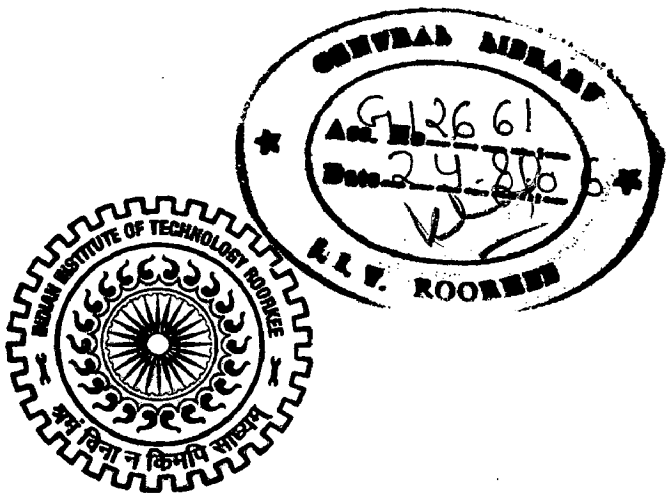
GLUCONIC ACID PRODUCTION UNDER SUBMERGED FERMENTATION CONDITION USING *ASPERGILLUS NIGER* IN AIRLIFT BIOREACTOR

A DISSERTATION

Submitted in partial fulfilment of the
requirements for the award of the degree
of
MASTER OF TECHNOLOGY
in
CHEMICAL ENGINEERING
(With Specialization in Industrial Pollution Abatement)

By

MEGHA AGRAWAL



DEPARTMENT OF CHEMICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)
JUNE, 2006

CANDIDATE'S DECLARATION

I hereby declare that the work, which is being presented in the dissertation entitled **"GLUCONIC ACID PRODUCTION UNDER SUBMERGED FERMENTATION CONDITION USING *ASPERGILLUS NIGER* IN AIRLIFT BIOREACTOR"** in partial fulfilment of the requirement for the award of the degree of **Master of Technology (M. Tech.)** in **Chemical Engineering** with specialization in **Industrial Pollution Abatement (IPA),** submitted in the **Department of Chemical Engineering** of **Indian Institute of Technology Roorkee**, is an authentic record of my own work carried out during the period from July 2005 to June 2006, under the kind guidance of **Dr. Bikash Mohanty**, Professor, Department of Chemical Engineering and **Dr. R. P. Singh**, Professor, Department of Biotechnology, Indian Institute of Technology, Roorkee.

The matter presented in this dissertation has not been submitted by me for the award of any other degree of this or any other Institute / University.

Date: June 26, 2006
Place: Roorkee

MEGHA AGRAWAL

CERTIFICATE

This is to certify that the above statement made by the candidate is correct to best of my knowledge.

(Dr. Bikash Mohanty)
Professor,
Dept. of Chemical Engineering,
I. I. T. Roorkee, Roorkee-247667

(Dr. R. P. Singh)
Professor,
Dept. of Biotechnology
I. I. T. Roorkee, Roorkee-247667

ACKNOWLEDGEMENT

I am greatly indebted to my guide **Dr. Bikash Mohanty,** Professor, Department of Chemical Engineering, Indian Institute of Technology Roorkee, for his kind support and guidance during the entire course of this work. His co-operation and in-depth knowledge have made my work possible.

I take the opportunity to pay my sincere regards to **Dr. R. P. Singh**, Professor, Department of Biotechnology Engineering, Indian Institute of Technology Roorkee, for his keen interest, constant valuable guidance and cheerful encouragement throughout this work. I would like to thank Dr. G. S. Randhawa, Head, Department of Biotechnology Engineering, Indian Institute of Technology Roorkee, for providing various facilities during the course of this project work.

I would like to express my sense of profound gratitude to Faculty members especially to Dr. R. Bhargava, Dr. Nidhi Bhandari and Staff of laboratories of Dept. of Chemical Eng. & Dept. of Biotechnology Engineering, for their co-operation and extended help.

I am indeed thankful to my friend Mr. Prashant Kardekar (M. Tech., Bioprocess Technology, UDCT Mumbai) for providing me technical as well as moral support and giving me constant motivation to work. I would also like to express my thanks to Mr. Amit Sharma (Ph. D., Department of Biotech. Engineering.), Mr. Mukesh Mayani (M. Tech., Department of Chemical Engineering) and all my classmates for their huge co-operation in completion of this project work.

I bow down to this institution for replenishing the state-of-art knowledge in me and exploring me to the astonishing field of research.

Last but not the least, it is all owed to the blessings of my parents that I have come up with this work in due time.

Megha.

MEGHA AGRAWAL

ABSTRACT

D- Gluconic acid is a simple dehydrogenation product of D- glucose which is widely used in the food, pharmaceutical, and chemical industries. Its market is now over 60,000 tonnes per annum and is still growing. Production of gluconic acid using several routes such as chemical, electrochemical, enzyme bioreactor, free and immobilized cells of either *Gluconobacter oxydans, penicillium chrysogenum* or *Aspergillus niger* have been extensively reviewed.

In the present work, we report on the bioconversion of glucose to gluconic acid under submerged conditions using mycelia of *A. niger* which has potential for better yield of acid. The aim of this work has been to optimize submerged fermentation process parameters. Experimental results showing the effects of variations in initial pH, $CaCO_3$% composition in media, initial glucose level and fermentation period on yield of gluconic acid and biomass production under submerged phenomena have been obtained. It has been concluded that all the investigated parameters significantly affect the production process and their optimal values were explored.

Experimental results obtained by process parameter optimization on batch process of fermentation of glucose to gluconic acid by *Aspergillus niger* show complex dynamic behaviour including a decline in yield. The data have been analyzed using an artificial intelligence based statistical analysis method to provide a regression model for predicting values of conversion, yield and biomass production. 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 glucose to gluconic acid.

Once the process conditions have been established, attempt has been made to make commercial scale production of gluconic acid in airlift bioreactors as in recent years, airlift bioreactors (ALRs) have been extensively investigated proficient bioreactors and

are most widely used for fermentation purposes. The airlift bioreactors possess diverse features, which make them more advantageous for various biotechnological applications. The intention of these works was to investigate the possible mode of commercial scale production of gluconic acid by biotransformation processes.

CONTENTS

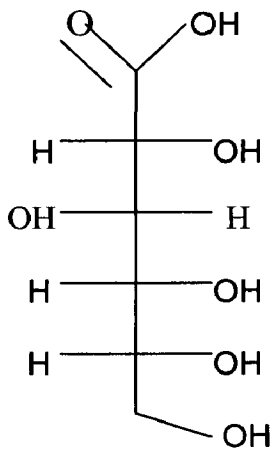
LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. GLUCONIC ACID

Gluconic acid (1,2,3,4,5-pentahydroxy-1-carboxylic acid, $C_6H_{12}O_7$, molecular mass 196.16) and its salts find diverse applications due to their extremely low toxicity, low corrosivity and capability of forming water soluble complexes with divalent and trivalent metal ions [Hustede et al.,1989]. They are important materials used in pharmaceutical, food, textile, detergent, photographic, leather, biological and metal itching industries and many more. There are different approaches available for production of gluconic acid: Chemical, Electrochemical, Bio-electrochemical and Biochemical processes [Das et al.,1987; Kulkarni et al.,2002; Klein et al.,2002].



D-Gluconic acid

Bioconversion is the reactions in which reactant are converted into products by enzyme which acts as biocatalyst. It is one of the dominant routes available for manufacturing of gluconic acid at present, although it suffers from many drawbacks, including those associated with process conditions required for the fermentation and microorganisms used, which has limited its commercial applicability. Further, the oxygen transfer from the gas phase into the liquid phase was found to be a limiting step for the bioconversion [Markos et al.,2002; Klein et al,2002]. For this reason, a great attention has to be paid for the optimization of the process conditions as well as the bioreactor operation (mixing, aeration and bioreactor design) in order to enhance the oxygen transfer rate and, hence, maximizing the product at optimized condition.

Different types of microbial species such as *Aspergillus niger, Penicillium chrysogenum* and *Gluconobacter oxydans* have been employed for the production of gluconic acid. It is an oxidative product of glucose, an abundantly available natural monosaccharide. Although other compounds or materials can also be used as the major carbon sources for gluconic acid production, as sucrose, fructose, other agro-food byproducts (like: grape must, banana must) [Singh et al.,2005; Singh et al.,2002], or whey [Mukhopadhyay et al.,2005] etc.

The success of the technology for biological production depends upon the well chosen process conditions, micro-organisms going to be used, choice of the bioreactor that will efficiently achieve mixing and contact of gas liquid phases, behavior of the organisms under prevailing conditions and medium components, hence from the different types of fermentation phenomena, (surface fermentation, submerged fermentation and solid state fermentation) and we have chosen the submerged fermentation phenomena for our present work because of much advantages involved of submerged fermentation over surface and solid state fermentation.

Microbial species such as *Aspergillus niger* [singh et al.,2001; Favela-Torres et al.,1998; Hatziuikolaou et al.,1995; Zand et al.,2004; Klein et al.,2002; Mukhopadhyay et al.,2005], *Gluconobacter oxydans* [Velizarov et al.,1995] and *Penicillium chrysogenum* etc. have been employed for gluconic acid production, while in the present work *Aspergillus niger* is used because it is capable of producing high activity of enzymes namely glucose oxidase (GOD) and catalase for gluconic acid production.

Here in the present work, we report on the bioconversion of glucose to gluconic acid under submerged conditions using mycelia of *Aspergillus niger* and as in any biochemical process or bioconversion, process conditions are having always great impact on productivity, so one of the main aim of the present work becomes to optimize the fermentation parameters including initial pH, $CaCO_3$ composition, initial glucose level and fermentation period under batch process condition with a view to obtain higher productivity of gluconic acid and biomass production. For efficient bioconversion, an optimum biomass production is necessary. Increasing quantum of biomass beyond this value results in an overgrown biofilm, which affects productivity adversely. All factors examined are having great impact on gluconic acid productivity.

For any bioconversion, pilot scale production condition, using submerged fermentation phenomena, different types of bioreactors are available such as: bubble column bioreactors, stirred tank bioreactors and airlift bioreactors. Although mechanically and pneumatically stirred reactors are normally used, but the choice nowadays is given to the pneumatically agitated – Airlift reactors because of its advantages such as energy efficient operation in comparison to stirred fermenter, better for heat sensitive cultures and less prone to the leakages [Nakao et al.,1997].

In spite of too much importance of gluconic acid, biotransformation processes have not been received any great attention although some work has been done, may be due to lack of knowledge, much sensitivity and lesser flexibility involved in fermentation processes, lack of uniformity and reproducibility of the methods adopted in various laboratories, which may not be surprising because of the fact that very few research laboratories worked with the same strain under similar conditions, etc.

In the present work, optimization of process parameters (initial pH, $CaCO_3$ composition, substrate concentration and fermentation period) for the bioconversion of glucose to gluconic acid under submerged fermentation condition, using *Aspergillus niger* as the fermentation micro-organism, is done. For the sake of design of experiment, mathematical regression modeling of the process has also been done with obtained real experimental results of parameter optimization using the software "Minitab" and best suitable regression model with obtained experimental results, based on four process variables (pH, $CaCO_3$ composition, substrate concentration and fermentation period), has been proposed for gluconic acid production, yield and biomass production. After this, the task of gluconic acid production, using *Aspergillus niger* under submerged cultivation and optimized process parameter conditions, in the airlift bioreactor, was carried out.

1.2. APPLICATIONS OF GLUCONIC ACID AND ITS DERIVATIVES

Gluconic acid is regarded as a bulk chemical and the primary application of it follows from its most important characteristics: it is a weak acid capable of dissolving the oxides, hydroxides and carbonates of polyvalent cations without attacking metallic or non-metallic surfaces. It has very low toxicity and corrosivity. Gluconic acid, its lactones and salts are all destroyed effectively and quickly by biological waste water

treatment processes [Hustede et al.,1989]. Consequently, gluconic acid and its derivatives have found a variety of applications:

1. It is used as an additive in the food, beverage and pharmaceutical industries.
2. For removal of paint and varnishes without damaging underlying surfaces.
3. In textile industry, for desizing polyamide fabrics, for finishing natural cellulose fibers and to prevent iron deposition.
4. In backing aids, as a leaving agent.
5. With gelatin, as sizing agent in paper industry.
6. Sodium salt as gluconate forms complexes with metal cations in alkaline solution and stability of such complexes often increases considerably with increasing pH.
7. For coagulation of soyabean protein in the manufacturing of tofu.
8. In removing calcareous and rust deposits from surfaces.
9. In leather industry, e.g. tanning of lather.
10. In the preparation of cured meat products, especially in the processing of ground meat for sausage manufacture.
11. In concrete, as an agent for regarding the curing the process, to produce homogeneous concrete with high resistance with water, frost and cracking, better flow properties for wet concrete mixtures, increased wettability with respect to iron reinforcing structures and maintenance of plasticity despite reduced water content.
12. Sodium salts are used in the galvanic deposition of nickel-cobalt brazing surfaces on the aluminum or in the baths required for preparing smooth, shiny surface plating of nickel, tin and zinc.
13. Ionization of hydroxyl groups allowing use of sodium gluconate in washing of glass bottles.
14. Calcium gluconate is used to treat diseases caused by a deficiency of Ca in the body.
15. Ferrous phosphorgluconate is used chemotherapeutically.

1.3. SCOPE OF THE PRESENT WORK

Gluconic acid, an oxidative product of D-glucose, today is largely produced by various biological and non-biological sources. The numerous advantages of gluconic acid and its salt make it commercially viable product, which indicates the need of cost effective commercially applicable production techniques. According to the recent estimates, the annual worldwide production of gluconic acid is more than about 50,000-60,000 tones. Some companies, e.g. Prathsta Industries Ltd., Secunderabad, produce calcium gluconate (Source: Chemical Weekly, September, 2004) in small amount, against large requirement, using traditional chemical process. It is not being produced on large scale in our country by fermentation process and is mostly imported nowadays because traditional chemical production processes give not only much increased cost but also the poor yield and quality of the product. While opposite to this, bioconversion process for commercial scale production of Gluconic acid is potential as well as economic. This in turn, indicates potential for future research initiative in this area and encourages us to develop the proper biochemical technology for large scale, cost-effective production of gluconic acid.

1.3 OBJECTIVES OF THE PRESENT WORK

As after realizing the potential of gluconic acid production by fermentation techniques, which has tremendous scope for the country like ours, the present study for its production has been taken up with these following objectives:

1. To carry out experiments for optimization of basal nutrients and major fermentation process parameters for gluconic acid production under submerged fermentation condition using *Aspergillus niger*.

Parameters to be optimized: **(a).** pH

(b). $CaCO_3$ concentration

(c). Substrate (glucose) concentration

(d). Fermentation Period

2. To develop the mathematical (Regression) model for fermentation process by statistical analysis of observed experimental results.

3. To carry out fermentation process for the production of gluconic acid under submerged condition using *Aspergillus niger* in the airlift bioreactor with optimized operating conditions.

2. LITERATURE REVIEW

The review of the literature is actually a pioneer of our dissertation work: Gluconic acid production under submerged fermentation condition using *Aspergillus niger* in airlift bioreactor. Looking towards the literature reviewed it is found that production of gluconic acid by bioconversion process is main function of operating and kinetic parameters, micro-organisms going to be used for the biotransformation and reactor design. The operating and kinetic parameters include pH, temperature, pressure, cultivation period, basal nutrients, superficial gas velocity, dissolved oxygen concentration and oxygen transfer from gas phase to liquid phase while design parameters are like: diameter and height of the reactor down comer design etc. The objectives of this literature review is to develop the best possible production procedure to carry out fermentation for gluconic acid under submerged condition using *A. niger* in chosen experimental set up of airlift bioreactor with optimized operating conditions. Actually this part of the report reviews the current status of available literature or prose on the work done on biotransformation process and its development for economic commercial scale production of gluconic acid with high yield.

2.1. GLUCONIC ACID

D-gluconic acid, an oxidation product of D-glucose finds its wide application in different process industries. Demand for this acid is mainly fulfilled by microbial fermentation. [Mukhopadhyay et al.,2005].

2.1.1. Crystal structure of d-gluconic acid:

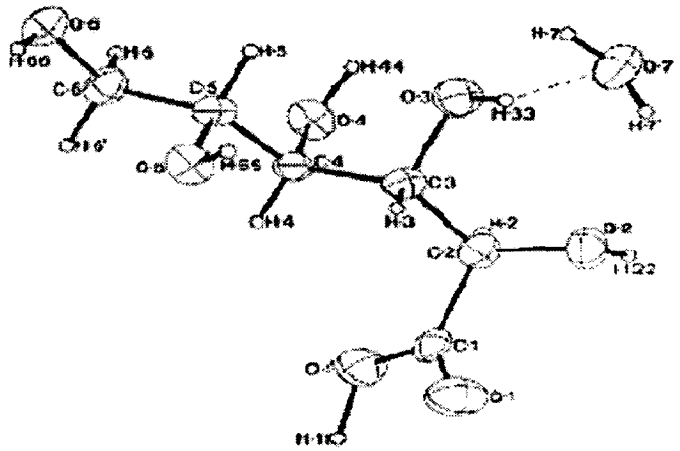
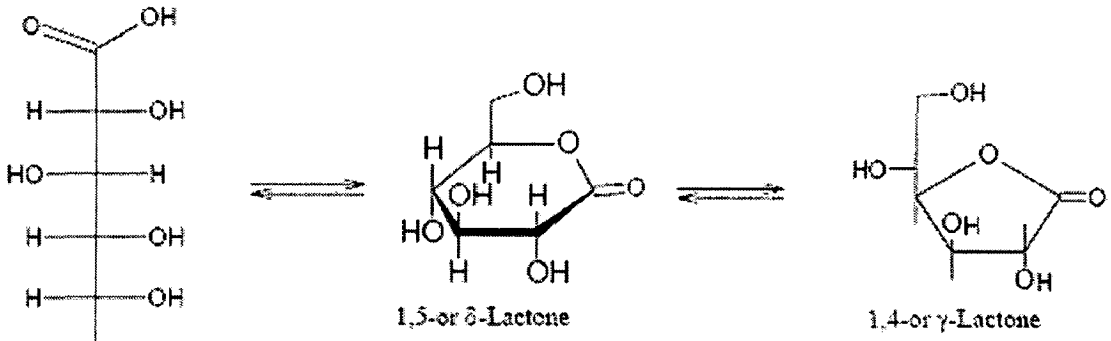


fig-2.1.1. *Atomic structure for D- gluconic acid monohydrate* [Lis T,1983].

