CHAPTER – I

INTRODUCTION

[A] GLUCOSE ISOMERASE

Glucose isomerase (EC 5.3.1.5) catalyzes the isomerisation of glucose into fructose. This is a thermostable enzyme having wide applications. It is produced by a number of microorganisms to take up available sugar and channelize it into their metabolic pathways. This enzyme also converts xylose into xylulose. Xylose is present in abundance as monomeric units of the polymer xylan in plant residues. Glucose isomerase is one of the popular enzymes of future market. This happens to be in demand due to the increasing requirement of health care products. High Fructose Corn Syrup (HFCS) is formed by converting corn starch into glucose and further isomerising it to fructose. It is extremely sweet and clear syrup, refined by carbon and ion exchange systems to assure the highest food standards in terms of colour, clarity, composition, flavour and ash. Fructose, also known as fruit sugar, is the sweetest natural sugar and is found in fruits, vegetables, and honey. HFCS has wide applications in pharmaceutical and food industries. It is added in medicated syrups, beverages, baking, canning, and confectionary items as a sweetening agent.

Glucose isomerase (GI) is commercially available as Sweetzyme produced by \textit{Bacillus coagulans} of Novo Nordisc industries, Maxazyme produced by \textit{Actinoplanes missouriensis} of Gist Brocades N V, Takasweet F produced by \textit{Flavobactrium arborescens} of Miles laboratories, Ketozyme produced by \textit{Actinoplanes missouriensis} of Universal Oil Products, Optisweet 22 produced by \textit{Streptomyces rubiginosus} of Kalie Chemie \cite{Jao, 1985}.

Fructose has higher sweetening index than glucose and sucrose. HFCS an equilibrium mixture of glucose and fructose (1:1) is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose.
Glucose in spite of being the metabolically most preferred sugar has few drawbacks as a sweetening agent. Glucose syrup at the final commercial concentration [71% (w/w)] must be kept warm to prevent crystallisation or diluted to concentrations that are microbiologically insecure. Fructose is 30% sweeter than sucrose, on a weight basis, and twice as soluble as glucose at low temperatures so a 50% conversion of glucose to fructose overcomes both problems giving stable syrup that is as sweet as a sucrose solution of the same concentration.

Glucose is normally isomerised to fructose during glycolysis but both sugars are phosphorylated. The use of this phosphohexose isomerase may be ruled out as a commercial enzyme because of the cost of the ATP needed to activate the glucose and because two other enzymes (hexokinase and fructose-6-phosphatase) would be needed to complete the conversion. Only an isomerase that would use underivatised glucose as its substrate would be commercially useful but, until the late 1950s, the existence of such an enzyme was not suspected.

They are remarkably amenable enzymes in that they are resistant to thermal denaturation and will act at very high substrate concentrations, which have the additional benefit of substantially stabilising the enzymes at higher operational temperatures. It would be useful to lower the pH optima of xylose isomerase to prevent sugar browning in the production of high-fructose syrup at elevated temperatures.

Different manufacturers have developed different methods for isomerisation according to the producer organism they are using. The medium components, growth requirements and the types of reactors vary with the technology. All processes start with 45% (w/w) glucose syrup and produce 10000 tonnes per month of 42% fructose dry syrup.

It is essential for efficient use of immobilised glucose isomerase that the substrate solution is adequately purified so that it is free of insoluble material and other impurities that might inactivate the enzyme by chemical (inhibitory) or physical (pore-blocking) means. In effect, this means that glucose produced
by acid hydrolysis cannot be used, as its low quality necessitates extensive and costly purification. Insoluble material is removed by filtration, sometimes after treatment with flocculants, and soluble materials are removed by ion exchange resins and activated carbon beads. This done, there still remains the possibility of inhibition due to oxidised by-products caused by molecular oxygen. This may be removed by vacuum de-aeration of the substrate at the isomerisation temperature or by the addition of low concentrations (< 50 ppm) of sulphite.

Purified glucose syrup is heated to remove dissolved $O_2$ and increase glucose concentration to 40%. The pH is adjusted between 7 and 8. The syrup is passed through a column containing immobilized bacterial cells with glucose isomerase activity; the temperature is kept at 60°C.

After isomerisation, the pH of the syrup is lowered to 4 - 5 and it is purified by ion-exchange chromatography and treatment with activated carbon. Then, it is normally concentrated by evaporation to about 70% dry solids.

The performance parameters of a biocatalyst in view to its large-scale application, e.g. concerning the rate of substrate diffusion to the active sites of a biocatalyst pellet, are of no less importance, as they influence the overall rate of isomerization.

Easily available, cheap and disposable starchy material is digested by enzymes. The saccharified product, glucose is isomerised by GI. The final product is a corn syrup containing a mixture of glucose and fructose and with a greater sweetening capacity than that of sucrose. Other sources of starch such as wheat, tapioca, and rice are used to a minor extent in other parts of the world. The by-products of the corn-milling industry are important in deciding the economics of HFCS production. Today HFCS is used to sweeten jams, condiments like ketchup, and soft drinks. It is also a favorite ingredient in many so-called health foods. This enzyme also finds use in medicated syrups, beverage, baking, canning, and confectionary items [Bhosale et al., 1996].

Since the adverse effects of sucrose are being realized on health, D-fructose can be used for diabetic patients as a sweetener because it is slowly
Introduction

absorbed by the stomach and does not influence the glucose level in blood. Fructose metabolism is different from glucose and is rapidly removed from blood and has a low renal threshold value in humans.

1.1 Enzymatic versus chemical isomerization

The chemical conversion of glucose to fructose [Fig. 1.1] has been known for the past 100 years and constitutes one of a group of reactions collectively known as the Lobry de Bruyn-Alberda van Ekenstein transformation. These reactions are usually carried out at high pH and temperature. The possibility of producing fructose chemically from glucose has been studied by Barker (1973). The reaction is nonspecific and leads to the formation of nonmetabolizable sugars such as psicose and other undesirable colored products. This method yields a maximum fructose concentration of 40%. Moreover, chemically produced fructose has off flavors and reduced sweetness, which cannot be easily remedied which makes it commercially unfit. On the other hand, enzymatic conversion of glucose to fructose offers several advantages, such as specificity of the reaction, requirement of ambient conditions of pH and temperature, and no formation of side products. Therefore, enzymatic conversion is preferred to chemical isomerization of glucose to fructose, and today the process involving GI has undergone considerable expansion in the industrial market.

Fig. 1.1: Reactions catalysed by GI a: In vitro reaction; b: In vivo reaction.
Moliner et al., (2010) proposed isomerisation by a highly active heterogeneous inorganic catalyst, a large-pore zeolite that contains tin (Sn-Beta) is able to isomerize glucose to fructose in aqueous media with high activity and selectivity.

1.2 Glucose Isomerase producing microorganisms

Glucose Isomerase was first reported to be produced by Pseudomonas hydrophila by Marshall and Kooi in 1957 since then many microbes have been screened for the production of the enzyme. Streptomyces have been the organisms of choice of many investigators for Glucose Isomerase production. Some members of Actinomycete group like Actinomyces olivcinereus, A. phaeochromogenes, Actinoplanes missouriensis, Streptomyces olivochromogenes have been reported to produce good amounts of the enzyme. Other bacteria Bacillus stearothermophilus, B. megabacterium, B. coagulans, Bifidobacterium sp. Brevibacterium incertum, B. pentosoaminoacidicum, Lactobacillus brevis, L. buchneri, L. fermenti, L. bifermantans are also reported to produce this enzyme. There are reports on enzyme being extracellular as well as intracellular [Givry and Duchiron, 2008]. The list of known GI producing microorganisms is mentioned in Table 1.1 and commercially used microorganisms for production of GI are given in Table 1.2.

Among various microbes studied Actinomycetes are the richest source of GI. Production of GI by Streptomyces was initially reported by Tsumura and Sato (1965). Extracellular GI has been reported to be produced by Streptomyces glaucescens and S. flavogriseus [Chen et al., 1979; Srinivasan et al., 1983]. Streptomyces have been an organism of choice for the production of GI by various researchers. Al-Tai (1985) reported GI with 70°C and 8.0 as optimum temperature and pH respectively. GI production in majority of the cases requires xylose as an inducer in the medium. Arthrobacter sp. are reported to be synthesizing the enzyme constitutively [Sapunova et al., 2004].
### Table 1.1: List of GI producing microorganisms

<table>
<thead>
<tr>
<th>GI-producing Microorganisms</th>
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<tbody>
<tr>
<td>Actinomyces olivocinereus, A. phaeochromogenes</td>
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<tr>
<td>Actinoplanes missouriensis</td>
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<tr>
<td>Aerobacter aerogenes, A. cloacae, A. levanicum</td>
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<tr>
<td>Arthrobacter spp.</td>
</tr>
<tr>
<td>Bacillus stearothermophilus, B. megabacterium, B. coagulans</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
</tr>
<tr>
<td>Brevibacterium incertum, B. pentosoaminoacidicum</td>
</tr>
<tr>
<td>Chainia spp.</td>
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<tr>
<td>Corynebacterium spp.</td>
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<tr>
<td>Cortobacterium helvolum</td>
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<tr>
<td>Escherichia freundii, E. intermedia, E. coli</td>
</tr>
<tr>
<td>Flavobacterium arborescens, F. devorans</td>
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<tr>
<td>Lactobacillus brevis, L. buchneri, L. fermenti, L. manitopoeus, L. gayonii, L. fermenti, L. plantarum, L. lycopersici, L. pentosus</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
</tr>
<tr>
<td>Microbispora rosea</td>
</tr>
<tr>
<td>Microellobosporia flavea</td>
</tr>
<tr>
<td>Micromonospora coerula</td>
</tr>
<tr>
<td>Mycobacterium spp.</td>
</tr>
<tr>
<td>Nocardia asteroides, N. corallia, N. dassonvillei</td>
</tr>
<tr>
<td>Paracolobacterium aerogenoides</td>
</tr>
<tr>
<td>Pseudonocardia spp.</td>
</tr>
<tr>
<td>Pseudomonas hydrophila</td>
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<tr>
<td>Sarcina spp.</td>
</tr>
<tr>
<td>Staphylococcus bibila, S. flavovirens, S. echinatus</td>
</tr>
<tr>
<td>Streptococcus achromogenes, S. phaeochromogenes, S. fractiae, S. roseochromogenes, S. olivaceus, S. californicos, S. venueus, S. virginial</td>
</tr>
<tr>
<td>Streptosporangium album, S. oulgare</td>
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<tr>
<td>Thermopolyispora spp.</td>
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<tr>
<td>Thermus spp.</td>
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<tr>
<td>Xanthomonas spp.</td>
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<tr>
<td>Zymomonas mobilis</td>
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</table>

