Introduction
1. Introduction

Microorganisms are ubiquitous in nature. The success of microorganisms lies in their ability to produce a wide range of enzymes enabling them to use many organic compounds as nutrient sources. For a long time Microorganisms, had an extremely important place in realms of microbiology and biochemistry due to their ability to produce useful enzymes. Microbial enzymes have been employed for rapid oxidation and decomposition of proteins, carbohydrates and fats. The peptides and proteins from microorganisms have been recently reviewed. Microorganisms are capable of producing large amounts of specific enzymes although the levels are relatively too low for commercial exploitation in the naturally occurring wild type strains. These strains may be mutated or the genes coding for the enzymes of interest are cloned and over expressed in suitable hosts.

1. Extremozymes from Thermophiles

An extremozyme is an enzyme often created by extremophiles that can function under extreme environmental conditions such as very high pH, very low pH, high temperature, high salinity or other factors. This feature makes these enzymes of interest to a variety of biochemical application.

Some extremophiles are subject to multiple stress conditions. Extremophiles are structurally adapted at the molecular level to withstand these harsh conditions. The biocatalysts, called extremozymes, produced by these microorganisms, are proteins that function under extreme conditions. Due to their extreme stability, extremozymes offer new opportunities for bio catalysis and biotransformation. Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes. Currently, only 1–2 % of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of extremophiles. However, the renewed interest that is currently emerging as a result of new developments in the cultivation and production of extremophiles. The success in the cloning and expression of their genes in mesophilic hosts will increase the bio catalytic applications of extremozymes.

Thermophilic microorganisms have attracted most attention and are amongst the most studied of the extremophiles. The bio catalytic potential of Thermophiles and their enzymes has been reviewed by Adams et al (1995). Thermophiles can be generally classified into
moderate thermophiles (growth optimum 50–60°C), extreme thermophiles (growth optimum 60–80°C) and hyperthermophiles. Among the extreme thermophiles, multicellular animals or plants cannot tolerate temperatures above about 50°C and the eukaryotic microbes that have been discovered so far cannot survive at temperatures higher than 60°C. Thermophilic fungi are potential source of thermo stable enzymes with novel properties, for applications in industry. Thermophily in fungi is not as extreme as eubacteria or archaea, some species of which are able to grow near 100°C. Thermophilic fungi have growth temperature minimum at or above 20°C and maximum at or above 50°C. Perhaps because their moderate degree of thermophily and because their habitats are not exotic, thermophilic fungi have not received much publicity and attention. Based on literature survey it has been observed that mainly it is a tendency of fungi to produced glucose oxidase dominantly as compare to other microbes.

2. Economic production by fermentation

If a fermentation process is to yield a product at competitive price, the chosen microorganism should give the desired end-product in predictable and economically adequate quantities. A number of basic objectives are commonly used in developing successful economically viable processes are:

- Investment on Fermentor and ancillary equipment should be minimum. The equipments thus used should be reliable.
- Raw materials should be as cheap as possible and utilized efficiently. A search for possible alternative material should be made.
- When raw materials form a major part of total costs, it is obvious that media and microbial strain improvement should form a major part of development programme.
- There should be savings in labour and process should be automated.
- When a batch process is operated, the growth cycle should be as short as possible to obtain the highest yield of the product and allow for maximum utilization of equipment.
- Recovery and purification procedure should be simple.

In any process it is important to know the cost breakdown, so that it may be seen where the biggest potential savings may be achieved. In a review of a number of processes, Nyiri and Charles (1977) concluded that four basic components contributed to the process cost in the following decreasing order: raw materials, fixed costs, utilities and labour. When
raw materials are major part of the total cost it is obvious that the media and microbial strain improvement research should form major part of development programme.

It is most important to build equipment as large as possible because of the economy scale. There is an empirical relationship between cost and size of items of equipment. According to this relationship, as facility size increases, its cost increases thus:

\[
\frac{\text{Cost}_1}{\text{Cost}_2} = \left(\frac{\text{Size}_1}{\text{Size}_2}\right)^n
\]

Where \( n \) is an exponent or scale factor. Scale factors have been estimated to be 0.6 for brewing, 0.7 to 0.8 for a single cell protein, 0.6 for antibody production process and 0.75 for fermentation process.

3. Strain Improvement

Strain improvement using mutagenesis to of different microorganisms as a strategy for the improvement of desired production make process cost effective. Historically, mutation programme contribute significantly increase yield of desired products by four fold reported in streptomycin, chlortetracycline and erythromycin (1970-85). It is always very important to decide if strain improvement programme can be justified on economical grounds to improve overall economy process.

4. Impact of immobilisation on production of Enzyme

Immobilization of microbial cells in biological processes can occur either as a natural phenomenon or through artificial process. While the attached cells in natural habitat exhibit significant growth, the artificially immobilized cells are allowed restricted growth. Since the time first reports of successful application of immobilized cells in industrial applications was published, several research groups all over the world attempted whole-cell immobilization as a viable alternative to conventional microbial fermentations. Various immobilization protocols and numerous carrier materials were tried. The cell immobilization process has also triggered interest in bioreactor design. Using immobilized cells, different bioreactor configurations have been reported with variable success. The study on the physiology of immobilized cells and development of non-invasive measuring techniques have remarkably improved understanding on microbial metabolism under immobilized state.

