MICROBIAL ENZYMES : GLUCOSE (XYLOSE) ISOMERASE FROM A THERMOPHILIC STREPTOMYCES SP.

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BY

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Dedicated to
my Parents

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DECLARATION

Certified that the work incorporated in the thesis entitled " **Microbial enzymes : Glucose (xylose) isomerase from a thermophilic *Streptomyces* sp.** " submitted by Mrs. Sumedha S. Deshmukh was carried out under my supervision. Such material as has been obtained from other sources has been acknowledged in the thesis.

(Dr. V. Shankar)

Research Guide.

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SUMMARY

Glucose (xylose) isomerase (EC 5.3.1.5) which catalyses the reversible isomerization of glucose to fructose is an industrially important enzyme and is used for the production of high fructose syrup. Glucose isomerase is produced by a variety of bacteria but the enzymes of commercial interest originate mainly from *Streptomyces* sp.. Moreover, most of the glucose isomerase producing organisms, reported so far, are mesophilic and only a few are thermophilic. Since thermophilic organisms grow at high temperature, their use not only helps to avoid microbial contamination but also permit optimum enzyme production in a shorter time. Enzymes from mesophilic organisms exhibit moderate thermostability and allows the isomerization reaction to be carried out at comparatively high temperatures (55 - 65 °C), a condition which apart from yielding 42% fructose, also discourages microbial contamination. Since isomerization reaction is reversible, by raising the temperature to 95 - 100°C, the yield of fructose in the syrup can be increased to approximately 55% and this has great economic importance. However, high operational temperature coupled with the alkaline pH optima of most of the industrially used glucose isomerases enhance the formation of by-products. On this background, thermophilic strains capable of producing glucose isomerase, having pH optima near neutrality, will have considerable industrial potential. Hence, a thermophilic strain of *Streptomyces thermonitrificans* which exhibited good intracellular

glucose isomerase activity, when grown at pH 7.0 and 50 °C was selected for further studies. The present investigation was then carried out to (a) optimize the medium for maximum enzyme production (b) purify and characterize the enzyme and (c) develop an immobilization procedure to obtain a highly active and stable immobilized preparation suitable for commercial application.

Chapter 1. General introduction.

This part comprises of literature survey pertaining to glucose isomerases, their properties and immobilization.

Chapter 2. Medium optimization for *S. thermonitrificans* glucose isomerase production.

Streptomyces thermonitrificans produced high levels of intracellular glucose isomerase activity (12 U/ml), when grown at 50 °C, in a medium containing 1 % (w/v) xylose supplemented with 2% (w/v) sorbitol as the second carbon source. Organic nitrogen supplements like cornsteep liquor, casamino acids, peptone, tryptone and yeast extract supported both growth and enzyme production. However, none of the inorganic nitrogen sources tested could support growth, when added in the absence of organic supplements. Though, maximum enzyme activity was obtained in a medium containing yeast extract, casamino acids and ammonium nitrate, comparable activities could be obtained in the absence of casamino acids and ammonium nitrate. Influence of metal ions showed that high levels of glucose isomerase activity was obtained in presence of 0.2% Mg^{2+} or Mn^{2+}. Unlike mesophilic

Streptomyces sp. *S. thermonitrificans* did not require Co^{2+} for growth and enzyme production. Our studies show that the productivity of *S. thermonitrificans* is the highest reported among thermophilic strains.

Chapter 3. Purification and characterization of glucose isomerase.

Glucose isomerase from *S. thermonitrificans* was purified to homogeneity by sonication, heat treatment, ammonium sulfate precipitation and preparative polyacrylamide gel electrophoresis. The purified enzyme is an oligomeric protein with a M_r of 1,66,000 and consists of 4 identical subunits of M_r 42,000. It is an acidic protein with a pI of 3.8. The optimum pH and temperature were 7.0 and 85 °C respectively. The enzyme required Co^{2+} and Mg^{2+} for its optimal activity and no activity was observed in the absence of metal ions. Substrate specificity studies showed that *S. thermonitrificans* glucose isomerase could isomerize only D-xylose and D-glucose. The K_m for D-xylose, D-glucose and D-fructose were 7 mM, 250 mM and 400 mM, respectively. The enzyme was inhibited competitively by D-xylitol, D-sorbitol, D-mannitol, D-galactose, L-arabinose and tris(hydroxymethyl) aminomethane. Moreover, glucose and xylose isomerase activities were competitively inhibited by D-xylose and D-glucose, respectively. Modification of histidine residues of the purified enzyme resulted in the loss of glucose and xylose isomerase activities associated with the enzyme. Kinetic and spectral analysis of diethylpyrocarbonate mediated inactivation

revealed the involvement of a single histidine residue in the catalytic activity of the enzyme. Furthermore, histidine modification was accompanied by a concomitant loss of both activities associated with the enzyme, suggesting the presence of a common catalytic site responsible for the isomerization of D-glucose and D-xylose.

Chapter 4. Immobilization of glucose isomerase.

Partially purified glucose isomerase from *S. thermonitrificans* when coupled to glutaraldehyde activated Indion 48-R, retained 30 - 40 % activity of the soluble enzyme. However, an approximately two-fold increase in the activity could be achieved by binding the enzyme in presence of glucose. Binding the enzyme to matrices presaturated with either glucose or fructose and influence of lysine modification on the activity of soluble enzyme revealed that the comparatively low activity observed in case of the enzyme bound in the absence of substrate is due to the nonspecific binding of either substrate or product to the matrix. Immobilization did not affect the pH and temperature optima of the enzyme but it lowered the temperature stability. Immobilization resulted in a marginal increase in the K_m and a three-fold decrease in the V_{max}. Substrate concentrations as high as 36% glucose could be converted into 18.5 % fructose in 5 h at pH 7.0 and 70 °C. The bound enzyme, however, showed inferior stability to repeated use and lost approximately 40% of its initial activity after five cycles of

use. Indion 48-R bound glucose isomerase could be stored, in wet state, for 30 days without any apparent loss in its initial activity.

Publications.

1. Deshmukh, S. S., Deshpande, M. V. and Shankar, V. (1994). Medium optimization for the production of glucose isomerase from thermophilic *Streptomyces thermonitrificans*. *World J. Microbiol. Biotechnol.* **10**, 264-267.

2. Deshmukh, S. S., Dutta Choudhury, M. and Shankar, V. (1993). Preparation and properties of glucose isomerase immobilized on Indion 48-R. *Appl. Biochem. Biotechnol.* **42**, 95-104.

Chapter 1

General Introduction

GLUCOSE ISOMERASES

Glucose (xylose) isomerase (EC 5.3.1.5) has attracted considerable attention largely due to its ability in the conversion of glucose to fructose. This enzyme also catalyzes the isomerization of xylose to xylulose and prefers xylose as substrate. Hence, the recommended name for this enzyme is xylose isomerase. Xylose isomerase has also been evaluated for its potential application in the conversion of cellulosic biomass to ethanol. However, due to the extensive application of this enzyme in the industrial production of high fructose syrups, it is generally referred to as glucose isomerase.

Prior to enzymatic isomerization, conversion of glucose to fructose was carried out under alkaline conditions (Tsao *et al.*, 1969; Parrish, 1970; Katz *et al.*, 1972; Barker, 1976; Vieth *et al.*, 1976). Based on this approach, the yield of fructose was relatively low (40%) and the syrup contained nondextrose and nonfructose degradation products as well as coloured impurities resulting in syrups with reduced sweetness. On the other hand, enzymes being specific, their use in isomerization of glucose not only gives high yields of fructose but also allows the production of isomerized syrups of varying fructose content.

