.1)$$

Where: A=temperature

B=carbon source concentration

C=initial pH

D=incubation time

4.4.6. Model adequacy checking

The model accuracy was checked by analysis of variance (ANOVA) that identifies the correct correlation coefficients of the developed regression model. The determination of the correlation coefficients for adequacy R-Squared, adjusted R-Squared and predicted R-Squared are listed in the following table.

Table4.8: Model adequacy measures

Std.Dev	5.30	R-squared	0.9702
Mean	23.28	Adj R-squared	0.8612
C.V.%	22.78	Pred R-squared	0.7193
PRESS	1591.93	Adeq precision	11.241

The quality of the model developed could be evaluated from their coefficients of correlation. The value of R-squared for the model developed correlation is 0.9702. This result shows 97.02% of the total variation in the percentage yield of gluconic acid is attributed to the experimental variables studied. The "pre R- squared" value of 0.7193 is in reasonable agreement with the "Adj R- square" value of the 0.8612 in minimum difference of 0.1419 as

one might expect. This indicated a close fit of the model to the actual response data. “Adeq precision” measures the signal to disturbance ratio due to random error. A ratio greater than 4 is desirable. Here the ratio of 11.241 indicates an adequate signal. Therefore, the model can be used to navigate the design space. The close fitting relation of predicted values to actual value was also indicated by graph. The graph of the predicted values obtained using the developed correlation versus actual values is shown in Figure 4.15.

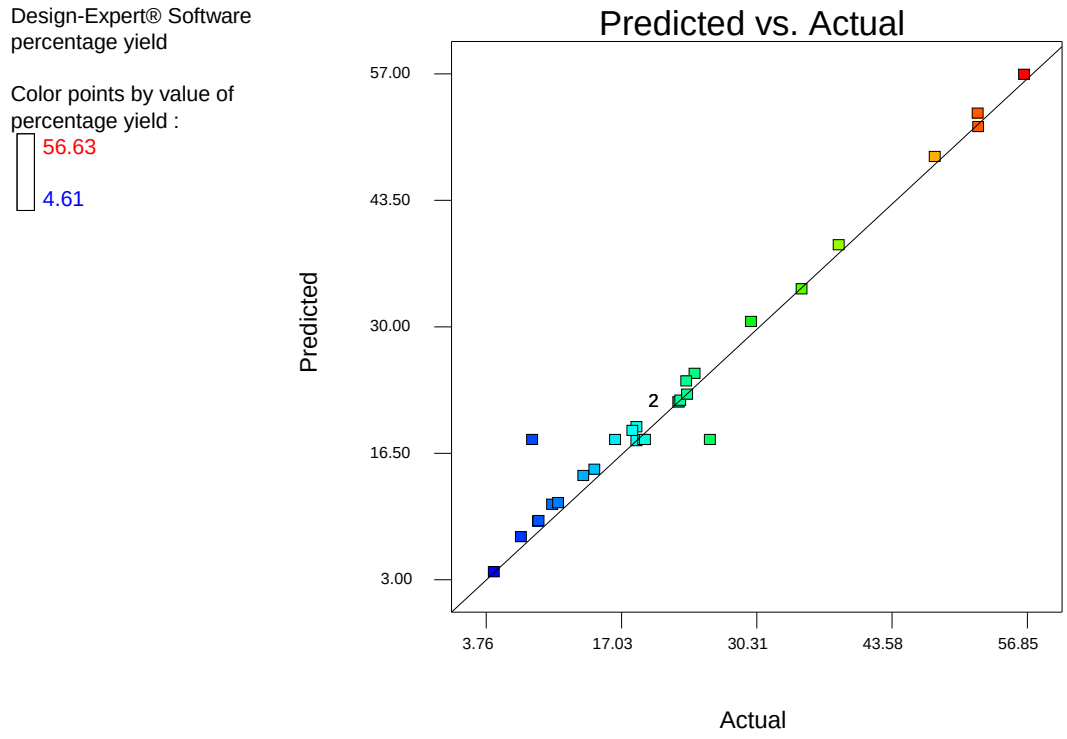


Figure4.15: Predicted versus actual percentage yield of gluconic acid production

Figure 4.15 demonstrated that the regression model equation provided a very accurate description of the experimental data, in which all the points are very close to the line of perfect fit. This result indicates that it was successful in recording the correlation between the four fermentation process variables to the percentage yield of gluconic acid. This model processes high reliability, high fitting degree, and deviation with coefficient of determination R^2 = 0.9702 and adequate precision of 11.241 as shown from Table4.8.

