EFFECT OF METAL IONS ON THE PRODUCTION OF GLUCONIC ACID BY *ASPERGILLUS NIGER*

BY

NABILA JAVED

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CERTIFICATE

This dissertation by Nabila Javed is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in Biology (Biochemistry/Molecular Biology).

INTERNAL EXAMINER: _____

Dr. Salman A.Malik

EXTERNAL EXAMINER: _____

Dr. Aftab Iqbal

CHAIRPERSON: _____

Dr. Samina Jalali

Dated: September 24, 2005

DEDICATION

I am dedicating this dissertation to my
Teachers and my Family

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All praises for **ALLAH** Almighty, who blesses the humanity with His countless blessings and who blessed me with the strength and potential to successfully complete my present work. Prays for His Last Prophet **Hazrat Muhammad (P.B.U.H.)** enlightening our conscience with the essence of faith in one Allah, converging all His kindness and mercy upon us.

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LIST OF ABBREVIATIONS

A	*Aspergillus*
A TCC	Nucleotide Sequence
C	Centigrade
g/L	Gram per liter
GOD	Glucose Oxides
MEA	Malt Extract Agar
pH	log [H]+
PDA	Potato Dextrose Agar
Sc	Surface Culture
SMF	Submerged Fermentation
SSC	Semi Solid Culture
SSF	Solid State Fermentation
T	Temperature
TSCA	Toxic Substance Control Act
U.V	Ultra Violet
Wt	Weight

ABSTRACT

Different cultures of *Aspergillus niger* isolated and selected in biochemistry lab, Quaid-i-Azam University, Islamabad were studied for gluconic acid production using shake flask culture methods. Cane molasses was provided as a carbon source. Other culture conditions such as pH 5.5, temperature $30^{O}C$ and shaking speed of 200 rpm were optimized. Addition of different metal ions Zn^{++} and Mg^{++} in their salts stimulated gluconic acid production when used in minimal quantities i.e. 0.05g/L, 0.062g/L respectively. At higher concentrations of metal ions the gluconic acid production is adversely effected but the fungal growth increased because gluconic acid production is an oxidative process and increased metal concentrations resulted in lack of control on activity of enzymes of the oxidative pathway.

INTRODUCTION

FUNGUS *ASPERGILLUS NIGER*

The filamentous *Aspergillus niger* is a multi-purpose industrial organism. Since it is used for production of many heterologous and homologous, extra cellular enzymes and large number of organic acids such as citric, gluconic and oxalic acid (Katarina and Matic, 2002).

Fungi comprises of organisms that exist as saprophytes or often as symbionts. These are heterogenous collections of organisms which display a wide variety of Morphological forms ranging from unicellular microscopic forms to macroscopic multi cellular forms (Berry *et al.*, 1977). *Aspergillus niger* is a member of genus *ASPERGILLUS* which includes a set of fungi that are generally considered a sexual. *Aspergillus* are ubiquitous in nature, they are geographically widely distributed and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. *Aspergillus niger* is commonly found as a saprophyte growing on dead leaves, stored grains, compost piles and other decaying vegetation. The spores are wide spread, and are often associated with organic materials and soils (Cappucino *et al.*, 1987).

STRUCTURE

The vegetative structure of fungi consists of hyphae. The mass of hyphae constitutes the thallus is known as **'mycelium'**. In fungi, the cell wall is a complex, dynamic structure and is the site of enzyme activity. The cell wall is composed of chitin, cellulose and

polysaccharides together with proteins. Reproduction of fungi is both sexual and asexual (Smith and Berry, 1975). Fungi produce spores that are highly specialized for reproduction, survival and dispersal. They can be classified into two different groups, which are Yeasts and Molds. Molds can be separated into four classes based on their method of sexual reproduction.

True fuugi are separated into four classes on their sexual mode of reproduction.

PHYCOMYCETES

Water, bread and terrestrial molds. Reproductive spores are sexual and uncovered.

ASCOMYCETES

Yeasts and molds. Sexual spores, called ascospores, are produced in a sac like structure called an ascus.

BASIDIOMYCETES

Fleshy, fungi, toadstools, mushrooms, puff balls, and bracket fungi. Reproductive spores, basidiospores, separate from specialized stalks called basidia.

DEUTEROMYCETES

Also called fungal imperfecti because no sexual reproductive phase has been described, (Cappucino *et al.*, 1987).

Aspergillus niger belongs to Molds and is a member of DEUTEROMYCETES or fungi Imperfecti which is a term (technically) reserved for fungi for which there is no known sexual state (Berry *et al.*, 1977). *Aspergillus niger* is commonly called BLACK MOLD.

Most of the pathogenic fungi are deuteromycetes and can be divided into two groups based on site of infection. The superficial mycoses, cause infections of the skin, hair and nails, for example, ringworm infections. The systemic mycoses cause infection of the subcutaneous and deeper tissues such as lungs, genital areas, and nervous system (Cappucino *et al.*, 1987).

Nutritionally the fungi are heterotrophic, eukaryotic organisms that are enzymatically capable of metabolizing a wide variety of organic substrates. Fungi can have beneficial or detrimental effects on humans. Those that inhabit the soil play a vital role in decomposing dead plants and animal tissues thereby maintaining a fertile soil environment. The fermentative fungi are of industrial importance in producing beer and wine, bakery products, cheese, industrial enzymes and antibiotics. The detrimental activities of some fungi include spoilage of foods by rots, mildews and rusts found on fruits, vegetables and grains. Some species are capable of producing toxins (aflatoxin) and hallucinogens. A few fungal species are of medical significance because of their capacity to produce disease in humans. a few species cause diseases in birds, cattle, horses and sometimes in humans. This disease is known as aspergillosis. *A. flavis* infect products such as groundnut meals and dried foods and produces a carcinogenic toxin

called AFLATOXIN which is known to induce liver cancer in humans (Miall and Rose, 1978).

The primary uses of *Aspergillus niger* are for the production of enzymes and organic acids by fermentation, while the foods, for the preparation of which some of the enzymes may be used, are not subject to TSCA. These enzymes may have multiple uses, many of which are not regulated except under TSCA. Fermentation to produce these enzymes may be carried out in vessels as large as 100,000 liters. Fungi are used for the production of secondary metabolites including antibacterial, antifungal and anti carcinogenic agents (Conde *et al.*, 1989, Gasset *et al.*, 1989). *Aspergillus niger* is also used to produce organic acids such as citric acid and gluconic acid.

GLUCONIC ACID

Gluconic acid ($C_6H_{12}O_7$) is an organic acid having a molecular weight of 196.16 gm. Pure gluconic acid is brown liquid and easily soluble in water. It is stable under ordinary condition. Gluconic acid, a common naturally occurring substance found in humans and other organisms, is a multifunctional carbonic acid. It is regarded as a bulk chemical and is important for the chemical, pharmaceuticals, foods, beverage, textile, and other industries. Gluconic acid can be used for cleaning purposes and in the construction industry, where it can be use as a cement resistance and stability under extreme climatic conditions, e.g. frost and floods (Hustede *et al.*, 1989). Gluconic acid is an oxidative product of D-glucose, Due to its low toxicity, low corrosively and completing abilities with divalent and trivalent metals ions the gluconic acid has found its extensive used in

the leather industries (Milson and Meere, 1985). Hlasiwetz and Habermann discovered gluconic acid in 1870 (Rohr *et al.*, 1983). Fungi of the genera *Aspergillus* and *Penicillium* (May *et al.*, 1927, 1929, 1934; Herrick and May 1928; Blom et al., 1952). Yeast-like Mycoderma (Walker and Ramachandran, 1949) and many bacteria, such as Gluconobacter sp, Pseudomonas, Phytomonas, Achromobacter, Acetobacter, Klebsiella, Zymomonas, mobilis, Acetobacter methanolicus, etc., have already been reported to form gluconic acid (Perwozwansk, 1930; Rohr *et al.*, 1983; Hustede *et a*l., 1989). Chemical synthesis of gluconic acid (chemical, electrochemical and catalytic) is possible; however, it is uneconomical for industrial production due its low selectivity (60.80%; Hustede *et al.*, 1989). Today, microbial fermentation processes are exclusively used for commercial gluconic acid production from glucose. Improved strains of (predominantly) *Aspergillus niger* with recycled mycelia or Gluconobacter suboxidans are now used in discontinuous submerged fermentations in industry (Rohr *et al.*, 1983). Today's sodium gluconate fermentation using A. niger in submerged fermentation is based on the process developed by Bloom *et al.*, 1952.

IMPORTANCE OF GLUCONIC ACID

Several studies have shown the importance of gluconic acid (Rohr *et al.*, 1983). In dairy industries it finds applications as a cleaning agent to remove cloudiness, improves taste and complexes heavy metals. In the meat industry; it is used as a mild, acidulant. Due to inherent capacity of gluconic acid to form complexes with metal ions like calcium and iron, it is used in the treatment of calcium deficiency and anemia. D- Gluconic acid is found in many foodstuffs and beverages, such as wine, soft drinks, vinegar, meat, fruit

juice, dairy products, rice and honey. This non-volatile organic acid imparts a sour, but refreshing taste, and given its occurrence in many natural foods, and in human metabolism, has been granted. The ratios of ethanol and glycerol to D-gluconic acid are a quality indicator in the wine industry. Botrytis infected grapes exhibit increased levels of this acid that can reach 1-2 g/L. There are many other applications of D-gluconic acid, including dietary supplements, detergents and in the pickling of foods. D-Glucono-o-lactone is found in association with D-gluconic acid, e.g. in wine, and is also widely used in the food industry. Foodstuffs containing D-glucono-o-lactone include bean curd, yogurt, cottage cheese, bread, confectionery and meat. Titanium gluconate has provided to be very useful in the preparation of white leather. Gluconic acid is an excellent acid catalyst and hence also employed in textile industry. Thus the over all demand of this organic acid is estimated to be 50,000-60,000 tons/Y and is still growing on. Thus the compound and its derivatives are mainly obtained (Singh *et al.,* 2003).

GLUCONIC ACID FERMENTATION

Fermentation can be defined as the process of converting simple sugar into different biochemical compounds by the action of microorganisms in an environment of decreased oxygen.

The world wide demand for gluconic acid production mainly involve the filamentous fungi particularly *Aspergillus niger* which is commonly called BLACK MOLD (Usami *et a.l,* 1996). Many species of *Aspergillus niger* are well known for their capacity to produce citric acid under suitable conditions (Banik, 1975, Kapoor *et al.*, 1982). Fermentation proceeds in two phases; **Growth Phase,** when gluconic acid is not

accumulated. Followed by **Acidogenic Phase** (stationary phase) when gluconate accumulates in the apparent absence of growth, or at least when the growth rate has been greatly reduced. The production of gluconate is extremely sensitive to conditions of culture; those that tend to restrict growth are generally most favorable to acidogenesis.

Gluconic acid fermentation is a sensitive fermentation process and depends upon the right combination of following factors.

- The Organism
- Medium Composition
- pH
- Temperature
- Inoculum
- Aeration

THE ORGANISM

Pre selection of *Aspergillus Niger* is done for rapid production of gluconic acid. (Benuzzi and Segovia, 1989). Special strains of *Aspergillus niger* are used for successful gluconic acid fermentation. Every industrial organization or research laboratory maintains its own selection program of high gluconic acid yielding strains of *Aspergillus niger* obtained from nature or by mutation technique. (Yuill, 1951) did extensive work on the isolation of *Aspergillus niger* strains.