Xylose isomerase activity was initially reported in the extracts of *Lactobacillus pentosus* (Mitsuhashi and Lampen, 1953), *Pseudomonas hydrophila* (Hochster and Watson, 1954) and *Pasteurella pestis* (Slein, 1955). However, the presence of an enzyme capable of converting glucose to fructose was first

demonstrated by Marshall and Kooi (1957) in the extracts of *P. hydrophila*. This discovery generated considerable excitement since glucose could be isomerized to fructose without prior phosphorylation. Subsequently, it was found that though the glucose isomerase described by Marshall and Kooi (1957) could convert unphosphorylated glucose to fructose, it required arsenate for isomerization. Similarly, glucose isomerases from *Aerobacter cloacae* (Tsumura and Sato, 1961), *Aerobacter aerogenes*, *Escherichia freundii* and *Escherichia intermedia* (Natake and Yoshimura, 1963; 1964) required arsenate for their activity, thus making them unsuitable for commercial exploitation. Hence, attempts were made to look for glucose isomerases which do not require arsenate for their activity. Finally, Yamanaka (1963) demonstrated that several lactic acid bacteria viz. *Lactobacillus pentoaceticus*, *L. brevis*, *L. fermenti*, *L. mannitopoeus*, *L. gayonii*, *L. buchneri* and *Leuconostoc mesenteroides* are also capable of producing glucose isomerase. Amongst them, maximum glucose isomerase activity was observed in *L. brevis*. Glucose isomerase from *L. brevis* seemed to have desirable properties for commercial exploitation but its low pH optimum and inferior stability at higher temperature prevented its further commercial development. Since then, several *Bacillus* and *Streptomyces* species have been studied extensively for their ability to produce glucose isomerase (Antrim *et al.*, 1979; Chen, 1980a). Other genera namely, *Nocardia*, *Micromonospora*, *Microbispora*, *Microellobospora* and *Actinomyces* have also been

shown to produce glucose isomerase (Chen, 1980a). Apart from aforementioned species, glucose isomerases from *Actinoplanes missouriensis* and *Arthrobacter* (Crueger and Crueger, 1984) are also of commercial importance. However, enzymes from *Bacillus megaterium* A_1 and *Paracolobacterium aerogenoides* (Takasaki and Tanabe, 1962; 1966) required NAD and ATP respectively, as cofactors.

Thus, early discoveries revealed that among the three types of glucose isomerases namely, (1) arsenate requiring (2) cofactors like NAD and ATP requiring and (3) those which do not require arsenate or cofactors, only the third type of enzymes are of commercial importance. The other factors which favour their commercial use is their desirable properties like low pH optimum and high optimum temperature. Some of the commercially important glucose isomerase producing organisms are listed in Table 1.1.

PRODUCTION

Glucose (xylose) isomerases are widely distributed in microorganisms (Chen, 1980a) but they are also present in yeast (Tomoyeda and Horitsu, 1964) and yeast like organisms (Zemek, 1986) and plants (Bartfay, 1960; Pubols, 1963). *Aspergillus oryzae* is probably the only fungus reported to have glucose isomerase activity (Antrim *et al.*, 1979). Glucose isomerase is an intracellular enzyme, though reports exist on extracellular glucose isomerases from *Streptomyces glaucescens* (Weber 1974), *Chainia* (Srinivasan *et al.*, 1983) and *S. olivaceus* M_{13} (Mikhailov *et al.*, 1986). Majority of wild strains, producing

Table 1.1: **Commercially important glucose isomerase producing organisms**

Name of the organism	Reference
Bacillus coagulans	Yoshimura *et al.* (1966) Diers (1976)
Arthrobacter NRRLB-3728	Lee *et al.* (1972) Meers (1977)
Actinoplanes missouriensis	Anheuser-Busch Inc. (1975)
Streptomyces olivaceus	Armbruster *et al.* (1974)
Streptomyces murinus	Jørgensen *et al.* (1988)
Streptomyces rubiginosus	Weidenbach *et al.* (1983)
Streptomyces olivochromogenes	CPC International Inc. (1975)
Streptomyces phaeochromogenes	Strandberg and Smiley (1971)
Streptomyces albus	Takasaki *et al.* (1969a)
Streptomyces griseofuscus	Jensen and Rugh (1987)
Flavobacterium arborescens	Lee (1981)

glucose isomerase, require xylose as an inducer and glucose cannot serve as an alternate inducer. However, pure xylose being expensive, cannot be used for the commercial production of this enzyme. Hence, efforts were directed towards selecting organisms capable of producing the enzyme on cheap carbon sources like xylan or xylan containing materials like wheat bran, corn cobs, corn hulls, bagasse pith and straw hemicellulose. Mutants of *S. albus* YT-4, YT-5 and YT-6 (Takasaki, 1966) could produce glucose isomerase on xylan or wheat bran whereas, *S. flavovirens* (Japan Bureau of Industrial Technics, 1966) and *S. bikiniensis* (Park and Toma, 1974) produced glucose isomerase in presence of xylan. However, *S. flavogriseus* (Chen *et al.*, 1979) and *Streptomyces* sp. S41-10 (Lai, 1975) showed high levels of enzyme activity when grown on straw hemicellulose or acid (H_2SO_4) hydrolysate of straw and hydrolysate of bagasse pith, respectively. On the contrary, *S. venezuellae* and *S. olivochromogenes* ATCC 21114 (Iisuka *et al.*, 1971), *S. oulgare* (Nonomura and Kaji, 1974) could produce glucose isomerase in a medium containing xylose and starch and xylose and dextrin, respectively.

Glucose is commonly used as a carbon source for constitutive producers of glucose isomerase. However, when xylose is obligatory, a supplementary carbon source like, sorbitol (Callens *et al.*, 1985a) glycerol or lactate (Wovcha and Brooks, 1980) are used. Some commercially important strains like *A. missouriensis* (Shieh, 1977) , *Arthrobacter* NRRL B-3728 (Meers, 1977) and *F.*

arborescens (Whitted and Boguslawski, 1985) produced the enzyme in presence of simple sugars like beet molasses, glucose and lactose respectively. Horitsu *et al.*(1992) reported a new type of glucose isomerizing enzyme from human intestinal bacterium namely, *Bifidobacterium adolescentis*, which could be induced by glucose.

Various organic nitrogen sources required for enzyme production include corn steep liquor, peptone, tryptone, yeast extract, beef extract and soyflour. Among them, corn steep liquor was found to be a good and cheap nitrogen source for many glucose isomerase producing organisms. However, its seasonal and batch to batch variation has proved to be an unreliable factor in enzyme production (Verhoff, 1985; Shieh, 1976). Some organisms like *A. cloacae* (Tsumura and Sato, 1961) *A. aerogenes* (Natake and Yoshimura, 1963) could utilize inorganic nitrogen sources like ammonium phosphate while, a mutant of *Bacillus coagulans* (Outtrup, 1974), *E. intermedia* (Natake and Yoshimura, 1964) and *P. aerogenoides* (Takasaki and Tanabe, 1966) utilized ammonium sulfate and ammonium chloride respectively, for enzyme production. In addition, amino acids like glycine and histidine are also known to enhance enzyme production in *Streptomyces* sp. (Vandamme *et al.*, 1981; Callens *et al.*, 1985a).

Most of the glucose isomerase producing organisms require mineral salts like $MgSO_4$, $MnSO_4$ or $CoCl_2$. Usually, two or more mineral salts are added for optimal enzyme production. Use of appropriate metal ions was important because, in case of

Streptomyces YT-5, Co^{2+} stimulated the enzyme production (Takasaki, 1966) while, *B. coagulans* HN-68 (Yoshimura *et al.*, 1966) and *L. brevis* (Yamanaka, 1968) required Mn^{2+} for maximum enzyme production. On the contrary, Co^{2+}, Cu^{2+} and Zn^{2+} repressed the growth of *B. coagulans* HN-68 (Yoshimura *et al.*, 1966). Interestingly, presence of Co^{2+} in the fermentation medium influenced the optimum pH of *S. phaeochromogenes* glucose isomerase. The enzyme showed comparable activity at pH 7.5 and 9.0 whereas, the enzyme from the same organism, grown in absence of Co^{2+}, had an optimum pH of 9.0 (Tsumura *et al.*, 1967). Similarly, *A. missouriensis* was reported to be insensitive to the presence of Co^{2+} but addition of Cu^{2+} and Co^{2+} stimulated enzyme production (Anheuser-Busch Inc., 1975). In case of *S. flavogriseus*, addition of Mg^{2+}, Mn^{2+} and Fe^{2+} significantly enhanced enzyme production but the organism did not require Co^{2+} for growth and enzyme production (Chen *et al.*, 1979). Other organisms which do not require Co^{2+} for growth and enzyme production are *Arthrobacter* and *S. olivaceus* (Reynolds, 1973), *S. olivochromogenes* (CPC International Inc., 1975), *Streptomyces* sp. (Chou *et al.*, 1976), *Streptomyces* sp. CCM4102 (Hasal *et al.*, 1992) and *Streptomyces violaceus-ruber* (Callens *et al.*, 1985a). Elimination of Co^{2+} in the fermentation medium is of great importance, as Co^{2+} is toxic and disposal of spent media can cause environmental hazards.

