KWARA STATE UNIVERSITY, MALETE, NIGERIA

SCHOOL OF POSTGRADUATE STUDIES (SPGS)

PRODUCTION OF GLUCONIC ACID FROM SWEET POTATO PEELS USING NATURALLY OCCURRING FUNGI BY SUBMERGED FERMENTATION

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(19/57MMB/00010)

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A M.Sc. THESIS SUBMITTED AND PRESENTED

BY

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JUNE, 2022.

DECLARATION

I hereby declare that this thesis titled **'Production of Gluconic Acid from Sweet Potato Peels Using Naturally Occurring Fungi by Submerged Fermentation'** is a record of my research. It has neither been presented nor accepted in any previous application for higher degree.

Rukayat Olaitan Said

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CERTIFICATION PAGE

This is to certify that this thesis by Rukayat Olaitan Said has been read and approved as meeting the requirements of the Department of Microbiology for the award of the degree of Masters of Science (M.Sc.) degree in Microbiology.

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DEDICATION

This project is dedicated to Almighty Allah, the source of wisdom, knowledge and understanding.

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ABSTRACT

Gluconic acid is an important organic acid, resulting from the oxidation of glucose. The aim of the study was to produce gluconic acid from sweet potato peels, using naturally occurring fungi isolated from soil retain by submerged fermentation. Isolation and identification of fungi were done using standard microbiological methods. Screening of fungal isolates for gluconic acid production was done using standard procedures. Proximate analysis of substrate was carried out using standardized methods. Total titrable acid was determined using titration method, and reducing sugar using DNSA method. Gluconic acid yields were determined using High Performance Liquid Chromatography. A standard gluconic acid producer, *Aspergillus niger* ATCC 10577, was used as a control. Proximate composition of the sweet potato peels showed percentage carbohydrate of 20.81 ± 0.07. A total of six different fungal species were isolated and identified. They included *Aspergillus niger*, *Aspergillus flavus*, *Penicillum* sp., *Cladosporium* sp., *Rhizopus stolonifer* and *Aspergillus terreus*. *Aspergillus niger* showed the highest zone of clearance and was further identified as *Aspergillus niger* UFMGCB 14248. Results of total titrable acid and reducing sugar showed that a substrate concentration of 50 g/L, carbon source starch, incubation day 7, pH 6 had the highest % total titrable acid, while substrate concentration of 50 g/L, carbon source glucose, incubation day 7, pH 6 had the highest reducing sugar (mg/ml). Gluconic acid concentrations (mg/ml) was highest at substrate concentration of 50 g/L, carbon source starch, incubation day 7 and pH 6 for both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577. The findings showed that the two isolates used in this study were good gluconic acid producers, which could be scale up for industrial production.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Gluconic acid has the chemical formula $C_6H_{12}O_7$ and is an organic molecule; a simple dehydrogenation reaction catalyzed by glucose oxidase produces gluconic acid from glucose. Gluconic acid creates the gluconate ion in an aqueous solution with a neutral pH; Gluconates refers to the salts of gluconic acid because such molecules develop from the oxidation of glucose. Gluconic acid, gluconate salts, and gluconate esters are abundant in nature (Mao, 2016). Gluconic acid is a moderate organic acid that has sparked a lot of attention due to its numerous industrial uses in the pharmaceutical, food, animal feed, textile, and leather industries (Singh *et al.*, 2003; Mao, 2016). It is also applied as an additive in cement to control the setting time, increase strength and water resistance. Gluconic acid can have further applications for the solubilization of phosphate (Ashraf *et al.*, 2014).

Gluconic acid is a non-corrosive, non-volatile, non-toxic, mild organic acid that gives a refreshing acidity to many foods such as wine and fruit juices. Sodium gluconate is an excellent chelating agent with high isolation at alkaline pH. Its effectiveness is superior to ethylene-diamine-tetra-acetic acid (EDTA), di-ethyltriacetic acid (NTA), and other chelating agents. Aqueous sodium gluconate solution is resistant to oxidation and reduction at high temperatures, also functions as a plasticizer and hardening inhibitor, is highly biodegradable (98 % in 48 hours), and has excellent properties of preventing food bitterness. The concentrated gluconic acid solution contains certain lactone structures (neutral cyclic ester) showing antiseptic properties (Ramanchandran *et al.,* 2006).

Gluconic acid is abundantly available in plants, fruits, and other foodstuffs such as rice, meat, dairy products, wine (up to 0.25 %), honey (up to 1 %), and vinegar. It is produced by different microorganisms which include bacteria such as *Pseudomonas ovalis, Acetobacter methanolicus*, *Zymomonas mobilis*, *Acetobacter diazotrophicus*, *Gluconobacter oxydans, Gluconobacter suboxydans, Azospir illum brasiliens* (Kim *et al.,* 2000) fungi such as *Aspergillus niger, Penicillium funiculosum, P. variabile*, *P. amagasakiense* (Rodriguez *et al.*, 2004) and yeasts such as *Aureobasidium pullulans* formerly known as *Dematium* or *Pullularia pullulans* (Anastassiadis *et al.,* 2005).

Sweet potato, *Ipomoea batatas* L. (Lam.), in many places, sweet potatoes are an important crop. In terms of annual production, sweet potatoes are the fifth most important food crop in the tropics and the seventh most important food production in the world, after wheat, rice, corn, potatoes, barley and cassava (FAO, 2016). Sweet potatoes perform several fundamental roles in the global food system, all of which are fundamental in meeting food needs, reducing poverty, and enhancing food security (El-Sheikha and Ray, 2010). Sweet potato roots are excessive in vitamins and feature an extensive variety of taste, texture, and flesh colour (white, cream, and yellow, orange, purple). Sweet potatoes exhibited various organic activities, together with defensive antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, and hepatoprotective interest outcomes (Hu *et al.,* 2016). Sweet potatoes have great potential to contribute to better nutritional quality in our diets around the world. Sweet potato skins generated by industrial processes generate a huge amount of waste; an estimated 30 % of the raw materials used in the sweet potato canning operation are abandoned and not used for human consumption (Schaub and Leonard, 2017). The pods are often used as fertilizer or fodder if used.

1.2 Statement of Research Problem

Surprisingly, nearly all agricultural activities produce wastes, which are produced in significant quantities in many nations, including Nigeria. However, these wastes may constitute a serious threat to human health through environmental pollution, and handling them may result in huge economic loss. Sadly, in many poor countries where substantial amount of these wastes are generated results into indiscriminate burning of agricultural solid wastes which causes climate-relevant emissions, indiscriminate dumping, and have resulted in pollution, which is a threat to human lives as well as other environmental problems, calling for global attention, although these wastes can be recycled.

1.3 Justification for the Study

A large number of food materials during storage decompose and create waste that can pollute the environment. The use of these wastes can, on the one hand, be part of the control of environmental pollution and, on the other hand, the production of value-added products of commercial importance, thereby changing their status from waste to wealth. In addition, using these agricultural wastes as natural substrates for gluconic acid production is cheaper than using convectional carbohydrate.

The global demand for gluconic acid has been growing for almost 20 years and recently the production has reached more than 60,000 tons per year and continues to increase (El-Enshasy, 2003). Commercially, gluconic acid is produced through a fermentation process in which specific microorganisms are grown in a medium containing glucose and other ingredients (Hill and Robinson, 2000). Microbial fermentation processes offer attractive techniques for gluconic acid production in order to minimize chemical related problems such as unavoidable side reactions and also to further economize the biological process. A large group of filamentous microorganisms are particularly capable of producing gluconic acid.

1.4 Aim of the Study

The aim of the study is to produce gluconic acid from sweet potato peels using naturally occurring fungi by submerged fermentation.

1.5 Objectives of the Study

The objectives of the study were to:

i. isolate and identify naturally occurring fungi from the fermented substrate (sweet potato peels) and soil;
ii. screen for gluconic acid-producing fungi;

iii. determine the pH, total titrable acid and reducing sugar of the fermented sweet potato peels; and
iv. determine optimum parameters for gluconic acid production.

1.6 Significance of the Study

Gluconic acid being an organic acid can be harnessed in pharmaceutical, hygienic products and in the formulation of food. It is also used as a mineral supplement to prevent deficiency of calcium, iron, and so on. and as buffer salt. Different salts of gluconic acid have different applications depending on their properties. This research is important because it produces gluconic acid for many industrial applications.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Gluconic Acid

A simple dehydrogenation reaction catalyzed with the aid of glucose oxidase produces gluconic acid (penta-hydroxycaproic acid) from glucose. The glucono-d-lactone ($C_6H_{10}O_6$) and hydrogen peroxide are produced while the aldehyde group at the C-1 of b-D-glucose is oxidized to a carboxyl group. Glucono-d-lactone is in addition hydrolyzed to gluconic acid either spontaneously or with the aid of a lactone hydrolyzing enzyme, and hydrogen peroxide is degraded with the aid of peroxidase to water and oxygen. The conversion technique may be completely chemical, but fermentation is the most broadly used approach. The enzymatic method can also be carried out in the absence of cells containing glucose oxidase and catalase generated from *Aspergillus niger*, under the right circumstances, almost all the glucose is converted to gluconic acid (Godjevargova *et al.,* 2004).

2.1.1 History of Gluconic Acid

Gluconic acid has been produced since 1870, when Hlasiwetz and Habermann discovered it (Rohr *et al.,* 2000). Boutroux *et al.* (2000) discovered for the first time in 1880 that acetic acid bacteria can produce sugar acid. Molliard (2000) discovered gluconic acid in *Sterigmato cystisnigra*, now *Aspergillus niger*, in 1922. Later, gluconic acid synthesis was discovered in bacteria like *Pseudomonas*, *Gluconobacter*, *Acetobacter*, and several fungal species. Bernhauer (2001) observed that when *Aspergillus niger* was neutralized by calcium carbonate, it produced significant amounts of gluconic acid, and that the production was pH-dependent. When compared to *Aspergillus niger*, the pH dependence of *Penicillium* sp. was found to be less critical, indicating that there was some correlation between the amount and time-dependent appearance of organic acids such as gluconic acid, citric acid, and oxalic acid, which were formed under different conditions. Mayer *et al.* (2002) used *Aspergillus niger* to investigate the synthesis of gluconic acid, *Penicillium luteum* was also used. Currie *et al.* (2005) submitted a patent for gluconic acid production of up to 90 % in 48 – 60 hrs using submerged culture. Later, Moyer *et al.* (2002) employed *A. niger* in pilot plant tests and achieved theoretical yields in glucose solution of up to 95 % of 150 to 200 g/L in 24 hrs. Porges *et al.* (2003) discovered that the procedure may be conducted semi-continuously by reusing the mycelium nine times in a row and recovering the inoculums using filtration or centrifugation. Moyer *et al.* (2002) discovered that adding 250 g/L glucose and boron compounds (1 % in a solution of 250 g/L glucose) at later phases of fungal growth, along with the reuse of mycelium in 24 hrs cycles, may reach efficiency of more than 95 %. Submerged fermentation with *Aspergillus niger* is being used in commercial sodium gluconate synthesis, and it is based on a modified techniques (Blom *et al.*, 2005). It entails fed-batch culture with gluconic acid injections on a periodic basis and the use of sodium hydroxide as a neutralizing agent. The pH is kept between 6.0 and 6.5, and the temperature is around 34^0C, with glucose transformed at a rate of 15 g/l, this process has a high productivity.

2.1.2 Properties of Gluconic Acid

Gluconic acid has been said to be the oxidized version of D-glucose (or dextrose), a sugar, polysaccharide, and cellulose building ingredient. It cyclizes in solution like glucose, but instead of forming a hemiacetal, it forms an ester (glucono—lactone). Gluconic acid is found in abundance in nature, particularly in fruits and sucrose-containing foods like honey. Alkaline hydrolysis and Hypobromite oxidation were two early ways for producing gluconic acid from glucose. It is now produced by microorganisms through fermentation process (Ramachandran, 2006).

Gluconic acid is a moderate organic acid that is noncorrosive, nonvolatile, and nontoxic. It gives many foods, including wine and fruit juices, a delightful sour flavor. Sodium gluconate has a strong capacity for sequestration. At alkaline pH, it is a good chelator; its action is superior to that of EDTA, NTA, and other chelators. At high temperatures, sodium gluconate aqueous solutions are resistant to reduction and oxidation. It works well as a plasticizer as well as a set retarder. It is biodegradable in a short amount of time (98 % at 48 hrs). It has the unusual virtue of preventing bitterness in foods. Certain lactone structures (neutral cyclic ester) are found in concentrated gluconic acid solutions showing antiseptic property (Ramachandran, 2006).

2.1.3 Production of Gluconic Acid

There are several methods of gluconic acid production, including chemical, electrochemical, biochemical, and bio-electrochemical methods (Isabell *et al*., 2000). This process appears to be more costly and inefficient than fermentation. Although the conversion is a simple one-step process, chemical methods are not preferred. Therefore, fermentation is one of the effective and mainstream techniques for gluconic acid production. The approach involving the fungus *Aspergillus niger* is one of the most widely used among the many microbial fermentation processes. On the other hand, the process involving *Gluconobacter oxidans* is becoming increasingly important. The magnitude of the resulting product, such as sodium gluconate or calcium gluconate, is independent of the use of fungi or bacteria. As a result of the reaction, an acidic product is produced, for example sodium gluconate or calcium gluconate and so on.

The acidity of the fermentation medium must be neutralized by adding neutralizing agents; otherwise, the acidity will inactivate the enzyme glucose oxidase, stopping the synthesis of gluconic acid. The fermentation conditions for the formation of calcium gluconate and sodium gluconate differ in a number of ways, including glucose concentration (both initial and final) and pH adjustment. Adding calcium carbonate slurry to calcium gluconate production helps control pH. The solubility of calcium gluconate in water (4 % at 30°C) is another relevant factor. Super-saturation occurs at high glucose concentrations above 15 %, and if the limit is exceeded, calcium salts precipitate on the mycelium, inhibiting oxygen transfer. To avoid the Lobry de Bruyn-van Ekenstein reaction, which changes the structure of glucose and reduces yield by around 30 %, the neutralizing agent should be sterilized separately from the glucose solution. The sodium gluconate method, on the other hand, is far superior because glucose concentrations of up to 350 g/l can be employed without any problem, the automatic addition of NaOH solution regulates the pH and sodium gluconate dissolves rapidly in water (Pfizer, 2001).

2.1.4 Solid-State Fermentation (SSF) Synthesis of Gluconic Acid

Solid-state fermentation (SSF) has been extensively described for the large scale organic acid and enzyme production (Pandey *et al*., 2011). However, only a few researchers use SSF in gluconic acid synthesis. Roukas (2005) reported solid state fermentation as a gluconic acid production method with a yield of approximately 63 %; the maximum content of gluconic acid was 490 g/kg. The addition of 6 % methanol to the substrate could increase the yield of gluconic acid from 490 to 500 to 685 g/kg. Singh *et al*. (2003) SSF was performed using bagasse pretreated with HCl, and the highest level of gluconic acid (107 g/l) was obtained with 95 % yield. In Comparism with submerged culture, solid state fermentation (SSF) has a higher degree of metabolism. Changes in osmotic pressure, water and dissolved oxygen content may be responsible for the increased product rates. In a study by Moksia *et al*. (2001) the first step was isolating *Aspergillus niger* spores on buckwheat seeds using SSF, and the second step was the bioconversion of glucose to gluconic acid using spores obtained from SSF medium. The spores could not germinate because the bioconversion medium contained no nitrogen source, which was an intriguing part of the study. The spores act as a biocatalyst, producing 200 g/l of gluconic acid with a yield of 1.06 g per mass of glucose, which is very close to the stoichiometric value (Ramachandran *et al.*, 2006). The general characteristics and structure of gluconic acid are illustrated in figure 1 and figure 2 respectively.

2.1.5 Submerged Fermentation Synthesis of Gluconic Acid

Aerobic submerged fermentation are used for the commercial synthesis of bi-molecular such as 2-keto-L-gluconic acid, D-sorbitol (Hancock *et al.*, 2002), gluconic acid and di-hydroxyacetone (Hekmat *et al.*, 2007). *Aspergillus niger* exhibits a wide variety of morphologies when grown in submerged culture, ranging from hyphae at low concentrations to clumps of interwoven hyphae at high concentrations known as pellets (Lu *et al.*, 2015). During submerged fermentation, mass transfer is influenced by the rheological properties of the fermentation medium, which are influenced by biomass concentration and mycelium morphology. Pelleted mycelia are chosen for fermentation to prevent large biomass concentrations and thus high viscosity, which impedes nutrient delivery in the fermentation broth (Riley *et al.*, 2000). However, the supply of oxygen between the large pellets is sometimes insufficient; leading to autolysis of the fungus, which interferes with fermentation (Mafra *et al.*, 2014). Besides agitation and aeration, the shape of the mycelium is also responsible for stimulating gluconic acid synthesis by increasing oxygen supply. Oxygen transfer rate is a limiting factor in bioreactors for catalyzing the conversion of glucose to gluconic acid due to insufficient carbon input concentrations. Lu *et al*. (2015) investigated the effect of the dispersed pattern of *A. niger* AN151 mycelia morphology on gluconic acid yield (1.05 g/g) in a shorter fermentation time (14 hrs and 5 mins) due to improved oxygen transfer rate in submerged fermentation compared to pellet fermentation.