2.1.2. Physical and chemical properties

Free D-gluconic acid can be prepared as a solid product. This product is light amber in colour and has faint acetous odor. Crystallization of the anhydrous gluconic acid is possible below 30°C. Anhydrous gluconic acid is white, odorless, crystalline powder with specific rotation $[\alpha]\ D^{22} - 6.7°$. The melting point of gluconic acid falls in the range 120 – 131° C.

Gluconic acid is extremely soluble in water, slightly soluble in alcohol and virtually insoluble in other organic solvents. In aqueous solution, equilibrium exists among gluconic acid and its two lactones, gamma and delta; the lactones exist in both, solid state and aqueous solution [Hustede et al., 1989].



D-gluconic acid

Fig-2.1.2. Structure of lactones of gluconic acid

Free gluconic acid is well tolerated in daily food by animal and human organisms without injury and disturbance of the digestive organs. Gluconic acid and its salts effectively lowers the pH of the urine in man without producing any evidence of pathological renal changes, thus indicating the utility of urinary acidifying agents. Gluconic acid and its derivatives are capable of forming water-soluble complexes with certain metallic ions. This conversion of divalent and trivalent ions to a de-ionized but water-soluble form has been defined as sequestering. The gluconate is an outstanding sequestrant for calcium in the presence of sodium hydroxide.

Two main salts of gluconic acid are calcium gluconate and sodium gluconate. Calcium gluconate, $Ca(C_6H_{11}O_7)_2$, is fine, white crystalline solid without odor or taste. it has very low solubility in water (3.5 gm in 100 ml water at 25°C) and is insoluble in ether or alcohol. Sodium gluconate, $NaC_6H_{11}O_7$, is irregularly fragmented, odorless, colorless crystal, soluble in water to the extent of 59gm in 100ml at 25°C but insoluble in organic solvents.

2.2. INDUSTRIAL PRODUCTION PROCEDURES

Gluconic acid can be produced by different methods e.g. chemical, electrochemical, bio-electrochemical and biological.

2.2.1. Chemical oxidation

D-gluconic acid [Penta-hydroxy-caproic acid, $(CH_2OH(CHOH)_4COOH)$] was discovered by Hlasiwetz and Habermann during the oxidation of glucose with chlorine. The catalysts generally employed in chemical oxidation have been finely

divided platinum group metals suspended on activated charcoal; aluminum oxide, hydrogen peroxide, ozone and oxygen have been oxidants of choice. The economic viability of chemical catalysis is dependent largely on the activity, selectivity, lifetime, cost of the catalyst, measures required for product purification and energy demands. 'Kawaken fine chemicals company limited, Japan' prepared gluconic acid by air oxidizing monosaccharide in aqueous alkali solution in presence of catalyst containing palladium (Pd) and selenium (Se). This reaction produced 98.5% conversion.

Park et al. (1986) obtained gluconic acid in 50% aqueous solution by treating calcium gluconate, prepared by electrochemical oxidation of glucose with oleum to precipitate calcium sulphate ($CaSO_4$), followed by passage over cataion exchange resin and concentration of the resulting solution. The product was 95.6% pure. Calcium gluconate was reported to react with sulphuric acid to form gluconic acid. This was treated with ferric carbonate ($FeCO_3$) to form ferrous gluconate, which is used in food products for enrichment of iron.

Disadvantages of chemical oxidation:

1. Limited specificity even under carefully controlled and optimized reaction conditions.
2. Unsatisfactory yields.
3. Undesirable by products.
4. Difficult isolation and purification of the product.
5. Rapid decrease in the catalyst activity and.
6. Need for highly purified glucose solutions.

2.2.2. Electrochemical oxidation

Electrochemical oxidation electrolyzes a glucose solution with an insoluble anode in the presence of water–soluble iodide at a current density of 1-20 A/dm^2. Carbonates or hydroxides are added to neutralize the resulting acid. The product yield based on glucose is 80-97%.

The catalytic oxidation of glucose in alkaline solution at a gold (Au) electrode was studied by the cyclic voltameter at a rotating disk electrode as a function of

glucose concentration and rotation velocity. The rate-determining step was concluded to be the transfer of electron form adsorbed carbohydrate radical species to the electrode. The mechanism was explained by a decrease in no. of interactions between each glucose molecule and the catalytic hydrous 'Au' oxide as the glucose concentration was increased. Different workers investigated the adsorption and electro-oxidation of glucose on a smooth platinum electrode. The adsorption of glucose by Pt was accompanied by dehydrogenation of adsorbed molecules in neutral and alkaline solutions glucose electro-oxidation rates on Au were reported to considerably exceed those on 'Pt'. Pintauro *et al.*(1984) studied paired electrochemical synthesis of gluconic acid and sorbitol in undivided flow cells. 50% reduction in energy consumption was achieved compared to the conventional synthesis. The synthesis of gluconic acid was carried out in parallel plate and packed bed cells. The optimum electrode materials and operating condition for the synthesis were: an amalgamated zinc (Zn) cathode, graphite anode and initial glucose concentration of 0.8 mol/dm3 sodium bromide (NaBr) supporting electrolyte, electrolyte flow rate of 0.81 L/min. and electrolyte pH of 7.

In other works, a catalyst was made by entrapping glucose oxidase in a polymeric film deposited on platinum electrode surface by anodic electro polymerization of pyrrole 0.1 mol/L in phosphate buffer containing perchlorate ions 0.1 mol/L as counter anions and glucose oxidase 1.5 mg/ml. The electrochemical step affects the enzymatic catalysis in two ways, the reactant oxygen was regenerated and the product, hydrogen peroxide (H_2O_2), which is glucose oxidase inhibitor, was consumed. When no potential was applied, the electrode only acted as an inert support. When a suitable potential was applied, H_2O_2 underwent oxidation at the electrode. The electrochemical step could occur throughout the polymeric structure rather than being limited to the electrode surface.

Disadvantages of Electrochemical oxidation:

Cost of the electricity is very high; in such a situation it is costly to use electrochemical method for oxidation of gluconic acid. The other disadvantages are the poisoning of the electrodes and low oxidation potentials of halogens.

2.2.3. Bio-electrochemical oxidation

Biocatalyst electrodes (oxidoreductase immobilized electrodes) in which the electrode behaved as a substitute for a chemical electron acceptor or donor of a oxidoreductase reaction were used in novel applications such as biosensors, bioreactors and biofuel cells. A film-coated glucose oxidase-immobilized 'Au' minigrid electrod with P-benzquinone-mixed paste and a film coated glucose oxidase-immobilized 'Pt' mesh electrode with oxygen permeable membrane were designed. These electrodes worked satisfactorily as biocatalyst electrodes oxidizing D-glucose electro-catalytically to D-gluconate. Glucose was converted to gluconic acid and hydrogen by glucose oxidase from *Aspergillus niger.*

Glucose oxidase and baker's yeast invertase catalyzed the conversion of sucrose to gluconic acid, fructose and hydrogen. Finally, glucose oxidase and cellulose transformed cellulose to gluconic acid and hydrogen. Another potential source of glucose for the bio-electrochemical cell, starch was degraded to glucose by the combined action of amylase and amyloglucosidase (Laane *et al.*, 1984).

A bio-electrochemical cell containing either glucose oxidase or xanthine oxidase with dichlorophenol indophenol as electron acceptor in one half and chloroperoxidase in the other half of the cell was described by Laane *et al.* (1984). Due to the combination of chemical, biochemical and electrochemical reactions, specific biochemicals could be produced in the cell simultaneously and in both the compartments. Furthermore, H_2O_2 did not inactivate the oxidases in a bio-electrochemical cell and as a result, the operational lifetime of both the oxidases increased.

2.2.4. Biochemical oxidation

The conversion of glucose to gluconic acid by biochemical methods can be classified into microbial and enzymatic routes. In microbial synthesis, the main strains used have been *Aspergillus niger, Gluconobacter suboxydans* and *Zymomonas mobilis*. The pathway by which gluconic acid is produced using these strains depends upon the type of strain used. Fermentation processes can broadly be classified in three categories: surface fermentation, submerged fermentation and solid-state fermentation. In which submerged fermentation processes are undoubtedly the most

frequent fermentation technique because they offer simple operations and inexpensive to conduct, low fermenter cost etc. Although they are having some weaknesses too, like: limited maximum oxygen transfer rate, and not always best fermentation method for all types of micro-organisms.

The present work is based on the microbial synthesis phenomena of *Aspergillus niger* using submerged fermentation. We will see microbial pathways for gluconic acid production by microbes, other than *Aspergillus niger*, in brief because our work is based on *Aspergillus niger* synthesis only.

2.3. MICROBES USED UNDER BIO CHEMICAL OXIDATION

2.3.1. *Gluconobacter suboxydans*

In *Gluconobacter suboxydans* process, glucose oxidized to D-gluconic acid mainly by a particulate membrane-bound D-glucose dehydrogenase. D-gluconate and 2-Oxo-D gulconate are transported into the cytoplasm where the later is reduces to D-gluconate by an NADP-depended 2-Oxo-D-gluconate reductase. The cytoplasmic D-gluconate is further metabolized through the pentose phosphate pathway. This process is distinguished by a high affinity for oxygen. Simple aeration is known to give high rate of glucose conversion. The process displays greater tolerances with respect to acidity, permitting the isolation of free gluconic acid directly form the fermentation solution [Velizarov et al.,1994].

2.3.2. *Zymomonas Mobilis*

Sorbitol and gluconic acid production by permeabilized cells of *Zymomonas mobilis* has been extensively studied. Such efforts can be justified by relatively mild operation conditions of the biosynthesis when compared to the chemical route and by the enzyme specificity, which minimizes by-product formation, besides the importance of both products.

The presence of glucose-fructose oxidoreductase in *Zymomonas mobilis* is utilized to convert mixtures of glucose and fructose to sorbitol and gluconic acid, provided that gluconic acid formed is not further metabolized. This is accomplished by breaking the cells with consecutive loss of essential coenzymes such as ADP and ATP as well as NAD (P). The procedure for cell treatment is: permeabilisation of

cells with toluene, freezing of cells at -200C, drying of cells and applications of cationic detergents. In a subsequent step, glucono-lactone is hydrolyzed to gluconic acid, both spontaneously and under the action of the enzyme glucono-lactonase.

2.3.3. *Aspergillus niger*

Gluconic acid can be produced by *A. niger* strain. It is selected for our present work because it has good ability to cause rapid fermentation for Gluconic acid production with high yield [Singh et al.;2005; Zand et al.,2004; Klein et al,2002; Sankpal et al.,1999; Torres et al.,1998].

Gluconic acid fermentation by the filamentous fungi *Aspergillus niger* belongs to aerobic fermentations with a high oxygen demand. This fungal fermentation, which belongs to the group of bioconversions, possesses indisputable advantages for the purpose of research of the oxygen transfer in bioreactors [Klein et al,2002].

The biotransformation of glucose to gluconic acid represents a simple dehydrogenation reaction without involvement of complex metabolic cell pathways, which is realized with a high selectivity, high yield and rate yield of conversion. Gluconic acid is formed by the hydrolysis of the gluconolactone ($C_6H_{10}O_6$); the by-product of the reaction (H_2O_2) is decomposed to water and oxygen by enzyme catalysis, which is present in the living cells. The overall reaction mechanism can be described as follows [Zand et al.,2004]:

Cell Growth:

$$C_6H_{12}O_6 + \text{Biomass} + O_2 \longrightarrow \text{Biomass}$$

Glucose Oxidation:

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

Gluconolactone hydrolysis:

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

H_2O_2 Decomposition:

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

The initial step of pathway is the catalytic oxidation of β–D-glucose to glucono–δ-lactone, which is catalyzed by flavoprotein glucose oxidase. Subsequent hydrolysis of the lactone to gluconic acid is to some extent spontaneous but the enzyme gluconolactonase also plays a role. Glucose oxidase contains two oxidized forms of flavine-adenine dinucleotide (FAD) as prosthetic groups. These are responsible for the abstraction of hydrogen atoms for glucose and reduce FAD to $FADH_2$, which ultimately combine with oxygen and produce hydrogen peroxide. This in turn is cleaved to water and oxygen by the enzyme catalase [Hustede et al.,1989].

Oxygen is one of the main direct substrates of the bioconversion, where during gluconic acid fermentation, GOD of *A. niger* uses molecular oxygen to oxidize glucose. Oxygen is required for bioconversion, as well as mycelial respiration. Oxygen is usually provided in the form of air, but pure oxygen and hydrogen peroxide are also options reported for efficient conversion.

Due to the high oxygen demand of the fungi *A. niger*, the oxygen transfer from the gas phase into the liquid phase was found to be a limiting step of the bioconversion. For this reason, a great attention has to be paid for the optimization of the bioreactor operation (mixing, aeration and bioreactor design) in order to enhance the oxygen transfer rate. some investigators have obtained high volumetric productivity of gluconic acid using a relatively high pressure (2–6 bar) resulting in an increase in dissolved oxygen up to 150 mg but with increased operating cost.

Medium composition

Growth and production of microorganism are strongly affected by the medium composition such as concentrations of carbon, nitrogen, phosphorous, potassium, trace-elements and stimulators. Thus, gluconic acid productivity by *A. niger* can be improved by optimizing the medium composition.

Carbon source

The commercial scale production of gluconic acid using *A. niger* is strongly affected by the type of substrate and its level of available carbon. For the production of gluconic acid, sucrose, fructose and glucose are the carbon substrates for *A. niger* (Sassi *et al.,* 1991). Among them, as glucose doesn't need any future modification for the metabolites, it is readily used by fungus. Glucose is a principal inducer of the glucose oxidase gene. Also, apple peels and pomace, grape pomace, banana extract, sugar cane baggasse and sugar beet molasses have been used to produce gluconic acid. Glucose is the crucial factor affecting acid production. According to Leangon *et al.* (2000), an increased glucose flux through glycolysis may be the reason for over-production of gluconic acid in fermentation. However, low glucose concentration may cause oxalic acid accumulation.

Calcium carbonate

Calcium carbonate is an insoluble salt frequently used in gluconic acid and glucose oxidase production. It was suggested as a mechanical support of fungal growth and addition of $CaCO_3$, in the growth medium substantially induced glucose oxidase activity [Hatziuikolaou D.U.,1995]. More importantly, this salt was found to be essential for the production of elevated levels of the enzyme from both sucrose and molasses. Although highly insoluble in water, $CaCO_3$, proved to work better at higher concentrations, leaving no other explanation than the direct effect of solid salt phase on the mechanism of enzyme production.

Nitrogen sources

The effect of nitrogen source on gluconic acid production has been intensively studied in submerged fermentation. Ammonium chloride, ammonium sulphate, ammonium nitrate, pepton and yeast extract were the most suitable nitrogen source for production

of citric acid by fungus. The limitation or starvation of nitrogen during the fermentation resulted in the limited growth of *A. niger* and to the enhancement of gluconic acid production.

Fermentation parameters:

Initial pH

The metabolic activity of fungus is very sensitive to pH level of media. The initial pH of the media is having a great impact on gluconic acid production by *A. niger*. Most filamentous fungi are observed to grow well under slightly acidic conditions, ranging from 3 to 6. For the production of gluconic acid by *A. niger*, the initial pH range from 2 to 6 is commonly used in solid substrate and submerged fermentation [Watanabe *et al.*, 1998; Adham, 2002; Lesniak *et al.*, 2002].

Fermentation period

The effect of cultivation time on glucose oxidase production is quite important. In general, the level of extracellular glucose oxidase activity varies from about 10% of the total at the initial stages of the fermentation to about 25% at the final stages. Determination of an optimum growth time for maximum total enzyme production is always necessary.

2.5.3.2 Fermentation temperature

Optimal fermentation temperature must be maintained despite the large amount of heat generated by the metabolic activity of microorganisms (Miller, 1999). Cultivated under temperatures other than ideal, cells show signs of adverse growth and metabolic production [Ellaiah *et al.*, 2003]. Higher than optimal temperatures result in enzyme denaturation and inhibition, excess moisture losses and growth arrest while lower temperatures lead to lower metabolic activity [Adinarayana et al., 2003]. Although most filamentous fungi are mesophilic requiring optimal temperatures between 25 and 35°C, A temperature of 30-32°C was identified as optimum for metabolite production and sugar utilization by *A. niger* [Hatziuikolaou D.U.,1995].

In the further clause some more literature has been given showing the sturdy base prepared with the significant jobs done by many workers in the field of gluconic acid production via submerged cultivation phenomena.

[Kelin et al.,2002]. In the present paper a glucose–gluconic acid biotransformation system was suggested with *Aspergillus niger* strain for the experimental study of oxygen transfer in bioreactors. This biosystem was used for the investigation of the effect of the flow rate and biomass concentration on the volumetric oxygen transfer coefficient in a $10dm^3$ internal-loop airlift bioreactor. For this purpose, the fermentation broth of the mycelial strain *Aspergillus niger* was employed, representing a three-phase system, where bubbles come into contact with dense rigid pellets. The results showed that the presented biotransformation system could be successfully utilized for the determination of the oxygen transfer rate in internal loop airlift bioreactors.

[Zand et al.,2004]. Here batch fermentation of glucose to gluconic acid was conducted using *Aspergillus niger* under growth and non-growth conditions using pure oxygen and air as a source of oxygen for the fermentation in 2 and 5 l stirred tank reactors (batch reactor). Production of gluconic acid under growth conditions was conducted in a 5 l batch reactor. Production and growth rates were higher during the period of supplying pure oxygen than that during supplying air, and the substrate consumption rate was almost constant. For the production of gluconic acid under non-growth conditions, conducted in the 2 l batch reactor, the effect of the pure oxygen flow rate and the biomass concentration on the gluconic acid production was investigated and an empirical equation suggested to show the dependence of the production rate rp on the biomass concentration Cx and oxygen flow rate Q, at constant operating conditions (30°C, 300 rpm and pH 5.5).

[Nakao et al.,1997]. In the given work an external loop airlift bubble column (ELBC), an internal loop airlift (ILBC) and a normal bubble column (NBC) were employed as promising bioreactors to carry out the immobilized glucose oxidase catalyzed oxidation of glucose with air to produce gluconic acid as a model of immobilized enzyme reactions. Optimal reactor design and operating conditions were

searched for in relation to its accumulation based on the reaction kinetics and reactor characteristics. A useful determination of optimal operating condition was proposed for each reactor type. ILBC and NBC were found to give higher GA productivity and lower GO activity decay due to their better mass transfer than ELBC.

[Sankpal et al.,2002]. The work which was described here was the optimization of gluconic acid fermentation using immobilized *Aspergillus niger* on a highly porous cellulose support. 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. Morphological characteristics of immobilized *A. niger* have also been investigated. Higher levels of glucose and DO enhance the productivity of gluconic acid. Oxygen enriched air can substitute pure oxygen and reduce the fermentation period substantially.