The adequacy of the model was further checked with analysis of variance (ANOVA) as shown in Table, based on a 95% confidence level, F – value is a test for comparing model variance with residual (error) variance. If the variances are close to each other, the ratio will be close to one and it is likely that any of the factors have a significant effect on the response

with the p – value less than 0.05. It is calculated by model mean square divided by residual mean square. Thus, On the whole, this model could be used for evaluation, optimization, and prediction of gluconic acid fermentation process variables.

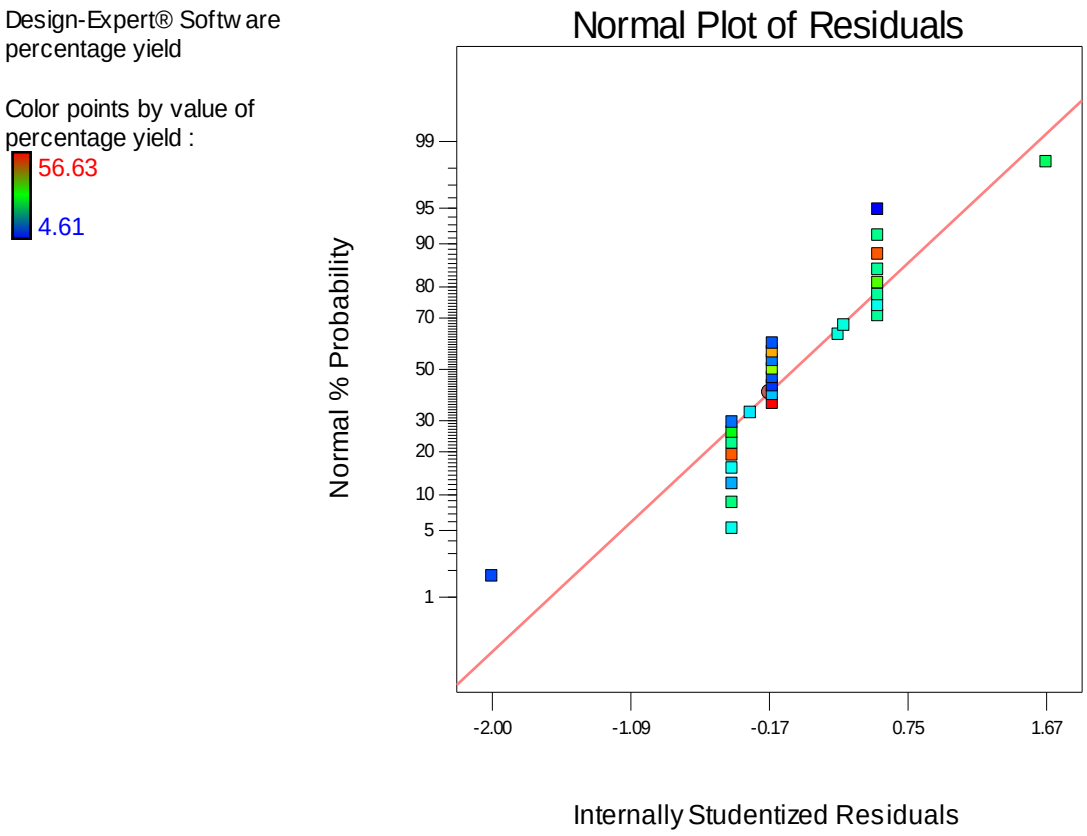


Figure4.16: Normal plot of residuals

4.4.7. Model validation

According to the Box-Benhken design result using Design-Expert® 7.0.0 software, an experiment with temperature, carbon source concentration, pH and incubation time were conducted in order to study the effect of the variables on gluconic acid yield. To get the highest outcome, the experiment was carried out at the optimized conditions. Based on the predicted model, numerical optimization was carried out to maximize the yield of gluconic acid, using the response optimizer in Design expert®7.0.0. The optimal values of variables were30.13℃ temperature, 250.00g/L carbon source concentration, 7 pH and 6.86 days of incubation time obtained from desirability Table 4.7. Gluconic acid yield of 61.96% was obtained with desirability value of 1 and in good agreement with the experimental result of 56.63%.

To validate the optimum conditions predicted by the model using desirability ramp response of model solution were conducted as shown figure 4.17. Therefore, the model is considered to be valuable, accurate and reliable for predicting the yield of gluconic acid. Hence, this study shows that molasses can be used for gluconic acid production with optimum fermentation process variables by using *Aspergillus carneus*.