2.1.5.1 Cheaper Raw Materials as Substrates for Gluconic Acid Production

Glucose is commonly utilized as a carbon source for microbial gluconic acid synthesis. Hydrolysates of a variety of raw materials, such as agro-industrial waste, have been employed as substrates in the past. In media containing glucose or starch hydrolysate as the sole carbon source, Kundu and Das (2001) obtained a significant yield of gluconic acid by immobilizing *Aspergillus niger* in hydrosol (corn starch hydrolysate as the fermentable sugar). Rao and Panda (2003) employed Indian cane molasses as a source of glucose. Acid treatment, potassium ferrocyanide treatment, salt treatment, and other pre-treatments were used on the cane molasses Treatment with potassium ferrocyanide yielded encouraging results. Copper, zinc, magnesium, calcium, iron, and other metal ions influenced the synthesis of gluconic acid. Deproteinized whey was employed as a nutritive medium for gluconic acid synthesis as reported by Mukhopadhyay *et al.* (2005). Lactose was employed as a carbon source. *Aspergillus niger* immobilized on polyurethane foam

Table 1 General Characteristics of Gluconic Acid

Gluconic Acid	Characteristics
Nature	Noncorrosive, mildly acidic, Less irritating, non odorous, Non toxic, easily biodegradable, Non volatile organic acid
Relative molecular mass	196.16
Chemical formula	$C_6H_{12}O_7$
Synonym	2,3,4,5,6-pentahydroxyhexanoic acid
pKa	3.7
Melting point (50 % solution)	Lower than 12 °C
Boiling point (50 % solution)	than 100 °C
Density	1.24 g/ml
Appearance	Clear to brown
Solubility	Soluble in water

Source: (Röhr *et al.,* 2000).

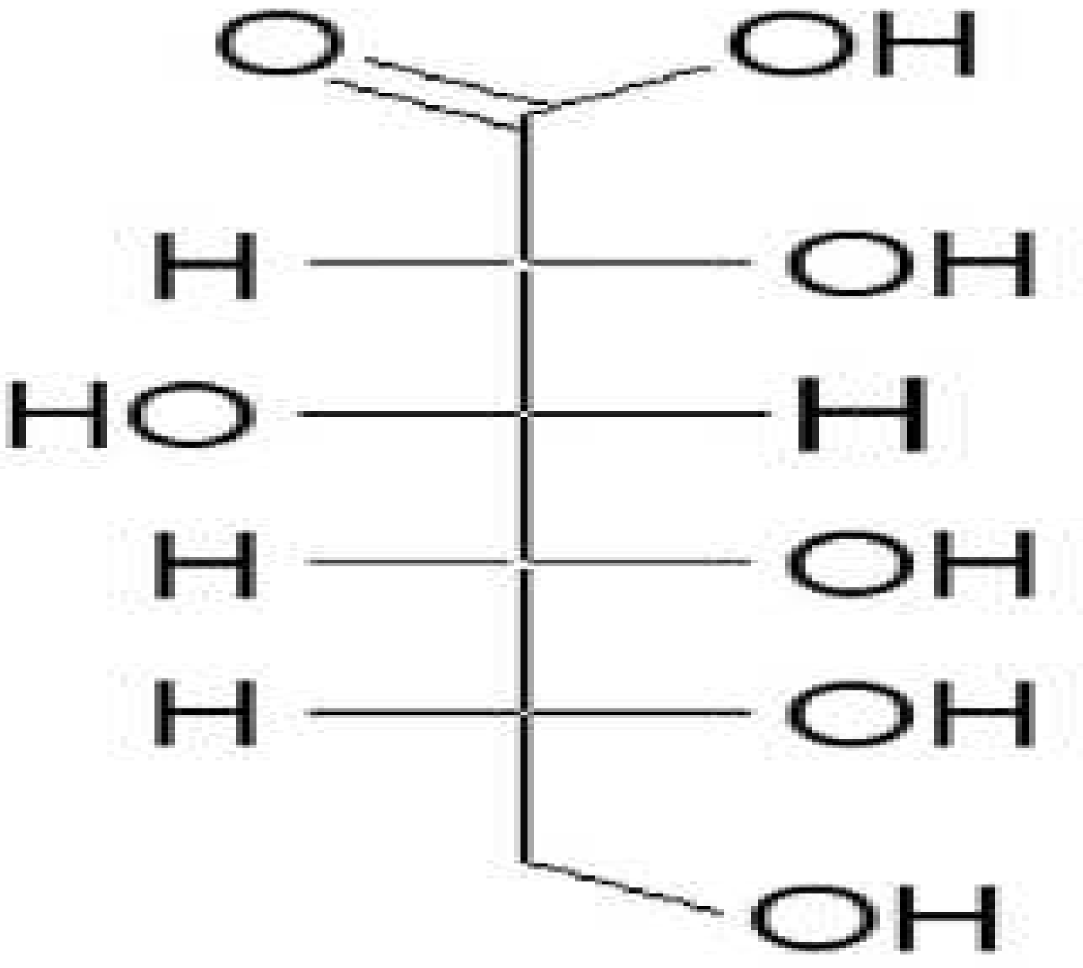

Figure 1: Gluconic Acid Structure

Source: (Ramachandran, 2006).

generated 92 g of gluconic acid from 1l of whey containing 0.5 % glucose and 9.5 % lactose.

In the bioconversion of gluconic acid using *Aspergillus niger* Ikeda *et al.* (2006) employed a saccharified waste paper solution with glucose concentration adjusted to 50 – 100 g/l. In Erlenmeyer flasks, yields were 92 %, while in the turbine blade reactor with 800 ml working capacity, yields were 60 %. Another interesting aspect of the research was when xylose and cellobiose were utilized as the only carbon sources; yields of gluconic acid were 83 % and 56 %, respectively. According to Singh *et al.* (2003), grape must and banana must produce considerable amounts of gluconic acid, 63 and 55 g/L, respectively. Grape and banana purification should result in 20 – 21 % increase in gluconic acid output. They also employed molasses, which produced 12 g/L of gluconate, but after treating the molasses with hexacyanoferrate, there was a huge rise in output of 60 g/L with a yield of 61 %. Rectified grape must appeared to be the best-suited substrate, yielding 73 g/L of gluconic acid with 81 % yield after 144 hrs, compared to 72 % yield from rectified banana must. Buzzini *et al.* (2005) employed grape must as well and rectified grape must and they found that the latter substrate was better, with a production of 67 g/L and a yield of 96 % in 72 hrs. Citric acid was also observed as a by-product.

2.1.6 Applications of Gluconic Acid

Gluconic acid is a non-acidic organic acid used in the food industry. It is found naturally in juices and honey, and is used to marinate meals. Its basic ester, glucono-d-lactone, has a sweet taste that gradually becomes slightly acidic. It is found in meat and dairy products, as well as in baked foods, where it is used as a leavening ingredient in baked foods. It is used as a flavoring additive as well as a fat absorption inhibitor in doughnuts and cones. Beans, yogurt, cheese, bread, candy, and meat are examples of foods that include D-glucono-d-lactone. Generally, gluconic acid and its salts are used in the formulation of food, pharmaceuticals and hygiene products. They are also used as mineral supplements to prevent deficiency of calcium, iron, and so on. and buffer salt. Various gluconic acid salts find different applications based on their properties (Ramachandran *et al.,* 2006).

The sodium salt of gluconic acid has an excellent ability to chelate calcium and other divalent and trivalent metal ions. It is used in cleaning bottles to prevent scale build-up and remove deposits from the glass. Great for removing lime deposits from metals and other surfaces Such as milk or beer stone on galvanized iron or stainless steel. Its ability to bind iron over a wide temperature and pH range is utilized in the textile industry. Used to prevent iron buildup and reduce the size of polyester and polyamide fabrics. It's also used for alkaline derusting in metallurgy, as well as washing painted walls and removing metal carbonate precipitates without producing corrosion. It can also be used as a cement additive to adjust the setting time and improve the strength and water resistance of cement. It helps in the production of concrete that is resistant to frost and cracks. It’s also included in household cleaning products such as mouthwash (Ramachandran *et al.,* 2006).

Calcium gluconate is a source of calcium used in the pharmaceutical industry to treat calcium deficiency by oral or intravenous administration. It is also used in animal feed. Iron gluconate and iron phosphogluconate are used in iron therapy. Zinc gluconate is used to treat common colds, wound healing, and various disorders caused by zinc deficiency. This includes delayed sexual development, mental drowsiness, skin changes, and susceptibility to infections (Ramachandran *et al.,* 2006).

2.2 *Aspergillus niger*

All the enzymes needed to convert glucose to gluconic acid are produced by *Aspergillus niger*, including glucose oxidase, catalase, lactonase, and mutarotase. Alpha-type crystalline glucose monohydrate is naturally converted to beta-type in solution, while *Aspergillus niger* produces the enzyme mutarotase, speeding up the process. *Aspergillus niger* glucose oxidase undergoes self-reduction by removing two hydrogen atoms during the glucose conversion process, and the reduced enzyme is further oxidized by molecular oxygen to produce hydrogen peroxide as a by-product of the process. Catalase is produced by *Aspergillus niger* and reacts with hydrogen peroxide to release water and oxygen. Lactonase helps hydrolyze glucono-d-lactone to gluconic acid. The reaction can occur spontaneously due to the rapid lactone cleavage at near-neutral pH induced by the addition of calcium carbonate or sodium hydroxide. Lactones need to be removed from the medium as their presence affects the rate of glucose oxidation and the formation of gluconic acid and its salts. *Aspergillus niger* reportedly contains the enzyme glucono-lactonase, which promotes the conversion of glucono-lactonase to gluconic acid (Ramachandran *et al.*, 2006).

The activity of glucose oxidase is closely associated with the formation of gluconic acid. Fermented broth containing sodium gluconate or calcium gluconate is prepared by adding sodium hydroxide or calcium carbonate solution for neutralization, depending on the application. The general ideal conditions for gluconic acid production are: Very high oxidation rate at (4 bar) with glucose at a concentration of 110-250 g/L, a very low concentration (20 mM) of nitrogen and phosphate supply, a medium pH of 4.5-6.5 (Sakurai *et al*., 2018).

Gluconic acid synthesis is affected by two important factors. Since glucose oxidase uses molecular oxygen for the biological conversion of glucose, the availability of oxygen and the pH of the medium are two factors, and oxygen is one of the major substrates in the oxidation of glucose. The concentration of oxygen gradient and the volumetric oxygen transfer coefficient are the critical factors, which monitor the availability of oxygen in the medium. These two factors highly influence the rate of the transfer of oxygen from gaseous to an aqueous phase; several reports are available on this particular aspect (Velizarov *et al*., 2017).

Table 2: Applications of Gluconic Acid and its Derivatives

Gluconic Acid and Derivatives	Applications of Gluconic Acid
Gluconic acid	Prevention of milkstone in the dairy industry Cleaning of aluminum cans
Glucono-d--lactone	Latent acid in baking powders for use in dry cakes and instantly leavened bread mixes Slow acting acidulant in meat processing such as sausages Coagulation of soybean protein in the manufacture of tofu In the dairy industry for cheese curd formation and for improvement of heat stability of milk
Sodium salt of gluconic acid	Detergent in bottle washing Metallurgy (alkaline derusting) Additive in cement Derusting agent Textile (iron deposits prevention) Paper industry
Calcium salt of gluconic acid	Calcium therapy Animal nutrition
Iron salt of gluconic acid	Treatment of anemia

Source: (Ramachandran *et al.*, 2006).

Aeration rate and agitation rate are two parameters that affect the availability of oxygen in the medium. Gluconic acid production is a highly oxygen consuming process and has a high oxygen demand for bio-transformation reactions that are strongly influenced by dissolved oxygen concentration. However, some studies also provided pure hyperbaric oxygen depending on the dissolved oxygen concentration. In most cases, oxygen is administered in the form of atmospheric air; nevertheless, high-pressure pure oxygen has been used in several investigations (Philip *et al.*, 2003).

Cho *et al*. (2005) detected high pressure oxygen at a pressure of about 6 bar while maintaining the dissolved oxygen at 150 ppm. It was observed that immobilized *Aspergillus niger* mycelium cultured in pure oxygen produced higher levels of gluconic acid than mycelium cultured in air. Kapat *et al*. (2001) discovered that the dissolved oxygen concentration for glucose oxidase synthesis was highest at a rate of 420 rpm and aeration of 0.25 vvm. Glucose oxidase oxygen levels are within water saturation (Schmid *et al*., 2007). Lee *et al*. (2001) using relatively high pressure (2–6 bar), achieved high volume productivity of gluconic acid and dissolved oxygen increase by 150 mg/L. As the number of bubbles increases during fungal growth, the distribution of oxygen becomes uneven and the supply of oxygen becomes inadequate (Philip *et al*., 2003).

The viscosity of the culture medium affects oxygen uptake, and as mycelium concentration increases, oxygen uptake decreases rapidly (lookwood, 2004).

Another important factor is the pH, which affects the production of gluconic acid. Citric acid, gluconic acid, and oxalic acid are one of the weak organic acids produced by *Aspergillus niger*, and their accumulation is affected by the pH of the growth medium (Znad *et al*., 2004). Below 3.5, the tri-carboxylic acid circuit is triggered and citric acid production is promoted. The pH range for fungal gluconic acid synthesis is between 4.5 and 7.0. For *Aspergillus*, pH 5.5 is generally considered ideal. Franke (2003) collected data on the relative activity of glucose oxidase at various pH values and reported 5 % and 35 % activity at pH 2.0 and 3.0, respectively, based on 100 % activity at pH 5.6. Heinrich and Rehm (2006) claimed that even at pH 2.5, gluconic acid synthesis takes place in the presence of manganese in fixed-bed and agitated-bed reactors. This is probably due to the difference in intracellular and extracellular pH.

2.3 Sweet Potato

Sweet potato (*Ipomoea batatas*) is one of the world`s most frequently cultivated food crops. It is grown in approximately 111 nations, with a total production of 110.75 million tons in 2013. China contributes roughly 71 % of global output. In 2011, the global amount of sweet potato trash amounted to about 7 % of the total crop (FAO, 2016). Sweet potato is a carbohydrate and mineral rich root tuber with high moisture content. On a dry basis, sweet potatoes contain 80-90 % carbohydrate, with 60 – 70 % of that being starch. However, the ratio of starch to other carbohydrates like sugars, as well as the root tuber's overall relative composition, fluctuates depending on cultivar, maturity, and storage period. Proteins, vitamins, carotenoids, flavonoids, and minerals are among the other beneficial components found in sweet potatoes. Sweet potatoes have become a staple cuisine in many nations due to their high carbohydrate content (Sun *et al*., 2014). The processing of the root tuber into starch, alcoholic drinks, ethanol, flour, purees, and other commodities produces by-products that have little contemporary use and typically end up in landfills or bodies of water. The agricultural stage of sweet potato cultivation has other important implications, including: vines and leaves. The vines and leaves are rich in proteins, vitamins, minerals, pigments and polyphenols by-products of vines and leaves are of little use, except for small-scale farmers who use them to feed their livestock (Truong *et al.,* 2010).

2.4 Sweet Potato Peels

Sweet potato peels generated by industrial processes generate a tremendous amount of garbage. Approximately 30 % of the raw material used in sweet potato canning operations is abandoned and not used for human consumption, according to estimates (Schaub and Leonard, 2017). The peels are usually utilized for fertilizer or animal feed if they are used at all. However, given regional shortages, the protein content may be the most important factor to consider. Sweet potato has a total protein level of roughly 5 % by dry weight, with sporamin being the most abundant soluble protein. According to research, the tuber's protein content is not equally distributed, with larger concentrations observed on the outer layer of the flesh closest to the peel. In preparation for further processing, 4.4 % - 12 % of the tuber's protein content may be removed, depending on the manner of peeling. Sweet potato protein is a useful component in processed food applications because of its strong solubility and emulsifying capabilities. Maloney *et al.* (2014) investigated the protein content of sweet potato peels produced at two stages of the sweet potato puree processing. Sweet potato peels could be used as fish feeding in subsistence aquaculture systems.

Fish fed with sweet potato peels grew at a slower rate than fish on a control diet, according to Omoregie *et al.* (2009). However, there was no growth depression when the peels were confined to 5 – 20 % of the diet. The meals containing sweet potato peels had a crude protein content of around 31 %, which was within the needed protein levels for juvenile herbivorous fish. Sweet potato peels can be used to make soluble fiber, which is an important nutritional supplement. Sweet potatoes could be used to make low-cost soluble fiber, which could provide a new supply of low-cost dietary fiber. When compared to untreated peels, micronizing sweet potato peels resulted in a larger proportion of soluble to insoluble fiber, as well as a more beneficial, nutritional product for human consumption (Huang *et al.*, 2010a). Sweet potato peels have been successfully fermented to make lactic acid using lactic acid bacteria such as *Lactobacillus rhamnosus*. Pagana (2014) produced 10 grams (10 g) of lactic acid per liter of sweet potato trash. Based on 30 g of glucose available in the sweet potato waste media, there is 40 % of the possible yield.

Biogas can be produced via anaerobic digestion of sweet potato food waste. Ge *et al.* (2014) examined methane production of tropical forestry (albizia) wastes with food wastes such as sweet potato, taro, and papaya, and discovered that while albizia waste had the largest volumetric production, food wastes, especially sweet potato, had the best yield.