[Source: Bhosale et al. 1996]
Table 1.2: List of commercial producers of GI

<table>
<thead>
<tr>
<th>Organism</th>
<th>Trade name</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td>Actinoplanes missousriensis</td>
<td>Maxazyme</td>
<td>Gist Brocades and Anheuser-Busch Inc.</td>
</tr>
<tr>
<td>Bacillus coagulans</td>
<td>Sweetzyme</td>
<td>Novo-Nordisk</td>
</tr>
<tr>
<td>Streptomyces rubiginosus</td>
<td>Optisweet</td>
<td>Miles Kali-Chemie</td>
</tr>
<tr>
<td></td>
<td>Spezyme</td>
<td>Finnsugar</td>
</tr>
<tr>
<td>Streptomyces phaeochromogene</td>
<td>Swetase</td>
<td>Nagase</td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>-</td>
<td>Reynolds Tobacco</td>
</tr>
<tr>
<td>Streptomyces olivaceus</td>
<td>-</td>
<td>Miles Laboratories Inc.</td>
</tr>
</tbody>
</table>

[Source: Bhosale et al. 1996]

Recent publications have been devoted to a purposeful change of properties of this enzyme by means of selection and adaptation of the respective wild-type producer strains. Authors have described the properties of GI extracted from hyper-thermopiles of the genera *Thermotoga* sp., which is active at temperatures above 100°C; at 90°C, productivity of the enzyme extracted from *Thermotoga neapolitana* is 1000 kg of fructose/1 kg of enzyme. However, it should be taken into account that the reaction of fructose degradation occurs at a temperature of ≥65°C in the presence of oxygen. GI from *Streptomyces olivaceoviridis* E86 displays high activity and is stable at pH 5.0. The enzyme so extracted retains initial activity at 60°C and pH 5.0 for 30 h. The use of acid stable GI will allow the unification of the stage of starch saccharification and glucose isomerization. Glucose fructose syrups (GFS) obtained via such one-stage technology contains ~50% fructose and less than 1.5% unspecified oligosaccharides.

Sapunova et al., (2004) proposed a screening method for GI producers. The cultures producing the enzyme were grown on xylose containing medium. This was then flooded with 0.1% 2,3,5-triphenyltetrazolium solution in 1 M NaOH at 30°C for 1 min in the dark. The screening could be done on the basis of capacity of D-xylulose to oxidize colorless 2,3,5-triphenyltetrazolium chloride
in an alkaline medium with the formation of formazan having a dark pink color. Dark brown halo around the light colonies indicated the production of glucose isomerase.

GI from some sources is reported to isomerise other sugars also eg. D-ribose, L-arabinose, L-rhamnose, D-allose, and 2-deoxyglucose widen their applicability. Pastinen et al., (1999) and Sanchez and Smiley, (1975) reported enzyme’s capacity to isomerise ribose, arabinose, rhamnose and allose. These sugars were isomerised to their respective keto forms. Xylose, arabinose, allose have hydroxyl groups on equatorial positions on carbon 2 whereas hydroxyl groups in lyxose and mannose are in axial position. The spatial arrangement of atoms must be interfering in the attachment of enzyme and substrate molecules. The enzyme definitely has differential affinities for different substrates. It’s high affinity for glucose shall be beneficial for industrial applications [Pastinen et al., 1999].

1.3 Structure of Glucose Isomerase

GI characterized from different microbial sources vary in molecular mass from 80 to 195 kDa and are composed of two or four identical subunits. Glucose isomerase from Streptomyces is a tetramer composed of four identical polypeptide chains of 43,000 Daltons each. The structure of GI from Streptomyces rubiginosus is shown in Fig. 1.2.

Fig. 1.2: Glucose isomerase (1OAD) from Streptomyces rubiginosus, in P21212 crystal form [Adapted from www.pdb.org/pdb/explore/images.do?structureId=1OAD]
It is a heat stable enzyme. Optimum temperature and pH for enzyme activity ranges from 60 – 70°C and 7 - 8 respectively. It requires magnesium, manganese and cobalt ions for optimum activity. Chou et al., (1976) reported glucose isomerase to be heat stable, with a half-life of 120 h at 70°C. The inactivation occurs only when reversible conformational changes in the apoenzyme which leads to the formation of more stable and inactive tetramer that is incapable of binding to metal ions [Ogbo and Odibo, 2007]. The isoelectric point for the GI from various sources ranges between pH 4.1 to 4.7.

Since the fructose/glucose equilibrium increases with temperature, a higher-temperature isomerization would yield a sweeter syrup. However, it would then be necessary to operate at lower pH, since undesirable 'browning reactions' occur when glucose is heated at alkaline pH. Hence enzymes with pH optima below 6 and stability above 80 °C are useful [Smith et al., 1991].

The N-terminal amino acid sequence and amino acid analysis shows 73–92% homology with xylose–glucose isomerases from other sources [Raykovska et al., 2001].

The structure of glucose isomerase has been resolved by X-ray crystallography by various researchers [Kozak and Taube, 2009]. According to Visuri et al., (1999) GI exhibits exceptionally high stability and is used industrially at high temperature of about 60°C with a half life of hundreds of days. The stability can be further increased by cross linking the crystalline glucose isomerase in presence of xylitol. Kozak (2005) proposed GI to be a potential molecular weight standard for small angle X-ray scattering studies as it has good stability on storage and exposure to radiations as compared to Bovine Serum Albumin. GI do not have any disulphide bridges, which is advantageous for its stability on exposure to synchrotron radiation.

The overall structure of each monomer consists of two domains: a large N-terminal domain (residues 1-320), and a small C-terminal loop (residues 321-387). The monomers of the enzyme are identical. The enzymes from Streptomyces species show large sequence homologies of up to >90% [Zhu et al., 2000]. Glucose Isomerase containing nine sulphur atoms is one among the
less sulphur containing proteins [Ramagopal et al., 2003]. The tetramer appears to be a dimer of dimers. The interactions between the two monomers forming the tight dimer are much more extensive than the interactions between the two tight dimers which form the tetramer [Gregory et al., 1987].

GI from Streptomyces sources is irreversibly inactivated when tetrameric enzyme forms are converted to monomeric forms. The loss of activity is accounted by the incorrect folding [Ghatge et al., 1994].

Reaction of lysine residues with the substrate has been suggested for Actinoplanes missouriensis glucose isomerase. The enzyme contains 20 lysine residues, of which 2 (positions 253 and 294 of the protein) are located at the interface between the dimers in the tetrameric enzyme. Genetic modification to remove one lysine residue was shown to improve the stability of the enzyme [Visuri et al., 1999].

The essential active-site histidine residue in the GI from Clostridium thermosulfurogenes was identified by substituting histidine residues at four different positions. Substitution of His-101 by phenylalanine abolished the enzyme activity, whereas substitution of other histidine residues had no effect.

Treatment with SDS (Sodium Dodecyl Sulphate) and guanidine hydrochloride denatures the enzyme only at high temperatures; the enzyme is quiet stable at mild conditions [Kasumi et al., 1981]. Cobalt ions are responsible for providing stability to enzyme structure [Callens et al., 1988]. Zhu et al., (2000) reported that the xylose isomerase from S. violaceoruber had less than 10% of its maximum activity with one equivalent of Co$^{2+}$ ion per monomer and had over 75% of its maximum activity with two equivalents of Co$^{2+}$ ion per monomer, and that the Co$^{2+}$ ion was superior to Mg$^{2+}$ in protecting the enzyme against thermal denaturation. The active site of xylose isomerase derived from Streptomyces diastaticus has two Co$^{2+}$ ions ligated by the side chains of seven amino acids which are conserved in all known xylose isomerases [Zhu et al., 2000].
1.4 Production of Glucose Isomerase

Glucose isomerase is produced and marketed by various companies. The enzyme is mainly available in immobilized forms. The most important standpoint is the cost effectiveness of the process. The development of a viable process requires high yielding strain, easily available and low cost production media ingredients. Both the requirements need extensive screening.

GI titres from 1,000 to 35,000 U/L have been reported. The yield can be improved by strain improvement through mutagenesis or recombinant DNA technology. Mutagenesis has increased the enzyme yield from 10 U/mL to 1500 U/mL. UV irradiation of Streptomyces olivochromogenes resulted in a mutant strain with 70% increased activity. Development of a Streptomyces acidodurans constitutive mutant has also been attempted which has resulted in increased enzyme yields.

GI is known to be produced in submerged aerated fermentation process. The production should be done essentially using GRAS (Generally Recognized as Safe) ingredients. A variety of production media have been devised by investigators. The thrust is on the determination of a suitable carbon and nitrogen source. Divalent cations like magnesium and cobalt are required by most of the GI producing organisms. Elimination of cobalt ions from the production media is also required considering it’s ill effects on health.