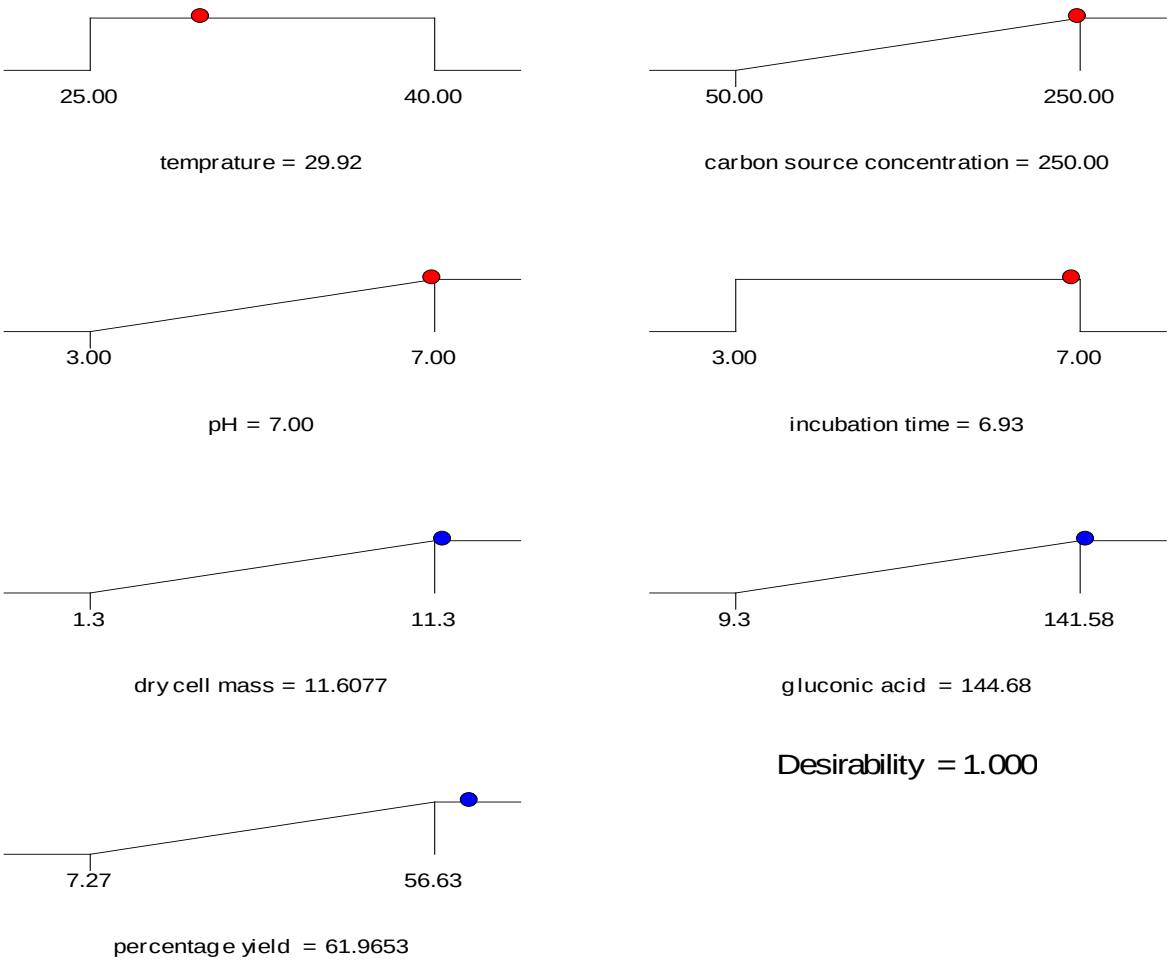


Figure 4.17: Ramp response of model solution

4.5. Fourier transform infrared spectroscopy analysis of gluconic acid

From the FTIR spectrum of gluconic acid figure4.18, it can be observed that the gluconic acid structure contains one broad band of high intensity in the range of 3200 to 3700 cm^{-1} with centroid at 3420 cm^{-1} which could be attributed to valance vibration of hydroxyl group, OH originating from the water present in gluconic acid(Nikolić et.al, 2014). The bands of valance vibrations of primary and secondary hydroxyl groups from gluconic acid also present in this area. There is another broad band peak originate from valance vibration of carboxyl groups(C=O). The band shown from figure 4.18 also exhibits broad band peaks 1600-1700 cm^{-1}were C=O stretching vibration peaks. The other peaks of band which show 600 to 700 cm^{-1} were the presence of cis-disubstituted alkenes (C-H) with strong appearance.