[Sankapal et al.,1999]. Bioconversion of glucose to gluconic acid at low pH using *Aspergillus niger* immobilized on cellulose fabric was investigated. Glucose solution (100 g/l) 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.

[Blom et al.,1952]. This paper is the first real representation of submerged fermentation process carried out in 1952 by a team of eight people. It had described pilot plant experiments in which sodium gluconate was produced directly by continuous neutralization of gluconic acid formed during submerged culture fermentation of glucose with *A. niger* in stirred fermenter. Many companies used this production technique in the 1970s.

[Singh et al.,2003]. The production of gluconic acid with respect to varying substrate concentrations in submerged (SmF), semisolid-state (SmSF), surface (SF) and solid-

state fermentation (SSF) fermentation was analyzed. Under the various fermentation conditions the biomass and specific growth rate varied with different concentration of glucose. The gluconic acid production and change in pH were analyzed at varying time intervals and it was observed that the SmF and SmSF 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.

[Tripathi et al.,2003]. Calcium gluconate production by *Aspergillus niger* was investigated in shake flask, rolling shaker, airlift reactor and stirred reactor at different initial glucose concentration. They investigated that success of the technology for biological products depends on the choice of bioreactor efficiently achieving mixing and contact of liquid and gas phases. Further, found that high calcium gluconate production was achieved in airlift reactor with pellet form of cell growth at moderate specific growth rate and biomass concentration, while in stirred reactor pulpy mycelial growth was obtained and hence calcium gluconate production was poor.

[Favela Torres et al.,1998]. In the present paper the effect of initial glucose concentration (30-450 gm/l) on the growth of *Aspergillus niger* 10 in submerged (SmF) agar surface (ASF) and solid state fermentation was examined. Biomass production and specific growth rate were considerably less sensitive to changes in initial glucose concentration in SSF compared to either SmF and ASF or both. In all the cases, maximum specific growth rate were obtained with the lowest initial glucose concentration employed (50gm/l-SSF, 30gm/l-SmF and ASF).

[Zand et al,2004]. A tanks-in-series model was applied for mathematical modeling of the unsteady state performance of a semi batch operation in a 10.5 dm^3 internal loop airlift bioreactor for the production of gluconic acid by fermentation.

The model has been validated with experimental data. The model is simple enough to be used in design studies and it can be adapted to airlift system configurations and fermentation systems other than gluconic acid. For gluconic acid fermentation an optimum range of airflow rate (9–45 dm^3 min^{-1}) is suitable in a 10.5 dm^3 internal loop airlift bioreactor beyond which the process will not be economical.

[Vunjak-Novakovic et. al.,2005]. Air-lift reactors (ALRs) have great potential for industrial bioprocesses, because of the low level and homogeneous distribution of hydrodynamic shear. In this paper, we discuss the requirements for photosynthetic biomass growth in an ALR. The effects of the operating variables are analyzed using a mathematical model. We describe the design and operation of this novel bioreactor and present the first series of experimental data obtained for two different algal species in a pilot-scale unit supplied with flue gases from a small power plant.

[Moresi et. al.,1991]. Gluconic acid production from corn starch hydrolysates by immobilized mycelia of *Aspergillus niger* was studied in a laboratory-scale stirred fermenter at different concentrations of glucose (So) 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 airlift fermenters. In particular, increasing so in the range 70-160 g/l, although uninfluential upon the yield coefficient, resulted in an exponential decrease in the gluconic acid formation rate constant.

[Jurascík et. al.,2000]. Gluconic acid fermentation by the filamentous fungi *Aspergillus niger* belongs to aerobic fermentation with high oxygen demand. The biotransformation of glucose (Glc) to gluconic acid (Glu) represents a simple dehydrogenation reaction without involvement of complex metabolic cell pathways, which is carried out with a high selectivity, high rate and high yield of conversion.

This study deals with glucose-gluconic acid fermentation by *Aspergillus niger* in internal loop airlift reactors (ILALR). The fermentations were carried out in three ILALR (10.5 L, 40 L and 200 L). Growth fermentations (in 40 L and 200 L ALR) were performed under constant air flow rate, and non-growth fermentations were carried out under various gas flow rates in order to study circulation velocity of the system.

[Sankpal et al.,2001]. Experimental data on continuous fermentation of sucrose and glucose solution at low pH to gluconic acid by *Asprgillus niger* immobilized on cellulose fabric show complex dynamic behaviour 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 5, 10 and 15 h 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.

[Bandaru et al.,2005]. Statistical experimental design was used to optimize the conditions of simultaneous saccharification and fermentation (SSF), viz. temperature, pH and time of fermentation of ethanol from sago starch with co-immobilized amyloglucosidase (AMG) and *Zymomonas mobilis* MTCC 92 by submerged fermentation. The optimum conditions were found to be a temperature of 32.4 °C, pH of 4.93 and time of fermentation of 17.24 h. Thus, by using SSF process with co-immobilized AMG and *Z. mobilis* cells MTCC 92, the central composite design (CCD) was found to be the most favorable strategy investigated with respect to ethanol production and enzyme recovery. CCD offers a number of important advantages. For instance, the researchers could easily determine factor effects with considerably less experimental effort, identify factors, find optima, offer greater precision and facilitate system modeling. Thus, the present study using the RSM with CCD enables to find the importance of factors at different levels. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for ethanol production.

[Liu et al., 2003]. Response surface methodology (RSM) was applied to optimize the speed of agitation and the rate of aeration for the maximum production of glucose oxidase (GOD) by *Aspergillus niger*. A 22 central composite design using RSM was employed in this investigation. A quadratic model for GOD production was obtained. Aeration had more negative effect on GOD production than agitation. Significant negative interaction existed between agitation and aeration. The quadratic term of agitation presented significant positive effect. The fermentation kinetics of GOD by *Aspergillus niger* were also studied in a batch system. A simple model was proposed using the Logistic equation for growth, the Luedeking–Piret equation for GOD production and Luedeking–Piret-like equation for glucose consumption. The model appeared to provide a reasonable description for each parameter during the growth phase.

Table-2.1 Summary of different methodologies used for production of gluconic acid.

Sr No.	Research paper	Micro-Organism Used	Aim of the Experiment	Experimental System	Parameters Studied	Conclusion of the work
1.	Blom et al. (1952)	*A.niger*	Sodium gluconate production:	Stirred reactor in batch fermentation	Antifoam agents, Sugar concentration	Fermentation is the better method for GA production.
2.	Hatziuikolaou et al. (1995)	A. niger	Max^{n}of total glucose activity, optimization of growth media and condition:	2 l Erlenmeyer flask, 650 ml working vol, pH 5.5, 24 hr, 175 rpm, 30°C	Carbon source, growth temperature, growth time for GOD activity	Glucose-the better carbon source, opti. Enzymatic activities obtained at-27.5°C, and 70 hrs is the optimum growth time for maximum total enzyme production
3.	Nakao et al. (1997)	*Glucose oxydase*	To study performances in three types of airlift bubble columns.	External, internal and normal loop bubbling columns.GOD immobilized on calcium alginate gel beads.	Effect of Ug on k_La and k_So. Effect of operating conditions (Conc. Of G, GA, gas hold up, Ug)	Optimum operating conditions have been recommended. ILBC & NBC have given higher gluconic acid productivity and lower glucose oxidase activity decay due to their better mass transport than ELBC.
4.	Velizarov et al. (1998)	Glucono-bacter oxydans (NBIMCC 1043)	To study the substrate and product inhibition situations in free GA production from G.	0.4 lit batch bioreactor (Bioflo C-30, New Brunswick) Aeration rate: 2 vvm Speed: 1000 rpm 32 °C for 8 hr Antifoam: silicon emulsion	Diff. G and GA conc., kinetic parameters and yield. Process kinetics was evaluated by comparing different inhibition models.	Substrate inhibition is linear inhibition of growth described by Tseng and Wayman. Product inhibition is found non-linear inhibition kinetics fitting well with Levenspiel model .
5.	Tripathi et al. (1999)	Aspergillus niger (NRRL - 3)	To investigate most suitable fermentation condition for calcium gluconate	Shake flask, rolling shaker, air-lift bioreactor (1.5 lit, 1 vvm) and Stirred tank bioreactor	Effect of G on biomass concentration. Spec. Growth rate and	In all system except stirred tank, pellets were produced and calcium gluconate production was higher.

			production.	(0.5 lit, 1 vvm, 300 rpm), 28 °C	Calcium gluconate prod.	
6.	Singh, O.V., Ph.D. Thesis (2000)	Aspergillus niger (ORS-4.410)	Bioconversion of agro-food by-products to GA in surface, submerged and Solid-state conditions.	Shake flasks Cells immobilized on calcium alginate. 12% Glucose conc. 1-2% inoculation level 28 - 32 °C, pH 4.5 - 6.5	Various substrates (Grape must, Banana must and Molasses). Effect of regulators (H_2O_2, starch, vegetable oil) Effect of Glucose	Rectified Grape must is better higher GA production. H_2O_2 enhance GA production. Surface or SSF gives better yield then submerged, but at large scale, it is not claimed.
7.	Kulkarni et al. (2002)	*A. niger* (NCIM545)	To study and optimize fermentation conditions using *A. niger* immobilized on cellulose microfibrils.	Batch bioreactor with recirculation. Air (25 ml/min) mixed with pure oxygen (135 ml/min). Wowen cellulogic fabric support for *A. niger*,30 °C, pH 6.0	Initial conc. of glucose. Effect of DO and biomass conc. on the support under continuous recirculation.	Optimum biomass conc. on the cellulose support is 0.234 mg/cm^2. Oxygen enriched air increase productivity and reduces the fermentation time.
8.	Markos et al. [31] (2002)	A.niger (CCM 8004)	Biotransformation of G to GA by *A. niger*- study of mass transfer in airlift bioreactor.	10 and 34 l internal loop bioreactor pH 6.5 - during growth phaseand 5.5 – during production phase	Effect of airflow rate and biomass concentration on k_La. Specific growth rate of GA production.	Increase of airflow rate (i.e. Enhancing liquid circulation velocity), intensity of mixing and mass transfer coefficient are increased. Specific prod. Rate decreased with increasing biomass concentration.
9.	Klein et. al, (2000)	A. niger	To suggest and test an experimental technique for studying the oxygen transfer in airlift bioreactor	Internal loop airlift bioreactor, working vol-10 l and 34 l, 30°C, 48 hrs, airflow rate-34 lpm, pH-5.5 during production phase	Time dependence, effect of rate of oxygen transfer in Gluconic Acid production,	Higher the oxygen transfer rate in airlift bioreactors, better is the performance, ALBRs are possible alternative design of submerged bioreactors for guconic acid production.

3. EXPERIMENTAL WORKING PROCEDURE

In 1870, Gluconic acid was identified first time by Hlasiwets and Haberman. While its production by microorganisms was observed in 1880 by Boutroux in the course of studies on lactic acid fermentation and was verified as the action of acetic acid bacteria. In 1922, Molliard detected gluconic acid first time in cultures of *Sterigmatocystis nigra* now known as *Aspergillus niger*. After that in 1928 Bernhauer selected a strain of *Aspergillus niger* which under specified condition produced only gluconic acid. Semi plant scale surface process was published in 1929 by May and his Co-workers who gave rather low yields (about 57 % of the theoretical values) and low productivities (about 2.3 g/l.hr). Subsequently, a submerged process was developed comprising a revolving drum equipped with internal baffles and enabling the introduction of air under pressure (May *et al.* 1934).

Systematic studies by Moyer *et al.* (1940) revealed that *Aspergillus niger* could convert Glucose to gluconic acid with high yields if the acid formed is neutralized by the addition of calcium carbonate. It was found that the concentration of glucose could be increased up to 35% (w/v) by the addition of boron compounds as complexing agent. In 1952, a process of gluconic acid production by fermentation using *A. niger* described by Blom et al.,1989 is essentially used by most manufacturers in the 1980s and 1990s. Hartmeir and Doppner (1983) entrapped mycelium of *Aspergillus niger* containing glucose oxidase and catalase with a thin layer of excess catalase.

Roukas and Harvey (1988) studied the effect of pH on the production of Gluconic acid from beet molasses by *Aspergillus niger* using continuous culture. Sakurai *et al.* (1989) immobilized free enzyme and mycelia of *Aspergillus niger* on a non -woven fabric. A sustained level (220 gm/l) of gluconate was reproducible. The immobilisate had a much longer durability and more gluconate producing activity.

3.1. SUBMERGED FERMENTATION PHENOMENA AND WORKING PROCEDURE FOR PARAMETER OPTIMIZATION

3.1.1. Strain development

Among the variety of fungii producing Gluconic acid, *Aspergillus niger* is the microorganism of choice for its efficient productivity and rapid fermentation process.

121°C for 15 min at 15 psi, was done. After autoclaving the media was left for being cool up to room temperature.

Next step was of doing inoculum in media flasks under laminar condition in laminar chamber. Sterilized $CaCO_3$ was added to the medium just before inoculation. After that all flasks were be put up in orbital shaker with specified temperature, rpm condition and cultivation period for fermentation.

Analytical methods:

(1). Estimation of Gluconic acid:

The gluconic acid formed in fermentation broth was determined by hydroxamate method of Hestrin as modified by Lien. The estimation of gluconic acid is based on the reaction of hydroxylamine with gluconolactone to form hydroxamic acid that reacts with iron in acidic solution resulting in colored solution.

Reagents:

1. 4 M Hydroxylamine hydrochloride.
2. 4 M Sodium hydroxide.
3. Hydroxylamine reagent: Solution (1) and (2) were mixed in equal volumes and pH adjusted to 8.0. This reagent is stable for about 4 hours only.
4. 4 M Hydrochloric acid.
5. Ferric chloride: 10% solution in 0.1N Hydrochloric acid.

Assay:

One milliliter of approximately diluted sample was acidified with hydrochloric acid to pH 1.5-2.0 and autoclaved at 15 psi.. The sample was then cooled in water bath and 2 ml of hydroxyl amine reagent was added. This was followed by the addition of 1ml of 4 M hydrochloric acid, and 1 ml of ferric chloride solution. At this point the pH of the reaction mixture is 1.2 ±0.2. The optimal density was read in Beckman model DU-6 spectrophotometer at 540 nm within 10 min after addition of the ferric chloride solution. Estimations for acid were done against a calibration curve made of suitable standard solution of gluconic acid.

3. EXPERIMENTAL WORKING PROCEDURE

In 1870, Gluconic acid was identified first time by Hlasiwets and Haberman. While its production by microorganisms was observed in 1880 by Boutroux in the course of studies on lactic acid fermentation and was verified as the action of acetic acid bacteria. In 1922, Molliard detected gluconic acid first time in cultures of *Sterigmatocystis nigra* now known as *Aspergillus niger*. After that in 1928 Bernhauer selected a strain of *Aspergillus niger* which under specified condition produced only gluconic acid. Semi plant scale surface process was published in 1929 by May and his Co-workers who gave rather low yields (about 57 % of the theoretical values) and low productivities (about 2.3 g/l.hr). Subsequently, a submerged process was developed comprising a revolving drum equipped with internal baffles and enabling the introduction of air under pressure (May *et al.* 1934).

Systematic studies by Moyer *et al.* (1940) revealed that *Aspergillus niger* could convert Glucose to gluconic acid with high yields if the acid formed is neutralized by the addition of calcium carbonate. It was found that the concentration of glucose could be increased up to 35% (w/v) by the addition of boron compounds as complexing agent. In 1952, a process of gluconic acid production by fermentation using *A. niger* described by Blom et al.,1989 is essentially used by most manufacturers in the 1980s and 1990s. Hartmeir and Doppner (1983) entrapped mycelium of *Aspergillus niger* containing glucose oxidase and catalase with a thin layer of excess catalase.

Roukas and Harvey (1988) studied the effect of pH on the production of Gluconic acid from beet molasses by *Aspergillus niger* using continuous culture. Sakurai *et al.* (1989) immobilized free enzyme and mycelia of *Aspergillus niger* on a non -woven fabric. A sustained level (220 gm/l) of gluconate was reproducible. The immobilisate had a much longer durability and more gluconate producing activity.

3.1. SUBMERGED FERMENTATION PHENOMENA AND WORKING PROCEDURE FOR PARAMETER OPTIMIZATION

3.1.1. Strain development

Among the variety of fungii producing Gluconic acid, *Aspergillus niger* is the microorganism of choice for its efficient productivity and rapid fermentation process.

It has been observed by many researchers that glucose, a cheap C-source, is mostly converted to gluconic acid under aerobic fermentation by *A. niger* strains. Therefore, for the present work of fermentative production of this acid the A. niger strain is undertaken. The microbial strain of *Aspergillus niger*, isolated from the dumping sites of the sugarcane industry, had maintained on potato-dextrose-agar (PDA-2%Dextrose, 2%-Agar, in potato extract) slants by periodical transformation by incubating at 30°C for 72 hours before storing at 4°C. Culture was renewed every month in slant forms.

3.1.2 Batch fermentation procedure

In the light of well sighted literature, gluconic acid fermentation, under submerged fermentation phenomena using the microorganism *Aspergillus niger*, was carried out with the fermentation media based on guesses. After optimizing the major parameters for the fermentation process, all studies were performed on the basis of that only. Fermentation process was carried on batch process for the lab work of parameter optimization. The step wise working procedure is described here.

Fermentation media:	**variation**	**initial guess**
$C_6H_{12}O_6$	8%-16%	110-120 gm/lit
$(NH_4)_2HPO_4$	0.1%	1 gm/lit
KH_2PO_4	0.05%	0.5 gm/lit
$MgSO_4.7H_2O$	0.015%	0.15 gm/lit
$CaCO_3$	1%-6%	30 gm/lit

Lab working procedure: First of all the pre-decided quantity of distill water in a suitable volume beaker was taken. The desired amount of media (except $CaCO_3$) weighed and added in the beaker one by one properly with the help of magnetic stirrer.

$CaCO_3$ weighed separately and raped in aluminum foil. The media was divided with suitable volume in few conical flasks (amount of media in a conical flask was approx. 1/10 of the flask volume). Conical flasks and aluminum foil of $CaCO_3$ (in a beaker) were covered-up properly with cotton plugs. pH of the medium had to be adjusted for decided value at 30°C. Autoclaving of the media and $CaCO_3$ foils, at

121°C for 15 min at 15 psi, was done. After autoclaving the media was left for being cool up to room temperature.

Next step was of doing inoculum in media flasks under laminar condition in laminar chamber. Sterilized $CaCO_3$ was added to the medium just before inoculation. After that all flasks were be put up in orbital shaker with specified temperature, rpm condition and cultivation period for fermentation.

