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N° D.U. 1837 — ANNEE 2008

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Présentée à l'Université Blaise Pascal
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DOCTEUR D'UNIVERSITE

GÉNIE DES PROCÉDÉS

soutenue le 1er juillet 2008

SUMITRA RAMACHANDRAN

Use of spores of *Aspergillus niger* obtained by Solid-state fermentation for the production of gluconic acid.

Président : **M. DUSSAP Claude-Gilles, Professeur, Univ. Blaise Pascal**

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M. ROUSSOS Sevastianos, Directeur de Recherches IRD, Marseille

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The origin of Life, the God on earth…**my Amma**… I dedicate this work at your feet!!!!

List of abbreviations

α	alpha
β	beta
°C	degree celsius
Å	angstrom
AAGR	average annual growth rate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
a_w	water activity
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
C/N	carbon/nitrogen
CPS	copper sulphate pentahydrate
D	dalton
DNS	dinitrosalicylic acid
DO	dissolved oxygen
Ds	dryweight of spore
EDTA	Ethylene diamine tetra acetic acid
EMP	Embden Meyerhof pathway
FAD	flavin adenine dinucleotide
$FADH_2$	flavin-adenine. dinucleotide (reduced form)
g	relative centrifugal force
G_0	no. of grains corresponding to dry weight at time zero
GADH	gluconic acid dehydrogenase
GDH	glucose dehydrogenase
Gox	glucose oxidase
GRAS	generally recognized as safe
Gt	no.of grains in the sample at time t
IDM	initial dry matter
IDW	initial dry weight
IU	international unit
kb	kilobar

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kDa	kilo dalton
KDG	2-keto 3-deoxygluconate
KDPG	2-keto 3-deoxyphosphogluconate
KGDH	2-ketogluconate dehydrogenase
K_m	Michaelis constant
LD	lethal dose
M	molarity
MIC	minimum inhibitory count
mM	millimolar
MPa	megapascal
MW	molecular weight
N	normality
NADP	nicotinamide adenine dinuleotide phosphate
NADPH	reduced nicotinamide adenine dinuleotide phosphate
ND	not determined
nm	nanometer
NRRL	Northern Regional Research Laboratory
NTA	nitrilotriacetic acid
PCA	phenazine -1- carboxylic acid
PCN	phenazine-1-carboxamide
PDA	potato dextrose agar
PGDH	phosphogluconate dehydrogenase
pI	isoelectric point
ppm	parts per million
PQQ	pyrroloquinoline quinine
psi	pounds per square inch
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SEM	scanning electron micrograph
slpm	standard litre per minute
SmF	submerged fermentation
SPAH	spores permeabilized after heat treatment
SPBH	spores permeabilized after heat treatment
SSF	solid-state fermentation

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TCA	tricarboxylic acid
TMS	trimethylsilyl
US FDA	Food and Drug Administration
v/v	volume per unit volume
vvm	volume of air per unit volume of medium per minute
Y p/s	yield (w/w) of product from substrate

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INTRODUCTION

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Bioconversion can be defined as the application of a biocatalyst to achieve a desired conversion under controlled conditions in a bioreactor. A biocatalyst can either be an enzyme or an enzyme complex, a cell organelle or a whole cell. The former can be free or immobilized and the latter can be viable (growing or non-growing) or non-viable. Biocatalysis has its roots in ancient China and Japan in the manufacture of food and alcoholic drinks. Europe also has a long history of applied biocatalysis. Cheese making has always involved the use of enzymes. The first company (1874) based upon applied biocatalysis was Christian Hansen's Laboratory, Denmark for the production of rennet.

General prerequisities for production of biocatalysts are as follows.

The biocatalysts must be available as pure culture

The biocatalyst must be genetically stable

Storage of biocatalyst without loss of quality should be possible

Growth on inexpensive media is preferable and growth should be rapid

Production of the desired metabolite or enzyme has to take place

High productivities are desirable

Production of a biocatalyst must be possible on a large scale

The biocatalysts should not be pathogenic

In this study fungal spores were exploited as biocatalyst in the bioconversion. To be precise, the work is dedicated on the production of gluconic acid using spores of *Aspergillus niger*. Spores acted as reservoir of glucose oxidase and thus were able to catalyse the bioconversion of glucose to gluconic acid.

Gluconic acid is derived from glucose through a simple dehydrogenation reaction. It is a multifunctional organic acid used as bulk chemical in the food, feed, pharmaceutical, textile, metallurgy, detergent and construction industries. Glucose oxidase (EC 1.1.3.4., β-D-glucose: oxygen-oxidoreductase) catalyses the oxidation of β-D-glucose to glucono-δ-lactone ($C_6H_{10}O_6$) and hydrogen peroxide using molecular oxygen as the electron acceptor. The worldwide production of gluconic acid is nearly about 87,000 tonnes/year and costs about 1.20 – 8.50/kg of gluconic acid (Business Communication Co., Inc.2004). There are different approaches such as chemical, biochemical and electrochemical available for its production, but microbial fermentation processes using filamentous fungus *A. niger* are the most widely used (Rohr et al 1996). These systems, although very efficient suffer from general drawbacks of mycelial cultivations, i.e. high viscosity of the medium and problems associated with mixing and aeration. Continuous operation of vegetative cells or immobilized enzymes as biocatalyst in the bioreactor systems cause their inactivation due to its exposure to various chemicals, physicochemical factors, and the release of degrading enzymes (Murata 1993). To overcome these

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limitations, Larroche & Gros 1997 proposed the use of fungal spores as an alternative for gluconic acid production, which could avoid mycelial proliferation.

Although spores were considered as dormant and metabolically inert, several studies suggested that the spores contained enzymes as in the vegetative cells at a comparable level of activity (Shigematsu et al 1992). Thus, the interest of exploiting spores as enzyme bags and promising biocatalysts developed. Replacement of vegetative cells by spores certainly possesses several advantages. In fermentation or biotransformation processes, use of spore instead of mycelium results in less viscous medium, further leading to easier aeration and product recovery. Moreover, spores are generally easier to store, and the lack of pellet formation results in greater homogeneity of the biocatalyst (Larroche & Gros 1997). In some cases, biotransformation reactions are carried out exclusively by spores; in others, vegetative cells perform the biotransformation less efficiently and for a considerably shorter period of time. Other significant advantages include easier recovery of the products, elimination of the lag period, reuse of spores as catalyst for many cycles, and elimination of the production of other undesired products.

This study has two aspects.

1. Production of biocatalyst (spores of *Aspergillus niger*)
2. Utilization of the biocatalyst in the bioconversion

Firstly, solid-state fermentation was carried out to produce spores. Later demonstration of spores as reservoir of glucose oxidase and the feasibility of the direct use of *A. niger* spores as biocatalyst in the conversion of glucose to gluconic acid was studied.

The first chapter is a review of the information and resources available from the literature on the various aspects of the study. Methods and techniques followed for the experimental part of the study is detailed in chapter two. The third chapter throws light on the production aspects of spore by solid-state fermentation. The fourth chapter describes various treatment procedures of spores and optimization studies for the use of spores as a catalyst. Advantages of using spores as biocatalyst are also briefed in the same. Finally highlights of the study are summarised.

Chapter 1

Literature survey

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1.1. Spores

1.1.1. Introduction

Spores are generally defined as small reproductive bodies that get detached from the microbial species (bacteria and fungi) to produce a new offspring. In the process they do not fuse with other reproductive bodies, but get germinated sooner or later. They widely vary in origin, size, shape, pigmentation, biological functions, etc. Microbial spores have a diameter ranging between 1-50 µm. However, the largest spores are the fungal ascospores, for eg. *Varicellaria microsticta*, measuring 340 x 115µm; the smallest spores are bacterial endospores measuring about 0.25 µm in diameter. Bacterial endospores remain dormant in the same place of origin and help the species to pass through an unfavourable period; such spores are termed as resting spores. Also, there are dispersal spores, those of Ascomycetes, whose explosive asci burst due to hydrostatic pressure and get scattered even upto a distance of 40 cm (Wolken et al 2003).

All bacteria do not produce spore. A few species of bacteria such as *Bacillus* and *Clostridium* have the ability to produce highly resistant structures known as endospores which resist a range of hazardous environments.

Fungal spores play an important role in the lifecycle of fungi (Fig. 1.1). They produce spores as agents of dispersion, reproduction and survival. Dispersal spores are produced in large numbers. They facilitate strain migration and get germinated readily. Survival spores are formed under unfavourable conditions and are comparatively low in number. Fungi produce **sexual and/or asexual** spores. The main classes of fungi producing **sexual** spores are Zygomycetes (*Rhizopus*), Ascomycetes (*Neurospora, Talaromyces*), Basidiomycetes (*Puccinia, Agaricus*) and their spores are called zygospores, ascospores, basidiospores respectively (Fig. 1.2). There is another class of fungi, Deuteromycetes (*Penicillium, Aspergillus*) which produce only **asexual spores called conidia**. There are many fungi which are able to produce more than one kind of spores, e.g., *Neurospora crassa* produces two kinds of spores such as microconidia and macroconidia. Fig 1.3 shows the details of some fungal and bacterial spores.

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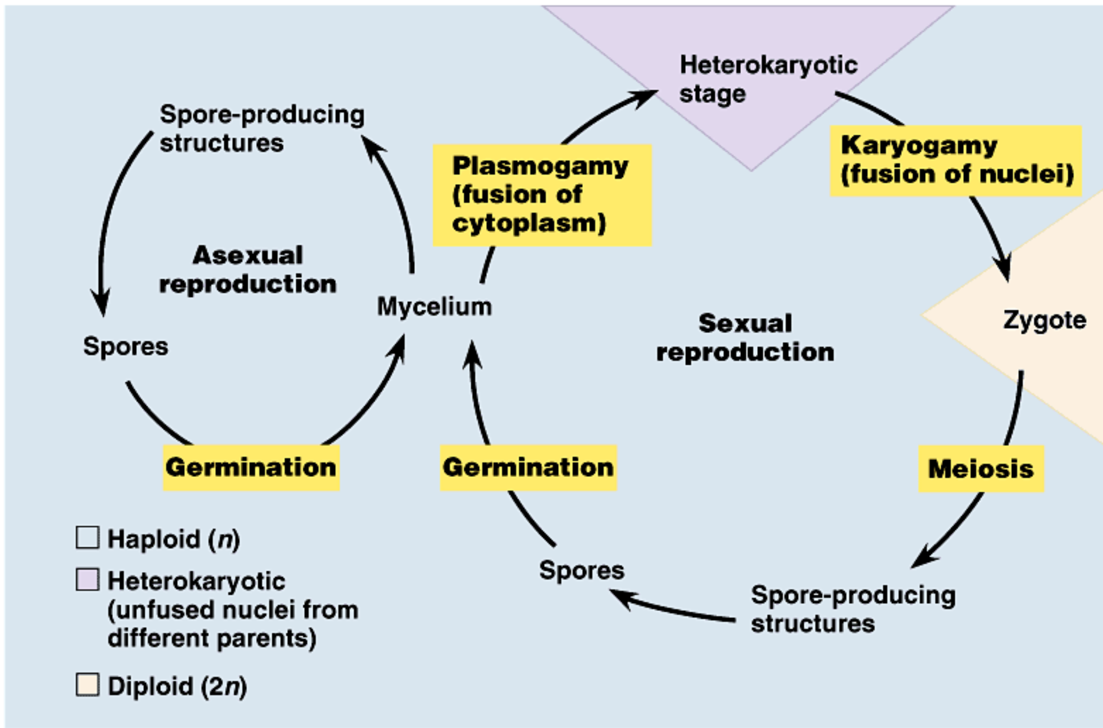

Fig. 1.1. Generalized Fungal Life Cycle, from http://www.biology.lsu.edu/heydrjay/1002/Chapter24/lifecycles/lifecycle.html

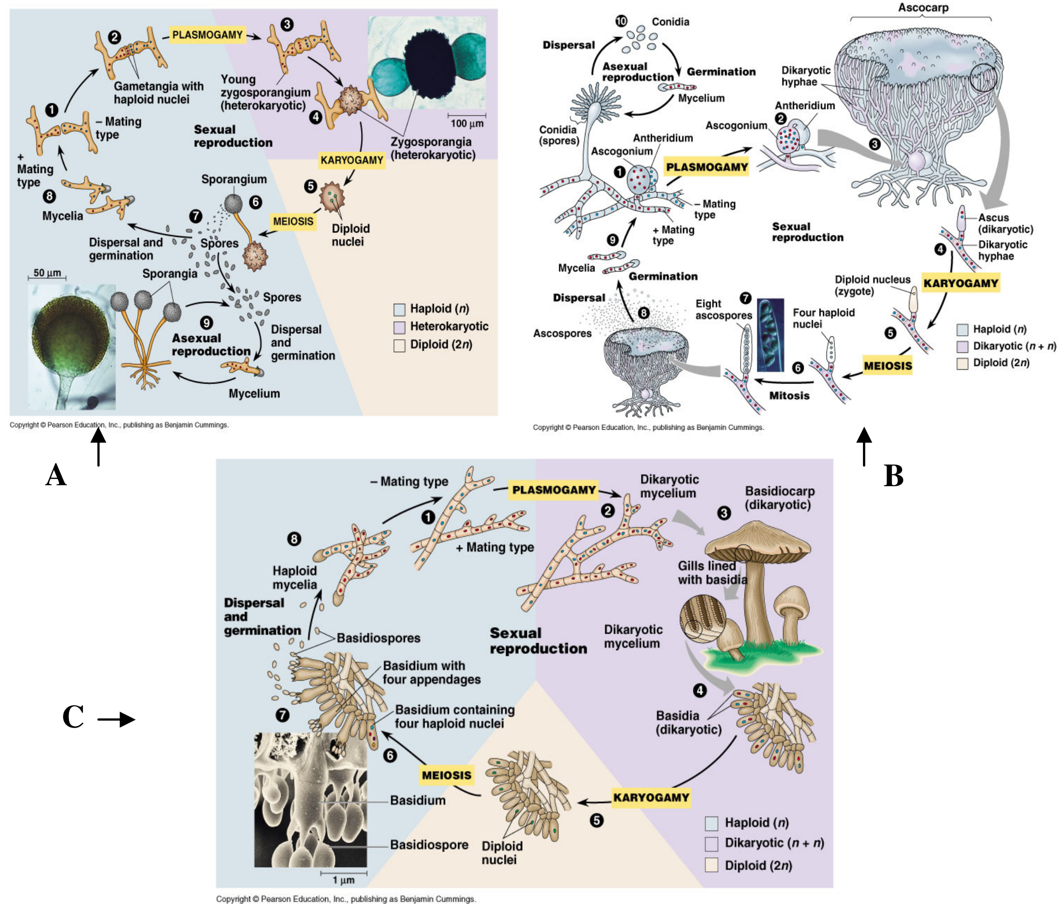

Fig. 1.2 Zygomycetes (A), Ascomycetes (B) and Basidiomycetes (C) life cycles, from http://www.biology.lsu.edu/heydrjay/1002/Chapter24/lifecycles/lifecycle.html

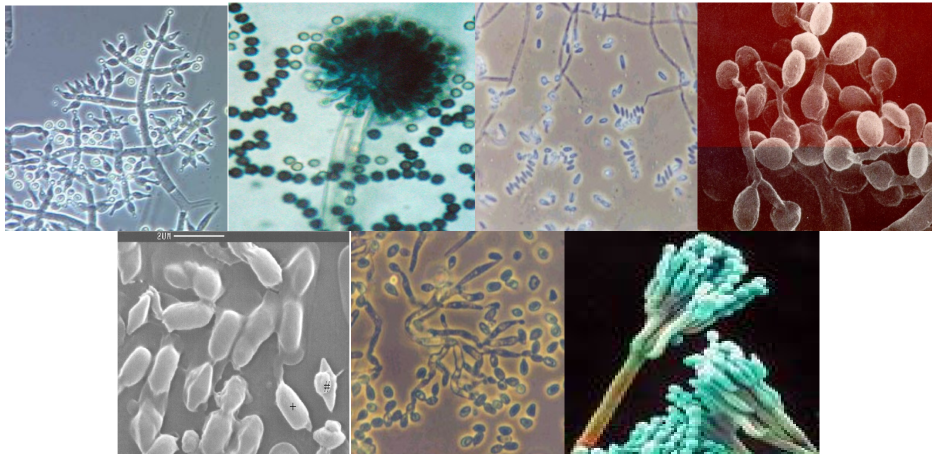

Fig. 1.3. Fungal and bacterial spores. From left to right *Trichoderma harzianum* (www.ngi-net.de), *Aspergillus niger* (www.moldbacteria.com), *Verticillium lecanii* (www.invasive.org), *Beauveria bassiana* (www.vertigo.uqam.ca), *Bacillus thuringiensis* (www.gre.ac.uk) *Metarhizium anisopliae* (www.invasive.org), *Penicillium roquefortii* (www.biocentrum.dtu.dk)

1.1.2. Significance of spores

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Spores are generally regarded as agents for disease, contamination and biological warfare. However, they possess several advantages when compared to their corresponding vegetative cells. Spores have interesting properties such as resistance to high temperature, toxic compounds, desiccation, radiation, etc. Some yeast spores survived heat treatment up to 350 times compared to their vegetative cells (Dijksterhuis & Samson 2002). Spores of *Metarhizium flavoviride*, known as green muscle®, the commercial biocontrol agent is stable for more than 42 months at 4 °C. Spores of various *Bacillus* species are generally 10 to 100-fold more resistant to UV than the corresponding vegetative cells (Setlow 2001). Apart from these, spores have the ability to germinate in response to external triggers such as plant hormones, host surface wax, or pressure. Another significant advantage of using spores is that they show higher resistance towards toxic effects of bioconversion substrates and products. Spores of *Penicillium digitatum* were found to be 2.5 times more resistant towards various toxic compounds (geraniol, citral, etc.) when compared to their mycelium (Wolken et al 2002). Spores are also highly resistant to mechanical forces. These characteristics apply to the products within the spores, as they too show tolerance to various chemical compounds and crucial conditions. The longevity that spore products have is the direct consequence of their higher resistance towards external factors (Wolken et al 2003).

1.1.3. Applications of spores

Spores possess some interesting properties which make them a candidate member for various applications in the bioprocess technology. Spores of fungi are used as inoculum for most of the fermentation process including small scale and large scale level. Apart from these, mainly spores are used as biocontrol agents (insecticide, fungicide, pesticide), biocatalysts in various bioconversion reactions.

Spores of *Bacillus thuringiensis* (a bacterium) produced by SSF are applied as control agent against plant pests such as caterpillars of Lepidoptera, mosquito larvae, locusts, grasshoppers, etc (Devi et al 2005, Capalbo et al 2001). Spores of *C. minitans* produced by SSF are used against the control of fungal pathogen *Sclerotinia sclerotiorum* that reduces yields of many crops. Spores of *Colletotrichum truncatum* protects soybean crop against *Sesbania exalta* (Silman et al 1993. Spores of *V. lecanii* control white fly invasion of various crops (Barranco-Florido et al 2002). Spores of *Beauveria bassiana* is used to control sugarcane borer and European corn borer, invading maize crop (Arcas et al 1999, Desgranges et al 1993).

Spores are also known to possess high biocatalytic activity because they generally exhibit high catalytic activity in terms of dry matter. Thus they are used in various biotransformation reactions. Spores of *Penicillium roquefortii* carried out transformation octanoic acid → 2-heptanone and produced blue cheese flavour (Larroche & Gros 1989). Spores of *Aspergillus niger* produced by SSF showed stable glucose oxidase activity even after long storage time and carried out biotransformation reaction of glucose to gluconic (Moksia et al 1996). Table 1.a shows some reactions catalysed by spores.

Spores are also applied in various other fields. Spores carry out steroid and antibiotic transformation. The first reaction discovered to be catalysed by the spore is the hydroxylation. Spores of *Aspergillus ochraceus* produced 11α-hydroxy-progesterone from progesterone. Spores of *A. niger* convert several sugars to citric acid, spores of *Mucor rouxii* reduce β-ketoesters, spores of *Aspergillus sydowi* convert soluble starch to glucose. There are also various other potential applications of the spores. Spores of *Bacillus* are used as bioindicators in the validation of thermal sterilization processes (Spicher et al 2002), also as biosensors (Rotman 2001). Spores also play an important role in the field of tumour detection and treatment (Mose 1970, Minton et al 1995), etc. Spore suspensions are also administered as probiotics (Casula & Cutting 2002). In agricultural industry, spores are receiving increasing attention as potential alternatives to antibiotics for use as growth promoters (Wolken et al 2003).

1.2. *Aspergillus niger*

Aspergillus niger is a well known producer of organic acids, enzymes, plant growth regulators, mycotoxins and antibiotics. *A. niger* is a prodigious exporter of species of homologous proteins and is able to produce certain enzymes in quantities of kg/m3 under right conditions. It is also generally regarded as safe (GRAS) and has long history of usage in the fermentation industry.

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Table 1.a Spore catalysed reactions (http://archive.bmn.com/supp/tibtec/Wolken-Tables.pdf).

Reaction	Microorganism
Blue cheese flavour production	
Fatty acid → 2-ketones	*Penicillium roquefortii, Paecilomyces varioti, Scaputariopsis brevicqulis, Aspergillus niger, Trichoderma koningi*
Triglycerides → 2-ketones	*P. roquefortii*
Alkyl esters of fatty acids → 2 –alkanones	*P. roquefortii*
Dispersed blue cheese → blue cheese flavorant	*P. roquefortii*
Terpenes	
Geraniol → methylheptanone	*P. digitatum*
Citronellal → citronellol	*P. digitatum*
Linalool → alpha terpineol	*A.niger*
Limonene → alpha terpineol	*A.niger*
Steroids	
Progesterone → 11 alpha hydrooxy-Progesterone	*Aspergillus ochraceus*
Progesterone → 16 alpha hydroxy-Progesterone	*Streptomyces argenteolus*
Progesterone → androstadienedione 1- dehydrotestololactone	*Fusarium solani*
Antibiotics	
Phenoxy methyl penicillin → 6 –amino penicillanic acid	*Fusarium moniliforme*
Antimycin A → inactive acids	*A. ochraceus*
Carbohydrates	
Sucrose → levan	*A. syndowi*
Starch → glucose	*A. wentii*
Sucrose → citric acid	*A. niger*

1.2.1.Ultra structure of conidial walls

In *Aspergillus niger*, the hyphal germ tube wall develops as an extension of the spore wall or an inner layer of the spore wall and are considered as Type I spores. Type II spores are those which have de novo formation of a wall on a naked protoplast (occurs only in zoosporic fungi). In type III spores, a vegetative cell wall forms de novo under the spore wall (Mucorales). Conidial walls generally are found to have two or three layers. According to Mangenot & Reisinger (1976), spore walls are three layered structures, although all three layers are not always easily discerned. The outermost layer is generally 5-20 nm in thickness. It is a glyco-protein lipid complex which accounts for approximately 25% of wall weight and gives a single band when studied by disc gel electrophoresis. This could be extracted by phosphate buffered solution containing urea, mercaptoethanol, SDS. Freeze etching also could remove this layer. In *A. niger* outer wall fractions contains less polysaccharide and considerably more protein than the inner wall fraction. Partial extraction of this layer results in reduced germination rates; however it does not reduce spore viability suggesting that outer wall components have some physiological and enzymatic functions in germination.

Middle wall is electron dense layer 30-50nm in thickness. It contains proteinaceous rodlets along with the polysaccharide and melanin. Rodlets make upto 10% wall weight. Hot alkali treatment makes this wall and rodlet disappear. Rodlets of *A. niger* occur in the outer wall fraction and are resistant to various chemical and enzymatic agents. However they are digested by elastase.

Inner wall is 200-300 nm in thickness, accounts for approximately 50% wall weight. This wall remains intact even after following the treatments that could remove outer and middle walls. Chitin microfibrils in an amorphous β- glucan matrix constitute the inner wall. Glucans are approximately 50% solubilized by digestion with β-1, 3 D-glucanase. Hot dilute acid causes rapid hydrolysis. Cole et al 1977 treated spore with 10% NaOH, leaving an alkali-resistant residue containing chitin and glucan. They separated glucan from chitin by acetylation, a procedure which separates a large portion of the total glucan as a chloroform soluble acetate, and by treatment of the alkali-insoluble residue with nitrous acid, a procedure which was found to render the complex soluble in dimethylsulfoxide and amenable, therefore, to permethylation. The data suggested that the preparation is an essentially linear glucan containing 85–95% $1 \rightarrow 3$ linkages and 10–15% $1 \rightarrow 4$ linkages. An analysis of the

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glycosidic linkages using NMR spectroscopy indicate that both α and β linkages are present in the ratio of 4:1 (Stagg & Feather 1973).

1.3 Gluconic acid

Gluconic acid (pentahydroxy caproic acid, Fig1.4) is produced from glucose through a simple dehydrogenation reaction catalysed by glucose oxidase. Oxidation of the aldehyde group on the C-1 of β- D-glucose to a carboxyl group results in the production of glucono-δ-lactone ($C_6H_{10}O_6$, Fig.2) and hydrogen peroxide. Glucono-δ-lactone is further hydrolysed to gluconic acid either spontaneously or by lactone hydrolysing enzyme, while hydrogen peroxide is decomposed to water and oxygen by peroxidase. The conversion process could be purely chemical too, but the most commonly involved method is the fermentation process. The enzymatic process could also be conducted, where the conversion takes place in the absence of cells with glucose oxidase and catalase derived from *A. niger.* Nearly 100 % of the glucose is converted to gluconic acid under the appropriate conditions. This method is an FDA approved process. Production of gluconic acid using enzyme has the potential added advantage that no product purification steps are required if the enzyme is immobilised, *e.g.* the use of a polymer membrane adjacent to anion-exchange membrane of low-density polyethylene grafted with 4-vinylpyridine (Godjevargova et al 2004).

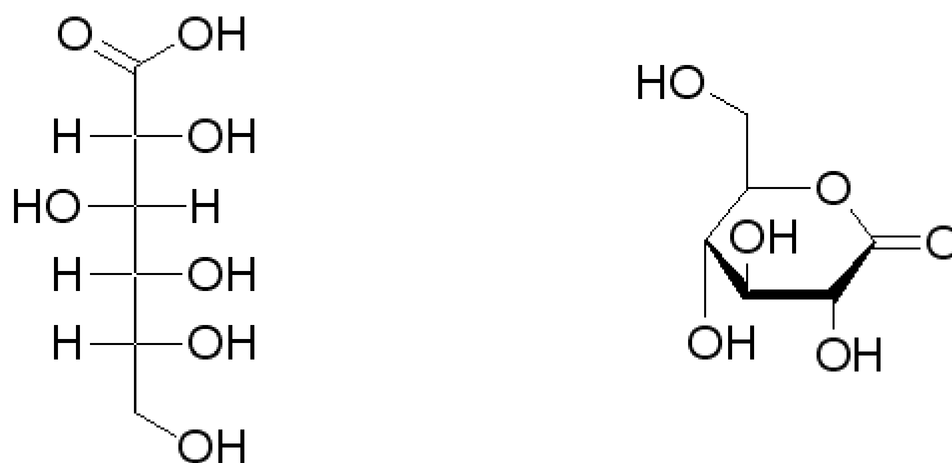

Fig.1.4. Formulae of gluconic acid (A) and glucono-δ-lactone (B)

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1.3.1.Properties

Gluconic acid is a non-corrosive, non-volatile, non-toxic, mild organic acid. It imparts a refreshing sour taste in many foods items such as wine, fruit juices *etc*. Sodium gluconate has a high sequestering power. It is a good chelator at alkaline pH; its action is comparatively better than EDTA, NTA and other chelators. Aqueous solutions of sodium gluconate are resistant to oxidation and reduction at high temperatures. It is an efficient plasticizer and a highly efficient set retarder. It is easily biodegradable (98 % at 48 h). It has an interesting property of inhibiting bitterness in foodstuffs. Concentrated gluconic acid solution contains certain lactone structures (neutral cyclic ester) showing antiseptic property. Main physical and chemical characterisitics are given in Table 1.b.

Table 1.b Physical and chemical properties of gluconic acid

Formula	$C_6H_{12}O_7$,(2,3,4,5,6-pentahydroxyhexanoic acid
Nature	Non corrosive, mildly acidic, less irritating, nonodorous, nontoxic, easily biodegradable, non volatile organic acid.
Molecular weight	196.16
Chemical formula	$C_6H_{12}O_7$
Synonym	2,3,4,5,6-Pentahydroxyhexanoic acid
pKa	3.7
Melting point (50 % solution)	Lesser than 12 °C
Boiling point (50 % solution)	Greater than 100 °C
Specific gravity	1.24-1.26
Appearance	Clear to brown (50% aqueous solution)
Soluble	Soluble in water
Sourness (Degree of sourness: Sourness of citric acid is regarded as 100)	Mild, soft refreshing taste Degree of sourness: 29-35
Toxicity	Oral rat LD 50 greater than 90 ml/kg

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In the European Parliament and Council Directive No. 95/2/EC, gluconic acid is listed as a generally permitted food additive (E 574). The US FDA (Food and Drug Administration) has assigned sodium gluconate a GRAS (generally recognized as safe) status and its use in foodstuff is permitted without limitation (*www.jungbunzlauer.com, Gluconic acid, 2005).*

1.3.2. History

Gluconic acid production dates back to 1870 when Hlasiwetz and Habermann discovered gluconic acid (Rohr 1983). In 1880 Boutroux found for the first time that acetic acid bacteria are capable of producing sugar acid. In 1922 Molliard detected gluconic acid in the *Sterigmatocystis nigra*, now known as *Aspergillus niger*. Later, production of gluconic acid was demonstrated in bacterial species such as *Pseudomonas, Gluconobacter*, *Acetobacter*, and various fungal species. Studies of Bernhauer showed that *A. niger* produced high yields of gluconic acid when it was neutralised by calcium carbonate and the production was found to be highly pH dependent. However it was found that with *Penicillium* sp., the pH dependence is not as critical when compared to *A. niger* indicating that there exists some correlation between the amount and time dependent appearance of organic acids, such as gluconic acid, citric acid, oxalic acid, which are formed under different conditions. Gluconic acid production has been extensively studied by May *et al.* 1934, Moyer 1940, Wells *et al.* 1937, Stubbs *et al.*1940 using *A. niger*. Using *Penicillium luteum* and *A. niger* Currie *et al.* 1931 filed a patent employing submerged culture, giving yields of gluconic acid up to 90 % in 48-60 h. Later Moyer *et al.* 1937 used *A. niger* in pilot plant studies and produced as high as 95 % of theoretical yields in glucose solution of 150 to 200 g/L at 24 h. Porges *et al.* 1940 found that the process could be run semicontinuously, by the reuse of the mycelium for nine times. Findings of Moyer *et al.* 1937 showed that efficiency of more than 95 % could be achieved by the addition of glucose at 250 g/L and boron compounds (1 % in solution of 250 g/L glucose) at later stages of the fungal growth with the reuse of mycelium in cycles of 24 h each. Current commercial production of sodium gluconate uses submerged fermentation with *A. niger* and is based on the modified process developed by Blom *et al.* 1952. It involves fed-batch cultivation with intermittent glucose feedings and the use of sodium hydroxide as neutralising agent. pH is held at 6.0-6.5 and the temperature at about 34 °C. The productivity of this process is very high, since glucose is converted at a rate of 15 g/(L.h.). Some of the important patents on gluconic acid production are summarised in Table 1.c.

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Table 1.c. Some patents on gluconic acid production

Year	Process	Number	Inventor(s)
1976	Process for the conversion of glucose into gluconic acid	3935071	Bergmeyer, HU, Jaworek, D
1984	Glucose oxidation with immobilized glucose oxidase-catalase	4460686	Hartmeier W
1988	Method for the production of sorbitol and gluconate	4755467	Scopes RK, Rogers, PL, Leigh DA
1995	Novel method for culture of filamentous fungi employed for production of GA	EP64899	Kiyoshi A, Yosuo M
1996	GA production by *Zymomonas* sp. with controlled ethanol production and selective precipitation	BR9403981	Jonas RHHH, Moura de Silveira M, Castilho-Lopes de Costa JP
1996	High yield enzymatic conversion of glucose to GA	WO9635800	Vroemem AJ, Beverini M
1997	An enzymatic method that allows high conversion rates of glucose to GA without expensive down stream recovering procedures	WO9724454	Lantero OJ, Shetty JK
1999	Process for the production of GA with a strain of *Aureobasidium pullulans* (de bary) Arnaud	5962286	Anastassiadis S, Aivasidis A, Wandrey C
1999	Enzymatic production of gluconic acid or its salts	5897995	Vroemen, A J. Beverini, Marc
2002	Production of gluconate salts	6416981	Chatterji C, Chatterji NP, Furtado ED
2004	Production of gluconate salts	6828130	Chatterji C, Chatterji NP, Furtado ED
2005	Process for the preparation of GA and GA produced thereby	6942997	Lantero OJ, Shetty JK

1.3.3. Measurement

There are several methods for the determination of D-gluconic acid and D-glucono-δ-lactone. Among them, isotachophoretic method (Everaents et al 1976) and hydroxamate method (Lien 1959) are the most commonly used ones for the determination of gluconic acid. The concentration of gluconic acid is also determined by gas chromatography of their

trimethylsilyl (TMS) derivatives prepared according to Laker and Mount (1980) with inositol as internal standard.

A widely used enzymatic method (Moellering & Bergmeyer 1988) is based on the following principle: D-gluconic acid is phosphorylated to D-gluconate-6-phosphate by ATP in the presence of the enzyme gluconate kinase with the simultaneous formation of ADP. In the presence of NADP, D-gluconate-6-phosphate is oxidatively decarboxylated by 6-phosphogluconate dehydrogenase to ribulose-5-phosphate with the formation of reduced NADPH. The NADPH is stoichiometrically formed and its measurement allows direct determination of the amount of D-gluconic acid.

1.3.4. Occurrence

Gluconic acid is abundantly available in plants, fruits and other foodstuffs such as rice, meat, dairy products, wine (up to 0.25 %), honey (up to 1%) and vinegar. It is produced by different microorganisms as well which include bacteria such as *Pseudomonas ovalis* (Bull & Kempe 1970), *Acetobacter methanolicus* (Loffhagen & Babel 1993), *Zymomonas mobilis* (Kim & Kim 1992), *Acetobacter diazotrophicus* (Attwood et al 1991), *Gluconobacter oxydans* (Olijve & Kok 1979, Weenk et al 1984, Silberbach et al 2003*), Gluconobacter suboxydans* (Nishimura et al 1992, Shirashi et al 1989), *Azospirillum brasiliense* (Rodriguez et al 2004), fungi such *Aspergillus niger* (Rao & Panda 1993, Buzzini et al 1993, Moksia et al 1996, Klein et al 2002, Liu et al 2003, Znad et al 2004, Mukhopadyay et al 2005*), Penicillium funiculosum* (Kundu & Das 1984)*, P. variabile* (Petruccioli et al 1995), *P. amagasakiense* (Milsom & Meers 1985), and various other species such as *Gliocladium, Scopulariopsis, Gonatobotrys, Endomycopsis* (Milsom & Meers 1985) and yeasts such as *Aureobasidium pullulans* (formerly known as *Dematium* or *Pullularia pullulans,* Anastassiddis et al 1992, 2003), *Saccharomyces cerevisiae* (Kapat 2001). Ectomycorrhizal fungus *Tricholoma robustum,* which is associated with the roots of *Pinus densiflora,* was found to synthesise gluconic acid (Iwase 1991). Gluconic acid producing fungi and bacteria are listed in Tables 1.d & 1.e.