Xylose works as an inducer for the production of enzyme in majority of the cases [Lobanok et al., 1998; Dhungel et al., 2007]. Xylose is expensive and cannot be used at commercial scale. There is a need to find a suitable agro residue which can substitute xylose. Researchers have tried substituting starch, glucose, glycerol and sorbitol etc. in place of xylose. Peanut shell is a good source of xylose and corn cob is reported to have high xylan content [Chandrakant and Bisaria, 2000]. The production medium containing such substitutes can make the process economic.

Luxurious growth and high enzyme yield is also observed in presence of wheat bran [Manhas and Bala, 2004; Srinivasan et al., 1983]. The case where
enzyme is intracellular high biomass indicates high enzyme production [Bok et al., 1984]. Chen et al., (1979) reported good enzyme yields by S. flavogriseus in presence of xylose, xylan as well as straw hemicelluloses a cheap media ingredient. Straw hemicelluloses can be hydrolyzed by acid or alkali treatment before adding it into the medium. Paik and Dewey, (1980) reported GI production by using xylan as an inducer in place of xylose.

As the agro residues are rich in cellulose, starch and xylan, availability of cellulase, amylase or xylanase is a crucial point for using such material. A cocktail of two enzymes (a polysaccharide hydrolyzing enzyme with isomerases) can improve the efficiency of the process. Teeradakorn et al., (1997) developed a Streptomyces fusant using Streptomyces cyaneus as GI producer and Streptomyces griseoruber as xylanase producer for production of xylulose from xylan. There are reports on Bacillus being used for GI production on xylan containing media also [Calik et al., 2009].

Another important aspect is the selection of a high yielding and cheap nitrogen source. Soy flour, corn steep liquor, yeast extract and various peptones can be used for GI production. Among inorganic sources ammonium compounds have proved to be useful but organic nitrogen sources are indispensible. Addition of certain amino acids improves the yield of enzyme by some strains of Streptomyces.

The temperature required for production ranges for most of the organisms between 27°C to 30°C. Some thermophiles are also reported to produce the enzyme having optimum temperature for production between 50°C to 60°C. Fan et al., (2011) produced GI from Thermoanaerobacter ethanolicus and Lee and Zeikus, (1991) from thermoanaerobic bacteria C. thermosulfurogenes and Thermoanaerobacter.

Optimum pH required for production is from 7 to 8 for majority of the producers. Kim and Pyong-Su, (1992) reported high yielding Streptomyces strain with low pH optima and high thermostability.
Time period required for maximum accumulation of enzyme varied for different strains. Chen et al., (1979) reported maximum accumulation of intracellular GI in 36 h and extracellular in 72 h for *S. flavogriseus* whereas Dhungel et al., (2007) and Chou et al., (1976) observed the same in 96 hrs for a psychrotolerant and unidentified *Streptomyces* sp.

### 1.5 Influence of magnesium and cobalt ions on GI production and activity

Influence of divalent ions (magnesium and cobalt) on the GI production has been stated differently for different strains. Most of the glucose isomerases are reported to be active only in presence of both the metal ions. There are some reports on the enzyme being active with only one metal site occupied in contrast to the cases where mutating one metal binding site makes the enzyme inactive. These ions are required for production as well as activity of the enzyme. GI shows maximum activity in the presence of both Mg$^{2+}$ and Co$^{2+}$ at ratio of 5 : 1, respectively, or higher. The reasons for the differences in the metal preference for specific enzymes and substrates are not clear, but this is most likely related to subtle conformational changes in the active site that vary depending on the specific metal present in each site [Epting et al., 2005].

Mg$^{2+}$ ions increase the enzyme yield and are also known to work as activator in isomerisation. Co$^{2+}$ ions do have any significant effect on production [Hasal et al., 1992; Chou et al., 1976] but is reported to enhance GI thermostability. Mg$^{2+}$ is superior to Co$^{2+}$ as an activator. Co$^{2+}$ can be used as a shield against denaturation by urea, guanidium chloride, heat and acid. Co$^{2+}$ has an essential role in holding the ordered conformation, especially the quaternary structure of the enzyme. Thermal dissociation of the active dimer into inactive monomer can be prevented to some extent by Co$^{2+}$. Two Co$^{2+}$ ions for each molecule of enzyme are required for optimum functioning [Gaikwad et al., 1992; Callens et al., 1988; Park et al., 1976]. Mg$^{2+}$ and Co$^{2+}$ have separate binding sites on the enzyme as they do not affect each other’s influence [Sanchez and Smiley, 1975]. It has been suggested that cobalt functions by modifying the enzyme structure to permit the accommodate glucose as well as xylose [Deshmukh et al., 1994].
Treatment of the purified xylose isomerase with EDTA resulted in the loss of 95% of enzyme activity. However, the activity could be restored by the addition of metal ions. In particular, increasing amounts of Mn$^{2+}$, Mg$^{2+}$, or Co$^{2+}$, each up to 10 mM, were able to restore only 60 to 70% of the original xylose isomerase activity.

The sequence of enzyme stabilization and activity by metal ions is Mg$^{2+}$ if considered 100%, 45% is for Co$^{2+}$ and 28% for Mn$^{2+}$. Detailed studies on metal ion binding sites have been done by various researchers [Bogumil et al., 1993; Sudfeldt et al., 1990]. Presence of ZnSO$_4$, FeSO$_4$ and K$_2$HPO$_4$ besides MgSO$_4$ in the production medium gives better yields [Debnath and Majumdar, 1987].

From the reported crystallographic data and binding studies, two metal-ion binding sites (1 and 2)/monomer are present. The first metal-ion site, M1, is co-ordinated to four carboxylates (Glu-181, Glu-217, Asp-245, Asp-292). The second, M2, is liganded by three carboxylates (Glu-217, Asp-255, Asp-257) and further by His-220 and a water molecule. Alteration of amino acids on these sites leads to change in pH activity profiles and substrate binding [Bastelaere et al., 1995].

On analyzing the thermal stability of the enzyme, many researchers have reported the loss of activity in presence of substrate to be accelerated as compared to its presence in an environment without the substrate [Giovenco et al., 1973].

Requirement of metal ions was also noticed for non actinomycetal sources like *Lactobacillus* sp. The inactivation caused by other ions like calcium was found to be reversed by Mg$^{2+}$ or Mn$^{2+}$ ions in these cases also [Givry and Duchiron, 2008].

**1.6 Purification of GI**

The intracellular enzyme can be extracted from the cells by treatment with detergents like CTAB, SDS and physical methods like sonication, osmotic shock, applying high pressure as in X-press or French press. Ultrasonication for 30 minutes and treatment with CTAB are efficient methods for extraction
Various purification procedures have been tried for both extracellular and intracellular enzyme. The purification of enzyme should be of high degree by a less expensive methodology. The economic implications of Glucose Isomerase have been studied by Ladisch et al., (1977). They designed a method by using lesser number of reagents and high productivity. The enzyme extracts were prepared in water. Precipitation of enzyme can be performed by acetone fractionation or ammonium sulphate fractionation. Cost factors to be considered include enzyme, purification and immobilization reagents, carrier and the cost of reactor. The cost affectivity of the process can be increased by also optimizing reagent quantities and reclamation of the reagents.

Glucose isomerase produced from *Streptomyces* sp. can be purified by fractionation with (NH$_4$)$_2$SO$_4$ and its subsequent separation from other proteins can be done by chromatography on diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex A-50 columns. The homogeneously purified enzyme can be analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis [Chen and Anderson, 1979].

1.7 Immobilization of GI

In order to increase the productivity of a process involving enzyme technology, immobilization is a very useful method. Immobilization of the enzyme aids in increasing it’s stability and reusability. The development of immobilized glucose isomerase (IGI) and it’s application for the production of HFCS has been a subject of great interest. With the growing demand of HFCS the increased reusability of the enzyme would be a boon for industrial processes. This will help in reducing the cost of HFCS production.

Immobilization techniques of GI and cells containing glucose isomerase shall increase the efficiency and economics of the process. It may also prolong the half life of the enzyme. Affectivity of the process can be ascertained as it’s substrate is a low molecular weight compound as compared to those having
high molecular weight substrates where the enzymes on or near the surface of gels are only available for the reaction.

Several techniques for immobilization of glucose isomerase have been reported by researchers. Whole cell as well as enzyme immobilization is investigated. *Streptomyces phaeochromogenes* cells have been immobilized with collagen in membrane form, and its enzyme in polyacrylamide. Crosslinking with gluteraldehyde, entrapment in gelatin, acrylamide [Strandberg and Smiley, 1971] and collagen membrane and chitosan beads, Indion-R, cobalt hydroxide gels, silica xerogel were tried [Kovalenko et al., 2011; Sapunova et al., 2003; Pawar and Deshmukh, 1994].

It has also been immobilized by covalently bonding the ruptured cells on gelatin or casein [Gong et al., 1980]. In evaluating an immobilized-enzyme system, the important factors to consider are the amount of enzyme loaded per unit of reactor column, the flow properties of, and the stability of, the enzyme complex.

Immobilization of whole cells containing GI rather than GI itself has advantages especially in the industrial perspective. This process saves the isolation and purification of the enzyme.

The enzymatic activity increased with repeated use at the early stages and then reached a constant value. This initial increase is one of the characteristics of cells immobilized by radiation induced polymerization. The increase can be attributed to the swelling effect of the polymer matrix by water, which increases the diffusivity of the substrate into cells entrapped in the inner part of the matrix. It was ascertained that swelling of the polymer matrix by water occurred at the initial stage of repeated use and then reached equilibrium after two or three batch reactions. Thus, swelling of the matrix has the apparent effect of increasing enzymatic activity of the whole cells. It is assumed that the diffusion of the substrate to the position of trapped cells is promoted by the swelling of the polymer matrix. In addition, the mobility of trapped cells might increase by the swelling, taking the more favored configuration for contact and reaction with substrate in the matrix [Kumakura et al., 1979].
Immobilization of nongrowing microbial cells inside xerogel of silicium dioxide containing insoluble hydroxyl compounds of cobalt (II) is developed by Kovalenko et al., (2011). They produced a recombinant *Escherichia coli* strain using xylose isomerase gene of *Arthrobacter nicotianae* producing glucose isomerase. This process increased activity and stability of the glucose isomerase to 3–5 times.