The broad band obtained from figure between 3200 to 3750cm^{-1} were confirmed by FTIR correlation table from appendix D which indicates the lactone(C=O) intensity is 1735cm^{-1}. Furthermore our FTIR spectrum of gluconic acid was excellent agreement to(Abdul Qadir et.al, 2012)and the FTIR spectrum of NIST gluconic acid from appendix E. Generally from this study the produced fermented broth product had C-H, OH-, C=O and CH_2 bonds which confirms the presence of gluconic acid and correlative with the standard.

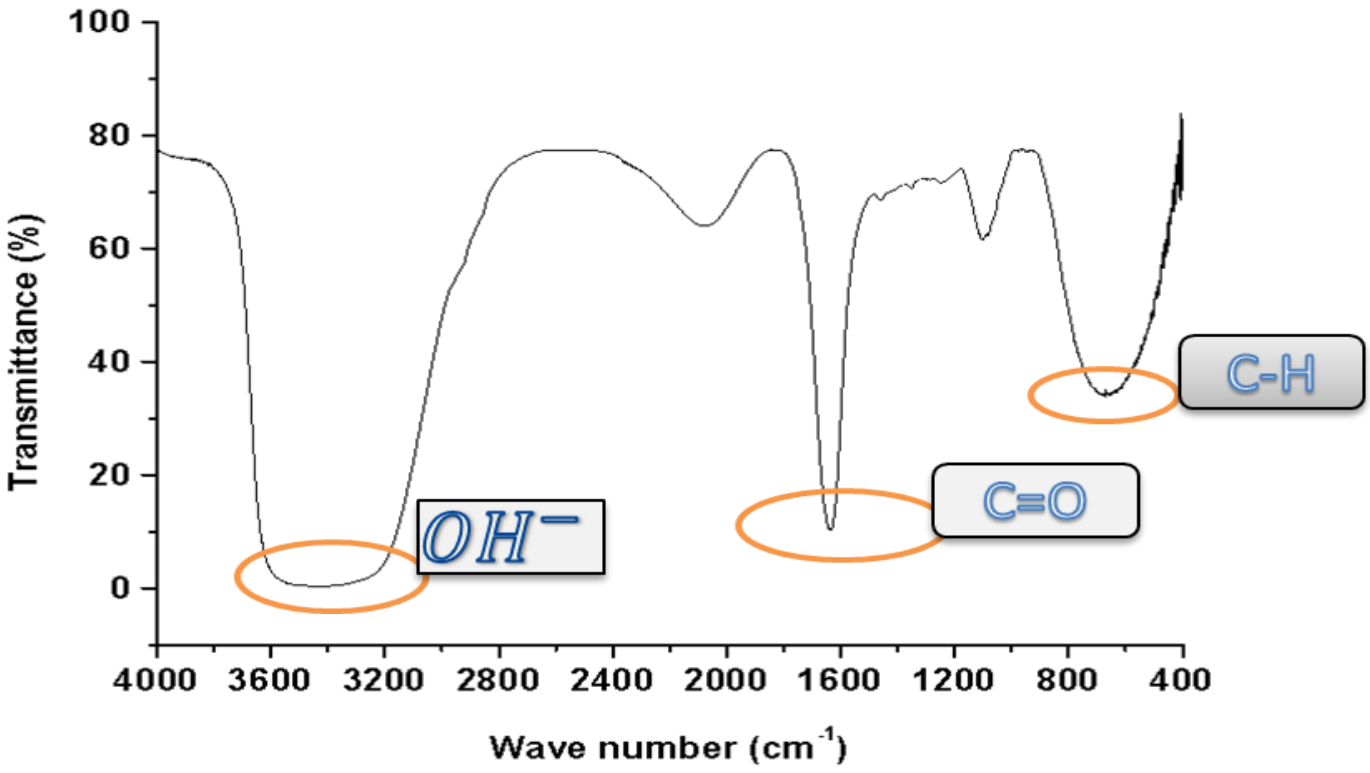


Figure4.18: FTIR spectrum of gluconic acid produced at temperature (32.5℃), Carbon source concentration (250g/L), pH (7) and incubation time (5days).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Gluconic acid was manufactured from glucose was used in food, pharmaceuticals, beverages, leather, textile, diary, cement Industries and metal chelating agent. Gluconic acid was produced in a large capacity from large sugar containing substrates. The production of gluconic acid by fermentation process involving filamentous fungi is well established commercially. However the mechanism of fermentation process, efficient microorganism to give high yield, more economical process, efficient conversion of carbon source to gluconic acid with longer shelf life, use of cheap substrate would be up to date research. This study illustrated the utilization of molasses for valuable production of gluconic acid. the process which; includes pretreatment of crude molasses by using potassium ferrocyanide that gives good result as characterized by ash content, conversion of molasses to gluconic acid by glucose oxidase enzyme was carried out in incubator shaker. Utilization of cane molasses improves the waste minimization, reduction of environmental pollution, use of cheap and local available raw material.

Based on the analysis of experimental results, the process variables temperature, Carbon source concentration, pH and inoculation time are exhibited significant effect on the production of gluconic acid yield percentage. This shows that the analysis of experimental variables in design experiment has been determined successfully. The highest product (141.57g/L medium) were obtained at temperature (32.5℃), Carbon source concentration (250g/L), pH (7)and inoculation time(5 days). The optimum variables to produce high percentage yield of gluconic acid (61.96%), the experiment should proceed at temperature (30.13℃), Carbon source concentration (250.00g/L), pH (7) and inoculation time (6.86days).