With growing consumer demand, the food processing industry is hastily substituting herbal colours derived from fruits, flowers, and veggies for synthetic colouring. The colour in the flesh and peel of distinct sweet potato cultivars is one of their defining traits. This pigmentation is due to the presence of anthocyanins and carotenoids which produce the purple, orange, and yellow colouring seen in tubers. The purple sweet potatoes are noted to have higher anthocyanin content than the other colours, there are several investigations in extracting the pigments from the tubers, while the major research is utilizing the tuber for extracting the pigments, and the colored peels of the sweet potato are equally good source of the compound. Studies have shown those anthocyanins are more concentrated in the peels than the flesh or whole tubers. Peels of purple-fleshed sweet potatoes generally contained higher anthocyanins than the flesh and could be a potential source for extracting the compound anthocyanin than the flesh (Zhu *et al.*, 2010). Steed also discovered that sweet potato peels had approximately twice as much anthocyanin as the flesh, with 174 mg /ml (Steed and Truong, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

The substrate (sweet potato peels) was obtained from Oja Oba (Market), Ilorin, Kwara State, Nigeria.

3.2 Soil Sample Collection

The soil samples were collected from two different sites within the Ilorin metropolis namely: sweet potato processing factory and agricultural soil of sweet potato, at the depth of 15 inches from the top using a sterile spatula. The samples were collected in sterile polythene bags, each and labeled appropriately then immediately transported to the Microbiology laboratory (Makut *et al.,* 2021).

3.3 Preparation and Pre-treatment of Substrate.

Substrates (sweet potato peels) were cleaned by washing with sterile distilled water, air-dried and further kept in hot air oven at 60^{o}C for 2 hrs to reduce the moisture content. After drying, it was chopped into small pieces and pulverized into powdered form with the aid of a Philips blender, sieved with a fine mesh of 0.05 mm in diameter, and stored in an air-tight sample container for further use (Ashraf *et al*., 2014).

3.4 Proximate Analysis of Sweet Potato Peels

Proximate analysis of the substrate (sweet potato peels) was carried out following the method of the Association of Official Analytical Chemists

(AOAC, 2000), to determine the carbohydrate concentration. The total carbohydrate was determined by differential method that is by subtracting total protein, moisture content, ash, lipid, and fiber from 100.

Thus: Carbohydrate (%) = (100-(moisture (%) + ash (%) + fat (%) + protein (%) + fiber (%))

3.4.1 Moisture Content

This was done by the method described by AOAC (2000). Five (5) grams of the potato peels was measured into a previously measured weighing moisture can. The sample in the can was dried in the oven at 105ºC for 3 hrs. It was cooled in desiccators and weighed. It was then returned to the oven for further drying. Drying, cooling, and weighing were done repeatedly at an hourly interval. The weight of moisture lost was calculated and expressed as a percentage of the weight of the sample analyzed. Moisture content (%) = $W2-W3 / W2- W1\times 100$

Where:

W1 = Weight of empty moisture can

W2 = weight of empty can+ Sample before drying

W3 = weight of can + sample dried to constant weight

3.4.2 Determination of Total Ash

Total ash content was determined as total inorganic matter by incineration of a sample at 600°C (AOAC, 2000). One (1) gram of the potato peel was weighed into a pre-weighed crucible and incinerated in a muffle furnace at 600ºC. The crucible was removed and cooled in desiccators and weighed. Ash content was calculated according to the following

Formula: Ash = W2−W1 /Weight of sample × 100

Where:

W1 = Weight of empty crucible

W2 = Weight of crucible + Ash

3.4.3 Determination of Crude Protein

This was determined by mixing two (2) grams of the potato peels with 25 ml of sulphuric acid in the digestion flasks, followed by the addition of 5 g of sodium sulphate and 1 g of copper sulphate. A little amount of selenium powder was also added before it was heated under a fume cupboard until a clear solution was obtained (the digest). The digest was diluted with water to a level of 100 ml in a volumetric flask and used for the analysis. 10 ml of the digest was mixed with 10 ml of 40 % NaOH and dispensed into the conical flask and a conical flask containing 10 ml of boric acid was attached to the condenser outlet and distillation was carried out for 4 mins. A total of 5 ml of the distillates was collected and titrated with 0.1 M HCl to a purplish–grey endpoint. The nitrogen and protein content was calculated (AOAC, 2000).

3.4.4 Determination of Crude Lipid

Five (5) grams of sample was wrapped in filter paper and put in a thimble. The thimble was put in soxhlet reflux flask and mounted into a weighed extraction flask containing 60 - 40 of petroleum ether. The upper of the reflux flask was connected to a water condenser. The solvent (petroleum ether) was boiled, vaporized and condensed into the reflux flask, filled with water. Soon the sample in the thimble was covered with the solvent until the reflux flask was filled upon and siphoned over, carrying its oil extract down to the boiling flask. This process was allowed to go on repeatedly for 4 hrs before the defatted sample was removed. The solvent was recovered and oil was left in the flask. The flask (containing the oil extract) was dried in the oven at 60^0C for 30 mins to remove any residual solvent. It was cooled in the desiccators and weighed (AOAC, 2000). The weight of oil (fat) extract was calculated as a percentage of the weight of sample analyzed Fat (%) = Digested sample (W2) − Ashed sample (W1)/weight of sample × 100

3.4.5 Determination of Crude Fiber

The crude fiber was determined by the method described by AOAC (2000). The potato peels was boiled with 150 ml of 1.25 % H_2SO_4 solution for 30 mins. The boiled sample was washed in several portions of water. It was returned to the flask and boiled again in 150 ml of 1.25 % of 150 ml NaOH for another 30 mins under the same condition. After washing several portions, the sample was allowed to drain before being transferred to a weighed crucible where it was dried at 105 ºC to a constant weight. It was thereafter taken to a muffle furnace where it was burnt, only ash was left out of it. The weight of the fiber was determined by difference and calculated as a percentage of the weight of the sample analyzed.

Crude fiber (%) = W2−W1/Weight of sample × 100

Where:

W1= weight of empty extraction flask

W2 = weight of flask + oil (fat) extract

3.5 Media Preparation

All media used potato dextrose agar (PDA) and Czakpek's Dox broth were prepared according to the manufacturer's instructions.

3.5.1 Preparation of Potato Dextrose Agar

Potato dextrose agar was prepared by weighing thirty-nine (39 g/l) of commercialized potato dextrose agar powder and dissolving in an equivalent volume of sterile distilled water in (ml). The media was homogenized completely on the hot plate, and then sterilized at 121^0C for 15 mins (Aryal and Sagal, 2018).

3.5.2 Preparation of Czapek-Dox Broth

Czapek-dox agar was prepared by weighing thirty-nine (35.01 g/l) of commercialized Czapek-dox agar powder and dissolving in an equivalent volume of sterile distilled water in (ml). The media was homogenized completely on the hot plate, and then sterilized at 121^0C for 15 mins (Makwin and Ade-Ibijola, 2021).

3.6 Isolation of Fungi

Fungi were isolated from soil samples and fermented substrate (sweet potato peels) by serial dilution method. Serial dilutions were carried out by putting 1 g of soil and 1 ml of fermented substrate separately into 9 ml of sterile distilled water in test tubes and dilutions were made up to 10^{-5}. An aliquot of 0.1 ml were taken from each test tube of soil and substrate suspension. The dilutions were plated on Potato Dextrose Agar (PDA) containing 0.1ml of streptomycin and incubated at 28°C for 7 days using the spread plate technique (Fawole and Oso, 2004). The fungi cultures grown on the medium were subcultured repeatedly until pure culture was obtained and then transferred on to PDA slants and maintained at 4^{o}C for further use. A typed strain of *Aspergillus niger* ATCC 10577 was obtained from the Federal Institute of Industrial Research, Oshodi, (FIIRO), Lagos state, for comparison of gluconic acid productivity. The control fungus was maintained on potato dextrose agar slants and kept at 4°C prior to further use.

3.7 Characterization and Identification of Fungal Isolates

3.7.1 Colonial Morphology

Physical features of the colony on the plates were used for the fungal identification according to Frank (2017). These include the colour of the colonies' colour of its reverse side, the height of the mycelia, and the pattern of growth.

3.7.2 Cellular Characteristics

For microscopic identification, a drop of lactophenol cotton blue was placed on a clean microscopic glass slide. A loopful of the fungal growth was removed using a sterile wire loop and placed in the drop of lactophenol blue. It was covered with a cover slip and observed under the microscope using x10 and x40 objective lens (Frank, 2017).

3.8 Inoculums Preparation

Spore suspensions of the fungal isolates were prepared by adding 10 ml of sterilized distilled water containing 2 drops of 0.1 % tween 80 to the sporulated 7days old culture. A sterile wire-loop was used to dislodge the spore clusters under sterile conditions and then mixed thoroughly to prepare a uniform spore suspension. The number of spores 2.0×10^5 spores per ml were counted using Neubauer's counting chamber as described by Grigoryev (2013).

3.9 Screening of the Fungal Cultures for Gluconic Acid Production

The isolated fungi were screened for gluconic acid production on calcium carbonate plates, the media used for the preparation of calcium carbonate plates consisted of, glucose, 10 g; $(NH_4)_2HPO_4$, 0.4 g; $MgS0_4.7H_2O$, 0.2 g; KH_2PO_4, 0.2 g; $CaCO_3$ 50 g and agar 20 g dissolved in one liter of double distilled water and autoclaved at 15 Ib for 15 mins. $CaCO_3$ was sterilized separately and added to the medium at the time of plating. The medium was inoculated with 1ml of spore suspension following incubation at 30^0C for 5 days. Clear zones were observed around distinct colonies on the plates indicating the production of calcium gluconate (Makwin and Ade-ibijola, 2021). Fungi-producing maximum clear zone was further used for gluconic acid production and also identified molecularly using PCR and gel electrophoresis to ascertain its strain.

3.9.1 Molecular Identification of Fungal Isolate

3.9.1.1 Genomic DNA Extraction

The well grown fungus (mycelia) from the fermented potato peel substrate, which was free from contamination, was filtered in filter set connected to the vacuum pump and transferred into a sterile mortar. Fungal tissue was grounded to a fine powder under liquid nitrogen using a sterile mortar and pestle. The tissue powder and liquid nitrogen was then transferred into 1.5 ml microcentrifuge tube. The extraction of genomic DNA was done using DNA extraction kit (QIAGEN-DNeasy Plant Mini Kit). The DNA extraction process was done following the manufacturer's procedures (Shamala *et al*., 2014).

3.9.1.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyse the quantity and quality of extracted genomic DNA, PCR product and purified PCR product. 1 % of agarose gel in 1X TBE buffer was used in DNA electrophoresis. The agarose was weighted and added with 30 ml 1X TBE buffer. The agarose solution was heated in microwave in high temperature. After that, the agarose gel was poured in the electrophoresis tank. The comb was removed after the gel solidified. The 1X TBE buffer was added into the tank until covering the gel. After preparations of agarose gel, 5 µl of λ Hind III marker was mixed with 2 µl of loading dye and 5 µl of the mixture was loaded into the first well and then followed by loading of DNA sample into the next well. For PCR product and purified PCR product, marker VC 1kb was used. The electrophoresis gel was run at 80 volts for 45 minutes. The process of gel electrophoresis was carried out with the aid of power supply machine (Powerpac Basic). After the gel electrophoresis, the gel was visualized using ethidium bromide under UV illumination and documented by Bio Imaging System using the GeneSnap software (Shamala *et al.*, 2014). A homology search of the sequence was conducted using the BLAST program at the NCBI database.

3.10 Gluconic Acid Productions by Submerged Fermentation

Gluconic acid fermentation was carried out by submerged fermentation in 250 ml cotton wool plugged Erlenmeyer flasks with 100 ml of fermentation media of Czapek's Dox broth consisting of (g/L) sucrose 30.0, $NaNO_3$ 3.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, KCl 0.5 and $FeSO_4.7H_2O$ 0.01 having pH 6.0. The medium was modified by substituting sucrose with thirty grams of a powdered substrate (sweet potato peels) which was dispensed into an Erlenmeyer flask. The medium was autoclaved at 121°C for 15 mins. After cooling to room temperature, the flasks were inoculated with 1 ml of fungal spore suspension, following incubation at 30^0C on a rotary shaker (200 rpm) (Ashraf *et al.*, 2014).

3.11 Optimization for Gluconic Acid Production

3.11.1 Effect of pH

Thirty gram of the sweet potato peels powder was weighed into 100 ml of Czapek dox broth. The pH of the medium was adjusted to different values (2, 4, 6, 8, and 10). An aliquot of fungi spore suspension (1 ml) containing $2.0\text{x}10^5$ spores/ml was inoculated into the flasks with each pH values. The flasks were incubated at 30^0C on a rotary shaker at 200 rpm for 7 days (Ashraf *et al.,* 2014).

3.11.2 Effect of Substrate Concentrations

Different masses/quantities of the sweet potato peels powder (10, 20, 30, 40, and 50g) were dispensed into 100 ml of Czapek Dox broth autoclaved, and cooled. An aliquot of fungi spore from soil suspension (1 ml) containing $2.0\text{x}10^5$ spores/ml was inoculated into each flask and incubated at 30^0C on a rotary Shaker at 200 rpm for 7 days (Ashraf *et al.,* 2014).

3.11.3 Effect of Incubation Time on Gluconic Acid Production

Thirty gram of the sweet potato peels powder was weighed and dispensed into 100 ml of Czapek Dox broth, autoclaved, and cooled. The pH of the media was adjusted to 6.0. An aliquot of fungi spore suspension (1 ml) containing $2.0\text{x}10^5$ spores/ml was inoculated into the flask. The flasks were incubated on a rotary shaker at 200 rpm for days (1, 3, 5, 7, 9, and 11) at 30^0C (Ashraf *et al.,* 2014).

3.11.4 Effect of Carbon Source

Thirty gram of each carbon sources (glucose, sucrose, lactose, and starch) was dispensed into 100 ml of Czapek Dox broth seperately, autoclaved, cooled, and inoculated with 1 ml fungi spore suspension containing 2.0 x 10^5 spores/ml. The flasks were incubated at 30°C on a rotary shaker at 200 rpm for 7 days (Ashraf *et al.,* 2014).

3.12 Separation of the Fermented Medium

The suspended extract and fungi biomass were separated by centrifugation at 4000 rpm for 10 mins. The clarified supernatants were filtered with the use of a filter paper and used for further analysis (Singh *et al.,* 2005)

3.13 Determination of Reducing Sugars

The reducing sugar obtained due to the enzymatic reaction was determined by dinitro salicylic acid (DNS) method (Miller, 1959; Singh *et al*., 2005). A spectrophotometer was used for measuring the absorbance. Reducing Sugar contents were determined by taking a 1.0 ml diluted solution (1 ml sample in 100 ml distilled water) with 3.0 ml of DNS reagent in a test tube. A blank containing 1.0 ml of distilled water and 3.0 ml of DNS was run parallel. The tubes were heated in a boiling water bath for 5 mins. After cooling the tubes to 37°C, 1 ml of 40 % Rochelle salt solution was added to each, and absorbance was read at 510 nm.

Sugar concentration was determined from the standard curve of glucose (Appendix 9).

3.13.1 Preparation of Glucose Standard Curve

A Stock solution of glucose was prepared by dissolving 0.1 g of glucose in 100 ml of sterile distilled water. Dilutions of different concentrations 100 mg/ml, 200 mg/ml, 400 mg/ml and 600 mg/ml were made by pippeting out 0.1 % stock solution into different test tubes. Final volume of each test tube was made up to 10 ml with the addition of distilled water followed by addition of 1.5 ml DNS reagent. The test tubes were boiled for 5 mins and cooled in ice; the absorbance was noted at 510 nm. The concentration of glucose (mg/ml) was plotted against their respective absorbance values to get the standard curve for glucose (Ali *et al*., 2014).

3.14. Assay for Total Acidity (T.A.)

The total acidity of the culture filtrates was determined by titration against standard alkaline solution, 0.1 N NaoH (Ashraf *et al.,* 2014), using phenolphthalein as an indicator. Two drops of phenolphthalein was added to 25 ml of filtrate from the fermented sweet potato peels and was titrated against 0.1 N NaoH until the colour changes to pink.

3.15. Assay for Gluconic Acid Production

The gluconic acids formed were qualitatively analyzed by high performance liquid chromatography, (Waters, Milford, USA) using the C18 ODS2 column. Elution was performed with an isocratic solvent (0.8 ml/min) using acetonitrile: H2O (3:7

v/v) and detected at 210 nm. A standard solution of gluconic acid (Sigma) was prepared and eluted similarly. The elution times of peaks were compared to the elution time of a standard peak (Lien, 1959; Singh *et al*., 2001).

3.16 Statistical Analysis of Data

The data were statistically processed to obtain the mean ± standard deviation (SD) and analysed using the one-way analysis of variance (ANOVA). All data were analyzed according to the Statistical Package for Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL). A Probability value of < 0.05 was considered to be statistically highly significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Composition of Sweet Potato Peels

The findings obtained showed percentage moisture 64.02 ± 0.27, percentage ash 3.24 ±0.16, percentage carbohydrate 20.81 ± 0.07, calorific value 402.02 ±1.20, percentage lipid 0.31 ± 0.01, percentage crude fibre 0.31 ± 0.01 and crude protein 2.55 ± 0.04% (Table 3).