The industrial biotechnology processes using microorganisms are generally based on the exploitation of the cells in the fermentation medium. The classical fermentations suffer from various constrains such as low cell density, nutritional limitations, and batch-mode
operations with high down times. It has been well recognized that the microbial cell density is of prime importance to attain higher productivity. The continuous fermentations with free-cells and recycled cells options aim to enhance the cell population inside the fermented broth. However, the free-cell systems cannot operate under chemostatic mode that decouples specific growth rate and dilution rates. During the last 20–25 years, the cell immobilization technology, with its origins in enzyme immobilization, has attracted the attention of several research groups. This novel process eliminates most of the constrains faced with the free-cell systems. The remarkable advantage of this new system is the freedom it has to determine the cell density prior to fermentation. It also facilitates operation of microbial fermentation on continuous mode without cell washout. The whole-cell immobilization process decouples microbial growth from cellular synthesis of favoured compounds.

Immobilization commonly is accomplished using a high molecular hydrophilic polymer gel such as alginate, carrageenan, agarose, etc. In these cases, the cells are immobilized by entrapment in the pertinent gel by a drop-forming procedure. When traditional fermentations are compared with the microbial conversions using immobilized cells, the productivity obtained in the latter is considerably higher, obviously partly due to high cell density and immobilization-induced cellular or genetic modifications.

**Immobilization methods**

Many methods namely adsorption, covalent bonding, cross linking, entrapment, and encapsulation are widely used for immobilization. These categories are commonly used in immobilized enzyme technology. However, due to the completely different size and environmental parameters of the cells, the relative importance of these methods is considerably different. The criteria imposed for cell immobilization technique usually determine the nature of the application.

**5. Enzyme activity**

Immobilization technique of an enzyme is for commercial use, as they possess many benefits to the expenses and processes of the reaction. In this, an enzyme is attached to an inert, insoluble material e.g. calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again. This is a more efficient process and so is widely used in
industry for enzyme catalysed reactions. An alternative to enzyme immobilization is whole cell immobilization. Enzymes are biological catalysts that increase the rate of reactions without themselves getting consumed. They may be used repeatedly as they remain active for longer duration. However, in most of the process, enzymes are mixed in solution with substrates and cannot be economically recovered. Post reaction they are generally wasted. Thus, there is an incentive to use enzymes in an immobilized or in solubilised form so that they may be retained in biochemical reactor for further catalysis.

Enzymes are able to catalyze the most complex chemical process under the mildest experimental and environmental conditions. Enzymes could be excellent industrial catalyst for sustainable development. However in general, enzyme does not fulfil the requirements for industry-as they are:

- instable,
- soluble;
- they undergo inhibitions;
- poorly selective on non-natural substrates.

The utilization of these necessary immobilization techniques to develop protocol to improve other enzyme properties seems to be a very exciting goal.

**Advantages of Enzyme immobilization:**

a) The fixation of enzyme on porous supports prevents them from aggregation, proteolysis, and interrelations with hydrophobic interfaces.

b) The multipoint covalent attachment of enzyme on highly activated supports may promote an intense regidification of 3D structure of enzymes. The relative distances among all residues involved in the multipoint covalent immobilization have to be preserved unaltered during any conformational changes induced by any distorting agent. Thus these conformational changes may become strongly reduced and these immobilized enzymes are much more stable than the one-point immobilized ones.

c) Multi-subunit immobilization of multimeric enzymes should promote dramatic stabilization under conditions where dissociation of subunits is the main mechanism of inactivation. After a correct orientation of the enzymes should promote dramatic stabilization under condition of enzyme on the support surface, involving the maximum amount of the enzyme subunits. An additional regidification of each subunit may exert additional beneficial effect for the enzyme stability.

d) Selectivity of enzymes can be greatly improved by promoting multipoint covalent immobilization on different enzyme regions.

e) In many instances, enzymes may be immobilized, stabilized and purified in just one step using tailor made supports.

f) Enzymes can be reversibly immobilized on ionized polymeric layers attached to the support surfaces.

**Technique of enzyme immobilization**

2. Cross linking
3. Entrapment: Occlusion within a cross linked gel and microencapsulation.

**Physical adsorption:** This method is based on physical and adsorption of protein on the surface of water-insoluble carriers. Examples of suitable adsorbent are ion-exchange matrices, porous carbon, clay hydrous metal oxides, glasses and polymeric aromatic resins. The bond between enzyme and carrier molecule may be ionic, covalent, hydrogenated and coordinated covalent or combination of any these.