The selected model was cubic model which correlates the operating variables with response. Based on the analysis of variance ($p<0.05$) the model is significant, adequate and reliable to fit the data of response variable. Chemical characterization of gluconic acid produce was evaluated using FTIR. From the result it can be seen that the gluconic acid produce from molasses contains OH^{-1}, C=O, CH and CH_2 functional groups which confirms the presence of gluconic acid in the product of fermentation process.

5.2. Recommendation

Further recommended investigations are listed below to understand the commercial application of the gluconic acid, such as:

- Gluconic acid increases the biotechnological value of Wine. Because it is one of the proposed quality parameter of wine. Future investigation of gluconic acid on wine is an interesting concept.
- Economic feasibility study of the overall conversion process is necessary for the purpose of commercialization
- Future studies should include the optimization of pretreatment process of molasses,
- The kinetics study of *Aspergillus carneus* growth and also finding the optimal growth that reduce fermentation parameter would be another interesting aspect of fermentation process.
- Downstream separation and purification from fermentation broth is the known technological barrier so nano-filtration method which concentrates the product needs to be further investigated.
- Finally, it is recommended that further researches should be done on gluconic acid production from different types of substrates to commercialize it in our country.

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APPENDICES

Appendix A: Properties of gluconic acid

Properties	Description
chemical name	gluconic acid; D-gluconic acid; dextronic acid
IUPAC name	2,3,4,5,6-pentahydroxyhexanoic acid
molecular formula	$C_6H_{12}O_7$
molecular weight	196.155 g/mol
Density	1.24 @ 25 ℃/4 ℃
physical state	*Liquid*-colourless to light yellow, clear syrupy liquid *Solid*-white crystalline powder.
Solubility	freely soluble in water; slightly soluble in alcohol, insoluble in ether and most other organic solvents
melting point	131 ℃
Taste	mild acid taste
Color	needles from ethanol & ether
hydrogen bond donor count	6
hydrogen bond acceptor count	7
rotatable bond count	5
spectral properties	✓ specific optical rotation: -3.49 to +12.95 deg @ 25 ℃/d (water); ✓ specific optical rotation: -6.7 deg @ 20 ℃/d (concn= 1 g/100 ml water) ✓ ir: 21802 (sadtler research laboratories grating collection) ✓ nmr: 11270 (sadtler research laboratories spectral collection)
dissociation constants	k= 2.5x10-4 @ 25 ℃
Toxicity	non-toxic
food additives	acidity regulator; raising agent; sequestrate
first aid measures	if there is body contact, rinse skin with plenty of water or shower
fire hazard	Combustible.