1.3.5. Applications

Gluconic acid is a mild organic acid, which finds applications in the food industry. As stated above, it is a natural constituent in fruit juices and honey and is used in the pickling of foods. Its inner ester, glucono-δ-lactone imparts an initially sweet taste which later becomes

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Table 1.d Gluconic acid producing fungi

Molds and yeasts	References
Aspergillus niger	Molliard 1922, Sakurai et al 1989, Rosenberg et al 1992, Rao & Panda 1993, Buzzini et al 1993, Moksia et al 1996, Klein et al 2002, Liu et al 2001, Znad et al 2004, Mukhopadyay et al 2005
Penicillium chrysogenum	Richter 1983, Pazur 1966
Penicillium notatum	Richter 1983, Pazur 1966
Penicillium glucum	Richter 1983, Pazur 1966
Penicillium amagasakiense	Milsom & Meers 1985
Penicillium funiculosum	Kundu & Das 1984
Penicillium luteum purpurogenum	Mattey 1992
Peniciilium variable	Petruccioli et al 1995
Phanerochaete chrysosporium	Kelly & Adinarayana 1986
Talaromyces flavus	Kay et al 1990
Aspergillus foetidus	Mattey 1992
Candida tenius	Haug et al 1990
Tricholoma robustum	Iwase 1991
Aureobasidium pullulans	Anastassiddis et al 1992, 2003
Saccharomyces cerevisiae	Kapat 2001

Table 1.e Gluconic acid producing bacteria

Bacteria	References
Bacillus subtilis	Rubio 1990, Roche 1992
B. megaterium	Roche 1992
Acetobacter methanolicus	Loffhagen & Babel 1993
Gluconobacter oxydans	Olijve & Kok 1979, Weenk et al 1984, Silberbach et al 2003
Gluconobacter suboxydans	Nishimura et al 1992, Shirashi et al 1989
Azospirillum brasiliensis	Rodriguez et al 2004
Pseudomonas ovalis	Bull & Kempe 1970
P. fluorescens	Haug et al 1990
P. acidovorans	Huag et al 1990
P Putida	Huag et al 1990
Rhodotorulla rubra	Huag et al 1990
Streptococcus ocidovorans	Huag et al 1990
S. faecium	Huag et al 1990
Zymomonas mobilis	Huag et al 1990, Kim & Kim 1992, Mattey 1992
Escherichia coli	Krishnaraj & Goldstein 2001

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slightly acidic. It is used in meat, dairy products, particularly in baked goods as a component of leavening agent for preleavened products. It is used as a flavouring agent (for example, in sherbets) and it also finds application in reducing fat absorption in doughnuts and cones. Foodstuffs containing D-glucono-δ-lactone include bean curd, yoghurt, cottage cheese, bread, confectionery and meat.

Generally speaking, gluconic acid and its salts are used in the formulation of food, pharmaceutical and hygienic products (Table 1.f). They are also used as mineral supplements to prevent the deficiency of calcium, iron *etc*., and as buffer salts. Different salts of gluconic acid find various applications based on their properties. Sodium salt of gluconic acid has the outstanding property to chelate calcium and other di- and trivalent metal ions. It is used in the bottle washing preparations, where it helps in the prevention of scale formation and its removal from glass. It is well suited for removing calcareous deposits from metals and other surfaces, including milk or beer scale on galvanised iron or stainless steel. Its property of sequestering iron over a wide range of pH is exploited in the textile industry, where it prevents the deposition of iron and for desizing polyester and polyamide fabrics. It is also used in metallurgy for alkaline derusting as well as in the washing of painted walls and removal of metal carbonate precipitates without causing corrosion. It also finds application as an additive to cement, controlling the setting time and increasing the strength and water resistance of the cement. It helps in the manufacture of frost and crack resistant concretes. It is also used in the household cleaning compounds such as mouth washes.

Calcium gluconate is used in pharmaceutical industry as a source of calcium for treating calcium deficiency by oral or intravenous administration. It also finds a place in animal nutrition. Iron gluconate and iron phosphogluconate are used in iron therapy. Zinc gluconate is used as an ingredient for treating common cold, wound healing and various diseases caused by zinc deficiencies such as delayed sexual maturation, mental lethargy, skin changes, and susceptibility to infections.

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Table 1.f Applications of gluconic acid and its salts

Components	Applications
Gluconic acid	Prevention of milkstone in dairy industry Cleaning of aluminium cans
Glucono-δ-lactone	Latent acid in baking powders for use in dry cakes and instantly leavened bread mixes Slow acting acidulant in meat processing such as sausages Coagulation of soybean protein in the manufacture of tofu In dairy industry for cheese curd formation and for improvement of heat stability of milk
Sodium salt	Detergent in bottle washing Metallurgy (alkaline derusting) Additive in cement Derusting agent Textile (iron deposits prevention) Paper industry
Calcium salt	Calcium therapy Animal nutrition
Iron salt	Treatment of anaemia Foliar feed formulations in Horticulture
Zinc salt	Treatment of zinc deficiencies and common cold

1.3.6. Market

Organic acids represent the third largest category after antibiotics and amino acids in the global market of fermentation. The total market value of organic acid will rise to $3

million in 2009. Citric acid dominates the market of organic acid due to its various application fields. The market of gluconic acid is comparatively smaller. Worldwide consumption of gluconic acid was about 87,000 tonnes in 2004 with the total market value of about US$ 333 millions (Business communication Co. 2004). Tables 1.g & 1.h show the global consumption and market expenditure of gluconic acid based on application.

Table 1.g Global consumption and market expenditure of gluconic acid based on application

Field of application	**Quantity consumed (tonnes)**	**Total consumption (%)**	**Global market expenditure (US$ million)**		**AAGR estimate (%)**
			2004	**2009 estimate**	**2004-2009**
Construction	40,000	46	60	76	4.8
Food	30,000	34.5	150	153	0.4
Pharmaceutical	8,000	9.2	63	51	-5.6
Other	9,000	10.3	55	31	-10.8
Total	**87,000**	**100.0**	**333**	**311**	**-1.4**

Table 1.h Market value of gluconic acid and its derivatives based upon major industrial application (Singh & Kumar 2007)

Product	**Application**	**Average cost ($/kg)**
Gluconic acid	Cement	1.20
Sodium gluconate	Cement	2.00
Pure sodium gluconate	Food	3.00
Calcium gluconate	Food and pharmacy	8.50

The main product among the gluconic acid derivatives is the sodium gluconate due to its properties and applications. Manufacturers of gluconic acid and its salt in the United States are Pfizer Inc., New York, Bristol–Meyers Co., New York, Premier Malt Products Inc., Wisconsin. European gluconate producers include Roquette Frères in France, Pfizer in Ireland, Benckiser in Germany. Fujisawa and Kyowa Hakko are the manufacturers of

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gluconate in Japan. Calcium gluconate is also an important product among the derivatives of gluconic acid and it is available as tablets, powder, and liquid for dietary supplements.

1.3.7. Production of Gluconic Acid

1.3.7.1 Introduction

There are different approaches available for the production of gluconic acid, namely, chemical, electrochemical, biochemical and bioelectrochemical (Isabell et al 1932, Pfizer 1957, de Wilt 1972). There are several different oxidising agents available, but still the process appears to be costlier and less efficient compared to the fermentation processes. Although the conversion is a simple one-step process, the chemical method is not favoured. Thus fermentation (Fig. 1.5) has been one of the efficient and dominant techniques for manufacturing gluconic acid. However, bioconversion has advantages over fermentation for the production of gluconic acid which is summarised in Table 1.i.

Table 1.i Advantages of bioconversion over fermentation in gluconic acid production

Fermentation	**Bioconversion**
1. Change of biomass concentration	Constant biomass concentration
2. Oxygen consumption for biomass growth biotransformation	Oxygen consumption for and biotransformation
3. Complex composition of media	Simple composition of media
4. Need for sterile environment	No need for sterile environment

Among various microbial fermentation processes, the method utilising the fungus *A. niger* is one of the most widely used ones. However, the process using *G. oxydans* has also gained significant importance. Irrespective of the use of fungi or bacteria, the importance lies on the product which is produced, for example, sodium gluconate or calcium gluconate, *etc*. As the reaction leads to an acidic product, it is required that it is neutralised by the addition of neutralising agents, otherwise the acidity inactivates the glucose oxidase, resulting in the arrest of gluconic acid production.

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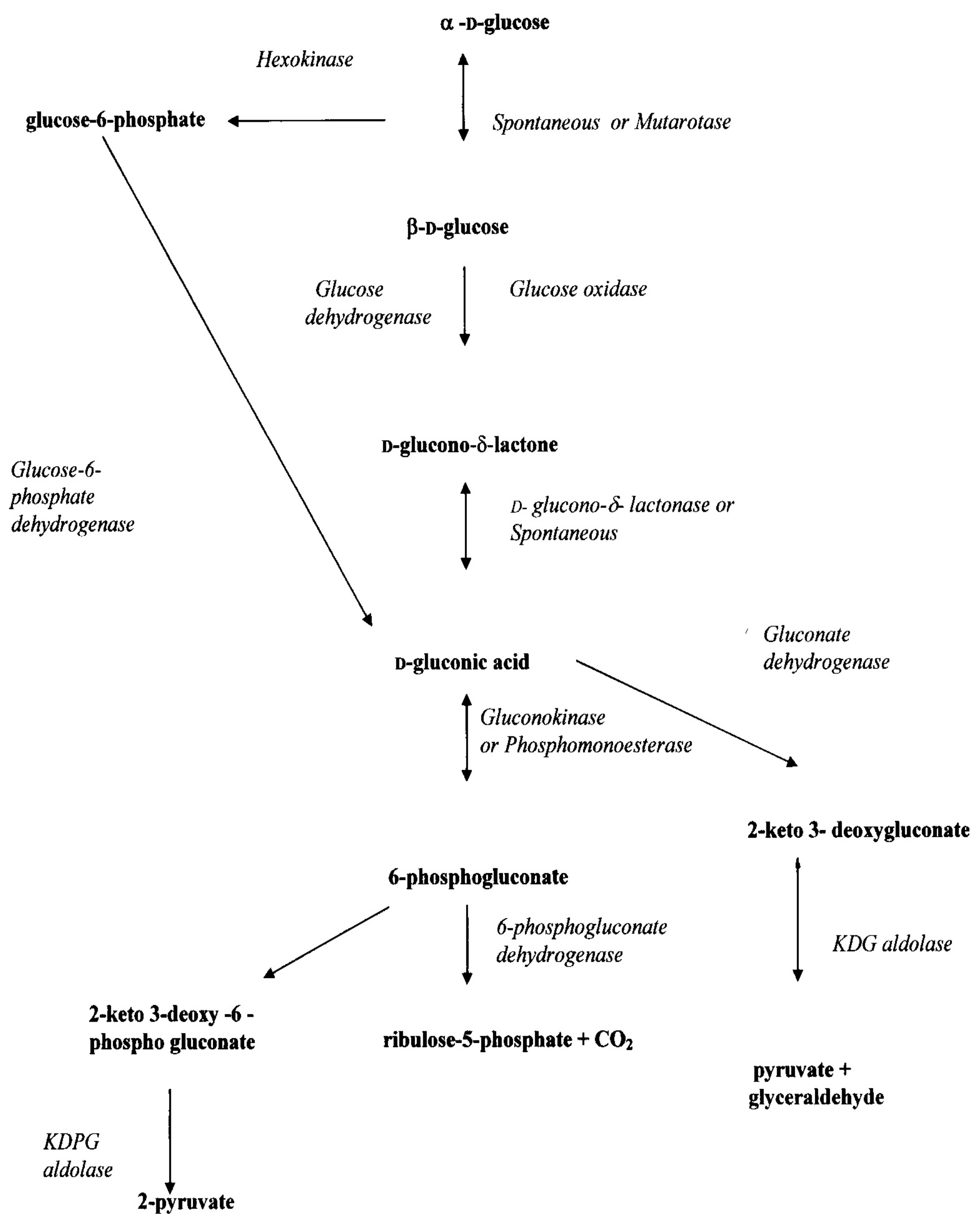

Fig.1.5. General gluconate pathway

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The conditions for the fermentation processes in the production of calcium gluconate and sodium gluconate differs in many aspects such as glucose concentration (initial and final) and pH control. In the process involving calcium gluconate production, the control of pH results from the addition of calcium carbonate slurry. Another important point to be noted is about the solubility of calcium gluconate in water (4 % at 30 °C). At high glucose concentration, above 15 %, supersaturation occurs. And, if it exceeds the limit, the calcium salt precipitates on the mycelia and inhibits the oxygen transfer. The neutralising agent should also be sterilized separately from the glucose solution to avoid Lobry-du Bryn Ekenstein reaction, which alters the conformation of glucose, which results in the reduction of yield about 30 %. On the contrary, the process for sodium gluconate is highly preferable as the glucose concentration of upto 350 g/L can be used without any such problems. pH is controlled by the automatic addition of NaOH solution. Sodium gluconate is readily soluble in water (39.6 % at 30 °C).

1.3.7.2. Gluconic acid production by filamentous fungi - Aspergillus niger

A. niger produces all the enzymes required for the conversion of glucose into gluconic acid, which include glucose oxidase, catalase, lactonase and mutarotase. Although, crystalline glucose monohydrate which is in the alpha form is converted spontaneously into beta form in solution, *A. niger* produces the enzyme mutarotase which serves to accelerate the reaction. During the process of glucose conversion, glucose oxidase present in *A. niger* undergoes self reduction by the removal of two hydrogens. The reduced form of the enzyme is further oxidised by the molecular oxygen, which results in the formation of hydrogen peroxide, a byproduct in the reaction. *A. niger* produces catalase which acts on hydrogen peroxide, releasing water and oxygen. Hydrolysis of glucono-δ-lactone to gluconic acid is facilitated by lactonase. The reaction can be carried out spontaneously as the cleavage of lactone occurs rapidly at pH near neutral, which are brought about by the addition of calcium carbonate or sodium hydroxide. Removal of lactone from the medium is recommended as its accumulation in the media has a negative effect on the rate of glucose oxidation and the production of gluconic acid and its salt. There are reports stating that the enzyme gluconolactonase is also present in *A. niger* (Cho & Bailey 1977) which increases the rate of conversion of glucono-δ- lactone to gluconic acid.

Production of gluconic acid is directly linked with the glucose oxidase activity. Depending on the application, the fermentation broths containing sodium gluconate or calcium gluconate are produced, by the addition of solutions of sodium hydroxide or calcium carbonate respectively, for neutralisation. The general optimal condition for gluconic acid production is as follows (Rohr et al 1983):

Key parameters which influence the gluconic acid production are given below.

Glucose at concentrations between 110-250 g/L Nitrogen and phosphorus sources at a very low concentration (20 mM) pH value of medium around 4.5 to 6.5 Very high aeration rate by the application of elevated air pressure (4 bar)

Among them, oxygen availability and pH of the culture medium are critical. Oxygen is one of the key substrates in the oxidation of glucose as glucose oxidase uses molecular oxygen in the bioconversion of glucose. The concentration of oxygen gradient and the volumetric oxygen transfer coefficient are the critical factors which monitor the availability of oxygen in the medium. These two factors highly influence the rate of the transfer of oxygen from gaseous to aqueous phase. Several reports are available on this particular aspect. The aeration rate and the speed of agitation are the two parameters which affect the availability of the oxygen in the medium. Gluconic acid production is an extremely oxygen-consuming process with a high oxygen demand for the bioconversion reaction, which is strongly influenced by the dissolved oxygen concentration. Oxygen is generally supplied in the form of atmospheric air, however, in some studies high pressure pure oxygen has also been provided. For example, Sakurai *et al.* 1989 supplied high pressure oxygen at 6 atm and maintained dissolved oxygen at 150 ppm. They found that immobilised mycelium of *A. niger* grown using pure oxygen produced hightitres of gluconic acid in comparison with mycelium grown in air. Kapat *et al.* (2001) found that at an agitation speed of 420 rpm and aeration of 0.25 vvm, the dissolved oxygen concentration was optimal for glucose oxidase production. The K_m value of glucose oxidase for oxygen lies in the range of air saturation in water (Schmid & Karube,1988). Lee *et al.* 1987 obtained high volumetric productivity of gluconic acid using relatively high pressure (2–6 bars), resulting in an increase in dissolved oxygen (DO) up to 150 mg/L. Generally during the course of fungal growth, the distribution of oxygen becomes uneven, as the size of gas bubbles increases, resulting in insufficient oxygen supply. The oxygen absorption rate is also

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influenced by the viscosity of the culture. A rapid decrease is observed in the absorption rate of oxygen with an increase in mycelial concentration (Steel & Maxson 1962).

pH is another important parameter that influences gluconic acid production. *A. niger* produces weak organic acids such as citric acid, gluconic acid and oxalic acid, and their accumulation depends on the pH of the medium (Lockwood, 1979). pH below 3.5 triggers the TCA cycle and facilitates the citric acid formation. The pH range of the fungi for the production of gluconic acid is around 4.5 to 7.0. pH= 5.5 is generally considered as optimum for the *Aspergillus niger* strain (Znad et al 2004). Franke (1963) had collected some data concerning the relative activity of glucose oxidase at different pH levels and reported 5 and 35 % activity at pH=2.0 and 3.0 respectively, based on 100% activity at pH=5.6. Report by Heinrich and Rehm (1982) states that gluconic acid production even occurs at pH 2.5 in the presence of manganese in fixed bed and stirred bed reactors, possibly because of the difference in intracellular and extracellular pH. Gluconic production by *Aspergillus niger* is summarised in Table 1.j

Table 1.j Brief literature survey on the gluconic production.

Culture method	**GA (g.L-1)**	**Volumetric productivity (g.l-1.h-1)**	**References**
Repeated batch culture	143	1.44	Vassilev et al 1993
Batch culture in air lift bioreactor	150	2.3	Klein et al 2002
Continuous reactor	158	6	Sankpal & Kulkarni 2002
Batch	80	0.5	Singh et al 2003
Batch culture in turbine blade reactor	100	1.13	Ikeda et al 2006
Batch	150	4.58	Znad et al 2004

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1.3.7.3. Cheaper raw materials as substrates

Glucose is generally used as carbon source for microbial production of gluconic acid. However hydrolysates of various raw materials such as agro-industrial waste have also been used as substrate. Kundu and Das (1984) obtained a high yield of gluconic acid in media containing glucose or starch hydrolysate as the sole carbon source. Vassilev *et al.* (1993) used hydrol (corn starch hydrolysate) as the fermentable sugar to produce gluconic acid by immobilized *A. niger*. Rao and Panda (1994) in their study used Indian cane molasses as a source of glucose. The cane molasses was subjected to different pre-treatments such as acid treatment, potassium ferrocyanide treatment, salt treatment *etc*. Potassium ferrocyanide treatment gave a promising result. Gluconic acid synthesis was influenced by various metal ions such as copper, zinc, magnesium, calcium, iron *etc*. Mukhopadhyay *et al.* (2005) used deproteinised whey as a medium for gluconic acid production. Lactose was used as a substrate and had found that 92 g of gluconic acid was produced from 1 L of whey containing 0.5 % glucose and 9.5 % lactose, by *A. niger* immobilized on polyurethane foam. Ikeda *et al.* 2006 used saccharified solution of waste paper with glucose concentration adjusted to 50 -100 g/L for bioconversion with *A. niger*. The yields were 92 % in Erlenmeyer flasks while 60 % in repeated batch cultures in the turbine blade reactor with 800 mL working volume. Another striking feature in the study was when xylose and cellobiose were used as the sole carbon sources, yields of gluconic acid obtained were 83 and 56 % respectively.

Singh *et al.* (2005) in his findings observed that grape must and banana must, resulted in significant levels of gluconic acid production, *i.e.* 63 and 55 g/L, respectively. The purification of grape and banana must, lead to a 20–21 % increase in gluconic acid yield. They also used molasses, where the gluconate production was 12 g/L, but a significant increase in production of 60 g/L with a yield of 61 % was observed following treatment of the molasses with hexacyanoferrate. Rectified grape must, appeared to be the best suited substrate which after 144 h resulted in 73 g/L of gluconic acid with 81 % yield when compared to the value of 72 % obtained from the rectified banana must. There is also a report which uses grape must and rectified grape must by Buzzini *et al.* (1993). They found that the latter substrate was better, with a production of 67 g/L and a yield of 96 % in 72 h. Citric acid was also observed as a by-product.

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1.3.7.4 .Use of solid-state fermentation (SSF)

SSF has been widely described for the production of industrial enzymes and organic acids (Pandey 1992, Pandey & Ramachandran 2005, Pandey et al 2000, Pandey et al 1999, Pandey et al 2001). However, for the production of gluconic acid, there are only a few reports using SSF. Roukas (2005) reported the production of gluconic acid by solid state fermentation on figs. The maximal gluconic acid concentration was 490 g/kg of dry fig with 63 % yield. The addition of 6 % methanol into the substrate helped to increase the production of gluconic acid from 490 to 685 g/kg. Singh *et al.* (2003) performed SSF by using HCl pretreated sugarcane bagasse and the highest level of gluconic acid (107 g/L) with 95 % yield was obtained. In comparison with the submerged culture, the degree of conversion was higher in SSF. The increased rate of product formation might be due to the variations of osmotic pressure, water content and dissolved oxygen. A study by Moksia *et al.* (1996) used a two-step process, the first being the production of spores of *A. niger* by SSF on buckwheat seeds, and the second step, the bioconversion of glucose to gluconic acid by the spores recovered from the SSF medium. The interesting aspect about this work was that the spores were not allowed to germinate as the bioconversion medium did not contain any nitrogen source. The spores acted as a biocatalyst, producing 200 g/L of gluconic acid with a yield of 1.06 g of gluconic acid/g of glucose, very close to the stoichiometric value.

1.3.7.5. Production of gluconic acid by bacteria

Acetic acid bacteria and *Pseudomonas savastanoi* were the cultures initially observed to produce gluconic acid. Unlike in fungi, in bacteria the reaction is carried out by glucose dehydrogenase (GDH, E.C.1.1.99.17) which oxidises glucose to gluconic acid, which is further oxidised to 2-ketogluconate by gluconic acid dehydrogenase (GADH). The final oxidation step to 2,5-diketo gluconic acid (DKG) is mediated by 2-ketogluconate dehydrogenase (KGDH). All three enzymes are localised in the membranes of the cells and are induced by high glucose concentrations (>15 mM) (Velizarov & Beschkov 1994). GDH is an extracellular protein and has PQQ (pyrroloquinoline quinine) as a coenzyme. Also there is an intracellular enzyme, a $NADP^+$-dependent glucose dehydrogenase, which is less involved in the gluconic acid formation when compared to the extracellular enzyme. Gluconic acid produced is exported to the cell and further catabolised via the reactions in pentose phosphate pathway. When the glucose concentration in the medium is greater than 15mM, pentose phosphate pathway is repressed and thus gluconic acid accumulation takes place.

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Gluconobacter oxydans is an obligate aerobic bacterium that oxidises glucose via two alternative pathways. The first pathway requires an initial phosphorylation followed by oxidation via the pentose phosphate pathway. The second is the 'direct glucose oxidation' pathway which results in the formation of gluconic acid and ketogluconic acid (Stadler-Szoke 1980).

Specific pathway for oxidation of glucose by *Gluconobacter G. oxydans* converts D-glucose into 2,5-diketogluconic acid by the action of three membrane-bound $NADP^+$-independent dehydrogenases as mentioned in Fig. 1.6. The acidotolerant acetic acid bacterium, *Acetobacter diazotrophicus* exhibited high rates of gluconic acid formation. Glucose oxidation by the organism was less sensitive to low pH values than glucose oxidation by *G. oxydans.* Both the phosphorylative and direct oxidative pathways of glucose metabolism appeared to be operative. In addition to a pyridine nucleotide (strictly NAD^+) - dependent glucose dehydrogenase, *A. diazotrophicus* contained a pyrrolo-quinoline quinone (PQQ)-dependent glucose dehydrogenase which was primarily responsible for gluconic acid formation. Bacterial gluconic acid production has limited success at industrial scale, as the oxidation proceeds with the secondary reactions leading to oxogluconic acids. The ability of *Pseudomonas* and *Gluconobacter* spp. to produce gluconolactone and gluconic acid has been exploited and the process is used commercially mainly in the production of lactone.

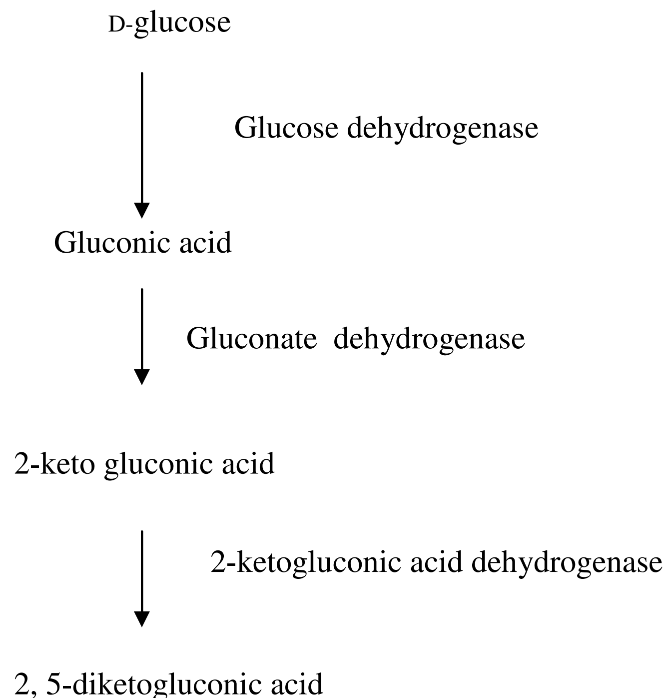

Fig 1.6. Specific pathway for oxidation of glucose by *Gluconobacter*.

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A. methanolicus is also used to catalyze the conversion of glucose into gluconic acid. The key advantage of using this facultatively methylotrophic microorganism as catalyst are that the gluconic acid formed is a metabolic dead end product unlike other bacterial fermentation processes, organism uses methanol, a cheap raw material as a substrate. Further in the process glucose is not assimilated or consumed for growth, so consequently the maximum theoretical yield coefficient is achieved. A patent was filed by Currie and Carter (1930) where the medium containing 200 g/L of glucose with other nutrients and a neutralising agent was allowed to flow through a tower packed with wood shavings or coke which had been inoculated with *Acetobacter suboxydans*, while air was passed upwards through the packing. Tsao and Kempe (1960), working with *Pseudomonas ovalis,* and found that the particular strain could convert glucose to gluconic acid with a yield of 99 %, and the rate was directly related to the efficiency of aeration.

1.3.7.6. Yeast

Research carried out by Anastassiadis et al (2005, 2003, 1999) utilised *Aureobasidium pullulans,* a yeastlike form of the dimorphic fungi, for the production of gluconic acid. Various process for the continuous and the discontinous production of gluconic acid such as pH, oxygen, temperature and medium composition, air saturation, *etc.* were studied (Anastassiadis et al 2005, 2003, 1999). The highest glucose conversion of 94 % and product yield of 87.1 % was achieved at an optimum pH of 6.5. At pH=4.5, the product selectivity and yield were very poor reaching 67.8 and 20.7% respectively. Temperature range of 29 to 31° C was found to be the suitable temperature for the production of gluconic acid by the yeast. Increase of temperature by 1 °C namely, to 32 °C dramatically influenced the reduction in steady state concentration of biomass and product.

1.3.8 .Immobilisation

Immobilisation techniques are involved where the biomass is immobilised onto the support and in some cases, the enzyme isolated from the culture is immobilised. It enables repetitive use of the high biomass to carry out biochemical reactions rapidly leading to process economy and stability. Immobilisation seems to be an attractive method for accomplishing high cell densities in order to achieve rapid carbohydrate conversion to organic acids (Vassilev et al 1993). Matrix immobilization is a simple and easy technique by which mycelia are retained on a matrix by mycelial entanglement. The type of support, cell

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retention, stabilization of enzyme or the mycelia and the quantum of biomass *etc*. play important roles.

In the past there were several investigations related to the production of gluconic acid with immobilised cells of *A. niger*. There are also reports of *A. niger* pellets immobilisation by flocculation with polyelectrolytes (Lee & Long 1974), calcium alginate (Rao & Panda 1994), glycidyl ester co-polymers (Nelson 1974) and entrapment in gels (Cai & Yin 1989). Glass rings were used to immobilise *A. niger* for the production of gluconic acid by Heinrich and Rehm (1982). Sakurai *et al.* (1989) adopted a novel method for the immobilisation of *A. niger* using a support of non-woven fabric. Vassilev *et al.* (1993) and Mukhopadhyay *et al.* (2005) reported the immobilisation of the same filamentous fungi on polyurethane foam. Different carriers such as calcium alginate agar, polyurethane sponge, pearlite and activated carbon were used for the immobilisation of *Penicillium variabile* by Petruccioli *et al.* (*1994*).

Free gluconic acid was continuously produced in an aerated tubular immobilized-cell bioreactor using *G. oxydans* for at least 6 months, with a volumetric productivity of at least 5 g/(L.h) per 100 g/L of glucose and the concentration of gluconic acid of about 80 g/L (Seiskari et al 1985). Spores of *A. niger* were immobilised on sintered glass, pumice stones, polyurethane foams and mycelia which developed on the pumice stone carrier produced high extracellular glucose oxidase (80 %) when compared to the enzyme activity on free cells (Sankpal & Kulkarni 2002).

An attempt was made by Sankpal *et al.* (1999) to study the bioconversion of glucose to gluconic acid using *A. niger* immobilized on cellulosic fabric as a support matrix. 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. The system was found to run continuously for a period of 61 days utilizing the entire available glucose. The emerging broth contained a product concentration of 120–140 g/ L of gluconic acid which is higher than the expected (maximum of 109 g gluconic acid/ 100 g glucose) as a result of evaporative concentration during the downward flow. Sankpal and Kulkarni (2002) found that the optimum biomass requirement on a porous cellulose support was 0.234 mg/ cm^2 for efficient bioconversion. Increasing the quantum of biomass beyond this value resulted in an overgrown biofilm which affected productivity adversely. Morphological characteristics of immobilized *A. niger* have also been investigated.

1.3.9. Recovery

The recovery process depends on the method followed for broth neutralisation and the nature of carbon sources used. Generally the downstream process is similar for the fermentation processes using fungal and bacterial species. Gluconic acid, glucono-δ-lactone, calcium gluconate, sodium gluconate are some of the important products and their extraction process is briefly mentioned below.

For the recovery of free gluconic acid from calcium gluconate the broth is clarified, decolorized, concentrated and exposed to -10 °C in the presence or absence of alcohol. Thus the calcium salt of gluconic acid crystallizes which is recovered and further purified. Gluconic acid may be also obtained by precipitating the calcium gluconate from hypersaturated solutions in the cold and released subsequently by adding sulphuric acid stoichiometrically, removing the calcium as calcium sulphate. Another method of passing the solution through a column containing a strong cation exchanger is also practised where the calcium ions are absorbed.

For obtaining calcium gluconate as a product, calcium hydroxide or calcium carbonate is used as the neutralising agent. They are added to the fermentation broth accompanied by heating and vigorous stirring. The broth is concentrated to a hot supersaturated solution of calcium gluconate, followed by cooling at 20 °C, and adding water miscible solvents, which crystallises the compound. A treatment with activated carbon facilitates the crystallisation process. Finally they are centrifuged, washed several times and dried at 80 °C.

Sodium gluconate, the principal manufactured form of gluconic acid, is prepared by ion exchange. In the process developed by Blom *et al.* 1952, the sodium gluconate from the filtered fermented broth is concentrated to 45 % (w/v) followed by the addition of sodium hydroxide solution raising the pH to 7.5, and drum drying. Carbon treatment of the hot solution before drying process is practised for obtaining a refined product. Glucono-δ-lactone recovery is a very simple process. Aqueous solutions of gluconic acid are an equilibrium mixture of glucono-δ-lactone, glucono-λ-lactone and gluconic acid. At a temperature between 30-70 °C the crystal which is separated from the supersaturated solution is glucono-δ-lactone. At temperature below 30°C, gluconic acid results even above 70 °C the resulting product would be glucono-λ-lactone.

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1.4. Glucose oxidase

Enzymes are the tools of bioconversion. Glucose oxidase (beta-D-glucose: oxygen 1-oxidoreductase, E.C. 1.1.3.4) belong to oxidoreductase class of enzyme. It is a flavoprotein which contains one very tightly but non-covalently bound FAD cofactor per monomer and is a homodimer with a molecular mass of 130-320 kDa depending on the extent of glycosylation. It catalyses the reaction where glucose is dehydrated to glucono-δ-lactone and hydrogen is transferred to FAD. The resulting $FADH_2$ is regenerated to FAD by transmission of the hydrogen to oxygen to form hydrogen peroxide (Fig. 1.7).The reaction involving the conversion of glucose to gluconic acid by filamentous fungi is catalysed by the enzyme glucose oxidase (beta-D-glucose: oxygen 1-oxidoreductase, E.C. 1.1.3.4). The enzyme was first isolated from a press juice obtained from *Penicillium glaucum* by Muller (1928). The enzyme was crystallised by Kusai *et al.* (1960) from *P. amagasakiense*. The enzyme was previously known as notatin. Glucose oxidase is a glycoprotein. The native enzyme is glycosylated, with a carbohydrate mass percentage of 16-25 % (Pazur & Kleppe 1964, Swoboda & Massey 1965). The enzyme from *A. niger* contains 10.5 % carbohydrate, which is believed to contribute to the stability without affecting the overall mechanism (Kalisz et al 1991). The carbohydrate moiety is designated as high mannose type with 80% (w/w) of the carbohydrate being mannose. The mannose is N and O glycosidically linked to Asn, Thr and Ser.

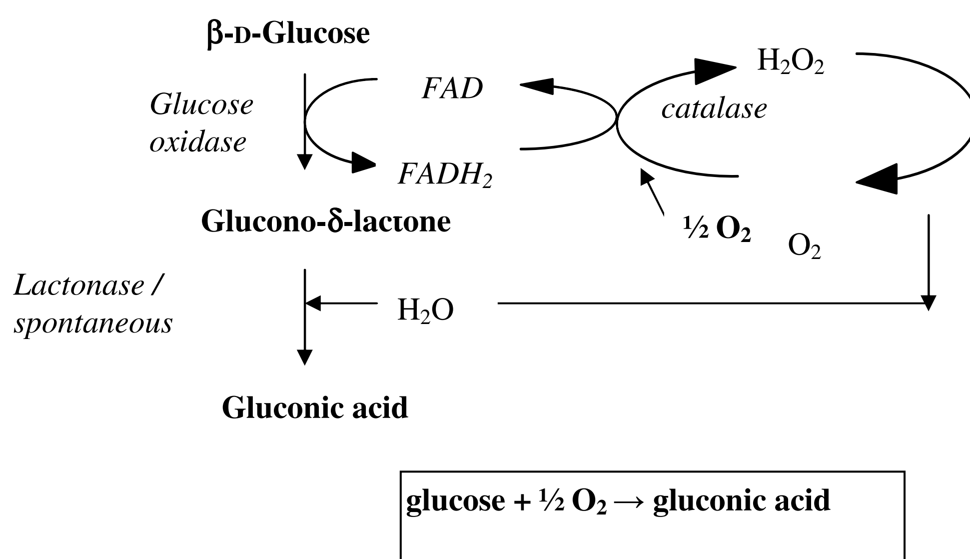

Fig.1.7. Oxidation of glucose by glucose oxidase.

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The holoenzyme is made up of two identical subunits of MW 80,000 D. The monomers are connected non covalently via a long but narrow contact area. There are 120 contact points between the dimmers centred around 11 residues which form either salt linkages or hydrogen bonds. The monomeric molecule is a compact spheroid with approximate dimensions 60Å x 52Å x 37Å. The monomer folds into two structural domains. One of the domains binds FAD and the other is involved with substrate binding. The subunit contains one disulphide bridge. The corresponding dimensions of the dimmer are 70Å x 55Å x 80Å. The minimum distance between the flavin and the surface of the monomer is 13Å. The two isoalloxazine moieties are separated by a distance of about 40Å, a distance which excludes any electrical communication between them. The general properties of glucose oxidase of *Aspergillus niger* are summarized in Table 1.k.