Dispersing agents like agar, diatomaceous earth, carboxymethylcellulose have also been used to increase glucose

isomerase yield by promoting filamentous rather than spherical growth of the organism (Dworschack *et al.*, 1972). A glucose oxime resistant mutant of *F. arborescens* showed 2 - 3-fold increase in glucose isomerase production. Moreover, this mutant when grown in presence of carbon sources other than xylose, showed an additional 5-fold increase in enzyme levels compared to the wild strain. The ability of glucose oxime, in increasing the glucose isomerase activity, was correlated to its interference with an aspect of catabolite repression, leading to increased enzyme synthesis (Whitted and Boguslawski, 1985).

In general, pH of the culture medium employed for glucose isomerase production ranges from 6.5 - 7.5. However, the exceptions are *S. acidodurans* (Bok, *et al.*, 1984) and *S. acidophilus* 10168 (Lu *et al.*, 1992) which grow at pH 4.0 - 5.0 and 5.2, respectively.

Glucose isomerase producing organisms can be broadly classified into mesophiles and thermophiles. Mesophiles generally grow at 30°C while thermophiles such as *B. coagulans* HN-68 (Yoshimura *et al.*, 1966), *B. stearothermophilus* (Suekane *et al.*, 1978), *Bacillus* TX-3 (Kitada *et al.*, 1989), *Streptomyces thermoviolaceus* (Hafner, 1985) and *Thermoanaerobacter* (Lee *et al.*, 1990) grow between 45 - 60°C. However, *Clostridium thermohydrosulfuricum* (Dekker *et al.*, 1991), *Thermus aquaticus* HB-8 (Lehmacher and Bisswanger, 1990) and *Thermotoga maritima* (Brown *et al.*, 1993) are capable of growing and producing glucose isomerase at 65, 70 and 80°C respectively.

Fermentation, at high temperatures, offers advantages like optimum enzyme production in shorter time and decreased chances of microbial contamination. Isomerization of glucose is a reversible process and reaction at high temperatures would result in high yield of fructose. Hence, thermostable enzymes obtained from thermophilic strains will have great commercial potential.

PURIFICATION

Glucose (xylose) isomerases are produced by large number of organisms but only a few of them have been purified and extensively characterized. Since majority of the enzymes are intracellular, most of the purification procedures, irrespective of the source, involve steps like lysis of cells, concentration of the crude extract by salt or solvent precipitation and heat treatment followed by conventional purification methods like ion exchange chromatography and gel filtration. In most of the cases, though sonication is used for disrupting the cells (Bengston and Lamm, 1973), lysozyme, toluene or detergents like N-cetylpyridinium chloride (Danno, *et al.*,1967; Takasaki *et al.*, 1969a) have also been used. Other methods of cell breaking include, grinding with aluminium oxide or sand (Kent and Emery, 1973; Natake and Yoshimura, 1964) and use of French Press (Kitada *et al.*, 1989). In most of the cases, ammonium sulfate or acetone have been used for the concentration of crude extract however, magnesium ions (Cory, 1978; Kralova *et al.*, 1985) and manganese chloride (Yamanaka and Takahara, 1977) have also been used.

Glucose isomerases are thermostable enzymes and a brief exposure of the crude extract to high temperature (50 - 60°C) has proved to be beneficial, as it not only helps in the inactivation of proteases, if any, but also in the removal of heat labile proteins. Though DEAE-cellulose has been widely used for the purification of glucose isomerases, hydroxyapatite has also been used in certain cases. For example, Basuki *et al.* (1992) separated two isozymes of glucose isomerase from *S. phaeochromogenes* on hydroxyapatite. Fujita *et al.* (1980) used a porous anion exchange resin namely, trimethylammonium type SO_4^{2-} form, for the purification of the enzyme whereas, Callens *et al.* (1985b) used phenyl-Sepharose. Affinity matrices like xylitol-Sepharose and xylose-agarose have also been employed for the purification of glucose isomerases (Lee,1977; Lehmacher and Bisswanger, 1990).

Apart from the above methods, other techniques like isoelectric precipitation (Jackson and Tsuda, 1980), preparative PAGE (Gaikwad *et al.*, 1988; Pawar *et al.*,1988; Khire *et al.*, 1990) and FPLC (Brown *et al.*, 1993) have proved beneficial for the purification of glucose isomerases from various sources.

CATALYTIC PROPERTIES

Assay procedures

Glucose (xylose) isomerases are usually assayed by measuring the fructose and xylulose produced, spectrophotometrically, following the isomerization of glucose and xylose, respectively. Xylose isomerase activity is also measured by coupled enzyme

assays using D-arabitol (Yamanaka, 1969), D-xylitol (Smith, 1962) or D-sorbitol (Callens *et al.*, 1985a) dehydrogenases while that of glucose isomerase using mannitol dehydrogenase (Sakai and Tamanaka, 1968). Unit of the enzyme is defined on the basis of µmoles of fructose/xylulose produced.

Optimum pH and pH stability: In general, the pH optima of glucose (xylose) isomerases are in the range of 7.0 - 9.0 (Table 1.2). However, the enzyme from *L. brevis* showed a pH optima between 6.0 - 7.0 (Yamanaka, 1968) whereas, glucose isomerase from *T. aquaticus* HB-8 was active over a broad range of pH i.e. 5.5 - 8.5 (Lehmacher and Bisswanger, 1990). The pH optima for different substrates need not necessarily be the same. For example, *B. coagulans* HN-68 glucose isomerase showed an optimum pH of 7.0 - 7.5 when assayed with D-glucose and D-ribose but with D-xylose it was 8.0 - 8.5 (Danno, 1970b). On the contrary, the enzyme from *L. brevis* exhibited the same optimum pH for the isomerization of D-xylose, D-glucose and D-ribose (Yamanaka, 1968). A similar behaviour was observed with *T. aquaticus* HB-8 xylose isomerase (Lehmacher and Bisswanger, 1990).

Isomerization is a reversible process and by increasing the temperature of isomerization, it is possible to obtain high yield of fructose. However, high isomerization temperature coupled with alkaline pH optima of most of the industrially important glucose isomerases, can lead to the formation of undesirable coloured by-products. In this respect, enzymes having pH optima on the acid side will be of commercial importance.

pH stability of most of the well characterized glucose isomerases are between 5.0 - 11.0 and they are unstable below pH 5.0 (Chen, 1980b). However, the enzyme from thermophilic *Bacillus* sp. namely, *B. coagulans* HN-68 (Danno, 1970a) and *Bacillus* TX-3 (Kitada *et al.*, 1989) showed a narrow pH stability and was stable between 5.0 - 7.0 and 7.0 - 9.0, respectively. However, *S. albus* glucose isomerase showed high pH stability and retained its activity for 3 h between pH 4.0 - 11.0 (Takasaki *et al.*, 1969a).

Optimum temperature and temperature stability: The temperature optima of most of the glucose isomerases are in the range of 70 - 80°C (Table 1.2). The enzymes from *T. aquaticus* HB-8 (Lehmacher and Bisswanger, 1990) and *S. griseofuscus* (Kasumi *et al.*, 1981a) showed an optimum temperature of 85°C. The highest optimum temperature of 105 - 110°C was reported for the enzyme from an extremely thermophilic bacterium namely, *T. maritima* (Brown *et al.*, 1993). In contrast, the optimum temperature of Alkalophilic *Bacillus* KX-6 was 60°C (Kwon *et al.*, 1987). Horitsu *et al.*(1992) noted that glucose isomerase from *Bifidobacterium adolescentis* exhibits a very low optimum temperature of 40°C. Most of the well characterized glucose isomerases show comparable temperature stability and retain significant amount of their activity upto 70°C for a brief period (10 - 15 min). However, *B. stearothermophilus* enzyme was stable at 75°C for 1 h (Suekane *et al.*, 1978) whereas, *S. griseofuscus* was stable at 80°C for 30 min (Kasumi *et al.*, 1981a). The thermophilic glucose isomerase from *T. maritima* retained its full activity at 100°C for 10 min

(Brown *et al.*, 1993). As mentioned earlier, isomerization is a reversible process and high isomerization temperature would favour fructose formation. However, most of the industrially used glucose isomerases show an optimum temperature in the range of 80 - 90°C, in the presence of substrate. Volkin and Klibanov (1989) investigated the thermoinactivation of *S. olivochromogenes* glucose isomerase and noted that the destabilization at high temperatures is probably caused by the oxidation of cysteine residues and to the presence of degradation products of glucose and fructose in the isomerized syrup. Site directed mutagenesis has been successfully used to obtain thermostable glucose isomerases (Luiten *et al.*, 1989; Drocourt *et al.*, 1988). In case of *A. missouriensis* glucose isomerase, replacement of lysine 253 by arginine enhanced the thermostability of the enzyme (Quax *et al.*, 1991). However, Sicard *et al.* (1990) opined that the enhancement of the thermostability of *S. olivochromogenes* enzyme (by replacement of cysteine 306 by a more stable amino acid like alanine) would be of little practical interest as isomerization at high temperature (90°C) would lead to chemical alterations of both substrate and products.