Two step UV irradiation and chemical mutagenesis were used for improving the gluconic acid levels produced by *Aspergillus niger* (Gupta & Sharma, 1995). The optimal conditions for mutagenesis were derived by variations in U. V and chemical mutagen exposures and by evaluating the conidial survival and frequency of positive and negative mutants. The range of dosage used for first and second stage U. V irradiation, the second stage of UV irradiance (450 J/m^2) appeared to ensure the maximum overproducing mutants (25 % of the tested colonies) with 59 % of cell survival. Mutagenic treatment had resulted into glucose oxidase overproducing mutant of *P. variable* and *Aspergillus niger* (petruccioli *et al.*, 1995; Witteveen *et al.*, 1985). It was observed that *Aspergillus niger* obtained after second stage of U. V treatment (450 j /meter sq.) had gluconic acid production, 87 % higher than the wild type.

MEDIUM COMPOSITION

The medium composition for gluconic acid fermentation depends upon the *Aspergillus-niger* strains and source of raw materials (Anastassiadis *et al.*, 2003). The basic medium was which was developed by (Currie, 1917) and is in general today can be described as follow.

- Glucose 150 g/l
- NH_4Cl 0.3 g/l
- KH_2PO_4 0.3 g/l
- $MgSO_4.7H_2O$ 0.2 g/l
- Yeast extracts 0.05 g/l

- Thiamine-HCL 0.1 mg/l
- $CaCO_3$ 20 -40 g/l
- pH 5.5 -6.5

CANE MOLASSES AS A CARBON SOURCE

Molasses is a by-product of sugar industry. The residual syrup is often referred to as blackstrap molasses. Today citric acid is mainly produced from molasses or starch hydrolysate by submerged fermentation process with the filamentous fungus *Aspergillus niger* (Kirimura *et al.*, 1999).

Molasses are the raw material used in submerged fermentation; it should be cleared before use. Activated charcoal, wood ash and potassium ferrocyanide is used for this purpose. Specific incubation period was very important (Khan *et al.*, 1975).

COMPOSITION OF CANE MOLASSES

It is difficult to predict the exact composition of cane molasses. Soil and climatic conditions, the variety and maturity of the cane and the processing conditions in the factory all influence molasses composition. Consequently, considerable variation may be found in nutrient content, flavor, color and viscosity.

The ranges with indicative averages of the composition of molasses can be found in the following table (Wang *et al.*, 2000).

Table No. 1

Typical nutrient analysis (%) of Cane Molasses

Components	Indicative average	Usual range
Water	20	17-25
Sucrose	35	30-40
Dextrose (Glucose)	7	4-9
Levoluse (Fructose)	9	5-12
Other reducing substances	3	1-5
Other carbohydrates	4	2-5
Ash	12	7-15
Nitrogen compounds	4.5	2-6
Non-nitrogenous acids	5	2-8
Wax sterol and phospholipids	0.4	0.1-1
Pigments	-	-
Vitamins	-	-

SUGAR

Soluble carbohydrates (disaccharide and monosaccharide haxose sugars) account for the major proportion of molasses, sucrose being a major sugar present. A characteristic of cane molasses is a relatively high proportion of reducing sugars. During the crystallization cycle the reducing sugars increase to such an extent that no more sucrose can be crystallized, because reducing sugars decrease the solubility of sucrose. Mineral matter tends to hold sucrose in solution, so it is in the balance of reducing sugars and

mineral matter that determines the theoretical yield of sucrose from sugar cane (Hossain *et al.*, 1984).

NON-SUGAR ORGANIC MATTER

The non sugar organic matter of molasses accounts for many of its physical properties, in particular viscosity. It consists mainly of carbohydrate nitrogen compounds and organic acids. The proportion of crude protein in standard cane molasses is very low averaging about 3.5%. There is also a significant quantity of organic acids in cane molasses, the major one being aconitic acid. Molasses also contain volatile fatty acids averaging about 1.3%.

MINERAL MATTER

Molasses is a rich source of minerals. The calcium content of cane molasses is high (up to 1%) where as the phosphorous content is low. Cane molasses is also high in sodium, potassium (which are present as chlorides) magnesium and sulphur.

Molasses also contain significant quantities of trace minerals for example copper (7ppm), zinc (10 ppm), iron (200 ppm) and manganese (200 ppm).

METAL IONS

Of the factors tested the source and concentration of carbon and nitrogen in the medium exerted maximum effect on growth and acid production. Glucose (15%) and urea (0.14%) induced glucose oxidase synthesis and optimum yield of calcium gluconate. Potassium

dihydrogen phosphate (0.2%) and magnesium sulphate (0.06%) stimulated glucose oxidase activity and calcium gluconate production. Borax at a concentration, of 1.5 g/L induced maximum glucose oxidase and calcium gluconate production with increased glucose utilization.

The ability of commercially-available citric, oxalic and gluconic acids to bind Co super(2+) and Zn super(2+) was investigated and compared with culture filtrates from *Aspergillus niger*, a fungus capable of citric, gluconic and oxalic acid production, grown in the presence and absence of cobalt or zinc phosphate. This work demonstrated that citric and oxalic acid and the A. *niger* culture filtrates can bind Co super(2+) and Zn, super(2+) and in some cases, the culture filtrates were more efficient than commercial organic acids. Gluconic acid did not bind Co super(2+) or Zn super(2+) under the conditions used in this study. The presence of insoluble metal phosphates in the growth medium was found to markedly influence the production of organic acids and, while large concentrations of gluconic acid were produced in the presence of Co sub(3) PO sub(4)) sub(2), the culture filtrate was unable to bind Zn super(2+). The production of oxalic acid by *A. niger* when grown in the presence of Zn sub(3) (PO sub(4)) sub(2) led to the precipitation of insoluble zinc oxalate, a phenomenon with implications for metal tolerance and toxicity (Sayer and Gadd, 2001).

pH

The pH of medium is of great importance for the success of gluconic acid fermentation; high pH is has been usually reported to be favorable (Rhodes and Fletcher, 1966). High

pH is reported to prevent contamination, suppress citric acid formation and make the sterilization process successful. The optimum pH value differs for various strains of *Aspergillus niger* used for fermentation and has to be carefully evaluated by workers. At low pH values citric acid is produced where as high pH gluconic acid is formed. It is well known that pH has ultimate effect on glucose oxidase activity (Kubicek and Rohr, 1986).

TEMPERATURE

Gluconic acid production is directly related with the temperature of the fermentation medium but only up to a certain extent. The optimum temperature for gluconic acid fermentation varies with strains of *Aspergillus niger*. Temperature between 25°C and 30°C are usually practiced. The maximum production of gluconic acid has been achieved at 30°C. When temperature of the medium was increased above 30°C, the production of gluconic acid decreased. Temperature has an inhibitory effect on gluconic acid formation. (Srivasta and Kamal, 1979).

INOCULUM

The size and type of inoculums is of great importance in industrial fermentation. Successful gluconic acid fermentation is also dependent upon the age of the inoculum. The effect of sporualting media for inoculums preparation on gluconic acid yield has been reported (Schweiger , 1961). The individual pellets of 0.2 -0.5 mm in diameter with dense enter composed of mycelia are desirable for gluconic acid fermentation (Rhodes and Fletcher , 1966).

AERATION

Perhaps the most critical parameter to success of gluconic acid fermentation is the transfer of adequate oxygen to the *Aspergillus niger*. The few values reported for dissolved oxygen tension of gluconate production. With an increase in effective aeration rate, the fermentation time decreased and the yield of gluconic acid increased actually (Hang *et al.*, 1987).

EFFECT OF DISSOLVED OXYGEN CONCENTRATION ON REPEATED PRODUCTION OF GLUCONIC ACID BY IMMOBILISED MYCELIA OF *ASPERGILLUS NIGER*

Gluconic acid production from corn starch hydrolysates by immobilized mycelia of *Aspergillus niger* was studied in a laboratory-scale stirred fermentor at different concentrations of glucose(S sub(0)) and dissolved oxygen (DO) in the culture broth. Its evolution was simulated quite well by applying the same unstructured model set up in previous experiments using stirred and air lift fermentors in particular, increasing sub(0) in the range 70-160g/L, although uninfluential upon the yield coefficient, resulted in an exponential decrease in the gluconic acid formation rate constant, nevertheless ,the greater the oxygen transfer rate used in the fermentor, the smaller the inhibitor effect of the higher concentrations of glucose on gluconate productivity became. (Moresi *et al.*, 1991).

CULTURE CONDITIONS

SUBMERGED FERMENTATION (SmF)

The most common method of commercial production involves submerged fermentation using filamentous fungus (Brook, 1994).

In the submerged fermentation (smf) process 100cm^3 of the fermentation medium was inoculated in Erlenmeyer flasks (500 cm^3) with mutant *Aspergillus niger* and incubated at 30°C in an orbital shaker with constant shaking (150 rpm) for up to 8 days. After completion of fermentation, the biomass was separated by filtration under suction and the fermentation broth was analyzed for residual sugar and gluconic acid concentration (Singh *et al.*, 2003).

SEMISOLID-STATE FERMENTATION (SmSF)

For the semisolid -state fermentation process (SmSF) Erlenmeyer flasks (500 cm^3) containing 100 cm^3 of fermentation media were inoculated with *Aspergillus niger* ORS-4.410. Chopped sugarcane bagasse (10g, 15-20mm, 70% moister, sterilized separately at 10 lb pressure for 20 min) was then added to the medium and incubated at 30°C with shaking (150 rpm) for uniform distribution of the spores (Singh *et al.*, 2003).

SURFACE FERMENTATION (SF)

In the surface fermentation processes (SF) cultures flask containing 100 cm^3 of the medium were inoculated and kept under stationary condition at 30°C with occasional shaking for pH uniformly. The organism was allowed to grow on the surface of the

medium as a mycelia mat when needed, a water suction pump was used to remove waste gases from the fermentation flask and also to circulate the air (1.5 to 2.0 dm^3 min-1) over the mycelia mat after fermentation, the culture was autoclave, biomass separated by filtration and the broth analyzed for the gluconic acid and residual sugar concentrations (Singh *et al.*, 2003).

SOLID-STATE SURFACE FERMENTATION (SSF)

Solid-state surface fermentation (SSF) was performed by using bagasse as the solid support. Before use, the finally powered bagasse (obtained from a local sugar mill) was treated over night with 2 mol dm^3 HCL at room temperature, followed by thorough washing with doubly distilled water until the washing were completely neutral. The medium (100 cm^3) containing 15% bagasse (70 % moisture content, sterilized separately) was sterilized and inoculated with *Aspergillus niger* ORS-4.410. Flasks were incubated at 30°C with occasional shaking once or twice in 24 hours, maintaining the pH of the medium uniform. Spores are allowed to grow on the surface of the bagasse and the waste fermentation gases were removed by connecting the flasks to the suction pump. This also provided the circulation of air over the solid surface. After the required time period for fermentation, the reaction was terminated by autoclaving; the mycelia mat separated and the broth was analyzed for gluconic acid and the residual sugar concentration (Cappucino *et al.*, 1987).