Table 1.k. Summary of main characteristics of the glucose oxidase from *Aspergillus niger.*

- The molar extinction coefficient of a 1% (w/v)solution at 280 nm is 13.8 (in 0.1M potassium phosphate pH 7.0, yellow solution)
- The native protein is acidic, having an isoelectric point (pI) of 4.2
- The diffusion coefficient of the holo-enzyme in 0.1M NaCl is 4.94 x 10^{-7} cm^{2} s^{-1}
- The GOx native dimmer has the molecular weight of 160kDa (composed of two subunits of 80 kDa each)
- The GOx (native dimmer) dimensions are 70Å x 55Å x 80Å. Each of the subunit is a compact spheroid with approximate dimensions 60Å x 52Å x 37Å
- The minimum distance between the flavin and the surface of the monomer is 13Å. The two isoalloxazine moieties are separated by a distance 60Å x 52Å x 37Å
- Michaelis constant (apparent) Km = 33 mM
- Molecular activity (turn over number) = 2.28 x 10^{4}

The enzyme synthesis is induced in the presence of high levels of glucose in the medium, pH around 5.5 and elevated oxygen levels. At pH 3.0 the reaction proceeds slowly and at pH 8.0 on the other hand, it proceeds with a half life of approximately 10 minutes. The

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enzyme is stable between pH = 4.0 and 6.0 at 40 °C for 2 h but is unstable above 50 °C. Liu *et al.* (2001) conducted a study on the effects of metal ions on simultaneous production of glucose oxidase and catalase and found that calcium carbonate induced the synthesis of both enzymes. The induction of calcium carbonate was accompanied by a metabolic shift from the glycolytic pathway (EMP) to direct oxidation of glucose by the enzyme (Fig 1.7). The enzyme is found to be inhibited by hydrogen peroxide, the by-product of gluconic acid production (Kleppe 1966). A study on glucose oxidase inactivation showed that only the reduced form of glucose oxidase is highly sensitive to hydrogen peroxide (Greenfield et al 1975).

The purified enzyme is used in various fields such as food, clinical analysis, mainly as glucose sensor, in the quantitative determination of glucose in body fluids and urine. It is used in food processing in the removal of glucose prior to the preparation of products such as dried eggs to reduce the non-enzymatic browning. It is also used in removing residual oxygen from fruit juices, beer, and wine and also from dehydrated packaged foods.

Reports on glucose oxidase localization are ambiguous. van Dijken (1980), Witteveen *et al.* (1993) reported that the enzyme of *A. niger* is intracellular and found in peroxisomes, where as Mischak *et al.* (*1985*) reported it as extracellular. There are also reports which have stated that it is intracellular prior to fungal autolysis (Zetalaki 1968). These varying reports on its location in the cell could be attributed to the differences of parameters and conditions adopted for the growth or due to the age of the fungal cultures. A little is known about the mechanisms of glucose oxidase export. Zetelaki (1970) associated export with autolysis of the fungus, whereas Mischak *et al.* (1985) reported that the glucose oxidase of *A. niger* was excreted after synthesis.

The gene encoding glucose oxidase of *A. niger* (goxA) has been cloned, and its amplification resulted in a 2-3 fold increase in activities (Witteveen *et al.*1993, Swart *et al* 1999, Fowler *et al* 1993). *A. niger* secretes multilple forms of catalases to shield itself against the arising hydrogen peroxide (Fiedurek & Ilezuk 1991), among which one has been cloned and characterised (Fowler et al 1993). Swart et al. (1999) described nine different complementation groups of glucose oxidase overproduction mutants. Gox B, gox C, and gox F belong to linkage group 11, gox 1 to linkage group 111, gox D and gox G to linkage group V, gox A and gox E to linkage group VII, and the linkage of gox H is unknown. Their study

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also indicates that gox A overproduction is regulated by the carbon source and oxygen in an independent manner. Knowledge about gene encoding lactonase is very narrow.

The gox-encoding gene of *P. variabile* P16 was isolated and characterized to identify the molecular bases of its high level of expression and in view of improving enzyme production by developing a process based on heterologous expression (Pulci, 2004).

There are some works carried out on the bacterial enzyme. A Tn*5*-induced glucose dehydrogenase (GDH) deficient mutant of *Gluconobacter oxydans* IFO 3293 was characterised. DNA sequencing showed that the insertion site occurred in an open reading frame with homology to the *pqqE* gene. It was shown that acid production could be restored by addition of the coenzyme pyrroloquinoline quinone (PQQ) to the medium. The *pqq* cluster of *G. oxydans* ATCC 9937 was cloned and sequenced. It has five genes *pqqA–E*. The cluster could complement the Tn*5*-induced mutation in IFO 3293. Pulsed-field gel electrophoresis suggested that the *pqq* genes are not closely linked to the *ribF* gene that produces the riboflavin cofactor for the gluconic acid dehydrogenase (Felder et al, 2000).

1.5. Solid-state fermentation

Solid-state fermentation process is defined as growth of micro-organism in the absence of free water. Solid-state fermentation (SSF) has been widely exploited for the production of many value-added microbial products and bulk chemicals (Pandey 1992, Pandey 1994). SSF offers various advantages when compared to SmF. This is partly because solid-state processes have lower energy requirements, produce lesser wastewater and are environmental-friendly as they resolve the problem of solid wastes disposal (Pandey, 2003). There has been much developments in the application of SSF in various areas such as bioremediation and biodegradation of hazardous compounds, biological detoxification of toxic agro-industrial residues, biotransformation of crops and crop residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites, etc (Pandey et al 2000). One among the various applications of SSF is the production of spores. With regard to this aspect, SSF is the role model technology and dominates SmF as it yields high quantity of spores.

Growth and proliferation of microorganisms cultured in SSF highly resemble their natural environments (i.e. growth on a solid support in the presence of limited free water)

enabling in producing spores. SSF being a cheaper and well adapted process meets the requirements of spore production with higher productivity, higher end concentration, higher product stability, etc. It also presents several advantages over SmF as it gives better yields of homogenous and pure spores (Larroche & Gros 1992). Research carried out reveal that SSF yields spores with desired properties when compared to SmF. Figure 1.8 shows some of the microbial spores commonly produced by SSF.

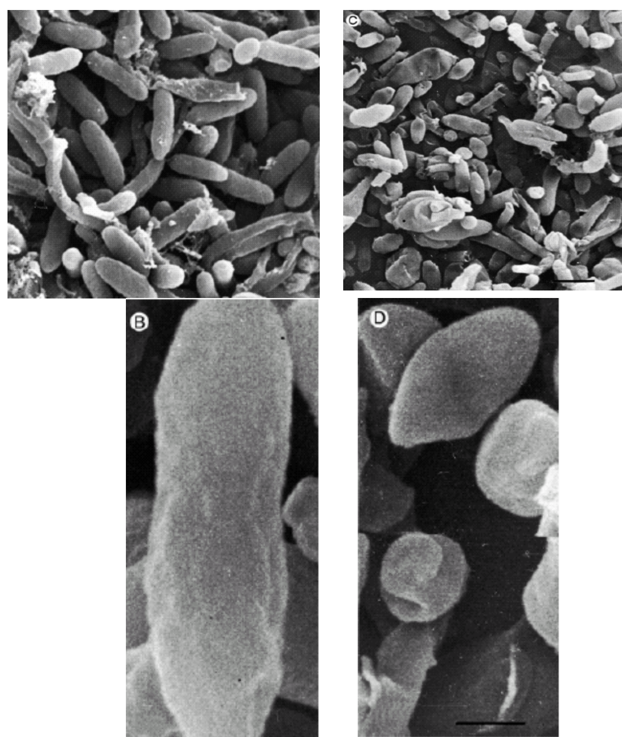

Fig. 1.8 Comparison of the spores produced by SSF and SmF - Scanning electron micrographs of spores of *V. lecanii* grown on rice (SSF) and submerged spores.
Top - left to right spore produced by SSF (low magnification), submerged spore (low magnification), low magnification ¼ 7000·, bar ¼ 2 lm. Bottom - left to right spore produced by SSF (high magnification), submerged spore (high magnification) where; high magnification ¼ 35,000·, bar ¼ 0.5 lm.[Courtesy Feng et al, 2002].

1.5.1. Production of spores by SSF

Spores of the microorganisms are generally used as inoculum in many fermentation processes. Also, spore form is most commonly used for maintenance and preservation of most of the fungi. These spores are developed in agar plates or slants containing nutrient medium. However for large-scale spore production, SSF using cheaper substrate is carried out.

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1.5.1.1. Bioreactors used for spore production

Several types of equipment are used for spore production in SSF such as petri dishes, bottles, wide-mouth Erlenmeyer flasks, etc which are simple without forced aeration and agitation, with temperature regulation by placing them in incubators. Generally, these are used for screening of substrates or micro-organisms in the preliminary stage. Scale up of the process for large-scale production of spores are carried out in bioreactors, mostly using non-mixed reactors such as packed-bed column, tray bioreactors and mixed reactors such as drum, stirred aerated bed reactors (Oostra et al 2000, Larroche & Gros 1989, Durand et al 1994). Autoclavable bags provided with micro strips for aeration are also used for spore production.

Packed-bed column reactors provide adequate temperature control (wall cooling) and aeration (forced aeration from bottom). However, scale-up of spore production in packed bed reactors is difficult. One of the severe problems encountered in large scale SSF spore production is the heat accumulation; high temperatures adversely affect the microbial growth and sporulation. Although forced aeration acts also as a convective cooling, water loss occur due to evaporation. This makes the substrate dry and critically affects the water activity, which in turn affects the sporulation process. Conductive cooling (wall cooling) is not efficient if the diameter of the reactor is large. Therefore, mixing is done to improve the heat removal. However, as discussed previously, intensive mixing impedes the fungal growth and affects the spore formation as mycelium is damaged and spore content is drastically reduced.

There are reports which use mixed system bioreactors which give high yield of spores. Scraped drum reactor was used for the production of *C. minitans* spores. A hollow scraper was situated over the length of the reactor near the wall for mixing at a rate of 0.2 rpm. Relatively high sporulation yield was observed, and the authors claimed that mixing improved the sporulation and spore production of *C. minitans* in large scale could be effectively scaled up using mixed bioreactor.

A comparative study of mixed and non mixed packed bed reactors was carried out by Durand et al 1994 for the production of *C. minitans* spores. In packed bed reactor (15 L) without mixing spore yield of 9×10^{14} conidia/m^3 was produced in 18 days. Reactor was well insulated; it mimicked a large-scale packed bed reactor concerning the absence of conductive cooling. The spore number was comparable with that of spores produced in mixed packed bed reactor of 50 L (6×10^{14} conidia/m^3) produced in 13 days (Durand et al 1994). This is because

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C. minitans tolerated the shear and collision forces due to mixing. This is not true with all the microorganisms.., for e.g. mixing of *Penicillium roquefortii* had a negative effect on the sporulation (Larroche et al 1986). A large scale solid-state fermentor was designed called Zymotis, in which the substrate bed remains static. This system is aerated and has an internal heat transfer plates for cooling. Productivity of conidiospores of *Trichoderma harzianum* using this bioreactor was five times higher as compared to the spores produced in Erlenmeyer flask containing agar medium (Roussos et al 1993).

Besides, these types of bioreactor, a reactor consisting of hollow metallic tubes coated with solidified medium containing extract of nutrient solution (wheat bran, corn flour) at both outer and inner surface was designed and exclusively used for spore production (Pandey et al 1996). Temperature and aeration was controlled and it was incubated at 28° C for 12-60 h. *Aspergillus niger* spores thus produced were found to produce 18% more glucoamylase enzyme than the spores produced in agar slants. Table 1.l gives a summary of various reactors used in spore production and spore yield.

Table 1.l. Various bioreactors used for spore production.

Bioreactor	**Spores**	**Spore Number**	**Ref**
Scraped drum reactor	*C. minitans*	$5x10^{12}$ /kg dry substrate	Oostra et al 2000
Zymotis bioreactor	*T. harzianum*	$5x10^{10}$/g substrate	Roussos et al 1991
Mixed packed bed reactor	*C. minitans*	$6x10^{14}/m^3$	Durand et al 1994
Packed bed column reactor without mixing	*C. minitans* *B. bassiana*	$9x10^{14}/m^3$ $1.07x10^{10}$/g dry weight	Durand et al 1994 Santa et al 2005
Drum reactor	*P. roquefortii*	$9x10^8$/ g dry substrate	Larroche et al 1986
Autoclavable bags	*B. bassiana* *C. minitans* *Clonostachys rosea*	$2x10^{12}$spores/g dry substrate 7.5×10^{12} spores/kg dry material 1.1×10^8 spores/g dry substrate	Tarocco et al 2005 Mc Quilken & Whipps et al 1995 Viccini et al 2006

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1.5.1.2. Fungal spores produced by SSF

Conditions and parameters for spore production often differ based on the microorganism used. Production parameters governing spore production of some commonly used fungi are discussed here. *Penicillium roquefortii* was cultivated on buckwheat seeds with initial moisture content of 1.5 gH_20/g IDM which resulted in spore yield of 1.5x 10^{10}/g IDM with productivity of 10^7 /h/g IDM (Larroche et al 1992). Maximal average productivity of these spores were 4x10^6, 4.4x10^6, 5.08x10^6, 6x10^6external spores/g IDM/h when cultivation was carried out in drum fermentor, column fermentor, non agitated 1-L bottle and semi-continuous 1-L bottle respectively. External spores are considered as useful spores as it is easy to extract as grinding the substrate is required to recover the spores formed inside the substrate (Larroche et al 1986). .

Conidia of *Coniothyrium minitans* was produced using oats as substrate and resulted in spore yield of 5x10^{12} spores/ kg dry oats after 450 h (Oostra et al 2000). Spore yield of 9 x 10^{14} conidia/m^3 packed bed of C. *minitans* was achieved in 18 days, using hemp impregnated with a solution containing 100 g/ dm^3 glucose and 20 g/dm^3 potato extract (Weber et al 1999). McQuilken & Whipps (1995) carried out cultivation of *C. minitans* in spawn bags (22.5 x 56 cm with microporus strip for aeration) containing oats at 18-20 °C and reported spore yield of 7.5x10^{11} spores /kg air dried material after 28 days of cultivation. Response surface methodology was performed to optimize the medium components for spore production of *C. minitans* (Chen et al 2005). They used wheat bran as substrate and carried out cultivation for 8 days, with the supplementation of nutrient solution achieved in producing spores of 1.04x10^{10} conidia/ g IDM.

Beauveria bassiana spores were produced using refused potatoes, coffee husks and sugarcane bagasse as substrates (Santa et al 2005). They reported that inoculum concentration of 10^7 spores/ g dry weight, initial moisture of 75% and temperature of 26°C allowed best spore production, which resulted in the spore yield of 1.07x10^{10} spores/ g dry weight in 10 days. Response surface methodology was carried out to optimise conditions for the maximal production of spores of *B. bassiana*. Conidial concentration of 2x10^{12} was obtained using parboiled rice (70 g) at pH 5-6 and a_w 0.999 (Tarocco et al 2005). Alves & Pereira (1989) obtained a yield of 2x10^{11} spores of *B. bassiana* / g powdered preparation using rice as a basic

growth substrate. Each 100 kg rice generated 3 kg conidial powder, i.e. 6x 10^{12} conidia / kg rice.

Spores of *Trichoderma harzianum* was produced by SSF using bagasse and cassava flour as substrates. Spore yield of $5x10^{10}$ spores/g cassava flour was achieved with a load of 21 kg moist medium. They reported that inclusion of feather meal to the medium resulted in 5 times increase in the spore production and use of bagasse absorbs the conidiospores and imparts protection during vacuum concentration (Roussos et al 1991).

SSF of *V. lecanii* on wheat bran and beet pulp mixture resulted in spore yield of 3.2 x 10^9 spores/g dry matter (Grajek 1994). Similar spore yields (1.5 $x10^9$ spores/g) were obtained when SSF was carried on 100 g cooked rice cultivated at 24° C for 2 weeks (Feng et al 2002). Spores of *Metarhizium anisopliae* was produced using rice bran and rice husk (1:1 on dry weight basis) as substrate with a initial moisture content of 47% at a flow rate of 0.34 l^{-1} g^{-1} for two weeks at 27°C (Dorta & Arcas 1998). Production of *Penicillium nalgoviensis* conidia on bread took 18 days to reach a spore count of $1x10^9$ /g solid substrate (Holker 2000). Spores of *Mucor bacilliformis* was produced on polyurethane foam containing nutrient solution (C/N=5) at an initial moisture of 90% with spore yield of 6x10^{8} spores/g inert support (Lareo et al 2006). Table 1.m gives a summary of various substrates used for the production of spores by SSF.

1.5.2. Advantages of SSF over SmF process in spore production

It is a well known fact that the properties of some products produced by SSF are found to be superior to those produced by SmF processes. For e.g. fungal glucosidase produced by SSF is more thermotolerant than the enzyme produced by SmF (Deschamps & Huet 1984). Similarly, with respect to the production of spore, SSF dominates the fermentation processes, this attraction prevails as the SSF system truly resembles the natural way of microbial life (especially that of fungi). This factor is responsible for the differences in morphological, functional and biochemical properties of spores produced by SSF and SmF, enabling the spores produced by former fermentation process to survive longer at natural and drastic environmental conditions. In SmF, some fungi are unable to sporulate (*Penicillium* spp), most fungi imperfecti do not produce conidia, but produce thin-walled mycelial fragments called blastospores, which are short lived in adverse conditions (Ferron 1978, Mc Coy et al 1984); some fungi produce only low amounts of spores e.g. *C. minitans* (McQuilken *et al* 1997). On the contrast, SSF has been reported to yield not only high quantity of spores, but better

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qualities such as greater resistance to UV-radiation and dessication during recovery process. Further more, they are highly viable (Munoz et al 1995).

Table 1.m. Various substrates used for spore production by SSF.

Spores	Substrate used	Ref
Metarhizium anisopliae	Mixture of rice bran and rice husk (1 :1)	Dorta & Arcas 1998
Coniothyrium minitans	Oats Wheat , wheat bran	Oostra et al 2000, Chen et al 2005 McQuilken & Whipps 1995 Durrand et al 1994 Weber et al 1999
Verticillium lecanii	Rice, rice bran, rice husk	Feng et al 2000, Feng et al 2002
Beauveria bassiana	Clay microgranules refused potatoes, coffee husks, sugarcane bagasse parboiled rice, caupi, sorgo, broad beans, beans, cassava bagasse, rye flour, cassava flour, wheat bran, rice husk	Desgranges et al 1993 Santa et al 2005 Tarocco et al 2005 Burtet et al 1997, Soccol et al 1997 Calderon et al 1995 Vilas Boas et al 1996
Mucor bacilliformis	Polyurethane foam	Lareo et al 2006
Penicillium roquefortii	Buckwheat seed	Larroche & Gros 1989, Desfarges et al 1987, Larroche et al 1988, Larroche & Gros 1992, Larroche et al 1986
Penicillium nalgoviensis	Bread	Holker 2000
Trichoderma harzianum	Bagasse	Roussos et al 1991
Aspergillus niger	Buckwheat seed	Moksia et al 1997
Clonostachys rosea	Rice	Viccini et al 2006
Bacillus thuringiensis	Wheat bran, rice, ground soybean, rice husk, lime powder	Devi et al 2005, Capalbo et al 2001, Foda et al 2002, Wang 1988,
B. sphaericus	Cottonseed meal, linseed meal, wheat bran, sesame seed meal	Foda et al 2003

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Morphology of the *V. lecanii* spores produced by SSF was compared with the spores produced by SmF (Feng et al 2002). Figure 6 shows the SEM images of the spores produced by SSF and SmF. Spores produced by SSF were cylindrical, ellipsoid and relatively uniform in size with rough brittle surface, while spores from SmF were of irregular in shape and size (oval to oblong) and had smooth surface. It is considered that the warty, brittle outer layer of aerial conidia renders strong protection and resistance to adverse environmental conditions (Gooday 1981). Spores produced by SSF are hydrophobic and are resistant to stress and are highly durable when compared to submerged spores which are hydrophilic (Munoz *et al* 1995). They reported that the increased concentration of a large (14 kDa) hydrophobin-like protein excreted to the surface of aerial spores is responsible for the hydrophobicity of spores produced using solid culture.

Pascual *et al* (2000) found that spores of *Penicillium oxalicum* obtained by aerial conditions had a higher surface hydrophobicity, better rate of survial after 27 weeks of storage, were less damaged by freeze-drying, and had higher biocontrol activity when compared to submerged conditions and thus concluded that SSF is the most suited system for the mass production of spores. There are some studies which use non agitated SmF process for spore production (McQuilken 1997), however this technique is unsuitable for scale-up as it requires large surface area, thus SSF stands as the appropriate spore production technique (Oostra et al 2000). Spores of bacteria produced by SSF also are comparatively superior to those produced by SmF. Devi et al 2005 claimed that *Bt* spores produced by SSF was not only effective against larvae of castor semilooper, but the material cost for production was cheaper too (one kg of Bt would be US$ 0.70). Apart from being simple and economical, spores production by SSF when compared to SmF does not require higher cost for down stream processing, as it can be used directly. Spores of *B. thuringiensis tolworthii* were used directly in maize fields to control fall armyworm (Capalbo *et al* 2001).

1.5.3. Applications of spores produced by SSF

Spores possess some interesting properties which make them a candidate member for various applications in the bioprocess technology. Spores of fungi are used as inoculum for most of the fermentation process including small scale and large scale level. Apart from these, mainly spores are used as biocontrol agents (insecticide, fungicide, pesticide), biocatalysts in various bioconversion reactions.

Spores are also known to possess high biocatalytic activity because they generally exhibit high catalytic activity in terms of dry matter. Thus they are used in various biotransformation reactions. Spores of *Penicillium roquefortii* carried out transformation octanoic acid → 2-heptanone and produced blue cheese flavour (Larroche & Gros 1989). Spores of *Aspergillus niger* produced by SSF showed stable glucose oxidase activity even after long storage time and carried out biotransformation reaction of glucose to gluconic (Moksia *et al* 1996). Table 1.n shows some reactions catalysed by spores of fungi produced by SSF.

Table 1.n Reported applications of spores produced by SSF as biocatalyst.

Spores	**Substrate used in SSF**	**Bioconversion reaction**	**Ref**
Penicillium roquefortii	Buckwheat seed	Octanoic acid → 2-heptanone	Larroche & Gros 1989 Creuly et al 1990
Aspergillus niger	Buckwheat seed	Glucose → gluconic acid	Moksia et al 1996
Penicillium digitatum	Agar medium	geraniol, nerol, citral, and geranic acid → methylheptenone.	Wolken & Werf 2001

Spores of *Bacillus thuringiensis* produced by SSF is applied as control agent against plant pests such as caterpillars of Lepidoptera, mosquito larvae, locusts, grasshoppers, etc (Devi et al 2005, Capalbo et al 2001). Spores of *C. minitans* produced by SSF are used against the control of fungal pathogen *Sclerotinia sclerotiorum* that reduces yields of many crops. Spores of *Colletotrichum truncatum* protects soybean crop against *Sesbania exalta* (Silman *et al* 1993. Spores of *V. lecanii* control white fly invasion of various crops (Barranco-Florido *et al* 2002). Spores of *Beauveria bassiana* is used to control sugarcane borer and European corn borer, invading maize crop (Arcas *et al* 1999, Desgranges *et al* 1993). A list of microbial spore produced by SSF applied as biocontrol agents is illustrated in Table 1.o.

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Table 1.o Application of spores produced by SSF in biological control uses.

Spores	Substrate used	Target organism	Crop protected	Ref
Beauveria bassiana	Wheat bran, rice husk (1:1)	*Diatraea saccharalis* (sugarcane borer)	Maize	Arcas et al 1999
Beauveria bassiana	Clay microgranules (inert support)	European corn borer	Corn	Desgranges et al 1993
Bacillus thuringiensis	Wheat bran	Larvae of castor semilooper	Castor beans	Devi et al 2005
Bacillus thuringiensis	Rice	Fall armyworm	Maize	Capalbo et al 2001
Verticillium lecanii	Sugarcane bagasse	White flies	Various crops	Barranco-Florido et al 2002
Coniothyrium minitans	barley, barley-rye-sunflower, bran-vermiculite, bran-sand, maizemed-perlite, millet, oats, peat-bran, rice and wheat	*Sclerotinia sclerotiorum*	Lettuce	McQuilken & Whipps 1995
Colletotrichum truncatum	Perlite corn meal agar Vermiculite	*Sesbania exaltata*	Soybean	Silman et al 1993
Clonostachys rosea	Rice	*Botrytis cinerea*	Strawberries	Viccini et al 2007

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Chapter 2

Materials and Methods

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2.1. Microorganisms and maintenance

Strains of *Aspergillus niger* NRRL 3, *A. niger* NRRL 567, *A. niger* NRRL 599, *A. niger* NRRL 67, *A. niger* NRRL 1737 (Fig 2.1) were used in the study. They were maintained on Potato Dextrose Agar medium at 30°C for 7 days and stored in freezer (-20°C) after development. Microorganisms were sub-cultured fortnightly.

Fig 2.1. Strains of *A.niger* used in the study. (From left to right - *A. niger* NRRL 567, *A. niger* NRRL 599, *A. niger* NRRL 67, *A. niger* NRRL 1737, *Aspergillus niger* NRRL 3).

2.2. Substrates

Buckwheat seeds, rice, corn, cassava, jack fruit seeds, wheat bran and cassava bagasse (Fig 2.2) were used as substrates in solid-state fermentation for the production of spores. Buckwheat seeds were obtained from Fertinature Semences, Montluçon (France). Corn was purchased from a local shop in Mumbai (India). All other substrates were obtained from local shops in Trivandrum (India).

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Fig 2.2. Substrates used in the study. (From left to right 1st row - buckwheat seeds, rice, corn; 2nd row - jack fruit seed, cassava bagsse, wheat bran).

2.3. Selection of fungal spores for the use as catalyst in the bioconversion of glucose to gluconic acid

Spores of *Aspergillus niger* NRRL 3, *A. niger* NRRL 567, *A. niger* NRRL 599, *A. niger* NRRL 67, *A. niger* NRRL 1737 were produced by solid-state fermentation. Solid state fermentation was carried out using buckwheat seed as substrate as mentioned below. Spores of these strains were then screened for the utilisation as catalyst in the bioconversion of glucose to gluconic acid.

2.4. Solid-state fermentation (SSF)

2.4.1. Preparation of inoculum

Ten ml of distilled water containing 0.1% Tween-80 was transferred to a sporulated (7-days old) PDA slant culture or a PDA petridish. The spores were dislodged using the inoculation needle under aseptic conditions and the suspension, with appropriate dilution was used as inoculum.

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2.4.2. Preparation of media

2.4.2.1. In Erlenmeyer flask

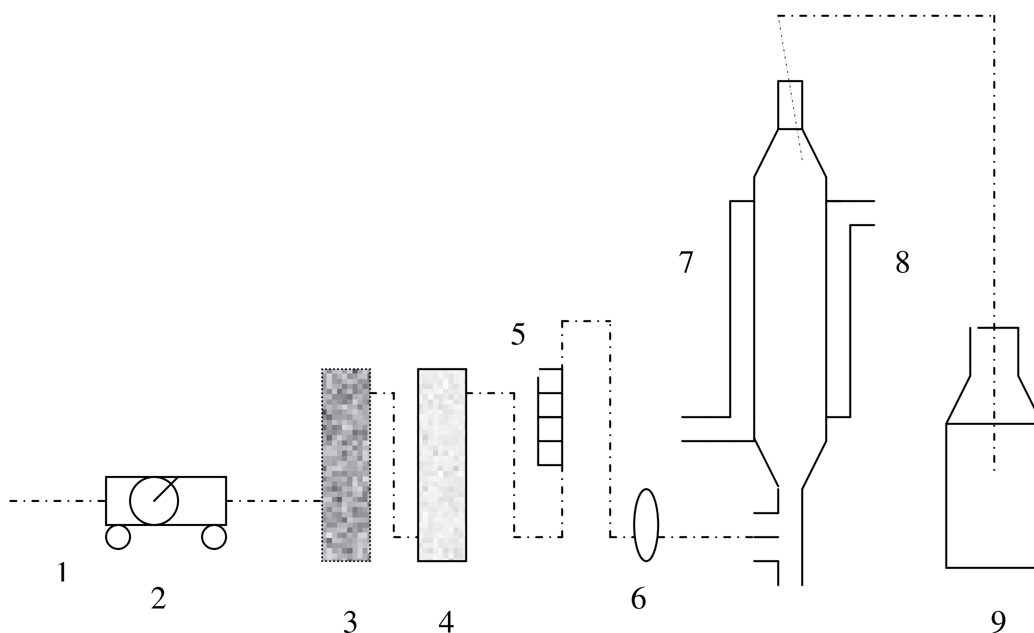
Twenty grams of dry substrates were taken into a 500 ml Erlenmeyer flask and distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121°C at 15psi for 15 min. This process also enabled the softening of the substrates for the penetration of mycelia. Autoclaved media were inoculated with approximately 3 x 10^6 spores/ g of substrate. Fermentation was carried out at 30°C for 200 h. Initial moisture of the substrate was 0.5g H_20/g IDW (Initial dry weight). Sampling was performed at regular intervals of 24 h. All the experiments were done in two sets and averages values are reported.

2.4.2.1. In Fixed bed column reactor

Buckwheat seeds (300 g) were washed with distilled water and allowed to cook with equal amount of distilled water in a boiling water bath (100 °C for 15 minutes) to soften the tissues. After this, the water was drained off; the heat resistant antibiotic chloramphenicol (1 mg/g dry buckwheat seed) was added to it and the seeds were autoclaved (121 °C at 15 psi for 15 min.). Thermostated fixed bed glass column ((inner diameter- 5 cm; height- 21 cm) fermentor with a jacket for circulation of water to control the temperature was used for the study. A suspension containing 10^6 spores/ml was prepared and used as inoculum (10^8 spores/ 100 g of buckwheat seeds). It was aseptically transferred into the SSF medium. The column was packed with pre-inoculated substrate with a bed height of 21 cm. The fermentor was supplied with continuous aeration from the bottom of the bioreactor at 0.075 *l* min^{-1} (free of CO_2, by passing it through a KOH solution column). Schematic representation of the column reactor is shown in Fig 2.3. Cultivation was carried out at 30 °C for 200 h. Sampling was performed at regular intervals of 24 h. The exhaust gas was collected in a bottle containing 0.5moles/L KOH to detect the carbondioxide evolved. Initial moisture of the substrate was 0.5g H_20/g IDW (Initial dry weight). Production of spores by SSF in column fermentor is shown in Fig 2.4.

2.5. Recovery of spores for bioconversion

SSF medium was harvested at different time periods (every 24 h). Spore suspension was obtained by adding 0.1% (v/v) Tween 80 solution (ratio 1 g/ml) and shaken at 180 rpm for 1 h. It was then filtered using gauze cloth to remove the substrate from the spore



1	Air
2	Air regulator
3	Drierite
4	KOH
5	Flowmeter Brooks R215 AAA
6	Filter 0.5µm
7	Glass column
8	Double jacket
9	Exhaust gas (CO_2)collected in KOH bottle

Fig 2.3 Schematic representation of the fixed column bed reactor.

suspension. The filtrate was collected and centrifuged at 10,000 g for 10 min. The supernatant was discarded and the pellet was used as spore source.

2.6. Analysis of SSF medium

Samples collected from SSF culture at intervals were appropriately diluted with 0.1 % Tween 80 and homogenised with an ultraturrax blender. This homogenised mixture was utilised for further anlaysis. Diagrammatic representation of the analysis carried out during SSF is shown in Fig 2.5.

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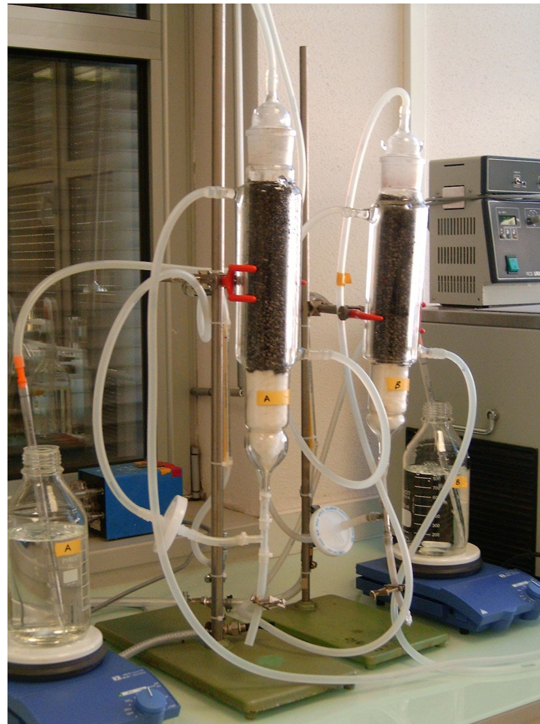

Fig 2.4. Production of spores by SSF in column fermentor.

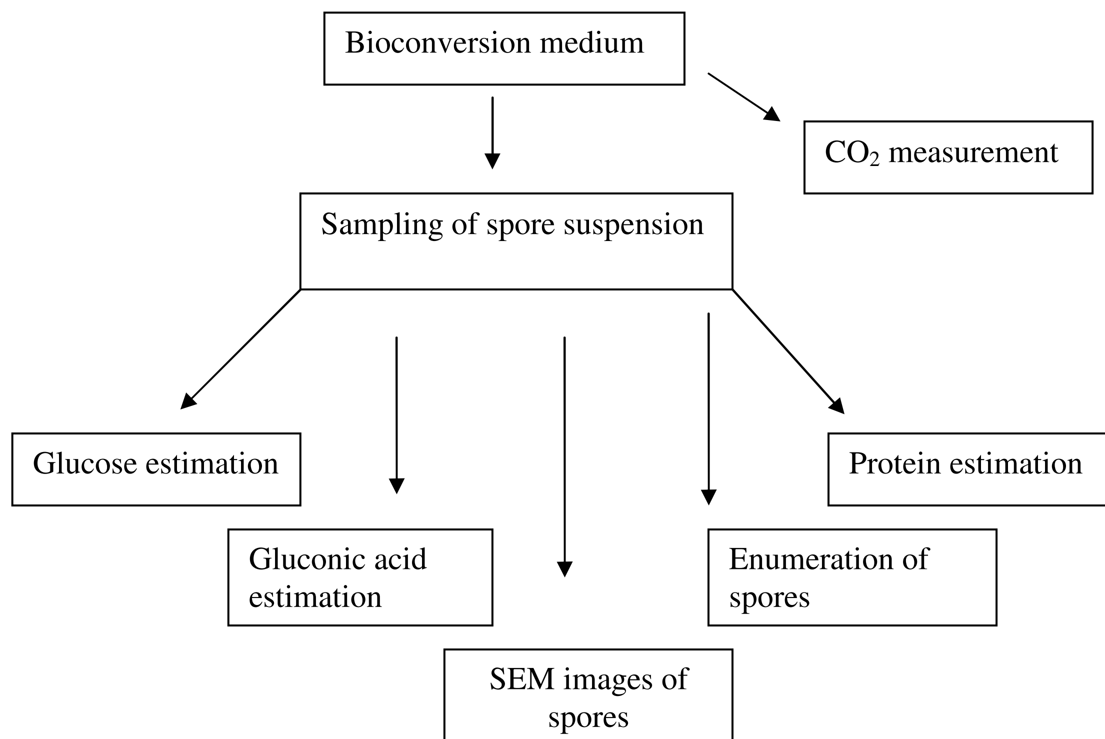

Fig 2.5. Flow chart of the analysis carried out during bioconversion.

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2.6.1. Total sugar

For the determination of starch, the homogenised mixture was treated at 100 °C for 15 min, followed by the hydrolysis at 55 °C for 20 min with 0.1 ml of 1g/L of amyloglucosidase (SIGMA). This was centrifuged at 10 000 g for 5 min and the supernatant was collected and assayed for reducing sugar DNS method (Sumner 1925).