Metal ion requirement: Almost all known glucose isomerases require divalent cations like Mn^{2+}, Co^{2+} and/or Mg^{2+} for their catalytic activity. While enzymes from *Streptomyces* sp., in general, require both Mg^{2+} and Co^{2+} for their optimal activity, those from *Bacillus* sp. require Mn^{2+} for maximum activity. On

the contrary, *S. phaeochromogenes* glucose isomerase was stimulated only by Mg^{2+} (Basuki *et al.*, 1992). Similarly, *Arthrobacter* strain NRRL B-3728 glucose isomerase was stimulated by Mg^{2+}, Co^{2+} and Mn^{2+} and amongst them, Mg^{2+} was the best (Smith *et al.*, 1991). Enzymes from *A. missouriensis* (Scallet *et al.*, 1974; Lloyd *et al.*, 1974) and atypical *B. coagulans* (Aschengreen, 1975) required the Mg^{2+} and Co^{2+} for their activity. In case of *A. missouriensis* enzyme, it required Co^{2+} in presence of low concentrations (10^{-3}M) of Mg^{2+} but with increase in the Mg^{2+} concentration (10^{-1}M), Co^{2+} was not necessary. The enzyme showed 90% of its optimal activity in presence of 100 mM Mg^{2+} (Gong *et al.*, 1980). In general, Mg^{2+} is required for activity and Co^{2+} for thermostability. Danno (1970b) noted that Mn^{2+} and Ni^{2+} also protected *B. coagulans* HN-68 glucose isomerase from thermal denaturation.

Inhibitors: Since most of the glucose (xylose) isomerases are metal requiring enzymes, they are strongly inhibited by metal chelators like EDTA. Moreover, they are also inhibited by polyols like xylitol, sorbitol, mannitol and arabitol (Table 1.2). In case of *B. coagulans* HN-68 (Danno, 1970b), *L. brevis* (Yamanaka, 1969) and *S. griseofuscus* (Kasumi *et al.*, 1981a) enzymes, inhibition by polyols was competitive. Inhibition of *Arthrobacter* strain NRRL B-3728 glucose isomerase with xylitol was competitive whereas, sorbitol showed mixed inhibition (Smith *et al.*, 1991). In addition, tris (hydroxymethyl) aminomethane, has been shown to inhibit *B. coagulans* HN-68 (Danno, 1970b) and

S. griseofuscus (Kasumi *et al.*,, 1981a) glucose isomerases competitively whereas, in case of *P. pestis* enzyme, it was non-competitive (Slein, 1955). Furthermore, sugars like D-mannose, D-galactose and L-arabinose inhibited the enzyme from *S. griseofuscus* competitively (Kasumi *et al.*, 1981a) while D-lyxose acted as a competitive inhibitor for *L. brevis* enzyme (Yamanaka, 1969).

Divalent cations namely, Hg^{2+}, Ag^{2+} (Takasaki *et al.*, 1969b) and Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} or Ca^{2+} (Danno, *et al.*, 1967) have been shown to inhibit glucose isomerase activity. (Table 1.2). In case of *Arthrobacter* strain NRRLB-3728, Ca^{2+} inhibited the enzyme competitively (Smith *et al.*, 1991).

PHYSICAL PROPERTIES

Molecular weight and subunit structure: Molecular weights of glucose (xylose) isomerases are in the range of 52 - 200 kDa and majority of them fall between 120 - 200 kDa (Table 1.2). However, enzymes from *S. bikiniensis*, (Park and Toma, 1974) *Chainia* (Khire *et al.*, 1990) and *A. missouriensis* (Gong *et al.*, 1980) are comparatively low molecular weight proteins, with a molecular weight of 52,000, 71,000 and 80,000 Da, respectively.

Most of the glucose isomerases are high molecular weight proteins and are made up of 4 identical subunits (Table 1.2). On the contrary, the enzyme from thermophilic alkalophile *Bacillus* TX-3 consists of 3 identical subunits of 45 kDa (Kitada *et al.*, 1989) However, the enzyme from *S. olivochromogenes*

(Suekane *et al.*, 1978) and Alkalophilic *Bacillus* KX-6 (Kwon *et al.*, 1987) are made up of 2 identical subunits of 56 & 58 kDa. Interestingly, the high molecular weight glucose isomerase from *B. stearothermophilus* is a single polypeptide chain of 130 kDa (Suekane *et al.*, 1978).

Isoelectric point: Isoelectric focusing studies on glucose isomerases from a variety of sources revealed that they are acidic proteins and their pI are between 4.0 - 5.0 (Pedersen, 1993).

STRUCTURE AND FUNCTION

Structure: The three dimensional structure of glucose (xylose) isomerases from *A. missouriensis* (Rey *et al.*, 1988), *S. olivochromogenes* (Farber *et al.*, 1989), *S. rubiginosus* (Carrell *et al.*, 1989; Collyer *et al.*, 1990), *S. albus* (Dauter *et al.*, 1989), *S. violaceoniger* (Sicard *et al.*, 1990), *Arthrobacter* (Henrick *et al.*, 1989), *S. murinus* and *B. coagulans* (Rasmussen *et al.*, 1994a, b) have been reported. Reports also exist on protein crystals with bound substrate-analogs or inhibitors and in presence of different metal ions (Collyer *et al.*, 1990; Carrell *et al.*, 1994).

Glucose (xylose) isomerases from different species crystallized as tetramers with tightly bound dimers of equivalent subunits. Each subunit folds into $(\beta\alpha)_8$-β barrel as observed in the case of triose phosphate isomerase. The active site is located deep inside the barrel. Each subunit consists of

a major and a minor domain. The minor domain of one overlaps with the major domain of the second molecule to form the dimer (Carrell *et al.*, 1989). Crystal structures have also helped in characterizing the two divalent metal binding sites in the protein molecule. The metal ions in one site is coordinated to 4 carboxyl groups while in the other site, the coordination is with 3 carboxylate and an imidazole group (Carrell *et al.*,1994). Probably, two alternate mechanisms exist for aldose/ketose isomerization. Based on the known mechanisms of similar enzymes, a pathway involving a base catalyzed proton transfer with *cis*-enediol intermediate was initially proposed (Rose *et al.*, 1969). Subsequently, crystallographic studies using various substrate analogs or inhibitor bound enzyme suggested a hydride shift mechanism mediated by metal ions.

Active site: In case of most of the glucose (xylose) isomerases, histidine has been implicated in the catalytic activity (Gaikwad *et al.*, 1988; Pawar *et al.*, 1988; Vangrysperre *et al.*, 1988; 1990 and Batt *et al.*, 1990). The role of carboxylate groups in the catalytic activity of glucose isomerases from *S. violaceus-ruber*, *Streptomyces* sp., *L. xylosus*, *L. brevis*, *B. coagulans* (Vangrysperre *et al.*, 1989a; 1990) and *Arthrobacter* (Siddiqui *et al.*, 1993) has also been demonstrated.

Danno (1970b), by competitive inhibition studies, demonstrated that the D-glucose, D-xylose and D-ribose isomerizing activities of *B. coagulans* HN-68 glucose isomerase is catalyzed by same active site. Similar observations were made by

Gaikwad *et al.* (1989) while studying the active site nature of *Streptomyces* sp. NCIM 2730 glucose isomerase.