The production of gluconic acid, extracellular glucose oxidase and catalase in submerged culture by a number of biochemical mutants has been evaluated. Optimization of stirrer

speed, time cultivation and buffering action of some chemicals on glucose oxidase, catalase and gluconic acid production by the most active mutant, AM-II, grown in a 3-L glass bioreactor was investigated. Three hundred rpm appeared to be optimum to ensure good growth and best glucose oxidase production, but gluconic acid or catalase activity obtained maximal value at 500 or 900 rpm, respectively. Significant increase of dissolved oxygen concentration in culture (16-21%) and extracellular catalase activity were obtained when the traditional aeration was employed together with automatic dosed hydrogen peroxide (Cappucino *et al.*, 1987).

GLUCONIC ACID PRODUCTION UNDER VARYING FERMENTATION CONDITIONS

The production of gluconic acid with respect to varying substrate concentrations in submerged (SmF), semisolid-state (SmSF), surface (SF), and solid- state surface (SSF) fermentations was analyzed. Under the various fermentation conditions the biomass and specific growth rate varied with different concentrations of glucose. The highest level of gluconic acid (106.5 g dm super (-3)) with 94.7% yield was obtained under (SSF) conditions. In all cases maximum degree of gluconic acid conversion was observed at on initial substrate concentration of 120 g dm super (-3). The rate of glucose uptake increased on increasing the initial glucose concentration, and glucose utilization was observed to be highest (89-94%) in the SmSF process and was comparable with the SSF and SF processes. The maximum rate of cell growth was obtained in all processes at an initial glucose concentration of 120 g dm super (-3). The gluconic acid production and change in pH were analyzed at varying time intervals and it was observed that the SmF

and (SmSF) processes were completed within 6 days of incubation whereas the highest yield was observed after 12 days of incubation and continued thereafter in the SSF process (Singh *et al.*, 2003).

FACTORS INFLUENCING PRODUCTION OF GLUCONIC ACID

Moresi *et al.*, (1991) investigated an economical medium that can give maximum yield of gluconic acid by the submerged culture technique, using *Aspergillus niger* NRRL3. It was found that the highest yield of acid could be secured when a medium of the following ingredients was used: 13.0% glucose syrup, 0.1% $NaNO_3$, 0.025% calcium super phosphate, 0.007% KCl, 0.025% $MgSO_4$- $7H_20$, and 0.5% $CaCO_3$. It was also found that glucose syrup, which represents an available and cheap carbohydrate source for gluconic acid production by fermentation, can replace glucose powder which is more expensive.

The production of citric and gluconic acid from fig extract by *Aspergillus niger* ATCC 10577 and *Aspergillus niger* B60 in surface fermentation was investigated. The strain 10577 gave higher concentration of citric and gluconic acid than strain B60. A maximum citric and gluconic acid concentration (20.5 g/L and 97.0 g/L, respectively), citric acid yield (12%), biomass dry weight (18.5 g/L), and sugar utilization (85%) was achieved at an initial sugar concentration of 200 g/L and a pH of 7.0. On the other hand, the highest gluconic acid yield (79.7%) was obtained in culture grown at initial sugar concentration 100 g/L. The addition of 4% (v/v) methanol in the fig extract increased the concentration of citric and gluconic acid from 20.5 and 97.0 g/L to 30.7 and 145.5 g/L, respectively.

Citric acid was separated from the gluconic acid by extraction with diethyl ether or ethyl acetate (Roukas., 2002).

The optimization of gluconic acid fermentation using immobilized *Aspergillus niger* on a highly porous cellulose support was studied. Experimental results showing the effects of variations in oxygen partial pressure, glucose concentration and biomass concentration obtained with a continuous recirculation reactor. Levels of dissolved oxygen and glucose concentrations during fermentation significantly affected the production and fermentation time. The optimum biomass requirement on a porous cellulose support has been estimated to be 0.234 mg cm^{-2} for efficient bioconversion. Increasing the quantum of biomass beyond this value resulted in an overgrown biofilm, which affected productivity adversely. Morphological characteristics of immobilized *Aspergillus niger* have also been investigated. (Sankpal *et al.*, 2001).

Aspergillus niger strain when grown on a synthetic medium containing urea as the sole source of nitrogen at pH 5.2, formed a mixture of citric and gluconic acids. On growing the organism at pH 2.0 the gluconic acid content was reduced but citric acid yield remained low. Addition of NH_4NO_3 to the medium lowered the gluconic acid yields to undetectable levels with a simultaneous increase in the citric acid content. Of the sugars used for the production of citric acid, sucrose in a unautoclaved medium was found to be the best carbon source. Sucrose medium if autoclaved at pH 2.0, or a mixture of glucose and fructose instead of sucrose gave lower yields of citric acid. Under optimum

conditions only citric acid was produced and the yield was 66-68 per liter after a growth period of about 10 days.

By extensive microbial screening, about 50 strains with the ability to secrete gluconic acid were isolated from wild flowers. The strains belong to the yeast-like mould *Aureobasidium pullulans* (de Bary) Arnaud. In shake flask experiments, gluconic acid concentrations between 23 and 140 g/L were produced within 2 days using a mineral medium. In batch experiments, various important fermentation parameters influencing gluconic acid production by *Aureobasidium pullulans* isolate 70 (DSM 7085) were identified. Continuous production of gluconic acid with free-growing cells of the isolated yeast-like microorganisms was studied. About 260 g/L gluconic acid at total glucose conversion could be achieved using continuous stirred tank reactors in defined media with residence times (RT) of about 26 hours. The highest space-time-yield of 19.3 g/L super (-1) h super (- 1) with a gluconic acid concentration of 207.5 g/L was achieved with a RT of 10.8 h. The possibility of gluconic acid production with biomass retention by immobilized cells on porous sinter glass is discussed. The new continuous gluconate fermentation process provides significant advantages over traditional discontinuous operation employing *Aspergillus niger*. The aim of this work was the development of a continuous fermentation process for the production of gluconic acid. Process control becomes easier, offering constant product quality and quantity (Anastassiadis et *al.*, 2003).

Experimental data on continuous fermentation of sucrose and glucose solution at low pH to gluconic acid by *Aspergillus niger* immobilized on cellulose fabric show complex dynamic behavior including a decline in yield. The data have been analyzed using an artificial intelligence based symbolic regression technique to provide a mathematical model for predicting values of conversion 5h, 10h and 15 hours ahead values of conversion. These predictions can be used during continuous operations to monitor the bioprocess and adjust the residence time of fermentation to get complete and more efficient conversion of sucrose or glucose to gluconic acid (Sankpal *et al.*, 2001).

DIAUXIC PRODUCTION OF GLUCOSE OXIDASE

On studying the production of glucose oxidase (GOD) by *Aspergillus niger*, we find that both growth and production are diauxic processes, with logistic and linear phases. The gluconic acid that is produced from glucose by means of enzyme action can therefore be considered as a useful source of carbon for growth, and does not interfere with the biosynthesis of GOD in spite of being a product of its activity. From a kinetic viewpoint, the enzyme is a primary metabolite, but this can only be proved by including the Luedeking and Piret equation in a dynamic model that takes into account the effects of hydrogen peroxide on GOD, as well as those of the catalase, which is also produced by the microorganism, on the hydrogen peroxide. (Liu *et al.*, 2001).

IMMOBILIZATION

IMMOBILIZED ON CELLULOSE MICRO FIBRILS

The optimization of gluconic acid fermentation using immobilized *Aspergillus niger* on a highly porous cellulose support is described. Experimental results showing the effects of variations in oxygen partial pressure, glucose concentration and biomass concentration have been obtained with a continuous recirculation reactor. Levels of dissolved oxygen and glucose concentrations during fermentation significantly affect the production and fermentation time. The optimum biomass requirement on a porous cellulose support has been estimated to be 0.234 mg cm super(-2) for efficient bioconversion. Increasing the quantum of biomass beyond this value resulted in an overgrown biofilm, which affected productivity adversely. Morphological characteristics of immobilized *Aspergillus niger* have also been investigated (Sankpal *et al.,* 2001).

IMMOBILIZED IN PUMICE STONE

Gluconic acid was produced in repeated batch processes with *Aspergillus niger* immobilized in pumice stone particles using an unconventional oxygenation of culture media based on the addition of H sub(2) O sub(2), decomposed by catalase to O sub(2) and water. The highest gluconic acid productivity of 8.2 g/L super(-l) h super(-I) was reached with 30gm immobilized mycelium per 150 ml, 10 % (w/v) glucose, at 24°C and pH 6.5, with sub(2) at 100% saturation. The immobilized mycelium was successfully reused up to 8 times in l-hour batches with only a slight loss (11%) of gluconic acid productivity (Fiedurek, 2001).

IMMOBILIZATION ON POLYURETHANE FOAM

Production of gluconic acid by cells of *Aspergillus niger* immobilized on polyurethane foam was studied in repeated-batch shake-flask and bubble-column fermentations. For passive immobilization, various amounts of polyurethane foam and spore suspension were tested in order to obtain a suitable combination for optimal concentration of immobilized biomass. Immobilized cells were successfully reused with higher levels of product formation being maintained for longer period (65-70 hours) than free cells. The highest gluconic acid concentration of about 143 g/L was reached on hydro-based production medium with 0.3 cm^3 foam cubes in the bubble column, where the effect of more suitable aeration and particle volume: medium volume ratio scheme was also investigated (Vassilev *et al.*, 1993).

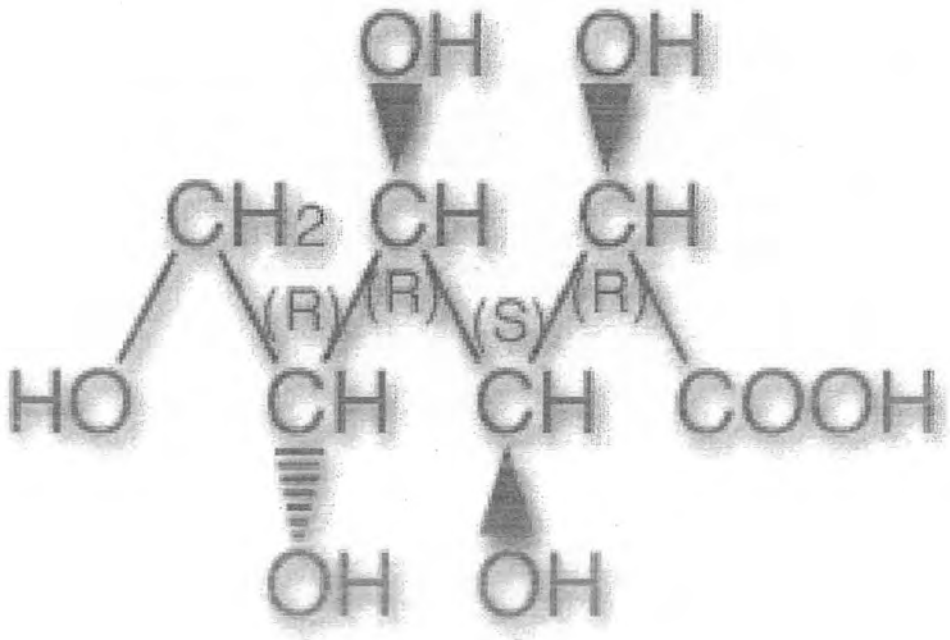
BIOCONVERSION OF GLUCOSE TO GLUCONIC ACID

Bioconversion of glucose to gluconic acid at low pH using *Aspergillus niger* immobilized on cellulose fabric was investigated. Glucose solution (100 g/L super (-1)) was made to flow through capillaries of a vertical fabric support, used for immobilization and is oxidized to gluconic acid at the interface. Conditions of temperature, humidity, airflow, and glucose feed rate have been optimized. The system could be run continuously for a period of 61 days utilizing the entire available glucose. The emerging broth contained a product concentration of 120-140 g/L super (-1) of gluconic acid which is higher than the expected [maximum of 109 g gluconic acid 100 g glucose super (-1)] as a result of evaporative concentration during the downward flow.