Azin et al., (1997) immobilized partially purified GI on DEAE (Diethylaminoethyl) Cellulose and entrapped whole cells in calcium alginate. These preparations were used for continuous isomerisation in packed columns. Glucose concentration of 45% was used as feed in the column for isomerisation. Paik and Dewey, (1980) also fixed the enzyme inside the whole cells by heat fixing for reusing it in insoluble form.

Porous TMPS (*p*-trimethyl- amine- polysterene) beads can be used for immobilization of GI through a molecular deposition technique. TMPS beads have good mechanical and flow properties. They are resistant to enzyme and microbial attack. The enzyme can be single layered or double layered to increase the isomerisation efficiency. Ge et al., (1998) developed a process for continuous production of HFS by IGI (Immobilised Glucose Isomerase) column continuously operated at 60°C, pH 8.2 – 8.5 for 45 days with a 50% (w/v) glucose solution as substrate and 0.01M Mg$^{2+}$ as an activator. This process was also carried out at a larger scale where 10,000 g of glucose was isomerised in 45 days to 42% HFS by 1 g dry bilayer IGI.

Immobilisation of an enzyme for industrial application requires optimisation of various factors. A low cost support, immobilized enzyme product with high activity per weight of support material, adequate operational stability and efficient use of the product are important considerations. The pH and temperature optima of the immobilized enzyme may vary a little bit from the soluble enzyme. In some cases it also influences the Michaelis constant ($K_m$) for the isomerisation reactions. The pretreatment of the biomass also affects the efficiency of the immobilized enzymes [Vlaev et al., 2009; Sapunova et al., 2003].
The substrate of immobilized GI, glucose can be produced from a cheap and disposable non sweet source starch or cellulose. As we are agricultural dominated country, huge amount of cellulosic agro-residues are generated every year. Thus for HFS production both the enzymes can be brought in close association by performing co-immobilization as done by Ge et al., (1997). They used macroporous $p$-trimethyl- amine- polysterene beads for co-immobilization of cellulase and glucose isomerase through molecular deposition technique.

Whole cell mycelium of *Streptomyces* spp. can also be immobilized by entrapment in gelatin matrices crosslinked with gluteraldehyde. This technique markedly increases the heat stability of the enzyme. The immobilized enzyme requires Mg$^{2+}$ ions for optimum activity and Co$^{2+}$ for heat stability just like the cell free enzyme. This process provides good recovery yield (40%) of the activity, long stability during storage (19 months) and isomerisation reactions. The operational half life can be prolonged from 80 days without cobalt ions to 260 days with the addition of cobalt ions. This IGI pellet preparation can be used in continuous packed bed column operation [Park et al., 1980].

Glucose isomerase from a rare Actinomycete *Chainia* was immobilized on Indion – 48 – R. It is a weak basic anionic exchange resin. The resin contains tertiary ammonium groups and based on cross-linked polystyrene bead-form. This is an inexpensive method, does not require cross linking agents and results in firm binding of the enzyme with the resin. This preparation also requires Mg$^{2+}$ and Co$^{2+}$ for optimum functioning as non immobilized enzyme. Removal of cobalt ions depletes the activity by 60% and unavailability of Mg$^{2+}$ reduces the activity by 89% This method also increased the resistance to heat denaturation [Gaikwad et al., 1992; Pawar and Deshmukh, 1994].

Chitosan is an ideal support material for enzyme immobilization because of its hydrophilicity, biocompatibility, biodegradability, and anti-bacterial property. The macromolecule is derived chemically by deacetylation of natural polymer chitin. Furthermore, chitosan exhibits a considerable protein binding capacity and the immobilized enzyme remains considerably active [Dolia and Gaikar, 2006].
Inhibitors like sorbitol, mannitol, sodium arsenate, cysteine and calcium ions have a reduced influence on the immobilized enzyme as compared to free enzyme. This property was observed with enzymes from other sources also eg. *Lactobacillus* sp. These cultures can also be successfully immobilized on glass plates which show increased heat stability [Shukla and Prabhu, 1985].

Benzyl DEAE-cellulose, triethylaminoethylcellulose, and DEAE-cellulose are also effective for immobilization of partially purified glucose isomerase. The properties of purified soluble enzyme, immobilized enzyme (DEAE-cellulose-glucose isomerase), and heat-treated whole cells may vary. Anionic exchangers are effective in adsorbing glucose isomerase, whereas cationic exchangers were not. Benzyl DEAE-cellulose and TEAE-cellulose retained more enzyme activity and protein than other ion exchangers. However, these two supports are much more expensive than DEAE-cellulose. DEAE-cellulose suspensions can adsorb high amounts of protein and thus high enzyme titre, 105 mg of protein per g of support and 1,052 U/g of support respectively. This can aid in increasing the specific activity of immobilized enzymes [Chen and Anderson, 1979].

**1.8 Continuous isomerization of glucose**

Currently, the majority of isomerization processes are carried out in a plug flow reactor. Some basic parameters of the process of isomerization have a crucial importance in the balance of these two characteristics: temperature, pH, concentration of additives, impurities in the substrate, production of by-products, etc. In equilibrium, approximately 45 to 50% of glucose can be converted to fructose. On the other hand, the excessive time required for the equilibrium, as well as the presence of oligosaccharides in the substrate stream, limits the process to conversions in which HFS’s are produced with 39 to 42% w/w [Borges da Silva et al., 2006]. The performance of the reactors is largely influenced by both diffusion resistance and enzyme inactivation. In fact, although diffusion resistance seems to enhance the apparent stability of the immobilized enzyme, it obviously affects productivity mainly at low residence times. Enzyme inactivation, is influenced by several factors, among which pH, ionic strength, temperature, and poisonous substances, progressively reduces
productivity. Glucose isomerase is protected by substrate against inactivation, because of the stabilization of the tertiary structure exerted in the complex by the link between active site and glucose [Converti and Borghi, 1998]. Meers (1981) proposed continuous culture process for production of GI in carbon limiting conditions. Packed columns of DEAE-cellulose-glucose isomerase or heat-treated whole cells were continuously operated at 70°C for 5 days [Chen and Anderson, 1979]. Rahman et al., (2011) has developed a mathematical model for production of fructose by immobilised glucose isomerase in a batch type of stirred tank bioreactor.

1.9 Strain improvement by mutagenesis and genetic engineering

The enzymes and the genes encoding glucose isomerases have been isolated from many microorganisms and are well studied. The production of GI is widespread among microbes. The amino acid sequences of the active sites are conserved in different enzyme sources. The nucleotide sequences of a number of GI genes have been determined. Glucose isomerasers can be separated into two groups on the basis of their amino acid sequences. The Glucose isomerases of *E. coli* and *B. subtilis* constitute one group, and the other group includes the enzymes from *Actinoplanes*, *Ampullariella*, and *Streptomyces* sp. The enzymes in the second group are less similar and lack a stretch of 30 to 40 amino acids which are present in the enzymes from the first group. The enzymes from *E. coli*, *Clostridium thermosulfurogenes*, *Lactobacillus pentosus*, and *B. subtilis* are significantly homologous to each other and consist of approximately 440 amino acids. The GIs from *S. violaceoniger*, *S. griseofuscus*, *A. missouriensis*, and *Ampullariella* spp. are homologous and contain 390 amino acids (Bhosale et al., 1996).

There have been continuous attempts to clone isomerase gene in *E. coli* and yeast. The *xylA* gene encoding a thermostable glucose isomerase was cloned from *Streptomyces chibaensis* J-59 and expressed in *E. coli* and purified by Joo et al., (2005). Huges et al., (2009) engineered *Saccharomyces cerevisiae* strain using plasmid SUMO yeast expression system for improved xylose utilization. Brat et al., (2009) cloned bacterial GI gene in yeast to facilitate its growth on xylose containing raw materials for the production of
ethanol. The homodimeric family II xylose isomerases from polycentric fungus *Orpinomyces* was sequenced, cloned and expressed in *Saccharomyces cerevisiae* by Madhavan et al., (2009).

The *xylA* has been isolated and characterized from various organisms such as *E. coli*, *Salmonella typhimurium*, *Ampullariella* and *Bacillus*. The expression of *xylA* through its natural promoter is highly regulated. In order to overcome this problem the *xylA* structural gene has been fused to other heterologous strong promoters such as *tac* and *λP*l. The *λP*l from *E. coli* is a well known strong promoter. This promoter was fused to *xylA* gene and the plasmid construct was introduced into *E. coli* which resulted in 15 times increase in the expression of the enzyme [Park et al., 1997].

The *xyl* gene cluster contained a putative transcriptional repressor (*xylR*), xylulokinase (*xylB*), and xylose isomerase (*xylA*) genes. The *xylR* mutants produced the enzyme 4.6 times higher than wild type in xylose induced condition. Even in the absence of xylose, the mutant strain produce over 60% of enzyme compared with the xylose-induced condition. Gel mobility shift assay showed that *xylR* was able to bind to the putative *xyl* promoter, and its binding was inhibited by the addition of xylose in vitro. This result suggested that *xylR* acts as a repressor in the *S. lividans* xylose operon. This opens a wide opportunity for increasing the enzyme production manifold [Young et al., 2008].