Immobilization can be brought about coupling and enzyme either to external or internal surface of carrier. The external surface method is advantageous as it does not involve in condition like pore diffusion. The disadvantage includes exposure of enzyme to microbial attack, physical abrasion of enzyme to turbulence associated with bulk solution.
**Covalent bonding:** Covalent binding is the most widely used method for immobilizing enzymes. The covalent bond with support could be established should be non essential for enzymatic activity. The most common technique is to activate a cellulose-based support with cyanogens bromide, which is then mixed with cyanogens bromide. The protein functional group present on enzyme. The protein functional groups which could be utilized in covalent coupling include: Amino group, Carboxylic group, Phenol ring, Indole group and Imidazole group. On the other hand examples of polymeric supports include: Amino and related groups of polysaccharides and silica gel etc.

The polymers may be engaged in direct coupling as well as could be modified by other coupling groups or activating groups. The most commonly used polymers are polysaccharides, polyvinyl alcohol, silica and porous glasses. The advantages are strength of binding is very strong, so leakage of enzyme from support is absent or very little. This is a simple, mild and often successful method of wide applicability.

**Cross linking:** This method is based on the formation of covalent bonds between the enzyme molecules, by means of multifunctional reagents leading to three dimensional cross linked aggregates. The most common reagent used for cross linking is glutaraldehyde. The advantage of use of this method is very little desorption and best used with in conjunction with other method.

**Entrapment:** In entrapment, the enzymes or cells are not directly attach to support surface but simply entrapped inside the polymer matrix. Entrapment is carried out by mixing biocatalyst in monomer solution, followed by polymerization initiated by change in temperature or chemical reaction. Polymers like polyacrylamide, collagen, cellulose acetate and calcium alginate or carrageen are used as matrices. This method helps us in minimizing loss in enzyme activity.

6. Glucose Oxidase and Microbial Resources

Glucose oxidase is present in all aerobic organisms and has become a very useful enzyme for its wide applications especially in food and pharmaceutical industries. A large number of microbes including bacteria, actinomycetes and filamentous fungi are used for production of glucose oxidase. Glucose oxidase is produced on large scale using *Aspergillus niger* and *Penicillium amagasakiene*. Among eukaryotic organism, only few species of fungi have ability to thrive at temperature between 45 and 55°C.

Among 1486 mould strains isolated from natural sources (screened for extracellular glucose oxidase) only 119 (*Aspergillus and Penicillium*) showed this enzyme activity. The best glucose oxidase producer, *Aspergillus niger* have been isolated from decaying tree. A large number of filamentous fungi such as *Aspergillus niger* UAF-1, *Aspergillus terrus*, *Aspergillus flavus*, *Aspergillus oryzae*, *Penicillium Sp.CBS120262*, *Penicillium notatum*, *Penicillium amagasakiense*, *Penicillium chrysogenum* SRT-1, *Penicillium variabiles*, *Penicillium funiculosum*, *Penicillium fellutanum*, *Penicillium adamezii* LF F-2044.1, *Penicillium glaucum*, *F. lini*, white-rot fungus *Pleurotus ostreatus*, *Talaromyces flavus* and other genus *Gliocadium*, *Scopulariopsis*, *Gonatobotyrs* and yeast cell *Aureobasidium pullulans* are glucose-oxidase producers.

Many bacteria are involved in production of this enzyme; some of these are *Zymomonas mobilis*, *Micrococcus* and *Enterobacter* (Scott, 1975), *G. oxydans*, *A. methanolicus*, *Pullularia*, *Scopulariopsis*, *Pseudomonas fluoscence*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, and *Pseudomonas stutzeri*.
Pseudomonas aeruginosa and Cellulomonase. Actinomycetes genera include Actinomycetes, Streptomyces and Nocardia producing glucose oxidase.

The success of Aspergillus niger group for industrial production of biotechnological products is largely due to the metabolic versatility of this strain. Aspergillus niger is well known to produce a variety of enzymes, organic acids, plant growth regulators, mycotoxins and antibiotics. The industrial importance of Aspergillus niger groups are not limited on its 335 native products but also on development and commercialization of the new products which are derived by modern bioprocess and molecular biology techniques.

Glucose oxidase (GO) is purified from a range of different fungal sources, mainly from the genus Aspergillus and Penicillium. Despite the fact that GO has been produced by a variety of filamentous fungi, Aspergillus niger is the most common fungus utilised for the production of GO. The Penicillium species GO has shown to exhibit more advantageous kinetics for glucose oxidation than that of Aspergillus niger. The reported glucose oxidase was found to be produced at temperature between 30 to 40°C and pH up to 6.0. Cloning and over-expression of glucose oxidase genes in Saccharomyces cerevisiae and Escherichia coli from Aspergillus niger and Penicillium amagasakiense respectively, are successful done.

GO are used in large scale technological applications since the early 1950s. Major applications for GO include: the enzymatic determination of glucose using biosensors, for the production of gluconic acid and as food preservative. Implantable glucose sensors have found application with diabetes patients. GO in new forms with useful properties for in biotechnology continues to be of considerable interest despite the abundant availability of commercial Glucose oxidase.