ENZYMATIC CONVERSION OF GLUCOSE INTO GLUCONIC ACID

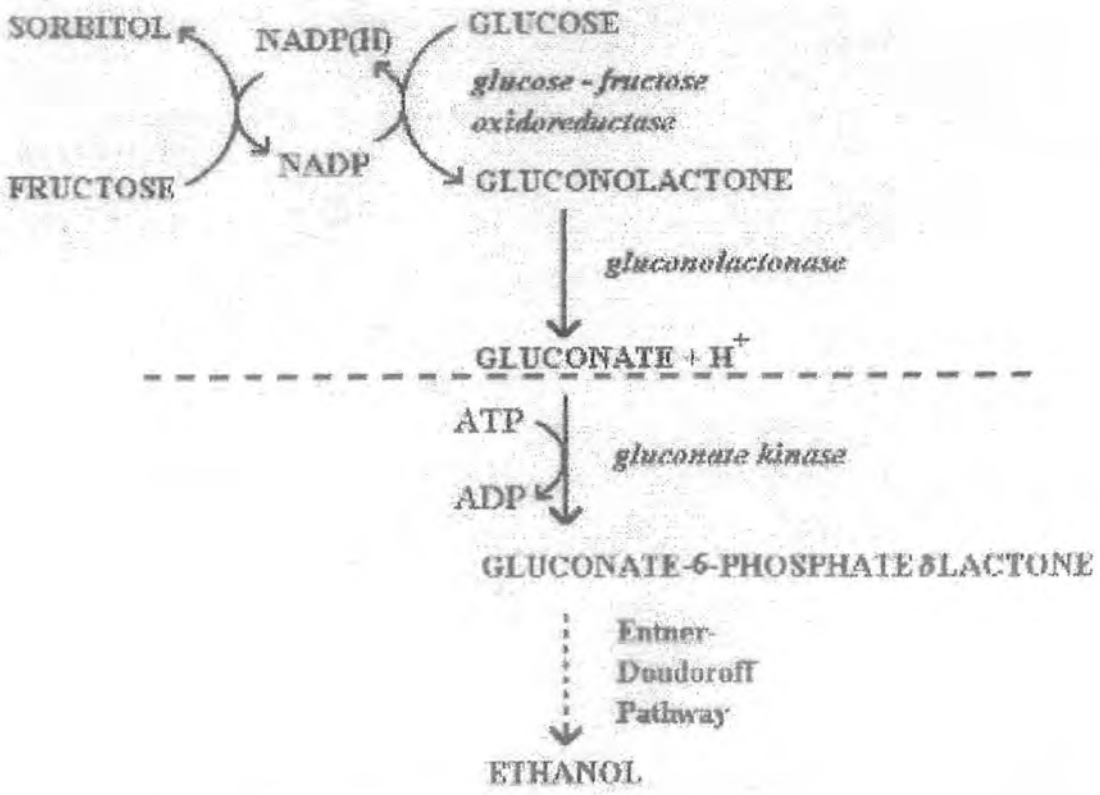
An enzymatic process for the conversion of glucose into gluconic acid uses concentrated glucose solutions. The process employs a combination of glucose oxidase and catalase enzymes which may be obtained from an *Aspergillus niger* strain and has a high ratio of catalase; glucose oxidase activity. The enzymatic process requires less time than conventional fermentation processes, the yield of the conversion is close to 100% and the obtained gluconic acid/gluconate solutions do not contain impurities.

Figure N0 1



Structure of Gluconic Acid

Figure No.2



Mechanism of and gluconic acid production by the GFOR and Gl enzymes and gluconic acid conversion to ethanol by the Enter-Doudoroff pathway.

MATERIALS AND METHODS

The present research was conducted in the Biochemistry Research Laboratory, Quaid-i-Azam University, Islamabad. The organism used in gluconic acid fermentation *Aspergillus niger*, was isolated and identified in the same laboratory.

CHEMICALS

All chemicals used in the study were of analytical grade and were made by either BDH Chemicals (Poole, England) or DIFCO laboratories (U.S.A) or E. Merck (Darmstadt, Germany) A.G (Switzerland).

STERILIZATION

All media were sterilized in an autoclave at 121°C and 15 Lbs/inch pressure for 20 minutes.

SAMPLE COLLECTION

Different samples of *Aspergillus niger* were collected from different ecological sources over Pakistan. These samples were taken from infected fruits (Oranges and Lemons), and from infected Breads.

ISOLATION OF ORGANISM

Five to seven loopfulls of spores were scraped from the surface of the fungal infected specimen and mixed in 50 ml of sterile distilled water. Then it was agitated with great

force, to obtain a homogeneous mixture. Three ml of this spore mixture was poured on the prepared plates of Saborauds Dextrose agar media and placed for incubation at 30°C for 96 hours.

There was another method that was used for isolation of organism. In this method a small portion of infected surface of fruits, vegetable or bread was positioned onto prepared plates of Saborauds Dextrose agar media and placed in the incubator at 30°C for 96 hours was used. Cultures of *Aspergillus niger* were isolated from soil samples of Sialkot by serial dilution method (Clark *et al.*, 1985).

COMPOSITION OF SABORAUDS DEXTROSE AGAR

- Dextrose 40.00 g
- Peptone 10.00 g
- Agar 15.00 g
- Distilled Water (Volume made up to) 1 L
- pH adjusted to 5.5

IDENTIFICATION OF ORGANISM

The plates were studied for physical characteristics after 96 hours growth. Slides were prepared to study the structure of spore mycelia along with spores. Spores were collected from the surface of the plate by light scraping them off by an inoculating loop. Then they were placed on a slide and covered with a drop of methylene blue stain. The slides were

studied under the microscope for morphological characteristics i.e. mycelium structure, conidiophores shape, conidia shape, vesicle structure and spores.

Those colonies, which show characters of Aspergillus niger, were then purified on fresh Saborauds agar plates.

STORAGE OF CULTURE

The isolated cultures were propagated on potato dextrose agar (PDA) slants, incubated at 35°C for 4 days and stored at 4°C and transferred monthly. The composition of PDA medium was as follows.

- Potatoes 200 g
- Dextrose 20 g
- Agar 15 g
- Distilled Water(Volume made up to) 1 L
- pH adjusted to 5.0

PREPARATION OF PDA MEDIA

Fresh potatoes were peeled off, sliced and transferred into glass-beaker containing 700 ml distill. H_2O boiled for 45-60 minutes until half amount left. The boiled potatoes were passed through muslin cloth. Now to the filtrate added 10g dextrose and 15g of agar and increases the volume with distill.H_2O up till liter.

The other medium used for storage of culture during study of sporulation medium was malt extract media.

MALT EXTRACT MEDIUM

Chemicals	Quantity
Malt extracts	20.00 g/L
Peptone	1.00 g/L
Agar	20.00 g/L
Glucose	20.00 g/L
pH adjusted to	5.2

PREPARATION OF MEA MEDIA

Weight accurately and dissolved above chemicals in one liter of distilled water to prepare the medium. The medium was heated to boiling, with constant stirring for 20 minutes. Now the media was ready for slant preparation.

SLANT PREPARATION

The prepared medium was warmed till homogenized; 10 ml of medium was transferred to the culture tube, cotton plugged and autoclaved for 15 min at 121°C and 151 lb pressure. After sterilization, culture test tubes were kept in a slanting position for overnight, till they got solidified.

CULTURING

The fungus was streaked on a slant and kept in incubator at 30± 1°C. After 96 hours, growth pattern was observed on the slants. These slants were used for the preservation of culture at 4°C in a refrigerator, till used.

INOCULUM PREPARATION

Spore inoculums was prepared by taking one loop full of fungal spores from the plates or storage slants; it was streaked on the PDA slants and incubated at 30± 1°C for 4-5 days till maximum sporulation. These slants were used for preparation of spore Suspension for inoculations shake flask fermentation.

SHAKE FLASK FERMENTATION

Submerged culture was mainly carried out in conical flasks containing 50 ml medium reciprocal shaker. The basal medium 2 was selected after considerable testing for shake flask fermentation. One to two loops full of spores were added to each flask. The flasks were rotated at 200 rpm in the rotary incubator shaker. The experiments were carried out in duplicate and only means values are reported as the data varied very little, all lying with in the range of mean± 5 %. The key results were confirmed again to establish the validity of data.

MEDIUM USED FOR GLUCONIC ACID FERMENTATION

- Glucose 120 g/L

- NH_4Cl 0.3 g/L
- KH_2PO_4 0.3 g/L
- $MgSO_4.7H_2O$ 0.2 g/L
- Yeast extracts 0.05 g/L
- $CaCO_3$ 40 g/L

EXPERIMENTAL DESIGN

Shake flask fermentation was done in 250 ml conical flasks. Spores inoculum was added to each flask containing 50 ml fermentation medium. The flasks were incubated in an orbital shaker, at 200 rpm and 30°C.

For optimization of gluconic acid production by fungus the effect of different parameters were studied. The parameters studied were pH, temperature, glucose concentration, agitation and effect of metal ion concentrations on the production of gluconic acid by *Aspergillus niger*.

The pH 4.0; 4.5; 5.0; 5.5; 6.0; 6.5 and 7.0 were investigated by keeping the molasses concentration and temperature constant, thus optimized the pH of the fermentation medium. The pH was adjusted with 1N HCl before sterilization. To optimize the temperature the production of gluconic acid at different temperature was carried out. Temperatures that were tested for this purpose are 28°C, 30°C, 35°C and 38°C. Taking the different initial concentration of molasses (50g, 100g, 150g and 200g) and did

optimization of concentration of molasses for gluconic acid production under optimal conditions.

Different concentrations of metals that are $ZnSO_4$ and $MgSO_4$ are used in fermentation mediums to study how the metal concentrations effect gluconic acids production by *Aspergillus niger*. $ZnSO_4$ in 0.025 g/L, 0.050 g/L, 0.10 g/L, and 0.20 g/L is used and $MgSO_4$ is taken in concentrations of 0.025 g/L, 0.062 g/L, 0.125 g/L, 0.187 g/L, and 0.20 g/L were added in fermentation medium under optimal condition. After fermentation the medium was centrifuged at 15000 rpm for 15 min. I took filtrate and discard.

ANALYSIS FOR GLUCONIC ACID

After centrifugation we ran the filtrate on HPLC for the (%) gluconic acid production by the fungus *Aspergillus niger*. Gluconic acid was quantitatively analyzed using C 18 column. Elution was performed with an isocratic solvent using acetonitrile: water (3:7) and detected at 210 nm. I took standard solution of gluconic acid which was 50 % water soluble. I made 0.025 % solution of gluconic acid by diluting with solvent and then used this solution as a standard to get peak on HPLC .