*Thermus thermophilus* is the most thermophilic organism from which a xylose isomerase gene has been cloned and characterized. Cloning of the gene using the *tac* promoter and production of enzyme in *Escherichia coli*, increased the yield by 45-fold and simplified the purification process. The enzyme also exhibited high thermostability [Dekker et al., 1991].

Typically, the pH optima of commercially available glucose isomerases range from 7.5 to 9.0. This limits the reaction temperature used in the industrial processes to 60°C because of the formation of browning products (mannose, psicose, and other acidic compounds) under the conditions of higher temperature and alkaline pH. However, higher temperature has the advantage of higher equilibrium concentration of fructose, faster reaction rate, and decreased viscosity of the substrate in the product stream. Therefore, a
thermostable glucose isomerase with acidic pH optima would permit higher reaction temperatures without the undesirable by-products and would have potential industrial application.

The strain, *Streptomyces thermovulgaris* 127, var. 7-86, was isolated as a result of mutagenic gamma-irradiation treatment of the grey form of the parental strain *Streptomyces thermovulgaris* 127 his residues are of importance for the enzymatic catalysis and their substitution with other residues caused a complete loss of enzymatic activity [Raykovska et al., 2001].

The strain of *A. nicotianae* BIM B5MG1 (B391 D) has been obtained and studied via the method of adaptive selection of the GI producing strain *A. nicotianae* BIM B_5 [Kovalenco et al., 2011].

Some microorganisms metabolize xylose to xylulose by xylose isomerase (*xylA*), xylulose is then phosphorylated by xylulokinase (*xylB*) to xylulose 5-phosphate, which enters either the pentose phosphate pathway or the phosphoketolase pathway. D-Xylose isomerase is essential for xylose catabolism which also has glucose isomerase activity [Fan et al., 2011].

The deduced amino acid sequence of the recombinant xylose isomerase was compared with those of several thermophilic anaerobes, fungi, and aerobic bacteria and found to exhibit between 26 and 97% identity to these enzymes. Amino acid sequence of the enzyme from various sources shows the presence of seven conserved domains and 15 conserved residues from the proposed active site commonly found in these enzymes. The length of the polypeptide chain encoded by *xylA* was the same as for these enzymes (439 amino acid residues). The *xylA* gene which exhibited extended acidic stability and activity was different from other enzymes by 7 (*T. saccharolyticum* B6A-RI) and 10 (*T. thermosulfurigenes*) amino acids. These relatively small differences take on more importance when one considers that the biochemical properties of these isomerases differ only with respect to the pH optima. The xylose isomerase from strain JW/SL-YS 489 has pH optima of 6.4 to 6.8, while the enzymes from *T. saccharolyticum* B6A-RI and *T. thermosulfurigenes* have pH optima of 7.0 to 7.5. This strongly suggests that the amino acid residue variations observed between
the sequences are responsible for the observed differences in pH optima. Cloning of such a gene into a suitable fast growing host shall be a very useful achievement for industrial purposes. This \( xyIA \) gene from \textit{Thermoanaerobium} strain JW/SL-YS 489 has been cloned in \textit{E. coli} and the complete nucleotide sequence was determined. Its expression, biochemical properties and purity have been determined. However, further studies, such as large-scale processes with immobilized enzyme, are necessary to assess the biotechnological use of this enzyme.

The GI gene from \textit{Streptomyces diastaticus} No. 7 strain M1033 (SM33) has been cloned, sequenced, and expressed in \textit{Escherichia coli}. A series of GI mutants were obtained in \textit{E. coli} by in vitro site-directed mutagenesis. Compared with wild-type GI, the optimum temperature of single mutant GIG138P (GI1) was increased by 10–12°C, and its thermostability was enhanced by 100%. Therefore, GI has a huge potential industrial production [Yang and Wang, 2001].

There have been trials for improvement of enzyme stability and activity by mutagenesis. Random PCR mutagenesis was applied to the \textit{Thermus thermophilus} \( xyIA \) gene encoding xylose isomerase. Three cold-adapted mutants were isolated. The wild-type and mutated \( xyIA \) genes were cloned and expressed in \textit{Escherichia coli HB101} using the vector \textit{pGEM-O-T Easy}. Compared with the wild-type, the mutants were active over a broader pH range. The mutants exhibited nine times higher catalytic rate constants \( k_{cat} \) for D-xylose compared with the wild-type enzyme at 60°C. For D-glucose, both the \( k_{cat} \) and the \( k_{cat}/K_m \) values for the mutants were increased compared with the wild-type enzyme. Furthermore, the mutant enzymes exhibited 255 times higher inhibition constants \( K_i \) for xylitol than the wild-type, indicating that they are less inhibited by xylitol. The thermal stability of the mutated enzymes was poorer than that of the wild-type enzyme [Lonn et al., 2002]. Glucose isomerase production was increased in different strains from 30 to 150% by mutagenesis by Ultra Violet light [Demnerova et al., 1979].
The production of GI is induced by xylose, and glucose exerts catabolite repression effect on the producing organism because of which constitutive production does not take place. Converting an inducible strain into constitutive producer can be helpful for commercial scale. Sanchez and Quinto, (1975) developed mutants of Streptomyces phaeochromogenes which constitutively produced the enzyme. The constitutive mutants so developed were insensitive to D-glucose repression. One of the wild-type strains of Actinoplanes missouriensis is able to produce GI constitutively and is used for commercial production of the enzyme by Gist Brocades. The cloning of xylA gene in front of a strong Streptomyces promoter can also serve the purpose. The P1-xylA gene has been integrated into the chromosome by using the integrative vector pTS55. The resultant strain (CBS1) gave about sevenfold higher activity in the absence of xylose compared with the wild-type strain fully induced by xylose.

1.10 Applications of Glucose Isomerase

GI can be used for various purposes like HFCS production, ethanol production, and conversion of biodegradable wastes of food and agro-industries into utilizable products.

1.10.1 High Fructose Corn Syrup production

The major application is High Fructose Corn Syrup production which has already been dealt earlier.

1.10.2 Ethanol production

Glucose isomerase also has great importance in production of ethanol from hemicelluloses. GI has been used to produce xylulose from xylose which can further be fermented to ethanol by yeasts [Maris et al., 2006]. The ever increasing demand of fuels can be served to some extent by bioconversion of renewable biomass to fermentable sugars. The biomass consists of cellulose (40%), hemicellulose (30%), and lignin (30%). The economic feasibility of biomass utilization depends on the hydrolysis of cellulose and hemicellulose to glucose and xylose respectively and their subsequent fermentation to ethanol by yeasts. Hemicelluloses currently represent the largest polysaccharide fraction.
wasted in most cellulosic ethanol pilot and demonstration plants around the world. The reasons are based on the heterogeneous polymeric nature of hemicelluloses and their low fermentation ability by the most common industrial microbial strains [Girio et al., 2010]. Constant strain improvement strategies using metabolic engineering are undertaken to improve the hemicellulosic fermentation by yeast [Van Vleet and Jeffries, 2009].

Xylan is a major constituent of hemicelluloses and consists of xylose units linked by a β (1,4) linkage. D-Xylose is easily produced by acid or enzymatic hydrolysis of xylan. Industrial yeast strains such as Saccharomyces cerevisiae generally ferment hexoses efficiently but D-xylose remains unutilized. A few yeasts such as Pachysolen tannophilus, Pichia stipitis, Candida utilis, and Candida shehatae are known to utilize pentoses through the oxidoreductive pathway, but the rates of fermentation are very low. Moreover, their low ethanol tolerance and catabolism of ethanol in the presence of oxygen limit their commercial application. GI has been used to produce xylulose from xylose, which otherwise represents a major metabolic block in the process of fermentation of xylose to ethanol by conventional yeasts such as Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Candida tropicalis. Although fermentation rates and product yields for ethanol production from D-xylose are significantly lower than from D-glucose, technology is now emerging to improve the process by transferring the GI gene to the yeast and conducting the isomerization and fermentation of xylose to ethanol simultaneously [Girio et al., 2010].

Pichia stipitis and Candida shehatae are the most successful xylose fermenting yeast species. Xylose fermentation was found to increase in the presence of biotin and thiamine. Xylose concentration above 40g/L has proved inhibitory to the growth of Pichia stipitis. Yeast needs to be grown in aerobic conditions which further produce ethanol in oxygen limiting environment. If totally anaerobic conditions are created the growth and ethanol production ceases. Growth and production are inversely related so it is not possible to find a rate of oxygen supply where both cell and ethanol production is maximized. In such conditions glucose isomerase can be used which also converts xylose into xylulose an easily fermentable sugar. Saccharomyces cerevisiae and
**Shizosaccharomyces pombe** can be used for this purpose which can ferment efficiently in aerobic conditions. The equilibrium of isomerisation reaction is towards xylose and only 10 to 30% of xylulose is formed. The equilibrium can be shifted towards xylulose by simultaneous isomerisation and fermentation. The challenge for such a process is difference in process optima, pH 7-8 and 70°C temperature for isomerisation and pH 4-6 and 35°C temperature for fermentation. Besides these difficulties there are reports on higher ethanol yields for the process as compared to direct fermentations [Preez, 1994].

### 1.10.3 Simultaneous isomerisation and fermentation

Researchers are trying to opt for simultaneous isomerization and fermentation of xylose (SIFX) against isomerization prior to fermentation, because the ratio of xylulose to xylose (1:5) is low at equilibrium. Removal of xylulose from the mixture facilitates conversion of xylose to xylulose, which is simultaneously converted to ethanol by the yeast [Chandrakant and Bisaria, 2000].

### 1.10.4 Cofermentation of Glucose and Xylose

The hydrolysed waste streams of agricultural residues contain both hexose and pentose sugars. Fermentation of xylose in presence of glucose is not efficiently feasible. An organism which can ferment both the sugars will be useful or co-immobilization of *Saccharomyces cerevisiae* and the enzyme glucose isomerase can be used for the production of ethanol [Grootjen et al., 1990].