4.2 Enumeration and Identification of Fungi

A total of six distinct fungi, namely, *Aspergillus niger, Aspergillus flavus, Penicillum* sp, *Cladosporium* sp, *Rhizopus stolonifer* and *Aspergillus terreus* were isolated and identified (Table 4), and their micrographs are shown in Plates 1 to 6.

4.3 Screening of Isolates for Gluconic Acid Production

Aspergillus niger from the soil showed the highest zone of clearance 6.70 ± 0.30 mm, *Penicillum* sp, showed the lowest zone of clearance 0.80 ± 0.30 mm, while *Aspergillus flavus*, *Cladosporium* sp. and *Rhizopus stolonifer* showed no zone. The observation is presented in Table 5.

4.4 Molecular Identification of Fungal Isolate

Blastering of the fungi genomic DNA sequence confirmed the fungi isolate to be *Aspergillus niger* UFMGCB 14248 as shown in Figure 2.

Table 3: Proximate Composition of Sweet Potato Peels

Parameters	Values (%)
Moisture	64.02 ± 0.27
Ash	3.24 ±0.16
Carbohydrate	20.81± 0.07
Calorific value (Kj/100 g)	402.02 ±1.20
Lipid	0.31 ± 0.01
Crude fibre	9.04 ±0.04
Crude protein	2.55 ± 0.04

Values are mean of duplicate readings and standard error of mean of proximate composition of sweet potato peels.

Table 4: Characteristics of Fungal Isolates

Fungal Isolates	Macroscopic and Microscopic Description	Probable Organisms
F_1	It appears black in colour powdery and reverse is yellow. It has smooth coloured conidiophore and the conidia are in chains.	*Aspergillus niger*
F_2	It is yellowish green in colour powdery and flat. It has aerial hypae bearing conidiophores, which are colourless, thick-walled, rough and bearing vesicles.	*Aspergillus flavus*
F_3	It appears white in colour and reverse is pale yellow; grow flat and cottony in texture. It has septate hyaline hyphae, branched conidiophores with conidia which appear round in shape.	*Penicullum* sp.
F_4	It appears powdery, green to black colour and the reverse is black. It has septate hyphae, pigmented conidiophores and conidia are in chains.	*Cladosporium* sp.
F_5	It is deeply cottony white in colour becomes grey on aged and reverse is pale white. It has non septate hypae, sporangiophores are brown in colour.	*Rhizopus stolonifer*
F_6	It appears as brown colour, reverse is yellow, grow flat on the plate. It has septate hyphae and hyaline; conidiophores are smooth-walled and have small conidia.	*Aspergillus terreus*

KEYS

F_1 –F_6 = Fungal Isolates

Table 5: Screening of Fungal Isolates for Gluconic Acid Production

Fungal Isolates	Zone of Clearance (mm)
Aspergillus niger	6.70 ± 0.30^{d}
Aspergillus flavus	0.00 ± 0.00^{a}
Penicillum sp	0.80 ± 0.30^{b}
Cladosporium sp	0.00 ± 0.00^{a}
Rhizopus stolonifer	0.00 ± 0.00^{a}
Aspergillus terreus	1.90 ± 0.30^{c}

Values are mean of duplicate readings and standard error of mean of screening of isolate for gluconic acid production. Values on the same column with different superscript alphabets are significantly different at $P < 0.05$.

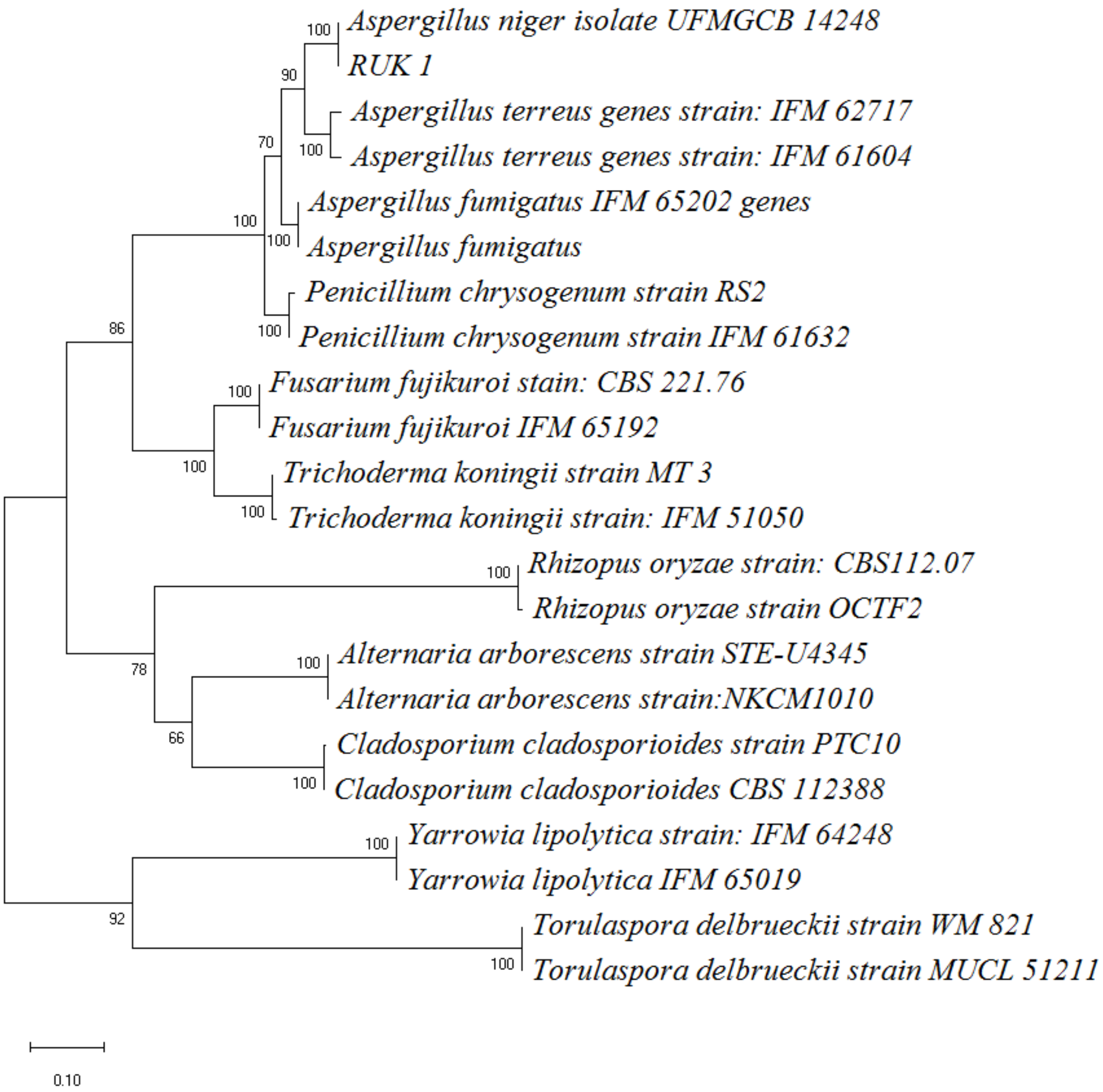

Figure 2: Phylogenetic Tree of *Aspergillus niger* UFMGCB 14248 from Soil.

4.5 Total Titratable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels at Different Substrate Concentrations

The total titratable acid (TTA) produced by *Aspergillus niger* UFMGCB 14248 at different substrate concentrations ranged from 0.90 ± 0.34 to 1.90 ± 0.11 % with the concentration 50 g/L having the highest and 10 g/l having the lowest. The TTA of the control *Aspergillus niger* ATCC 10577 ranged from 0.83 ± 0.31 % to 1.89 ± 0.24 % with concentration 40 g/l having the highest and 10 g/l having the lowest. For reducing sugar, the concentration, 50 g/L, showed the highest value of 13.20 ± 0.12 mg/ml and 10 g/L showed the least value of 9.73 ± 0.12 mg/ml for *Aspergillus niger* UFMGCB 14248 while concentration 40 g/l showed the highest value of 13.81 ± 0.33 mg/ml and 10 g/l showed the least value of 9.10 ± 0.08^{a} mg/ml for control *Aspergillus niger* ATCC 10577. The pH readings at different substrate concentrations for *Aspergillus niger* UFMGCB 14248 ranged from 5.89 to 6.19 while that of the control *Aspergillus niger* ATCC 10577 ranged from 6.06 to 6.22. These observations are presented in Table 6.

4.6 Total Titratable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels Using Different Carbon Sources

Starch showed the highest TTA value of 2.16 ± 0.17 % for *Aspergillus niger* UFMGCB 14248 while sucrose showed the highest TTA value of 2.32 ± 0.03 % for the control *Aspergillus niger* ATCC 10577. Glucose had the highest reducing sugar values of 13.75 ± 0.23 mg/ml for *Aspergillus niger* UFMGCB 14248 and

15.43±0.08 mg/ml for the control *Aspergillus niger* ATCC 10577. The pH readings at different carbon sources for *Aspergillus niger* UFMGCB 14248 ranged from 5.89 to 6.33 and those of the control *Aspergillus niger* ATCC 10577 ranged from 5.93 to 6.18 (Table 7).

4.7 Total Titratable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels at Different Incubation Days

At different incubation days the highest TTA value was observed at day 7 for both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577 with 1.96±0.11 % and 1.90 ± 0.03 % values, respectively. For the reducing sugar, the highest values were observed at day 7 for both *Aspergillus* niger UFMGCB 14248 and control *Aspergillus niger* ATCC 10577 with 13.43 ± 0.17 mg/ml and 12.11 ± 0.17 mg/ml respectively. The pH readings at different incubation days for *Aspergillus* niger UFMGCB 14248 ranged from 5.07 to 6.00 and those of the control *Aspergillus niger* ATCC 10577 ranged from 5.05 to 6.00 as shown in Table 8.

Table 6: Total Titrable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels at Different Substrate Concentrations

Substrate Conc (g/l)	TTA (%)		Reducing Sugar (mg/ml)		pH Values	
	Aspergillus niger UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577
10	0.90 ± 0.34^{a}	0.83 ± 0.31^{a}	9.73 ± 0.12^{a}	9.10 ± 0.08^{a}	6.09	6.06
20	1.10 ± 0.21^{a}	1.13 ± 0.08^{b}	12.61 ± 0.03^{d}	11.43 ± 0.03^{b}	5.98	6.11
30	1.80 ± 0.13^{b}	1.74 ± 0.11^{c}	10.93 ± 0.33^{b}	13.10 ± 0.17^{d}	6.19	6.11
40	1.86 ± 0.17^{b}	1.90 ± 0.24^{d}	11.43 ± 0.23^{c}	13.81 ± 0.33^{e}	6.12	6.08
50	1.90 ± 0.11^{b}	1.89 ± 0.17^{d}	13.20 ± 0.12^{e}	12.72 ± 0.11^{c}	6.01	6.22

Values are mean of duplicate readings and standard error of mean and values on the same column with different superscript alphabets are significantly different at $P < 0.05$.

Parameters: pH 6, Temperature, 30°C, Day 7.

Table 7: Total Titrable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels Using Different Carbon Sources

Carbon Sources (30g/l)	TTA (%)		Reducing Sugar (mg/ml)		pH Values	
	Aspergillus niger UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577
Glucose	2.12 ± 0.03^{b}	1.99 ± 0.11^{c}	13.75 ± 0.23^{d}	15.43 ± 0.08^{d}	5.89	5.93
Sucrose	2.11 ± 0.13^{b}	2.32 ± 0.03^{d}	12.61 ± 0.08^{c}	11.98 ± 0.18^{c}	6.33	5.94
Lactose	1.96 ± 0.21^{a}	1.84 ± 0.08^{a}	3.91 ± 0.03^{b}	3.91 ± 0.03^{b}	5.94	6.01
Starch	2.16 ± 0.17^{c}	1.90 ± 0.17^{b}	2.43 ± 0.13^{a}	2.10 ± 0.21^{a}	6.19	6.18

Values are mean of duplicate readings and standard error of mean. Values on the same column with different superscript alphabets are significantly different at $P < 0.05$.

Parameters: pH 6, Temperature, 30°C, Day 7, using different carbon sources.

Table 8: Total Titrable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels at Different Incubation Days

Incubation days	TTA (%)		Reducing Sugar (mg/ml)		pH Values	
	Aspergillus niger UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577
1	0.36 ± 0.13^{a}	0.53 ± 0.16^{a}	5.13 ± 0.13^{a}	5.73 ± 0.11^{a}	6.00	6.00
3	0.90 ± 0.08^{b}	1.13 ± 0.23^{b}	9.61 ± 0.08^{b}	9.23 ± 0.08^{b}	6.00	5.59
5	1.40 ± 0.06^{c}	1.74 ± 0.08^{d}	11.93 ± 0.03^{d}	11.80 ± 0.03^{e}	5.67	5.49
7	1.96 ± 0.11^{f}	1.90 ± 0.03^{e}	13.43 ± 0.17^{f}	12.11 ± 0.17^{f}	5.40	5.30
9	1.90 ± 0.17^{e}	1.89 ± 0.17^{e}	13.17 ± 0.22^{e}	11.73 ± 0.21^{d}	5.21	5.19
11	1.73 ± 0.21^{d}	1.62 ± 0.12^{c}	9.94 ± 0.13^{c}	9.34 ± 0.13^{c}	5.07	5.05

Values are mean of duplicate readings and standard error of mean. Values on the same column with different superscript alphabets are significantly different at $P < 0.05$.

Parameters: pH 6, Temperature, 30°C.

4.8 Total Titratable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels at Different pH Values

The highest level of TTA and reducing sugar at different pH values were observed for both isolates at pH 6. The pH readings at different pH values for *Aspergillus niger* UFMGCB 14248 ranged from 2.91 to 9.33 and the control *Aspergillus niger* ATCC 10577 ranged from 2.97 to 9.27 (Table 9).

4.9 Effect of Substrate Concentrations on Gluconic Acid Production

The findings obtained revealed that the highest concentration of gluconic acid produced by *Aspergillus niger* UFMGCB 14248 and control *Aspergillus niger* ATCC 10577 was at substrate concentration of 50 g/L with 69.01 ± 0.11 mg/ml and 70.67 ± 0.08 mg/ml respectively. This is presented in Figure: 3.

4.10 Effect of Carbon Sources on Gluconic Acid Production

The highest concentration of Gluconic acid (16.78 ± 0.13 mg/ml) was obtained by *Aspergillus niger* UFMGCB 14248, while the control *Aspergillus niger* 10577 produced 13.45 ± 0.27 mg/ml, using starch as carbon source. This is presented in Figure: 4.

Table 9: Total Titrable Acid, Reducing Sugar and pH of Fermented Sweet Potato Peels at Different pH

pH	TTA (%)		Reducing Sugar (mg/ml)		pH Values	
	Aspergillus niger UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577	*Aspergillus niger* UFMGCB 14248	*Aspergillus niger* ATCC 10577
2	0.26 ± 0.11^a	0.23 ± 0.26^a	4.13 ± 0.02^a	4.73 ± 0.17^a	2.91	2.97
4	0.40 ± 0.17^b	1.03 ± 0.03^b	5.61 ± 0.08^b	5.23 ± 0.08^b	4.97	4.76
6	1.90 ± 0.08^e	1.94 ± 0.08^e	10.93 ± 0.26^e	13.80 ± 0.03^e	5.94	6.33
8	1.36 ± 0.03^d	1.60 ± 0.21^c	7.43 ± 0.17^d	9.11 ± 0.11^c	7.71	7.71
10	1.13 ± 0.11^c	1.76 ± 0.24^d	7.17 ± 0.21^c	10.73 ± 0.26^d	9.33	9.27

Values are mean of duplicate readings and standard error of mean. Values on the same column with different superscript alphabets are significantly different at $P < 0.05$.

Parameters: Temperature, 30°C, Day 7.