PERCENTAGE OF GLUCONIC ACID

The percentage of gluconic acid was determined by using the following method

$$\text{Percentage of Gluconic Acid} = \frac{\text{Sample Peak Area} \times 100}{\text{Standard Peak Area}}$$

RESULTS

The fungus studied was identified as *Aspergillus niger*. Different strains of *A. niger* were collected from different ecological sources. Strains were isolated earlier and used in previous work in the same laboratory.

The isolated strains of *Aspergillus niger* were identified by observing the colonies on saborauds dextrose plates. Initially, growth of colonies was observed. The strains of *Aspergillus niger* were characterized by white fluffy mycelium and then dirty greenish colonies were formed with black spores. As colonies become more mature, the surface covered with lots of black spores and giving black pepper like effect. Under the microscope mycelia structure, conidiophores, conidia and spores were observed. Hyphae of mycelial mat were distinctly septate, while conidiophores were non-septate. Globose vesicles were covered with thick balls of spores and these spores were spherical and black in colour.

OPTIMIZATION OF DIFFERENT PARAMETER

Selected strain was used for optimization of different parameters. Production of gluconic acid was studied at different parameters for media containing $ZnSO_4$ as well as media containing $MgSO_4$.

pH

The production of gluconic acid was studied at different pH of the medium in the presence of $ZnSO_4$ and the results are given in Table 2 and Figure 3.

It is clear from Table 2, that initially there was a regular increase in gluconic acid production. According to the table at pH 4.0 the gluconic acid percentage was 29.49%, peak area was 309,266 and concentration of gluconic acid was 7.37 g/L. The gluconic acid production increases from 29.49% at pH 4.0 to a maximum of 64.40% at pH 5.5 with peak area 675,373 and gluconic acid production 16.09 g/L, where after there is a moderate decline till pH 6.5.

At pH 6.5 gluconic acid percentage was 59.90%, peak area was 628,180 and concentration of gluconic acid produced is 14.97 g/L. There after the drop of gluconic acid production is more pronounced and at pH 7.0 the gluconic acid production is only 33.62% with peak area 352,578 and gluconic acid concentration 8.40 g/L. These all results shown in Table 2 can be seen in a glance in Figure 3.

Similar results have been found in the presence of $MgSO_4$ (Table 3 and Figure 4). It is clear from Table 3, that initially there is a regular increase in gluconic acid production. According to the table at pH 4.0 the gluconic acid percentage was 17.71%, peak area was 185,727 and concentration of gluconic acid was 4.42 g/L. The gluconic acid production increases from 29.49% at pH 4.0 to a maximum of 58.67% at pH 5.5 with peak area 615,281 and gluconic acid production 14.66 g/L, where after there is a moderate decline till pH 6.5.

At pH 6.5 gluconic acid percentage is 38.06%, peak area was 399,141 and concentration of gluconic acid produced is 9.51 g/L. There after the drop of gluconic acid production is

Table No.2

Effect of pH on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

pH	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
4.00	309,266	29.49%	7.37
4.50	441,089	42.06%	10.51
5.00	501,286	47.80%	11.94
5.50	675,373	64.40%	16.09
6.00	652,615	62.23%	15.55
6.50	628,180	59.90%	14.97
7.00	352,578	33.62%	8.40

Total Molasses = 150 g/L
Temperature = 30± 1^{O}C
Concentration = 0.05 g/L

Figure No.3

Effect of pH on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

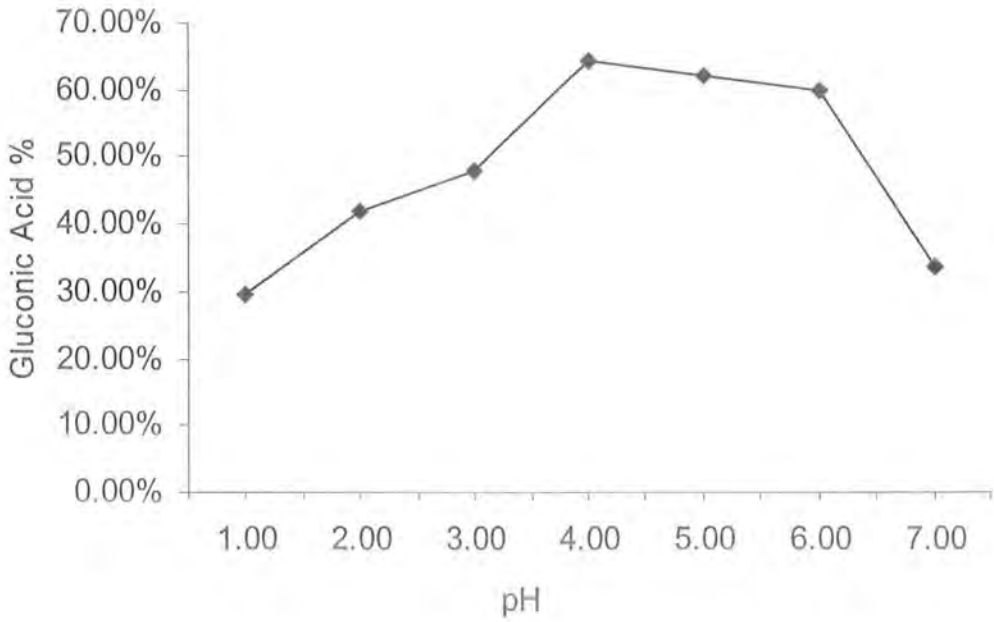


Table No.3

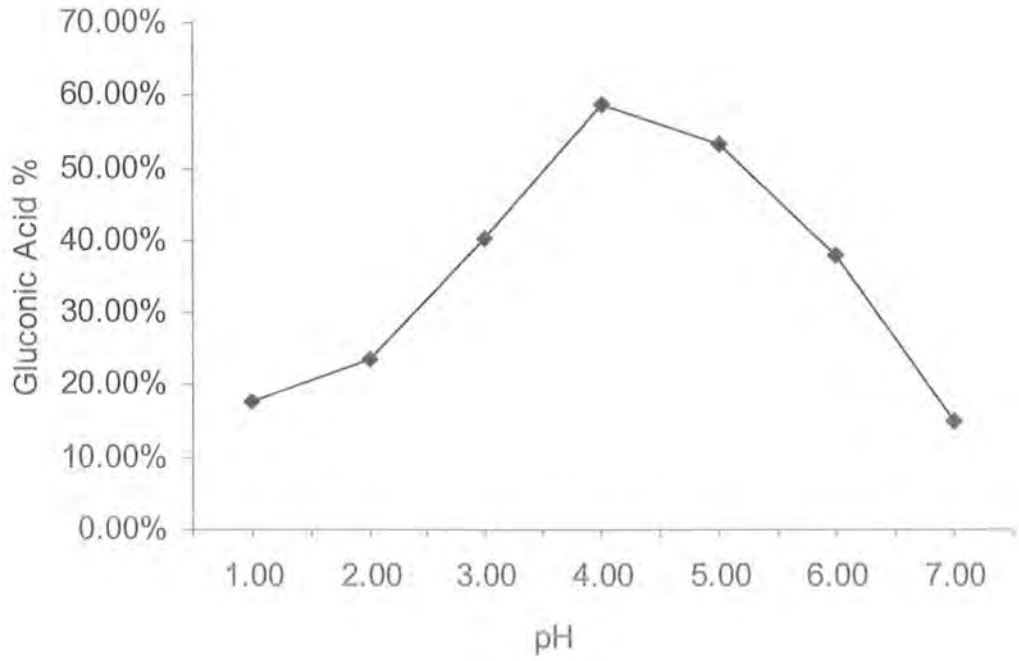
Effect of pH on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$

pH	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
4.00	185,727	17.71%	4.42
4.50	247,916	23.64%	5.90
5.00	423,891	40.42%	10.10
5.50	615,281	58.67%	14.66
6.00	558,441	53.25%	13.31
6.50	399,141	38.06%	9.51
7.00	158,356	15.10%	3.71

Total Molasses = 150 g/L
Temperature = 30± 1^{O}C
Concentration = 0.062 g/L

Figure No.4

Effect of pH on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$



more pronounced and at pH 7.0 the gluconic acid production is only 15.10%, peak area was 158,356 and gluconic acid concentration 3.71 g/L. These all results shown in Table 3 can be seen in a glance in Figure 4.

MOLASSES CONCENTRATION

Effect of Molasses concentration on gluconic acid production by *Aspergillus niger* was examined first in the presence of $ZnSO_4$ (Table 4, Figure 5) and then in the presence of $MgSO_4$ (Table 5, Figure 6).

In the presence of $ZnSO_4$ it was found that at molasses concentration of 150g/L maximum concentration of gluconic acid was obtained. At this concentration the gluconic acid production was 17.08g/L, percentage was 68.36% and peak area was 716,902.

It is clear from Table 4 that further increase in molasses concentration results in gradual reduction in gluconic acid production. As the table shows that when the molasses concentration increases from 150g/L to 200g/L, the gluconic acid concentration decreases from 17.08 g/L to 15.31g/L with decline in percentage from 68.36% to 61.27% and peak area from 716,902 to 642,548.

Similar results have been found in the presence of $MgSO_4$ (Table 5, Figure 6). Table 5 shows that at molasses concentration of 150g/L the maximum gluconic acid production was 14.05g/L, percentage was 56.22% and peak area was 589,588 and when the molasses concentration was increased from 150g/L to 200g/L the gluconic acid %age decreases.

Table No.4

Effect of Molasses Concentration on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

Molasses Conc. (g/L)	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
50.00	420,430	40.09%	10.2
100.00	677,470	64.09%	16.14
150.00	716,902	68.36%	17.08
200.00	642,548	61.27%	15.31

pH = 5.5
Temperature = 30±1^{O}C
Concentration = 0.05 g/L

Figure No.5

Effect of Molasses Concentration on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