Composition of the DNS reagent was as follows (g/L):

2,3 dinitrosalicylic acid, 10

NaOH, 16

Sodium potassium tartarate, 300

To the sample solution 0.6 mL of DNS reagent was added and kept in boiling water bath (100°C) for 5 min. The reaction was stopped by placing the reaction tubes in ice bath. After cooling, 6mL of water was added to the tubes and optical density was measured at 540 nm against the blank. The blank contained distilled water instead of sample solution and the reagent.

2.6.2. Soluble reducing sugar

Soluble reducing sugar of the sample was analysed using DNS method (Sumner 1925) as mentioned above.

2.6.3. Extracellular protein

Soluble protein content was measured by Lowry method modified by Peterson 1983.

Three stock solutions were prepared namely

1.CPS (Copper sulphate pentahydrate - 0.1%, Potassium tartarate - 0.2%, sodium carbonate – 10%),

2. NaOH (0.8N)

3.Folin Ciocalteu reagent (2N).

Solutions 1 and 2 were mixed with water in the ratio 1:1: 2 and named as solution A.

Solution 3 was diluted 6 times and named as solution B.

To 200µL of sample 850 µL of water and 100µL of solution A was added. It was agitated and incubated at 20°C for 10 min after which 500 µL of solution B was added. This was then shaken well and the coloration was read at 750 nm after 30 min of incubation. Bovine serum albumin (0-7g/L) was used as standard.

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2.6.4 Spore number

Spore count was performed using haematocyte counter (Malassez cell) under the microscope.

2.6.5. Dry weight of spore

Spore suspension (Tween 80 at 0.05%) made from spores recovered from solid-state fermentation was centrifuged for 15 min at 10 000 g. The supernatant was eliminated and washed two times with 10 mL distilled water. Pellets collected were dispensed in 5mL of distilled water. One millilitre of this suspension was used for the numeration of spore (N_1). Rest of the suspension (V_1) was collected in a preweighed aluminium pan (M_1). This was placed in oven at 110°C for 24h. Weight of the aluminium pan containing dried spore was measured (M_2).

Dry weight of the spore (Ds) was calculated using the formula

$$Ds = \frac{M_2 - M_1}{V_1 . N_1}$$

2.6.6. Determination of initial dry weight of a sample

A sample was divided into two aliquotes, one for initial dry weight (IDWt) measurement, the other for analytical purposes. IDWt was calculated by the following equation

$$IDWt = Gt \times IDW0$$

where IDW0 was the average dry weight of grains before inoculation and Gt the number of buckwheat grains.

The corresponding dry weight DWt was obtained by drying the same sample in an oven at 110°C for 24 h. These two data allowed calculation of the ratio DWt/IDWt which was used to convert analytical measurements which were obtained related to DWt .

2.6.7. Carbondioxide evolution

The outlet air from the fermentor was collected in 0.5M KOH. Periodic assay of this solution for its carbonate content using titrimetric method was done (Vogel 1961).

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$$2\ KOH + CO_2 \longrightarrow CO_3^{2-} + H_2O + 2K^+$$

It should be taken care that KOH be replaced once it gets exhausted. Otherwise carbondioxide gets collected in distilled water giving the following reaction

$$CO_2 + H_2O \longrightarrow HCO_3^{2-} + H^+$$

The method for determining the carbonate content involved two steps. Firstly, OH – and the carbonates were neutralised by 0.127 N HCl. This was done in the presence of phenolphthalein. Volume of the acid added (V_1) was noted when the indicator turns it color from pink to colorless at pH 8.3. The second reaction was started by adding bromophenol blue to the sample solution. Again HCl was added to neutralise till the color changes from blue to yellow at pH 4.3. Total volume of HCl (V_2) required to neutralise was noted. The quantity of KOH in the solution is calculated by the following equation

$$(CO2)\ \text{moles} = (CO_3^{2-})\ \text{moles} = N.V_S \frac{(V_2-V_1)}{Va}$$

N = normality of HCl (0.127 mole/L)

V_S = Total volume of the CO_2 trapped sample

Va = volume of the CO_2 trapped solution taken for the assay

V_2 and V_1 as mentioned earlier is the volume of HCl.

It should be noted that $V_1 < \frac{V_2}{2}$

If it was not the condition, the KOH solution should be replaced with fresh solution.

2.7. Bioconversion of glucose to gluconic acid

2.7.1. In Erlenmeyer flask

Bioconversion was carried out in 250 mL, 500 mL Erlenmeyer flask with 50 mL and 100 mL medium respectively containing (g/L) glucose 40 and sodium azide 0.01, unless and otherwise mentioned. Calcium carbonate slurry (40 g/L) was used as neutralising agent and was separately added to the medium after sterilization. The flasks were incubated at 30° C on a rotary shaker at 200 rpm. The pH of the media was maintained at 6.5. Spore concentration in the media was 1 x 10^{10}spores/ml.

2.7.2. In bioreactor

For sodium gluconate production, a bioreactor (Applikon, The Netherlands) of 1.5 L working volume was used (Fig 2.6). The medium contained (g/L) Glucose 100, sodium azide 0.01. pH was maintained throughout the process at 6.5 with 5 mol/L sodium hydroxide solution. Bioconversion reaction was carried out at 600 rpm, with aeration rate of 0.075 l min^{-1} and temperature of 30 °C. In this case too, spore concentration in the media was 1 x 10^{10} spores/ml.

Fig 2.6 Bioconversion carried out for the bioconversion of glucose to gluconic acid, neutralised by 0.5M NaOH in 1.5L bioreactor.

Fed-batch production of gluconic acid was carried out in 2-litre fermentor (Biostat Braun, Germany, see Fig 2.7). Medium contained only glucose with an initial concentration of 100 g/L and periodically glucose was added in powder form. pH was continuously adjusted to 5.8 with a 2M sodium hydroxide solution or potassium hydroxide. The reactor was fed with air free of CO_2 by passing it through a KOH pellets column. The aeration rate was 0.15slpm and stirring speed was 600 rpm. Spore concentration in the media was 1 x 10^9spores/ml.

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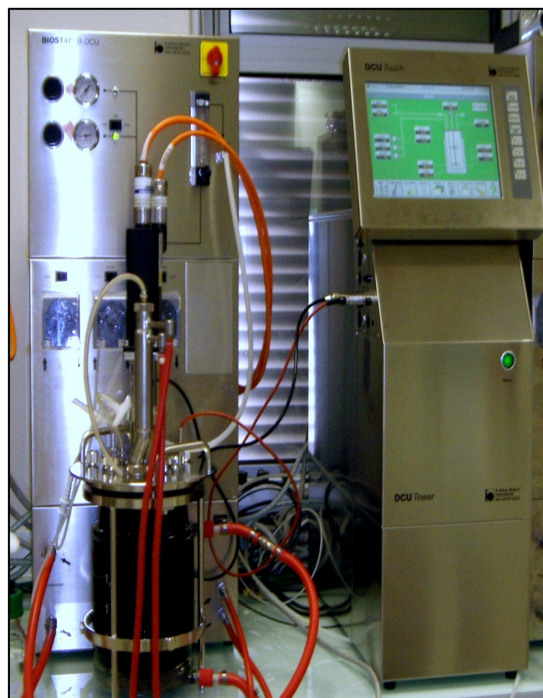

Fig 2.7 Fed-batch production of gluconic acid carried out in 2-litre fermentor

2.8. Analysis of bioconversion

Sampling was done at regular intervals by collecting the spore suspension from the bioconversion medium. It was subjected to centrifugation to remove the spore pellets. The supernatant collected was used for various analyses. Spore count was done by visualizing them using haematocyte counter under light microscope.

2.8.1. Glucose

Glucose was estimated by DNS method (Sumner 1925) as mentioned above.

2.8.2. Gluconic acid

Gluconic acid produced was measured by the method of Moellering & Bergmeyer (*15*) using the Boehringer assay kit (R-Biopharm France). D-Gluconic acid (D-gluconate) is phosphorylated to D-gluconate-6-phosphate by adenosine-5'-triphosphate (ATP) in the presence of the enzyme gluconate kinase with the simultaneous formation of adenosine-5'-diphosphate (ADP)

(D-Gluconate + ATP —gluconate kinase→ D-gluconate-6-P + ADP

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In the reaction catalyzed by 6-phosphogluconate dehydrogenase (6-PGDH), D-gluconate-6-phosphate is oxidatively decarboxylated by nicotinamideadenine dinucleotide phosphate (NADP) to ribulose-5-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH)

$$\text{D-Gluconate-6-P} + NADP^{+} \xrightarrow{\text{6-PGDH}} \text{ribulose-5-P} + NADPH + CO2 + H^{+}$$

The amount of NADPH formed in the above reaction is stoichiometric to the amount of D-gluconate. The increase in NADPH is measured by means of its light absorbance at 340nm.

Reagents:

Solution 1: 4.5 g of powder mixture, consisting of triethanolamine buffer, pH approx. 7,6; NADP, approx. 60 mg; ATP, approx. 150 mg; magnesium sulphate was dissolved in 27mL of distilled water;
Solution 2: 0.5 ml 6-PGDH suspension, approx. 110 U
Solution 3: 0.5 ml gluconate kinase suspension, approx. 13 U

To 1mL of solution 1, 100µL of the sample and 1.9 mL of distilled water was added and vortexed. 20µL of solution 2 was added and the absorbance (A1) was read at 340 nm after approx. 5 min. Solution 3 (20µL) was added, mixed and the absorbances of the solutions was read at 340 nm (A2) after the completion of the reaction (approx. 20 min). Blank contained 100µL of distilled water instead of the sample. The absorbance differences (A2-A1) for both, blank and sample was detrmined. The absorbance difference of the blank from the absorbance
difference of the sample was subtracted.

ΔA = (A2-A1)sample - (A2-A1)blank

It was made sure that the measured absorbance differences be at least 0.100 absorbance units to achieve sufficiently precise results
According to the general equation for calculating the concentration:
V = final volume [ml] = 3.040 mL
v = sample volume [ml] = 0.100 mL
MW = molecular weight of the substance to be assayed [g/mol] = 196.1

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d = light path [cm] = 1

ε = extinction coefficient of NADPH at 340 nm = 6.3 [l × mmol-1 × cm-1]

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

$$c = \frac{3.040 \times 196.1}{\varepsilon \times 1.00 \times 0.100 \times 1000} \quad X \quad \Delta A \quad \text{(g D-gluconic acid /L sample solution)}$$

2.8.3. Electronic images

Pictures of spores were recorded using scanning electron microscope JEOL –JSM 820 (Tokyo, Japan) equipped with secondary electron detector. Samples were mounted using a conductive carbon double sided sticky tape. A thin coating of gold sputter was deposited onto the samples to reduce the effects of charging.

2.8.4. Oxygen and carbondioxide analysis

Measurement of O_2 consumption and CO_2 production rates of the microorganism during bioconversion carried out in a fermentor were done by a gas analyser Servomex Xentra 4100 (Servomex Compagny Inc, Nerwood USA).

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2.9. Prevention of germination and growth to use spore as a reservoir of enzyme

Spores recovered from solid-state fermentation medium were stored at -20°C for 24h. They were subjected to repeated washings (5, 8, 15 times with 50 ml of distilled water each time) and used as catalyst in the bioconversion medium. To fully arrest the fungal growth and to ameliorate the yield, incorporation of different components such as sodium azide (0.01g l^{-1}) and 0.72 g NaCl l^{-1} with 0.01% of polysorbate (v/v) were also tried.

2.10 Permeabilization studies

Various chemicals tested were hexane (40 g l^{-1}), ether (40 g l^{-1}), toluene (40 g l^{-1}) and Triton X 100 (0.1 g l^{-1}). They were incubated with 50ml of spore suspension containing 10^{10}spores/ml for 8 h in a rotary shaker at 180 rpm. Then the medium components were added and the bioconversion was commenced by adding glucose (40 g l^{-1}), calcium carbonate (40 g l^{-1}) and sodium azide(0.01g l^{-1}). Spores were treated with enzyme, acid, detergent, high temperature, sonication to check whether these could help in permeabilization. Enzyme

cocktail from Novo Nordisk called Glucanex™ containing β (1,3) glucanase, β (1,3), (1,6) glucanase, chitinase at a very high concentration of 0.6g/L was added to the medium, shaken for 18h before starting bioconversion. Acid treatment of spore suspension was carried out by incubating them with 10% acetic acid for 2 h before bioconversion. Heat treatment of spores was done by incubating them at 60°C for 30 min before bioconverion. The effect of freezing -20° C, -80° C, 4° C, 8° C of spores was studied by exposing spores in dry state to different time period. Details are mentioned in Ch 4. Permeabilized and unpermeabilized spores (10^{10} spores/ ml, 10^7 spores/ ml) were exposed to pressure (1, 2, 2.7 kb) using One Shot Model Cell Disruptor (CDS, Montreal, Canada). At 2.7 kb pressure, 3 and 5 passages were carried out. Unpermeabilized spores after pressure treatment were permeabilized by freezing and some of the unpermeabilized spores were directly used in bioconversion reaction without freezing to check whether the pressure treatment aids in permeabilizing the spores.

2.11. Effect of PCA as spore germination inhibitor

Bioconversion was carried out as mentioned above with freeze-thawed spores (kept at -20°C for 48 h) treated with phenazine -1- carboxylic acid at 140 µg/ml.

2.12. Effect of sodium azide in the glucose oxidase activity

To 1g/L of glucose oxidase (EC 1.1.3.4. - type II from *A. niger.,* Sigma Chemical Co.) sodium azide (0.01 g/L) was added and bioconversion was carried out as mentioned above. A control was maintained without the addition of sodium azide.

2.13. Use of different terpenes as permeabilizing and spore inhibiting agent

Fresh spores (10^{10} spores/ml) after the recovery from SSF medium were treated with terpenes such as alpha pinene, alpha pinene oxide, beta pinene, beta pinene oxide, carvone, carveol, citral, citronellal, limonene, limonene oxide, linalool, myrcene, myrtenol, isonovalal, orcinol, terpeniol. Spores were treated with 10% of these chemicals for 18 h at 30°C in a rotary shaker at 200 rpm unless and otherwise mentioned. Bioconversion was carried out with 50 ml of medium by adding the substrate and neutralising agent as mentioned above.

2.14. Optimization of citral concentration

Fresh and frozen spores (10^9 spores/ml) were treated with different concentrations (2, 3, 4, 5, 6 %) of citral for different time period (3, 4, 5, 6 h) for the optimal bioconversion condition. Bioconversion was carried out in Erlenmeyer flask containing 50 ml of media by

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adding the substrate and neutralising agent. Bioconversion was carried out at 30°C in a rotary shaker at 200 rpm.

2.15. Optimization of spore concentration

Bioconversion was carried out with various concentrations of fresh and frozen spores (10^7-10^{10} spores/ml) using the optimized concentration of citral. Bioconversion was carried out in Erlenmeyer flask containing 50 ml of media by adding the substrate and neutralising agent. Bioconversion was carried out at 30°C in a rotary shaker at 200 rpm.

2.16. Effect of terpenes on the glucose oxidase activity at different temperature

Citral, limonene and isonovalal at the concentration of (1 and 10%) were added to the solution. Bioconversion was started by the addition of substrate and the neutralising agent as mentioned above**.** Also, bioconversion was carried out with citral treated spore at 50, 60 and 70°C. A control experiment was carried out with commercial glucose oxidase (1g/L) prepared in distilled water.

2.17. Bioconversion carried after exposure of spores to different temperature

Permeabilized frozen spore suspension after thawing were exposed to different temperatures of 60° C (10, 30, 60, 90 min), 70°C (5, 6, 7, 8, 9, 10, 15 min), 80°C (1, 2, 3, 4 min), 90°C for (1, 2, 3, 4 min). These spores are termed as permeabilized spore before heat treatment (SPBH). Unpermeabilized fresh spores were also exposed to temperatures such as 60° C (10, 30, 60, 90, 120, 150, 180, 210 min), 70°C (10, 15, 20, 30, 60, 75, 90, 105, 120, 135 min), 80°C (1, 2, 3, 4 min), 90°C (1, 2, 3, 4 min) and then were frozen at -20°C for 48 h. These spores are termed as permeabilized spore after heat treatment (SPAH). Commercial glucose oxidase of 1g/L concentration was prepared in distilled water and exposed to 70°C (5, 10, 12 min), 80°C (1, 2, 3, 4 min) and 90°C (1, 2, 3, 4 min).

2.18. Reutilization of spores as catalyst

Spores recovered from SSF were utilised as catalyst in the bioconversion for various cycles. Bioconversion was carried out in Erlenmeyer flask containing 50 ml of media. Spore count was maintained at 10^9 spores/ml. Glucose at a concentration of 50g/L was used and neutralization was carried out by the addition of calcium carbonate slurry. At the end of bioconversion, spores were recovered by centrifugation and washed with distilled water. Harvested spores were transferred into the fresh medium and bioconversion was carried out at 30°C in a shaker maintained at 200 rpm. Thus bioconversion was carried out till 14 cycles.

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2.19. Stability of enzyme activity of spores

Biocatalytic activity of *Aspergillus niger* spores stored at -20°C for one year was checked for its biocatalytic activity. Bioconversion was carried out in Erlenmeyer flask containing 50 ml of media. Spores (10^9 spores/ml) treated with 3% citral for 3h were used for the study. Glucose at a concentration of 50g/L was used and neutralization was carried out by adding calcium carbonate slurry. The bioconversion pattern was compared to that of spores stored at -20°C for 24 h.

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Chapter 3

Production of spores of *Aspergillus niger* by solid-state fermentation

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3.1. Introduction

Various studies have been carried out which use solid-state fermentation as a means for the production of fungal spores (Larroche et al 1994, Larroche & Gros 1992). Solid-state fermentation gives rise to better yields of homogenous and high volume of spores (Pandey et al 2000a). Going by theoretical classification based on water activity, SSF well supports the growth of fungi and its spore production (Pandey et al 2000a, Pandey 2003). Various advantages and significance of the production of spores by SSF is detailed in Ch 1.

Though spore production by SSF process is simple, many environmental factors such as moisture content, water activity, temperature, oxygen levels and concentration of nutrients are taken into consideration as they significantly affect the microbial growth and sporulation. Selection of substrate is one of the most critical steps in spore production process in solid substrate fermentation as it provides both nutrition and support. Nutritional requirement of the microorganism is a crucial parameter for its growth and sporulation. Sporulation is associated with the synthesis of macromolecules which are needed to produce the sporulation-specific structures and storage molecules needed for germination. Spore formation occurs without overall protein synthesis, but with the formation of wall. Thus chitin content at the end of growth is considered as a good indicator of spore formation in fungi (Desfarges et al 1989). Supplementation of starchy material and trace elements are considered to increase the spore production. However, it is believed that certain nutrient limitation must be maintained to achieve a good sporulation yield (Larroche et al 1988). Research showed that free reducing sugars accumulation (Moo Young et al 1983) or nitrogen supplementation such as NH_4 and urea (Raimbault 1981) does not induce sporulation. Cellulosic substrates are ideal for spore production as it provides better aeration, less compaction problems and greater growth surface area (Lonsane et al 1985). However, starchy substrates are the best suited for spore production. For e.g., buckwheat seed renders excellent mechanical properties (retention of structure, lack of agglomeration) along with high external sporulation (Larroche & Gros 1989. Wheat, wheat bran, rice, rice husk, rice bran, buckwheat seeds, oats, refused potatoes, soy bean flour, cassava flour, sesame seeds, linseed meal, cotton seed meal, etc. are some of the substrates are widely used for spore production. Certain substrates (oats, Weber et al 1999) shrink due to the evaporation of water, resulting inadequate heat removal from the bed, subsequently yielding low spore. Hence, apart from the nutritional aspect, physical properties of the substrate such as porosity, water uptake capacity, mechanical strength, etc, should be

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taken into consideration, such that it provides higher spore yield and facilitates easy extraction of spores. Many inert supports have been used for the spore production such as hemp, perlite, bagasse (Weber et al 1999), polyurethane foam (Lareo et al 2006), pozzolano particles, glass beads, wood shavings, Biogrog N (Desfarges 1988), etc. Roussos et al 1999 reported that bagasse absorbed the conidiospores and imparted protection during vacuum concentration. Hemp provided the best spore yields due to its high water uptake capacity among the other inert supports (bagasse, perlite) tested (Weber et al 1999). The colonization of the microorganism in the susbtrate generally leads to both internal and external spore production. It is preferable that the spores are produced in the external surface of the substrate as it is easier in the extraction process. It is also possible to modify this internal/external ratio of spore content by varying substrate pre-treatment (Larroche & Gros 1989).

Spores produced by SSF are mostly that of fungal species; however some bacterial spores are also produced. Spores of entomopathogenic fungi such as *Beauveria bassiana* (Santa et al 2005), *Cordyceps spp,* (Hussey & Tinsley 1981), *Verticillium lecanii* (Feng et al 2000), *Metarhizium anisopliae* (Dorta &Arcas 1998), *Colletotrichum truncatum* (Silman et al 1993), *Trichoderma harzianum* (Munoz et al 1995), *Penicillium oxalicum* (Pascual et al 2000), *Coniothyrium minitans* (Oostra et al 2000), *Clonostachys rosea* (Viccini et al 2007) etc., have been widely produced in SSF. Spores of bacteria such *Bacillus thuringiensis* (Capalbo et al 2001, Wang 1988), *B. sphaericus* (Foda et al 2003) are also produced by SSF for the use as insecticide. Fungal spores for applications in the food and other industries have also been been predominantly produced by SSF such as *Penicillium roquefortii* (Larroche & Gros 1992, Larroche & Gros 1989), *P. nalgoviensis* (Holker 2000), *Aspergillus niger* (Moksia et al 1996), *Mucor bacilliformis* (Lareo et al 2006), etc.

The aim of the present study was to investigate the kinetics of growth parameters and sporulation behaviour of *A. niger* in SSF using different substrates.

3.2. Buckwheat seed

Buckwheat (*Fagopyrum esculentum* Moench) grain is a basic food item in porridges, soups, and the preparation of "kasha" in Central and Eastern Europe. In Japan, buckwheat is used mostly for manufacturing a noodle, soba, which is prepared from a mixture of buckwheat and wheat flours. The mature buckwheat seed is an achene. Buckwheat seed is a starchy substrate, with excellent mechanical properties (retention of structure, lack of

agglomeration) and known to produce spores of *Penicillium roquefortii* (Larroche & Gros 1992).Apart from its starchy nature, it also has high protein content than rice, wheat, millet, maize and sorghum. Chemical composition of buckwheat seed is given in Table 3.a. Aminoacid in buckwheat protein is well balanced and is rich in lysine and arginine. Chemical composition of buckwheat seed is given in Table 3.b.

Table 3.a Chemical composition of buckwheat seed (Pomeranz, 1983).

Components	**mg/g of seed**
Protein	100-125
Starch	650 – 750
Fat	20-25
Mineral	20-25

Table 3.b Composition of aminoacid of buckwheat seed protein (Pomeranz, 1983).

Amino acid	**g/ 100g protein**
Lysine	6.1
Histidine	2.7
Arginine	9.7
Aspartic acid	11.3
Threonine	3.9
Serine	4.7
Glutamic acid	18.6
Praline	3.9
Cystine	1.6
Glycine	6.3
Alanine	4.5
Valine	5.1
Methionine	2.5
Isoleucine	3.8
Leucine	6.4
Tyrosine	2.1
Phenylalanine	4.8

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Sucrose is the major sugar accumulating in buckwheat achenes. The monosaccharides, glucose and fructose, are mostly in the pericarp and seed coat. Levels of glucose and fructose are similar. Except in embryo tissues, increasing glucose and fructose levels precede sucrose and starch accumulation (Obendorf et al 1993). The protein content of mature whole grain is 13.8%, dehulled groat 16.4%, pericarp 4%, endosperm 10.1% and embryo 55.9% (Pomeranz and Robbins 1972). The amino acid content of protein in the embryo and endosperm are quite similar. Embryo proteins are enriched slightly in arginine, serine, and glutamine/glutamic acid while endosperm proteins are slightly enriched in lysine, proline, alanine, methionine, and leucine. Buckwheat proteins are composed of about 18% albumins, 43% globulins, 1% prolamins, 23% glutelins, and 15% insoluble residue (Javornik et al. 1981).

3.2.1. Growth and sporulation behaviour of* A. niger *on buckwheat seed during solid-state fermentation

Growth of fungus on a solid substrate involves two phenomena. The first is the synthesis of cellular components, which takes place by transfer of medium compounds (carbon and nitrogen sources, other nutrients) to the biomass. The second phenomenon deals with the synthesis of volatile compounds due to oxidative process such as respiration and maintenance. Carbondioxide produced is generally removed from the cultivation vessel, especially in the case of aerobic growth with forced aeration. The second phenomenon leads to loss of more dry weight than the first one as the amount of water required as reactant for this process is very low (Larroche et al 1998). Initial dry weight was calculated using grain counting method as mentioned in Ch 2. Initial dry weight decreases with time as the substrate is periodically sampled. The ratio of DW/IDW decreases with increase in time. During the cultivation process, DW/IDW decreased from unity to 0.78 corresponding to 22% (w/w) dry matter lost (Fig. 3.1a). Results were expressed as IDW, which was calculated as mentioned in ch 2. For eg, in the study, at 50% moisture content, 100 grains of buckwheat seed measured 1.82 g IDW. Likewise 1g of dry matter measured 43-47 seeds. Water content of the biomass depends heavily on the moisture content of the solid substrate. Both growth and sporulation increase linearly according to the initial water content of the solid substrate when *Penicillium roquefortii* is cultivated on buckwheat seeds (Larroche et al 1994). This indicates that water is

the limiting factor for fungal development since neither carbon nor nitrogen sources were exhausted during these experiments. This feature validates the concept of available water for fungal growth, defined as initial water content of substrate minus its residual water content when vegetative growth stops.

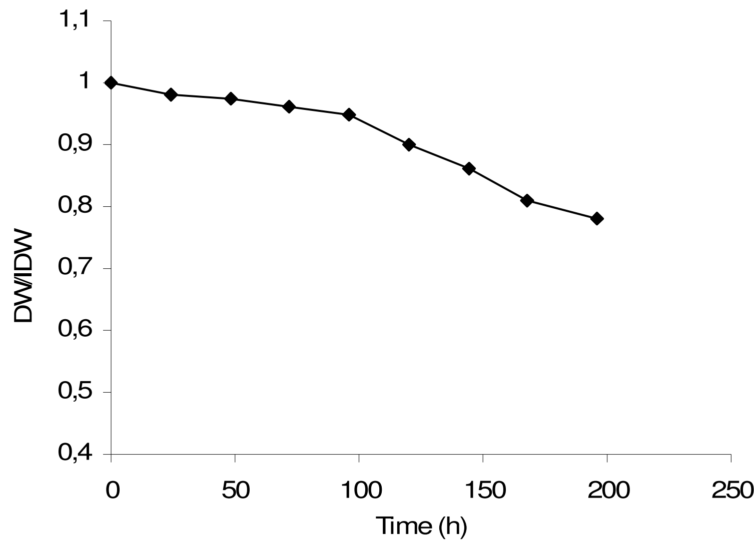

Fig 3.1a. Time-course of the ratio DW/IDW calculated from grain counting method.

The time course of the consumption of starch, free reducing sugar, protein content and carbondioxide evolution rate are given in Fig. 3.1b. Spore germination took place at initial hours of SSF, followed by the uniform colonization of the substrate with mycelium. There was active growth phase from 24-168 h, where utilization of starch took place. Reducing sugars liberated did not accumulate till 168 h. But starch and reducing sugar were no longer consumed after 168 h. This behaviour could not be attributed to poor digestibility of the remaining sugars, since significantly higher sugar consumption could be observed in the case of *Penicillium roquefortii* grown on the same substrate (Larroche & Gros 1992). Both growth and sporulation patterns were found to be homogenous in different segments of the column. Carbondioxide production could be associated with growth as its release corresponded to the fungal respiration. Its evolution rate continued after the aging of the mycelium too. This might be due to endogenous respiration and to spore formation since not only mycelium was formed during growth of fungi in SSF, but also spores were produced (Lareo et al 2006).

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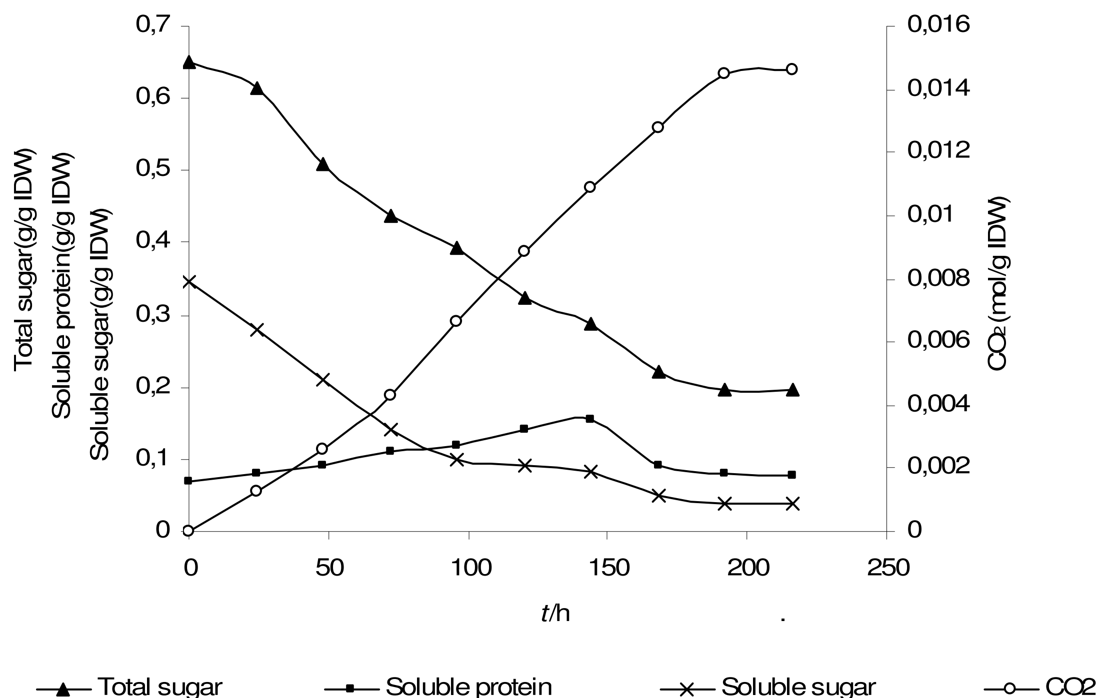

Fig 3.1b. Time course of various growth parameters during solid-state fermentation using buckwheat as substrate. Total sugar (▲), soluble protein (■), soluble sugar (*x*) and carbondioxide evolution(o). Experimental conditions: 300 g buckwheat seeds (wet weight), aeration 0.075 *l* min^{-1}, temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

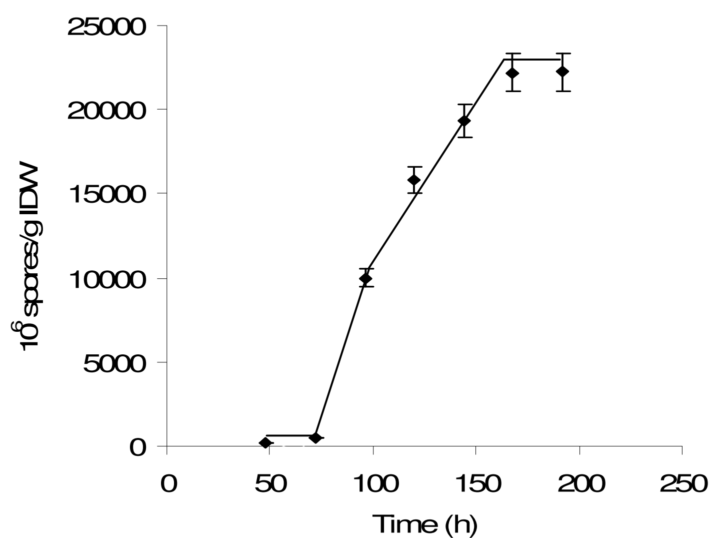

Fig 3.2. Sporulation of *A. niger* during solid-state fermentation on buckwheat seed. Experimental conditions: 300 g buckwheat seeds (dry weight), aeration 0.075 *l* min^{-1}, temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

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Sporulation behaviour of filamentous fungi generally depends on the nutrients and moisture content of the medium. It has been reported that nutrient limitation and increased moisture content aids in achieving good sporulation yield (Larroche & Gros 1992).

3.2.2. Bioconversion carried out with spores recovered at different time period of solid-state fermentation

Spores obtained at different periods of SSF cultivation (48 h-216 h) were frozen for 48 h and studied for their ability to catalyse the bioconversion of glucose to gluconic acid.

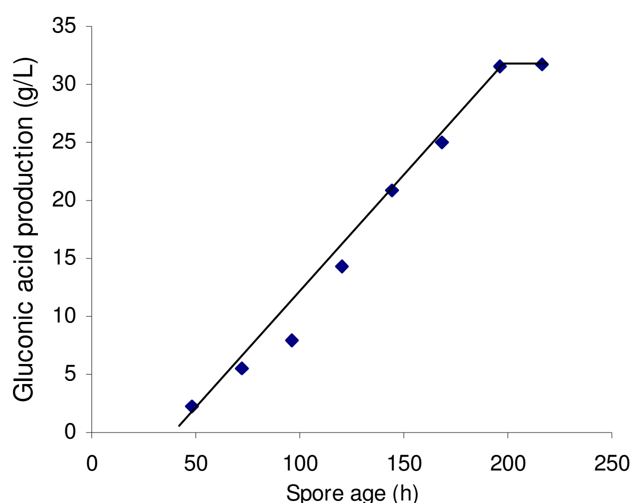

Fig 3.3 Gluconic acid production by spores recovered at different time period during SSF. Initial glucose concentration - 40 g/L. Spore content -10^{10} spores/ml.

It was interesting to note that the overall initial specific enzymatic activity increased with increase in the age of spores (Fig 3.3). This feature revealed heterogeneity in the conidial material which could result from the presence of at least two kinds of conidia bearing different enzymatic activity. A similar phenomenon has already been reported with *A. candidus* grown on buckwheat seeds, where spores were shown to have different protein contents (Moksia 1997). However, irrespective of the age of spores, product yield was nearly 100 %. As mentioned in Ch 3, bioconversion carried out with optimized conditions had average reaction rate of 5.26 g/L.h (Fig 3.16). Similar pattern was shown by spores harvested at 216 h. The enzyme produced by the mycelial stage of the fungus was transported to its spores without any loss of enzyme into the solid medium. The transport of the enzyme from the mycelial to the spores form was fully accomplished with the aging of the spore. Thus, older spores could better carry out the bioconversion reaction when compared with the

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younger ones. During SSF, glucose oxidase was likely to be present in both the fungal forms (mycelial stage or spores); however, gluconic acid was not produced in the medium at any time period, which indicated that no enzyme release in the medium took place during the cultivation.

3.3. Growth and sporulation behaviour of *A. niger* on rice during solid-state fermentation

Rice (*Oryza sativa*) is a staple food for a large part of the world's human population, especially in East, South and Southeast Asia, making it the second most consumed cereal grain. It is a complex carbohydrate, which means that it contains starch and fiber. It has low sodium content and contains useful quantities of potassium, the B vitamins, thiamin and niacin. Details of the chemical composition of rice are represented in Table 3c.

Table 3.c Chemical composition of rice (http://en.wikipedia.org/wiki/Rice)

Components	g/g (dry weight)
Carbohydrates	0.80
Fat	0.006
Protein	0.07
Fibre	0.0065

Small grain such as rice is as an excellent substrate with large surface areas per weight. Its particulate nature also adds advantage ruling out the formation of sticky mat formation of the substrate. In this study, initial moisture of the substrate was 50%. Over the range of initial moisture contents of 37–70% (w/w wet basis) maintained in the study on the spore production using rice as substrate, the optimum range for better sporulation was between 46%- 50% (Viccini et al 2007). When the moisture increases, the substrate becomes sticky and agglomerated, which would further restrict the diffusion of oxygen within the substrate bed. Low moisture level results in reduced solubility leading to low availability of nutrients to the culture. By increasing the water content, cultivation time becomes shorter and results in better spore yields. Sporulation of *Mucor bacilliformis* on polyurethane foam were studied at different initial moisture content of the substrate and maximum spore production ($6x10^8$ spores/g inert support) was obtained with the highest moisture content (90% w/w) (Lareo et al 2006). However, it is also possible to increase the spore content by maintaining low moisture content by adding an organic nitrogen source (Larroche et al 1988). Spores of

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Metarhizium anisopliae was produced on rice bran and rice husk with an initial moisture content of 47% (Dorta & Arcas 1998). Hence, at moisture level of 50%, there were no such problems and was suitable for the colonization and sporulation of *A. niger*.