### 1.10.5 Production of fructose syrup from whey permeate

Besides making use of agro residues GI can also be employed for converting dairy industry waste to useful syrup which is as sweet as sucrose. Lactase-hydrolyzed whey permeate can be used to convert glucose present in it to fructose. Excess of minerals should be removed from whey permeate before treatment with the enzyme. The optimum enzyme activity can be achieved by adding 250 and 100 ppm of Mg$^{2+}$ and Co$^{2+}$ ions respectively. Chiu and
Kosikowski (1986) reported a maximum conversion of 52% at 0.1 g enzyme/mL after 7 h of incubation at 60°C.

1.10.6 Xylitol Purification

Xylitol and sorbitol are the inhibitors of glucose isomerase. The enzyme has a capability to specifically bind to xylitol if present in a mixture. Xylitol is a natural sweetener can be industrially manufactured from hardwood pentosan arabinoxylan. Arabinoxylan is hydrolysed to monosaccharides by chemical pulping process. The process yields xylose, which is purified and reduced either chemically or microbially to xylitol. Glucose or arabinose present in it is also reduced to corresponding sorbitol or arabinitol respectively. Xylitol, sorbitol and arabinitol are difficult to separate from each other in industrial scale and a specific separation technique would be of great value. Cross-linked enzyme crystals have been used for many applications including chemical synthesis in polar solvents and chiral separations of several compounds. The first commercial cross-linked enzymes were prepared from glucose isomerase (D-xylose keto-isomerase, E.C.5.3.1.5.) by Visuri et al., (1989). These crystals were packed in a column and a mixture of arabinitol and xylitol were applied to it. Arabinitol did not bind and appeared in the void volume of the column. Part of the xylitol was bound and when the column was fully loaded also xylitol appeared in the effluent. Xylitol can be eluted with CaCl$_2$ after washing the column with water. More than 95% of both arabinitol and xylitol can be recovered in the effluent. The column can be reused on reactivation with 20 mM Mg$^{2+}$ [Pastinen et al., 1998].

The average crystal size of these crystals is 86 µm. They can be used in chiral separations and as an immobilized enzyme in a backed-bed or fluidised bed column. Cross-linking makes the enzyme insoluble but it retains its activity as water containing porous material. GI crystal [Fig. 1.3] formation can be enhanced by increasing the pressure [Maruoka et al., 2010].
Fig. 1.3: Cross-linked glucose isomerase crystals.
[B] ACTINOMYCETES

Actinomycetes are the richest source of glucose isomerase [Bhosale et al., 1996; Srinivasan et al., 1983]. They produce a wide repertoire of extracellular enzymes for breakdown of macromolecules like starch, cellulose, pectin, chitin, keratin and xylan etc and utilize the breakdown products. Some of these have unique substrate specificities and a higher stability, including temperature stability than similar enzymes from other bacteria or fungi. *Streptomyces* are generally considered to be non pathogenic and secrets several proteins. Unlike other organisms it does not produce metabolic by-products that inhibits it’s growth. Another interesting property of *Streptomyces* is its ability to produce secondary metabolites including antibiotics and bioactive compounds of high value in human and veterinary medicine, agriculture, and unique biochemical tools. Structural diversity is observed in these secondary metabolites that encompass not only antibacterial, antifungal, antiviral, and antitumor compounds, but also metabolites with immunosuppressant, antihypertensive, and antihypercholesterolemic properties [Alam et al., 2010; Baltz et al., 2008; Anderson et al., 2001]. Thus, *Streptomyces* is a rich source of the secondary metabolites in which common intermediates in the cell (amino acids, sugars, fatty acids, terpenes, etc.) are condensed into more complex structures by defined biochemical pathways.

Actinomycetes are a peculiar group of filamentous bacteria with heterogenous morphology consisting of unicellular *Corynebacterium* to filamentous *Streptomyces*. *Streptomyces* which belong to the bacterial order *Actinomycetales* are the filamentous spore forming Gram positive bacteria and are industrially popular microorganisms. *Streptomyces* evolved about 450 million years ago as branched filamentous organisms adapted to the utilization of plant remains. They posses versatile characters and produce numerous primary and secondary metabolites of commercial importance. They exhibit a typical growth pattern which resembles fungus. Their colonial morphogenesis is usually coordinated with the excretion of bioactive compounds that are often coloured. They have indispensible role in mineralization of all complex organic as well as inorganic matter. They are important decomposers of plant and
animal remains and recalcitrant compounds in the soil. They produce a large repertoire of enzymes for performing degradation. They are present in large numbers in soil and compost pits. They are slow growing prokaryotes which appear only after the typical bacteria and fungi have flourished. Actinomycetes have high G+C content ranging from 69 to 72% [Udwary et al., 2007; Ikeda et al., 2003; Bentley et al., 2002].

They are also known as ‘ray fungi’ because the first known example grew as fungus-like branching filaments, but Actinomycetes are now known to encompass various forms. The simplest are unicellular spheres and rods. The group includes pathogenic Corynebacteria, the causative agent of diphtheria as well as useful industrial species used to make amino acids for food supplementation [Chater et al., 2006].

Since the discovery of streptomycin from Streptomyces griseus by Selman A. Waksman, this group of microbes has been extensively explored. Majority of known antibiotics have been isolated from Streptomyces. Although thousands of antibiotics have been described these are thought to represent only a small fraction of the repertoire of bioactive compounds produced by Streptomyces. In addition, screening using various assays has revealed that Streptomyces culture supernatants contain other pharmaceutically active compounds such as antiviral and anticancer compounds, modulators of immune response, various enzyme inhibitors as well as herbicides, insecticides and anti-parasitic compounds. The chemical screening methods have uncovered the remarkable structural diversity of these compounds [Thompson et al., 2002].

Geosmin, trans-1,10-dimethyl-trans-9-decalol is an earthy smelling substance produced by many members of this group [Gerber and Lechevalier, 1965; Gerber and Lechevalier, 1977]. Geosmin comes from the Greek "ge" = earth and "osme" = odour. High numbers of geosmin producing organisms render the water with unacceptable taste and odour [Zaitlin et al., 2003]. The genus Streptomyces is widely used synonymously with odour-producing Actinomycetes but it is important to note that non-streptomycetal Actinomycetes such as Nocardia are also potent producers of geosmin, while
many *Streptomyces* are non-producers. A further key consideration is that not all *Streptomyces* produce geosmins *(Juttner and Watson, 2007)*.

They are placed in Group 22 to 29 in Bergey’s Manual of Determinative Bacteriology [1984]. All Actinomycetes are placed in Class *Actinobacteria* of Phylum 14 in Domain II, Bacteria in Volume 4 of Bergey’s Manual of Systematic Bacteriology [2001].

**1.11 Life cycle and morphology of Actinomycetes**

Actinomycetes exhibit a typical life cycle [Fig. 1.4] which is associated with the synthesis of elaborate biomolecules and provided environmental conditions. The growth cycle is usually slow so thick agar layers with humid incubators are required. Their detailed morphological pattern can be divided into following parts:

1- **Mycelium**: This is the filamentous network of the organism consisting of substrate and aerial hyphae. Spore outgrowths or mycelium fragmentation develops hyphae which penetrate into the agar medium. The hypha spreads on the substratum and branches intermittently. They anchor firmly into the medium developing tough and leathery colonies. The colonies may be covered with aerial mycelium which is characterized by free and erect hyphae surrounded by hydrophobic sheath that grow upwards. The aerial mycelium is less branched. These hyphae are usually white but assume a range of colours when spore formation begins. At this stage colonial appearance becomes chalky, velvety or powdery and can be distinguished from typical bacterial colonies. The development of aerial mycelium and its extent depends upon the media ingredients and the physiological conditions such as temperature and growth factors. Substrate mycelium is described as facultatively aerobic while the aerial growth was obligately aerobic. Above pattern of differentiation is unique among Gram-positive bacteria, requires the specialized coordination of metabolism and is more complex than other Gram-positive bacteria. A simple technique for studying the morphology of spores and mycelia is slide culture technique *(Williams et al., 1989)*.
Aerial growth is parasitic on the primary colony, which is digested and reused for aerial growth. The reproductive phase is coordinated with the secretion of antibiotics, which may protect the colony against invading bacteria during aerial growth.

Fig. 1.4: Life cycle of a typical Streptomyces.
2- **Conidia**: a general term used to refer to any asexual spores that are not intercalary chlamydospores or sporangiospores. Actinomycetes form conidia in a variety of ways.

   a- **Single conidia** – These are bacterial endospores possessing thermostability. Eg. *Thermoactinomyces*. Nonthermostable conidia are found in *Saccharomonospora* and *Promicromonospora*, *Micromonospora* and *Thermomonospora*.

   Members of genera *Frankia*, *Dactylosporangium* and *Intrasporangium* may form terminal vesicles which are not spores. *Actinomadura* may also form vesicles when grown under adverse conditions.

   b- **Pairs of conidia** – *Microbispora* possess longitudinal pairs of conidia on aerial mycelium where as *Faenia* sp. bear such structures both on aerial as well as substrate hyphae.

   c- **Short chains of conidia** – Spore chains up to 20 spores are considered as short chains belonging to *Nocardia*, *Pseudonocardia*, *Saccharomonospora*, *Streptoverticillium*, *Sporicthya*, *Actinomadura*, *Microtetraspora*, *Streptoalloteichus*, *Glycomyces*, *Amycolata*, *Amycolatopsis*, *Catellaspora*. Some *Microellobosporia* type form extremely short chains of spores, which are surrounded by an envelope that can be seen by light microscopy.

   d- **Long chains of conidia** – are formed by strains belonging to various genera. These may include *Nocardia*, *Nocardioides*, *Pseudonocardia*, *Sacchropolyspora*, *Actinopolyspora*, *Streptomyces*, *Streptoverticillium*, *Actinosynnema*, *Nocardiopsis*, *Streptoalloteichus*, *Kibdelosporangium*, *Kitasatosporia*, *Glycomyces* and *Sacchrothrix*.

   e- The conidia bearing hyphae may be united into synnemata releasing motile spores (*Actinosynnema*, Group 27)

3- **Sporangia** are spore containing bags. These may be borne on well developed aerial hyphae or on surface of colonies with little or no aerial hyphae [Actinoplanes, *Ampullariella*, *Pilimelia*, *Dactylosporangium* (Group 24)].
**Planobispora, Planomonospora, Spirillospora, Sterptosporangium** (Group 26). *Kineosporia* (Group 25) may develop sporangia within the agar.