7. Structure and Properties of Glucose Oxidase

Structure:

Enzymes are proteins, and the major constituent of proteins is an unbranched polypeptide chain consisting of amino acids linked together by amide bonds between the carboxyl group of one residue and the amino group of the next. Glucose oxidase (Gox, β-D-glucose: oxygen, 1-oxidoreductase, EC 1.1.3.4) is flavo-protein which catalyses the oxidation of β-D-glucose to glucono-δ-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor with the concomitant reduction Glucose oxidase. It is a dimeric protein composed of two identical subunits. Each subunit, or monomer, folds into two domains: one domain binds to the substrate, β-D-glucose, while the other domain binds non-covalently to a cofactor, flavin adenine dinucleotide (FAD), which it uses as a powerful oxidising
agent. FAD is a common component in biological oxidation-reduction (redox) reactions, in which there is a gain or loss of electrons from a molecule.

**Figure 1: Overall structure of glucose oxidase from Aspergillus niger**

![Figure 1: Overall structure of glucose oxidase from Aspergillus niger](image)

**Properties of Glucose Oxidase**

Glucose oxidase usually occurs as a glycoprotein, with a mannose-type carbohydrate content of around 16%. However, different forms of the enzyme have been isolated, including non-glycosylated enzyme from the fungus Phanerochaete chrysosporium. Glucose oxidase is secreted by the fungus, and is distributed between the extracellular fluid surrounding the mould, the cell wall, and in the slime mucilage. The synthesis of glucose oxidase can be induced by various substances, including molecular oxygen, which induces the transcription of the enzyme. Glucose oxidase may seem like an ordinary enzyme, but it has become commercially important in the last few years, gaining a multitude of different uses in the chemical, pharmaceutical, food, beverage, and other industries. In addition, gluconic acid, which is produced from the hydrolysis of D-glucono-1, 5-lactone has its own important industrial uses.

Glucose oxidase belongs to the large group of enzymes Oxidoreductase and is also called glucose aerodehydrogenase the molecular weight of GO ranges from approximately 130 kD (Kalisz et al., 1997) to 175 kD (Eriksson et al., 1987). The GO enzyme is highly specific for the β-anomer of D-glucose, while the α-anomer does not appear to be a suitable substrate. Low GO activities are exhibited when utilizing 2-deoxy-D-glucose, D-mannose and D-galactose as substrates. Inhibitors of GO include p-chloromecuribenzoate, Ag⁺,Hg²⁺,Cu²⁺,hydroxylamine, hydrazine, phenyl hydrazine, dimedone and sodium bisulphate .The carbohydrate and amino acid compositions of the enzymes has also investigated and compared, which indicates that similar carbohydrates were contained in both enzymes which consist mainly of glucose, mannose and hexosamine. The Aspergillus niger GO contained more mannose and hexosamine than that of Penicillium amagasakiense, but less glucose. The overall carbohydrate content are found to be 16% for A. niger and 11% for...
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P. amagasakiense. The amino acid contents of both enzymes showed that the A. niger GO contained more histidine, arginine and tyrosine and less lysine and phenylalanine than the P. amagasakiense GO.

Glucose oxidase has a molecular weight of 160,000 D and consists of two identical polypeptide chain subunits having nearly equal molecular weights linked by disulphide bonds and it is highly specific for β-D-glucose (Bentley, 1966). Each subunit of the glucose oxidase contains one mole of Fe and one mole of FAD (Flavin adenine dinucleotide) and it contains 74% protein, 16% natural sugar and 2% amino sugars. The Glucose oxidase enzyme in its purest form is pale-yellow powder. Dried enzyme samples are stable at 0°C for upto 2 years. White 0.1 to 0.2% aqueous solutions are stable for one week at 5°C and its enzyme activity was lost on heating at 39°C (Sidney and Northon, 1955).

In glucose oxidase, FAD acts as an electron acceptor, which causes it to be reduced to FADH$_2$; the FADH$_2$ is then oxidised by the final electron acceptor, molecular oxygen, with the oxygen being reduced to hydrogen peroxide (H$_2$O$_2$). The active site of glucose oxidase contains three important amino acids involved in catalysis: His516, and Glu412, which is hydrogen-bonded to His559.

![Diagram of Glucose Oxidase Reaction](image)

**Fig: 2 Representation of GO reaction**

9. **Glucose-oxidase production**

Recent researches are focused on factors that regulate the production of GO. There are several media constituents like carbon, nitrogen, CaCO$_3$ and environmental factors such as temperature, pH and agitation involved in the production of GO. The above factors have to be optimized for the effective production of GO. There are few reports concerned in the
optimization of cultural conditions for the production of GO from various fungi (Sandeep et al., 2008). Optimization of media composition and other factors by using statistical method have few advantages over the single factor analysis at a time. It can be used for easy determination of important parameter from a large number of factors and the study of the interactions between the variables of the media constituents.