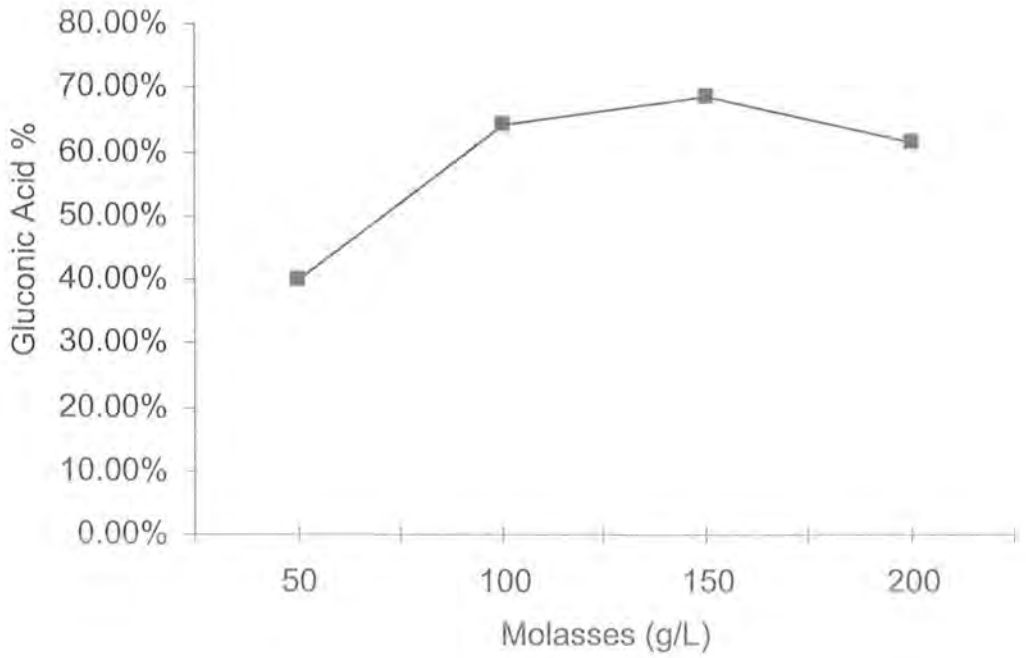


Table No.5

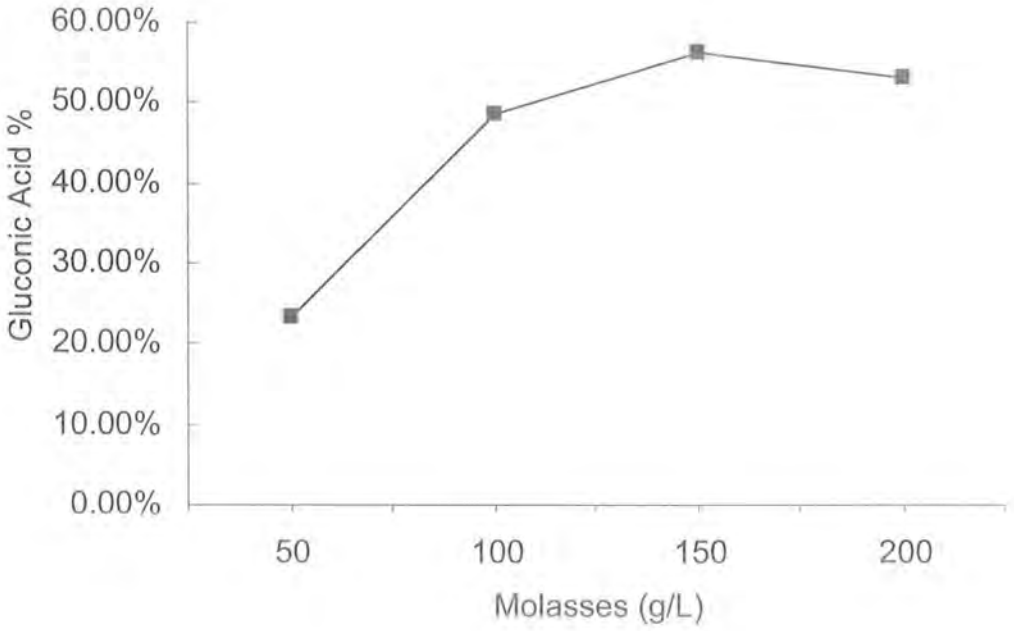
Effect of Molasses Concentration on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$

Molasses Conc. (g/L)	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
50	245,819	23.44%	5.85
100	508,837	48.52%	121.12
150	589,588	56.22%	14.05
200	554,141	52.84%	13.20

pH = 5.5
Temperature = 30± 1^{O}C
Concentration = 0.062 g/L

Figure No.6

Effect of Molasses Concentration on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$



At 200g/L the gluconic acid production was 3.20g/L, percentage was 52.84% and peak area was 554,141.

These results can also be seen in a glance in Figure 5 & 6 where the graphs shows that at 150g/L we obtain maximum gluconic acid production but then from 150g/L to 200g/L the gluconic acid production decreased as the molasses concentration increased although now strain uses more molasses than earlier but this increase in molasses concentration did not result in production of more gluconic acid after certain optimum molasses concentration.

TEMPERATURE

Gluconic acid production at different temperature was studied first in the presence of $ZnSO_4$ and then in the presence of $MgSO_4$.

In the presence of $MgSO_4$ (Table 6, Figure 7), it was found that at 30°C, the production was maximum i.e. 16.79g/L, percentage was 67.20% and peak area was 704,737. Table 6 shows that as the temperature was increased production was decreased significantly. A shift in temperature of the medium from 30°C results in decrease in gluconic acid production as at 35°C, the gluconic acid production was 10.52 g/L, percentage was 42.10% and peak area was 441,509. At 38°C the production of gluconic acid obtained was 5.35 g/L, percentage was 21.44% and peak area was 224,844.

Similar results have been obtained for $MgSO_4$ (Table 7, Figure 8). Table shows that at $28^{O}C$, the production of gluconic acid was 11.67g/L, percentage was 46.72% and peak area was 489,950. By increasing the temperature up to 30°C, the gluconic acid production obtained was maximum i.e.13.13g/L, percentage was 52.55% and peak area was 551,100. By increasing the temperature from 30°C the gluconic acid percentage decreases gradually i.e. 38.81% at 35°C and 27.40% 38°C. Figures 7 & 8 also illustrates that as the temperature increases, production of gluconic acid decreases significantly.

EFFECT OF SHAKING SPEED ON THE PRODUCTION OF GLUCONIC ACID

Shaking speed of agitation was another important factor, as the culture was subjected to shake flask fermentation. The effect of shaking speed on the production of gluconic acid was also studied first in the presence of $ZnSO_4$ (Table 8, Figure 9) and then in the presence of $MgSO_4$ (Table 9, Figure 10).

In the presence of $ZnSO_4$ it was found that maximum production was obtained at shaking speed 200 rpm and minimum production was obtained at 250 rpm of the shaker (Table 8, Figure 9). Table 8 shows that when shaking speed increases from 150 rpm to 200 rpm, the gluconic acid production increases. According to Table 8, at 200 rpm the gluconic acid production was 16.14 g/L, percentage was 64.57% and peak area was 677,151. At 250 rpm gluconic acid production was 9.34 g/L, percentage was 37.38% and peak area was 392,010.

Table No.6

Effect of Temperature on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

Temperature (^{O}C)	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
28^{O}C	623,776	59.48%	14.86
30^{O}C	704,737	67.20%	16.79
35^{O}C	441,509	42.10%	10.52
38^{O}C	224,844	21.44%	5.35

pH = 5.5
Total Molasses = 150 g/L
Concentration = 0.05 g/L

Figure No.7

Effect of Temperature on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

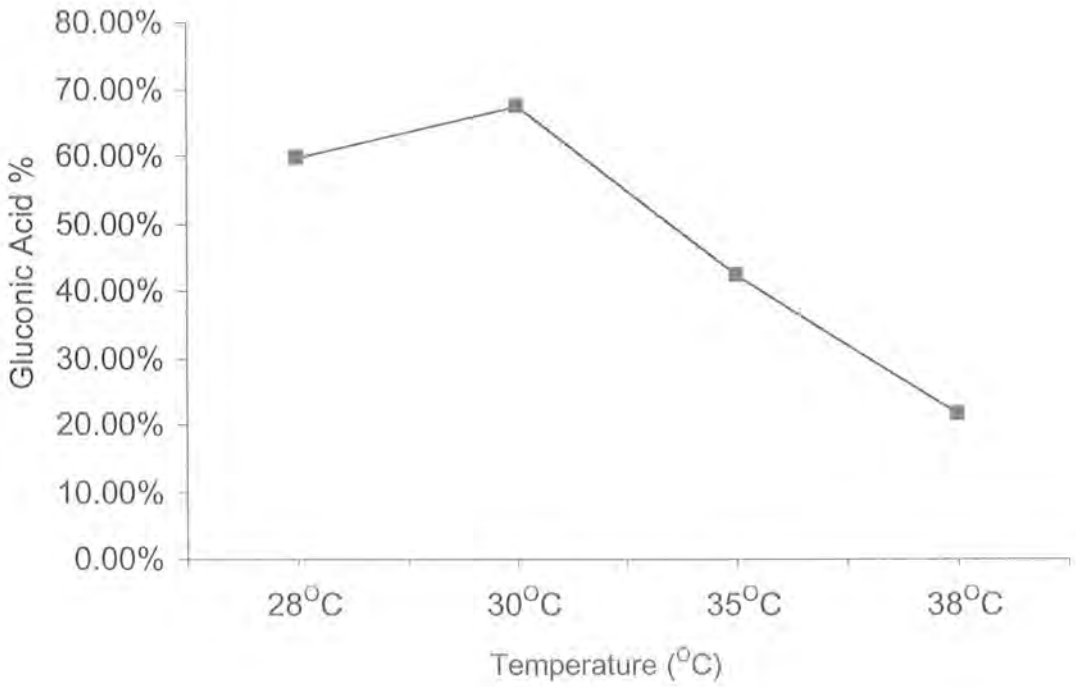


Table No.7

Effect of Temperature on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$

Temperature (^{O}C)	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
28^{O}C	489,950	46.72%	11.67
30^{O}C	551,100	52.55%	13.13
35^{O}C	407,006	38.81%	9.70
38^{O}C	287,348	27.40%	6.84

pH = 5.5
Total Molasses = 150 g/L
Concentration = 0.062 g/L

Figure No.8

Effect of Temperature on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$

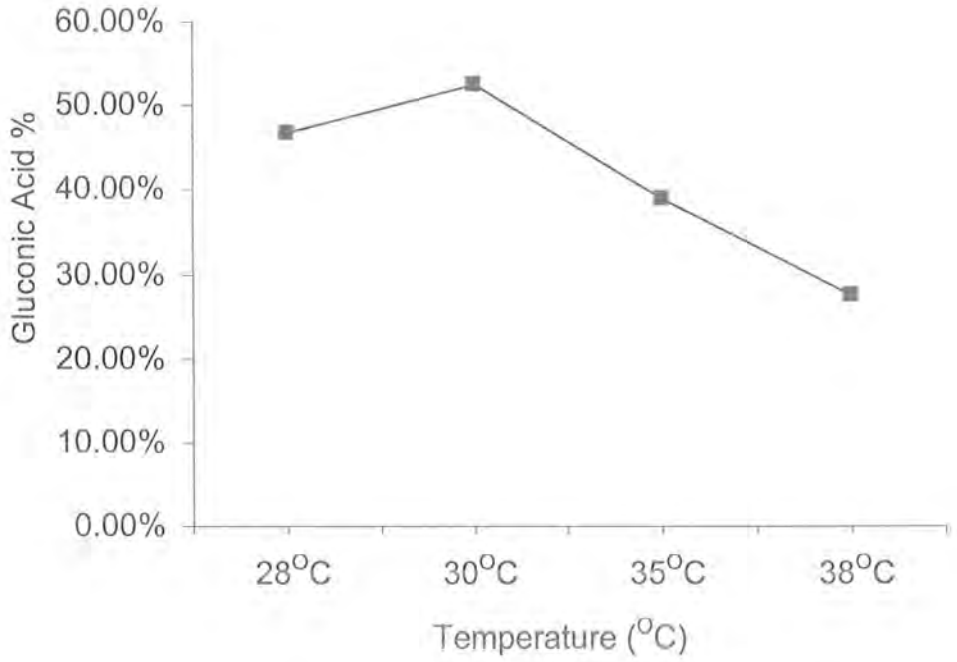


Table No.8

Effect of Shaking Speed on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

Shaking Speed (rpm)	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
150	421,059	40.15%	10.03
200	677,151	64.57%	16.14
250	392,010	37.38%	9.34

pH = 5.5
Total Molasses = 150 g/L
Temperature = 30± 1^{O}C
Concentration = 0.05 g/L