Analytical methods:

(1). Estimation of Gluconic acid:

The gluconic acid formed in fermentation broth was determined by hydroxamate method of Hestrin as modified by Lien. The estimation of gluconic acid is based on the reaction of hydroxylamine with gluconolactone to form hydroxamic acid that reacts with iron in acidic solution resulting in colored solution.

Reagents:

1. 4 M Hydroxylamine hydrochloride.
2. 4 M Sodium hydroxide.
3. Hydroxylamine reagent: Solution (1) and (2) were mixed in equal volumes and pH adjusted to 8.0. This reagent is stable for about 4 hours only.
4. 4 M Hydrochloric acid.
5. Ferric chloride: 10% solution in 0.1N Hydrochloric acid.

Assay:

One milliliter of approximately diluted sample was acidified with hydrochloric acid to pH 1.5-2.0 and autoclaved at 15 psi.. The sample was then cooled in water bath and 2 ml of hydroxyl amine reagent was added. This was followed by the addition of 1ml of 4 M hydrochloric acid, and 1 ml of ferric chloride solution. At this point the pH of the reaction mixture is 1.2 ±0.2. The optimal density was read in Beckman model DU-6 spectrophotometer at 540 nm within 10 min after addition of the ferric chloride solution. Estimations for acid were done against a calibration curve made of suitable standard solution of gluconic acid.

(2). Estimation of residual Glucose:

The total residual glucose was measured by the standard DNS method. The method is based on the principle, that the 3-5-dintrosalicyclic acid is reduced to 3-amino-5-ntrosalicyclic acid while, the aldehyde group gets oxidized to carboxyl group.

Reagents:

1. DNS reagent:

3-5-dintrosalicyclic acid	10 g/l
Phenol	2 g/l
Sodium sulphite	0.5 g/l
Sodium potassium tartarate	200 g/l

2. Standard Glucose solution

   100 μg/ml in double distilled water

Assay:

To 3 ml of aliquots solution, an equal volume of DNS reagent was added. The mixture was heated for 5 min in a boiling water bath and then cooled under running tap water.

The color intensities were measured in a Beckman model DU-6 spectrophotometer at 575 nm against a reagent blank. A calibration curve was made with suitable standard solution and tested accordingly for estimations.

(3). Estimation of dry Cell Mass:

For determination of dry weight of biomass, the culture fluid was filtered through Whatman No. – 4 filter paper. The filtered mycelia was washed thoroughly with acidified double distilled water (pH 2.5 with 4 N HCl) in order to convert the insoluble $CaCO_3$ to soluble $CaCl_2$. The mycelia was then washed several times with deionized water until the washing showed neutral pH. Mycelia were then dried to a constant weight. The dry weight of immobilized cells were determined by subtracting the predetermined average dry weight of matrix from the weight of matrix with mycelium at the end of fermentation after drying at 75°C to a constant weight.

3.2 Optimization of main parameters affecting the fermentation process for Gluconic acid production by submerged phenomena

Bioconversion processes are always very sensitive to their input operating conditions and parameters. So determination of optimized operating conditions is a major challenge, primarily because of varieties of microorganisms used, presence of multiple substrate conditions and variety of designs available for experimental set-up. There are many factors affecting the performance of the fermentation process, a few major parameters of them are optimized here. Working procedure for optimization is as follows while; there effects have been described in the next chapter.

(1). pH

Working condition:

Fermentation period:	8 days
Temperature	30°C
Rpm	150
pH variation	from 1 to 8

Fermentation media:	**Amount**
$C_6H_{12}O_6$:	12%
$(NH_4)_2HPO_4$	0.1%
KH_2PO_4	0.05%
$MgSO_4.7H_2O$	0.015%
$CaCO_3$	3%

Assay: Working procedure is same for fermentation as described earlier; the only new added step is the variation of pH of fermentation broths from 1 to 8 before autoclaving. After the completion of fermentation period analysis had been done by the analytical methods already given.

(2). $CaCO_3$

Working condition:

Fermentation period:	8 days
Temperature	30°C
Rpm	150
pH	5.5

Fermentation media:	**Amount**
$C_6H_{12}O_6$:	12%
$(NH_4)_2HPO_4$	0.1%
KH_2PO_4	0.05%
$MgSO_4.7H_2O$	0.015%
$CaCO_3$	1%-6%

Assay: Fermentation procedure is same. For optimization of parameter the amount of $CaCO_3$ added, at the time of inoculum, in each flask is different varying from 1% to 6%.

(3). Glucose concentration:

Working condition:

Fermentation period:	8 days
Temperature	30°C
Rpm	150
pH	5.5

Fermentation media:	**Amount**
$C_6H_{12}O_6$:	4%-20% (variation)
$(NH_4)_2HPO_4$	0.1%
KH_2PO_4	0.05%
$MgSO_4.7H_2O$	0.015%
$CaCO_3$	4%

(4). Fermentation period:

Working condition:

Temperature	30°C
Rpm	150
pH	5.5

Fermentation media:	**Amount**
$C_6H_{12}O_6$:	12%
$(NH_4)_2HPO_4$	0.1%
KH_2PO_4	0.05%
$MgSO_4.7H_2O$	0.015%
$CaCO_3$	4%

Assay: Fermentation procedure is same. For optimization of parameter, fermentation period, cultivation time varied from 2 to 14 days.

3.3 GLUCONIC ACID PRODUCTION IN AIRLIFT BIOREACTOR

3.3.1 Experimental set-up

Transport processes usually affect the productivity of bioprocesses and the efficiency of bioreactors. Thus, mass transfer phenomena belong to common problems, which a bioreactor engineer encounters during fermentation practice and bioreactor design. Gas–liquid (G–L) transfer is often a limiting factor of much aerobic fermentation. This is mainly caused by a low solubility of oxygen in fermentation media in comparison with the solubility of carbon sources and other nutrients.

These problems can be solved by different ways like: higher air flow rates can be applied; by increasing the partial pressure of oxygen in the inlet gas stream, etc. One of further possibilities is adjusting of the existing bioreactor or use of a more suitable type of bioreactor.

In recent years, airlift bioreactors (ALRS) have been extensively investigated as a possible alternative to conventional stirred tank bioreactors, which are most widely used for fermentation purposes. The ALRS possess features, which make them more advantageous for various biotechnological applications. However, an accurate description of the performance of airlift bioreactors is still difficult [Zand et al.,2004; klein et al.,2002].

For gluconic acid fermentation an optimum range of airflow rate (9–45 dm^3/ min) is suitable in a 10.5 dm^3 internal loop airlift bioreactor beyond which the process will not be economical. The shorter bioreactor shows relatively more uniform axial DO concentrations than the longer bioreactor, where a greater variation in DO concentrations with the height was observed [Zand et. al,2004].

For our further research work of gluconic acid production the bioreactor chosen is airlift bioreactor.

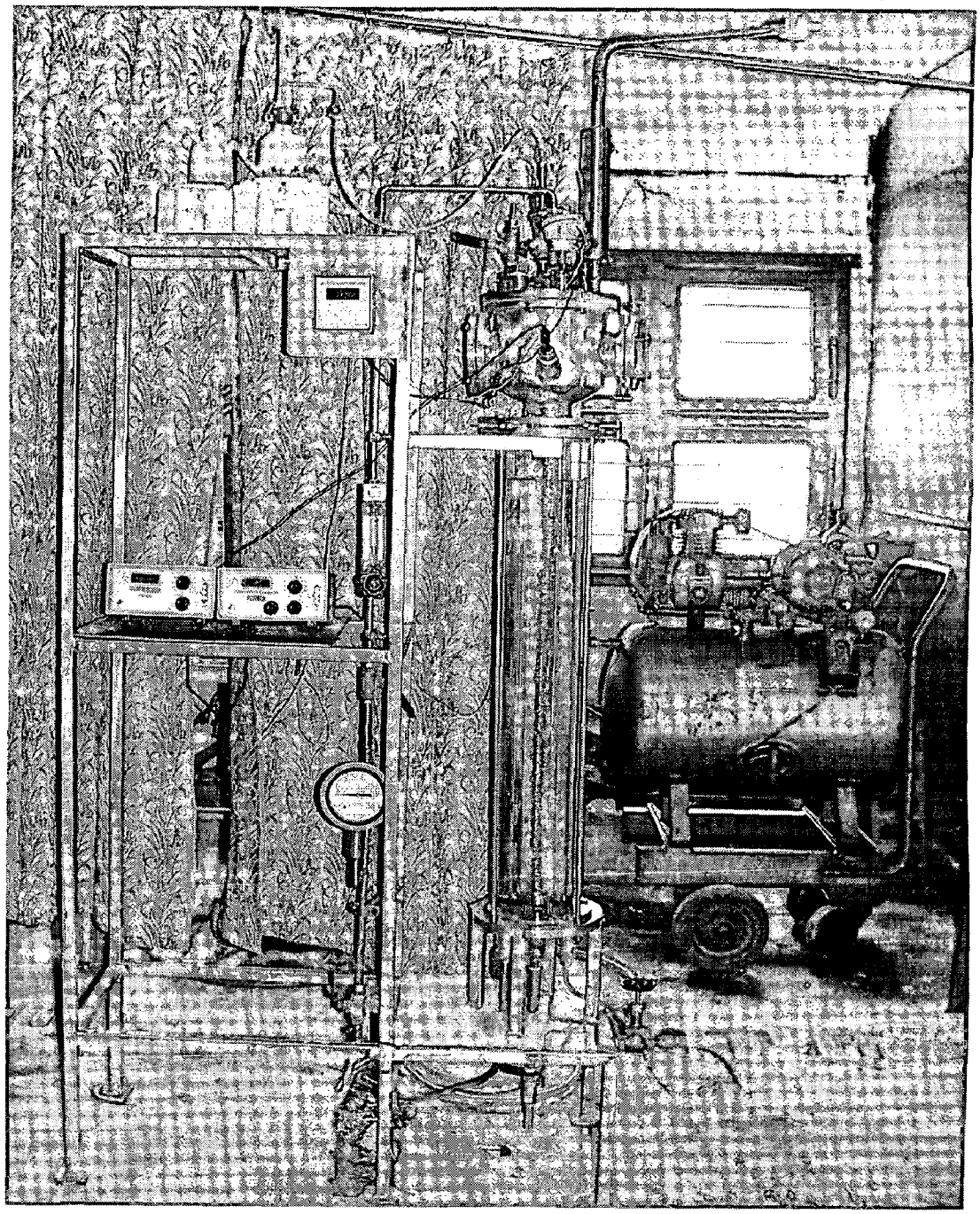


Fig-3.3.1. (a) Airlift bioreactor

Experimental set up for the present work, Departt. Of Chemical Engg, Roorkee

Selection criteria

Selection of airlift bioreactor relies on the following advantages and disadvantages:

Advantages

- A special property of ALR is a liquid circulation loop created by the interconnected aerating (riser) and recirculating sections (downcomer). Despite an absenting mechanical agitator, this well-defined flow pattern with high liquid velocities in line gives efficient mixing.
- Low uniformly distributed shear stresses can create an optimal environment for many productive microorganisms.
- An airlift bioreactor can also satisfy a high oxygen demand, especially in the large scale, where a high liquid level (40–50 m) creates high partial pressure of oxygen.
- Construction wise simple because there are no moving parts or agitators attached to it.
- Sterilization is easy in the reactor.
- When compared with bubble column, ALRS have increased heat and mass transfer capacity & less energy consumption for mixing.

Disadvantages

- Despite these advantages of airlift bioreactors, their implementation is still limited; mainly due to an insufficient knowledge of hydrodynamics and mass transfer phenomena in the ALRS.
- Inherently impossible to maintain consistent levels of substrate, nutrients and oxygen with the organisms circulating through the bioreactor and conditions changing.
- Inefficient gas/liquid separation when foaming occurs.

DESIGNING DETAILS

An airlift reactor consists of four distinct sections: riser, down comer, top and bottom sections. Each section of ALR exhibits different hydrodynamic and mixing behavior. Airlift reactors have no moving parts and mixing is caused pneumatically. Gas-liquid circulation is caused by the gradient in density between riser and down comer because the gas hold-up is different in riser and down comer.

The main specifications of the reactor are:

Draft tube (Riser tube):	ID = 0.042 m, Height = 100 cm
Airlift reactor tube (Down comer):	ID = 0.0725 m, Height = 1.032 m
Separator:	ID = 0.2 m, Height = 0.18 m

3D MODEL AND CONSTRUCTION DETAILS

3D model of concentric tube airlift bioreactor with actual dimensions is presented in the Figure 3 with important dimensions. Upper and lower parts of reactor are made up of SS 304, whereas middle parts including riser and down comer sections are made of glass for visualization of mixing. Entire stand is made from SS 304 and glass bottles are placed at the top for addition of base, acid or antifoam, etc. The working volume of the reactor is 4.5 l.

The overall size of the experimental set-up made of SS and glass is about 2 m x 1 m. The system is closed and designed such that it can be sterilized *in situ* using steam at about 121 °C for 15 min after replacing sensors and probes by blinds. Sparger can also be removed for cleaning purpose and replaced by another one to sparge in down comer. Water-cooling is provided to maintain the temperature of the system by manually controlling the flow rate. The size of the air filters provided at the inlet and outlet has pore size of 0.2 micrometer, which prevents entry of any microbes present in the atmospheric air.

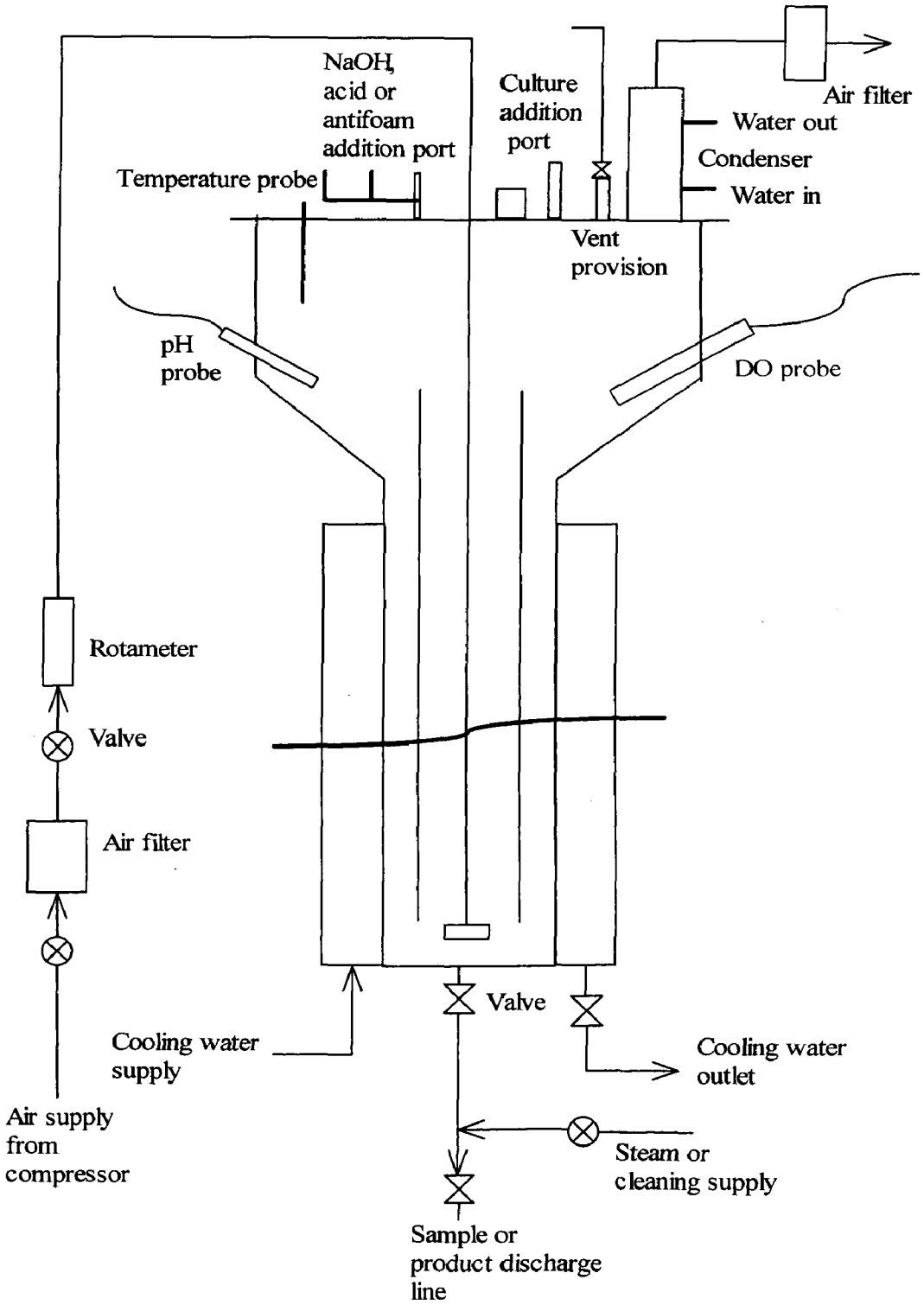


Fig-3.3.1. (b) Schematic diagram of experimental set-up with air supply and system accessories