(Source: https://pubchem.ncbi.nlm.nih.gov/compound/D-gluconic acid;Ramachandran et.al.2006)

Appendix B: Experimental result

Table B-1: Moisture content of crude cane molasses

Run	Weights(g)			Moisture content %	Average moisture %
	W0	W1	W2		
1	40.5	54.3	51.9	17.4	14.35
2	37.08	48.1	46.8	11.8	
3	35.02	44.4	43.1	13.86	

Table B-2: Ash content of crude cane molasses

Run	Weights(g)			Ash content %	Average ash content %
	W0	W1	W2		
1	26.4	34.4	27.6	15	13.45
2	27.3	34.2	28.1	11.59	
3	26.8	34.8	27.9	13.75	

Table B-3: Ash content of treated cane molasses

Run	Weights(g)			Ash content %	Average ash content %
	W0	W1	W2		
1	28.46	29.36	28.49	3.33	3.17
2	28.28	29.42	28.32	3.51	
3	28.36	29.48	28.39	2.68	

Table B-4: The absorbance of standard glucose solution reacted with benedict solution.

Standard glucose concentration(g)	Absorbances @540nm		Average absorbance
	Absorbance1	Absorbance2	
0	0	0	0
0.05	0	0.001	0.0005
0.1	0.03	0.02	0.025
0.4	0.18	0.13	0.155
0.8	0.262	0.241	0.2515
1	0.398	0.413	0.4055
1.25	0.417	0.430	0.4235
1.5	0.429	0.432	0.4305

Table B-5: The absorbance of molasses reacted with benedict solution

Run	Absorbances @540nm			
	Absorbance1	Absorbance2	First average	Average
1	0.849	1.064	0.9595	0.8983
2	1.038	0.921	0.9795	
3	0.716	0.802	0.759	

Appendix C: Calculation part

C-1: Proximate Analysis of crude molasses

Note: each value of numbers is the average value taken from triplicate samples.

- Moisture content

M0. Wt of empty crucible: 37.533g

M1. Wt of molasses and crucible: 48.93gm

M2. Wt of molasses and crucible after drying: 47.267gm

$$\%\, Moisture\ content = \frac{M1 - M2}{M1 - M0} * 100 = \frac{48.93 - 47.267}{48.93 - 37.533} * 100 = 14.5$$

- Ash content

M0. Mass of empty crucible before burning: 26.83gm

M1. Mass of sample and crucible: 34.467g

M2. Mass of sample and Crucible after burning: 27.867g

$$\%Ash\ content = \frac{M2 - M0}{M1 - M0} * 100 = \frac{27.867 - 26.83}{34.467 - 26.83} * 100 = 13.58$$

- Total dissolved solid

$$Total\ dissolved\ solid\ ,\% = 100 - moisture\ content, \%$$

$$= 100 - 14.5$$

$$= 85.5\%$$

- Total reduced sugar of molasses

  Average absorbance=0.8983

  Y-intercept=0.0018

  Slope=0.3151

  Gram of sample used =10g

  Volume of molasses=0.5ml

$$conc\ of\ unkwon\ sample = \frac{Ave.absorbance - y_{intercept}}{slope}$$

$$\%yield\ of\ TRS = \frac{conc\ of\ unkwon\ sample}{gram\ of\ sample\ used} * volume\ of\ molasses * 100$$

$$conc\ of\ unkwon\ sample = \frac{0.8983 - 0.0018}{0.3151} = \mathbf{2.8452}\frac{g}{ml}$$

$$\%TRS = \frac{2.8452\frac{g}{ml}}{10g} * 0.5ml * 100 = \mathbf{14.46}\%$$

C-2: Amount of gluconic acid

For each experiment, quantity of gluconic acid can be calculated as follows.

$$Amount\ of\ H2SO4\ acid\ titrated = amount\ of\ lactones\ hydrolised$$

$$= 5\%of\ gluconic\ acid$$

$$GA\ in\ ml = \frac{amount\ of\ lactones}{5} * 100$$

$$GA\ in\ gram = amount\ of\ lactones\ in\ ml * 20 * \frac{1.24g}{ml}$$

Where: density of GA=1.24g/ml

Total acidity in terms of gluconic acid

- Normality of gluconic acid = $\frac{normality\ of\ NaOH \times NaOH\ volume}{volume\ of\ gluconic\ acid}$
- Concentration of gluconic acid = $\frac{(gluconic\ acid\ normality \times equivalent \times 100)}{volume\ of\ gluconic\ acid\ used}$
- (Equivalent = 96, volume of gluconic acid used = 10)