4. **Other structures**: Organisms of Group 23 form masses of spores that are the result of division in several planes rather than division perpendicular to the axis of the hyphae. These spore bearing structures are called multilocular sporangia.

Many Actinomycetes form spherical structures on aerial hypha which are drops of condensed water that enclose a curled chain of spores or hyphae embedded in amorphous matrix. Eg. *Kibdelosporangium* (Group 29). Sclerotia are globose structures formed by some Streptomycetes which contain cells filled with lipids.

**Pridham et al., (1958)** proposed following 7 groups for classification on the basis of spore pattern:

1. Rectus-Flexibilis (RF).
2. Retinaculum-Apertum (RA).
3. Spira (S).
4. Monoverticillus (MV).

**1.12 Identification of Actinomycetes**

Streptomyces identification can be taken up as a stepwise procedure where one has to understand details of their growth pattern. Their characterization is definitely a tedious job as they are slow growers. The unknown isolate can be identified by determining the listed details in Bergey’s Manual.

1. Cultural Characters
2. Morphological analysis
3. Biochemical characterization and chemosystemics
4. Partial and whole genome sequencing
The cultural characters which must be observed in order to identify a Streptomyces strain is the aerial spore mass colour, colony reverse which represents the substrate mycelium colour, pigmentation and texture of the colony.

The observation of substrate, aerial mycelium and spore pattern is studied by slide culture technique. This helps in preliminary grouping of the organism on the basis of morphological characters.

The next stage is biochemical characterization where utilization of various sugars, oxidation fermentation tests and optimum growth conditions are examined. Carbon sources for this test could be arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose.

Chemosystemics or chemical taxonomy is a rapidly expanding discipline in which information derived from chemical analyses of whole cells or cell fractions is used for classification and identification. Presence of specific amino acids and sugars in the cell are convenient means of classifying the organisms. Sugar composition gives valuable information for the identification of Actinomycetes. Actinomycetes have a peculiar pattern of sugar in the cell wall besides muramic acid and glucosamine of peptidoglycon [Sivakumar et al., 2005].

The 16S rRNA sequencing has developed as a reliable tool for identification of Streptomyces. This has been picked up by researchers in all fields including taxonomic studies, agriculture, clinical isolates, industrial sectors and diversity studies [Clarridge III, 2004; Patel et al., 2003; Rintala et al., 2002; Ramos et al., 1997; Bowden et al., 1993]. It is a useful method for establishing the relatedness of taxa is comparative analysis of ribonuclease-resistant oligonucleotides if 16S rRNA. This macromolecule is particularly suitable for generating phonetic data that can be used to determine both close and very distant relationships because it is ubiquitous, highly conserved in sequence and genetically stable DNA - DNA hybridization can also be used for determining the relatedness between organisms [Kizuka et al., 1995].
Researchers have also developed other methods besides these routine identification techniques. Atalan et al., (2000) characterized Streptomyces using pyrolysis mass spectrometry which is a rapid and a practical way of determining the phenotypic species diversity of Streptomyces in natural habitats and the classical methods. They grouped the isolates from meadows of Northumberland, UK into three *Streptomyces* groups A, B and C on the basis of pigmentation, aerial spore mass colour and colony reverse. This categorization coincided with the results obtained by pyrolysis mass spectrometry. Zhao et al., (2006) used FT-IR for categorizing their isolates and compared the results with those of 16S rRNA sequencing. The selected *Streptomyces* isolates that were studied were all clearly classified to the same four clusters using colour grouping, 16S rDNA sequences analysis and FT-IR spectroscopy. It only takes 1–10 seconds to collect the FT-IR fingerprints from each sample, and this approach can be performed using automation. Thus FT-IR can be used as a rapid, high throughput screening technique for preliminary differentiation of isolates at least at species level. This is a rapid, non-destructive spectroscopic approach for whole organism fingerprinting. This technique is based on the absorption of IR light directed onto a sample. The amount of light absorbed depends on the molecules found within the sample. It measures dominantly vibrations of functional groups and highly polar bonds. Therefore it gives a lot of information about the total biochemical composition of a sample regarding the molecule composition, structure and interactions. FT-IR spectrometers record the interaction of IR radiation with samples, measuring the frequencies at which the sample absorbs the radiation and the intensities of these absorptions. *Streptomyces* isolates were grouped together based on macro-morphological characters, the production of distinct aerial spore mass, the colours of substrate mycelia and diffusible pigment colours on oatmeal agar plates.

1.13 Actinomycetal genes

As the popularity of Actinomycete group is increasing day by day because of their immense potential to produce commercially important products many of
them have been sequenced completely. The researchers are able to sense the capacity of the organism to produce experimentally undetected metabolites.

*Streptomyces* possess largest of the bacterial genome. Most *Streptomyces* chromosomal DNA molecules are about 8-Mb long. This accounts for it’s extensive capacity to produce wide range of primary and secondary metabolites. Complete genome have been sequenced for various members of this group namely the model organism *Streptomyces coelicolor* (%G+C = 72.1) and avermectin producer *Streptomyces vermitilis* (%G+C = 70.7) [Ikeda et al., 2003; Bentley et al., 2002]. Two important aspects of the genomes structures of *Streptomyces* were supported by sequence data. Firstly, that the genome size of *Streptomyces* is large compared to other bacteria; 8,667,507 base pairs for *S. coelicolor* (7,825 protein coding genes) and 9,025,608 bp (7,577 protein coding genes) for *S. avermitilis*. *S. avermitilis* has the highest proportion of predicted secondary metabolite gene clusters of all bacterial genomes sequenced. It contained 25 clusters for biosynthesis of melanin, carotenoid, siderophore, polyketide, and peptide compounds. The total lengths of these gene clusters were estimated to be about 560 kb. This analysis predicted that 6.43% of the *S. avermitilis* genome is occupied by genes concerned with the biosyntheses of secondary metabolites, a far higher proportion than has been found in other sequenced genomes. Secondly, that the genomes of these two species are linear and both ends contain unique terminal inverted repeats that probably covalently bind a terminal protein. The linearity of chromosomes in *Streptomyces* was first discovered in *Streptomyces lividans*. Terminal inverted repeats and covalently bound terminal proteins are not found in the limited number of other bacteria that have linear chromosomes such as *Borrelia burgdorferi* and Agrobacterium tumefaciens and, up to the present, seem to be unique to the *Streptomyces* and perhaps other Actinobacteria. Over 2,500 *Streptomyces* strains are present in the Ribosomal Database Project, over 1,500 are available at the American Type Culture Collection (http://www.atcc.org/) and many more are held in both public and private culture collections throughout the world [Hsiao and Kirby 2008; Omura et al., 2001].
K. rhizophila an industrially important organism, commonly used as a standard quality control strain for antimicrobial susceptibility testing. Sequencing and annotation of the genome of K. rhizophila DC2201 (NBRC 103217) revealed a single circular chromosome (2,697,540 bp; G+C content of 71.16%) containing 2,357 predicted protein-coding genes [Takarada et al., 2008].

Further incentive to explore Actinomycetes as a source of novel secondary metabolites comes from the genome sequences of Streptomyces coelicolor and Streptomyces avermitilis, both of which revealed many unanticipated biosynthetic gene clusters, thus demonstrating that even well studied taxa have the potential to yield new metabolites. Such genomic-based information has been used not only to predict the chemical structures of previously unobserved metabolites but also to develop fermentation methods that enhance their production. Bioinformatics-based approaches to natural product discovery have also been used successfully at the industrial level, where genome scanning has led to the discovery of significant new chemical entities. These methods have great potential to eliminate the redundant isolation of previously described compounds while allowing detailed fermentation studies or molecular cloning experiments to be focused on strains that possess a high probability of producing new chemical structures. Among the strains cultured from marine samples is the genus Salinispora, which was recently described as the first seawater-requiring marine actinomycete. Among bacteria sequenced to date, S. tropica devotes the largest percentage of its genome (9.9%) to natural product assembly S. tropica was that it is a producer of the potent anticancer agent salinosporamide A. The existence of so many different secondary metabolites probably indicates specific adaptations to different habitats [Borodina, 2009; Udwary et al., 2007].

1.14 Biotechnological importance of Actinomycetes

Actinomycetes have proved to be useful producers of a wide range of primary and secondary metabolites for human use. Some of these are listed here.
1.14.1 Enzymes

This filamentous group of bacteria has emerged as an inexhaustive treasure for a wide range of enzymes like glucose isomerase [Bhosale et al., 1996], amylase [Kar et al., 2011; Gulve and Deshmukh, 2011; Khosravi-Darani et al., 2008; Sharma and Shukla, 2007], protease [Guravaiah et al., 2010; Guangrong et al., 2008; Rifaat et al., 2006], lipase [Ko et al., 2005], cellulase [Nurkanto, 2009; Jaradat et al., 2008; Vonothini et al., 2008; Murugan et al., 2007], pectinase [Xu and Yang, 2009], xylanase [Bajaj and Singh, 2010; Kansoh and Nagieb, 2004] and many more. These enzymes have biotechnological applications in clinical chemistry and medicinal therapy also. They are preferred above other sources because they possess greater stability, activity at high temperature and unusual substrate specificity [Manivasagan et al., 2010; Mehta et al., 2006].