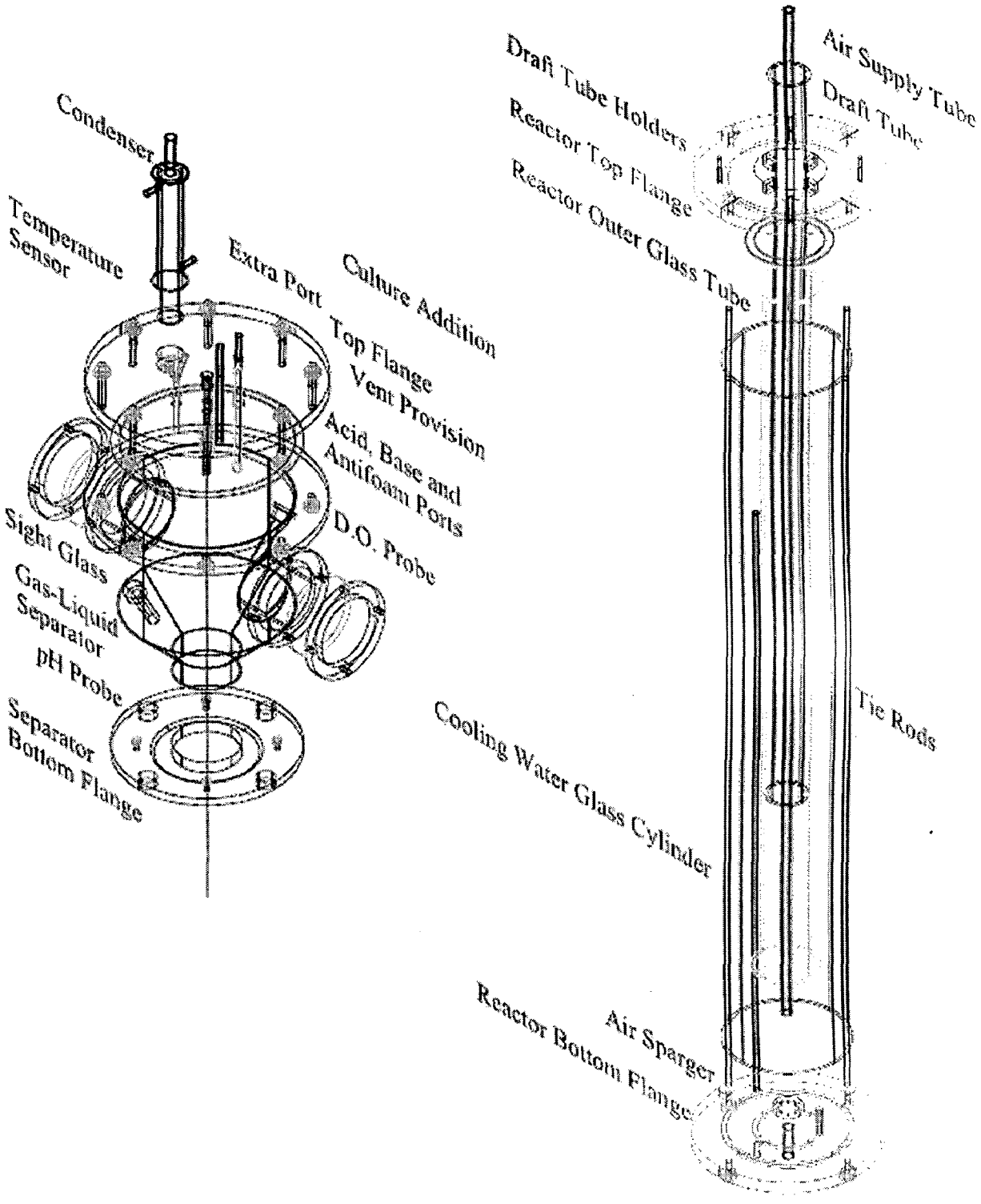


Fig-3.3.1. (c) 3D model view of an airlift bioreactor with important parts

3.3.2. Working Procedure:

Table:- 3.3.2(a) Operating parameters and conditions:

Parameters	Operating condition
Temperature	30-32°C
pH	Optimized value
Pressure	Atmospheric pressure
Working volume	4.5 l
Air flowrate	8 lpm
Inoculum level	2% of total working volume
Fermentation period	10 days
Antifoaming agent	Peanut oil

Table:- 3.3.2(b) Fermentation medium:

Compound	Composition	Amount
$C_6H_{12}O_6$	Optimized composition	-------
$(NH_4)_2HPO_4$	0.1%	1 gm/lit
KH_2PO_4	0.05%	0.5 gm/lit
$MgSO_4.7H_2O$	0.015%	0.15 gm/lit
$CaCO_3$	Optimized composition	-------

Procedure

1. The first fermentation stage is the growth of the vegetative inoculum, as described arlier. After that strain of *A. niger* prepared from a culture grown on a solid medium had introduced into the inoculum fermenter. A 2-3% (vvm) inoculum was used for the biotransformation.
2. Reactor was cleaned properly and checked whether all the joints were tightened properly or not.
3. Attachments did:
    a. Pipeline from the compressor to the air supply line of the reactor.
    b. Dissolved oxygen-meter and digital pH-meter plug to the switchboard.
    c. Steam supply pipeline was attached to its corresponding line provided in the reactor for steam sterilization.

d. Cooling water supply line was attached to its corresponding line, flowrate of which could be maintained manually.

e. Flask containing NaOH solution was provided on the stand and joined to the base addition port for maintenance of pH value. Knob was closed at that time.

f. Acid addition port was closed by blinds, but if addition is necessary, can also be attached with container having acid solution.

g. Antifoaming agent addition flask was provided to its corresponding addition port.

h. Feeding flask was provided over the stand but didn't join to the feed addition port, because before feeding sterilization was necessary.

4. All the valves and openings were checked whether they were closed or not.
5. After that DO and pH probes and temperature sensor were attached to their respective places and standardized with their respective standard solutions.
6. Next step was removal of the DO and pH probes and closing of their openings with blinds.
7. Steam sterilization was done at 121°C for 15 min and steam supply was get stopped after it.
8. Cooling water supply was provided by opening the valve of water supply.
9. Feed addition port was opened and feeding was done from fermentation media and inoculum flask, after feeding the feed addition port was get closed with a blind.
10. DO meter and digital pH meter was get switch-on, after attachment of DO and pH probes again.
11. Compressor was started and flowrate of air was adjusted for the desired value by rotating the compressor valve.
12. pH value of the media was adjusted to 5.5-6.5 by addition of base/acid from their addition port.
13. Temperature condition was maintained for the desired value by adjusting the cooling the water supply to the outer jacket of the reactor manually.
14. Once the operating parameters had been adjusted and the fermentation process had been started, the next task was to maintain the operating conditions constant up to the complete fermentation period of 10 days.

15. Samples of fermentation broth were taken time to time from the drain, product quality and % conversion of the substrate was analyzed.
16. After completion of the fermentation period, final product was taken from the drain and analyzed as per analytical procedure described earlier
17. Reactor got discharged and cleaned by removing sparger, draft tube holders etc.

4. STATISTICAL ANALYSIS OF THE EXPERIMENTAL DATA REGRESSION MODEL

In this work, we have used an Artificial Intelligence (AI) based tool performing regression model fitting, for the statistical analysis of the data obtained from the experiment done for parameters optimization, to monitor a bioprocess. This technique allows us to obtain automatically a mathematical expression which accurately fits a given set of experimental data.

Online determination of local production rates, substrate concentration etc. involves cumbersome procedures and may not be always feasible. We have, therefore, developed a mathematical expression using 30 plus observed experimental run data, predicting yield, gluconic acid production and biomass production as a function of initial pH, $CaCO_3$ concentration, substrate (glucose) concentration and fermentation period. Towards this end, we used the regression modeling technique through the software "Minitab", which is described below in detail. Once reasonable expressions predicting:- (1). The yield vs. fermentation parameters (initial pH, $CaCO_3$ concentration, glucose concentration and fermentation period), (2). Gluconic acid production vs. fermentation parameters (pH, $CaCO_3$ concentration, glucose concentration and fermentation period.) and (3). Biomass production vs. fermentation parameters (pH, $CaCO_3$ concentration, glucose concentration and fermentation period.) have been obtained, the same can be used as a process-monitoring tool obviating the need for too-frequent costly experimental run or measurements.

So finally the strategy is adopted for real experiments on fermentation to gluconic acid production under submerged cultivation using the micro-organism *Aspergillus niger* and results presented here.

Artificial intelligence based regression model

In general suppose that there a single dependent variable Y that depends upon k independent or regressor variables, for example X_1, X_2, X_3.....X_k, say $Y = f(X_1, X_2, X_3.....X_k)$. The relationship between these variables is characterized by a mathematical model called a regression model. The regression model is fit to a sample of data. As in most cases the true functional relationship is unknown, we chooses an

appropriate function to approximate it. In view of this, it becomes necessary to repeat the model fitting procedure many times by employing different liner and low order polynomial equation for arriving at an overall optimal solution. Low order polynomial models are widely used as approximation functions.

Regression Model implementation

In general we start with simple liner model, where the response Y can be related to k regressors or dependent variables by the multiple liner regression model as

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots\ldots + \beta_k X_k + \varepsilon \qquad \ldots\ldots(1)$$

Where the parameters are:

Xj – Regressor variables

βj – Regression coefficient, j = 0, 1, 2,……., k. ; it represents the expected change in response Y per unit change in Xj where all the remaining independent variables Xi ($i \neq j$) are held constant.

Models that are more complex in appearance and could not be fitted well by equation (1) may still be analyzed by multiple regression techniques. As taking another example in which second degree polynomials (Eq. (2)) is considered with three regressor variables to estimate the response or the dependent variable:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3 + \beta_{123} X_1 X_2 X_3 + \varepsilon \qquad \ldots\ldots(2)$$

Where Y is predicted response, X_1, X_2, X_3 the independent variables, β_0 the offset term, β_1, β_2, β_3 the linear effects, β_{11}, β_{22}, β_{33} the squared effects and β_{12}, β_{23}, β_{13}, β_{123} are interaction terms.

Here if we let $X_{11} = X_1^2$, $X_{22} = X_2^2$, $X_{33} = X_3^2$, $X_{12} = X_1X_2$, $X_{23} = X_2X_3$, $X_{31} = X_3X_1$ and $X_{123} = X_1X_2X_3$ then equation (2) becomes:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{11} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_{12} + \beta_{23} X_{23} + \beta_{13} X_{31} + \beta_{123} X_{123} + \varepsilon \qquad \ldots\ldots(3)$$

Equation (3) is another liner regression model. In general any regression model which is liner in parameters is liner regression model. The method of least square is typically used to estimate the regression coefficients (β) in a multiple regression model.

Design Expert system, 'Minitab' uses the method of least square method for regression analysis of the data obtained and to estimates the coefficients of the regression equation. The regression coefficients in the method are always chosen in such a way so that the sum of errors, ε can be minimized, and also the goodness of the model could be checked by different criteria.

The P values were used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. Another tool to check the proper fitness of the model is the "coefficient of determination"- R^2. The closer the value of R (multiple correlation coefficient) to 1, the better is the correlation between the observed and predicted values.

Here Statistical analysis of the experimented data obtained by fermentation parameters optimization and suitable model fitting for the experiment was done by the software- "Minitab's statistical analysis regression model" and the results are shown in chapter 5.

5. RESULTS AND DISCUSSION

5.1 Parameters optimization

Submerged cultivation was carried out for optimizing various parameters using soaring gluconic acid producing mutant strain *Aspergillus niger*. Glucose was used as the main substrate. The cultivation was done at 30°C for a maximum period of 14 days. The yield, gluconic acid production and biomass were analyzed, by the analytical methods described earlier, for getting the optimized values of the parameters.

5.1.1. Effect of initial pH

The initial pH of the nutrient solution was adjusted from 1 to 8. The results showed that nutrient solution pH had to have an important effect on gluconic acid production. The impact of initial pH of the fermentation medium on gluconic acid production under submerged condition has been presented in figer-5.1.1. A typical bell shaped curve has been obtained. The reason for this behavior may be given as different micro-organisms can grow under different and only certain specified environmental conditions; *Aspergillus niger* can grow well under temperature 30-32°C and pH is a function of temperature condition, so under the given temperature condition may be enzymatic activities of *Aspergillus niger* are best under only certain range of pH and giving the shape of the curve like this.

The best gluconic acid production was obtained at a pH 5-6. However, the highest tested initial pH of 8 did not lead to a maximum in gluconic acid production after 8days of fermentation. The yield was substantially lower above and below this pH range, so finally an initial pH of 5.5 (with the addition of $CaCO_3$), was considered suitable for gluconic acid production with 82.5% yield and 14.3 g/l biomass production.

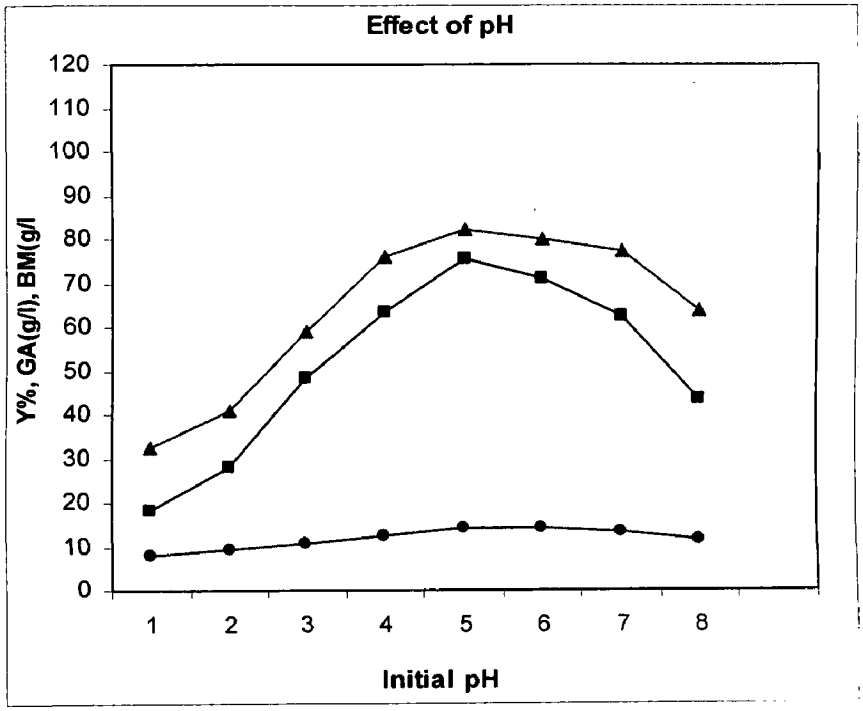


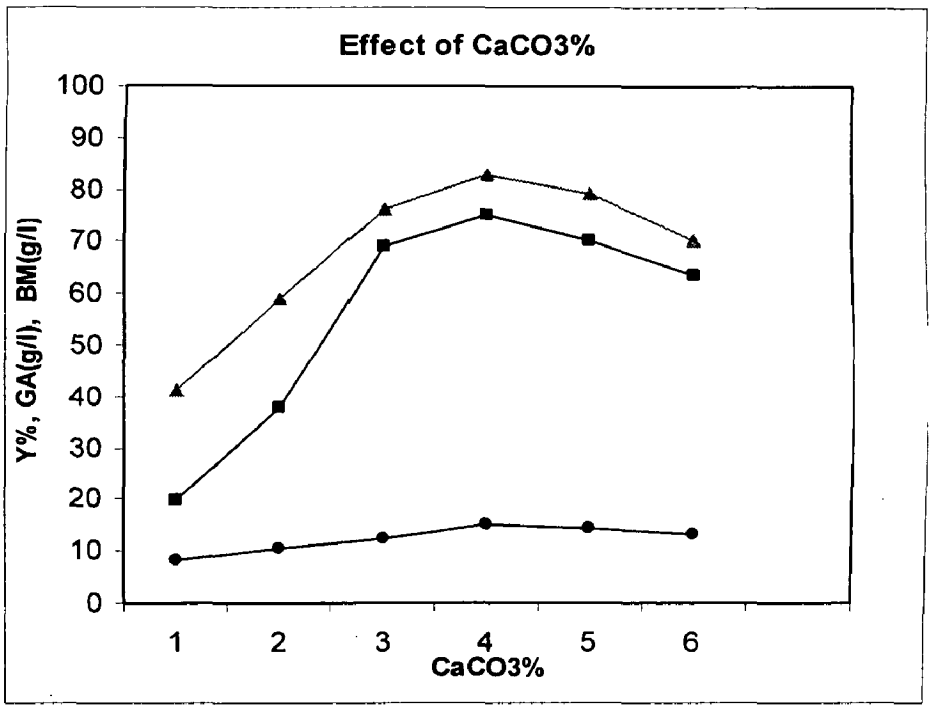
figure-5.1.1. Y%, GA(g/l), BM(g/l) vs. pH

(1). Yield%	[---▲---]	(Y)
(2). Gluconic acid (g/l)	[---■----]	(GA)
(3). Biomass (g/l)	[---●----]	(BM)

5.1.2. Effect of $CaCO_3$

Gluconic acid produced by *Aspergillus niger* strain was to found to improve with the inclusion of $CaCO_3$ from 2-4%. Although variation is given from 1 to 6% of $CaCO_3$ concentration, the excessive use of $CaCO_3$ retarded fungal growth as well as acid production. The observed optimum $CaCO_3$ concentration of 4%, with 83.00% yield and 14.8 g/l biomass production, was used in the subsequent experiments.

$CaCO_3$ is one of the major nutrients used for our experiment. Lesser % of $CaCO_3$ had not produced good food might be because of food starvation or nutrient starvation for the micro-organism, while excessive use also reduced the yield, wide change in pH ands substrate inhibition can be given the reason for this.



Figer-5.1.2. Varying amount of $CaCO_3$ from 1 to6% added with constant pH of 5.5±0.1 and 8 days fermentation period. Results are plotted for responses vs. $CaCO_3$%, where

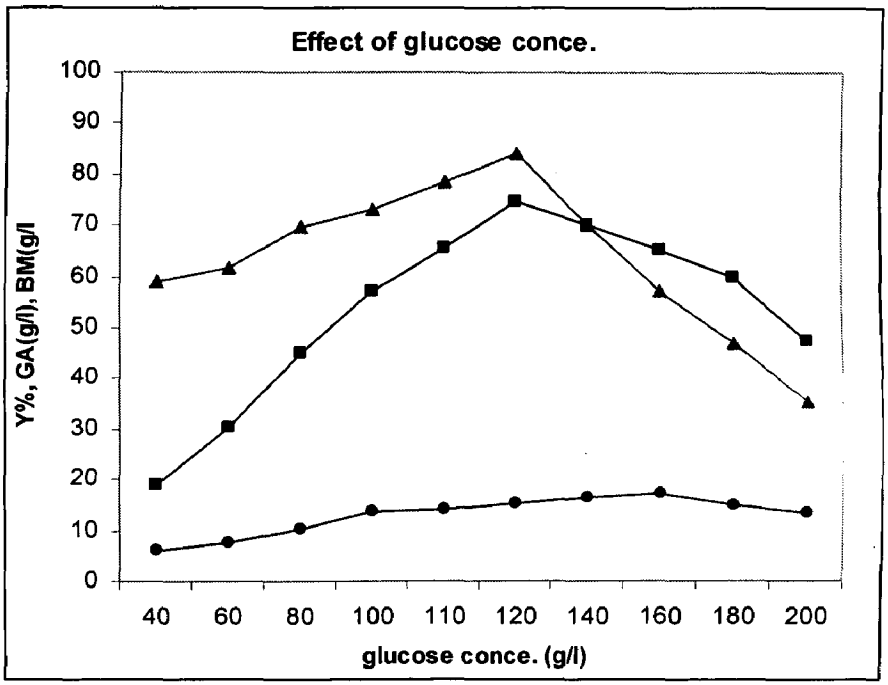
(1). Yield%	[---▲---]	(Y)
(2). Gluconic acid (g/l)	[---■----]	(GA)
(3). Biomass (g/l)	[---●----]	(BM)

5.1.3. Effect of glucose (substrate) concentration:

Since from the literature it has found that glucose is best substrate for gluconic acid production from *Aspergillus niger*, so used as the main carbon source. Concentration of glucose was varied from 4% to 20%, to decide its optimum level required in submerge cultivation for gluconic acid production. Initial glucose concentrations of 10%-14% were found to be suitable for gluconic acid production.