Figure 3.4 shows the fermentation kinetics of solid-state fermentation with a bed depth of 2.3 cm carried out in a 500-mL Erlenmeyer flask containing 20 g of the cooked rice. As seen in Figure 3.4, there was an immediate utilization of starch, resulting in the increase in the protein content due to the filamentous mycelial growth. Consumption of soluble sugars was comparable with that of total sugar. Sporulation started after 48 h of fermentation and flask was densely covered with spore mat after 120 h of fermentation. There was no significant increase in the protein content once the sporulation reached its peak. At the end of the fermentation, the starch content decreased by more than 60%, indicating that the fermentation was nearly complete. Spore count registered at the end of the fermentation was 1.9×10^{10} spores/ gIDW (Fig 3.5). Such a cultivation of spores on rice is Alves & Pereira (1989) obtained a yield of 2×10^{11} spores of *B. bassiana*/g powdered preparation using rice as a basic growth substrate. Each 100 kg rice generated 3 kg conidial powder, i.e. 6×10^{12} conidia/kg rice.

Bioconversion was carried out with the spores harvested after 200h of fermentation. Spores were treated (optimized conditions as detailed in Ch 2) before using it as a catalyst in the bioconversion of glucose to gluconic acid. Spores of *A. niger* (200 h old) were shown to fully retain all the glucose oxidase synthesized by the mycelium during solid-state fermentation (SSF). They acted as catalyst and carried out the bioconversion reaction effectively, provided spores were pretreated and bioconversion was carried out at optimized conditions (see Ch 3). Glucose oxidase activity was found retained in the spores even after repeated washings. 54 g/L of gluconic acid produced out of 50 g /L glucose consumed after approx. 10 h reaction, which corresponded to a molar yield close to 100%. Average rate of reaction was comparable with the spores produced by solid-state fermentation using buckwheat seed (see Fig 3.16).

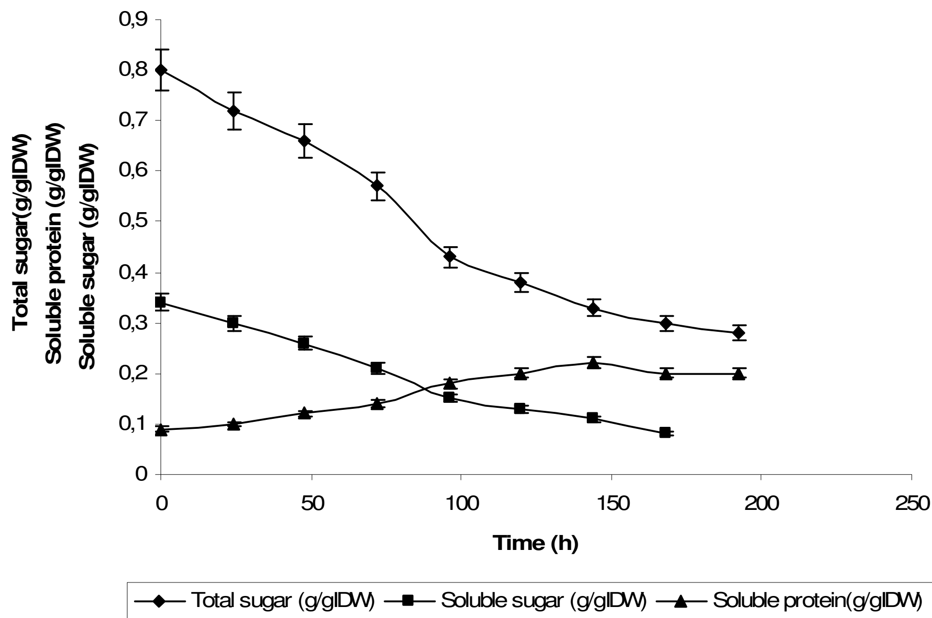

Fig 3.4. Time course of various growth parameters during solid-state fermentation using rice as substrate. Experimental conditions: 20 g rice (dry weight), temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

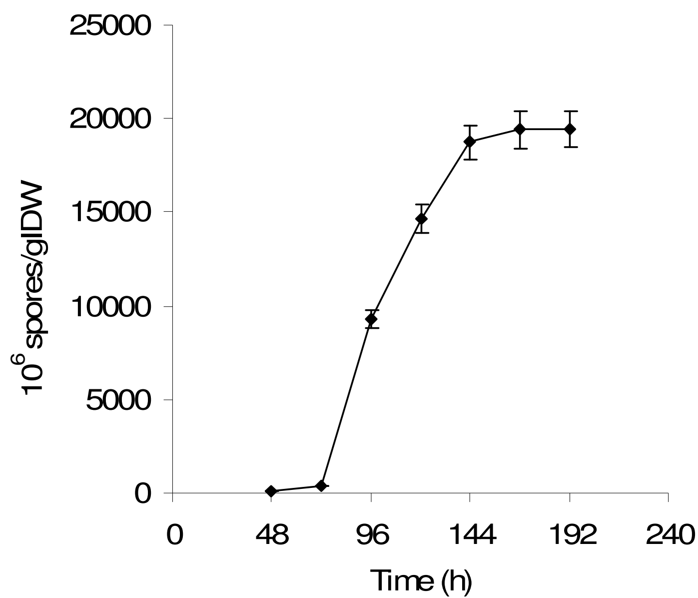

Fig 3.5. Sporulation behaviour of *A. niger* on rice during solid-state fermentation.

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3.4. Growth and sporulation behaviour of *A. niger* on jack fruit seed during solid-state fermentation

Jackfruit (*Atrocarpus heterophyllus* Lam.) is a monoecious evergreen tree that is popular in several tropical countries. Largest of all tree-borne fruits, the jackfruit could be 20–90 cm long and 15–50 cm wide, and the weight could be from 4.5 to 20 kg, or even as much as 50 kg (Morton, 1987). There may be 100, or up to 300 seeds in a single fruit. Seeds make up around 10–15% of the total fruit weight and have high carbohydrate and protein contents (Bobbio et al, 1978, Kumar et al, 1988). Seeds are normally discarded or steamed and eaten as a snack or dessert or used in some localdishes). However, in some locations is the world, it is not used as food material and allowed to go to waste. A single seed is enclosed in a white aril encircling a thin brown spermoderm, which covers the fleshy white cotyledon. Jackfruit cotyledon is fairly rich in starch and protein. Jackfruit seeds are discarded as waste from different agro-industries and higher percentage of fruits. Seeds also go waste from the fallen fruits. Since the seeds are rich in carbohydrate, protein and trace elements, it can be used as a potential substrate for the production of spores. The diverse chemical constituents of jack fruit mainly sugars and amino acids may be favourable to fungal growth and then to spore production. Chemical composition of jack fruit seed is represented in Table 3.d.

Table 3.d Chemical composition of jack fruit seed (Tulayathan et al 2002).

Components	(% dry weight basis)
Crude protein	11.17
Crude lipid	0.99
Crude fibre	1.67
Ash	3.92
Carbohydrate	82.2
Total starch	77.7

Figure 3.6 shows the pattern of sugar, protein consumption of *A.niger* cultivated on jack fruit seed. Starch and sugars were consumed in a comparatively faster rate till 96 h of cultivation, after which the utilization rate decreased. Spores were visually seen in Erlenmeyer flask after 36 h of cultivation. However, their number increased drastically from 70 – 120 h, which was the peak period of sporulation. Active growth, mycelial production and sporulation resulted in the increase of protein content (Fig 3.7); however it decreased when

sporulation reached its stationary phase. Production of spores was carried at 30°C as it was the optimal temperature for the mesophilic fungi. Temperature is considered as an independent process depending on the microorganism used; however it is one of the key parameters which should be controlled in spore production process. In SSF process, heat rise up is commonly observed as the system is usually a packed bed and sporulation response gets affected as consequence of the thermal gradients. However, when SSF was carried out in Erlenmeyer flask, where the bed depth is not too high, heat rise up was not found so crucial.

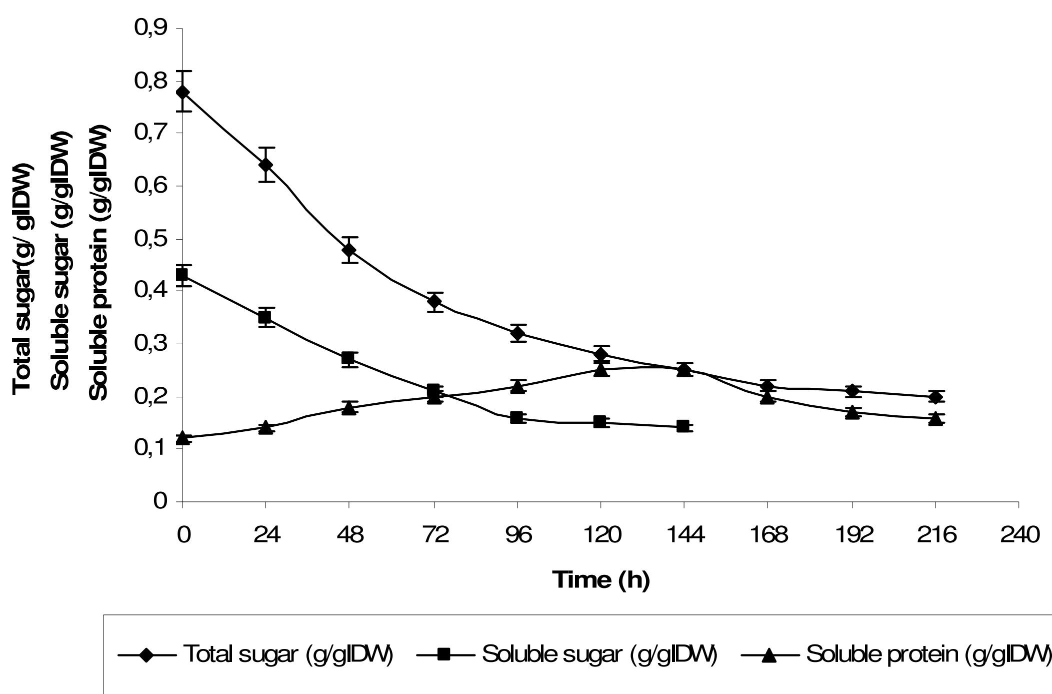

Fig 3.6. Time course of various growth parameters during solid-state fermentation using jack fruit seed. Experimental conditions: 20 g jack fruit seed (dry weight), temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

At the end of 200h of fermentation, spores were harvested and used as catalyst in the biconversion after treatment (optimized conditions as detailed in ch 2) before using it as a catalyst in the bioconversion of glucose to gluconic acid. As noticed before, spores of *A. niger* (200 h old) were shown to fully retain all the glucose oxidase synthesized by the mycelium during solid-state fermentation (SSF). Spores were active and acted as reservoir of glucose oxidase. Glucose oxidase excretion into the medium was not noticed as before. Average rate of reaction was lesser with the spores produced by solid-state fermentation using buckwheat seed and rice (Fig 3.16); however molar yield was close to 100%.

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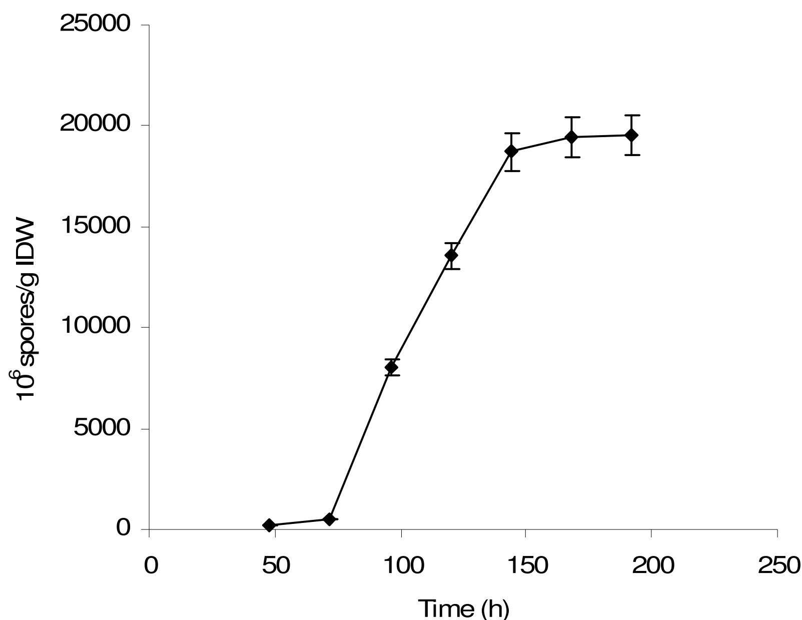

Fig 3.7. Sporulation behaviour of *A. niger* on jack fruit seed during solid-state fermentation

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3.5. Growth and sporulation behaviour of *A. niger* on corn during solid-state fermentation

The major chemical component of the maize kernel is starch, which provides up to 72 to 73 percent of the kernel weight. Other carbohydrates are simple sugars present as glucose, sucrose and fructose in amounts that vary from 1 to 3 percent of the kernel. After starch, the next largest chemical component of the kernel is protein. Protein content varies in common varieties from about 8 to 11 percent of the kernel weight. Most of it is found in the endosperm. Table 3.e shows the chemical composition of corn.

Table 3.e Chemical composition of corn (Cortez & Wild-Altamirano 1972).

Composition	g/100g dry wt.
Moisture	9.6
Protein	10.7
Carbohydrate	70.4
Fibre	2.2
Ash	1.7

The investigation of the cultivation pattern on corn showed that during the initial phase of growth, rapid sugar consumption and protein synthesis occurred from 0-96 h (Fig 3.8). There was an exponential sporulation phase from 72-150 h. The sporulation of *A.niger* on corn was very similar to that of rice and jack fruit seed. Sporulation started during the active growth phase and continued even after the mycelium synthesis stopped (Fig 3.9).

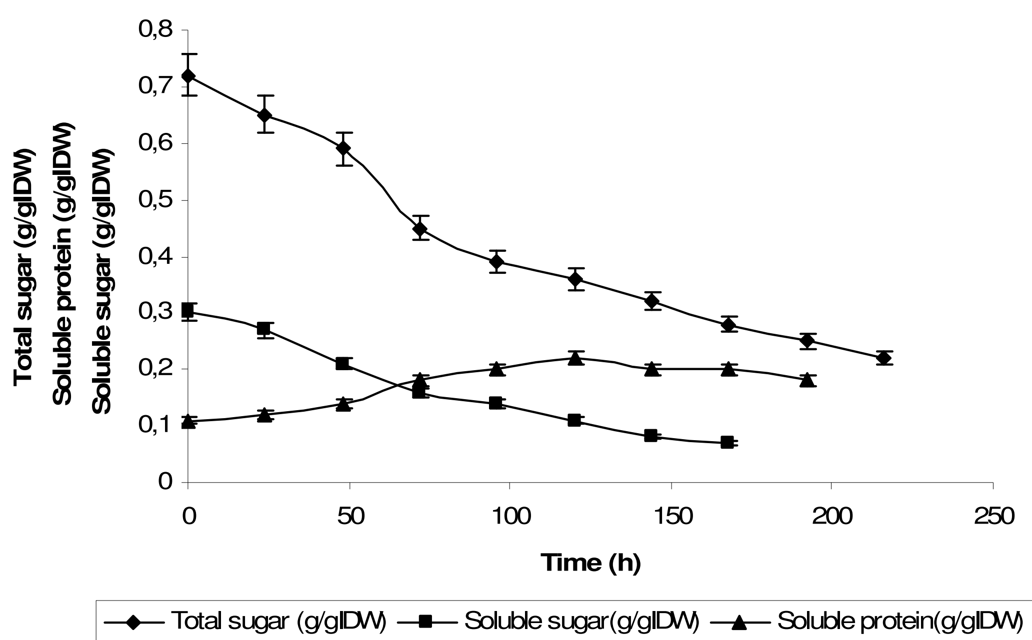

Fig. 3.8. Time course of various growth parameters during solid-state fermentation using corn as substrate. Experimental conditions: 20 g corn (dry weight), temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

The rapidly growing fungi, *A. niger* entirely invaded the corn and penetrated deep inside resulting in the production of spores inside and outside the substrate. Colonization of the fungi was homogenous and heavy deposit of spores was found at the top and even at the bottom of the flask. The final spore count on corn was close to 1.8 x10^{10} spores/ml. Substrate was of uniform size and was not densely packed. Medium packing density also governs the spore production. In densely packed bed, non-homogenous growth and sporulation occur if aeration and temperature are maintained uniform through out. The effect of packing density was studied by Dorta & Arcas 1998 .It was found that when apparent density increased upto 0.496 g/ml, sporulation was drastically affected. Their observation showed that on top of the solid

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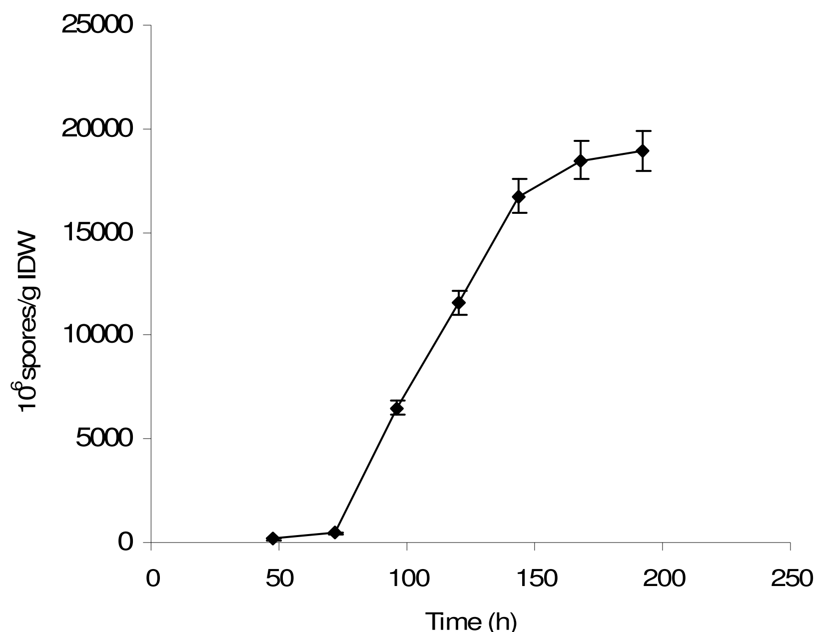

Fig 3.9. Growth and sporulation behaviour of *A. niger* on corn during solid-state fermentation

bed, spore yields were 35 times higher than those estimated at the bottom of the column. Also, spore yields were observed maximum at the periphery regardless of bed heights (Dorta & Arcas 1998). Sporulation response gets affected as a consequence of thermal and humidity gradients.

Oostra et al 2000 observed that metabolic heat production rate in SSF for spore production of *C. minitans* carried out Erlenmeyer flask was 500 watts/m^3 bed. Mixing of the medium also could improve heat removal via the reactor wall because it increases the heat transfer coefficient from solids to the wall (De Reu 1995). Mixed bioreactor was found to be superior to non-mixed bioreactor for the spore production of *Coniothyrium minitans* (Oostra et al 2000). There was no detrimental effect of mixing on spore production, instead resulted in high spore yields. However, this is not universally applicable, depends on the process and microorgansim used as mixing significantly inhibits the sporulation of the microorganism. The substrate bed must remain static in fungal spore production because agitation causes damage to the reproductive hyphae, greatly reducing spore yields. Thus, mixing was not done in all the SSF studies carried out in this chapter.

Spores were harvested and used as catalyst in the bioconversion after treatment (optimized conditions as detailed in ch 2) before using it as a catalyst in the bioconversion of

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glucose to gluconic acid. Average rate of reaction was lesser with the spores produced by solid-state fermentation using other starchy substrates such as buckwheat seed, rice and jack fruit seed (Fig 3.16); however molar yield was close to 100%.

3.6. Growth and sporulation behaviour of *A. niger* on cassava roots during solid-state fermentation

Cassava, *Manihot esculenta* is a woody shrub of the Euphorbiaceae (spurge family) native to South America that is extensively cultivated as an annual crop in tropical and subtropical regions for its edible starchy tuberous root, a major source of carbohydrates. Indeed, cassava is the third largest source of carbohydrates for human food in the world. As enlisted in Table 3.f, cassava roots are very rich in starch, and contain significant amounts of calcium (50 mg/100g), phosphorus (40 mg/100g) and vitamin C (25 mg/100g). However, they are poor in protein and other nutrients. In contrast, cassava leaves are a good source of protein if supplemented with the amino acid methionine. Cassava is famous for the presence of free and bound cyanogenic glucosides, linamarin and lotaustralin. They are converted to HCN in the presence of linamarase, a naturally occurring enzyme in cassava. Linamarase acts on the glucosides when the cells are ruptured. In the roots, the peel has a higher concentration than the interior. Cassava roots were chopped to reduce its size, washed, boiled, drained to eliminate the major contaminants such as cyanides. Finally it was cooked into a suitable form to increase its utilization by the microorganism. Fig 3.10 shows the profiles for starch, soluble sugar, protein for a culture with 50% (w/w) of moisture content. Initial protein content of cassava is very less. Sporulation starts after 40h of fermentation when active growth started (Fig 3.11). There is an increased protein content noted during the fermentation. It is generally considered that the proteins of the fungal spores are of hyphal origin, while the wall is synthesized from the substrate (Weber & Hess 1975).

Table 3.f. Chemical composition of cassava tuber (Pandey et al 2000b).

Composition	g/100g dry wt
Moisture	15.7
Protein	1.4
Lipid	0.5
Starch	80.6
Fibre	1.2
Ash	1.8
Cyanide	1.6

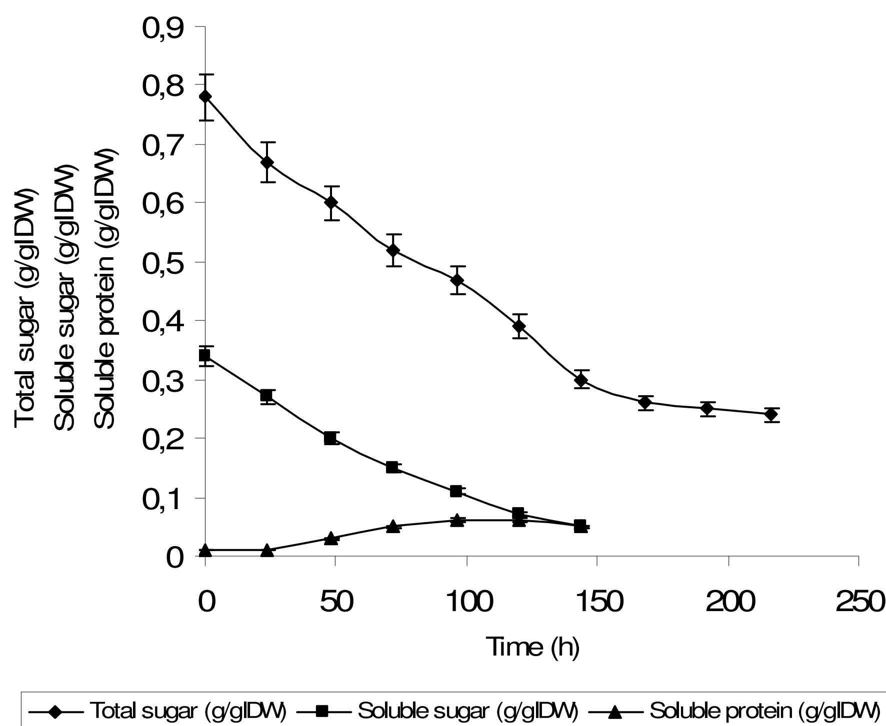

Fig 3.10. Time course of various growth parameters during solid-state fermentation using cassava roots as substrate. Experimental conditions: 20 g cassava (dry weight), temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

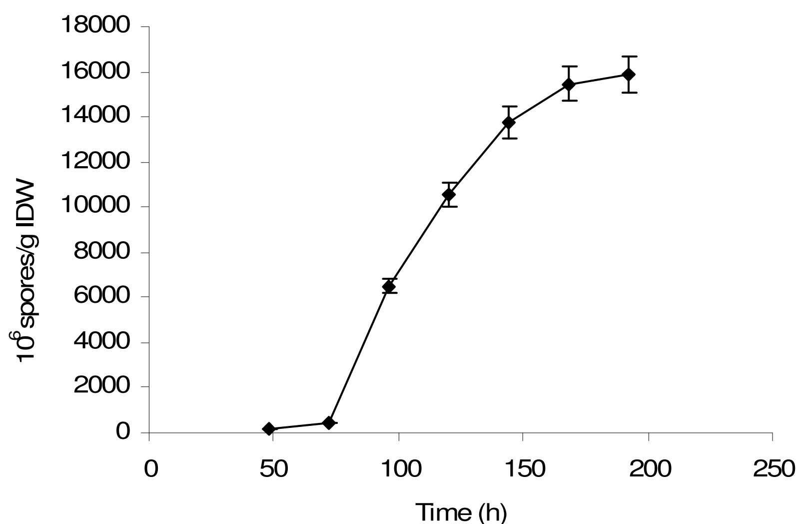

Fig. 3.11. Sporulation behaviour of *A. niger* on cassava during solid-state fermentation.

Spores recovered after 200 h of SSF was used as catalyst in the bioconversion reaction, with a productivity of 4g/L.h with 100% molar yield (Fig 3.16). There are reports which used cassava flour in SSF culture of filamentous fungi. Spores of *Trichoderma*

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harzianum spores were produced in disc fermenter, column fermenter and Zymotis (a large scale SSF fermenter designed at ORSTOM lab) by SSF using bagasse and cassava flour as substrates. Five times higher production of conidiospores ($5x10^{10}$ spores/g cassava flour) was achieved in Zymotis (with a load of 21 kg moist medium), as compared to the agar medium in Erlenmeyer flasks (Rossous et al 1991).

3.7. Growth and sporulation behaviour of *A. niger* on wheat bran during solid-state fermentation

Wheat bran is a potential source of protein and dietary fibre. It is cheap and readily available. It is a by-product of the flour milling industry used mainly for animal feed. In recent years, however, the use of wheat and other cereal bran has gained importance in the formulation of various food products. Wheat bran is rich in protein (~14%), carbohydrate (~27%), minerals (~5%), fat (~6%) and B-vitamins (Kent & Evers, 1994). It is nutritionally superior to endosperm protein (Miladi et al., 1972) and an abundant source of dietary fibre (Burkitt, 1988). Table 3.g. illustrates the chemical composition of wheat bran.

Table 3.g. Chemical composition of wheat bran (Haque et al 2002).

Composition	%
Moisture	11.2
Crude protein	12.8
Starch	8.5
Ash	4.9
Crude fibre	10.4
Phosphorus	1.2

Fig 3.12 shows the profiles for starch, soluble sugar, protein for a culture with 50% (w/w) of moisture content. Starch and sugar content present in wheat bran was comparatively low when compared to other substrates. Spore production started early at 30h of fermentation when residual starch and sugar were less than 0.1g/gIDW (Fig 3.13). Wheat bran is widely used for the production of spores of entomopathogenic fungi such as *Cordyceps* spp, *Beauveria bassiana* (Hussey & Tinsley1981), *Trichoderma* spp. Spore yields on SSF of *V. lecanii* on wheat bran and beet pulp mixture resulted in spore yield of $3.2x10^{9}$ spores/g dry

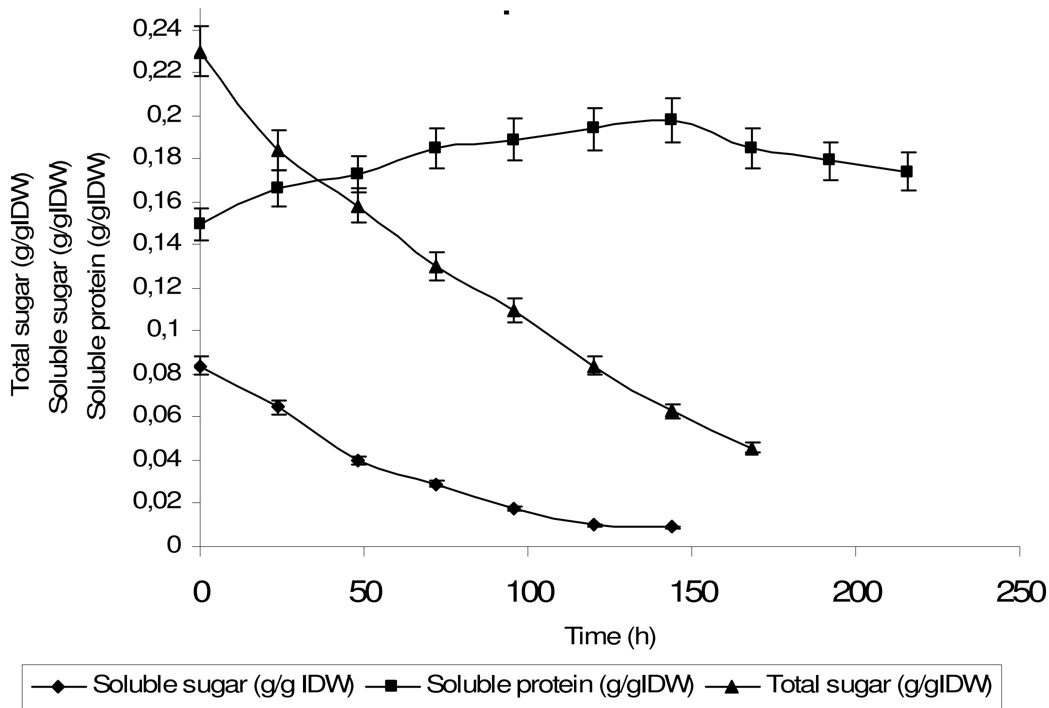

Fig.3.12. Time course of various growth parameters during solid-state fermentation using wheat bran. Experimental conditions: 20 g wheat bran (dry weight), temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

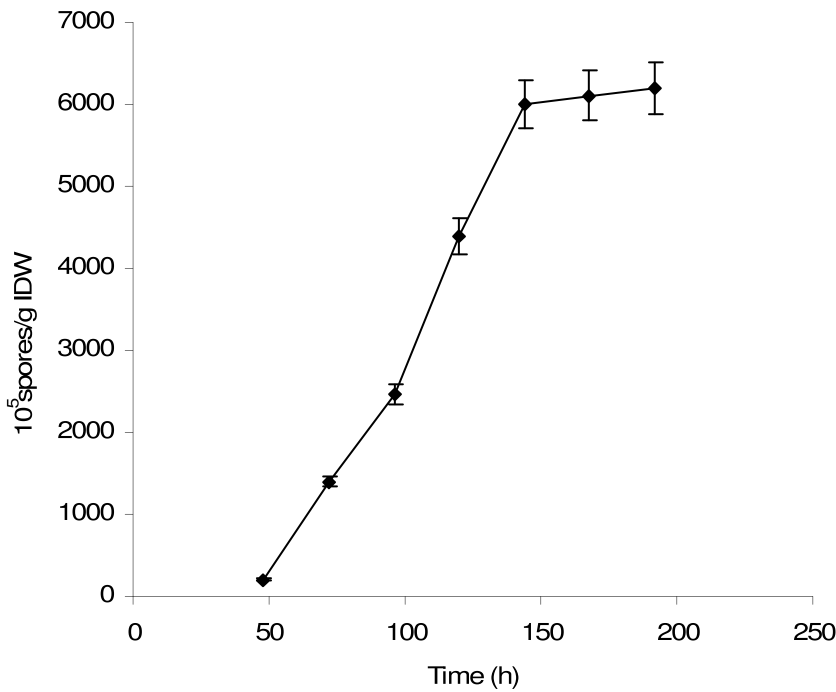

Fig.3.13. Sporulation behaviour of *A. niger* on wheat bran during solid-state fermentation.

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matter (Grajek 1994). Response surface methodology was performed to optimize the medium components for spore production of *C. minitans* using wheat bran as substrate (Chen et al 2005). Cultivation was carried in 250 ml Erlenmeyer flask for 8 days, with the supplementation of nutrient solution which resulted in 1.04×10^{10} conidia/ g IDM.

Spores harvested after fermentation intervals did not show glucose oxidase activity. Thus there was no bioconversion of glucose to gluconic acid by these spores harvested from wheat bran (data not shown). This might be due to the lack of carbon source in the medium. Experiments were carried out with the supplementation of 1% glucose. Spores harvested from this medium were found to contain active spores with glucose oxidase activity. Productivity of gluconic acid with spores cultivated in wheat bran supplemented with 1% glucose was 3.5g/L.h (Fig 3.16). This was lesser than the registered reaction rate of *A. niger* spores grown on other substrates.

3.8. Growth and sporulation behaviour of *A. niger* on cassava bagasse during solid-state fermentation

Cassava bagasse is a fibrous residue, which contains about 50% starch on a dry weight basis (Carta et al., 1999). Table 3.h shows the composition of cassava bagasse as determined by various authors. In addition, the composition may also differ due to the use of different crop varieties. Starch is the main component determined as carbohydrates. Cassava bagasse does not show any cyanide content. However, its poor protein content makes it unattractive as an animal feed.

Table 3. h Chemical composition of cassava bagasse (Pandey et al 2000b).

Component	**g/100g dry weight**
Protein	1.57
Lipid	1.06
Fibre	50.5
Ash	1.10

Substrate was not physically suitable, since the surface area was less, chances of agglomeration of substrate and stickiness was higher. Also, the nutrient requirement of the fungus was not met by the substrate. Growth stopped due to the exhaustion of the nutrients (Fig 3.14). The spore yield in the system was about 8.6×10^{7} spores /g after 150 hours of

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cultivation (Fig 3.15). A further increase in spore numbers is not likely to occur because substrate analysis showed complete starch depletion at this point in time. Visual observations showed that the fungal development was not appreciable. There was neither matty mycelial colonization nor dense sporulation as seen during SSF using other substrates.

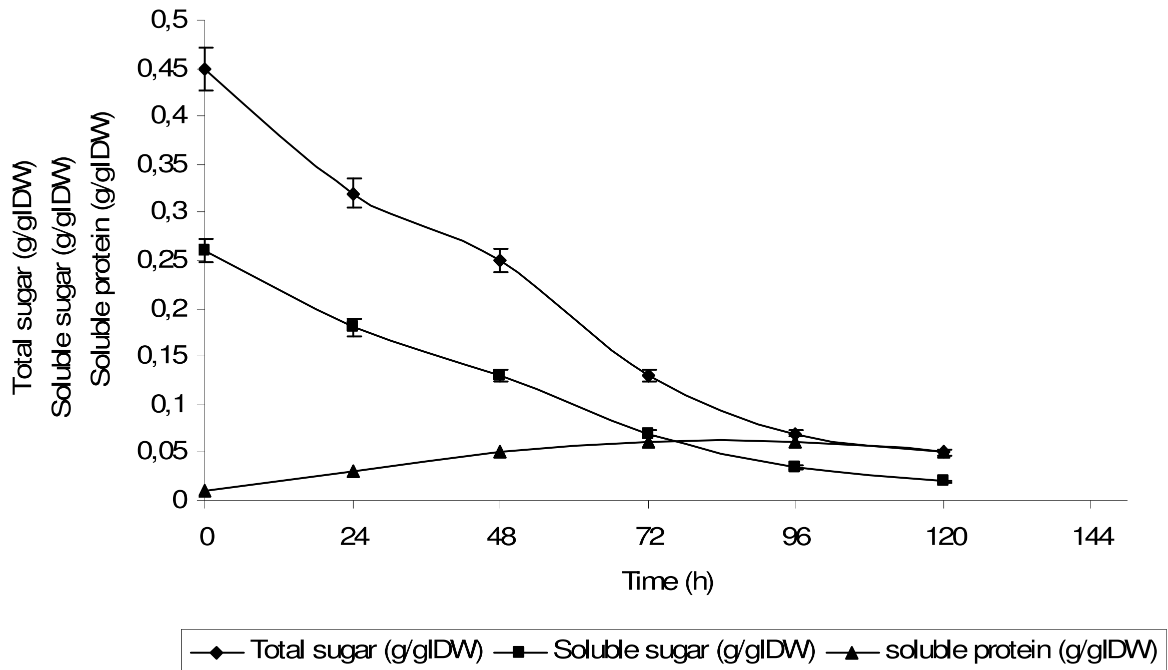

Fig.3.14. Time course of various growth parameters during solid-state fermentation using cassava bagasse. Experimental conditions: 20 g cassava bagasse (dry weight), temperature 30 °C initial moisture 0.5g H_20/g IDW (Initial dry weight).