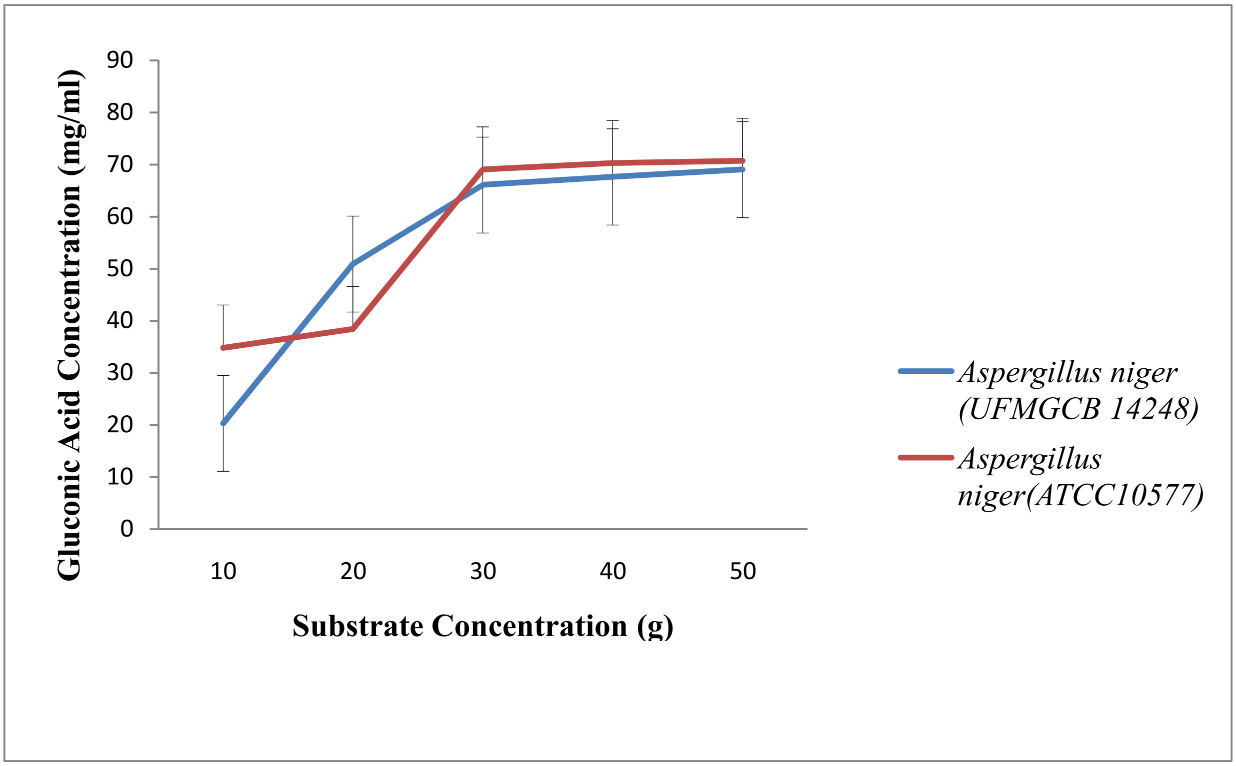

Figure 3: Effect of Substrate Concentration on Gluconic Acid Production

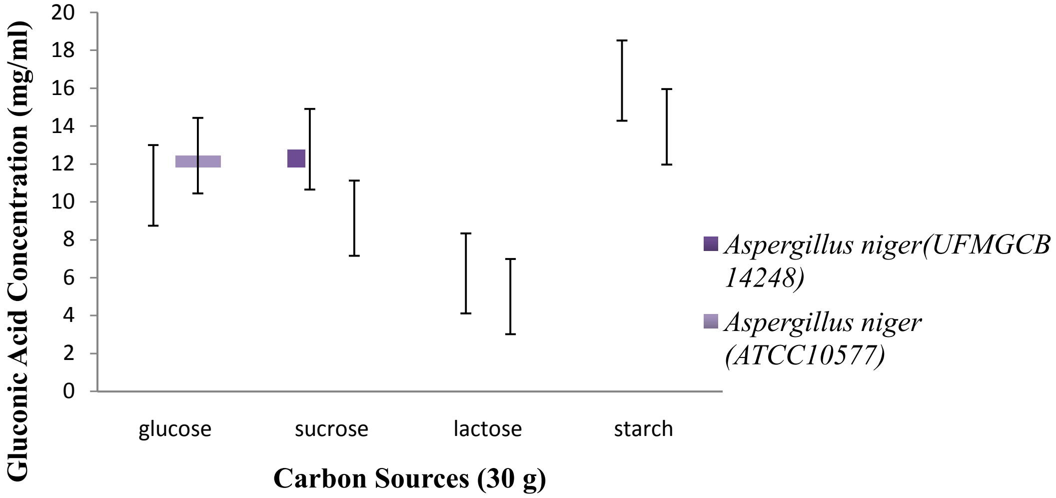

Figure 4:Effect of Carbon Sources on Gluconic Acid Production

Figure 4: Effect of Carbon Sources on Gluconic Acid Production

4.11 Effect of Incubation Days on Gluconic Acid Production

At different incubation days, the highest gluconic acid concentration was observed at day 7, with *Aspergillus niger* UFMGCB 14248 having 18.23±0.08 mg/ml, while the control A*spergillus niger* ATCC 10577 had 63.12 ± 0.17 mg/ml. This is presented in Figure 5

4.12 Effect of pH on Gluconic Acid Production

The highest concentration of gluconic acid 28.32 ± 0.08 mg/ml was obtained by the control *Aspergillus niger* ATCC 10577 and *Aspergillus niger* UFMGCB 14248 had 21.01 ± 0.13 mg/ml at pH 6 as presented in Figure 6.

4.13 Best Combined Parameters

Aspergillus niger UFMGCB 14248 produced TTA of 2.96 ± 0.06 %, reducing sugar value of 20.93 ± 0.08 mg/ml and gluconic acid concentration of 69.89 ± 0.21 mg/ml, while for the control *Aspergillus niger* ATCC 10577, TTA of 3.04 ± 0.28 %, reducing sugar 25.06 ± 0.14 mg/ml, and gluconic acid concentration of 75.54 ± 0.06 mg/ml were obtained (Table 10).

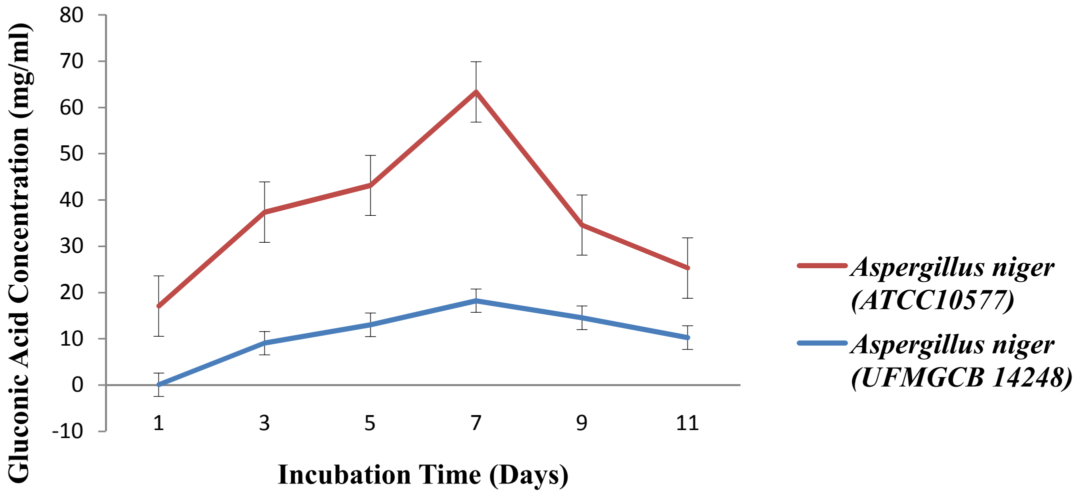

Figure 5: Effect of Incubation Time on Gluconic Acid Production

Figure 5: Effect of Incubation Time on Gluconic Acid Production

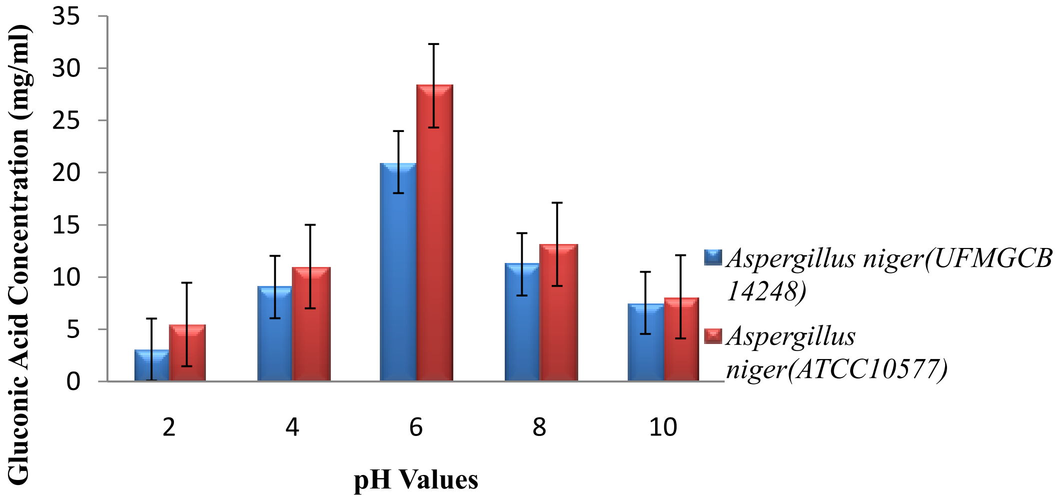

Figure 6: Effect of pH on the Gluconic Acid Production

Figure 6: Effect of pH on the Gluconic Acid Production

Table 10: Total Titrable Acid, Reducing Sugar and Gluconic Acid produced from Fermented sweet Potato peels

Isolates	TTA (%)	Reducing sugar (mg/ml)	Gluconic Acid Conc. (mg/ml)
Aspergillus niger (UFMGCB 14248)	2.96± 0.06^{a}	20.93 ± 0.08^{b}	69.89 ± 0.21^{c}
Aspergillus niger (ATCC10577)	3.04 ± 0.28^{a}	25.06 ± 0.14^{b}	75.54 ± 0.06^{c}

Values are mean of duplicate readings and standard error of mean and values on the same column with different superscript alphabets are significantly different at $P < 0.05$.

Best Combined Parameters: Temperature, 30^{o}C, pH 6.0, Substrate Starch, 30 g, Day 7.

CHAPTER FIVE

5.0 DISCUSSION

The production of gluconic acid by fermenting glucose or a glucose-containing substrate using suitable microorganisms is a mature biological process with the literature reporting very efficient processes dating back to 1940. Today, despite many approaches to the production of gluconic acid, microbial fermentation is still the method of choice because other methods are more expensive and less effective than fermentation with *Aspergillus niger* as the most commonly used microorganism (fungus) (Ramachandran *et al.*, 2006). This study was able to isolate fungi from the soil and fermented sweet potato peels. This is expected because fungi play an important role in breaking down most organic and inorganic wastes in the environment and as earlier reported by Nimkar *et al.* (2010).

Sweet potato peel was used as substrate for gluconic acid production. The proximate composition of the sweet potato peels revealed that the carbohydrate, protein and fat contents were high enough to serve as a good source of carbon and energy for gluconic acid production. The Protein content also indicated that it may be a good source of nitrogen for microbial metabolism. Nitrogen is one of the most essential components of fungal fermentation media and various studies have reported that the yield of high nitrogen-containing organic acids has increased (Betiku *et al.,* 2016). The findings from this study were similar to that of Katherine *et al*. (2014).

The screening of the distinct fungal isolates revealed that *Aspergillus niger* had the highest zone of clearance compared to other fungal isolates. This is similar to the reports from several researchers that *Aspergillus niger* is the most common producer of gluconic acid (Sharma *et al.*, 2015).

The *Aspergillus niger* that produced the highest zone of clearance was confirmed to be *Aspergillus niger* UFMGCB 14248. Evaluating the different parameters that is substrate concentration, carbon source, pH and incubation period for total titratable acid (TTA) and reducing sugar of fermented sweet potato peels, *Aspergillus niger* UFMGCB 14248 produced the highest TTA value at a substrate concentration of 50 g/L and lowest TTA value was at a substrate concentration of 10 g/L at day 7, while the control *Aspergillus niger* ATCC 10577 produced the highest TTA value at a concentration off 40 g/L and substrate concentration of 10 g/L showed the lowest TTA. For the reducing sugar, concentration of 50 g/L showed the highest and 10 g/L showed the least value for *Aspergillus niger* UFMGCB 14248, while a concentration of 40 g/L showed the highest value and 10 g/L showed the least value for the control *Aspergillus niger* ATCC 10577.

For the carbon sources, starch showed the highest TTA value for *Aspergillus niger* UFMGCB 14248, while sucrose was highest for the control *Aspergillus niger* ATCC 10577. Glucose had the highest reducing sugar for both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577. This finding is in line with the reports of Ikeda *et al.* (2006) and Shindia *et al.* (2006), who separately reported high titratable acidity using *Aspergillus niger* on different carbon sources. Sharma *et al.* (2015) also reported high yield of titratable acid by glucose compared to other carbon sources used in their study.

At different incubation periods, the highest TTA value was observed at day 7 for both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577, while for reducing sugar, the highest values were also observed at day 7 for both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577. This observation is in line with that of Mafra *et al.* (2014), who reported at high titratable acidity at high substrate concentration used in their study. This acidity could be as a result of the high substrate concentration used.

The ability of gluconic acid production in various amount demonstrated by the different fungal isolates in this study is in agreement with the findings reported by Shindia *et al*. (2006) on the ability of gluconic acid production by some local fungi which are known to utilized various substrates and release different secondary metabolites.

The highest gluconic acid concentration produced by both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577 was at substrate concentration of 50 g/L. This could be as a result of the amount of substrate concentrate, since the gluconic acid produced was increasing with an increase in the substrate concentration used. Makut *et al.* (2021) reported the highest gluconic acid production at different substrate concentrations by *Aspergillus niger* Bs2 at a substrate concentration of 25 %, followed by substrate concentrations of 20 %, 15 %, 10 % and 5 % substrate concentration.

Gluconic acid production varied with different carbon sources. Starch produced the highest concentration of gluconic acid by both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577. This might be as a result of starch being a polysaccharide. Sharma *et al.* (2015) contradicted this with the report of high gluconic acid concentration using glucose and sucrose by *Aspergillus niger,* athough glucose produced higher gluconic acid concentration than sucrose. It was also confirmed that the concentration of carbon sources plays an important role on the conversion of glucose into gluconic acid. The enhancement in the gluconic acid production can be attributed to the induction of glucose oxidase at increased glucose level in the fermented medium (El-Enshasy, 2003). At different incubation periods, the highest gluconic acid concentration was observed at day 7 for both *Aspergillus niger* UFMGCB 14248 and the control *Aspergillus niger* ATCC 10577. From this study, the active fungal growth began after day 2 of fermentation and was accompanied by increasing levels of gluconic acid production. The kinetics of growth and gluconic acid production were studied by Znad *et al.* (2004) and their findings supported the results obtained from this study. However, some researcher found that an incubation period of 6 days was optimal for gluconic acid production by *Aspergillus niger* strains (Sharma *et al.*, 2015). The effect of pH is an important parameter in the production of organic acids, or bio-acids, using fungal or any other microorganisms. At pH 6, both isolates produced the highest gluconic acid concentrations. This is in agreement with the findings obtained by Makut *et al.* (2021) who reported over 50 % yield of gluconic acid at pH range of 5 to 7. The best yield was at pH 6.0. However, the acid yield above and below this pH was poor (Dowdells *et al.,* 2010). The findings confirm that sweet potato skin is a good substrate for gluconic acid production, confirming the observations of Sharma *et al*. (2015) and Yi *et al*. (2017) on the suitability of industrial agricultural waste as a fermentable substrate

5.1 Conclusion

The data obtained in this study indicated that good yields of gluconic acid from fermented sweet potato peel depend on the presence of suitable physical conditions and nutritional requirements during fermentation. Therefore, it can be concluded from this study that the experimental *Aspergillus niger* strain can be used for the production of gluconic acid when grown on an optimized biological process.

5.2 Recommendations

The following recommendations are made based on the findings of this research.

Agricultural wastes such as sweet potato skins should be used as a natural substrate for the production of organic acids, such as gluconic acid, as a means of converting waste into wealth.

Gluconic acid should be produced through fermentation to reduce chemical problems. It can also be produced from natural substrates, as it is cheaper and easier to obtain than synthetic carbohydrates.

The government should encourage the use of agricultural wastes as source of gluconic acid to reduce environmental pollution.

The government should provide adequate equipment in various laboratory centers and research institutes for optimal production of gluconic acid.

5.3 Contribution to Knowledge

This study has shown that gluconic acid can be produced from sweet potato peels by naturally occurring fungi, *Aspergillus niger,* under optimal fermentation conditions. Agricultural wastes are good sources of carbon for the production of organic acids.