As glucose is the major substrate of the fermentation media, availability of its sufficient amount is always necessary for proper growth of the mycelia as well as for its enzymatic activities, so the lesser amount of substrate had not given much production of gluconic acid while; when greater amounts of glucose concentration were examined, the yield of the experiments were again decreased, "substrate inhibition" can be given as the major cause of these happenings.

One the basis of results obtained for gluconic acid production, it was found that 12% glucose concentration led to maximum yield of 84.10% with 15.2 g/l biomass production, so because of these results, glucose at the concentration of 12% was taken as optimal value, and used for all further experiments.



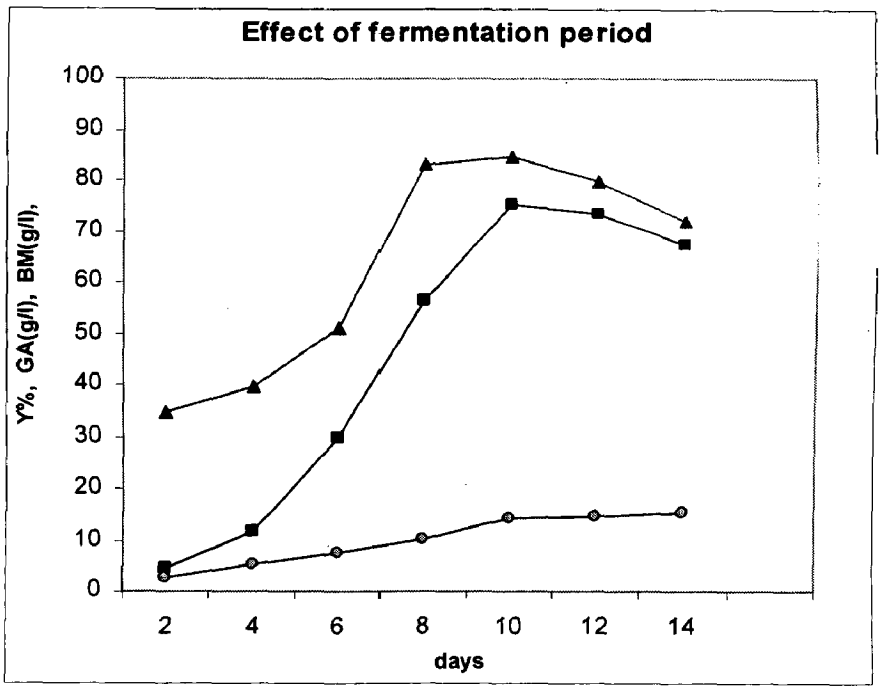
Figer-5.1.3. Varying amount of glucose concentration from 4% to 20% was added with constant pH of 5.5±0.1, $CaCO_3$-4% and 8 days fermentation period. Results are plotted for responses vs. $C_6H_{12}O_6$%, where

(1). Yield%	[---▲---]	(Y)
(2). Gluconic acid (g/l)	[---■----]	(GA)
(3). Biomass (g/l)	[---●----]	(BM)

5.1.4. Effect of fermentation period:

Finally, the effect of cultivation time on *Aspergillus niger* for gluconic acid production under the optimum conditions found above is depicted in figure 5.1.4 and showed an optimum fermentation time of 10 days for maximum total yield and biomass production. The maximum yield was 84.65% with gluconic acid production of 75.2g/l and maximum biomass production was 14.5g/l with 10 days optimal cultivation period.

Higher cultivation periods reduced the yield and mycelia production, may be because of starvation of nutrients or in other word unavailability of further more nutrients or substrate and 'product inhibition'.



Figer-5.1.4. Varying fermentation period from 2 to14 days was given with constant pH of 5.5±0.1, $CaCO_3$%-4% and substrate concentration of 12%. results are plotted for responses vs. fermentation period, where

(1). Yield%	[---▲---]	(Y)
(2). Gluconic acid (g/l)	[---■----]	(GA)
(3). Biomass (g/l)	[---●----]	(BM)

5.2. APPLICATION OF STATISTICAL ANALYSIS ON THE EXPERIMENTAL DATA TO FIT THE REGRESSION MODEL

The most important physical factors affecting the fermentative production of gluconic acid under submerged cultivation, optimized in the present work, are the initial pH (X_1), $CaCO_3$ concentration (X_2), substrate (glucose) concentration (X_3) and fermentation period (X_4). Regression model fitting is done by design expert system Minitab. The experimental data are given in appendix. Thirty-one experiments were

performed using different combinations of the variables as per the one variable at a time technique. (The one variable at a time technique is the most frequently used operation in optimization process. This technique is based on changing one parameter at a time, while keeping the others at fixed levels.)

The results were obtained for getting three parameters as responses (Yi) – yield (Y_1, %), gluconic acid production (Y_2, g/l) and biomass production (Y_3, g/l). As described in general methodology, several equations simulating the conversion of glucose, gluconic acid production and biomass production (Yi) as function cultivation parameters (X_1, X_2, X_3 and X_4) were obtained separately. Starting from the simple liner equation, regressor variables were given up to polynomial term for getting the best possible solution.

Using the results of the experiments and regression analysis we report here the best fitting expression as follows: Eq. (4), (5) and (6) which are second order polynomial equations giving the responses (yield, gluconic acid production and biomass production) as a function of cultivation parameters, initial pH (X_1), $CaCO_3$ concentration (X_2), substrate (glucose) concentration (X_3) and fermentation period (X_4).

Table-5.2(a) Regression Analysis: Yield versus X_1(pH), X_2($CaCO_3$%), X_3 (glucose concentration) and X_4(fermentation period).

The regression equation is

$$\text{Yield } (Y_1) = -190 + 21.7\,X_1(\text{pH}) + 28.5\,X_2(CaCO_3) + 1.18\,X_3(\text{g}) + 16.7\,X_4(\text{days}) - 1.86\,X_1^2 - 3.44\,X_2^2 - 0.00552\,X_3^2 - 0.790\,X_4^2 \quad \ldots(4)$$

Predictor	Coef	SE Coef	T	P
Constant	-189.58	25.74	-7.36	0.000
X_1(pH)	21.748	4.031	5.39	0.000
X_2(caco3	28.541	6.063	4.71	0.000
X_4(day)	16.667	2.330	7.15	0.000
X_3(g)	1.1843	0.1879	6.30	0.000
X_1^2	-1.8610	0.4514	-4.12	0.000
X_2^2	-3.4394	0.8490	-4.05	0.001
X_3^2	-0.0055162	0.0007595	-7.26	0.000
X_4^2	-0.7904	0.1399	-5.65	0.000

S = 6.867 R-Sq = 87.7% R-Sq(adj) = 83.2%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	8	7406.91	925.86	19.63	0.000
Residual Error	22	1037.56	47.16		
Total	30	8444.47			

Table-5.2(b) Regression Analysis: gluconic acid versus X_1(pH), X_2($CaCO_3$%), X_3 (glucose concentration) and X_4(fermentation period).

The regression equation is

$$\text{Gluconic acid } (Y_2) = -301 + 24.9\,X_1(\text{pH}) + 34.8\,X_2(caco_3) + 1.60\,X_3(g) + 23.0\,X_4(\text{day}) - 2.29\,X_1^2 - 3.99\,X_2^2 - 0.00579\,X_3^2 - 1.04\,X_4^2 \quad \text{.....(5)}$$

Predictor	Coef	SE Coef	T	P
Constant	-301.32	34.07	-8.84	0.000
X1(pH)	24.919	5.335	4.67	0.000
X2(caco3	34.806	8.024	4.34	0.000
X3(g)	1.6019	0.2487	6.44	0.000
X4(day)	22.958	3.084	7.45	0.000
X_1^2	-2.2928	0.5973	-3.84	0.001
X_2^2	-3.987	1.124	-3.55	0.002
X_3^2	-0.005786	0.001005	-5.76	0.000
X_4^2	-1.0371	0.1851	-5.60	0.000

S = 9.089 R-Sq = 87.0% R-Sq(adj) = 82.3%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	8	12194.0	1524.2	18.45	0.000
Residual Error	22	1817.3	82.6		
Total	30	14011.3			

Table-5.2(c) Regression Analysis: Biomass versus X_1(pH), X_2($CaCO_3$%), X_3(glucose concentration) and X_4(fermentation period).

The regression equation is

Biomass production (Y_3) = - 37.1 + 2.43 X_1(pH) + 4.03 X_2($CaCO_3$) + 0.204 X_3(g)
+ 3.61 X_4(day) - 0.205 X_1^2 - 0.442 X_2^2 -0.000620 X_3^2 -0.154 X_4^2

.....(6)

Predictor	Coef	SE Coef	T	P
Constant	-37.052	5.730	-6.47	0.000
X1(pH)	2.4272	0.8972	2.71	0.013
X2(caco3	4.031	1.349	2.99	0.007
X3(g)	0.20436	0.04182	4.89	0.000
X4(day)	3.6059	0.5186	6.95	0.000
X_1^2	-0.2048	0.1005	-2.04	0.054
X_2^2	-0.4419	0.1889	-2.34	0.029
X_3^2	-0.0006195	0.0001690	-3.67	0.001
X_4^2	-0.15394	0.03113	-4.95	0.000

S = 1.528 R-Sq = 86.2% R-Sq (adj) = 81.2%

Analysis of Variance

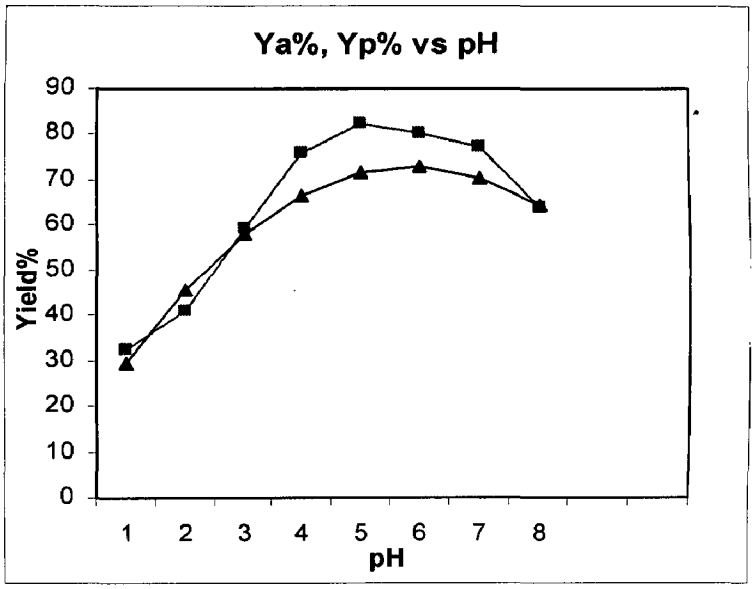
Source	DF	SS	MS	F	P
Regression	8	321.983	40.248	17.23	0.000
Residual Error	22	51.391	2.336		
Total	30	373.374			

The predicted levels of yield, gluconic acid and biomass using the above equation were given along with experimental data in appendix. The coefficients of the regression model [using Eq^n. (4), (5) and (6)] calculated are listed in Table 5.2(a) 5.2(b) and 5.2(c) in which they contain four linear, four quadratic one block term. Graphs are plotted for getting the strategy about the fitness of the model. Figures 5.2[(a) to (l)] show the variation of actual and predicted yield, gluconic acid and biomass production with change in each parameter separately.

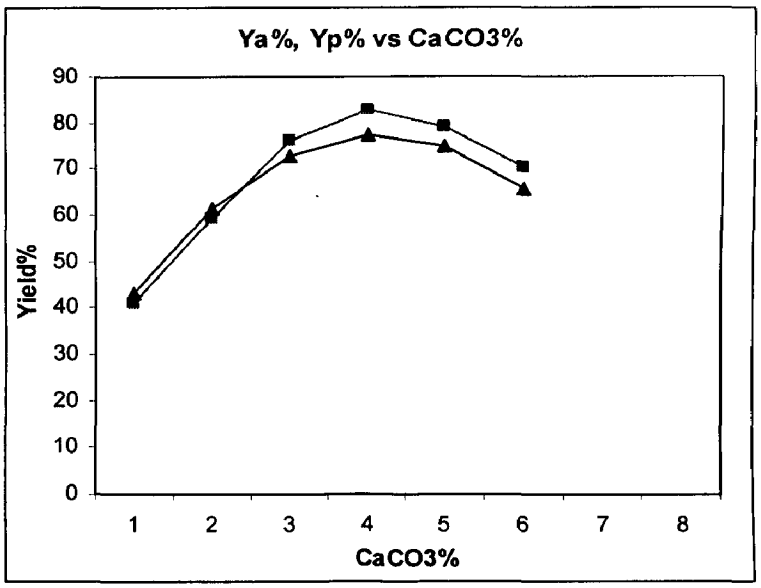
Figure-5.2 Actual and predicted yield obtained by varying fermentation parameters (X_1, X_2, X_3, X_4),

(1).Actual yield [---■---]
(2). Predicted yield [---▲--]

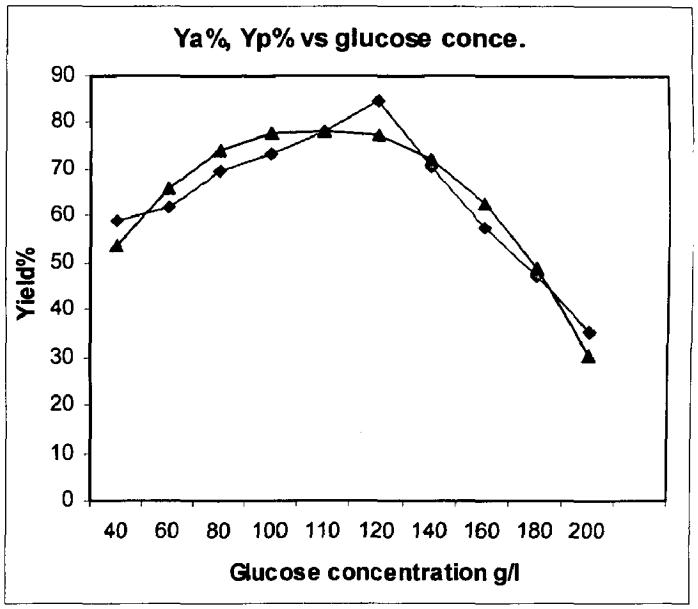
(a). Actual and predicted yield by varying pH



(b). Actual and predicted yield by varying $CaCO_3$ % in the media



(c). Actual and predicted yield by varying glucose concentration (g/l)



(d). Actual and predicted yield by varying fermentation period (days)

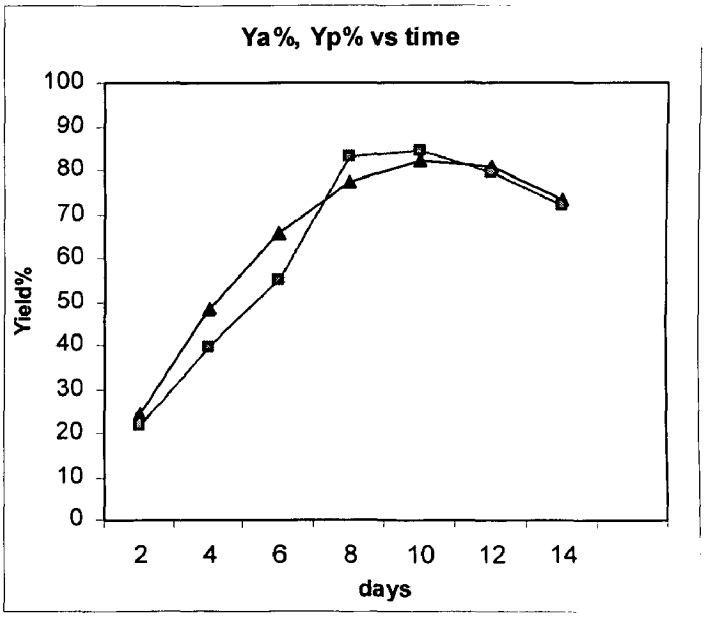
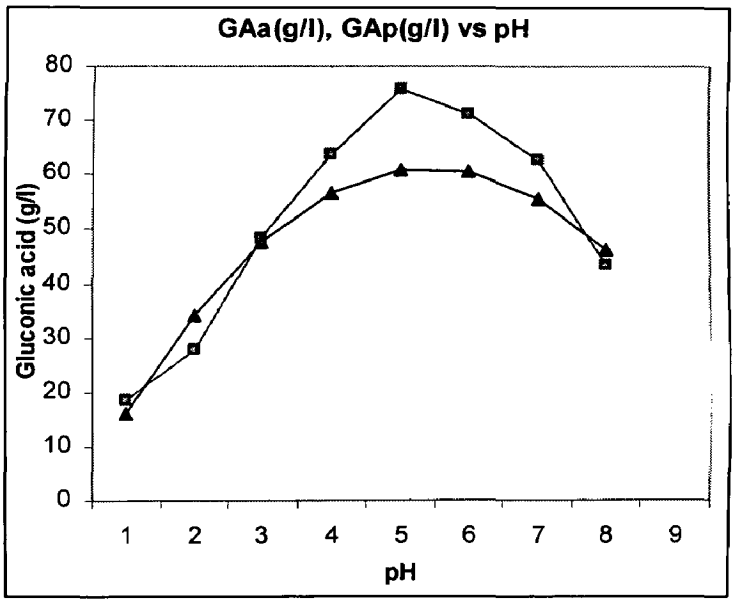


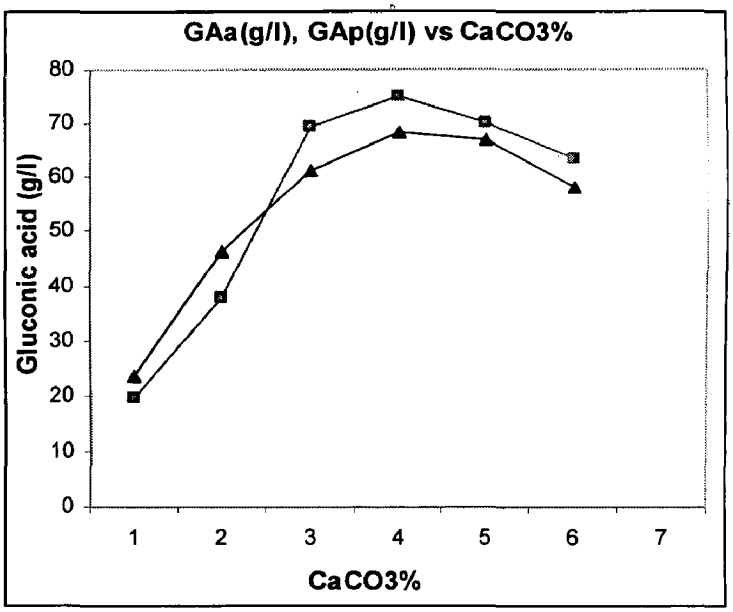
figure 5.2 [(a), (b), (c), (d)] showing the Ya% and Yp% vs. different parameters are quite self-explanatory that actual and predicted yield are following approximately the similar trend with each other for different parameter variation (X_1, X_2, X_3, X_4).