Figure No.9

Effect of Shaking Speed on the production of Gluconic Acid by *Aspergillus niger* in the presence of $ZnSO_4$

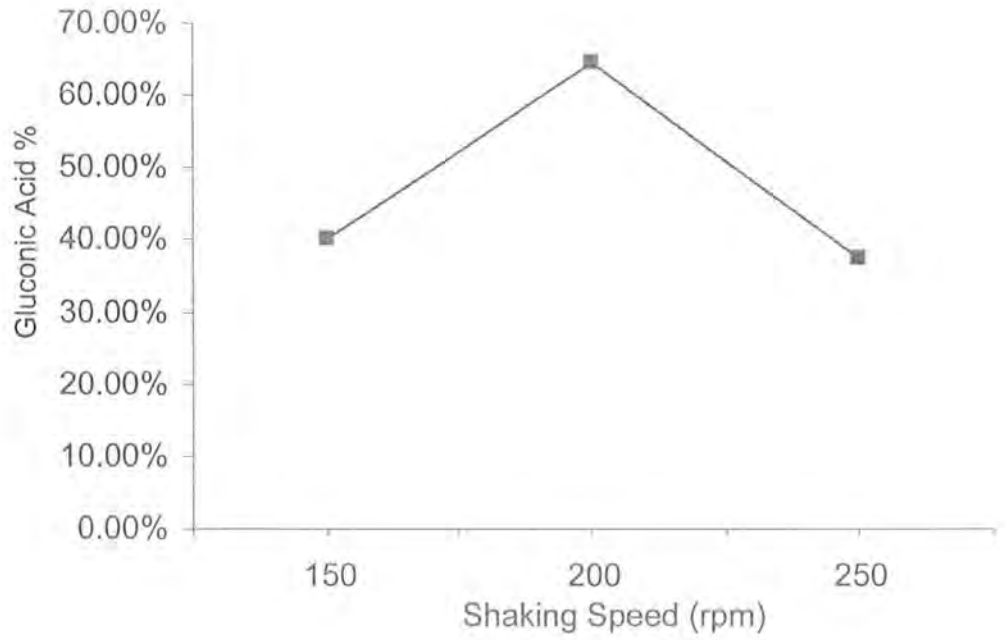


Table No.9

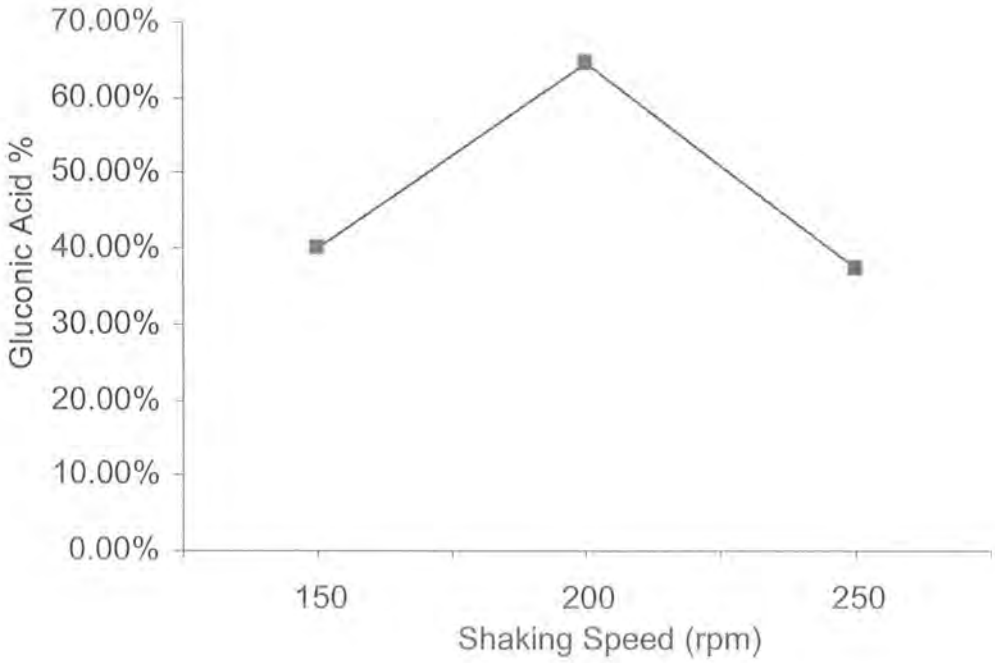
Effect of Shaking Speed on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$

Shaking Speed (rpm)	Peak Area	Gluconic Acid (%)	Gluconic Acid (g/L)
150	460,491	43.91%	10.97
200	551310	52.57%	13.14
250	403,126	38.44%	9.60

pH = 5.5
Total Molasses = 150 g/L
Temperature = 30±1^{O}C
Concentration = 0.062 g/L

Figure No.10

Effect of Shaking Speed on the production of Gluconic Acid by *Aspergillus niger* in the presence of $MgSO_4$



Effect of shaking speed in the presence of $MgSO_4$ also shows the same results (Table 9, Figure 10). Table 9 shows that at 200 rpm, maximum gluconic acid concentration i.e. 13.14 g/L, percentage was 52.57% and peak area was 551,310. After further increasing the speed at 250 rpm gluconic acid production decreased to 9.60g/L, percentage was 38.44% and peak area was 403,126.

The results can be seen in a glance in Figure 9 & 10 which illustrates that gluconic acid production increases gradually with increase in shaking speed up to 200 rpm but after that sudden drop in gluconic acid production was observed at 250 rpm.

EFFECT OF Zn CONCENTRATION ON THE PRODUCTION OF GLUCONIC-ACID

Addition of Zn enhances gluconic acid production to an extent (Table 10, Figure 11). Various concentrations of Zn were added to the fermentation medium for five days and it was found that low level of Zn, more gluconic acid was produced. Maximum percentage of gluconic acid was obtained at 0.050 g/L that is 68.32%, 67.73%, 64.90%, 62.71% and 62.63% respectively during the incubation period of 5 days. As the concentration increased, gluconic acid production decreased as shown in Figure 11.

EFFECT OF Mg CONCENTRATION ON THE PRODUCTION OF GLUCONIC-ACID

The effect of addition of different concentrations of Mg to the fermentation medium is presented in (Table 11, Figure 12). Maximum gluconic acid was produced at the

concentration of 0.062 g/L that is 42.57%, 64.86%, 69.54%, 64.08% and 46.75% respectively during the incubation period of 5 days. As the concentration of Mg in the medium was increased, gluconic acid production decreased as shown in Figure 12.

Table No.10

Effect of $ZnSO_4$ Concentration on the production of Gluconic Acid by *Aspergillus niger*

Concentration (g/L)	Gluconic Acid (%) 1st Day	Gluconic Acid (%) 2nd Day	Gluconic Acid (%) 3rd Day	Gluconic Acid (%) 4th Day	Gluconic Acid (%) 5th Day
0.025	50.76%	49.54%	53.51%	56.44%	47.12%
0.050	68.32%	67.73%	64.90%	62.71%	62.63%
0.075	39.08%	64.28%	60.79%	56.64%	45.37%
0.100	47.58%	50.19%	39.48%	47.92%	37.80%
0.200	38.95%	43.84%	26.21%	34.50%	22.39%

Figure No.11

Effect of $ZnSO_4$ Concentration on the production of Gluconic Acid by *Aspergillus niger*

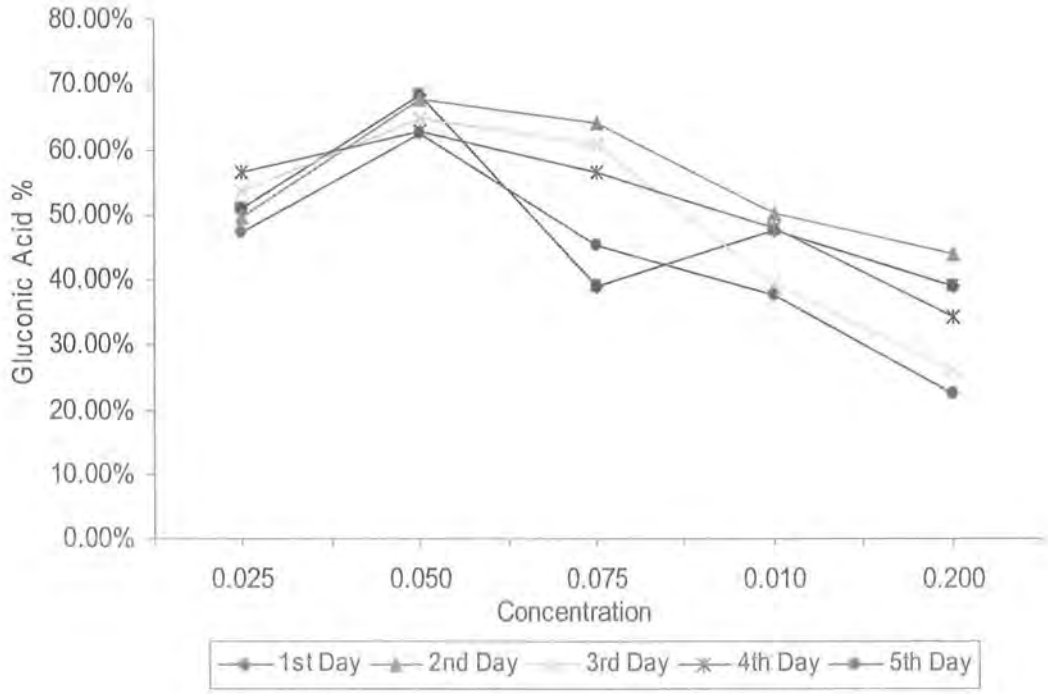


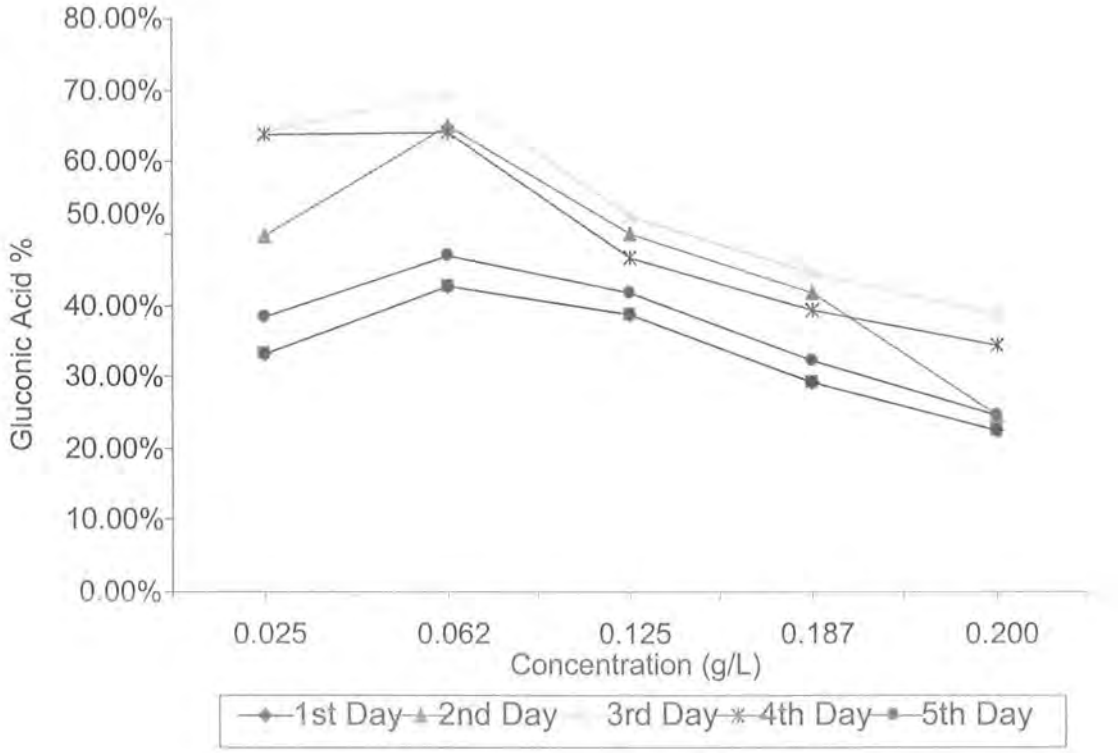
Table No.11

Effect of $MgSO_4$ Concentration on the production of Gluconic Acid by *Aspergillus niger*