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APPENDIX

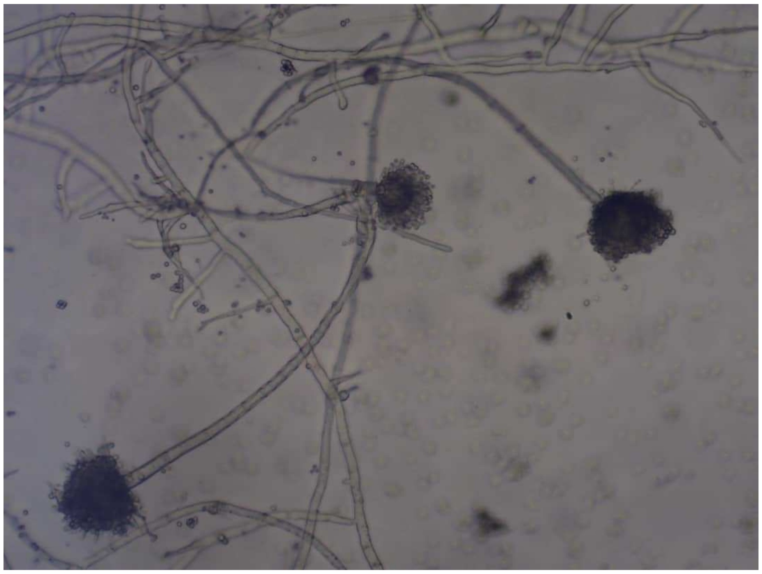

Plate1: Micrographic Features of *Aspergillus niger*

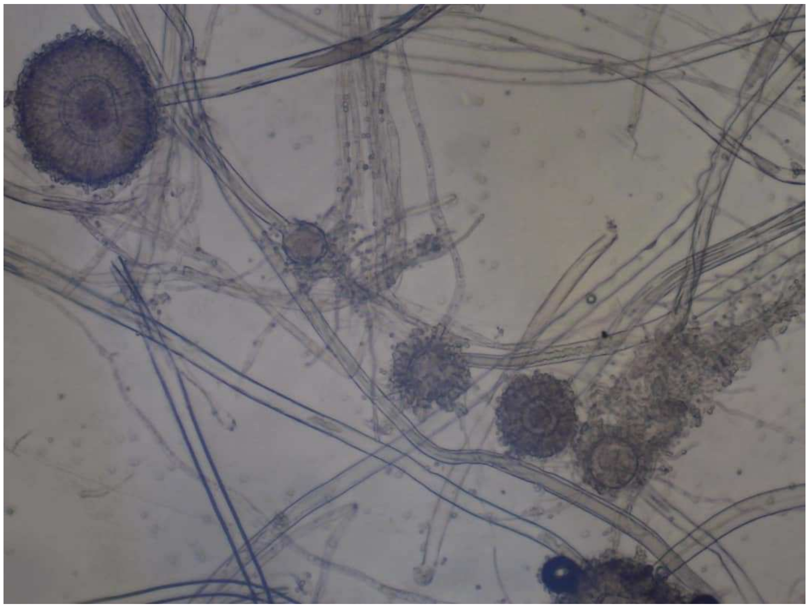

Plate 2: Micrographic Features of *Aspergillus flavus*

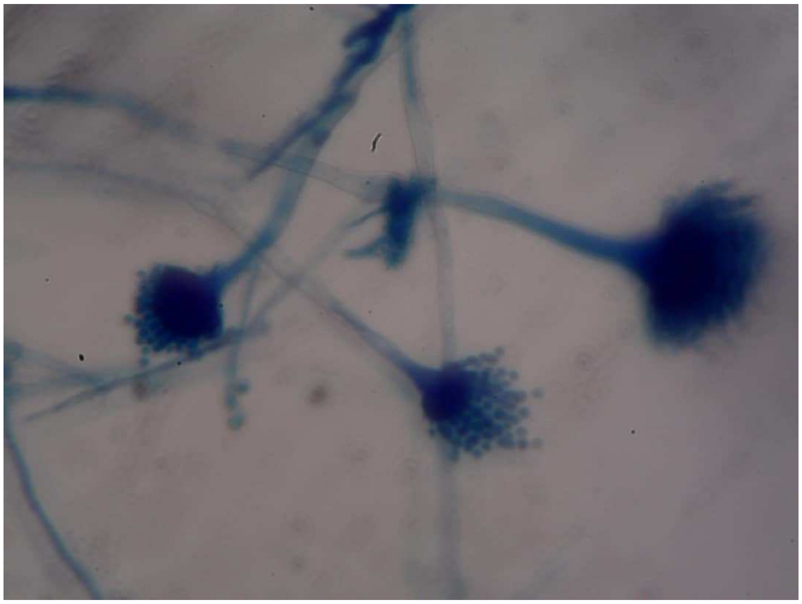

Plate 3: Micrographic Features of *Penicillum* sp.

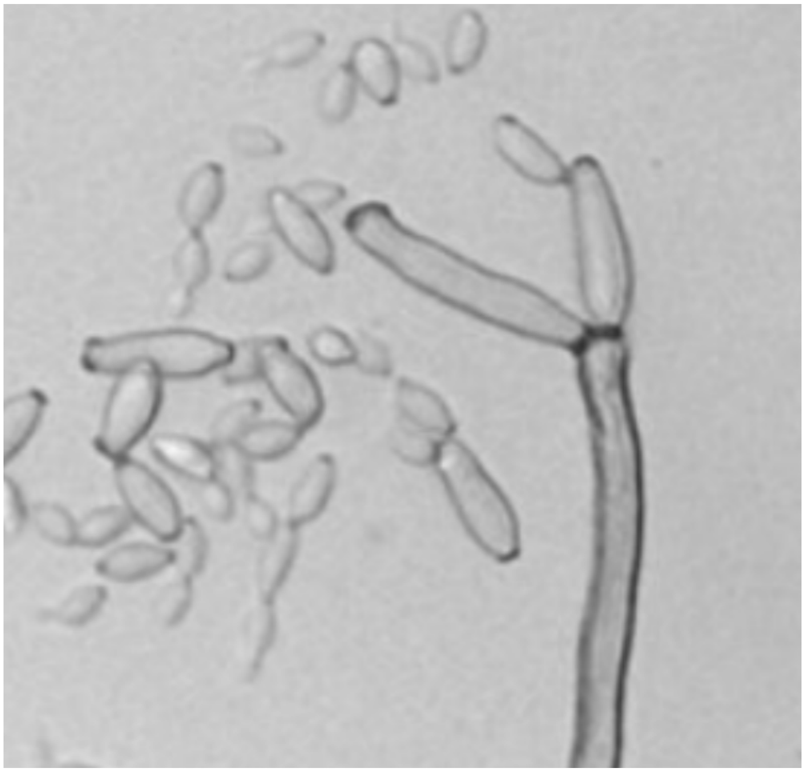

Plate 4: Micrographic Features of *Cladosporium* sp.

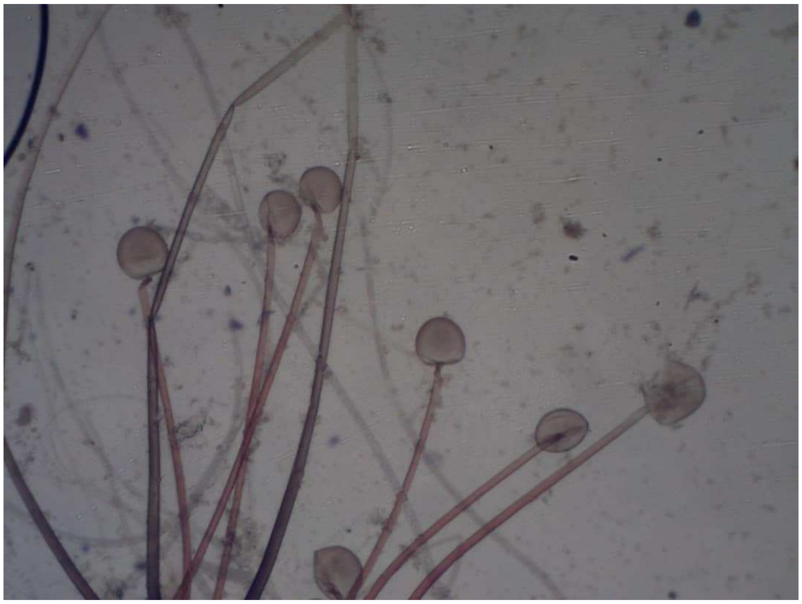

Plate 5: Micrographic Features of *Rhizopus stolonifer*

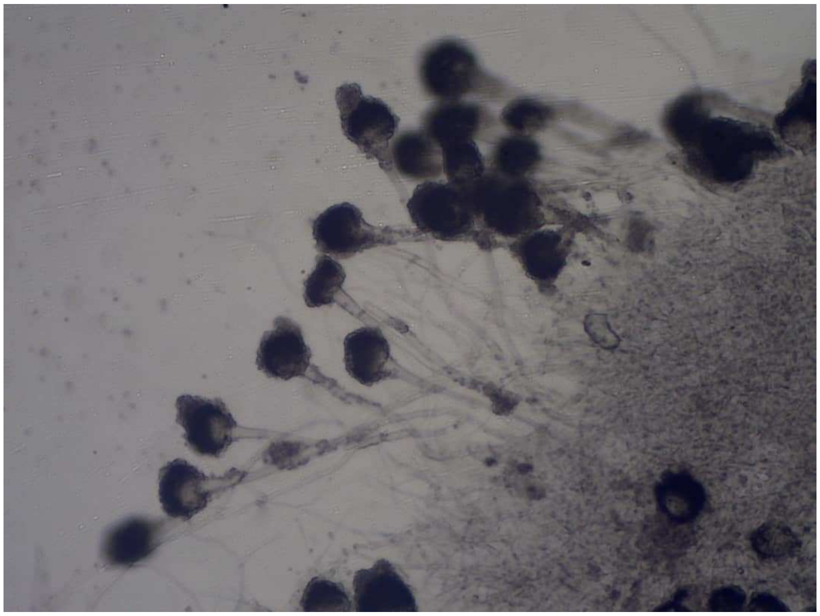

Plate 6: Micrographic Features of *Aspergillus terreus*

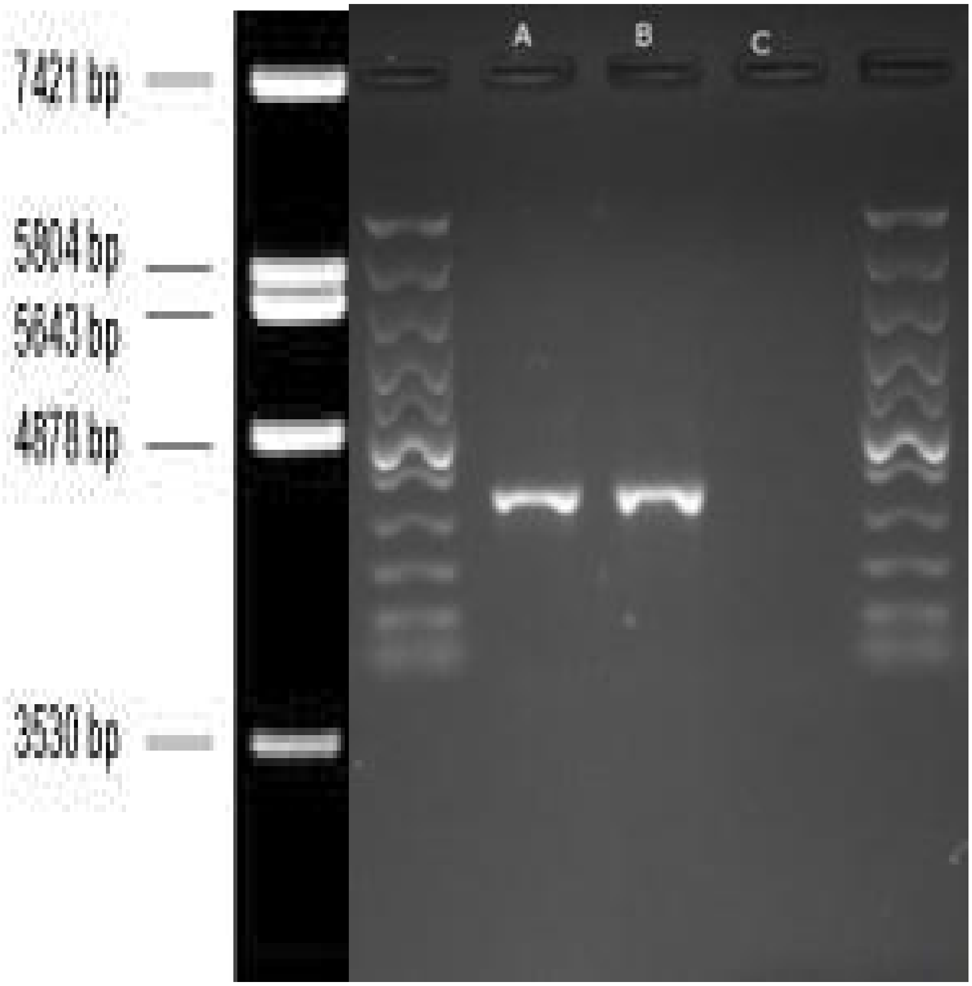

Plate: 7 Gel Electrophoresis of Fungi Isolate

Key

B = Identifying *Aspergillus niger*

AMCCTGATCCGAGGTCACCTGGAAAGAATGGTTGGAAAACGTCGGCAG
GCGCCGGCCAATCCTACWGAGCATGTGACAAAGCCCCATACGCTCGAG
GATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCCGGAGAG
GGGGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACG
CTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTC
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CCCCCCGGCGGCCGACAAGCGGCGGGCCCGCCGAAGCAACAGGGTACA
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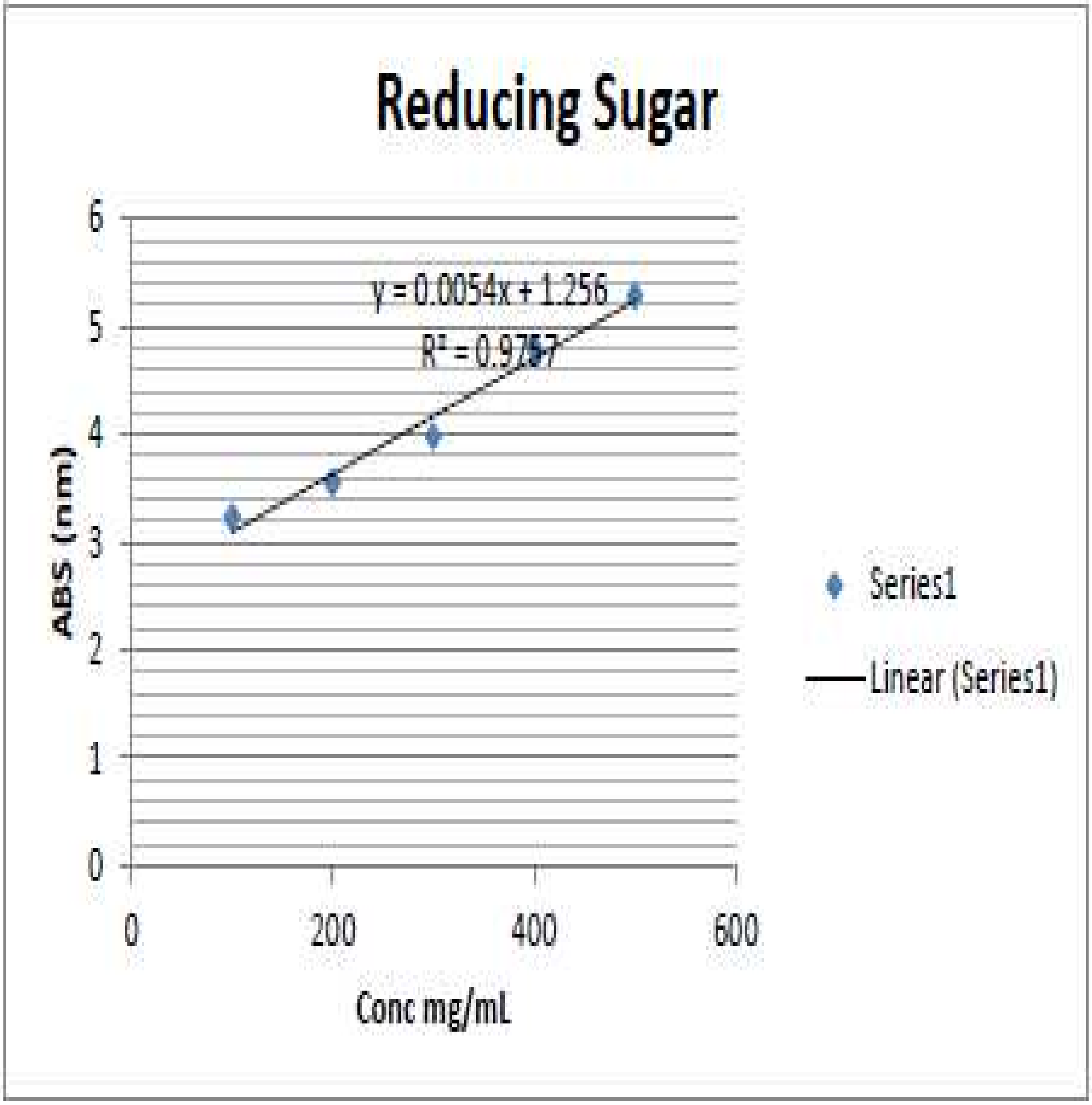
A