9.1 The influence of physical parameters on GO production:

The optimum pH ranges for the A.niger GO and P. amagasakiense GO production, are shown to be 3.5-6.5 and 4.0-5.5, respectively. A. niger GO is found to have a broader pH range than the P.amagasakiense. Glucose oxidase is being produced mostly by microorganisms such as Pencillium notatum, Pencillium chrysosporium, Aspergillus niger and Botrytis cinerea (Liu et al; 1998). The glucose oxidase enzyme is produced from Aspergillus niger in the submerged culture with continuous shaking at 180rpm (Fiedurck and Gromada, 1996). Industrially important fungi (Aspergillus niger) are isolated from soil samples using dilution and plating technique. Aspergillus niger can be identified by LCB staining. The isolated strain of A.niger is screened for Glucose oxidase production by plate assay. The high yielding strain of A.niger is isolated and maintained in agar slant as wild type. The strain of A.niger is checked for the Glucose oxidase production under different conditions, like pH, temperature, different carbon sources, nitrogen sources and CaCO₃ concentration. Temperature (60) and pH stability (8) of enzyme were also studied. The mycelia fungi Aspergillus and Penicillium serve as industrial producers of GO.

The glucose oxidase from Aspergillus niger is an intracellular enzyme, present in the mycelium of the organism and is released from the mycelium by means of cell disruption techniques (Zetelaki and Vas, 1968). The tertiary structure of glucose oxidase from Aspergillus niger has been determined by X-ray crystallography and results are drawn from experiments on electrical communication between the enzyme and the electrode. (Hecht et al, 1993).

9.2 The influence of organic and inorganic compounds on GO production:

Glucose oxidase is commercially prepared from two fungal sources, Aspergillus niger and Penicillium amagasakiense. Fiedurck et al (1986) screened the best glucose oxidase producer as Aspergillus niger from different fungi.

A. niger is considered to be the main producer of commercial glucose oxidase and many other enzymes, organic acids, growth regulators and other compounds which are generally recognized as safe (GRAS). Several carbon sources were used to propagate this mould such as hydrolyzed corn starch, glucose, sucrose, and molasses (Hatzinikolaou and
Macris, 1995) and many other hexoses and pentoses (El-Enshasy et al., 2001). Many workers have been studied the various methods of extraction and purification of the enzyme from cell-free extract.

For the production of GO predominate strains selected are Aspergillus niger and Penicillium spp. However, both the species are associated with some drawbacks. Aspergillus niger produces an intracellular enzyme, which requires opening of cell wall to release the enzyme, thus, ensures comparative high cost in recovery steps as compared to a extracellular enzyme. On the other hand, Penicillium spp produces an extracellular GO. However during fermentation, it produces a non-Newtonian fluid behaviour in poor mass transfer at high mycelium concentration. In view of this problem it is necessary to find an alternative strain which produces extracellular GO which is capable of maintaining good mass transfer at high mycelium concentration. There is also effect of metal ions on the glucose oxidase activity. At higher salt concentrations, the metal chlorides and metal gluconates show various degrees of inhibition (Yang et al., 1996).

Although A. niger grew on all the carbon sources that are tested, significant levels of GO has been only obtained using glucose, sucrose and molasses. Petruccioli et al (1993) studied GO production by 84 strains of the genus Penicillium and reported that P. expansum (1 strain), P. italicum (1 strain), P. chrysogenum (3 strains) and P. variabile (3 strains), are investigated for their ability to oxidize glucose, fructose, mannose, galactose, arabinose and xylose. Only one of the P. italicum strains (NRRL 983) displayed enhanced oxidizing activity towards mannose, galactose, and xylose being 32.38%, 17.90% and 26.40% compared to glucose (100%), respectively.

The fungus requires glucose as a carbon source and NaNO\(_3\) and peptone as nitrogen source to grow. Besides, the result is in good agreement with the studies done by Hatzinikolaou and Macris who reported that glucose is the principal inducer of glucose oxidase gene. NaNO\(_3\) as inorganic nitrogen source has been reported to have a stimulating effect on glucose oxidase production. Most gluconic acid and its derivatives are produced today by submerged fermentation with strain Aspergillus niger and product yields are close to the theoretical under well known process parameters.

**9.3 Formulation of media and designing of fermentation condition**

The most common microbial sources for GO production are selected strains of Aspergillus niger and Penicillium amagasakiense, although a high level of production have also been reported from Penicillium variabile. Typical problems that are usually encountered during their production are high cost of the substrate, low productivity and simultaneous
production of other enzymes such as catalase. To overcome these problems, it is suggested that economical and commercially available media be investigated to reduce the production costs. Some of the commercial substrates exploited for production of GO are molasses and corn steep liquor. The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are to be obtained. Rice polish is the flour taken from the basic brown rice during the process of making white rice. It contains parts of the rice germ and bran, and provides a high content of vitamins and iron. A reduction of the medium cost suggests that rice polish is a cost effective medium for fermentation.

During microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but is also used in the synthesis of polysaccharide and as energy source. The rate at which carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. Fast growth due to high concentration of rapidly metabolized sugars is often associated with high productivity of growth-associated products or primary metabolites.

Plackett–Burman designs are experimental design presented in 1946 by Robin L. Plackett and J.P. Burman. Their goal was to find experimental designs for investigating the dependence of some measured quantity on a number of independent variables (factors), each taking \( L \) levels, in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments. Plackett-Burman design has been employed to evaluate the significant of media constituents for glucose oxidase production.