Substrate specificity: Glucose isomerase is a multifunctional enzyme and mainly catalyzes the isomerization of D-glucose and D-xylose (Table 1.2). However, enzymes from *B. coagulans* HN-68 (Danno, 1970b), Thermophilic Alkalophile *Bacillus* TX3 (Kitada *et al.*, 1989) and *L. brevis* (Yamanaka, 1968) can isomerize D-ribose, in addition to glucose and xylose . Glucose isomerases from *B. stearothermophilus* and *S. olivochromogenes* (Suekane *et al.*, 1978), Alkalophilic *Bacillus* No. KX-6 (Kwon *et al.*, 1987), and *S. bikiniensis* (Park and Toma, 1974) were shown to isomerize arabinose in addition to glucose, xylose and ribose.

Table 1.2: Properties of the purified glucose isomerase

Organism	Optimum pH	Optimum temperature (°C)	Substrate specifi-city	Inhibitors	Molecular weight (kDa)	No. of sub-units	Reference
S. albus YT-5	8.0 - 8.5	80	Glucose Xylose	Ag^{2+}, Cu^{2+} Hg^{2+}	187	4	Takasaki *et al.*, (1969a; b)
S. bikiniensis	8.0 - 9.0	80	Glucose Xylose Ribose Arabinose	-	52	-	Park and Toma (1974)
S. flavogriseus	7.5	70	Glucose Xylose	Ag^{2+}, Cu^{2+}, Hg^{2+}	171	4	Chen *et al.* (1979)
S. griseofuscus S-41	8.5	85	Glucose Xylose Ribose	Sorbitol Mannitol Xylitol D-mannose L-arabinose D-galactose Tris	185	4	Kasumi *et al.* (1981a)
S. olivochromogenes	8.0 - 9.0	80	Glucose Xylose Ribose Arabinose	-	120	2	Suekane *et al.* (1978)

S. violaceus-ruber	7.5 - 9.5	80	Glucose Xylose	Xylitol Sorbitol Tris	171	4	Callens *et al.* (1986)
Streptomyces sp. (NCIM 2730)	7.5 - 8.0	75 - 80	Glucose Xylose	Xylitol	160	4	Gaikwad *et al.* (1989)
S. coelicolor A3 (2)	7.0	70	Glucose Xylose	Xylitol, Ca^{2+} Cu^{2+}, Hg^{2+}	160	4	Sailaja and Joseph (1993)
S. phaeochromogenes	9.0	80	Glucose	EDTA	160	4	Basuki *et al.* (1992)
A. missouriensis	7.0		Glucose Fructose	-	80	2	Gong *et al.* (1980)
L. brevis	6.0 - 7.0		Glucose Xylose Ribose	Xylitol Sorbitol Mannitol Arabitol D-lyxose	165	4	Yamanaka (1968; 1969)
Chainia (extracellular)	9.5	60	Xylose	Hg^{2+}, Cu^{2+}, Sn^{2+} Sorbitol Xylitol	71	-	Khire *et al.* (1990)
Chainia (intracellular)	7.5	75	Glucose Xylose Fructose	Cu^{2+}, Ca^{2+}, Tris, CTAB Triton X-100 Xylitol Sorbitol	158	4	Pawar *et al.* (1988)

B. coagulans HN-68	7.0	75	Glucose Xylose Ribose	Cu^{2+}, Zn^{2+} Ni^{2+}, Ca^{2+} Xylitol Sorbitol Mannitol Tris	175	4	Danno (1970a;b)
B. stearothermo-philus	7.5 - 8.0	80	Glucose Xylose Ribose Arabinose		130	-	Suekane *et al.* (1978)
Alkalophilic *Bacillus* No. KX-6	7.0 - 10.0	60	Glucose Xylose Ribose Arabinose	Hg^{2+}, Ag^{2+} Cu^{2+}	120	2	Kwon *et al.* (1987)
Thermophilic Alkalophile *Bacillus* TX-3	7.5 - 9.0	80	Glucose Xylose Ribose	-	140	3	Kitada *et al.* (1989)
Thermotoga maritima	7.5	105 - 110	Glucose Xylose	-	165	4	Brown *et al.* (1993)
Thermus aquaticus HB-8	5.5 - 8.5	85	Glucose Xylose	-	196	4	Lehmacher and Bisswanger(1990)
Bifidobacterium adolescentis	7.0	40	Glucose	-	-	-	Horitsu *et al.* (1992)
Arthrobacter NRRR B3728	8.0	60	Xylose Fructose	Ca^{2+}, Sorbitol Xylitol	185	4	Smith *et al.* (1991)

IMMOBILIZATION

Use of enzymes as catalysts for large scale industrial processes is hampered by their high cost of production and stabilization. Since they are soluble their recovery from the mixture of substrate and product for reuse is not economically practical and this renders the costly enzymatic process even more costly. However, with the advent of immobilized enzyme technology, increasing efforts are being made to replace the conventional enzymatic reactions with immobilized systems since, immobilization [a] enables the processing of large amounts of substrate as the enzyme can be easily removed from the mixture of substrate and product for reuse, [b] imparts greater stability to the enzyme thus allowing their use for the development of continuous process, [c] affords greater control of the catalytic process and [d] permits the economical utilization of an otherwise cost-prohibitive enzyme.

Glucose isomerase is an industrially important enzyme and is used for the production of high fructose syrup of various compositions. The enzyme itself is considered to be a major cost centre because of the high K_m for glucose and hence numerous attempts have been made to immobilize glucose isomerase to obtain a highly active and stable preparation suitable for commercial application.

Glucose isomerase has been immobilized on several inorganic adsorbents like silica, alumina, magnesium chloride and hydroxide in addition to organic supports like modified celluloses,

collagen, chitin and polystyrene resins. A few representative examples are given below.

Glucose isomerase from *S. phaeochromogenes* and *L. brevis* bound to DEAE-cellulose, at pH 8.5, exhibited high conversion efficiency and stability compared to its soluble counterpart. Immobilization also brought about a decrease in the optimum pH and temperature (Baxter Laboratories, 1972). Similarly, partially purified enzyme from *Streptomyces* ATCC 211175, bound to DEAE-cellulose, showed a half life of 198 h, at an initial conversion efficiency of approximately 50%. However, the enzyme bound to Amberlite IRA-938 exhibited higher stability (Sipos, 1973).

Messing (1974; 1975) immobilized glucose isomerase from *Streptomyces*, on control pore alumina of varying pore diameters (140 - 220 Å), and studied the effect of various inorganic ions on the operational stability of the immobilized preparation. It was observed that the half life of the immobilized preparation was dependent on the inorganic ion content in the glucose feed. Partially purified glucose isomerase from *S. olivochromogenes* immobilized on colloidal silica exhibited good flow properties. On continuous operation, the bound enzyme could convert 50% glucose to fructose with a conversion efficiency of 51% for 10 days (CPC, International Inc., 1977). The advantage of such an immobilized system is that it is relatively non-compressible and hence can be used in fixed bed reactors. Purified *S. olivocinereus* glucose isomerase bound to aminosilochrome

exhibited high retention of activity (58%) and stability (Ananichev *et al.*, 1978). Cory (1978) immobilized *S. phaeochromogenes* glucose isomerase on several matrices namely, controlled pore alumina, carbon, magnesium carbonate, DEAE-cellulose and polystyrene resins and noted that the enzyme bound to controlled pore alumina exhibited maximum efficiency and half life.

Bhatt *et al.* (1979) bound glucose isomerase to polystyrene sulfonate type of cation exchanger equilibrated with transition metal salts like, titanium (T^{4+}), zirconium (Z^{4+}) and vanadium (V^{5+}) and assessed their performance, in the production of isomerized syrup, in packed bed reactors. Among them, the enzyme bound to V^{5+} equilibrated resin showed high retention of activity and good conversion efficiency. Moreover, the particle size and degree of sulfonation of the resin did not affect the retention of the enzyme. It was also observed that the valency of the complexing cation influenced the retention and stability of the enzyme. *Streptomyces* sp. glucose isomerase adsorbed to Indion 48-R (a macroporous anion exchange resin) showed high retention of activity (60%)) and could convert high concentrations of glucose (25% w/v) to fructose. Immobilization brought about a decrease in the temperature and pH optima in addition to temperature stability (Gaikwad and Deshpande, 1992). Lehmacher and Bisswanger (1990) bound an extremely thermophilic xylose isomerase, from *T. aquaticus* HB-8, to several matrices and noted that the enzyme entrapped in hollow fibres exhibited very high

retention of activity. Use of inorganic and polystyrene based matrices is considered advantageous owing to their resistance to mechanical compression and microbial attack.