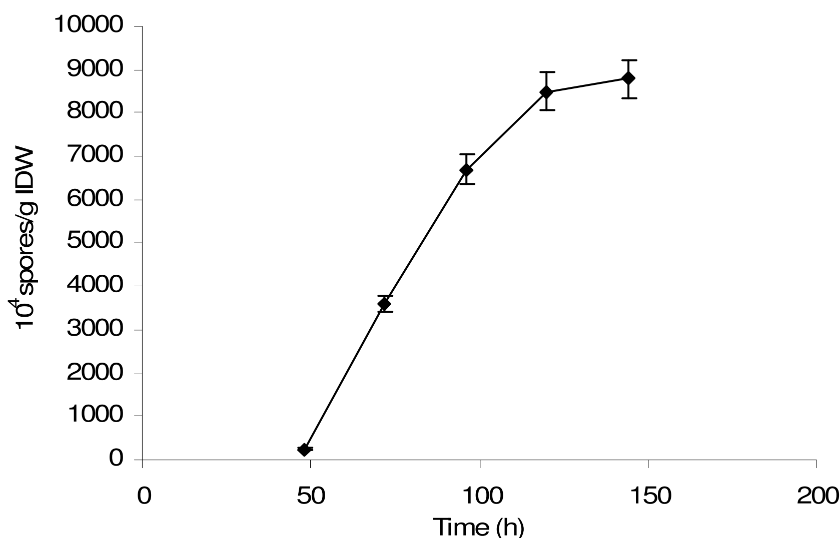

Fig 3.15. Sporulation behaviour of *A. niger* on cassava bagasse during solid-state fermentation.

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Since number of spores harvested was scanty, they were not utilised for the bioconversion process. Thus, cassava bagasse was found not suitable for the production of spores. Other than nutritional parameters, environmental conditions too could affect the mycelial growth and sporulation.

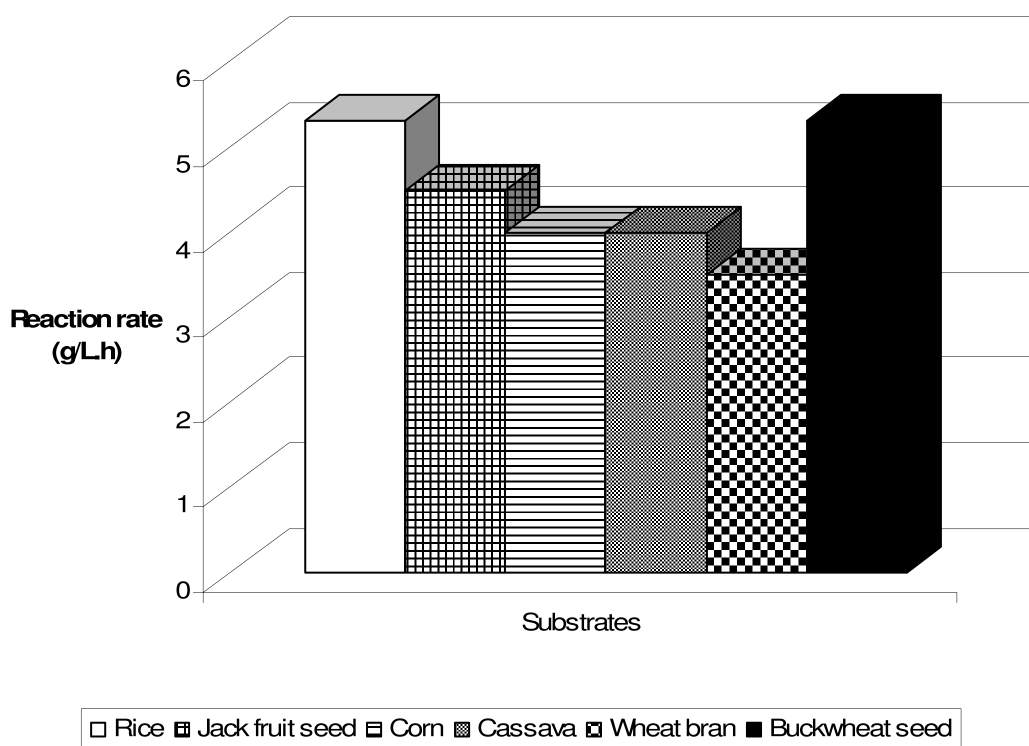

Fig 3.16. Bioconversion of glucose to gluconic acid by spores harvested from different substrates. Bioconversion conditions: Volume - 50 ml (Erlemeyer flask), Spores - frozen spores treated by 10% citral for 18h; Spore concentration - 10^{9} spores/ml; Glucose concentration - 50g/L; Stirring speed - 200 rpm, pH 6.5 (neutralised by calcium carbonate), Temperature - 30°C.

3.9. Dry weight of the spores

Average dry weight of *A. niger* spore was found to be 1.2×10^{-8} mg/spore, a value very close to that found for spores of *Penicillium roquefortii* (1.39×10^{-8} mg/spore, Moksia 1997). Interestingly, this value did not vary with the variation of the time or substrate used for SSF.

3.10. Conclusions

Inspection of these results shows that the use of complex cereal type, starchy substrates gives fairly good spore yields. Spores produced by solid-state fermentation using

particulate substrates having large surface area rich in starch (0.7-0.8g /g) was suitable for the production of spores to be utilized as a catalyst for the conversion of glucose to gluconic acid. The positive effect of starch might be caused by the availability of a higher amount of substrate to synthesize the spores.

Buckwheat seed was the best substrate among all other substrate used followed by rice. This point leads to consider that the enzyme glucose oxidase is inducible by glucose, a sugar easily found in starchy media since it is the main compound of soluble reducing sugars that accumulate during fungal development.

A. niger NRRL 3 was an efficient biocatalyst, exhibiting the highest reaction rate. Glucose oxidase synthesized by the mycelium of *A. niger* during SSF was not excreted to the SSF medium but transported to spores. Spores harvested at later period of SSF (196 h) produced high titres of gluconic acid in the bioconversion medium when compared to the spores harvested at early (48 h) stages of SSF.

Thus, it was demonstrated that spores of *A. niger* contained an active enzymatic system holding glucose oxidase and all the accessory enzymes such as peroxidase for the production of gluconic acid, similar to its corresponding mycelium. The spores could serve as enzyme reservoir and could be efficiently used as biocatalyst in the bioconversion reaction directly. Thus, role of spores as biocatalyst eliminated the extraction and purification process of the enzyme from the spores, making the process simple and economical.

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Chapter 4

Bioconversion of glucose to gluconic acid by spores of *Aspergillus niger*

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4.1. Introduction

Fungal spores are generally believed to be dormant and metabolically inert. However, they contain almost all the enzymes that are present in vegetative cells at a comparable level of activity. They serve as excellent enzyme bags and thus could be exploited as a promising biocatalyst in bioconversion processes (Murata 1993). The first spore-catalysed reaction to be described and used on a limited scale in industry was the 11α-hydroxylation of steroid catalysed by spores of *Aspergillus ochraceus* (Schleg and Knight 1962).

A number of pathways in vegetative cells have been found in extracts of spores and germinated spores, although the activities of spore enzymes were relatively low when compared with those in vegetative cell extracts. However for a few cases (Shigematsu et al 1993, Goldman & Bluementhal 1961, Simmons & Costilow 1962, Prasad et al 1972), the studies on spore enzymes are exceedingly fragmentary and are generally relied on indirect measurements, such as respiratory activity. The yeast spores contained almost all the activities for the same enzymes as vegetative cells. Among the enzymes examined, phosphatase (2-3 fold), oxidase (10- fold for D,L-glutamate, D,L- aspartate, and TCA cycle members) and two enzymes in glycolytic bypass namely methylglyoxal synthase (10-fold), glyoxalase I (3-fold) significantly increased in spores. On the other hand, dipeptidase, protease, DNase, RNase and TCA cycle enzymes decreased in spores almost by 50% of those in vegetative cells. The marked increases in glycolytic bypass enzymes, methylglyoxal synthase and glyoxalase I is related to the regulation of meiosis and sporulation of yeast cells (Shigematsu et al 1993, Shigematsu et al 1992). The lack of certain activities in spores might be advantageous in biocatalysis. For example, spores of *Aspergillus ochraceus* produce only the desired product (11α-hydroxyprogesterone) from progesterone, its mycelium produces 6 side products because of undesirable activities or side reactions (Zedan et al 1976).

Spores of *Penicillium roquefortii* carried out transformation of octanoic acid into 2-heptanone and produced blue cheese flavour (Creuly et al. 1994; Larroche et al. 1994). This demonstrated the ability of fungal spores to be the recipient of complex enzymatic systems that can be activated. It was also demonstrated that conidia could be stored for several months at - 20°C without any loss of activity (Larroche et al. 1994). This simplicity for storage enabled to consider this material, as being close to purified enzymes. Preliminary study

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carried out by Moksia et al. (1996) demonstrated that spores of the fungus *Aspergillus niger* could host the enzyme glucose oxidase (β-D-glucose:oxygen 1-oxido-reductase, EC 1.1.3.4). This enzyme catalyses the oxidation of β-D-glucose by molecular oxygen to glucono - δ - lactone, which yields in turn, gluconic acid. This result pointed out the feasibility of the use of *A. niger* spores in the biotransformation of glucose to gluconic acid.

This chapter is dedicated on the utilization of spores of *Aspergillus niger* produced by solid-state fermentation as a catalyst in the conversion of glucose to gluconic acid. It discusses various conditions, treatment procedures of spores, to exploit *Aspergillus niger* spores as biocatalyst. Fed-batch production of gluconic acid was tried using the optimal conditions. Various advantages of using spores in the gluconic acid production is highlighted.

Use of fresh spores from SSF did not carry out bioconversion. Instead, spores got germinated and mycelial growth took place. Glucose, supplied as the substrate for the bioconversion was used for the proliferation of biomass. Although there was no nitrogen source supplemented in the bioconversion medium, residual nitrogen traces from the solid substrate fermentation helped in the growth. Thus it was necessary to prevent the germination of spores and its further growth.

4.2. Prevention of germination and growth to use spore as a reservoir of enzyme

Spores were subjected to repeated washings (5, 8, 15 times with 50 ml of distilled water each time) and used as catalyst in the bioconversion medium. To fully arrest the fungal growth and ameliorate the yield, incorporation of components such as and 0.72 g NaCl l^{-1} with 0.01g l^{-1} of polysorbate (v/v) was also tried. Direct use of spores separated from the production medium by only one centrifugation step did not facilitate any bioconversion. However, subsequent washings improved the product yield (Table 4.a). This behaviour was attributed to the decrease in fungal growth due to removal of residual nitrogen. However, growth of the fungus could not be completely suppressed even after 15 washing cycles with distilled water and the resulting bioconversion yield were still low. Even high salinity could not stop the spores from germinating. Glucose supplied was utilized for germination and growth, instead of bioconversion.

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Table 4.a Comparison of product yields achieved with spores (stored at -20°C for 24h) washed with distilled water.

No. of washings	Y p/s[a] without sodium azide	Yp/s[a] with sodium azide[b]
0	0	0.92 (0.89) [c]
5	0.28	0.85
8	0.40	ND
15	0.76	ND

a - Yp/s is the yield (w/w) of gluconic acid production from glucose, determined from the slope of the linear plot of gluconic acid concentration as a function of glucose consumed. Average relative error of the slope was found close to 10%
b- Sodium azide (0.1 g l^{-1}) was added to the bioconversion media to stop the fungal vegetative growth.
c - Value obtained when spores were not separated from buckwheat, i.e. the whole SSF medium was used for the bioconversion

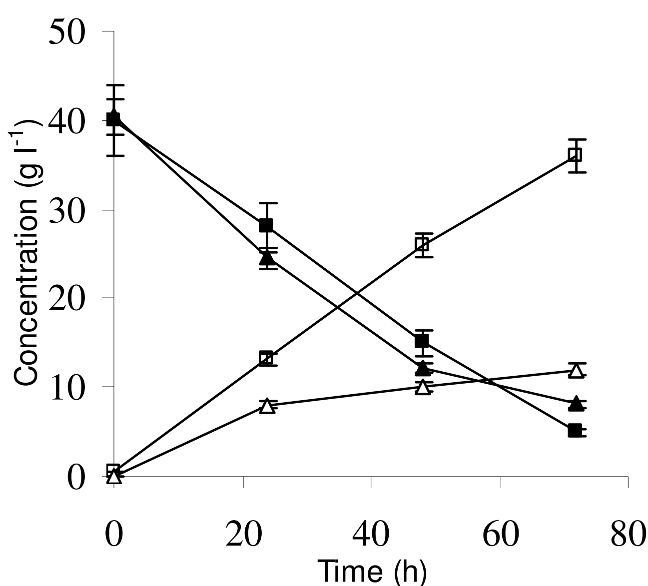

Fig 4.1. Time course of bioconversion of glucose to gluconic acid. Residual glucose (■) and gluconic acid produced (□) when sodium azide (0.01g l^{-1}) was added to the bioconversion media when spores were used unwashed. Residual glucose (▲) and gluconic acid concentrations (△) with spores washed 5 times with distilled water before bioconversion.

To control the growth of the fungus, sodium azide ($0.1 g\ l^{-1}$) was added to the medium. Use of sodium azide was found very efficient in controlling the germination and growth of *A. niger*, even without any washing step (Table 4.a). Sodium azide is a common preservative of sample and stock solutions in laboratories. It is a broad spectrum biocide and highly toxic compound which is a respiration inhibitor. The overall glucose consumption rate was similar with and without sodium azide (Fig. 4.1). This compound was thus systematically used in all subsequent experiments.

Analysis performed during the course of fungal spores production by solid-state fermentation on buckwheat seeds (see Materials and Methods section) revealed that no gluconic acid was produced during the process. Also, when the solid substrate (grains with mycelium and spores) were put in bioconversion medium, an enzymatic activity was evidenced. However, gluconic acid was not produced when the spores were removed from the media (Fig 4.2). Also, glucose oxidase activity was not detected in the supernatant fluid during the washing process. These data clearly revealed that the enzyme glucose oxidase produced by the mycelial part of the fungus was finally accumulated in the spores, and spores acted as reservoir of the enzyme. Results showed that the typical product yield was close to 1.01 g gluconic acid. g $glucose^{-1}$, near to the theoretical value of 1.09 g . g^{-1}.

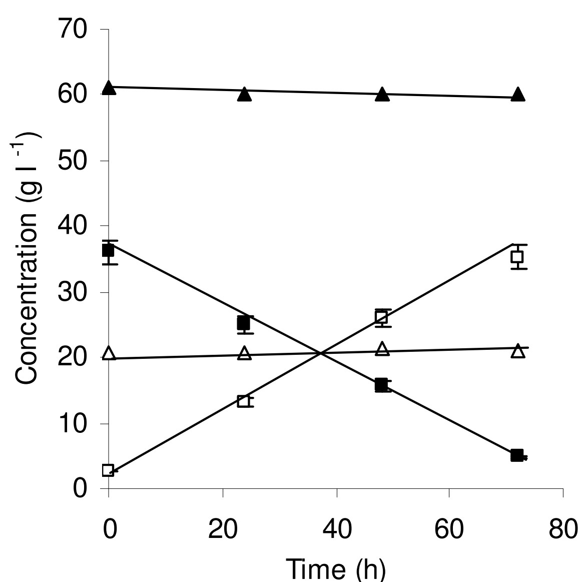

Fig 4.2. Residual glucose (■) and gluconic acid production (□) observed when spores were recovered after 24 h of bioconversion and reused into a fresh bioconversion medium. Glucose (▲) and gluconic acid (△) concentrations observed after removal of the spores from the reaction medium.

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Spores used for the previous study were frozen for 24 h. It was found that unfrozen spores do not carry out bioconversion (Table 4.b). Thus it was confirmed that freezing was necessary for bioconversion to take place since it helps in the transport of substrate and product in and out of the spore. Freezing incorporates structural changes in the spore, causes permeabilization of spores and thus facilitates bioconversion.

Fig 4.b Bioconversion of glucose to gluconic acid by unfrozen spores with addition of sodium azide (0.01g l^{-1}).

Time (h)	**Glucose (g/L)**	**Gluconic acid (g/L)**
0	40	0
24	40	0
48	40	0
72	40	0

Thus two key points for the use of *Aspergillus niger* spores as biocatalyst (Fig 4.3) are

1. Arrest of spore germination and growth
2. Permeabilization of spores.

Further studies were aimed at meeting these two points and improving the yield and rate of reaction. Studies were carried out to find various agents which could permeabilize spores. Also attempts were carried out to replace sodium azide and incorporate harmless non-toxic germination inhibitor.

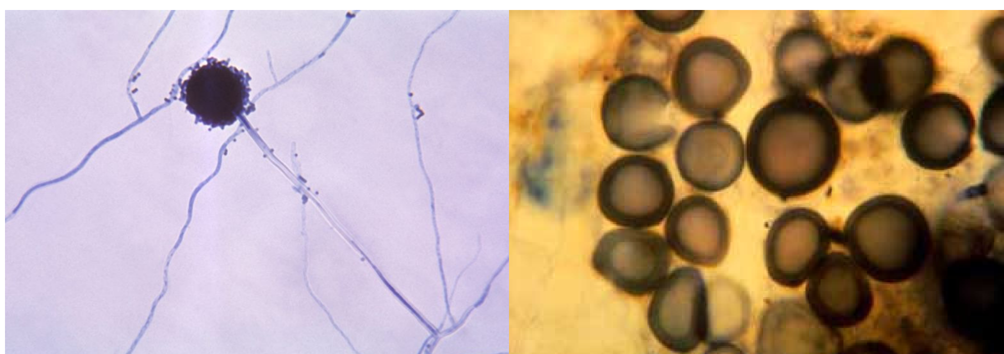

Fig. 4.3. Photographs depicting that control of spore germination and permeabilization are mandatory for bioconversion carried out by *Aspergillus niger spores*

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4.3 Permeabilization studies to use spore as a reservoir of enzyme

It was found necessary that cell membrane barrier should be removed so that the substrates and high molecular-weight chemicals are able to penentrate into the cells and to make contact with their target enzymes. However, in addition to the high resistance of microbial spores to physical and biochemical attack, the configuration and components of the coat and cortex of spores are thought to be largely different from the cellwall and cell membrane systems of vegetative cells. In order to render the fungal spore permeable to substrate, it was treated by physical (pressure, sonication), biochemical (treatment with organic solvents, detergents, lytic enzymes) approaches, and change in permeability of spores was judged by the bioconversion activity of spores.

4.3.1 Physical treatment

4.3.1.1 Effect of pressure

Spores were exposed to high pressure ranging from 1 to 2.5 kbar. This did not affect the glucose oxidase activity of the spores. It did not also help in their permeabilization. As given in the Table 4.c unpermeabilized (unfrozen) spores were not able to carry out bioconversion. Even results of various cycles of pressure application (3x 2.7 kbar, 5x 2.7 kbar) indicated that the permeability of substrate (glucose) across the spore structure was not improved by this physical treatment. Also, there was no inhibition of spore germination under pressures ranging from 1 to 2.5 kbar. No release of the enzyme from the spores could be evidenced, which was in agreement with microscopic examination of cells which appeared as intact.

High pressure strongly disturbs the cytoarchitecture by weakening the cell wall and altering the elasticity of plasma membrane (Zimmerman 1971). As a result, there is decrease in the cell membrane fluidity leading to thickness of the cell. High pressure causes enzyme inactivation, alterations of metabolism and cell morphology (Cheftel 1995). However, we noticed that glucose oxidase activity of pressure treated frozen spore was similar when compared to the control. Thus various cycles of pressure treatment upto 2.7 kbar did not affect the cell structure of *A. niger* and also did not inactivate the enzyme within it.

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Table 4.c Effect of pressure on glucose oxidase of permeabilized (frozen) and unpermeabilized (unfrozen) spores.

Pressure (kbar)	Gluconic acid produced by frozen spores (g/ L) after 24 h reaction	Gluconic acid produced by unfrozen spores (g/L) after 24 h reaction
1	30.4	0
2	29.6	0
2.7	30.1	0
2.7 (3 cycles)	30.7	0
2.7 (5 cycles)	30.1	0
Control	30.8	0

High pressure treatment is an excellent means to inactivate moulds in the vegetative stage, but spores are more resistant to this (Eicher and Ludwig 2002).On the contrast, there are reports that show that spores of *Colletotrichum gloeosporoides* was inactivated at 3.5 kbar (Palhano et al 2004). Spores of *Mucor plumbeus* were completely inhibited at 4.0 kbar (Fenice et al 1999). Inactivation of spores is dependent on the individual strains and its cultural conditions. Spores produced by SSF have rough warty brittle surface which renders protection to adverse conditions. Spores produced in this study also had the similar morphology (see later Fig. 4.6). Feng et al 2002 found that spores produced by SSF were cylindrical, ellipsoid and relatively uniform in size with rough brittle surface, while spores from SmF were of irregular in shape and size (oval to oblong) and had smooth surface. Spores cultured in SSF are hydrophobic and are resistant to stress and are highly durable when compared to submerged spores which are hydrophilic (Munoz et al 1995). Reasons for the stability of spore produced by SSF are unclear, but it can be stated that under SSF fungi is grown in the environment very closer to its natural habitat.

4.3.1.2 Sonication

Ultrasonic extract was also prepared (as below) from a spore suspension (frozen and unfrozen; 50 ml, 10^{10} spores/ml). The extract was cooled in an ice bath and was exposed to three 2min ultrasonic pulses at 3 min intervals with a microtip ultrasonifier (Model U 50

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Control, IKA Labrotechnik), followed by the bioconversion. Spores of *A. niger* was not disrupted by this process. The treatment did not cause any damage to the spore structure and the spores were found intact when the sonicated spores were examined under the microscope (data not shown). Also, spores after treatment were found resistant and did not lose its viability. This indicated that the sonicated spore structure is still a barrier for the transport of substrate (glucose) and or product (gluconic acid). Sonicated spores also did not carry out bioconversion (Table 4.d). This further confirmed that sonication was not efficient in permeabilizing fungal spores. Thus, spore structure was rigid and intact following the sonication. This was contrary to the results obtained by Murata 1993. Bacterial spores (*Bacillus subtilis*) were found to be fragile and completely disrupted by the sonication; however structure of yeast spores (*Saccharomyces cerevisiae*) was not seriously affected. But in both cases, alkaline phosphatase was significantly increased after sonication. The high alkaline phosphatase activity of the sonicated spores was caused by the sharp disruption of the inner structure of the yeast spores, and the disrupted materials, including enzymes are retained in the permeabilized spore coat (Murata 1993).

Table 4.d Effect of sonication on permeabilization and bioconversion carried out by frozen and unfrozen spores.

Spores	Gluconic acid produced (g/ L) after 4 h reaction	Gluconic acid produced (g/L) after 24 h reaction
Frozen	5.6	32
Unfrozen	0	1.2

4.3.1.3 Heat treatment

Heat treatment of spores was done by incubating them at 60°C for 30 min before bioconverison. Thermal treatment given to the spores prior to the bioconversion did not help in conversion of glucose to gluconic acid.

4.3.2 Chemical treatment

Various chemicals such as hexane (40 g l^{-1}), ether (40 g l^{-1}), toluene (40 g l^{-1}) and Triton X 100 (0.1g l^{-1}), bactinyl (20%, v/v) were incubated with 50ml of spore suspension containing 10^{10}spores. ml^{-1} (unfrozen) for 8 h in a rotary shaker at 180 rpm. Treatment with organic acid and detergent was neither effective in the activation of spores nor in enhancing the permeability. It also did not induce any structural changes in the spores. Bioconversion carried out with treated spores did not show glucose oxidase activity. Organic solvents and detergents are often used for improvement of permeability of microbial cell membranes. Inactivation of the enzyme occurs due to contact with organic solvents or detergents. To rule out this, commercial glucose oxidase from Sigma was treated with these chemicals. Results were negative indicating that organic solvents did not have any impact on the enzyme. Bactinyl is a disinfectant which contains peroxides, quaternary ammonium compounds (benzalkonium chloride) which acts on bacteria, fungi their spores. It did not have any effect on the spores of *A. niger* (Table 4.e).

Enzyme cocktail from Novo Nordisk called Glucanex™ containing β (1,3) glucanase, β (1,3), (1,6) glucanase, chitinase at a very high concentration of 0.6g/L was added to the medium, shaken for 18h before starting bioconversion. Acid treatment of spore suspension was carried out by incubating them with10% acetic acid for 2 h before bioconversion. Frozen spores (-20°C for 48 h) were also exposed to similar conditions. Bioconversion was started by adding glucose and sodium azide.

Use of enzyme or acid treatment neither facilitated the release of glucose oxidase, nor did it help in the gluconate production. They were unable to permeabilize the spores and thus could not help in the bioconversion (Table 4.e). Reports from literature supports the fact that yeast spores were found resistant to lytic commercial enzymes such as zymolyase, cellulase, lysozyme, snail enzyme, glucoronidase (Murata 1993). Generally, lytic enzymes are expected to render the microbial spores permeable to various chemicals through partial lysis of spore structure. Fenice et al 1999 reported chitinase (10U/ml) caused complete inhibition of spore germination of *Mucor plumbeus* with protoplast formation. Using a lower enzyme concentration (4U/ml), the spore germination was reduced to about 70%. All fungal structures (spores and hyphae) appeared damaged, excessive branching and cell lysis accompanied by protoplasm extrusion were also observed. A further reduction of the enzyme concentrations (1U/ml) did not lead to any reduction of spore germination, but the mycelium

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appeared damaged. It is known that fungal vegetative form is more sensitive to enzyme treatments than the spores.

Table 4.e. Influence of spore (unfrozen) permeabilization with different agents on the bioconversion behaviour. Initial glucose concentration : 40 g l^{-1}. Volume of spore: suspension: 50mL.

Permeabilizing agent	Residual glucose (g. L^{-1} at 24 h)	Gluconic acid produced (g. L^{-1} at 24 h)
Hexane	40	0
Toluene	38	1.8
Ether	40	0
Triton X 100	40	0
Glucanex™	40	2.5
Bactinyl ™	40	0
Acid treatment	40	0
Heat treatment	40	0
Control	40	0 .

Table 4.f Influence of spore (frozen) permeabilization with different agents on the bioconversion behaviour. Initial glucose concentration: 40 g l^{-1}. Volume of spore: suspension: 50mL.

Permeabilizing agent	Gluconic acid produced (g L^{-1} at 4 h)	Gluconic acid produced (g L^{-1} at 24 h)
Hexane	5.3	31
Toluene	5.6	32
Ether	5.5	32
Triton X 100	4.8	31.5
Glucanex™	6	34.0
Heat treatment	4.2	30.1
Control	5.6	32.5

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4.3.3 Freezing

It was confirmed from the above experiments (when comparing the controls, Tables 4.e & 4f) that freezing process was necessary to achieve an enzymatic activity (as mentioned earlier). This feature could be attributed to a membrane permeabilization of the cells. It was found that time of freezing also had an effect on the permeabilization of spore. Freezing of the spore for 48 h was the optimal time period, which increased the bioconversion rate. Freezing for more than 48 h did not bring out much additional change, and bioconversion rate and yield were comparable to those obtained after 48h storage at -20 °C (Fig 4.5).Results of bioconversion by spores which were placed at temperatures such as -80°C and 4°C for 48 h are shown in Table 4.g. As the temperature decreased (8°C< 4°C < -20°C < -80°C), there was better membrane permeabilization and resulted in high rate of reaction (Fig 4.4, Table 4.g). However, the images of the spores did not show any structural differences between frozen and unfrozen spores (Fig 4.6).

Table 4.g. Influence of freezing on permeabilization of spores and bioconversion behaviour. Initial glucose concentration 40 g l^{-1}. Volume of spore suspension: 50mL.

Temperature (°C)	Gluconic acid at 8h (g/L)	Gluconic acid at 24h (g/L)
- 80	12.5	39.7
+ 4	0	7.1

Spore permeabilization could not be achieved by any of the methods such as chemical or mechanical cell permeabilization methods such as use of organic solvent, enzyme, acid, surfactant, pressure or sonication (Table 4.e). It should be pointed out that the enzyme was never released in the extracellular medium, and it could be considered that the permeabilization by freezing allowed only low molecular weight compounds (glucose and gluconic acid) to cross the spore membrane.

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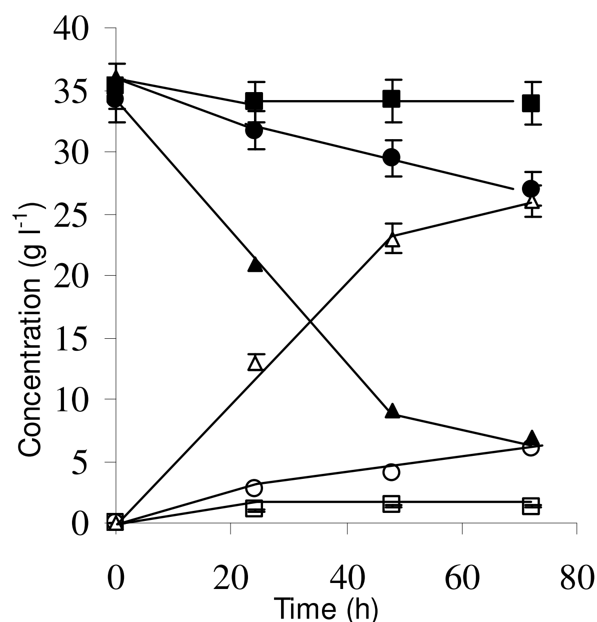

Fig 4.4. Influence of freezing on spores permeabilization. Residual glucose (▲) and gluconic acid (△) produced when the spores were frozen (-20°C) for 24 h. Residual glucose (•) and gluconic acid (○) production when spores were stored at 8° C for 24 h. Residual glucose (■) and gluconic acid (□) production when spores were not frozen.

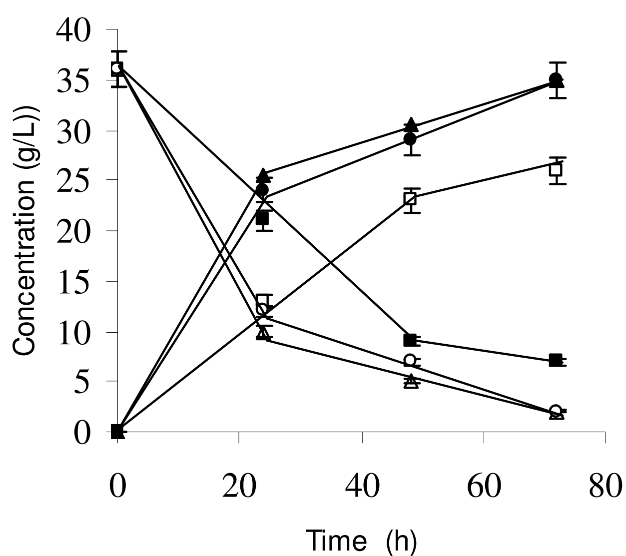

Fig 4.5. Effect of freezing time of the spores on the permeabilization of spore. Residual glucose (■) and gluconic acid (□) production with spores frozen for 24 h. Residual glucose (▲) and gluconic acid (△) production with spores frozen for 48 h. Residual glucose (•) and gluconic acid (○) production with spores frozen for 72 h.

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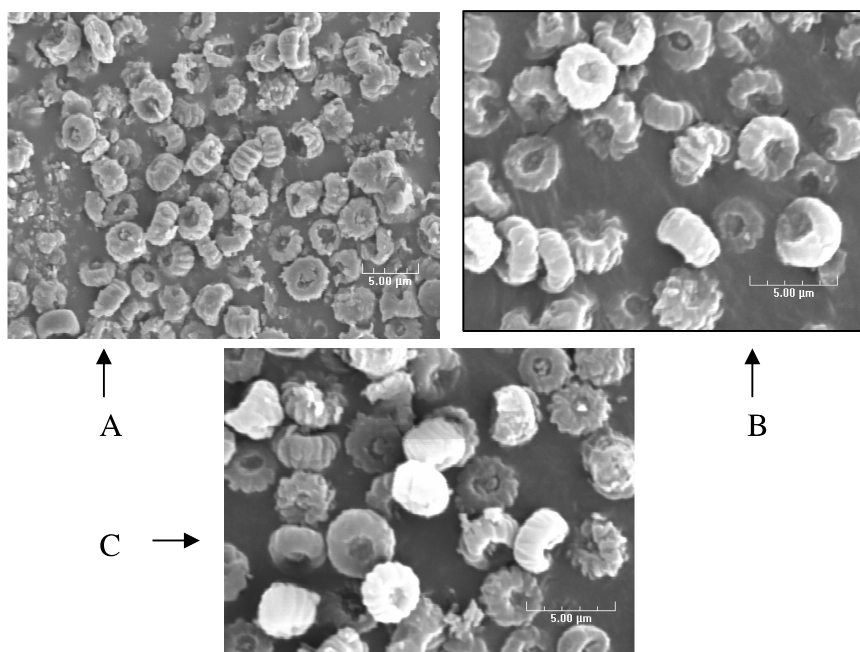

Fig 4.6. SEM of frozen spores exposed to -80°C (A), -20°C (B) and unfrozen spores (C).

With the optimized conditions (spore permeabilized by freezeing at -20°C for 48h and germination inhibited by the addition of sodium azide), bioconversion was done in bioreactor (Applikon, The Netherlands) of 1.5 L with 500 mL working volume. pH was maintained at 6.5 by the addition of NaOH. The conditions followed are mentioned in Ch 2 section 2.14.When initial glucose concentration was increased to 100 g L^{-1} and the bioconversion was carried out in a bioreactor, 102 g L^{-1} of gluconic acid were produced after 100 h (Fig 4.7). This result demonstrated the resistance of this system towards high glucose concentrations and its easiness for use at larger scale.

It was clear that glucose oxidase produced by the mycelial stage of the fungus during SSF was transported to spores without any loss of enzyme into the solid medium. Spore membrane appeared highly resistant such that even sonication and other permeabilizing agents were not able to facilitate the bioconversion reaction. Thus, glucose oxidase in the spore was naturally protected from the external conditions, and leakage of the enzyme from the spore was ruled out. Another interesting fact to be noted was that spores did not contain any other interfering components or inhibitors, which could affect the glucose oxidase activity.