10. Scale up of glucose-oxidase production:

10.1. Strain improvement:

Traditionally, strain development requires painstaking lengthy and tedious procedures to identify superior isolates among a mutagen-treated population. Rational selection procedures are considerably more efficient than random screening for selecting improved producers, and usually have a biochemical basis. Special environmental condition, toxic to the majority of cell types but less toxic or non-toxic to a desired minority of cells have been often employed to enrich a cell population to obtain desired mutants. The great advantage of this screening method is its simplicity; it does not require any profound understanding of molecular biology and physiology of the micro-organisms being manipulated. Strain improvement is a lengthy and laborious job, where we have to screen the better isolates among a mutagen-treated population. Various investigations have been conducted to improve glucose oxidase production by strain selection using classical screening and mutagenesis.
techniques. Physical and Chemical mutagens may induce mutations within a sequence originating mutagen-specific patterns of mutations. Still mutagenesis and selection are cost effective procedure for reliable short term strain improvement (Rowlands, 1984; Iftekhar et al., 2010). The greatest advantage of screening methods is the simplicity that does not require any profound understanding of the molecular biology and physiology of the microorganisms being manipulated (Gromada and Fiedurek, 1997).

Several attempts are made to improve glucose oxidase production through Aspergillus niger by strain selection using mutagenesis classical screening techniques (Fiedurek et al., 1986; Markwell et al., 1989; Witteveen et al., 1990).

Strain improvement studies are performed by using physical mutagenic agents (UV, gamma radiations) and chemical mutagenic agents (ETBR and Sodium azide). In last few decades, the exponential increase in application of glucose oxidase in various fields demand extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for overproduction of the enzyme as quantities produced by wild type are usually low. Irradiation by UV, gamma radiations may cause some mutations to genes of cells through the DNA repair mechanism within the cells. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialised desirable characteristics.

10.2. Immobilization of Aspergillus niger

The use of immobilized cell preparations in biotechnological process allows easier separations of the cells from fermentation broth, facilitates the isolation and refinement of products and allows repeated use of entrapped cells. Many papers and reviews on immobilization of microbial cells have been published. However, no ideal general methods applicable to be immobilization of all types of microbial cells have been worked out. The production of α-amylase was attempted in the batch system using cells of B.subtilis immobilized in polyacrylamide gel-lattice. In similar system immobilized Streptomyacin fradiae cells are used for protease production. Immobilization could result in enhanced protease secretion by entrapped cells in comparison with free cells. Reports are also available for pectinase production increased by 15 to 60 times after immobilization of spores of Aspergillus awamori compared with that of free cells. Fiedurek (1990) shown that pumice stone is an excellent support for immobilization of Aspergillus niger for production of glucoamylase.
Rogalski et al (1988) observed optimization of culture conditions for GO production by selected mutant of *Aspergillus niger* G-B and investigation of some properties of the enzyme are described. A simple method is described for immobilization of *Aspergillus niger* GIV-10 which produces extracellular GO. *Aspergillus niger* conidia are immobilized on sintered glass reaching rings, pumice stones or polyurethane foam. Mycelia growing out from spores produced extracellular GO the highest production was with the pumice stone carriers. This technique facilitates the growth of filamentous culture in spongy structure of a support with continuous accumulation of biomass.

Mainly GO is commercially available as a by product of gluconic acid fermentation process with selected strain of *Aspergillus niger* (Cruger and Cruger 1990). Fungal GO has been widely studied during past two decades and there is much literature on enzyme and cell immobilization for production of Gluconic acid. On the contrary only one paper is concerning the production of GO from immobilized growing fungal glucose oxidase (Fiedurek and Iczuk 1991). The production of a new and specific GO by selected strain of *Penicillium variable* (Petruccioli et al 1993; Garzillo et al 1994) immobilized in different carriers Ca-alginate, agar, polyurethane sponge, partite and active carbon used in repeated batch process.

A systematic study made in 1980s by Kokufuta et al has demonstrated that polyelectrolyte (PEs) or polyelectrolyte complexes (PECs) are very useful for the immobilization of enzymes and microbial cells. In the case of a PEC-immobilized mycelia organism (*Aspergillus terreus*), a marked improvement in sedimentation of mycelia was observed, although there was little difference in gluconic acid production between the immobilized and free cells in a small scale (100 ml) cultivation with an Erlenmeyer flask on a rotary shaker.

### 10.3 Downstream processing

GO has been purified for commercial application from different fungi including *A. niger* and *Penicillium species* such as *P. pinophilum, P. amagasakiense, P. chrysogenum, P. notatum*, and *P. funiculosum*. GOD is known to be produced intracellular or extracellular or sometimes as mycelia-associated enzyme. Hence cells have to be disrupted for complete release of GOD into the broth. The intra- or extracellular location of the enzyme of *A. niger* and *Penicillium* species has been the subject of numerous discussions. In the meantime, the periplasmic location of the *A. niger* GO has clearly demonstrated (Witteveen et al., 1992), which is in agreement with the presence of a signal sequence preceding the *A. niger* GO gene (Frederick et al., 1990). As a consequence of peripheral location, the release of the enzyme
from mycelium may be facilitated by mechanical and physical forces, e.g. agitation and/or sonication.