Katwa and Rao (1983) immobilized α-amylase, amyloglucosidase and glucose isomerase on cyanogen bromide-activated Sepharose-6MB and studied the continuous conversion of 5% soluble starch to fructose, at 60°C. The final concentration of fructose in the syrup, after 6 h, was approximately 40%. Though the multienzyme complex could be successfully used 7 - 8 times, amyloglucosidase lost 60 - 70% of its activity after 4 - 5 cycles of use. In an effort to develop a one step conversion of cellulose to fructose, Storey and Chakraborti (1990) co-immobilized glucose isomerase with cellulase and ß-glucosidase on polyurethane foam and noted that the co-immobilized system could produce fructose from soluble and insoluble cellulosic substrates. Though the fructose content in the isomerized syrup was comparatively low (30%), in the opinion of the authors, the system appears to have the potential for one step conversion of cellulose to fructose.

Jørgensen *et al.* (1988) immobilized glucose isomerase from a selected strain of *S. murinus* (Sweetzyme T) and the immobilized preparation showed high retention of activity and long half life. Compared to other commercially used immobilized systems namely, Sweetzyme S and Sweetzyme Q, Sweetzyme T exhibited very high productivity. Moreover, the by-product formation during isomerization was minimum. Immobilized glucose isomerase, from *Streptomyces kanamyceticus*, could be successfully

used for the conversion of high concentration of glucose (25% w/v) to fructose, for 16 days, with only 20% loss in its activity (Banerjee *et al.*, 1993).

Wei *et al.* (1994) immobilized glucose isomerase, in films and in porous p-trimethylaminopolystyrene beads, by a new procedure i.e. deposition and immobilization of enzyme multilayers by cationic-anionic attraction as the driving force. The enzyme activity could be maintained in multilayers.

Inspite of glucose to fructose conversion being a single enzyme transformation, whole cell immobilization has been the method of choice for immobilizing many of the commercially used glucose isomerases. Whole cell immobilization of glucose isomerase can be broadly classified into two types namely, (a) where the enzyme remains within the cell and (b) release of the enzyme from the cell prior to its immobilization.

Vieth *et al.* (1973) subjected *S. phaeochromogens* cells, containing glucose isomerase, to heat treatment (to fix the enzyme in the cell) and entrapped them in hide collagen by adjusting the pH of the mixture to 11.2, followed by casting it on an appropriate surface. The resulting membrane, after treatment with either glutaraldehyde or formaldehyde, when used for the continuous isomerization of glucose, exhibited a half-life of 40 days, at 70°C. *S. olivaceous* NRRL 3585 cells subjected to glutaraldehyde crosslinking could be used several times with minor losses in the enzyme activity (Miles Laboratories Inc., 1974). *B. coagulans* cells entrapped in

cellulose acetate showed poor operational stability due to leakage of the cells (Kolarik *et al.*, 1974). However, *A. missouriensis* after entrapped in glutaraldehyde crosslinked cellulose fibres showed good operational stability with a half-life of 40 days (Linko *et al.*, 1977). *Streptomyces* sp. glucose isomerase immobilized in glutaraldehyde crosslinked gelatin retained 40% of its initial activity and exhibited good operational and storage stability. On continuous operation, the immobilized preparation showed a half-life of 260 days in presence of 1 mM Co^{2+} and 80 days in the absence of Co^{2+} (Park *et al.*, 1980). Several *Streptomyces* sp., containing glucose isomerase, have also been immobilized in polyacrylamide, chitosan and casein to assess their potential for the conversion of glucose to fructose (Chen, 1980b).

In order to increase the fructose yield in the isomerized syrup Barker *et al.* (1983) added germanate anions to glucose feed and studied the conversion efficiency using immobilized *Arthrobacter* glucose isomerase. It was observed, that in presence of germanate anions, the immobilized system could convert glucose (40% w/v) to more than 90% fructose. In the opinion of the authors, use of germanate is more advantageous than that of borate since, the latter denatures the enzyme and in high concentrations, decreases the conversion efficiency. An isolate of *L. brevis* cells, after heat treatment, adsorbed onto glass rod or slides could be used for 5 cycles. Interestingly, at elevated temperatures, the cells showed

predominantly glucose isomerase activity and was free from xylose and ribose isomerase activity. The immobilized system was less sensitive to inhibitors like arsenate, L-cysteine, Ca^{2+} and polyols (Shukla and Prabhu, 1985). Hasal *et al.* (1990) developed a method for the preparation of a granulated immobilized glucose isomerase preparation for the isomerization glucose. *Streptomyces* CCM 4102 cells suspended in water was mixed with either polyethyleneimine or glutaraldehyde. The slurry was then homogenized, by maintaining the pH at 7.8, filtered, mixed with MgO and dried. The immobilized preparation showed high retention of activity and good conversion efficiency.

Other methods of cell immobilization include, adsorption on to anion exchangers, and entrapment in photopolymerizable resin (Chen, 1980b) and hydrophilic monomers (Kumakura, 1979). Some of the industrially used immobilized glucose isomerase preparations are given in Table 1.3.

Table 1.3: **Commercially immobilized glucose isomerases***

Enzyme source	Trade name	Immobilization method	Reference
S. olivochromogenes	G-zyme G994	Purified glucose isomerase adsorbed on an anion exchange resin.	Walon and Stouffs (1980)
S. olivochromogenes	Ketomax 100	Purified glucose isomerase bound to cross-linked PEI-treated ceramic alumina.	Rohrbach (1981)
S. rubiginosus	Spezyme	Purified enzyme adsorbed on an anion exchange resin consisting of DEAE-cellulose agglomerated with polystyrene and TiO_2.	Antrim and Auterinen (1986)
S. griseofuscus	AGI-S-600	Cells bound to glutaraldehyde treated chitosan and granulated	Jensen and Rugh (1987)

S. murinus	Sweetzyme T	Enzyme bound to cell material via glutaraldehyde and extruded	Jørgensen *et al.* (1988)
A. missouriensis	Maxazyme	Spherical particles of cells occluded in gelatin followed by glutaraldehyde cross-linking	Hupkes and van-Tilburg (1976)
F. arborescens	Takasweet	Polyamine-flocculated, glutaraldehyde cross-linked cells extruded and spheronized.	Lantero (1986)
B. coagulans	Sweetzyme	Lysed cells cross-linked with glutaraldehyde and granulated	Amotz and Thiesen (1973)

* adapted from Pedersen (1993).

PRESENT INVESTIGATION

Glucose (Xylose) isomerase is a commercially important enzyme, used for the production of high fructose syrup. Most of the glucose isomerase producing organisms, reported so far, are mesophilic and only a few are thermophilic. Since thermophilic organisms grow at high temperature, their use not only helps to avoid microbial contamination but also permit optimum enzyme production in a shorter time. Enzymes from mesophilic organisms exhibit moderate thermostability and allows the isomerization reaction to be carried out at comparatively high temperatures (55 - 65 °C), a condition which apart from yielding 42% fructose, also discourages microbial contamination. Since isomerization reaction is reversible, by raising the temperature to 95 - 100 °C, the yield of fructose in the syrup can be increased to approximately 55% and this has great economic importance. However, high operational temperature coupled with the alkaline pH optima of most of the industrially used glucose isomerases enhance the formation of by-products. On this background, thermophilic strains capable of producing glucose isomerase, having pH optima near neutrality, will have considerable industrial potential. Hence, a thermophilic strain of *Streptomyces thermonitrificans* which exhibited good intracellular glucose isomerase activity, when grown at pH 7.0 and 50 °C was selected for further studies. The present investigation was then

carried out to (a) optimize the medium for maximum enzyme production (b) purify and characterize the enzyme and (c) develop an immobilization procedure to obtain a highly active and stable immobilized preparation suitable for commercial application.