Similarly other trends of actual and predicted (by proposed regression model) gluconic acid (g/l) and biomass production (g/l) are given further which are also capable of describing the good fitness of the proposed model.

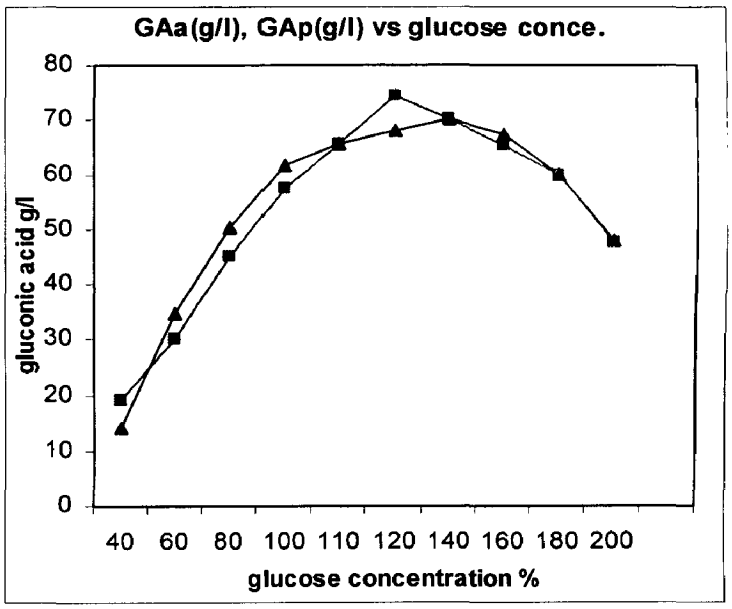
(e). Actual and predicted gluconic acid (g/l) production by varying pH



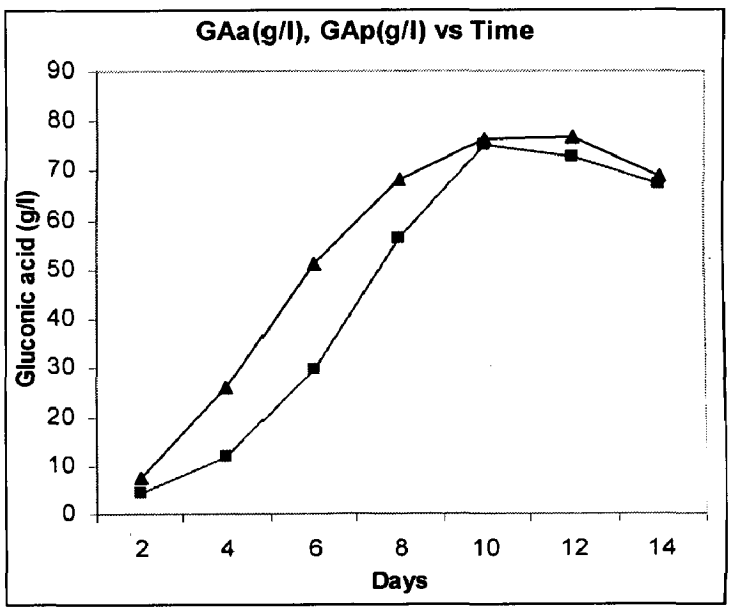
(f). Actual and predicted gluconic acid (g/l) production by varying $CaCO_3$%



(g). Actual and predicted gluconic acid (g/l) production by varying substrate concentration (g/l)

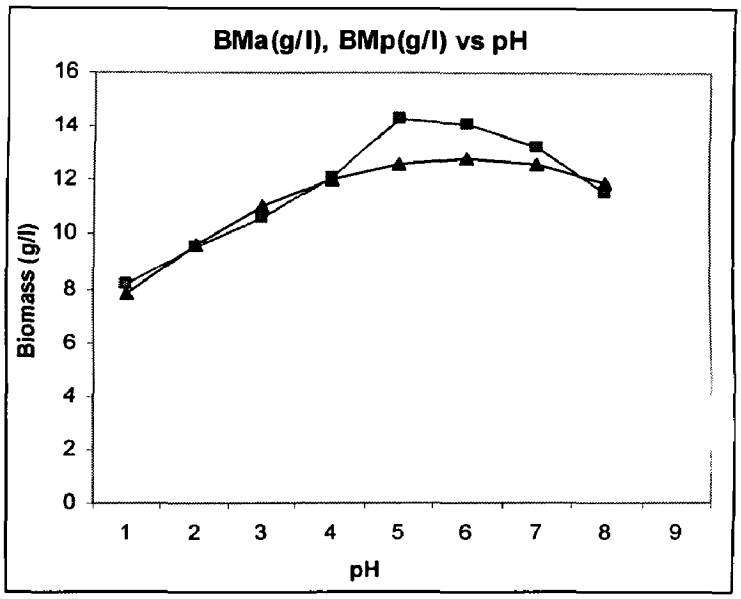


(h). Actual and predicted gluconic acid (g/l) production by varying fermentation period (days)

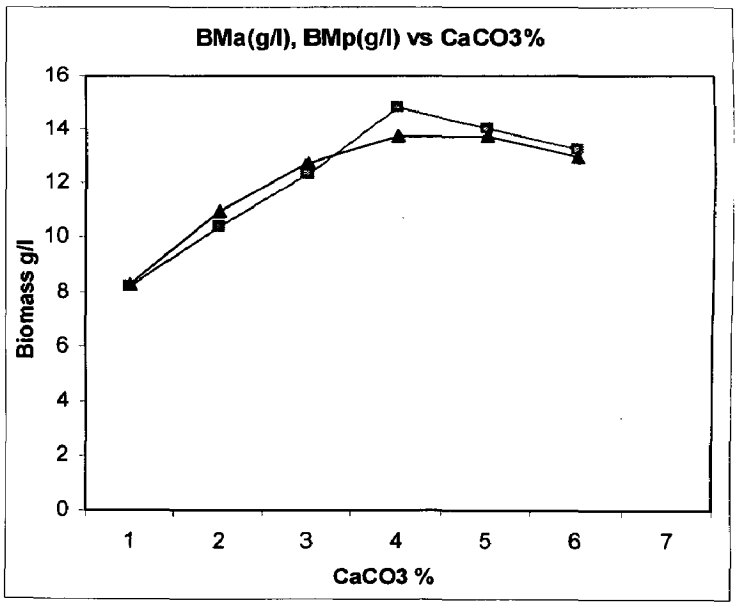


Further graphs from (i) to (l) show the actual and predicted (by the proposed model) biomass production by varying different cultivation parameters one by one.

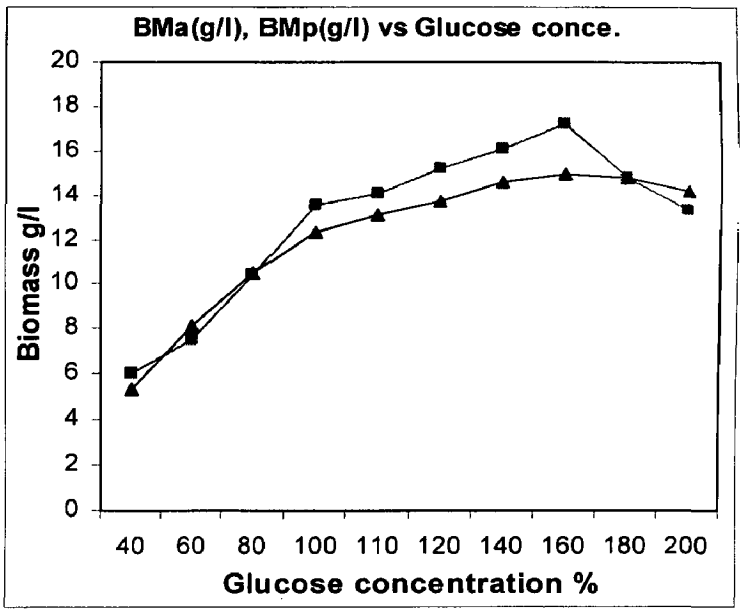
(i). Actual and predicted biomass (g/l) production by varying pH



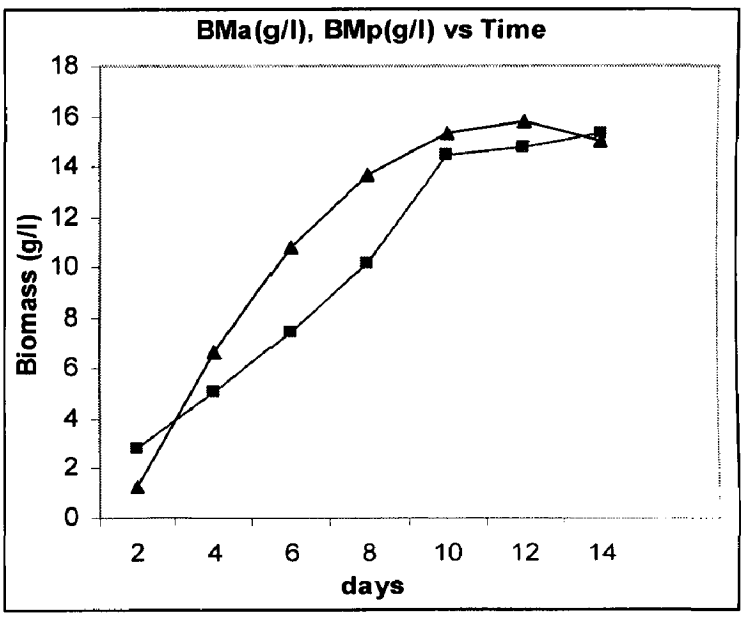
(j). Actual and predicted biomass (g/l) production by varying $CaCO_3$%



(k). Actual and predicted biomass (g/l) production by varying Glucose concentration (g/l)



(l). Actual and predicted biomass (g/l) production by varying fermentation period (days)



Small deviations obtained between the actual and predicted response curves can be analyzed by the fact that there will always be some-what a slight difference between experimental and predicted results.

However the goodness of the model could be checked by some other different criteria too. The P values were used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. The effects of all four parameters, i.e. pH, $CaCO_3$, substrate (glucose) and time of fermentation were found to be significant as ($p \leq 0.05$), but their interaction terms with each other have not been found any significant effect on acid production.

The parameter estimates and the corresponding P values [Table 5.2(a), 5.2(b), 5.2(c)] suggest that the quadratic terms of all parameters have significant negative effect on gluconic acid production while approximately all liner terms showed a significant positive effect.

Another fitness checking criteria of the proposed model is the coefficient of determination (high fitness score) R^2. It implies that closer the value of R^2 (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. Value of R^2 obtained from equations (4), (5) and (6) are-0.877, 0.87, 0.862, indicating a good agreement between the experimental and predicted value. Figures 5.2(m) and 5.2(n) are parity plot showing the distribution of experimental vs. predicted values of gluconic acid yield and biomass production.

Figure-5.2(m). Predicted vs. experimental yield

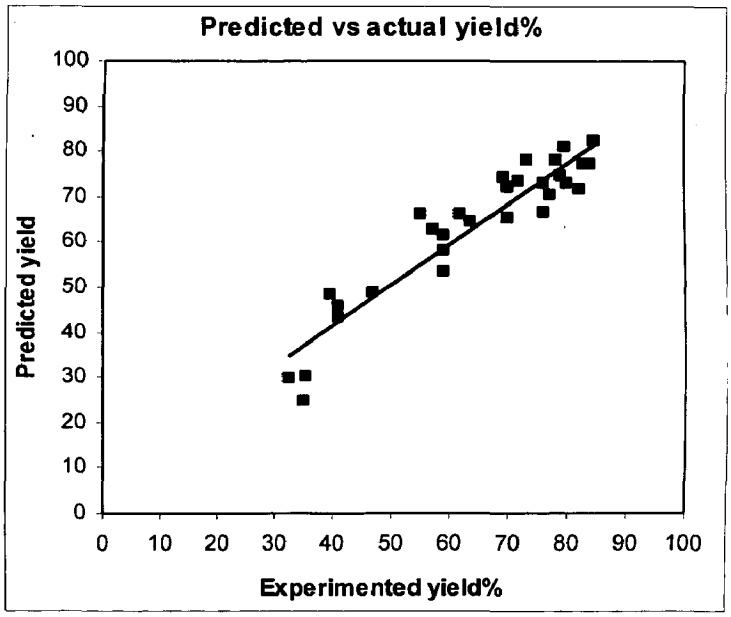
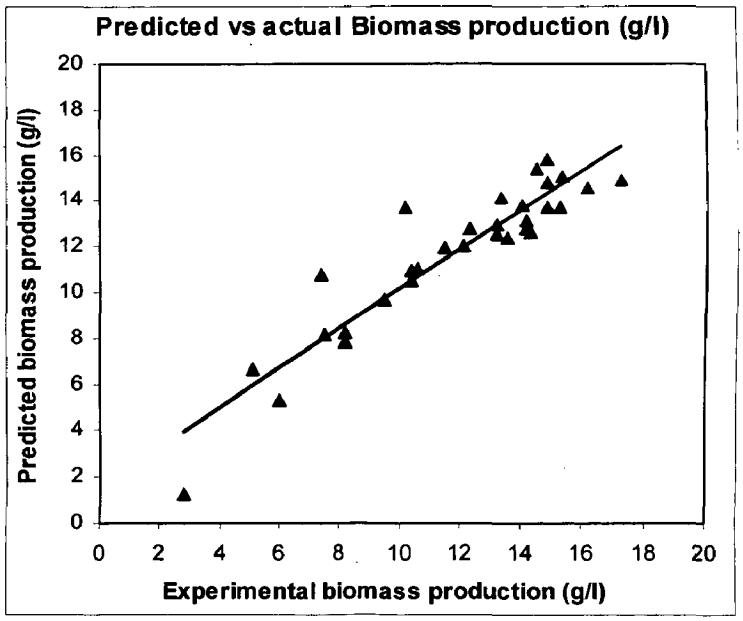


figure-5.2(n). Predicted vs. experimental biomass production.



The above parity plot [figure 5.2(m), 5.2(n)] showed a satisfactory correlation between the values of experimental values and predictive values wherein, the points clustered around the diagonal line are shown which indicates the good fit of the model, since the deviation between the experimental and predictive values was less.

Equation (4), (5) and (6) can be used on-line during fermentation as a bioprocess monitor. It provides the scenario earlier the work giving sufficient time to take remedial measures such as adjustment of media composition, parameter condition selection and residence time etc to ensure complete conversion of substrate and bioconversion efficiency. This is especially important since on-line measurement of concentrations of many substances is either not possible or quite often very cumbersome.

Thus, the present work of fitting the regression model by statistical analysis of real experimented data enables us to find the importance of factors at different levels. A quite well similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of fitted model to optimize the process for glucnic acid production under submerged cultivation.

5.3. Gluconic acid production in Airlift bioreactor

This part of work deals with glucose-gluconic acid fermentation by *Aspergillus niger* in internal loop airlift reactors (ILALR). The fermentations were carried out in three ILALR (4.5 l). Growth fermentations were performed for 10 days of fermentation period, experimental set-up, media composition and other operating parameter conditions (which were optimized earlier during lab work) have already been specified.

The concentration profiles of glucose, gluconic acid and biomass during fermentation are depicted in Fig. 5.3.1. In this figure, a fairly long lag phase was observed. From about the 5 days of fermentation, a linear production phase was observed until the carbon substrate totally exhausted. The maximal rate of Gluconic acid production was attained in the linear stage after 10 days. The average yield of the bioprocess was 67.5%.

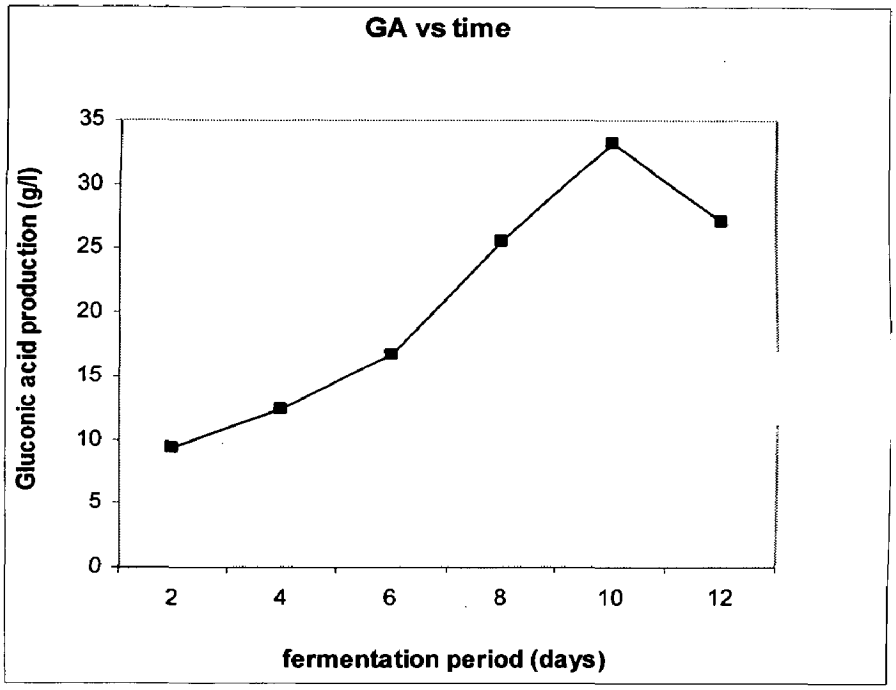


Fig. 5.3.1. Time dependence of Glconic acid production under submerged fermentation condition in airlift bioreactor.