Various methods of cell disruption have been used for filamentous fungi, including homogenization, sonication and a combination of both. A comprehensive study of different methods for the disruption of two filamentous fungi, *Ganoderma applanatum* and *Pycnoporus cinnabarinus* has performed and concluded that fungal cells are particularly resistant to some of the disintegration methods commonly used for yeasts and bacteria as well as the mechanical and non mechanical cell disruption methods described by Christi and Moo-Young (1986). For release of intracellular as well as cell-bound GO into the liquid broth, various methods like sonication, bead mill, homogenizer and freeze-thawing are applied. After the disruption of the cells, GO is released in the fermentation broth which may be separated from the cells either by differential centrifugation or by filtration.

Various precipitation techniques have been used for purification of GO including ammonium sulphate, potassium hexacyanoferrate and copper sulphate. Ammonium sulphate precipitation has been successfully employed to precipitate both intra- and extracellular GO with different percent cut of ammonium sulphate. The differences in ammonium sulphate precipitation characteristics for intra- and extracellular GO may be attributed to the fact that GO from *Penicillium* species are known to be glycosylated. GO from *P. amagasakiense* is a glycoprotein which contains 11–13% carbohydrate described as the high-mannose type (Kusai et al., 1960; Eriksson et al., 1987; Nakamura and Fujiki, 1968). Precipitation is followed by chromatographic separation techniques such as ion exchange chromatography.

On an average, the pI of GO has been found in the range of pH 4 to 5 (Eriksson et al., 1987; Kalisz et al., 1997; Kusai et al., 1960). Hence an anion exchange chromatography is commonly used for its purification (Swoboda and Massey, 1965; Dai et al., 2002). In column chromatography column of sephadex G200 have been used for purifying the concentrated dialyzed enzyme preparation (for gel filtration 40-120 μ fractionation range from 1000 to 200.000 MW) as mentioned by Shindia et al. (2001). The sephadex G200 column (2.6 × 70.0 cm) has been used. This sephadex is swollen in 0.1M citrate phosphate buffer, pH 5.6 and eluted again with the same buffer at a flow rate of 20 ml h-1 at room temperature. Fractions of 5 ml has collected and stored at (-20°C) until use. The glucose oxidase activity and protein content were examined for all different fractions separately exchange resin.
Initial sample
<Cell disruption, centrifugation>

Solid liquid separation
<Ammonium sulphate>

Precipitation
<filtration (salt removal)>

Ion exchange chromatography
<anion exchange>
<Diafiltration (salt removal)>

Lyophilization

Size exclusion chromatography
<Lyophilization>

Final product

Figure- 3 . General protocol for purification of glucose oxidase.

104. Gene regulation:

A new glucose oxidase from *Aspergillus niger* is isolated and characterized. The enzyme showed different kinetic and stability characteristics when compared to a commercially available batch of *A. niger* glucose oxidase. The gene encoding of the new glucose oxidase is isolated and DNA sequence analysis of the coding region showed 80% identity to the sequence of a glucose oxidase gene previously published. The new glucose oxidase is likewise induced by calcium carbonate. Observations concerning the effect of gluconolactone and the levels of glucose-6-phosphate isomerase upon calcium carbonate induction suggested that the enhancement of glucose oxidase biosynthesis by calcium carbonate is accompanied by a metabolic shift from glycolysis to the pentose phosphate pathway.
Factors important for optimal induction of glucose-oxidase are high glucose concentrations, a pH around 5.5, high level of dissolved oxygen, control of pH can be achieved by titration or by adding CaCO₃ to the medium. The presence of Mn³⁺ has been claimed to be important for GO induction. Nitrogen limitation is not necessary to obtain efficient induction of GO, although this was previously reported to stimulate the formation of GO in both *Penicillium* and *Aspergillus*. Induction of glucose-oxidase requires the presence of glucose and oxygen.

11. Applications of GO:

*From Biosensors to Food Preservative*

Glucose oxidase has become an important tool in several different industries, its uses ranging from a glucose biosensor for the control of diabetes, to a food preservative and colour stabiliser. GO is of considerable commercial importance due to its applications in food science, clinical chemistry and biotechnology. Raba and Mottola (1995) stated that GO is the most widely used enzyme as an analytical reagent due to its application in the determination of glucose, and furthermore it’s relatively low cost and good stability. Wilson and Turner (1992) also attribute the success of GO as a diagnostic reagent to the enzyme’s relative specificity. Raba and Mottola (1995) reviewed glucose oxidase as an analytical reagent and stated that the glucose/GO system was a convenient model for method development especially in the area of biosensors. Some of its current applications in industry are described below.

11.1 Glucose biosensor for diabetes monitoring

People with diabetes mellitus need to constantly monitor their blood glucose levels in order to detect fluctuations in glucose level that could lead to hyperglycaemia (high blood glucose levels) and hypoglycaemia (low blood glucose levels) so as to control the disease. Currently, such monitoring is done using finger-prick blood samples and a portable meter several times a day.