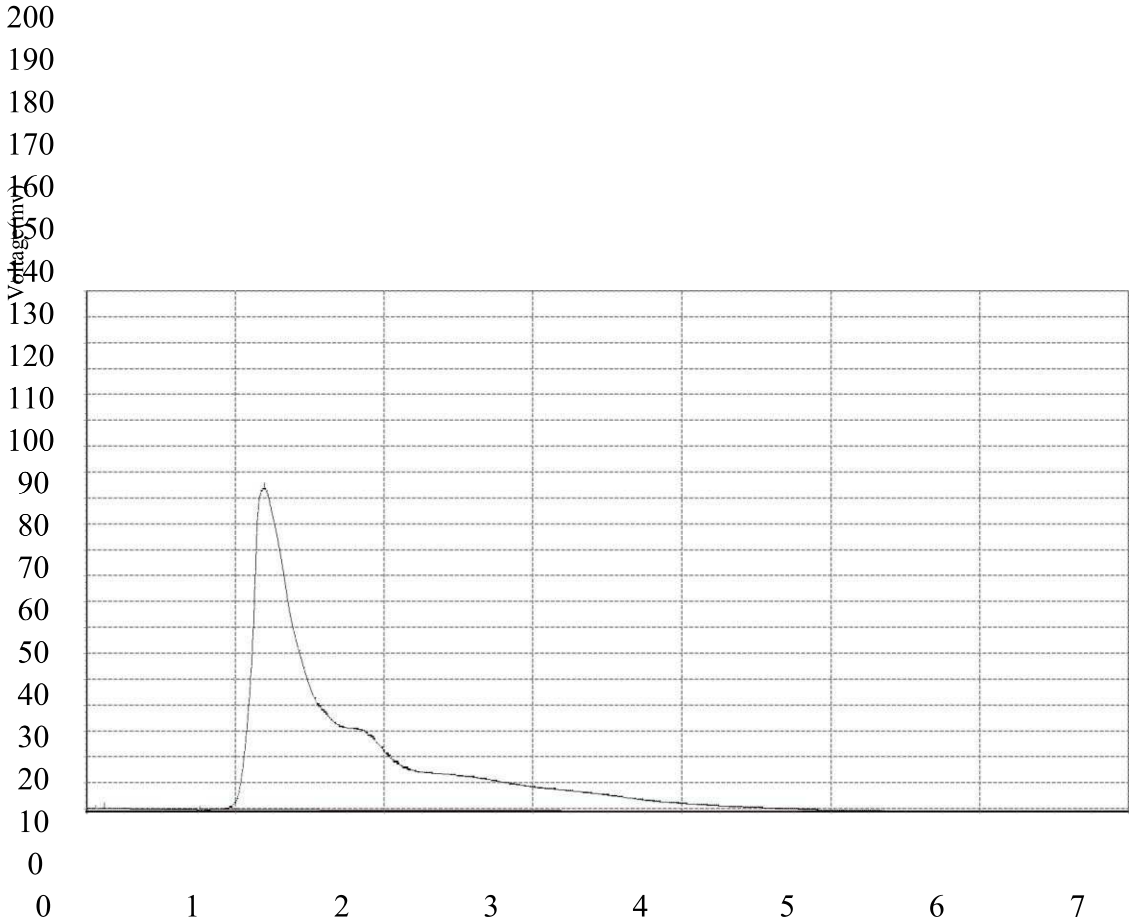


Reducing Sugar
y = 0.0054x + 1.256
R² = 0.9757
ABS (nm)
Conc mg/mL
0
1
2
3
4
5
6
0
200
400
600
Series1
Linear (Series1)

Instrument:LC Gradient:HighPressure Detector:DAD

Column Temp.(¡æ)£º40 Wavelength(nm)£º245

Chromatogram (GLUCONICACID Ret. Time (min)



RESULTS

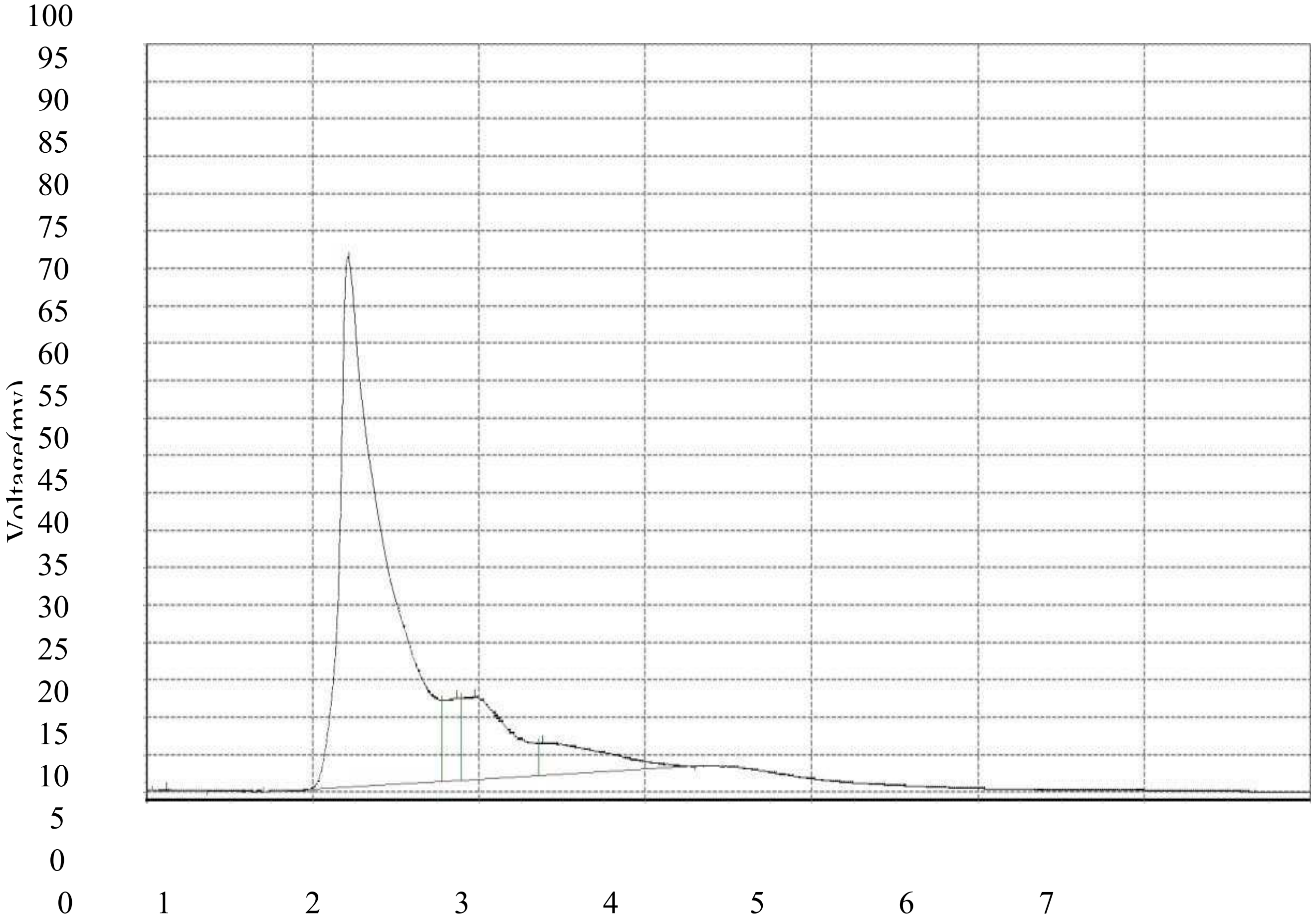
Peak No.	Peak ID	Ret Time	Height	Area	Conc mg/ml
1	Unidentified	0.115	252.625	1332.800	0.0295
2	Gluconic acid	1.190	124232.06	4512676.000	99.9705

System Evaluation

Instrument:LC Gradient: Detector:DAD

Column Temp.(¡æ)£º40 Wavelength(nm)£º245

Chromatogram Ret. Time (min)



RESULTS

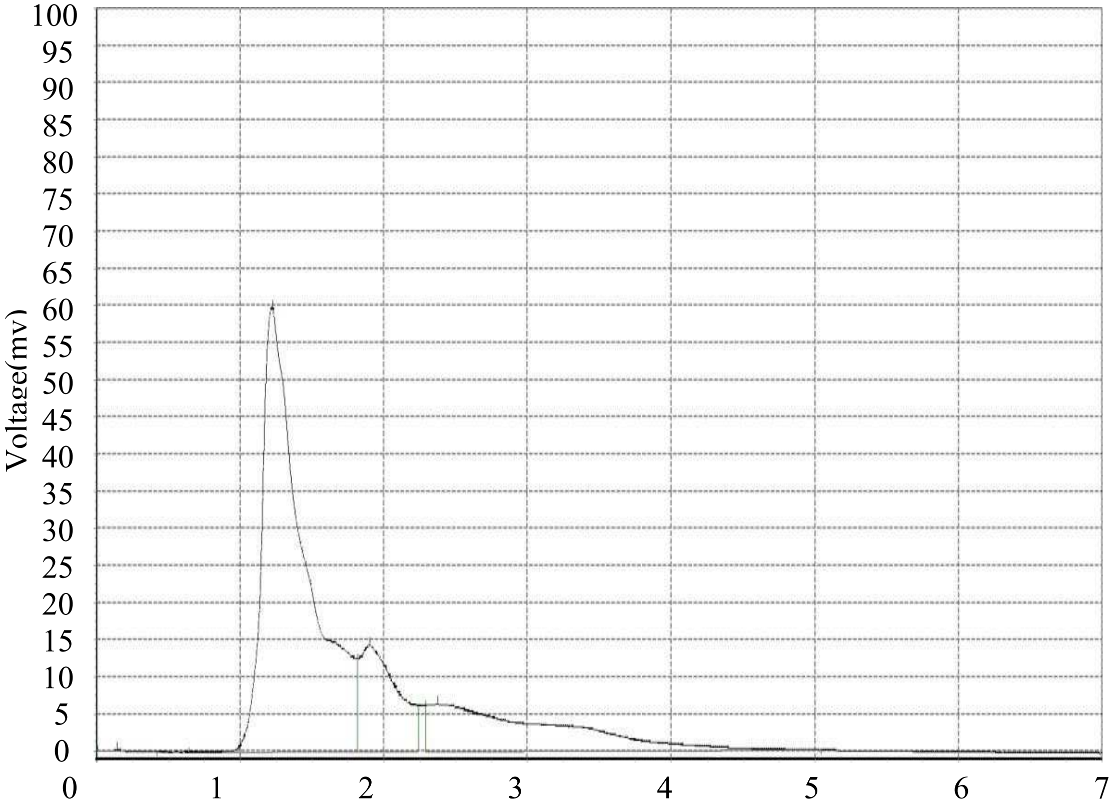
Peak No.	Peak ID	Ret Time	Height	Area	Conc mg/ml
1	Unidentified	0.115	147.256	912.700	0.0542
2	Unidentified	1.215	70646.734	1271036.000	75.4980
3	Unidentified	1.865	10993.230	76210.570	4.5268
4	Gluconic acid	1.973	11024.480	216748.000	12.8746
5	Unidentified	2.382	4267.961	118629.453	7.0464

System Evaluation

Instrument:LC Gradient: Detector:DAD

Column Temp.(¡æ)£º40 Wavelength(nm)£º245

Chromatogram Ret. Time (min)

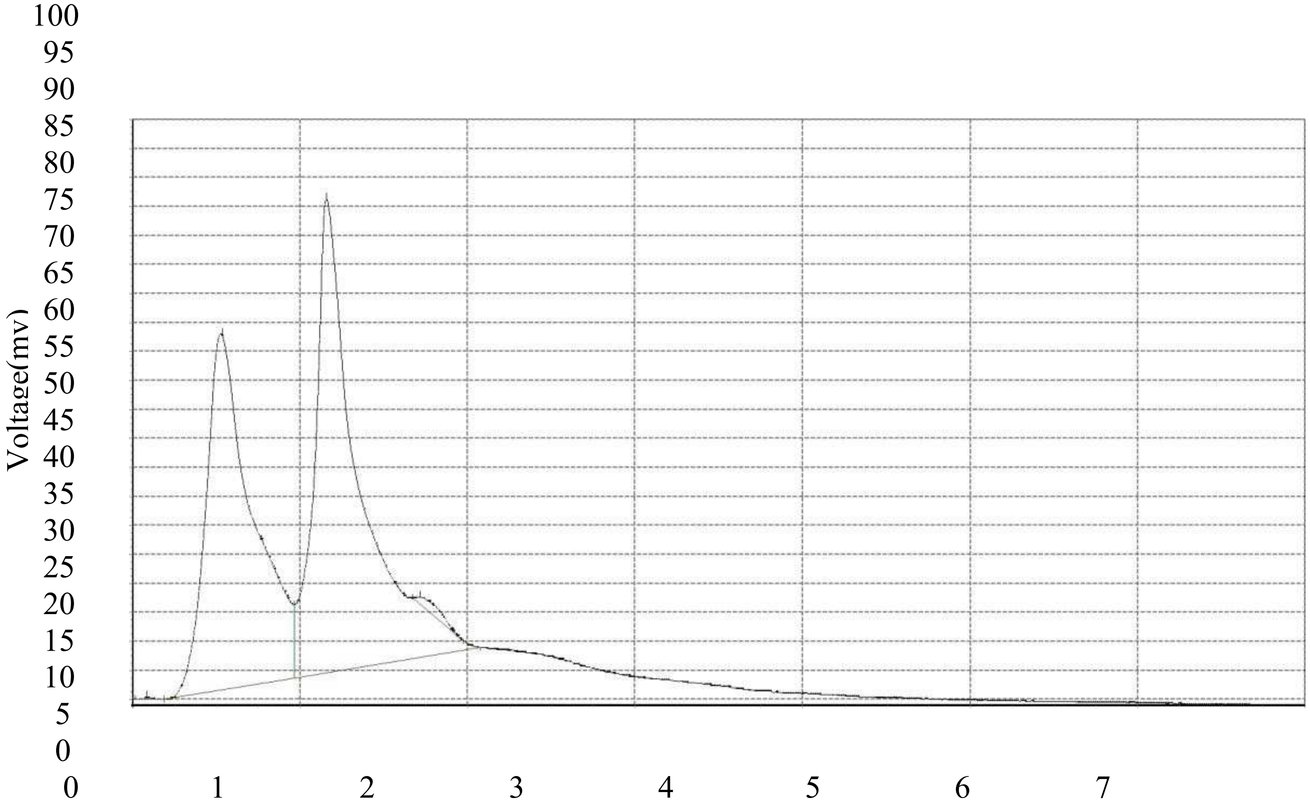


RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	C m
1	Unidentified	0.140	192.551	1188.100	0.0
2	Unidentified	1.223	59838.555	1225591.125	65.2
3	Gluconic acid	1.898	14357.064	262757.656	13.9
4	Unidentified	2.373	6382.758	390189.313	20.7

Instrument:LC Gradient: Detector:DAD
Column Temp.(¡æ)£º40 Wavelength(nm)£º245

Chromatogram Ret. Time (min)



RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	Conc mg/ml
1	Unidentified	0.082	379.200	1250.600	0.0521
2	Unidentified	0.532	61310.418	1186594.000	49.4435
3	Gluconic acid	1.197	81617.953	1193938.375	49.7495
4	Unidentified	1.715	1169.972	18118.199	0.7550