Although the experiments for gluconic acid production in airlift bioreactor were done with efforts of our level best but the obtained production of gluconic acid was not as much high as it was during batch process (lab work for process parameter optimization). Here are some reasons which can be given thoughts or recommended for the gaining of lesser yield in airlift bioreactor in comparison with the yield obtained in laboratory work during parameter optimization: Human error, instrumental errors during taking results in experiments, further more requirement of understanding of reaction kinetics in the airlift bioreactor or exploration of optimal levels of other fermentation conditions which may affect the process significantly etc.

6. CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION:

D-Gluconic acid, an oxidation product of D-glucose, finds wide application in food, pharmaceutical textile, detergent, leather, photographic and other biological industries. Several processes for the production of Gluconic acid by chemical means have been elaborated comprising simple chemical oxidation, electrochemical and bio-electrochemical. The disadvantages of these processes are lower yields of product due to inevitable side reactions and poor product quality; thus, the specificity of the biochemical reaction has made the fermentation as the preferable method where the biotransformation represents a simple dehydrogenation reaction without involvement of complex metabolic cell pathways.

The present work shows the production of Gluconic acid under submerged fermentation condition. The microbial species selected for the work is *Aspergillus niger*. From the results shown in chapter-5, following salient conclusions can be drawn:

i. In the present work the main submerged fermentation parameters optimized, for production of gluconic acid by *Aspergillus niger*, are: Initial pH, $CaCO_3$ composition in media, glucose concentration and fermentation period. Their optimal values, based on the high yield and better biomass production, are:

Parameter	Optimal value
Initial pH	5.5
$CaCO_3$%	4%
Glucose concentration(g/l)	120 (g/l)
Fermentation period	10 days

ii. The highest yield obtained with optimized parameter conditions, during laboratory work, was approximately 85%.

iii. Statistical analysis of obtained results was done and regression models, for yield, gluconic acid production and biomass production, were proposed. Actual and predicted responses were compared with each other showing the aptness of the proposed model.

iv. Further with these optimized parameter conditions production of gluconic acid under submerged fermentation condition was performed in airlift bioreactor with the approx. yield of 67.5%.

6.2. RECOMMENDATIONS FOR FUTURE WORK

Following future work has been recommended based on the efforts carried out at hand:

i. The fermentation parameters optimized here are initial pH, $CaCO_3$%, $C_6H_{12}O_6$ concentration and fermentation period. Some other parameters of the fermentation parameters like nitrogen source DO can be optimized for complete specificity of the process and for finding their significant effect in the phenomena.

ii. Parameters optimization work can be done with more improved strain of the micro-organism.

iii. Optimization of all the affecting parameters collectively can also be done by CCD using response surface methodology, or accuracy of the proposed model can further be improved by investigating other process parameters which may have significant effects in the process.

iv. As responses for gluconic acid production in the present experimental set-up of airlift are not so high, factors affecting the process (may be other process variables, reaction kinetics etc.) can be modified for the further action.

REFERENCES

1. Ambati P., Ayyanna C., Optimizing medium constituents and fermentation conditions for citric acid production from palmyra jaggery using response surface method, World J. Microb. Biotechnol. 17 (2001) 331-335.
2. Bandaru V. V. R., Somalanka S. R., Menduc D. R., Madicherla N. R., Chityala A., Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology, Enzyme Microb. Technol. 38 (2006) 209–214.
3. Blazej M., Kisa M., Markos M., Scale influence on the hydrodynamics of an internal loop airlift reactor, Chem. Eng. Proc. 43 (2004) 1519–1527.
4. Blom R. H., Pfeifer V. F., Moyer A.J., Traufler D. H., Convey H. F., Crocer C. K., Farison R. E., Hannibal D. V., Sodium gluconate production: Fermentation with *Aspergillus niger*, Ind. Eng. Chem., 44 (1952) 435-440.
5. Bao J., Koumatsua K., Furumotob K., Yoshimotoa M., Fukunagaa K., Nakaoa K., Optimal operation of an integrated bioreaction–crystallization process for continuous production of calcium gluconate using external loop airlift columns, Chem. Eng. Sci. 56 (2001) 6165–6170.
6. Burris V. L., McGinnis D. F., Little J. C., Predicting oxygen transfer and water flow rate in airlift aerators, Water Research 36 (2002) 4605–4615.
7. Favela-Torres E., Cordova-lopez J., Garcia-Rivero M., GutierrezRojas M., Kinetic of growth of *Aspergillus niger* during submerged, agar surface and solid state fermentations, Process biochem. 33 (1998) 103-107.
8. Ghosh P., Ghosh T. K., Oxygen transfer in gluconic acid fermentation, J. Fer. Technol. 56 (1978) 139-143.
9. Hatziuikolaou D. G., Macris B. J., Factors regulating production of glucose oxidase by *Aspergillus niger*, Enzyme Microb. Technol. 17 (1995) 530-534.
10. Hustede H., Benckiser J. A., Elvers B., Hawkins S., Ravenscroft M., Rounsaville J. F., Schulz G. W., Gluconic acid. "Ullmann's Encyclopedia of Industrial Chemistry", 5th Edn. (1989) VCH. A12: 449-456.

11. Jie B., Koumatsua K., Furumoto K., Yoshimoto M., Fukunaga K., Nakao K., Deactivation kinetics of immobilized glucose oxidase for production of calcium gluconate in an external loop airlift bioreactor, Biochem. Eng. J. 22 (2004) 33–41.
12. Kapat A., Jung J. K, Park Y. H., Hong S. Y., Choi H. K., Effects of agitation and aeration on the production of extracellular glucose oxidase from a recombinant Saccharomyces cerevisiae, Bioprocess Eng. 18 (1998) 347-351
13. Kawase, Y., Kanai, T., Uzumaki, Y. Simulation of airlift bioreactors: steady-state performance of continuous culture processes, Comp. Chem. Eng. 20 (1996) 1089–109.
14. Kelly S., Grimm L. H., Hengstler J., Schultheis E., Krull R., Hempel D. C., Agitation effects on submerged growth and product formation of *Aspergillus niger*, Bioprocess Biosyst. Eng. 26 (2004) 315–323.
15. Klein J., Rosenberg M., Markoš J., Dolgoš O., Krošlák M., Krištof'ıková L., Biotransformation of glucose to gluconic acid by *Aspergillus niger*—study of mass transfer in an airlift bioreactor, Biochem. Eng. J. 10 (2002) 197–205.
16. Laane C., Pronk W., Franseen M. and Veeger C., Use of bioelectrochemical cell for the synthesis of (bio) chemicals. Enzyme microb. Technol., 6 (1984) 165-168.
17. Liu J. Z., Huang Y. Y., Liu J., Weng L. P., Ji L. N., Effects of metal ions on simultaneous production of glucose oxidase and catalase by *Aspergillus niger*, Letters Appl. Microb. 32 (2001) 16-19.
18. Liu J. Z., Weng L. P, Zhang O. L.,, Xu H., Ji L. N., A mathematical model for gluconic acid fermentation by *Aspergillus niger*, Biochem. Eng. J. 3669 (2002) 1–5.
19. Liu J. Z., Weng L. P., Zhang O. L., Xu H., Ji L. N., A mathematical model for gluconic acid fermentation by *Aspergillus niger*, Biochem. Eng. J. 14 (2003) 137–141.
20. Liu J. Z., Weng L. P., Zhang Q. L., Xu H., Ji L. N., Optimization of glucose oxidase production by *Aspergillus niger* in a benchtop bioreactor

using response surface methodology, World J. Microb. Biotechnol. 19 (2003) 317–323.

21. Lu T., Peng X., Yang H., Ji L., The production of glucose oxidase using the waste myceliums of *Aspergillus niger* and the effects of metal ions on the activity of glucose oxidase, Enzyme Microb. Technol. 19 (1996) 339-342.
22. Mandal S. K., Banerjee P. C., Submerged production of oxalic acid from glucose by immobilized *Aspergillus niger*, Process Biochemistry 40 (2005) 1605–1610.
23. Mariam I., Nagra S. A., Production of calcium gluconate by *Aspergillus niger* in Shake flasks, Pakistan J. biolog. Sci. 5 (2002) 321-324.
24. Mirón. J., González M.P., Pastrana L., Murado M.A., Diauxic production of glucose oxidase by *Aspergillus niger* in submerged culture A dynamic model, Enzyme Microb. Technol. 31 (2002) 615–620.
25. Moksia J., Christian L., Gros J. B., Gluconate production by spores of aspergillus niger, Biotechnol. Letters 18 (1996) 1025-1030.
26. Moresi M., Parente E., Mazzatura A., Effect of dissolved oxygen concentration on repeated production of gluconic acid by imrnobilised mycelia of *Asperyillus niger*, Appl. Microbiol. Biotechnol. 36 (1991) 320-323.
27. Mukhopadhyay R., Chatterjee S., Chatterjee B. P., Banerjee P. C, Guha A. K., Production of gluconic acid from whey by free and immobilized *Aspergillus niger*, Inter. Dairy J. 15 (2005) 299–303.
28. Nakao K., Kiefner A., Furumoto K., Harada T., Production of gluconic acid with immobilized glucose oxidase in airlift reactors, Chem. Eng. Sci. 52(1997) 4127 4133.
29. Papagianni M., Fungal morphology and metabolite production in submerged mycelial processes, Biotechnol. Advances 22 (2004) 189–259.
30. Park K., Pintauro P.N., Baizer M.M. and Nobe K.J., Flow reactor stuidies of the paired electrooxidation and electroreduction of glucose, J. Appl. Electrochem. 16 (1986) 1850-1855.

31. Paula G. C., Priedeb M. A., Thomasa C. R., Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations, Biochem. Eng. J. 3 (1999) 121-129.
32. Roukas T., Citric and gluconic acid production from fig from *Aspergillus niger* using solid state fermentation, J. Industrial Microb. Biotechnol. 25 (2000) 298-304.
33. Sankpal. N. V., Joshi A. P., Sutar I. I., Kulkarni B. D., Continuous production of Gluconic acid by Aspergillus niger immobilized on a cellulosic support: study of low pH fermentative behaviour of *Aspergillus niger*, Process Biochemistry 35 (1999) 317–325.
34. Sankpal N. V., Cheema J. J. S., Tambe S. S., Kulkarni B. D., An artificial intelligence tool for bioprocess monitoring: application to continuous production of gluconic acid by immobilized *Aspergillus niger*, Biotechnology Letters 23 (2001) 911–916.
35. Sankpal N. V., Kulkarni B. D. Optimization of fermentation conditions for gluconic acid production using *Aspergillus niger* immobilized on cellulose microfibrils, Process Biochemistry 37 (2002) 1343-1350.
36. Singh O. V., Singh R .P., Gluconic acid production by *Aspergillus niger* ORS-4.410 in submerged and solid state surface fermentation, Ind. J. Experi. Boil. 39(2001) 691-696.
37. Singh O. V., Production of gluconic acid from alternative economic sources, Ph.D. Thesis, Indian Institute of Technology, Roorkee, 2002.
38. Singh O. V., Singh R. P., Utilization of agro-food by–products of gluconic acid production by *Aspergillus niger* ORS-4 under surface culture cultivation, J. Scientific and Industrial Research 61 (2002) 356-360.
39. Singh O. V., Jain R. K., Singh R.P., Gluconic acid production under varying fermentation conditions by *Aspergillus niger*, Chem. Technol. Biotechnol. 78 (2003) 208-212.
40. Singh O.V., Singh R. P., Kapur N., Evaluation of agro-food byproducts for gluconic acid production by *Aspergillus niger* ORS-4.410, World J. Microbiol. Biotechnol. 21(2005) 519-524.

41. Thibault J., Halsall-Whitney H., Tayler D., Multicriteria optimization of gluconic acid production using net flow, Bioprocess Biosyst. Eng. 25 (2003) 299-307.
42. Velizarov S., Beschkov V., Production of free gluconic acid by cells of *gluconobactor oxydans*, Biotechnology Letters 16 (1994) 715-720.
43. Zhang J., Greasham R., Chemically defined media for commercial fermentations, Appl. Microbiol. Biotechnol. 51 (1999) 407-421.
44. Znad H., Bales V., Markos J., Kawase Y., Modeling and simulation of airlift bioreactors, Biochem. Eng. J. 21 (2004) 73–81.
45. Znad H., Markoš J., Baleš V, Production of gluconic acid from glucose by *Aspergillus niger* growth and non-growth conditions, Process Biochemistry 39 (2004) 1341–1345.
46. Znidar P., Aleksander P., The Morphology of Filamentous Fungi in Submerged Cultivations as a Bioprocess Parameter, Food Technol. Biotechnol. 39 (2001) 237–252.

APPENDIX:-Data table for experimental and predicted results

Sr. No.	Parameter variation	Experimented Yield %	Experimented Gluconic acid (g/l)	Experimented Biomass Production (g/l)	Predicted Yield% (Y_1)	Predicted Gluconic acid (g/l, Y_2)	Predicted Biomass Production (g/l, Y_3)
A	**pH variation**						
1	1	32.5	18.4	8.2	29.5	16.2	7.8
2	2	41.0	27.8	9.5	45.6	34.2	9.6
3	3	59.1	48.5	10.6	58.0	47.6	11.0
4	4	76.1	63.7	12.1	66.7	56.5	12.1
5	5	82.3	75.6	14.3	71.7	60.8	12.6
6	6	80.0	71.0	14.1	72.9	60.5	12.8
7	7	77.2	62.5	13.2	70.5	55.7	12.6
8	8	63.8	43.5	11.5	64.3	46.2	11.9
B	**$CaCO_3$% variation**						
9	1	41.2	19.6	8.2	43.3	23.6	8.2
10	2	59.0	37.8	10.4	61.5	46.4	10.9
11	3	76.1	69.2	12.3	72.8	61.2	12.7
12	4	83.0	75.0	14.8	77.2	68.1	13.7
13	5	79.3	70.1	14.0	74.7	67.0	13.7
14	6	70.0	63.4	13.2	65.4	58.0	12.9
C	**Substrate composition%**						
15	4	59.0	19.0	6.0	53.5	14.2	5.3
16	6	61.9	30.2	7.5	66.0	34.6	8.1
17	8	69.5	45.0	10.4	74.2	50.4	10.5
18	10	73.2	57.3	13.5	77.9	61.6	12.4

19	11	78.2	65.4	14.1	78.1	65.4	13.0
20	12	84.1	74.5	15.2	77.2	68.1	13.7
21	14	70.2	70.2	16.1	72.1	70.0	14.6
22	16	57.1	65.3	17.2	62.6	67.3	15.0
23	18	46.9	59.8	14.8	48.7	59.9	14.8
24	20	35.2	47.4	13.3	30.3	47.9	14.1
D	**Fermentation time (days)**						
25	2	22.0	4.5	2.8	24.4	7.5	1.2
26	4	39.5	11.8	5.1	48.3	26.0	6.6
27	6	50.9	29.8	7.4	66.0	51.2	10.7
28	8	83.0	56.4	10.2	77.2	68.1	13.7
29	10	84.7	75.1	14.5	82.2	76.7	15.3
30	12	79.7	73.1	14.8	80.8	76.9	15.8
31	14	71.8	67.3	15.3	73.1	68.8	15.0

19	11	78.2	65.4	14.1	78.1	65.4	13.0
20	12	84.1	74.5	15.2	77.2	68.1	13.7
21	14	70.2	70.2	16.1	72.1	70.0	14.6
22	16	57.1	65.3	17.2	62.6	67.3	15.0
23	18	46.9	59.8	14.8	48.7	59.9	14.8
24	20	35.2	47.4	13.3	30.3	47.9	14.1
D	**Fermentation time (days)**						
25	2	22.0	4.5	2.8	24.4	7.5	1.2
26	4	39.5	11.8	5.1	48.3	26.0	6.6
27	6	50.9	29.8	7.4	66.0	51.2	10.7
28	8	83.0	56.4	10.2	77.2	68.1	13.7
29	10	84.7	75.1	14.5	82.2	76.7	15.3
30	12	79.7	73.1	14.8	80.8	76.9	15.8
31	14	71.8	67.3	15.3	73.1	68.8	15.0

19	11	78.2	65.4	14.1	78.1	65.4	13.0
20	12	84.1	74.5	15.2	77.2	68.1	13.7
21	14	70.2	70.2	16.1	72.1	70.0	14.6
22	16	57.1	65.3	17.2	62.6	67.3	15.0
23	18	46.9	59.8	14.8	48.7	59.9	14.8
24	20	35.2	47.4	13.3	30.3	47.9	14.1
D	**Fermentation time (days)**						
25	2	22.0	4.5	2.8	24.4	7.5	1.2
26	4	39.5	11.8	5.1	48.3	26.0	6.6
27	6	50.9	29.8	7.4	66.0	51.2	10.7
28	8	83.0	56.4	10.2	77.2	68.1	13.7
29	10	84.7	75.1	14.5	82.2	76.7	15.3
30	12	79.7	73.1	14.8	80.8	76.9	15.8
31	14	71.8	67.3	15.3	73.1	68.8	15.0

19	11	78.2	65.4	14.1	78.1	65.4	13.0
20	12	84.1	74.5	15.2	77.2	68.1	13.7
21	14	70.2	70.2	16.1	72.1	70.0	14.6
22	16	57.1	65.3	17.2	62.6	67.3	15.0
23	18	46.9	59.8	14.8	48.7	59.9	14.8
24	20	35.2	47.4	13.3	30.3	47.9	14.1
D	**Fermentation time (days)**						
25	2	22.0	4.5	2.8	24.4	7.5	1.2
26	4	39.5	11.8	5.1	48.3	26.0	6.6
27	6	50.9	29.8	7.4	66.0	51.2	10.7
28	8	83.0	56.4	10.2	77.2	68.1	13.7
29	10	84.7	75.1	14.5	82.2	76.7	15.3
30	12	79.7	73.1	14.8	80.8	76.9	15.8
31	14	71.8	67.3	15.3	73.1	68.8	15.0

19	11	78.2	65.4	14.1	78.1	65.4	13.0
20	12	84.1	74.5	15.2	77.2	68.1	13.7
21	14	70.2	70.2	16.1	72.1	70.0	14.6
22	16	57.1	65.3	17.2	62.6	67.3	15.0
23	18	46.9	59.8	14.8	48.7	59.9	14.8
24	20	35.2	47.4	13.3	30.3	47.9	14.1
D	**Fermentation time (days)**						
25	2	22.0	4.5	2.8	24.4	7.5	1.2
26	4	39.5	11.8	5.1	48.3	26.0	6.6
27	6	50.9	29.8	7.4	66.0	51.2	10.7
28	8	83.0	56.4	10.2	77.2	68.1	13.7
29	10	84.7	75.1	14.5	82.2	76.7	15.3
30	12	79.7	73.1	14.8	80.8	76.9	15.8
31	14	71.8	67.3	15.3	73.1	68.8	15.0