*Biosensors*

Biosensors are also being developed to measure blood glucose levels. Glucose oxidase is one of the possible enzymes that a biosensor can use. Biosensors work by keeping track of the number of electrons that pass through the enzyme by connecting it to an electrode and measuring the resultant charge. Alternatively, some biosensors use sensitive
fluorescence measurements, monitoring changes in the intrinsic FAD fluorescence of glucose oxidase.

11.2 Food and beverage additive

Glucose oxidase has been used successfully to remove residual glucose and oxygen in foods and beverages in order to prolong their shelf life. The hydrogen peroxide produced by the enzyme acts as a good bactericide, and can be later removed using a second enzyme, catalase, that converts hydrogen peroxide to oxygen and water. For example, glucose is removed from egg whites before they are dried for use in the bakery industry using the glucose oxidase/catalase system. GOx has also found application in the bakery industry, providing slight improvements to the crumb properties in bread and croissants (Rasiah et al., 2005).

Glucose oxidase can also be used to remove oxygen from the top of bottled beverages before they are sealed. In addition, glucose oxidase is used to prevent colour and flavour loss from foods and beverages. For example, they are used to reduce the discolouration occurring in wines and mayonnaises.

Wine production

Glucose oxidase has potential for use in the wine industry, where it can lower the alcohol content of wine through the removal of some of the glucose (by converting it to D-glucono-1, 5-lactone), which would otherwise be converted to alcohol. Tests showed that the glucose oxidase treatment of wine-must could reduce the potential alcohol content of wine by about 2%. In addition, glucose oxidase is able to inhibit wine spoilage through its bactericidal effect on acetic acid bacteria and lactic acid bacteria during the fermentation process. The bactericidal effect of the enzyme means fewer preservatives need to be added to wine. Some strains of Saccharomyces cerevisiae have been genetically engineered to carry the glucose oxidase gene itself.

As per Pickering et al. (1998) GO-CAT enzyme system is in reduction of the fermentation alcohol potential was achieved by pre-treating the grape juice with the GO-CAT enzyme system to convert the available glucose to gluconic acid (Scheme 1.5). The low pH of the grape juice was determined to be a limiting factor, which was subsequently overcome by the use of calcium carbonate prior to the enzymatic treatment. A glucose conversion of 87% was achieved with this system.
Grape juice rich in glucose and fructose

Gox –Cat enzyme system
  |
Acidified grape juice rich in gluconic acid and fructose
  |
Alcoholic fermentation with Saccharomyces cerevisiae
  |
Deacidification
| Reduced alcohol wine

Figure 4: Procedure for producing low alcohol wine (reproduced from Pickering et al., 1998)

11.3 Oral hygiene

Glucose oxidase, as well as lacto peroxidase, can be used as anti-microbial agents in oral care products. The oral cavity houses several species of Streptococci bacteria, such as Streptococcus mutans, which is a significant contributor to tooth decay and is carried by virtually everyone. The hydrogen peroxide produced by glucose oxidase acts as a useful bactericide. The ability of glucose oxidase to kill S. mutans appears to be enhanced by the fusion of the enzyme with heavy chain antibodies.

11.4 Gluconic acid

Glucose oxidase is also used as a commercial source of gluconic acid, which can be produced by the hydrolysis of D-glucono-1,5-lactone, the end-product of glucose oxidase catalysis. Gluconic acid has been used as a food additive to act as an acidity regulator (mild acidulate), in sterilization solution or bleaching in food manufacturing, and as a salt in chemical components for medication. It has even been used in the construction industry as an additive to cement to increase its resistance and stability under extreme weather conditions. It occurs naturally in honey, fruit and wine.

11.5 Other Applications:

- GO, usually in combination with CAT, is used to stabilize colour and flavour in Beer, fish, tinned foods, and soft drinks, by the removal of oxygen (Crueger and Crueger, 1990).
- GO is also used to remove glucose during the manufacture of egg powder, preventing browning during dehydration caused by the Maillard reaction (Crueger and Crueger, 1990).
• The use of GO has found application in the textile industry as a method for producing hydrogen peroxide for bleaching (Tzanov et al., 2002). Covalently immobilised GOx on alumina and glass supports, resulting in higher recoveries. Maximum hydrogen peroxide concentrations of 0.35 g/L and 0.24 g/L were reached after 450 minutes for GOx immobilised on the glass and alumina supports respectively (20g glucose in 50ml 0.1 M acetate buffer, pH 5, 35°C, and aerated at 5 L/min). The alumina support proved more stable at the 17 operational conditions and could be used for 3 consecutive runs. The hydrogen peroxide produced was tested for bleaching scoured woven cotton fabric and was found to be comparable to standard bleaching processes. No stabilisers were needed since the gluconic acid produced acted as stabilising agent.

• The applications for GO and GO-CAT enzyme systems are numerous and the need for microbial strains exhibiting enhanced GO production will continue to be of interest. The GO-CAT enzyme system was used by Isaksen and Adler-Nissen (1997) to scavenge oxygen in mayonnaises with different oxidative susceptibility. The investigation proved that the GO-CAT enzyme system could be used to retard the lipid oxidation in mayonnaise stored at 5°C and 25°C, in mayonnaises containing pure soybean oil and where up to half the vegetable oil had been supplemented with fish oil.

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