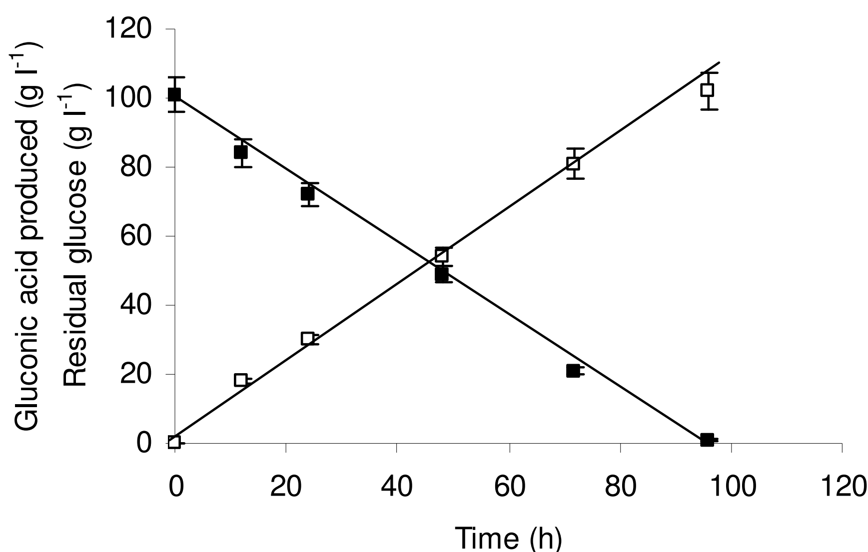

Fig 4.7. Bioconversion carried out with spores (frozen for 48 h) recovered at 200 h of solid-state fermentation. Residual glucose (■) and gluconic acid production (□) during the course of reaction.

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Stable glucose oxidase activity of 200 h-old spores might be due to the absence of protease activity. Thus, spores of *A. niger* containing an active enzymatic system when permeabilized, acted as efficient catalyst in the bioconversion giving high yields, close to the stoichiometric values. Once the spore germination process was effectively controlled, the spores served as enzyme reservoir and were efficiently used as biocatalyst in the bioconversion reaction directly. This also eliminated the extraction and purification process of the enzyme from the spore, making the process simple.

Spores, when permeabilized did not release proteins. This could be due to the fact that the pore size of the cell wall was too small to let them diffuse out (Visser et al 1994). When submerged fermentation was carried out, the enzyme was released in the extracellular medium, consistent with literature data (Visser et al 1994, Hatzinikolaou & Macris 1995, Mischak et al 1985). It should be mentioned that some authors have also reported that the enzyme could be intra-cellular and found in peroxisomes (van Dijken & Veenhuis 1980, Witteveen et al 1992). On the contrary in SSF, the enzyme synthesized by the mycelium was exported into spores. The differences and ambiguousness in the location of enzyme could be due to the parameters and conditions adopted for the growth. When cultivated by a submerged

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technique, *A. niger* did not sporulate. It could, thus, be considered that at least with this strain, the enzyme could not remain within the mycelium, but had to be released either outside (submerged conditions) or in the spores (SSF).

In literature, no information is available on the localization of enzyme in the spores. From the results obtained in this work, the possibility for glucose oxidase to be exocellular could be eliminated. It confirmed that the enzyme was intracellular or bound to the plasma membrane. It could be also a surface enzyme embedded to the cell outside the plasma membrane.

4.4. Effect of PCA as spore germination inhibiting agent

The interest was to find an agent which could replace sodium azide. Phenazine -1-carboxylic acid (PCA) was used to inhibit the spore germination and arrest growth of fungi. PCA is a water soluble compound produced by *Pseudomonas* sp. exhibiting broad spectrum activity against bacteria, fungi. It is a pigmented antibiotic that inhibits *G. graminis var. tritici* and other fungal root pathogens in vitro at less than 1 p.g/ml (Thomashow et al 1990). Minimal inhibitory concentration of PCA against mycelial form of *A. niger* is 50-60 µg/ ml. In this study, 140 µg/ml of PCA was incorporated in the bioconversion media to inhibit spore germination of *A. niger.*

PCA could not inhibit the spore germination. Glucose supplied in the media was utilized for growth of the fungus. Dense mycelial development was found which produced negligible amount of gluconic acid (Table 4.h). The conditions at which phenazine derivatives are active can be diverse (Turner & Messenger 1986). The PCA-producing strain *P. fluorescens* strains 2–79 (Thomashow & Weller 1988) and *P. aureofaciens* strain 30–84 (Pierson et al 1995) showed no significant biocontrol activity against *F. oxysporum*. Comparison of antifungal activity of PCN (phenazine-1-carboxamide) and PCA *in vitro* showed that the antifungal activity of PCA was at least 10 times lesser. Phenazines are toxic to a wide range of organisms including bacteria, fungi, and algae (Toohey et al 1965). However, the mechanisms of its action in antifungal interactions are poorly understood. It is assumed that they diffuse across or insert into the membrane and act as a reducing agent, resulting in the uncoupling of oxidative phosphorylation and the generation of toxic intracellular superoxide radicals and hydrogen peroxide which are harmful to the organism (Turner & Messenger, 1986, Hassett et al1992; Mahajan et al 1999).

Table 4.h Bioconversion pattern of spores treated by PCA.

Time (h)	Residual glucose (g/L)	Gluconic acid (g/L)
0	50	0
24	23	1.5
46	6.7	4

4.5. Effect of sodium azide

Sodium azide was used in the bioconversion medium in the previous studies. As it was found toxic, its effect on the enzyme was compared with commercial glucose oxidase. When sodium azide was added to the bioconversion medium containing commercial glucose oxidase, 50% of the activity was lost when compared to the control (Fig 4.8). Thus it was found that sodium azide is an inhibitor for glucose oxidase enzyme. There are reports which demonstrated that sodium azide inhibited peroxidase (Vernwal et al 2006) and laccase (Lu et al 2006).

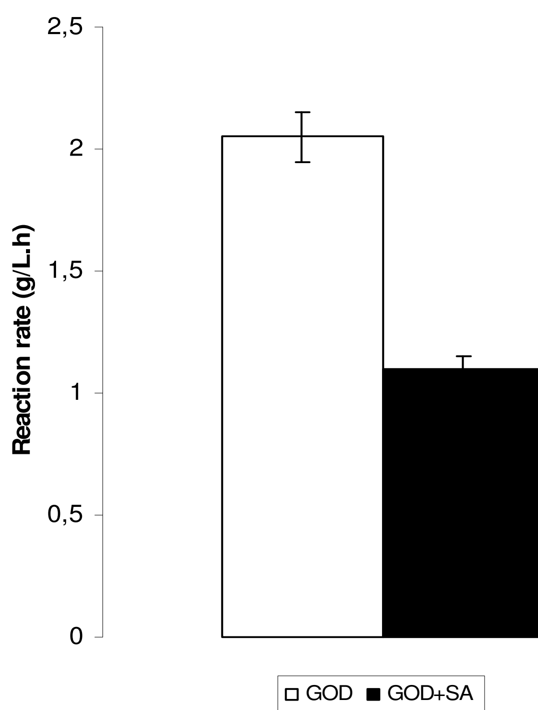

Fig 4.8. Bioconversion rate of commercial glucose oxidase (1g/L) with and without sodium azide (0.01g/L).

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The toxicity of sodium azide and most of its physiological effects can be traced to its reversible inhibitory effect on enzymes containing a coordinated divalent ion, such as those of cellular respiration (Kleinhofs *et al* 1978). Sodium azide binds metal sites in enzymes, either as the free acid HN3 or as the ionic compound. The azide anion functions in different situations as a reversible inhibitor, or irreversible inhibitor of catalytic hemoproteins like catalase and horseradish peroxidase (Ortiz de Montellano et al 1988).

4.6. Effect of monoterpenes as permeabilizing and spore germination inhibiting agent

Further research was focussed on the use of other monoterpenes as a means for both permeabilizing and inhibiting the germination of fungal spores. Monoterpenes such as myrcene, limonene, α- pinene, β-pinene, terpenoids such as linalool, orcinol, myrtenol, α-terpineol, carveol, carvone, isonovalal, citral, citronellal , α- pinene oxide, β- pinene oxide and limonene oxide were tested for their ability to permeabilize and inhibit spores for the use as catalyst in the bioconverion of glucose to gluconic acid (Figs 4.9 and 4.10). Out of all the terpenes tested citral, isonovalal and α-terpineol permeabilized the spores better and resulted in higher bioconversion rate. Fig 4.11 shows the SEM (Scanning Electron Micrograph) of spores treated with citral and isonovalal. Citral and α-terpineol are known to inhibit the growth of many bacterial pathogens. Minimum inhibitory count of MIC (minimum inhibitory count) of citral and α-terpineol tested in vitro conditions against food borne pathogens are given in Table 4.i. It was interesting to note that monoterpenoids (α- pinene oxide, β-pinene oxide, limonene oxide) permeabilized spores better than monoterpenes (α- pinene, β-pinene, limonene). It was seen that terpenoids could basically permeabilize and inhibit the germination of spores irrespective of its different functional group such as alcohol (terpineol), aldehyde (citral, isonovalal), etc.

Terpenes are hydrocarbon compounds consisting of multiple isoprene units and may or may not be cyclic. Knowledge of the inhibition action of purified terpene hydrocarbons is limited. The most common representative of cyclic terpenes, α – pinene together with β-pinene, limonene and terpinolene was shown to inhibit bacterial growth in agar plate diffusion tests (Sikkema 1995). Andrews et al 1980 studied the effects of α – pinene and some other terpenes produced by the Douglas fir on some *Bacillus* strains and *Saccharomyces cerevisiae*.

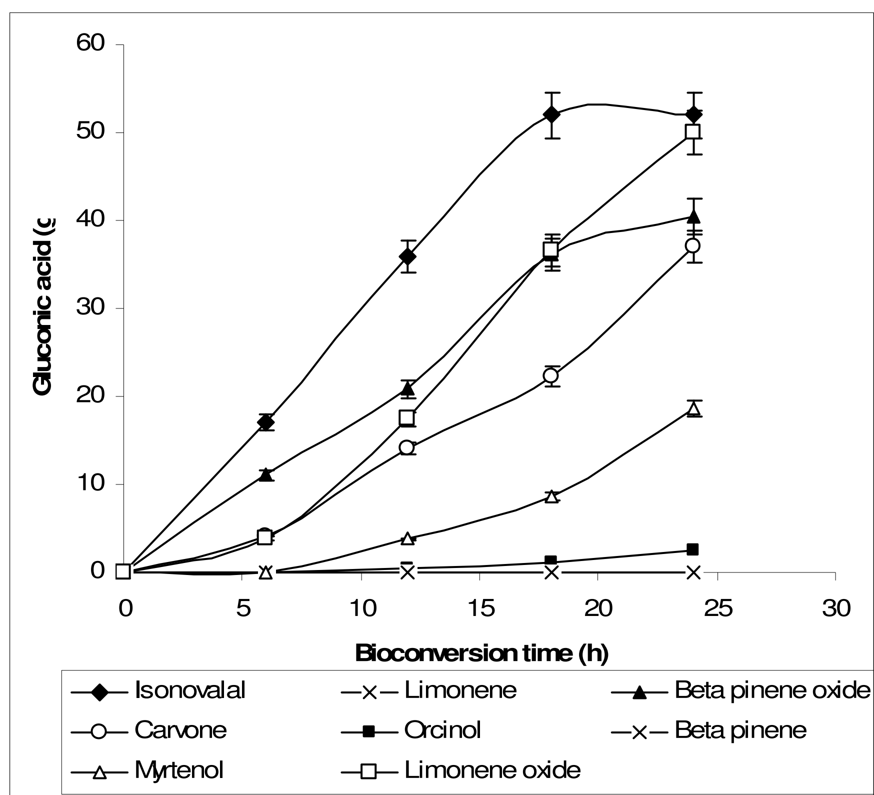

Fig 4.9. Bioconversion of glucose to gluconic acid by spores permeablized by monoterpenes and monoterpenoids.

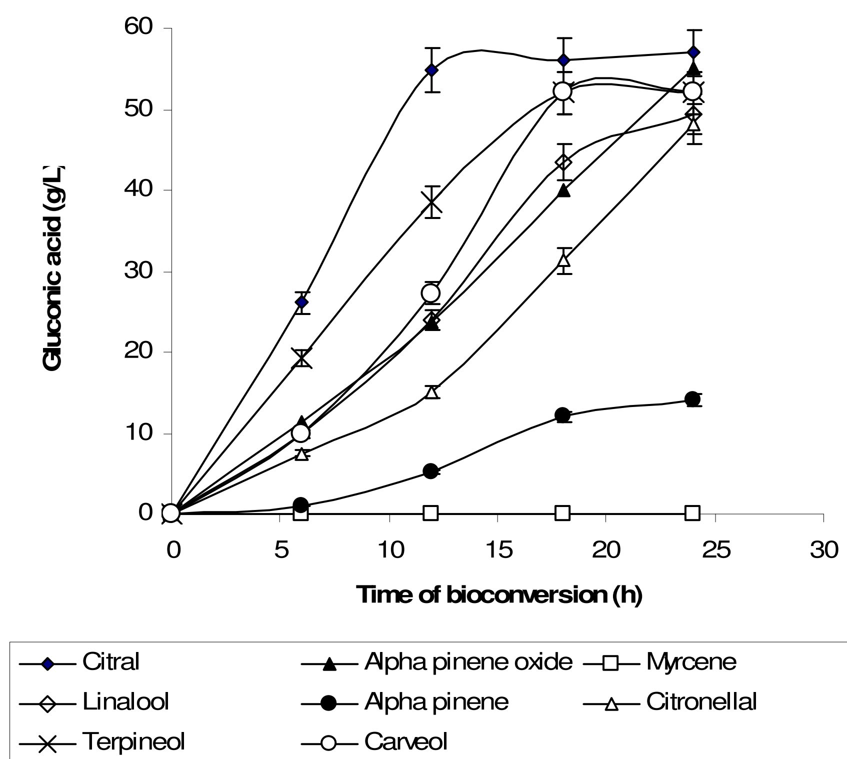

Fig 4.10. Bioconversion of glucose to gluconic acid by spores permeablized by monoterpenes and monoterpenoids.

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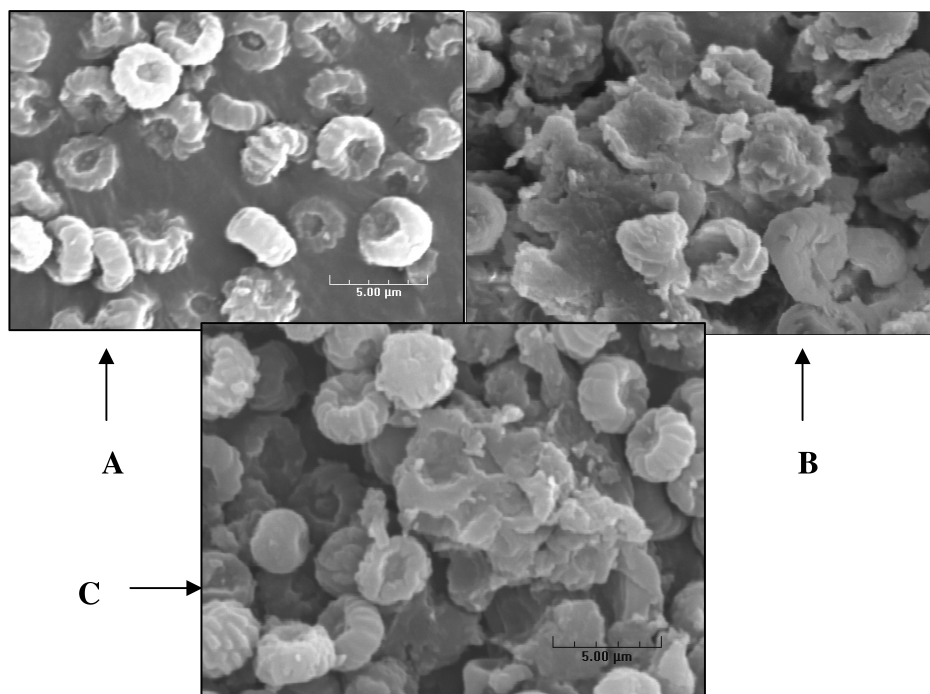

Fig 4.11. Scanning electron micrograph of unpermeabilized spores (A), citral treated spores (B), isonovalal treated spores (C).

It was shown that α – pinene, limonene, camphene and isobornyl acetate was inhibitory to *Bacillus thuringiensis* spores. β-pinene inhibited the respiration of both intact cells of *Saccharomyces cerevisiae* and mitochondria isolated from this yeast (Uribe et al 1985).The main target for the toxicity of lipophilic compounds such as monoterpene is the cell membrane. They affect the cell membrane of eukaryotes and causes increase in membrane fluidity leading to permeabilization and help in the contents to leak out (Piper et al 2001). Proteins and more specific enzymes are known to be affected by these compounds (Wolken et al 2002).

In this study, citral - a monoterpene, helped in permeabilization of spores and in carrying out the bioconversion of glucose to gluconic acid. Also it inhibited spore germination in the bioconversion medium. Replacement of sodium azide by citral resulted in high yield of 100% and a reaction rate 4.35 g/L.h. However the treatment did not release glucose oxidase out of the spore. The supernatant of the bioconversion medium did not contain glucose oxidase and when the spores were harvested and reused for the second cycle of bioconversion, gluconic acid production was noticed. This observation was similar with the earlier results when spores were permeabilized by freezing (Ramachandran et al 2007a). Thus it showed that citral could help in permeabilization without hindering the enzyme activity. There is not much literature on the toxic effect of citral on the enzyme activity of spores. However, there are

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reports where citral is tested as antifungal and antibacterial agent (Pattnaik et al 1997, Scora and Scora 1998). Spores are less susceptible to the toxicity of these compounds than vegetative cells, for example *Penicillium digitatum* spores were reported to be more resistant to the toxic effects of monoterpene geraniol and its products (Wolken et al 2002). An interesting feature noted by Palhano et al 2004 was that spores (10^6) treated with citral (0.15mg/ml) had 90% germination rate while untreated spores had only 70% germination rate. However, combination of pressure (350 MPa) and citral (0.75 mg/ml) treatment effectively inhibited and inactivated the spores of *Colletotrichum gloeosporioides.* Reports of Filtenborg et al 1996 supported this fact, stating that the germination of *Penicillium digitatum* conidia was stimulated by compounds such as limonene, β-pinene, sabinene, β-myrcene, etc. Thus the antimicrobial activity of terpenes is highly depended on their concentrations used. In this study where high concentrations of spores were used, the toxicity noticed was negligible even with high concentration was citral.

Table 4.i. MIC (minimum inhibitory count) of citral and α-terpineol tested in vitro against food borne pathogens.

Terpenes	**Bacteria**	**MIC (µl /ml)**	**References**
Citral	*Bacillus cereus*	0.1875 - >0.9	Cosentino et al 1999 Pol & Smid 1999
	Escherichia coli	0.5	Kim et al 1995
	Salmonella typhimurium	0.5	Kim et al 1995
	Staphylococcus aureus	0.5	Onawunmi 1989
	Listeria monocytogenes	0.5	Kim et al 1995
α-Terpineol	*Bacillus cereus*	0.9	Cosentino et al 1999
	Escherichia coli	0.450-0.9	Cosentino et al 1999
	Salmonella typhimurium	0.225	Cosentino et al 1999
	Staphylococcus aureus	0.9	Cosentino et al 1999
	Listeria monocytogenes	>0.9	Cosentino et al 1999

Basically, monoterpenes are of hydrophobic nature and studies on their antimicrobial action are not attributable to one specific mechanism but there are several targets in the cell (Skandamis et al 2001, Carson et al 2002). These include the degradation of cell wall (Helander et al 1998), damage to cytoplasmic membrane (Sikkema et al 1994, Ultee et al

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2000, 2002), damage to membrane proteins (Juven et al 1994, Ultee et al 1999), leakage of cell contents (Helander et al 1998, Cox et al 2000, Lambert et al 2001), coagulation of cytoplasm (Gustafson et al 1998) and depletion of the proton motive force (Ultee & Smid 2001, Ultee et al 1999).

However, in this case glucose oxidase was not excreted out from the spores. Terpenes acted on the spore membrane and increased the membrane fluidity, further helping in the transfer of the substrate and product in and out of the spore. Penetration of apolar compounds across the membrane is a simple diffusion process. Accumulation of such lipophilic compounds enhances their availability to the cell causing their toxicity. Even when citral was removed from the spores and resuspended in fresh glucose solution, spores were unable to germinate. This showed that citral was adhered to the hydrophobic spore and was able to stop the growth. However fungistatic effect was reversible when citral treated spores were plated onto PDA. Thus, citral was selected to treat spores before bioconversion for further studies. The mechanism of action of terpenes cited in the literature comments that terpenes affect the proteins and specific enzymes. But in this case, glucose oxidase activity of the spore was not affected. Terpenes had an effect only on the viablility of *Aspergillus niger* spores and not on their biocatalytic activity. This was contrary to the result of Wolken et al 2002, where terpenes were found to have effect on both the viability and the enzyme activity.

Further studies were carried out to optimize its concentration and time of exposure to inhibit germination and permeabilization. Both, spores initially permeabilized by freezing thawing process and unpermeabilized fresh spores were taken for study. Higher concentration of citral and longer time of exposure were required to permeabilize fresh spores (Fig 4.12a, b,c). On the contrary frozen-thawed spores required lesser concentration of citral and shorter duration to get permeabilized, yet resulted in comparatively better biocatalytic activity (Fig 4.13a,b). Treatment of freeze-thawed spores with 5% citral and 3% citral resulted in better bioconversion, however treatment of spores with later concentration for 5 h was adopted since the concentration was affordable and the exposure time was practical.

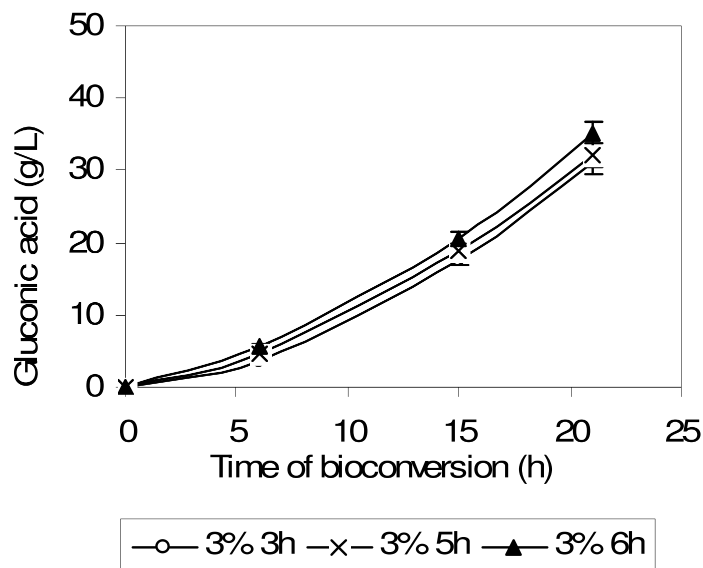

Fig 4.12 a. Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (fresh –unpermeabilized). Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

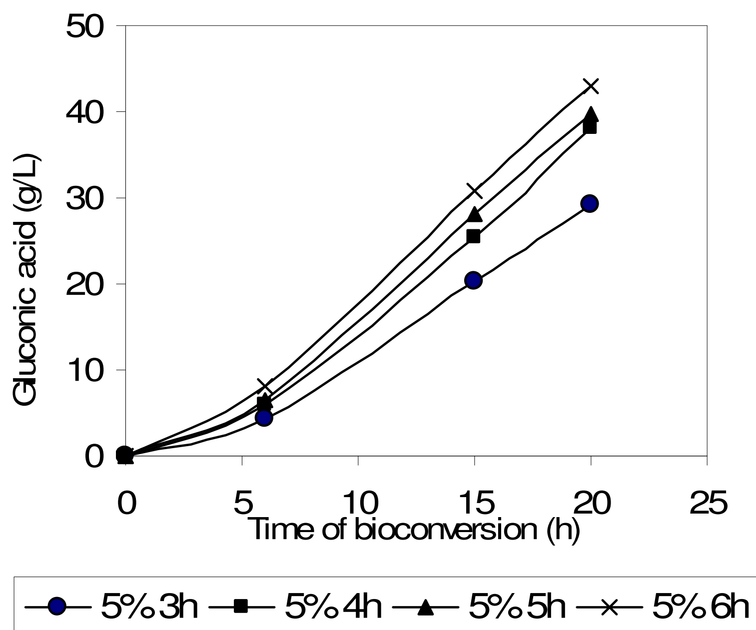

Fig 4.12. b Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (fresh –unpermeabilized). Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

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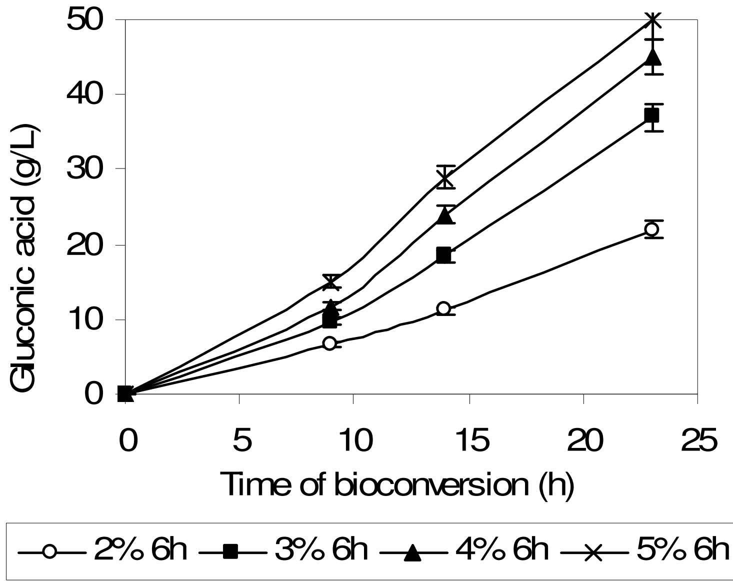

Fig 4.12c. Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (fresh –unpermeabilized). Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

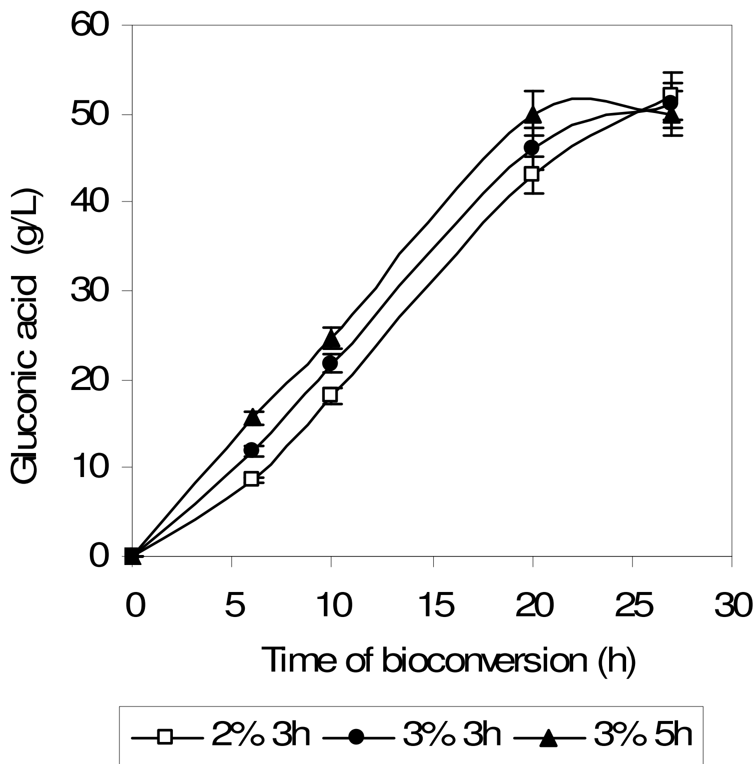

Fig 4.13a. Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (frozen–permeabilized). Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

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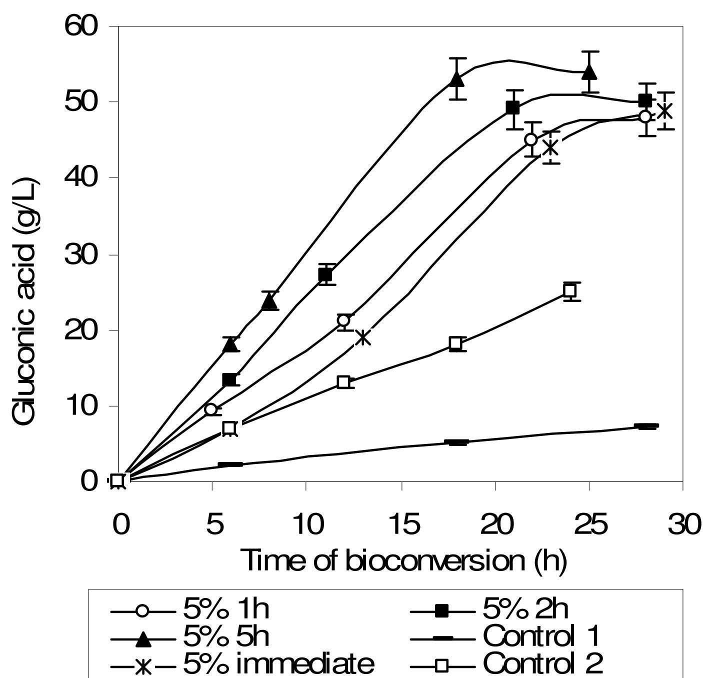

Fig 4.13b. Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (frozen–permeabilized). Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

Citral is a mixture of two geometric isomers, geranial (trans confirmation, approx.55-70 %) and neral (cis confirmation, 35-45 %). There are reports where citral was tested as antifungal and antibacterial agent (Pattnaik et al 1997, Scora & Scora 1998). Owing to their lipophilic nature, they appear to accumulate in the microbial cell membrane (hydrophobic part) and play an important role in the mechanism of the toxic action (Sikkema et al 1995) and also increase their permeability, resulting in leakage of enzymes and metabolites. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on cytoplasmic membrane. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Juven et al 1994, Sikkema et al 1995).

Different types (frozen and unfrozen), concentrations of spores (10^7-10^{10} spores/ml) were tested using this condition (3% citral for 5h). Too low numbers of spores (10^7 and 10^8 spores per ml) were not sufficient for the bioconversion. Bioconversion increased with increase of spore concentration. Increase bioconversion was comparatively better with 10^9

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spores per ml than 10^{10} spores per ml. With very high concentration (10^{10} spores/ml) of spores, treatment of citral resulted in paste-like spore suspension, which probably leads to transfer limitation. Thus it was noted that 10^9 spores/ml treated with 3% citral for 5h was the optimal spore concentration for the bioconversion reaction (Fig 4.14).

The extent of the inhibitory effect and permeabilization is depended strongly on the ratio between the terpene concentration and nature of the biomass (spores). Spores cultured by SSF are hydrophobic and are highly durable (Munoz et al 1995). Spores used in this study was found to be resistant to organic solvents, detergents, mechanical cell permeabilization methods such as use of sonication, high pressure. They were unable to permeabilize spores of *Aspergillus niger* (Ramachandran et al 2007a).

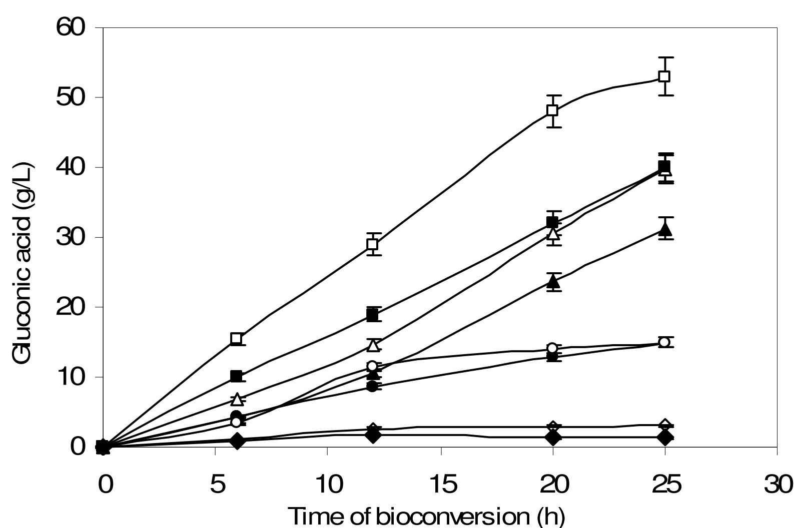

Fig 4.14. Bioconversion of glucose to gluconic acid with frozen and unfrozen spore at different spore concentration [10^{10} frozen (△), 10^{10} unfrozen (▲), 10^9 frozen (□), 10^9 unfrozen (■), 10^8 frozen (o), 10^8 unfrozen (•), 10^7 unfrozen (◇), 10^7 frozen (◆)].Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

Commercial glucose oxidase (Sigma) treated with citral and limonene was unaffected by the action of terpenes (Fig 4.15). The activity was similar to that of control (without addition of terpene in the medium). This proved that terpenes did not have any effect on the enzyme activity of glucose oxidase

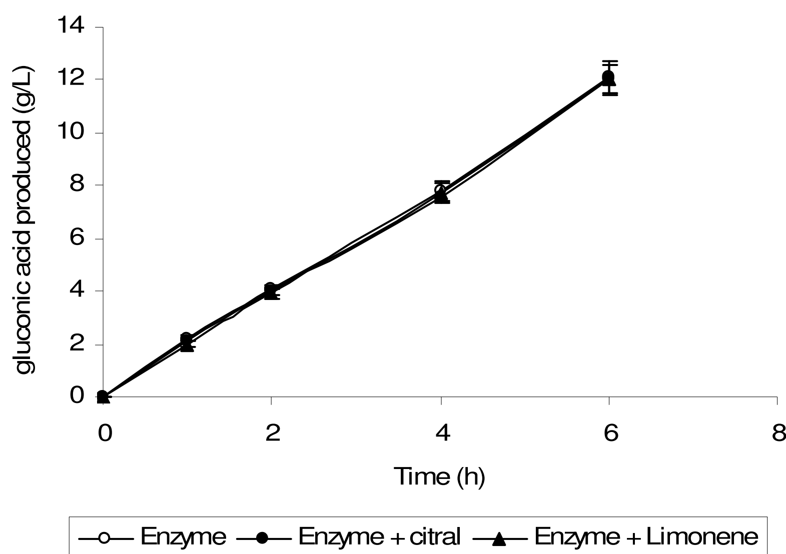

Fig 4.15. Effect of monoterpenes on the glucose oxidase activity. Commercial enzyme (1g/L) treated with citral and limonene at concentration of 3% for 5h before bioconversion.

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4.7 Fedbatch production

Attempts were carried out to scale-up the process in a bioreactor using the optimized conditions as above and feed the substrate once it gets exhausted. Before bioconversion, treatment of the spores with citral was carried out in the bioreactor. Bioconversion was commenced by the addition of glucose supplied in powder form and pH regulated by sodium hydroxide. Bioconversion started immediately with a very high reaction rate of 5.26 g/L.h (Fig. 4.16). This was comparatively higher with that of the reaction rate registered when bioconversion was carried out in Erlenmeyer flask. This could be explained because of the regulation of other parameters such as oxygen supply, high stirring speed etc. Reaction rate was slightly reduced when glucose was about to exhaust, and was restored once it was re-fed. At 18h of reaction 86 g/L of gluconic acid was produced with 14.4 g/L of residual glucose. There was neither protein synthesis nor carbon dioxide evolution noted.