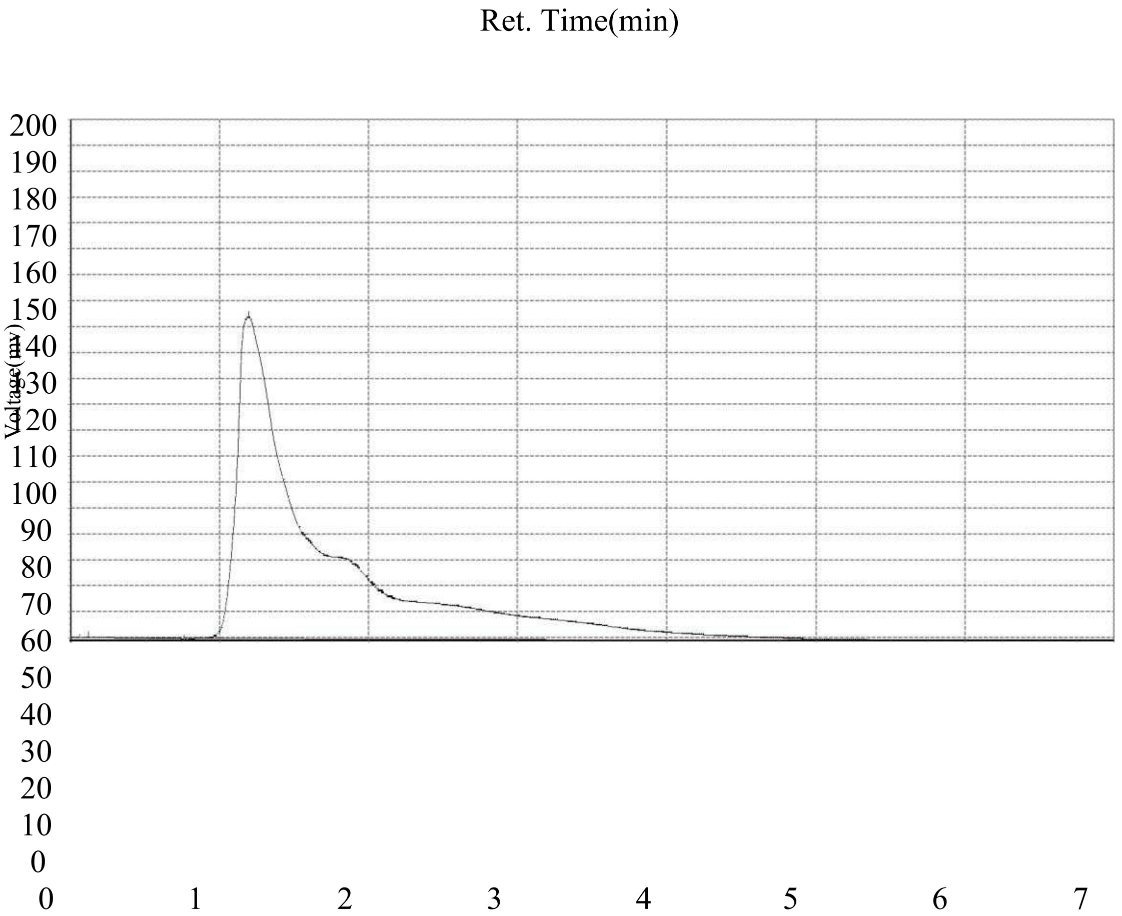
Instrument:LC

Column Temp.(¡æ)£º40

Gradient:High Pressure

Detector:DAD

Wavelength(nm)£º245



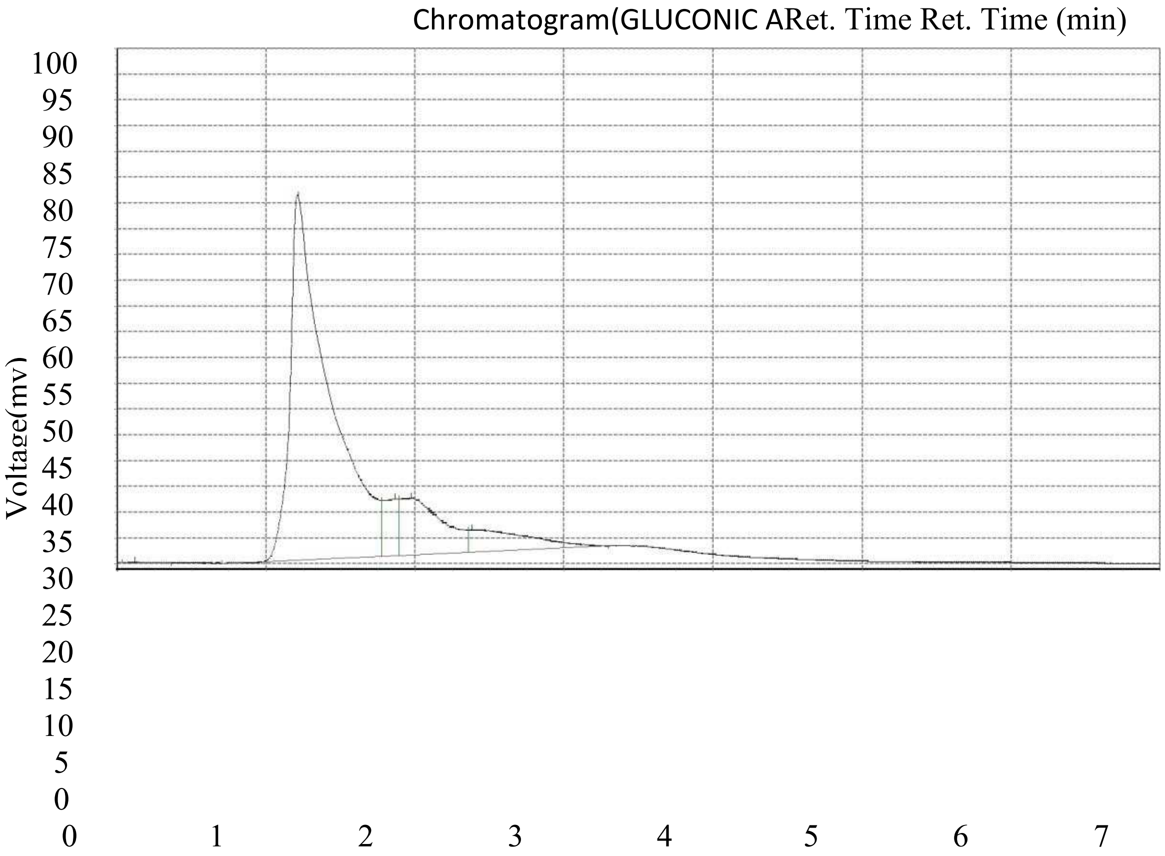
RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	Conc mg/ml
1	Unidentified	0.115	252.625	1332.800	0.0295
2	Gluconic acid	1.190	124232.063	4512676.000	69.9705

System Evaluation

Instrument:LC Gradient: Detector:DAD

Column Temp.(¡æ)£º40 Wavelength(nm)£º245



RESULTS

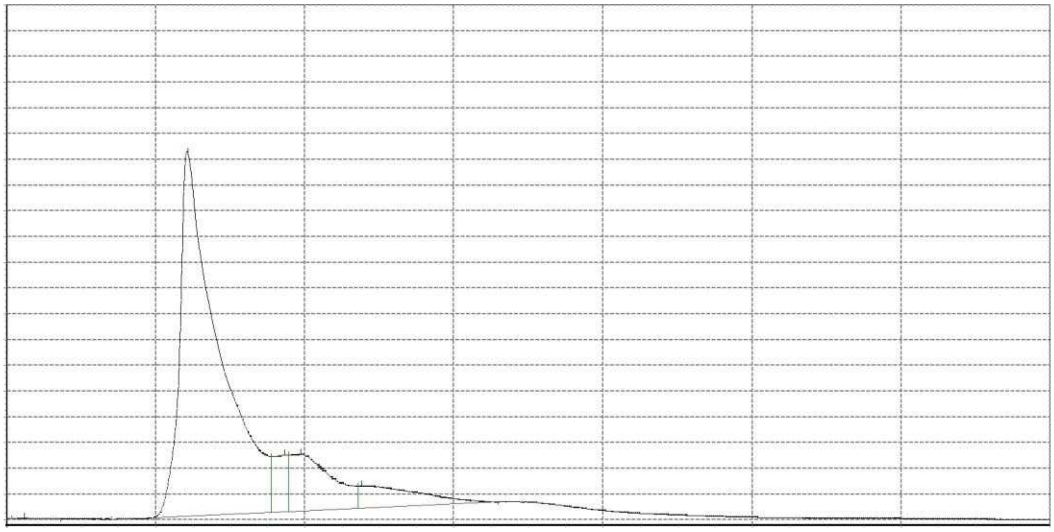
Peak No.	Peak ID	Ret Time	Height	Area	Conc mg/ml
1	Unidentified	0.115	147.256	912.700	0.0542
2	Unidentified	1.215	70646.734	1271036.000	75.4980
3	Unidentified	1.865	10993.230	76210.570	4.5268
4	Gluconic acid	1.973	11024.480	216748.000	12.8746
5	Unidentified	2.382	4267.961	118629.453	7.0464

System Evaluation

Instrument:LC Gradient: Detector:DAD

Column Temp.(¡æ)£º40 Wavelength(nm)£º245

Chromagram Ret. Time(min)

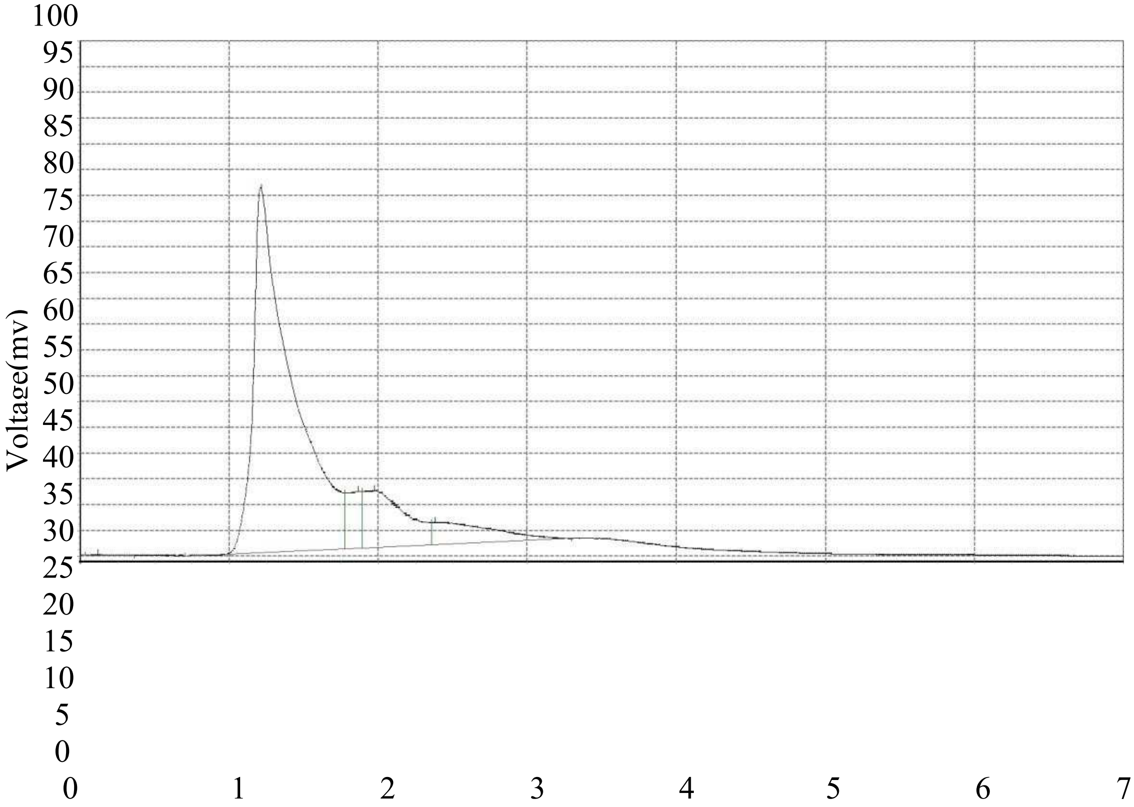


RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	Co m
1	Unidentified	0.115	147.256	912.700	0.0
2	Unidentified	1.215	70646.734	1271036.000	75.4
3	Unidentified	1.865	10993.230	76210.570	4.5
4	Gluconic acid	1.973	11024.480	216748.000	19.8
5	Unidentified	2.382	4267.961	118629.453	9.0

System Evaluation

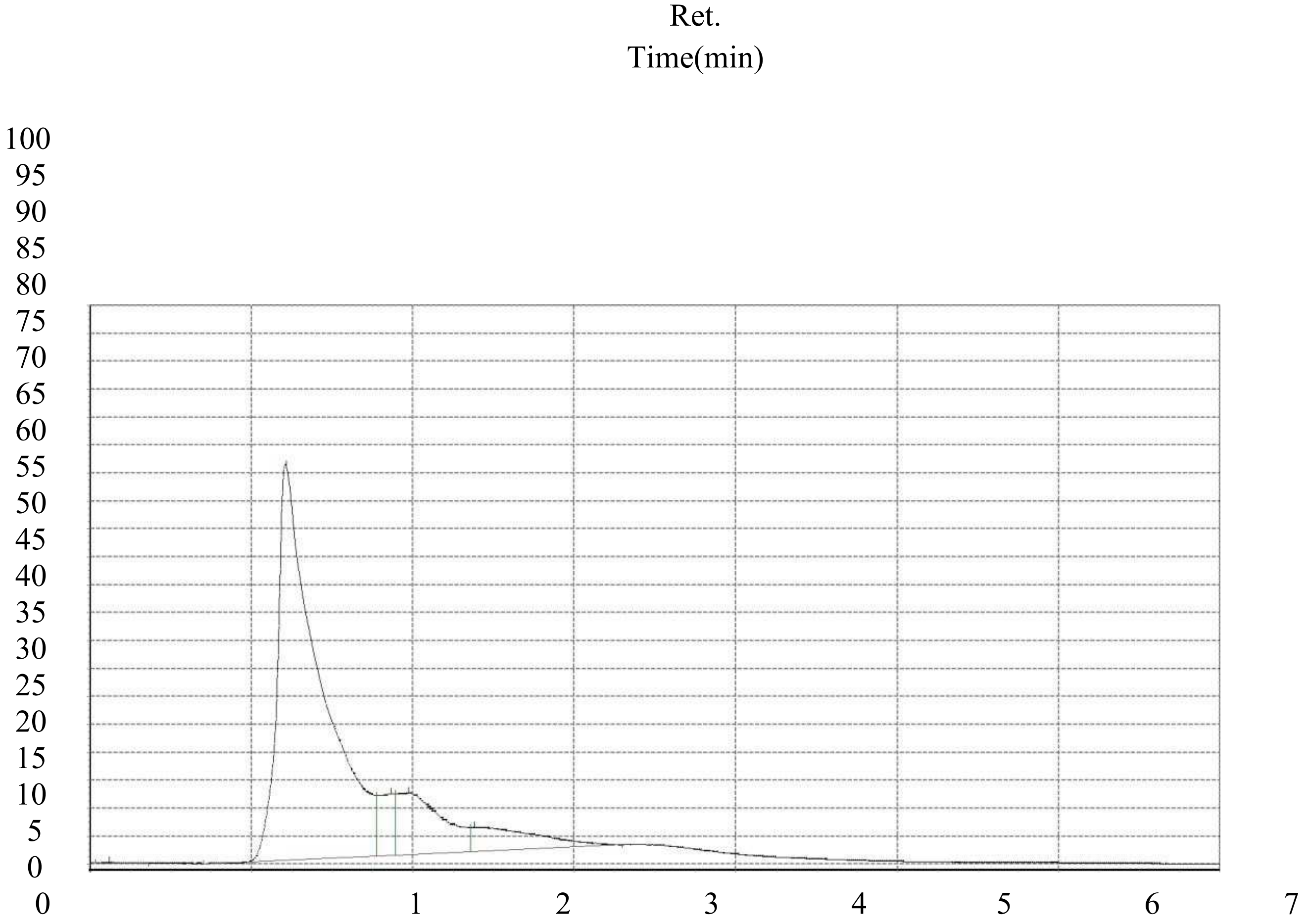
Instrument:LC Gradient: Detector:DAD
Column Temp.(¡æ)£º40 Wavelength(nm)£º245



RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	Co m
1	Unidentified	0.115	147.256	912.700	0.0
2	Gluconic acid	1.215	70646.734	1271036.000	75.4
3	Unidentified	1.865	10993.230	76210.570	4.5
4	Unidentified	1.973	11024.480	216748.000	12.8
5	Unidentified	2.382	4267.961	118629.453	4.0

System Evaluation



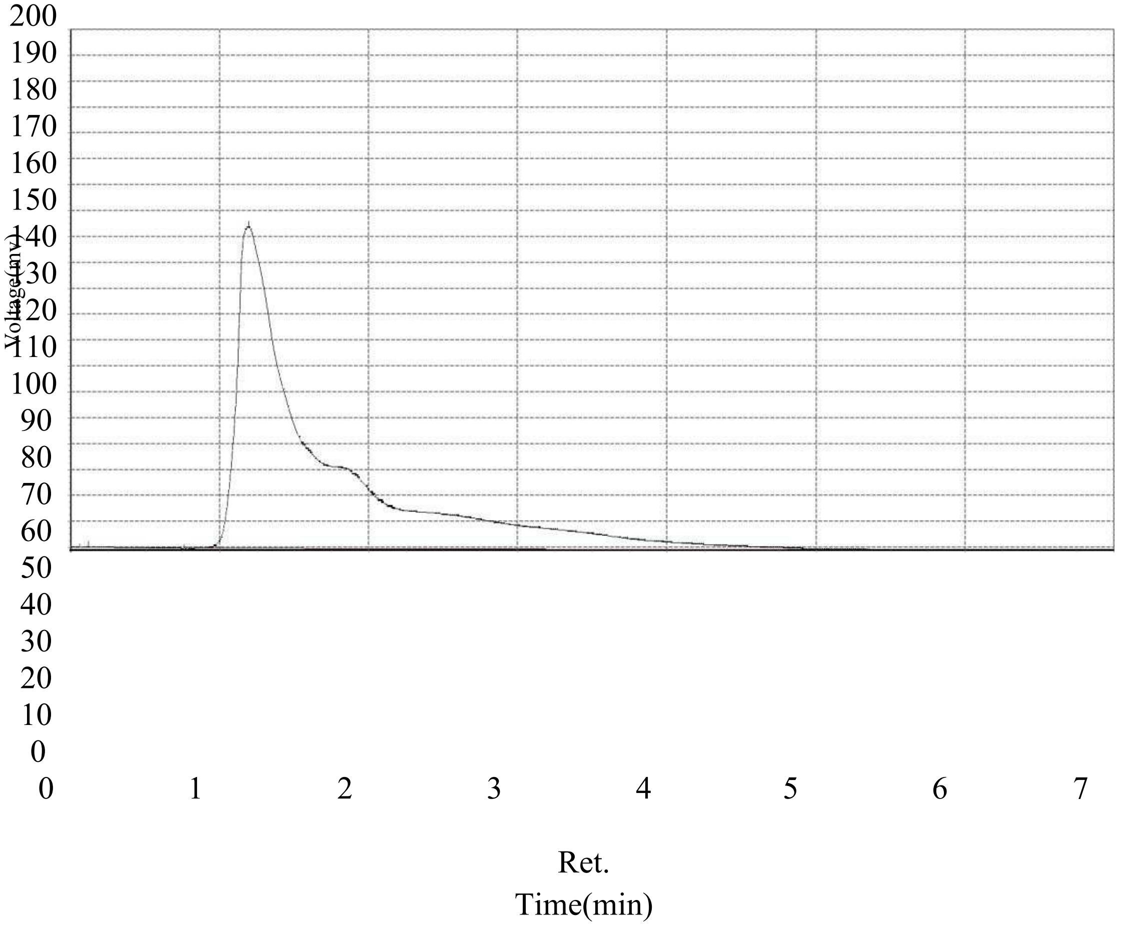
RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	Co m
1	Unidentified	0.115	147.256	912.700	0.0
2	Unidentified	1.215	70646.734	1271036.000	75.4
3	Unidentified	1.865	10993.230	76210.570	4.5
4	Unidentified	1.973	11024.480	216748.000	12.8
5	Gluconic acid	2.382	4267.961	118629.453	23.

System Evaluation

Instrument:LC
Gradient:High Pressure
Detector:DAD

Column Temp.(¡æ)£º40
Wavelength(nm)£º245



RESULTS

Peak No.	Peak ID	Ret Time	Height	Area	Co m
1	Gluconic acid	0.115	252.625	1332.800	15
2	Unidentified	1.190	124232.06	4512676.000	69.

System Evaluation