Chapter 2

Medium Optimization for *S. thermonitrificans* Glucose Isomerase Production

SUMMARY

A thermophilic strain of *Streptomyces thermonitrificans* produced high levels of intracellular glucose isomerase (12 U/ml) when grown in a medium containing 1% (w/v) xylose, supplemented with 2% (w/v) sorbitol as the second carbon source. Organic nitrogen supplements like corn steep liquor, casamino acids, peptone, tryptone and yeast extract supported both growth and enzyme production. However, none of the inorganic nitrogen sources tested could support growth, when added in the absence of organic supplements. Though maximum enzyme activity was obtained in a medium containing yeast extract, casamino acids and ammonium nitrate, comparable activities could be obtained in the absence of casamino acids and ammonium nitrate. Influence of metal ions showed that high levels of glucose isomerase activity was obtained in presence of 0.2% Mg^{2+} or Mn^{2+}. Unlike mesophilic *Streptomyces* sp. *S. thermonitrificans* did not require Co^{2+} for growth and enzyme production. Our studies show that the productivity of *S. thermonitrificans* is the highest reported among the thermophilic strains.

INTRODUCTION

Glucose isomerase (EC 5.3.1.5), which catalyses the reversible isomerization of glucose to fructose, is an industrially important enzyme and is used for the production of high fructose syrup. Though many organisms produce glucose isomerase (Chen, 1980a), most of them are mesophilic and only a few are thermophilic (Takasaki, 1974; Hafner, 1985; Kitada *et*

al., 1989). Since thermophilic organisms grow at high temperatures, it not only prevents microbial contamination but also permits optimum enzyme production in a shorter time as compared to mesophilic organisms. In view of this, several thermophilic organisms were screened to evaluate their glucose isomerase producing ability. Among them *S. thermonitrificans*, which exhibited high activity of intracellular glucose isomerase when grown at 50°C, was selected for optimization studies and the results are presented in this Section.

MATERIALS AND METHODS

Yeast extract, malt extract, peptone and casamino acids (DIFCO, USA); sorbitol (Sigma Chemical Co., USA); perchloric acid (Qualigens, India); L-cysteine hydrochloride (Loba-Chemie Indoaustranal Co., India) and carbazole (Fluka AG, Switzerland) were used. All other chemicals used were of analytical grade.

Microorganism and growth

The thermophilic strain of *S. thermonitrificans* (NCIM 2007) was routinely maintained at 50°C on MGYP slants (malt extract 0.3%; yeast extract 0.3%; peptone 0.5%; glucose 1.0% and agar, 2.0%).

Enzyme production

The inoculum was prepared by inoculating 50 ml of Callens's medium (Callens *et al.*, 1985a) [g/l : 20, sorbitol; 10, xylose; 10, casamino acids; 5, yeast extract; 2.7, KH_2PO_4; 5.2,

K_2HPO_4; 2, $MgSO_4.7H_2O$ and 3, NH_4NO_3, adjusted to pH 7.0] with a 4 day old well sporulated slant followed by incubation on a New Brunswick thermostated shaker (200 rpm)at 50°C for 24 h.

Optimization studies were carried out in 250 ml conical flasks, containing 50 ml of the medium, using inoculum medium as the control medium by transferring 10% inoculum followed by incubation at 50°C for 16 h.

Preparation of the crude extract

The cells, from 50 ml grown culture, were harvested by centrifugation (9000 g, 20 min), washed twice with distilled water and suspended in 25 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}. The cells were sonicated using a Ralsonic sonicator at 10,000 Kcyc for 6 min and the cell debris was removed by centrifugation (9000 g, 20 min). The supernatant was used for determining the enzyme activity.

Determination of glucose isomerase activity

The glucose isomerase activity was determined according to the method of Chen *et al.* (1979). The reaction mixture contained, 0.1 ml of 1 M potassium phosphate buffer, pH 7.0, 0.1 ml of 2 M glucose, 0.1 ml of 100 mM $MgSO_4.7H_2O$, 0.1 ml of 20 mM $CoCl_2.6H_2O$ and 0.1 ml of appropriately diluted enzyme, made upto 2 ml with distilled water. The reaction was initiated by the addition of the enzyme followed by incubation at 70°C for 30 min. The reaction was terminated by the addition of 2 ml 500 mM

perchloric acid and the fructose formed was measured spectrophotometrically, at 560 nm, according to Dische and Borenfreund (1951) modified by Marshall and Kooi (1957).

One unit of the enzyme is defined as the amount of enzyme required to liberate 1 µmole of fructose /min under the assay conditions.

Determination of protein

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin as standard. The blue colour developed after the addition of Folin's reagent was read at 550 nm.

RESULTS AND DISCUSSION

Effect of carbon sources

Influence of carbon sources on the production of glucose isomerase is given in Table 2.1. All the carbon sources tested except xylose and sorbitol supported growth but not enzyme production. In presence of sorbitol, only low activity of the enzyme could be detected. However, increased enzyme levels could be obtained when sorbitol was used in combination with xylose. Glucose isomerase is an inducible enzyme and xylose is the most potent inducer of the enzyme (Chen, 1980a). In the present studies, xylose not only supported growth but also the enzyme production. Additionally, the inoculum grown in presence of sorbitol when transferred asceptically to a xylose containing medium could produce the enzyme, suggesting that like most of the glucose isomerases, *S. thermonitrificans* glucose isomerase is

also an inducible enzyme. Influence of xylose concentration on intracellular glucose isomerase revealed that the optimal enzyme activity (0.4 U/mg) is obtained in presence of 1% (w/v) xylose (Table 2.1). Similarly, effect of sorbitol concentration (1 - 3%) on the enzyme levels showed that maximum enzyme activity is obtained when the culture is grown in a medium containing 2% sorbitol and 1% xylose. Since optimal enzyme production was obtained with 2% sorbitol and 1% xylose, further experiments were carried out with 2% sorbitol and 1% xylose.

Pure xylose, however, is expensive and therefore its use is not economical from a commercial point of view. Hence, considerable attempts have been made to replace xylose with cheaper carbon sources such as wheat bran and xylan. Several streptomycetes such as *S. albus, S. bikiniensis, S. flavogriseus, S. flavovirens* and *S. olivaceous* can grow and produce high activities of enzyme on medium containing xylan (Chen, 1980a). *S. thermonitrificans* however, could grow but not produce glucose isomerase in presence of these substrates. The inability of the culture to produce glucose isomerase in presence of wheat bran and xylan can be correlated to the absence of significant xylanase activity (data not shown). Moreover, our efforts to reduce the amount of xylose by supplementing the medium with other carbon sources such as glucose, glycerol and lactose failed to enhance the enzyme production (Table 2.1).

Table 2.1: Effect of different carbon sources on glucose isomerase production by *S. thermonitrificans**

Carbon source	Final pH	Specific activity (U/mg protein)
Sorbitol (2%)	9.0	0.06
Xylose + Sorbitol		
0.5% + 2%	9.0	0.20
1.0% + 2%	8.0	0.40
2.0% + 2%	7.5	0.30
Xylose + Glucose (0.5%) (0.5%)	7.5	0.30
Xylose + Glycerol (0.5%) (0.5%)	7.5	0.29
Xylose + Lactose (0.5%) (0.5%)	9.0	0.23

*Glucose, glycerol, lactose, starch, wheat bran and xylan supported only growth but not enzyme production in 16 h.

Effect of nitrogen sources

Influence of nitrogen sources revealed that none of the inorganic nitrogen sources tested, in the absence of organic supplements, supported growth. On the contrary, all the organic nitrogen sources namely, corn steep liquor, casamino acids, peptone, tryptone and yeast extract supported both growth and enzyme production (Table 2.2).

Table 2.2: **Effect of organic nitrogen sources on glucose isomerase production***

Nitrogen source (1.8% N)	Final pH	Specific activity (U/mg of protein)
Corn steep liquor	6.5	0.20
Casamino acids	6.5	0.20
Bacto peptone	6.5	0.28
Proteose peptone	6.5	0.22
Tryptone	6.5	0.34
Yeast extract + NH_4NO_3	7.5	0.36
Casamino acids + Yeast Extract + NH_4NO_3	8.0	0.40

*In 16 h.