There was no glucose oxidase excreted out from the spore. When glucose was supplemented at the concentration of 100 g/L for the second time, reaction rate again increased as before. However at the end of the reaction, spore concentration was found to decrease due to the addition of sodium hydroxide for neutralization. This was gradually reflected in the reaction rate at the end of the reaction. On the whole, 178 g /L of gluconic

acid was produced out of 164 g/ L glucose consumed after approx. 36 h reaction, which corresponded to a molar yield close to 100%

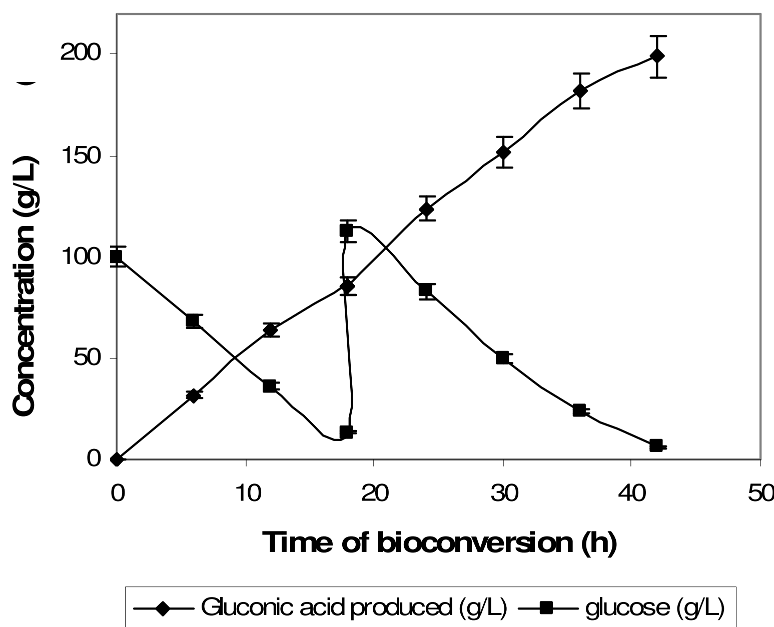

Fig 4.16. Fed-batch production of gluconic acid with permeabilized spores of *Aspergillus niger*. Spore concentration - 10^9 spores/ml; Initial glucose concentration -100 g/L; Temperature -30°C; Aeration - 0.15 slpm; Stirring - 600 rpm; Volume - 500mL.

Thus the difference in the reaction rate noticed was due to the exhaustion of glucose and the decrease in the spore concentration. Similar results were found when potassium hydroxide was used as neutralising agent (data not shown). It was noted that rate of reaction was increased with increase in initial glucose concentration of glucose however the yield always remained the same (100%).

4.8 Effect of temperature

Heat treatment of *A. niger* spores affected its glucose oxidase activity. Glucose oxidase activity of PSBH was drastically affected while the effect of PSAH was less with the temperatures 60 and 70° C studied (Figs 4.17, 4.18). With PSBH spores, glucose oxidase activity was unaffected at 60° C till 10 min. After this period, the enzyme activity of PSBH was found to decrease with increase in time. 35% of the activity was retained when they were exposed for 1 h at 60 °C. Further increase of exposure time drastically affected the enzyme

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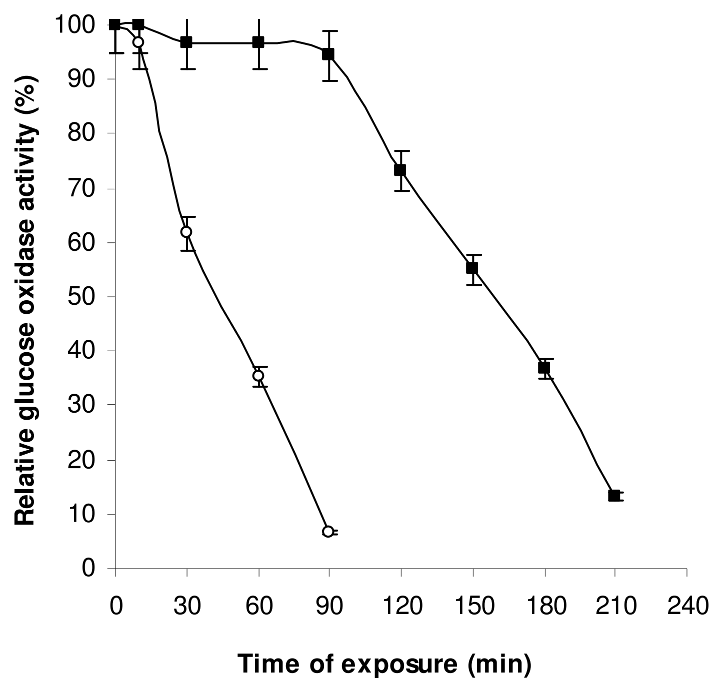

Fig 4.17. Relative glucose oxidase activity at 24 h of bioconversion with PSBH (permeabilized spore before heat treatment ○), PSAH spores (permeabilized spore after heat treatment ■) and commercial glucose oxidase ▲ exposed to 60°C.

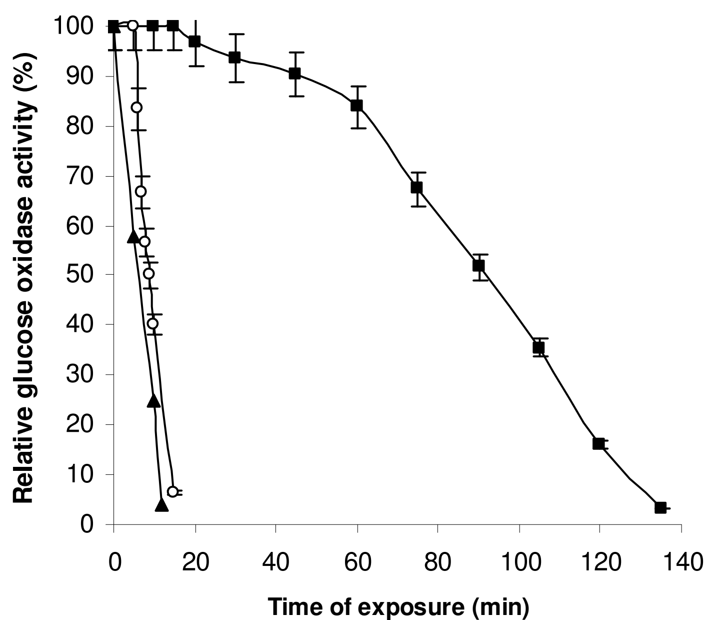

Fig 4.18. Relative glucose oxidase activity at 24 h of bioconversion with PSBH (permeabilized spore before heat treatment ○) PSAH spores (permeabilized spore after heat treatment ■) and commercial glucose oxidase ▲ exposed to 70°C.

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and only 6% of the original activity was found at 90 min. At 70°C, 83% of the original activity was retained when PSBH were exposed for 6 min. Beyond this time, the inactivation of the enzyme noticed was very rapid having only 6% of the original activity at 15 min. Glucose oxidase activity of PSAH was comparatively stable and the enzyme retained 94% of relative glucose oxidase activity at 60°C even after 90 min. Half of the original enzyme activity was retained at 150 min of exposure to 60°C, finally the enzyme activity reached 13% at 210h. At 70°C, 90% of the enzymatic activity was retained at 45 min. 50% of the activity was retained at 90 min and the enzyme got inactivated completely at 140 min. Commercial glucose oxidase could not resist high temperature and got inactivated and retained only 4% of original activity when exposed 12 min at 70°C (Fig 4.18).

PSAH could not resist further higher temperatures. Glucose oxidase activity of PSBH and PSAH were similar at 80 and 90°C (Figs 4.19, 4.20). The enzyme activity of both kinds of spores was completely inhibited after 4 min at 80 °C, 90°C and their activity at these temperatures were comparable with the commercial enzyme.

Variation of glucose oxidase activity of PSAH and PSBH was interesting. Cellular membrane structure was disturbed due to permeabilization by freezing, thus easily facilitating heat inactivation of enzyme when spores were frozen before exposure to high temperature. PSAH resisted high temperature and 100% of activity was retained even after its incubation for 15 min at 70°C, while commercial glucose oxidase (1 g/L) only retained 25% of relative activity when exposed for 10 min at 70°C. Thus, glucose oxidase inside the spore is well protected by the rigid structure and it resisted temperature till 70°C. It is interesting to note that the stability of spore is transferred to its products. Glucose oxidase inside spores is comparatively thermostable when compared to the mycelial enzyme as it is well protected by the rigid spore structure. Thus, enzyme in the spores resembles a naturally immobilized enzyme protected from adverse environments, even temperatures upto 70°C for few hours which the mycelial enzyme from *A. niger* cannot withstand. It is also necessary to remark that this protective effect of the spore structure is suppressed when the temperature becomes higher than 80°C, which is probably the result of ultrastructural changes.

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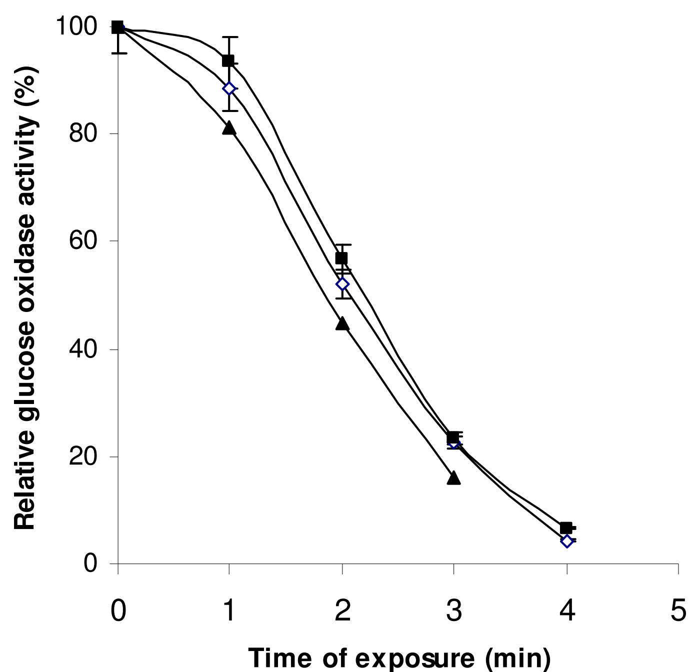

Fig 4.19. Relative glucose oxidase activity at 24 h of bioconversion with PSBH (permeabilized spore before heat treatment ○) PSAH spores (permeabilized spore after heat treatment ■) and commercial glucose oxidase ▲ exposed to 80°C.

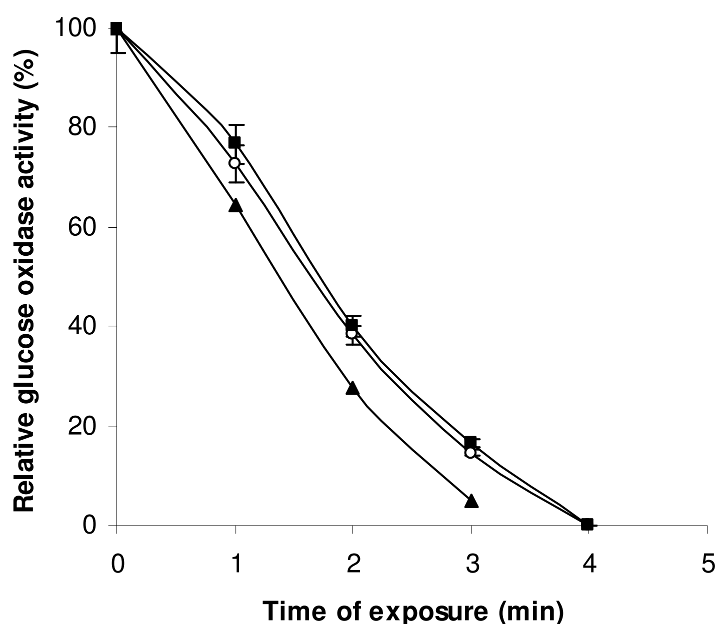

Fig 4.20. Relative glucose oxidase activity at 24 h of bioconversion with PSBH (permeabilized spore before heat treatment ○) PSAH spores (permeabilized spore after heat treatment ■) and commercial glucose oxidase ▲ exposed to 90°C.

Spores could carry out bioconversion reaction at temperatures 50 and 60°C (Fig 4.21); however, protective effect of the spore structure is suppressed when the temperature becomes higher than 60°C, which is probably the result of ultrastructural changes. On the other hand, commercial glucose oxidase was inactivated (not completely) with increase in temperature (Fig 4.22).

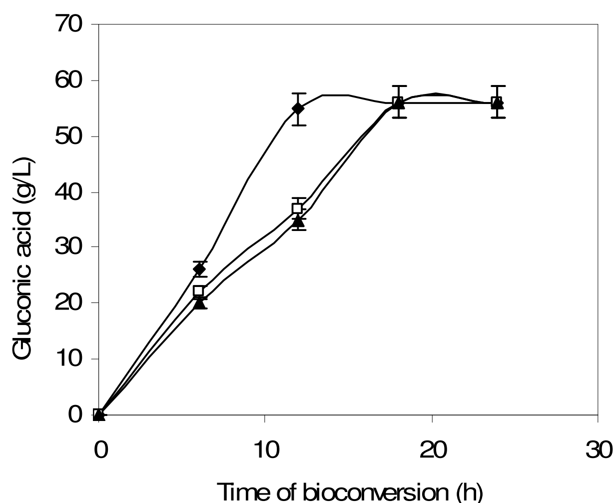

Fig 4.21. Effect of different temperatures (◆ 60°C, □ 50°C, ▲ 30°C) on biocatalytic activity of spores. Volume -100mL ; Spore concentration - 10^{10} spores/ml; Permeabilization - 10% citral for 18 h, Glucose concentration - 50g/L, Neutralization - calcium carbonate.

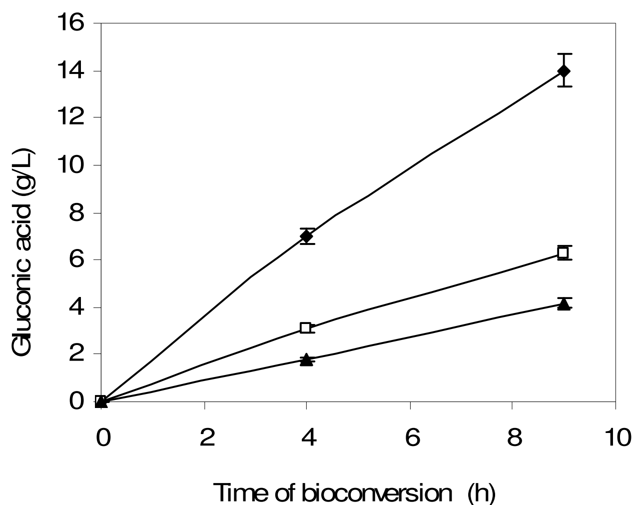

Fig 4.22. Effect of different temperatures (◆ 60°C, □ 50°C, ▲ 30°C) on biocatalytic activity of commercial glucose oxidase.

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Half life of commercial (Sigma) glucose oxidase from *A. niger* exposed to 67° C is 4.5 min (Gouda et al 2003). Optimum temperature of *Aspergillus niger* glucose oxidase is 40° C (Kalisz et al 1990) and the denaturation point of periodate oxidized enzyme of *A. niger* is as 72.8° C (Nakamura et al 1976). Heat resistance of enzymes is markedly enhanced when they are present in spores rather in vegetative cells (Murata 1993). Spore walls are resistant to extreme conditions and it is interesting to note that the stability of spore is transferred to its products. Glucose oxidase inside spores is comparatively thermostable and it is well protected by the rigid spore structure. It is also necessary to remark that yield was very similar to the bioconversion pattern of spores at 30°C (Fig 4.21).

4.9 Reuse of spores as catalyst

Spores were utilised repeatedly as biocatalyst for over 14 cycles of bioconversion. They were harvested after each cycle of bioconversion and the next cycle was started by transferring the washed spores into fresh glucose medium Gluconic acid production pattern was similar for each cycle without any appreciable loss of activity (Fig 4.23). This could be achieved as there is no enzyme leakage and enzyme in the spore closely resembled immobilized enzyme. Thus the biocatalyst could be used with considerable operational stability even after many cycles of bioconversion.

Vezina et al 1963 reported a minor decrease in steroid hydroxylation by spores of *A. ochraceus* with age and successfully reused the spores 13 times. Conversely, Johnson et al 1968 reported that the enzymes responsible for starch hydrolysis are lost or inactivated as the spore ages. Similarly, Mandels 1963 reported a decrease in respiration with increasing age of spores of *Memnoniella echinata* and *Myrothecium verrucaria.* The endogenous respiration of aging *M. verrucaria* spores was low, but constant and actually equalled the endogenous respiration of young spores after a period of equilibration amounting to about 6h. Therefore no generalizations can be made from the limited evidence available as to the reusability of fungal spores for successive use.

4.10. Stable enzyme activity of spores

Biocatalytic activity of *Aspergillus niger* spores stored at -20°C for one year was comparable with that of spores stored at the same temperature for one day (Fig. 4.24). This indicated that there was no loss of enzyme activity during long storage time. Spores of *A. ochraceus* were stable for one year at -20° C or 3 months at 4°C without any detectable loss of hydroxylating

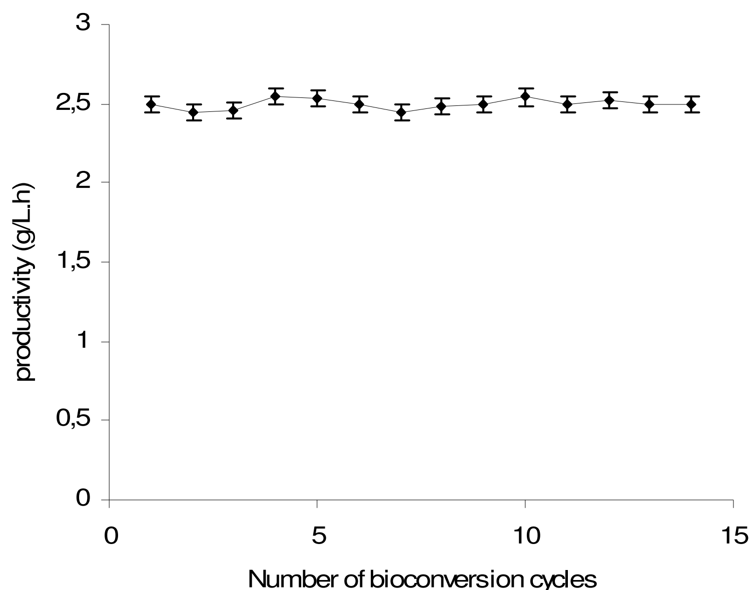

Fig 4.23. Reuse of spores as catalyst in the bioconversion of glucose to gluconic acid. Volume -100mL Spore concentration - 10^9 spores/ml; Permeabilization - 3% citral for 5h, Glucose concentration - 50g/L, Neutralization - calcium carbonate.

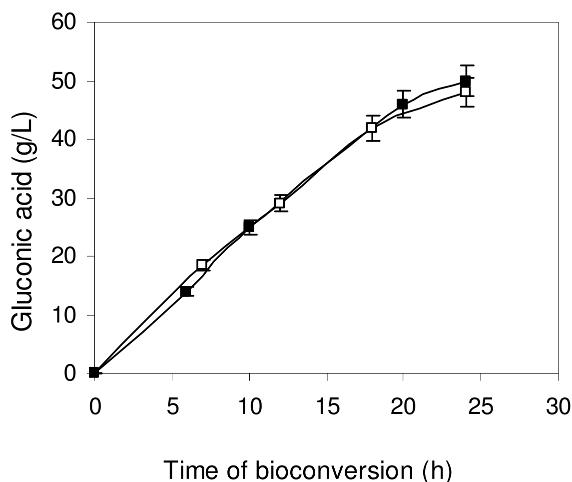

Fig 4.24. Bioconversion of glucose to gluconic acid by spore stored at freezer (-20°C) for 1 year further treated with 3% citral for 5h. (□); spores stored at freezer for 24 h further treated with 3% citral for 3 h (■). Spore concentration - 10^9 spores/ml; Initial glucose concentration -50 g/L; Temperature 30°C; Volume- 25 mL.

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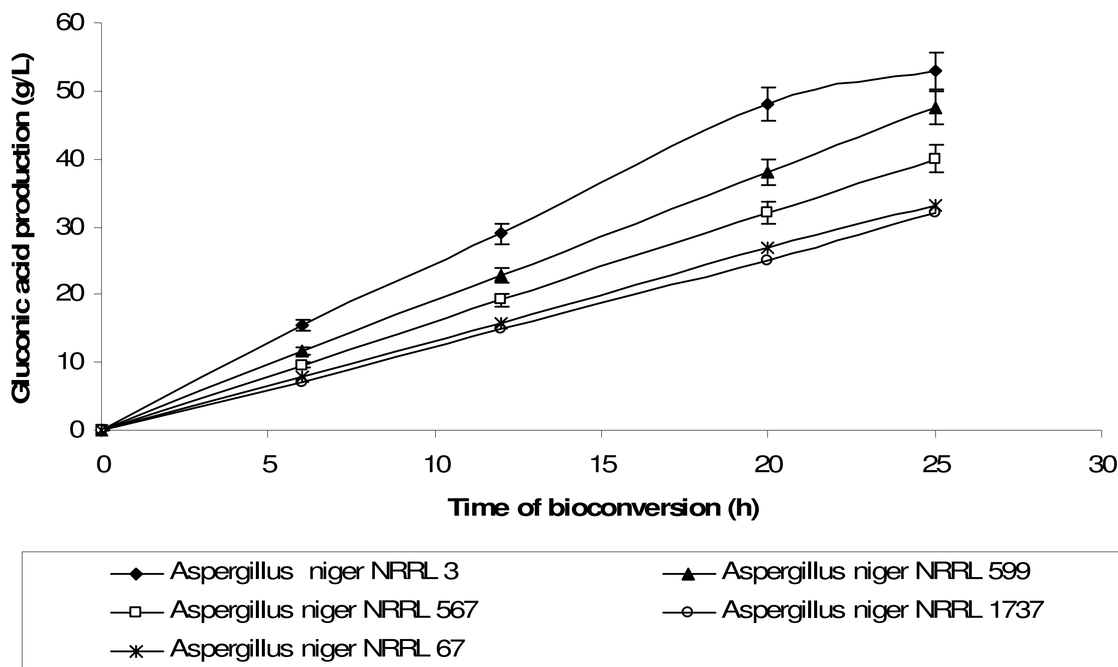

Fig 4.25 Bioconversion of glucose to gluconic acid catalysed by spores of various *Aspergillus niger* strains. Conditions: Volume - 50 ml (In Erlemeyer flask), Spores - frozen spores treated by 3% citral for 5h; Spore concentration - 10^9 spores/ml; Glucose concentration - 50g/L; Stirring speed - 200 rpm, pH 6.5 (neutralised by calcium carbonate), Temperature - 30°C.

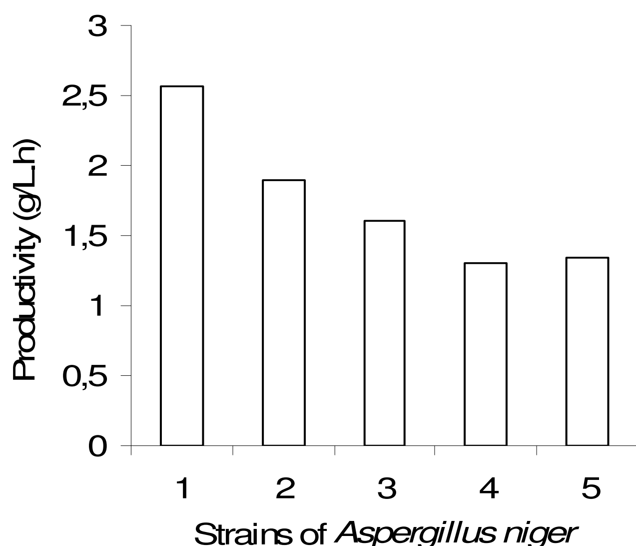

Fig 4.26. Productivity of gluconic acid by various strains of *Aspergillus niger* (**1** - *Aspergillus niger* NRRL 3, **2** - *Aspergillus niger* NRRL 599, **3** - *Aspergillus niger* NRRL 567, **4** - *Aspergillus niger* NRRL 67, **5** - *Aspergillus niger* NRRL 1737). Conditions: Volume - 50 ml (In Erlemeyer flask), Spores - frozen spores treated by 3% citral for 5h; Spore concentration - 10^9 spores/ml; Glucose concentration - 50g/L; Stirring speed - 200 rpm, pH 6.5 (neutralised by calcium carbonate), Temperature - 30°C.

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activity. Commercial biocontrol agent 'green muscle' (spores) lasts for 3 years at 4°C. The longevity of spore products has a direct consequence of their high resistance towards external factors (Wolken et al 2003). An extreme example of the longevity of spores was spores of *Bacillus sphaericus* that was revived and cultured after having been dormant for more than 25 million years embedded in piece of amber (Cano & Borucki 1995).

4.11. Assessment of other *A. niger* strains

Among the five *A. niger* strains, spores of *A. niger* NRRL 3 was the most efficient biocatalyst, exhibiting the highest reaction rate (2.56g/L.h.). Bioconversion carried out with other strains of *A. niger* also had a yield close to 100%, however the productivity was lesser when compared to *A. niger* NRRL 3 (Fig 4.25). With spores of *A. niger* NRRL 599 and A. *niger* 567, average reaction was 1.9 g/L.h and 1.6 g/L.h respectively (Fig 4.26). Bioconversion with spores of other *A. niger* strains such as NRRL 67 and NRRL 1737 had a reaction rate of 1.3 g/L.h with the production of 32 g/L of gluconic acid and a yield of 1.07 g/g at the end of 25 h. Thus the analysis showed that the spores were reservoir of glucose oxidase, active and were able to act as a catalyst with high yields. However, the best results were obtained with *A.niger* NRRL 3. Among the fungal strains evaluated for gluconic acid production, strains of *A. niger* NRRL 567, NRRL 599, NRRL 67, NRRL 1737 are known to produce considerable amounts of citric acid (Saad 2006, Barrington & Kim 2008, Saad 2004). *A. niger* NRRL 599 produced gluconic and citric acids in crude date syrups at concentrations of 6.0 and 1.47 g/litre, respectively. Gluconic acid concentration increased as pH increased from 3.5 to 6.5, reaching its maximum at pH 6.5 (Saad 2004).

4.12. Conclusions

The use of fungal spores as a biocatalyst needs four conditions to be met. They are:

- ability to produce large amounts of pure spores
- this material must harbor the enzyme(s) of interest
- germination of spores must be inhibited
- the wall/membrane complex must be permeabilized in order to ensure contact between the intracellular enzyme and the substrate.

The two first points are related to data reported in chap 3. This chapter clearly demonstrated that the last point was also met with spores of *A. niger* grown on buckwheat seeds. Hence, *A. niger* acted as reservoir of glucose oxidase since it acted as a biocatalyst in the conversion of glucose to gluconic acid when permeabilized and its germination was

arrested. When their germination was inhibited by sodium azide, in addition to cell permeabilization by freezing, productivity of 1.5g/L.h was achieved. Then, it was found that sodium azide had an inhibitory effect on glucose oxidase. The feasibility of the use of monoterpenes to both permeabilize the spores and inhibit their germination was examined. Results showed that they were able to have both effects, and it was possible to increase the initial rate of reaction up to 4.35 g/L.h with spores treated with 10% citral for 18h. Optimization studies showed that use of freeze- thawed spores at a concentration of 10^9 spores/ml treated with citral (3% v/v) for 5 h efficiently permeabilized the spores yielding high reaction rate. Fed-batch production of gluconate was carried out following the above mentioned conditions by sequential additions of glucose powder and pH regulated with a solution containing 2 mol/L of either NaOH or KOH. Average rate of reaction was 5.3 g/l.h with 178 g /L of gluconic acid produced out of 164 g/ L glucose consumed after approx. 36 h reaction, which corresponded to a molar yield close to 100%.

Bioconversion performance of the spore enzyme was compared with the commercial glucose oxidase at 50, 60 and 70°C. Results showed that the spore enzyme was comparatively stable at 60° C. It was also found that the spores could be reutilised for more than 14 cycles with almost similar reaction rate. Similar biocatalytic activity was rendered by spores after its storage of 1 year at -20°C. This study provided an experimental evidence of the significant catalytic role played by *A. niger* spore in bioconversion of glucose to gluconic acid with high yield and stability, giving protection to glucose oxidase.

The interesting fact about this system is its high reaction rate and its stability. Hence, the protection rendered by spores to glucose oxidase even at high temperature (60°C), their reusability and their easy storage add advantages to the process.

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CONCLUSION – FUTURE PROSPECTS

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The role of conidial spores as a reservoir of glucose oxidase and the suitability of this system as a biocatalyst in a bioconversion reaction for the production of gluconic acid was demonstrated. Solid-state fermentation (SSF) was carried out in column bed bioreactor for the production of *A. niger* spores. Growth parameters, sporulation and kinetics of gluconic acid production were analysed at different time intervals during the course of SSF. Spores harvested from the SSF was utilised as a catalyst in the bioconversion reaction. Important and significant results are summarised below :

- ✓ Starchy substrate was suited for the production of spores by SSF for its use as biocatalyst in the conversion of glucose to gluconic acid.

- ✓ Buckwheat seed was the best substrate among all other substrate used followed by rice.

- ✓ Among the five *A. niger* strains, spores of *A. niger* NRRL 3 was the most efficient biocatalyser, exhibiting the highest reaction rate.

- ✓ Glucose oxidase synthesized by the mycelium of *A. niger* during SSF was not excreted to the SSF medium but transported to spores.

- ✓ Spores acted as catalyst and carried out the bioconversion reaction effectively, once their germination was inhibited and spore cells were permeabilized.

- ✓ Spores harvested at later period of SSF (196 h) produced high titres of gluconic acid in the bioconversion medium when compared to the spores harvested at early (48 h) stages of SSF.

- ✓ Initially freezing and thawing was used for permeabilizing the spore cells; sodium azide was incorporated in the bioconversion medium for inhibiting germination of spores. By these treatments, spores could be used as catalyst in the bioconversion of glucose to gluconic acid and an initial rate of 1.5g/L.h.

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- ✓ The use of conventional chemical or mechanical cell permeabilization methods such as use of organic solvent, surfactant, sonication, enzyme treatment, acid treatment or heat treatment could not help in the permeabilization of the spore membrane.

- ✓ Permeabilization allowed only low molecular weight compounds (glucose and gluconic acid) to cross the spore membrane and the glucose oxidase was never released outside the spore. Thus, glucose oxidase in the spore was naturally protected from the external conditions, and leakage of the enzyme.

- ✓ Thus the possibility for glucose oxidase to be exocellular could be eliminated. It confirmed that the enzyme was intracellular or bound to the plasma membrane.

- ✓ There was no protein synthesis, or carbondioxide evolution noted during the bioconversion.

- ✓ It was found that sodium azide had an inhibitory effect on glucose oxidase.

- ✓ Spore germination inhibition was arrested by treatment with 15 different monoterpenes and terpenoids. They could also significantly permeabilize the spore membrane and increase the productivity.

- ✓ Citral was selected among all the terpenes tested and it was possible to increase the initial rate of reaction up to 4.35 g/L.h. with 10% citral treatment for 18 h before bioconversion.

- ✓ Optimization of citral concentration, time of exposure and spore concentration was carried which resulted in the selection of treatment of 10^9 spores/ml with 3% citral for 5h.

- ✓ Fed-batch production of gluconate was done in a bioreactor with the best conditions [(10^9 spores/ml) of freeze- thawed spores treated with citral (3% v/v) for 5 h] followed by sequential additions of glucose powder and pH regulated with a solution containing 2 mol/L of either NaOH or KOH.

- ✓ Average rate of reaction was 5.3 g/l.h with 178 g /L of gluconic acid produced out of 164 g/ L glucose consumed after approx. 36 h reaction, which corresponded to a molar yield close to 100%.

- ✓ Spores could carry out bioconversion reaction at temperatures 50 and 60°C. On the other hand, commercial glucose oxidase activity was found to decrease with increase in temperature (50, 60°C).

- ✓ The effect of the physical parameters such as pressure and temperature on spore permeability and its glucose oxidase stability was studied. Pressures up to 2.7 kbars had no effect. Exposure of spores to temperatures up to 70°C revealed that spores acted as a protective agent towards glucose oxidase activity. This protective effect was suppressed above 80°C, showing alteration of the spore ultrastructure.

- ✓ Spores could be reutilised for more than 14 cycles with almost similar reaction rate. This could be achieved as there is no enzyme leakage and enzyme in the spore closely resembled immobilized enzyme.

- ✓ Biocatalytic activity was rendered by spores even after its storage of 1 year at -20°C and was comparable with the spores stored for 24 h.

In conclusion, the results reported here demonstrated the feasibility of the use of conidiospores of *Aspergillus niger* as a vector for glucose oxidase extraction from solid media, and their direct use as biocatalyst in the bioconversion of glucose to gluconic acid. Use of spores in bioconversion adds advantages by eliminating purification, as enzyme within the spore resembles immobilized enzyme. Spores of *A. niger* served as efficient catalyst in the model bioconversion reaction after permeabilization. Glucose oxidase within is well protected from the adverse environmental conditions such as high temperature or pressure and whose activity was comparatively better than the commercial glucose oxidase. The biocatalyst was used with considerable operational stability even after many cycles of bioconversion. The stability of the enzyme, which can be attributed to the lack of active protease in the conidia, and the longevity of spore stability add advantage to the process. It is simple, versatile, easy to handle, reliable and economical process. To our knowledge, this is the first detailed study

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on the ability of *A. niger* spores to act as reservoir of enzyme synthesized during SSF without its release into solid media. The interesting fact about this system is its high reaction rate and its productivity. Spore stability, the protection rendered by spore to glucose oxidase even at high temperature (60°C), reusability, its storage and the simplicity of the process without much regulation of other parameters adds advantages to the process. This could serve as an innovative process and could compete efficiently with the conventional use of mycelial form of the fungus in gluconate production.

Future work could be aimed at increasing the productivity by studying the cellular transports and by establishing the localization of enzyme in spores. Investigation of the presence of other active enzymatic systems in fungal spores could also be a promising perspective of this work.

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ABSTRACT

Spores of *Aspergillus niger* resulting from asexual reproduction process was found to possess glucose oxidase. Solid-state fermentation was carried out for the production of spores using various agricultural crops and residues such as buckwheat seeds, rice, corn, cassava root, jack fruit seed, wheat bran and cassava bagasse. Glucose oxidase was found to be quantitatively translocated from the mycelium to conidia during the fungal development in solid-state cultivation. Spores acted thus as a reservoir of enzyme and could be used as a catalyst in the conversion of glucose to gluconic acid after their harvest. It was shown that expression of biocatalytic activity needed conidia permeabilization and prevention of their germination. The optimum conditions for the reaction involved the use of freeze - thawed spores (10^9 spores/ml) treated with citral (3% v/v) for 5 h at 30°C followed by sequential additions of glucose powder using a fed-batch strategy. Average rate of reaction was 5.3 g/l.h with 178 g /L of gluconic acid produced out of 164 g/ L glucose consumed after approx. 36 h reaction, which corresponded to a molar yield close to 100%. It was found that spores could be reused for 14 cycles with almost similar reaction rate. Similar biocatalytic activity was rendered by spores even after storage of 1 year at -20°C. Thus this process could efficiently compete with the conventional use of mycelial form of the fungus in gluconate production.

RESUME

Les spores *d'Aspergillus niger* résultant d'un processus de reproduction asexuée possèdent une glucose oxydase sous forme active. Ces spores ont été produites par fermentation en milieu solide en utilisant différents substrats tels que les graines de sarrasin, de riz, de maïs, les racines de manioc, les semences de jack fruit, le blé, la bagasse de manioc et la bagasse. La glucose oxydase est quantitativement transféré du mycélium aux spores au cours de développement fongique sur substrat solide. Les spores se comportent donc comme une source d'enzyme utilisable dans la conversion du glucose en acide gluconique après leur récolte. Il a été démontré que l'expression de leur activité biocatalytique nécessite une permeabilisation des conidies et la prévention de leur germination. Les conditions optimales de réaction impliquent l'utilisation de spores ayant subi un cycle congélation-décongélation (10^9 spores / ml) traités par du citral (3% v / v) pendant 5 h à 30 °C, la réaction se déroulant selon un mode fed-batch impliquant des ajouts successifs de glucose en poudre. La vitesse moyenne de réaction obtenue est de 5,3 g / l.h avec 178 g / L d'acide gluconique produit pour 164 g / L de glucose consommé après environ 36 h de réaction, ce qui correspond à un rendement molaire proche de 100%. Il a été constaté que les spores peuvent être réutilisées pendant 14 cycles de réaction sans perte d'activité décelable. De même, un stockage d'un an à -20 °C ne conduit à aucune perte d'activité. Ainsi, ce processus pourrait concurrencer efficacement les procédés classiques de production de gluconate, qui impliquent la mise en œuvre de la forme mycélienne du champignon.