Concentration (g/L)	Gluconic Acid (%) 1st Day	Gluconic Acid (%) 2nd Day	Gluconic Acid (%) 3rd Day	Gluconic Acid (%) 4th Day	Gluconic Acid (%) 5th Day
0.025	33.24%	49.79%	64.15%	63.71%	38.42%
0.062	42.57%	64.86%	69.54%	64.08%	46.75%
0.125	38.47%	50.05%	52.37%	46.72%	41.75%
0.187	29.14%	41.80%	44.34%	39.38%	32.16%
0.200	22.35%	24.46%	38.50%	34.41%	24.52%

Figure No.12

Effect of $MgSO_4$ Concentration on the production of Gluconic Acid by *Aspergillus niger*



DISCUSSION

In the present study the production of Gluconic acid by a fungal strain *Aspergillus niger* was examined using cane molasses as carbon source. Prior to investigation, strain of *Aspergillus niger* were isolated (from different ecological sources and different localities) in the biochemistry research lab of Quaid-e-Azam University. Selection method of strain of *Aspergillus niger* for gluconic acid production can be compared with the methods of Singh *et al.*, 2003 and with the findings of Mattey, 1992, Burgstaller and Schinner, 1993, Wolschek and Kubicek, 1999 who isolated *Aspergillus niger* and found that *Aspergillus niger* can produce significant quantities of citric, gluconic and oxalic acid along with other organic acids.

When effect of **pH** on gluconic acid production was studied using molasses as a carbon source, it was noted that decrease in production was more pronounced if pH was decreased rather than when pH was increased from optimum pH. From this it can be inferred that slightly acidic pH favors production of gluconic acid rather than strongly acidic pH. These results can be compared with the results of Sankpal *et al.*, 2001 as they studied low pH fermentative behavior of *Aspergillus niger* immobilized on cellulosic support. Mandal and Chatarjee, 1885 also reported that pH ranging from 5.5 to 6.5 was better for gluconic acid production, an initial pH of 6.5 was found to be suitable for both the growth of the fungal strain and gluconic acid production. Gluconic acid production by fungus *Aspergillus and Penicillium* is strongly pH dependent (Rehm *et al.*, 1980). Heinrich and Rehm, 1982 reported gluconic acid formation by *Aspergillus niger* at low

pH values during citric acid fermentation. This difference is due to the difference in strain and different fermentation conditions. Gluconic acid and its 2- and 2,5-keto derivatives, are produced by fungi *Aspergillus niger* and related genra as a result of external oxidative pathway effective on glucose and other aldose sugars (Whiting *et al.*, 1976, Babu *et al.*, 1995, Williams *et al.*, 1996). The gluconic acid is subsequently taken up by transport systems of the cell and utilized by cellular metabolic pathways. The external oxidation of glucose therefore produces only a transient increase in gluconic acid concentration (Drosinos and Board, 1994).

Glucose oxidase catalyses the key step during gluconic acid production by external oxidative pathway and it is well known that pH has very ultimate effect on glucose activity (Velizarov and Beschkov, 1994). So it can be presumed when initial pH was 6.0, the gluconic acid production increased due to higher glucose oxidase activity and any increase or decrease in pH greatly reduced gluconic acid biosynthesis. Glucose oxidase is probably deactivated at pH values below 5.0 and above 7.0 (Frank, 1963). This is in agreement with Roukas and Harvey, 1988 who reported at low pH values, citric acid was produced where as at high pH values gluconic acid was produced Sayer and Gadd, 2001 also reported citric acid as the only organic acid detected at low pH values.

Temperature has strong effect on gluconic acid production as indicated by the results obtained from present study. These are in good agreement with the findings of Moyer *et al.*, 1936. They reported that 30°C to 32°C was favorable for maximum yields of gluconic acid. Above this temperature gluconic acid production dropped, although the

growth was not substantially effected. Gluconic acid production is directly related with the temperature of fermentation medium up to a certain extent. Temperature above 30°C has inhibitory effect on gluconic acid production. It may be due to accumulation of other by products such as Oxalic acid takes place as reported by Lock wood, 1979. It can be assumed that might be higher than 30°C temperature becomes suitable for the growth of organism. Above the optimal temperature, it prefers to enter into growth phase rather entering into acidogenic phase. No increase in glucose was observed till the 30°C temperature but afterwards there is regular increase in glucose that was consumed by strain. The increase in utilization of glucose might be due to over growth of organism.

Regular increase in gluconic acid production was observed with increase in **molasses** concentration. These results are in agreement with the work of Subba *et al*, 1994 in their analysis of effect of metals on gluconic acid production using cane molasses. It is observed that as the molasses concentration increased the optimum level i.e. 150 g/l, the gluconic acid production decreased although now the strain used more sugar than earlier. The results of the present study are in agreement to the earlier studies where 15% of molasses concentration was found best (Fiedurek *et al.*, 1996, Khan *et al.*, 1970).

The decrease in gluconic acid production with increased molasses concentration may be due to decrease in porosity in the medium. As the porosity decreases air supply also decreases which is necessary for glucose production. As mentioned gluconic acid production is an oxidative process where oxygen serves as a substrate and is incorporated into glucose molecule increasing selectively (g/L) to over 100% (Anastassiadis *et al.*,

1999). Now the question arises why oxygen consumption is more, the answer is, this increase in glucose utilization was due to increased mycelia growth and its overgrowth leads to reduction in gluconic acid accumulation.

High product concentrations were achieved with a very low biomass in shake flasks. Gluconic acid production is a strictly aerobic process and a very good oxygen supply must be provided, which can be achieved by the addition of pure oxygen instead of air and vigorous shaking (Takao and Sasaki, 1964, Sasaki and Takao, 1970). **Agitation** of a culture broth can have different effects on filamaentous fungi, which includes in variation in efficiency of growth rate and also in the rate of product formation. Results show that with the increase in the shaking speed the product of gluconic acid was also increased in a gradual mode up till 200 rpm, maximum production was obtained at 200 rpm. It was due to agitation that improves bulk mixing, heat transfer and mass transfer. Improved bulk mixing is required to minimize nutrient gradient to ensure adequate flow rates of heat transfer and further increase in agitation speed result in sudden drop in gluconic acid production. This drop in production may be due to decrease in accumulation, which may be due to damaging of mycelial tips of Hyphae. Therefore, more extremes present cell of old age thus adequate production would be presented. But due to increase in shear force, damaging of extremes occurs. It is most likely that it causes denaturing of proteins and inactivation of some enzymes as well as the transport of nutrients into the cells (Reese, 1980). These results are in agreement with reports presented by other scientists (Elnaghy and Elkatany, 1981, Rao and Panda, 1993).

Metals have a pronounced effect on the glucose oxidation pathway of *Aspergillus niger*. Manganese is responsible for both gluconic acid and citric acid production (Heinrich and Rehm, 1982). Simine *et al.*, 2001 found that zinc concentration is required for glucose oxidation to produce gluconic acid.

The synthesis of gluconic acid has been observed to be influenced by the effect of various metal ions viz. copper, manganese, cobalt, magnesium, the yield of gluconic acid production is influenced more by combination of metal ions rather than individual ions (Subba *et al.*, 1994).

One of the factors that is considered most important is that only a certain concentration of metals is effective. Sayer and Gadd, 2001 found that only low concentration of $ZnSO_4$ is effective for gluconic Acid production. Metal concentrations are effective also on the malic enzyme activity and lipid in *Aspergillus niger (*Katarina and Malic, 2002).

Yield of Gluconic acid was greatly increased when iron, copper, zinc were used (Shu and Johanson, 1947). When the effect of various concentrations of metal ions i.e. $MgSO_4$ and $ZnSO_4$ was investigated and it was formed that certain minimal quantity gave higher gluconic acid yield. This result was found to be more or less same during the incubation period of five days which shows that metal concentrations strongly effect gluconic acid production and during the five days incubation period we are getting the result that at the concentration of 0.062 g/L we obtained optimum production and then there is a random decrease.

Similarly when the effect of **Magnesium** was studied it was found that 0.062 g/L is the optimum concentration for gluconic acid production and for the effect of **Zinc** 0.050g/l was found as the optimum concentration.

These results can be explained by giving reasons that at higher concentrations of metal ions gluconic acid production decreased while cell growth increased. These findings can be supported by the results of Wold and Suzuki, 1976 who reported that minimal quantities of metal ions were required to attain sufficient growth for maximum gluconic acid yield while over abundance leads to excessive growth at the expanse of gluconic acid accumulation.

So above the minimal 0.050g/L and 0.062g/L concentrations of $ZnSO_4$ and $MgSO_4$ more growth has occurred and resulted in higher values of dry weight in contrast at low levels of metal ions, there was no sufficient growth of mycelia for gluconic acid production so there was low gluconic acid yield. High concentrations have inhibitory effect on gluconic acid yield and favors growth. These finding are in agreement with the findings of Kubicek and Rohr, 1977 who studied the influence of Mn on enzyme synthesis and citric acid accumulation in *Aspergillus niger.*

Suzuki and Kirimura, 1996, Wold and Suzuki, 1976 reported that Zn favored growth at the expanse of acidogenesis metal ions influence the production of glucose oxidase and catalase by *Aspergillus niger* (Liu *et al.*, 2001).

The results may be supported by the findings of Sayer and Gadd, 2001, as an effect of lack of control on activity of enzymes of oxidative pathway, related to the toxic stress caused by increased metal concentrations.

CONCLUSIONS

The present study, therefore demonstrates that for industrial production of gluconic acid, submerged culture method would be beneficial with reference to the production scale performance and utilization of molasses. In submerged cultures of *Aspergillus niger*, fungal growth and metabolic activities related to accumulation of gluconic acid are affected by change in composition of culture media. Uses of certain concentrations of metals in the culture media are found to be very much effective in the production of gluconic acid by *Aspergillus niger* under optimum conditions.

However outcome through such a study could be used to plan a better yielding of gluconic acid by using heavy metals in culture media under optimum conditions. A better yielding strain could be established either by mutagenesis and selection or by recombinant DNA technology. Other agricultural byproducts, like molasses, rice bran, wheat bran, rice husk etc. can be utilized as a submerged culture. It is better economically for the production of gluconic acid at large scale.

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