Appendix D: Infrared spectroscopy correlation table

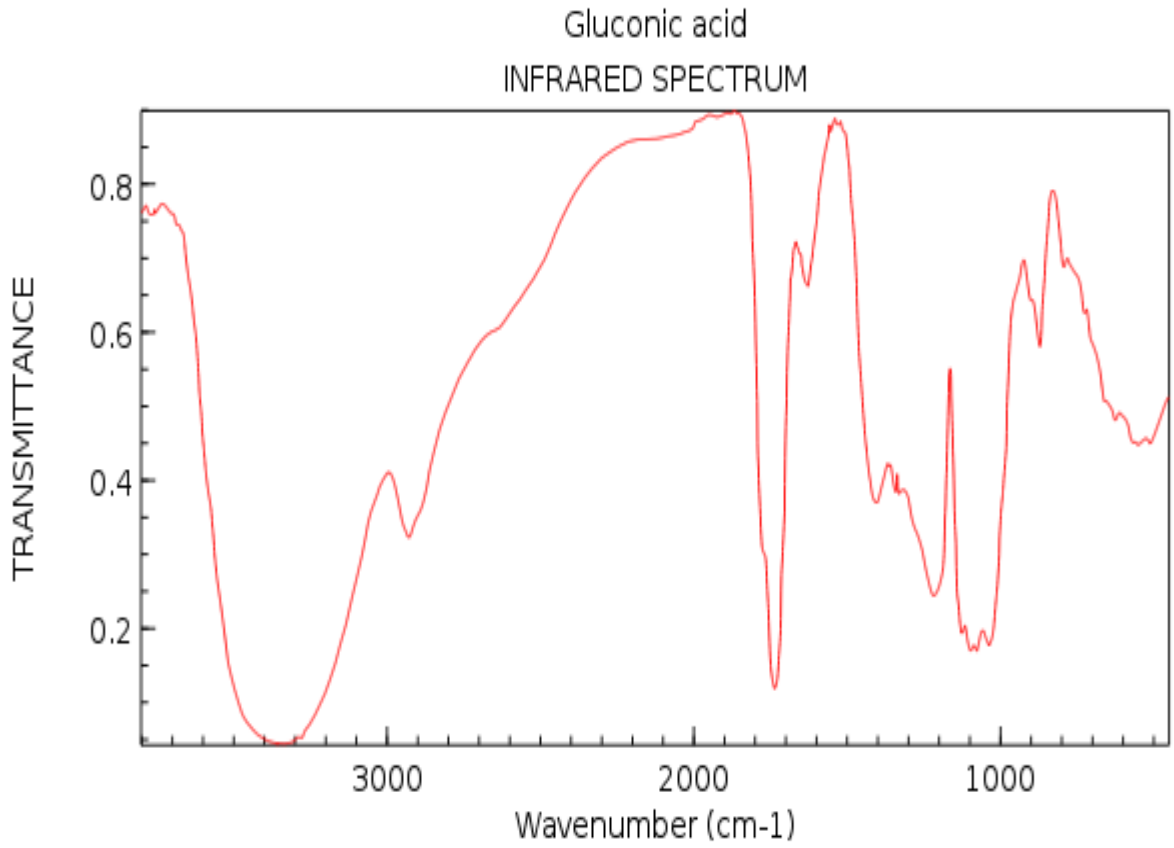
Bond	Type of bond	Specific type of bond	Absorption peak (cm−1)	Appearance
C—H	Alkyl	Methyl	1260	Strong
			1380	Weak
			2870-2960	Medium to strong
		Methylene	1470	Strong
			2850-2925	Medium to strong
		Methane	2890	Weak
	Vinyl	C═CH2	900	Strong
			2975-3080	Medium to strong
		C═CH	3020	Medium

		Mono substituted alkenes	900-990	Strong
		cis-disubstituted alkenes	670-700	Strong
		trans-disubstituted alkenes	965	Strong
		trisubstituted alkenes	800-840	Medium to strong
	Aromatic	Benzene/sub. Benzene	3070	Weak
		Benzenes	690-860	Strong
	Alkynes	Any	3300	Medium
	Aldehydes	Any	2720-2820	Medium
C═C	acyclic C═C	Alkenes	1645-1675	Medium
	Conjugated C═C	Dienes	1600-1665	Strong
	with benzene ring		1625	Strong
	with C═O		1600	Strong
	C═C (both sp2)	Any	1640-1680	Medium
	aromatic C═C	Any	1450-1600	Weak to strong
	C≡C	terminal alkynes	2100–2140	Weak
		disubst. Alkynes	2190–2260	Very weak
C=O	aldehyde/ketone	saturated aliph./cyclic 6-membered	1720	
		α,β-unsaturated	1685	
		aromatic ketones	1685	
		Aldehydes	1725	influenced by conjugation (as with ketones)
	carboxylic acids/derivates	saturated carboxylic acids	1710	
		unsat./aromatic carb. Acids	1680-1690	
		esters and lactones	1735	influenced by conjugation and ring size (with ketones)
		Anhydrides	1760-1820	
		acyl halides	1800	
		Amides	1650	associated amides
		carboxylates (salts)	1550-1610	
		amino acid zwitterions	1550-1610	
O─H	alcohols, phenols	low concentration	3610–3670	