1.14.2 Antibiotics

Actinomycetes are known for the largest number of secondary metabolites produced by the microbes. The complexity of genes for secondary metabolism accounts for the wide range of antibiotics produced by these organisms. Streptomycetes are responsible for the production of around half of the 10,000 known bioactive compounds. A strain is capable of producing more than one antibacterial substance. Numerous Actinomycetes have been isolated from, soil, marine environments, hot springs, alkaline environments and polar regions which have the capacity to produce potent antibiotics. Important antibiotics from Actinomycetes include anthracyclines, aminoglycosides, β-lactams, chloramphenicol, macrolides, tetracyclines, nucleosides and peptides. Recently efforts have been made to explore rare Actinomycetes like Actinomadura, Actinoplanes, Ampullariella, Actinosynnema and Dactylosporangium for search of new antibiotics. Target directed screening is done for searching new antibiotics [Bull et al., 2007; Chater, 2006; Challis and Hopwood, 2003; Anderson et al., 2001; Srinivasan et al., 1983]. Exploring new members of the group is a continuous process worldwide for discovering novel bioactive compounds [Basavaraj et al., 2010; Manjula et al., 2010].
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2009; Hong et al., 2009; Nakaew et al., 2009; Imada et al., 2007; Vasavada et al., 2006; Castillo et al., 2002; Huck et al 1991]. Regular strain improvement strategies at genetic levels have also enhanced the productivity of antibacterials [Tanaka et al., 2009]. Yadav et al., (2008) isolated *Streptomyces* sp. from Mt. Everest base camp which produced streptomycin like antibiotic. Thenmozhi et al., (2010) reported the production of antioxidants from *Streptomyces* sp.

Actinomycetes inhibit the growth of dermatophytic fungi eg. *Trichophyton rubrum*. Human pathogenic fungi are becoming increasingly resistant towards antimycotic drugs, therefore there is increasing demand for more potent, nontoxic and cost effective antifungal agents [Lakshmipathy and Krishnan, 2009].

1.14.3 Biotransformation of steroids

Actinomycetes are also involved in transformation of steroids. A mixed culture of *Arthrobacter simplex* and *Streptomyces roseochromogenes* is used to insert a double bond and hydroxyl group in the desired position of the substrate 9-α-fluorohydrocortisone [Kokare, 2008].

1.14.4 Agriculture

Actinomycetes also improve soil health by aiding in humification. Lignocellulose from wheat straw was subjected to degradation by *Streptomyces* and *Thermomonospora* which solubilised it completely and produced aromatic monomers, such as p-coumaric acid, protocatechuic acid, gallic acid, gallic acid methyl ester, 2,4- dimethoxybenzoic acid and 4-methoxyphenol. Phenolic acids are considered to play a major role in humification in soils. In fact, humic substances are predominantly formed by humic acid polymers which are synthesized from phenolic acids, amino acids and other large molecules through enzymatic and oxidative reactions [Trigo and Ball, 1994].
1.14.5 Biocontrol of phytopathogens

Besides curing human diseases *Streptomyces* have also contributed towards restricting growth of plant pathogens. Approximately 60% of antibiotics developed for agricultural use were isolated from *Streptomyces* sp. Halotolerant Streptomycetes isolated from Qaroon Lake were found to possess antiviral activities against tobacco mosaic tobamovirus (TMV) and potato Y potyvirus (PVY) [Mohamed and Galal, 2005]. *Actinomycetes* are important not only for pharmaceutical sector but also have wide applications in agriculture. They can be used as biocontrol agents for restricting the phytopathogens. *Streptomyces* producing chitinolytic enzymes can be used to control fungal phytopathogens and other insect pests. Several chitinolytic enzymes have been identified in various *Streptomyces* sp., including, *Streptomyces plicatus*, *S. lividans*, *S. virdificans* and *S. halstedii*. *Streptomyces viridodiasticus* also produced antifungal metabolite(s) that significantly reduced the growth of the pathogen in vitro [Hosny et al., 2010; Tahtamouni et al., 2006; El-Tarabily, 2000; Gomes et al., 2000; Ames et al., 1989; Williams and Robinson, 1981].

1.14.6 Bioremediation

Bioremediation can be done by means of transformation of xenobiotics into harmless compounds. The most characteristic reactions in transformation of xenobiotics are oxidative, reductive, hydrolytic, dehydration and condensation. The ability of actinomycetes to perform a variety of microbial conversions of organic compounds is an important factor in biodegradation of pollutants in soil and water. *Streptomyces* and *Nocardia* species have ability to perform highly selective chemical modifications of complicated compounds of natural and synthetic origin. *Nocardia* strains have been found to degrade aromatic hydrocarbons by hydroxylation [Kokare, 2008].

As stated earlier Actinomycetes have complex metabolome as a result of which they degrade varied substances, some members are also known for bioremediation. Bioremediation of chromium (VI) contaminated soil is reported by *Streptomyces* sp. MC1. The most stable and common forms in the environment are trivalent Cr (III) and hexavalent Cr (VI). Cr (III) is an essential
nutrient required for normal sugar and fat metabolism, whereas Cr (VI) is an established human carcinogen that can damage protein and cell structures, due to its strong oxidizing potential. *Streptomyces* sp. MC1 was able to reduce 30% of Cr (VI) after 96 h of incubation with 10 mg/L of Cr (VI) [Polti et al., 2009]. *Streptomyces* are also observed to perform bioremediation of zinc, which possess carcinogenic effect and toxicity for kidney [El Sayed et al., 2011]. *Streptomyces* are also known to produce biosurfactants which are used for bioremediation [Doshi, 2010; Lakshmipathy et al., 2010; Thampayak, 2008]. Actinomycetes are also able to degrade certain pesticides. The herbicide, dalapon is degraded by *Nocardia* strains isolated from soil [Kokare, 2008].

It is well said for Actinomycetes by Demain (1988) that "It is clear that *Actinomycetes* have served us and are serving us well beyond the call of duty; however *Actinomycetes* can never relax because we shall expect more from them in the future and this will ensure that actinomycete scientists and technologists would be around for a long time." [Srinivasan et al., 1991].
This work is an attempt to standardize the production of Glucose Isomerase (GI), an enzyme of future market. Enzymes bag a huge market share in the products being produced by microbes. According to a report by Business Communications Company (BCC 2008), the global demand of Industrial enzymes will reach $4.9 billion by 2013. Glucose isomerase occupies third highest position in world market in production of enzymes after amylase and protease. Glucose isomerase converts glucose into fructose. This enzyme is used commercially for the production of High Fructose Corn Syrup (HFCS). According to researchers GI shall be the enzyme in highest demand in future times to come. The world market of HFCS is expected to reach over 153 million tonnes [Quarterly Market Outlook, February 2009]. HFCS has almost completely replaced sucrose in the developed countries, and only a moderate (3 to 4%) growth rate in its production is expected on a global basis.

Fructose has higher sweetening index than glucose and sucrose. HFCS an equilibrium mixture of glucose and fructose (1:1) is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. The price of HFCS is 10 to 20% lower than that of sucrose on the basis of its sweetening power. The process for making the sweetener high fructose corn syrup (HFCS) from corn was developed in the 1970s.

Chemical isomerisation of glucose is possible but it is a set of tedious reactions which should be carried out at high pH and temperature. It also leads to some undesirable byproducts like coloured impurities and psicose which is nonmetabolizable sugar. Investigators have reported the production of Glucose Isomerase by fermentative process but there is a need to develop an
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Economically viable technology. Immobilized reactors are used at industrial scale for isomerisation, still the demand is higher than supply. Enzymatic glucose isomerization was first accomplished on an industrial scale in 1967 by Clinton Corn Processing Co. in the United States. Four companies control 85 percent of the $2.6 billion business—Archer Daniels Midland, Cargill, Staley Manufacturing Co. and CPC International. Currently, the HFC syrups are manufactured and used in several countries throughout the world. The product has an immense demand in the developing countries. The product is also gaining popularity in the Indian market. The various industries, such as soft drink, confectionary, and other fruit processing units are growing at a rapid pace. As HFC syrup has a derived demand in the market, thus there is huge market potential for the product. Since GI is a subject of great commercial importance, much of the information on new producer organisms and on developed processes is in the form of patents. Point of consideration is that most of the plants are situated overseas and we need to develop indigenous technology using our own isolates. As most of the methodologies are patented we may have to pay a huge amount of money to establish a plant in our country.

We need to standardize production media as there is no perfect combination of medium ingredients described so far which can produce huge amount of the enzyme at industrial level. Elimination of expensive xylose [inducer] and hazardous cobalt from the production media is crucially required. Searching a strain which can use crude source of xylose and independence of cobalt ions is a challenge for developing an indigenous technology.

The researchers have opted for submerged fermentation process for GI production. Xylose works as an inducer for the production of enzyme. There is a need to find a suitable agro-residue which can substitute expensive xylose. Peanut shell is a good source of xylose and corn cob is reported to have high xylan content. The production medium containing such substitutes can make
the process economic. Soy residues and corn steep liquor are reported to enhance GI production. Presence of divalent cations example magnesium, manganese and cobalt also increases the GI yield.

So, the present investigation was carried out with the following aims and objectives:

# Isolation and diversity study of Actinomycetes

# Enzyme profiling and screening for Glucose Isomerase (GI) producing Streptomyces

# Molecular characterization of GI producing Streptomyces isolates.

# Optimization of fermentation medium for enhancing GI production.

# Parametric optimisation for efficient production of GI.

# Partial purification and characterization of GI.

# Application of GI for HFCS production.