		high concentration	3200–3400	Broad
	carboxylic acids	low concentration	3500–3560	
		high concentration	3000	Broad
N─H	primary amines	Any	3400–3500	Strong
	secondary amines	Any	>3000	weak to medium
	ammonium ions	Any	2400-3200	multiple broad peaks
C─O	Alcohols	Primary	1040–1060	Strong, broad
		Secondary	~1100	Strong
		Tertiary	1150–1200	Medium
	Phenols	Any	1200	
	Ethers	Aliphatic	1120	
		Aromatic	1220-1260	
	carboxylic acids	Any	1250–1300	
	Esters	Any	1100–1300	two bands (distinct from ketones, which do not possess a C─O bond)
C─N	aliphatic amines	Any	1020–1220	often overlapped
	C═N	Any	1615–1700	similar conjugation effects to C═O
	R─N─C (isocyanides)	Any	2165–2110	
	R─N═C═S	Any	2140–1990	
C─X	Fluoro-alkanes	Ordinary	1000-1100	
		Trifluromethyl	1100-1200	two strong, broad bands
	Chloroalkanes	Any	540-760	weak to medium
	Bromoalkanes	Any	500-600	medium to strong
	Iodoalkanes	Any	500	medium to strong
N─O	nitro compounds	Aliphatic	1540	Stronger
		Aromatic	1520	lower if conjugated
P─C	Organophosphorus cpd	Aromatic	1440-1460	Medium
P─O	phosphorus oxide	Bonded	1195-1250	Strong

(Source: https://en.wikipedia.org/wiki/Infrared spectroscopy correlation table)

Appendix E: Gluconic acid infrared spectrum of NIST



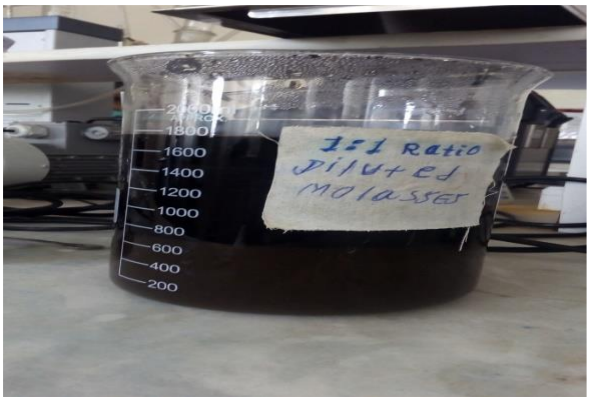
NIST Chemistry WebBook (https://webbook.nist.gov/chemistry)

(https://webbook.nist.gov/cgi/cbook.cgi?ID=C526954&Mask=80)

Appendix F: Sample laboratory work pictures

F1: Crude Molasses

F2: Refractometer Instrument



F3: 1:1 Ratio Diluted Molasses

F4: Spectrophotometer Instrument

F5: Chemicals for strain growth

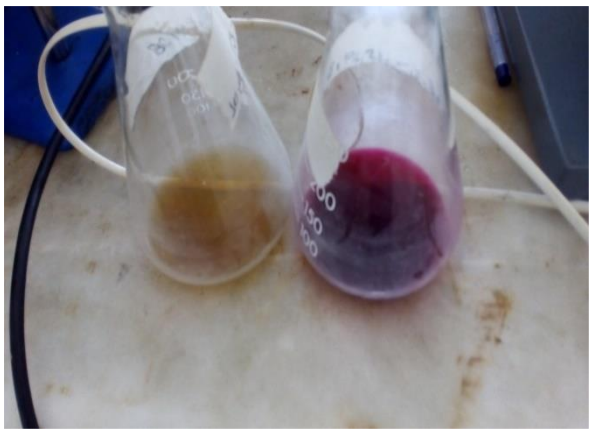
F6: Inoculated microorganism

F7: PH instrument

F8: Homogenized and sterilized mixture

F9: v-cabinet hood instrument(laminar flow)

F10: Incubator shaker instrument



F11: Fermented broth before and after titration

F12: FTIR Instrument