However, the addition of urea neither supported growth nor enzyme production. Though maximum enzyme activity was obtained in a medium containing yeast extract, casamino acids and ammonium nitrate, comparable activity could also be obtained in the absence of casamino acids and ammonium nitrate, suggesting that casamino acids and ammonium nitrate are not obligatory for obtaining high enzyme levels. Replacement of ammonium nitrate with other inorganic nitrogen sources, in combination with yeast extract, showed that the culture could grow and produce glucose isomerase only in the presence of ammonium chloride. Corn steep liquor is reported to be an excellent and cheap nitrogen source

for glucose isomerase producing organisms (Rehm and Reed, 1987). However, its batch to batch and seasonal variability has proved to be an unreliable factor in enzyme production (Verhoff *et al.*, 1985). In the present studies, corn steep liquor with and without pretreatment (to remove the sludge) failed to give optimum enzyme production.

Effect of metal ions

Majority of glucose isomerase producing organisms utilize mineral salts such as $MgSO_4$, $MnSO_4$ and $CoCl_2$ for growth and enzyme production (Antrim *et al.*, 1979). Additionally, strains like *Actinoplanes missouriensis* and *Corynebacterium candidus* showed high levels of enzyme activity in presence of Cu^{2+} and Fe^{2+}, respectively (Rehm and Reed, 1987). *S. thermonitrificans* could grow and produce enzyme in presence of low concentrations (0.005%) of divalent ions like Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}. However, high activities of glucose isomerase were produced only in presence of 0.2% Mg^{2+} or Mn^{2+} (Table 2.3). Since maximum enzyme activity could be obtained in a medium containing 0.2% Mg^{2+}, this concentration was maintained in further experiments.

Glucose isomerases from many strains contain Co^{2+} and it has been suggested that cobalt functions by modifying the enzyme structure to permit the active site to accommodate glucose as well as the "natural substrate" xylose. In general, though Co^{2+} salts have been used in the medium for mesophilic *Streptomyces* sp. the thermophilic species do not seem to require

Table 2.3: Effect of metal ions on glucose isomerase production*

Metal ions (mg/100 ml)	Final pH	Specific activity (U/mg protein)
Control (without metal ions)	8.0	0.2
Mg^{2+}		
5	7.5	0.20
50	7.5	0.29
100	7.5	0.29
200	8.0	0.40
300	8.0	0.30
Mn^{2+}		
5	8.0	0.20
200	8.0	0.30
Co^{2+}		
5	7.0	0.30
Zn^{2+}		
5	8.0	0.24
Fe^{2+}		
5	8.0	0.08
Cu^{2+}		
5	7.0	0.15

Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+} at 0.2% repressed growth as well as enzyme production.

*In 16 h.

it for enzyme production (Bucke, 1977). In the present case, the production of high levels of glucose isomerase in the absence of Co^{2+} can be correlated to the thermophilic nature of *S. thermonitrificans*. This observation is of importance since Co^{2+} is considered to be toxic and disposal of the spent media could present environmental hazards. Glucose isomerase production by different thermophilic organisms, in presence and absence of Co^{2+}, is given in Table 2.4. The data clearly shows that *S. thermonitrificans* exhibits higher productivity in Co^{2+} free medium.

Effect of initial pH

It was observed that *S. thermonitrificans* grew and produced the enzyme only from pH 7.0 - 8.0. However, at both these pH values, the specific activity of the enzyme was comparable and the organism failed to grow below pH 7.0 and above pH 8.0.

Effect of temperature

Influence of temperature on glucose isomerase production revealed that *S. thermonitrificans* could grow and produce the enzyme between 37 - 55°C. However, maximum enzyme activity was obtained when the culture was grown at 50°C (Table 2.5).

Table 2.4: Comparison of glucose isomerase production from thermophilic organisms

Strain[a]	Temperature (°C)	Time (h)	Productivity (U/l. h)	References
B. coagulans	50	16	0.250	Rehm and Reed (1987)
B. stearothermophilus	50	24	0.010	Rehm and Reed (1987)
Thermophilic alkalophile *Bacillus* TX-3	55	16	0.480	Kitada *et al.* (1989)
S. thermoviolaceus[b]	42	72	20.000[@]	Hafner (1985)
S. albus	45	20	0.095	Takasaki (1974)
S. thermonitrificans	50	16	0.750[*] 150.000	Present work

[a]Except *S. thermonitrificans* , all other organisms need Co^{2+} in production medium.

[b] Co^{2+} requirement not known.

[@] Productivity is expressed as U/g (dry wt. of cells).h

[*]Glucose isomerase activity was determined using 1 M glucose as substrate.

Table 2.5: Effect of temperature on glucose isomerase production*

Temperature (°C)	Final pH	Specific activity (U/mg of protein)
37	7.5	0.26
45	8.0	0.28
50	8.0	0.38
55	7.5	0.24

*In 16 h

Profile of growth and enzyme production

The time course of cultivation of *S. thermonitrificans* in the production medium is shown in Fig. 2.1. The gradual increase in the pH of the medium is characteristic of *Streptomyces* sp. (Chen *et al.*, 1979). Glucose isomerase production was maximal after 16 h. Concomitantly, the reducing sugar steadily decreased and at maximum enzyme activity (16 h), 60% of the reducing sugar was utilized. However, after 16 h the specific activity showed a marginal decrease though the reducing sugar was utilized at a rapid rate. The above results suggest that after 16 h the organism utilizes xylose for growth rather than enzyme production.

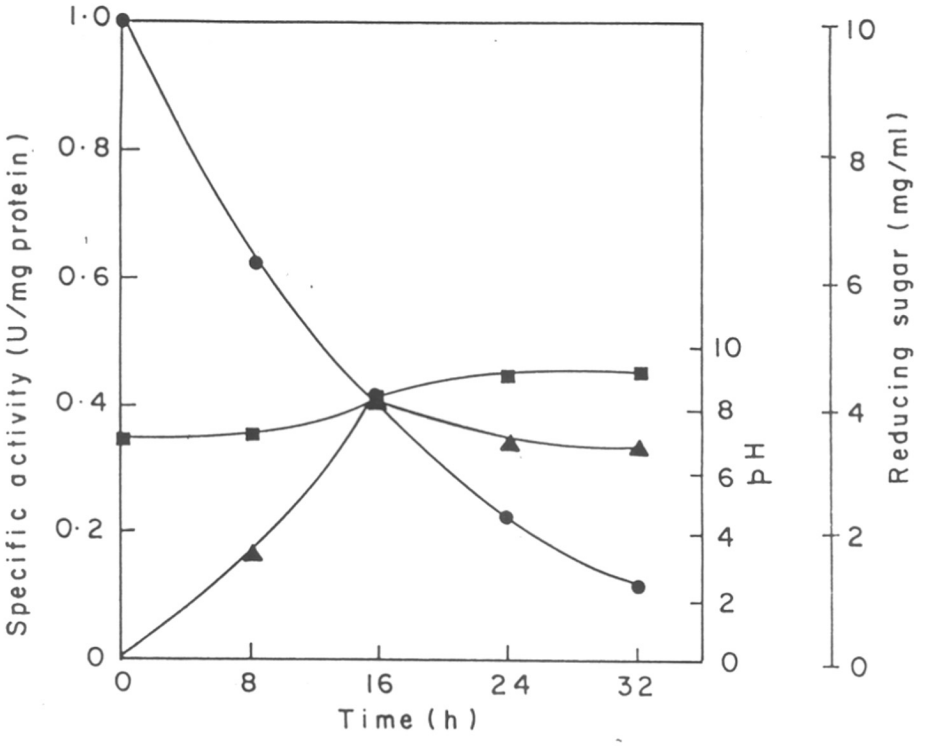

Fig. 2.1: Production profile of glucose isomerase by S. thermonitrificans.

Specific activity (▲), pH (■) and unutilized reducing sugar (●).

CONCLUSION

S. thermonitrificans produces high activity of glucose isomerase in the absence of casamino acids and Co^{2+}. Histidine could enhance the enzyme production in *S. violaceus-ruber* (Callens *et al.*, 1985a) but *S. thermonitrificans* could produce high activity of glucose isomerase in the absence of histidine. The above observations are of importance as they will help in reducing the cost of enzyme production. Moreover, the high enzyme activity obtained in Co^{2+} free medium are beneficial, as disposal of spent media will not cause environmental problems. Our study shows that the productivity of *S. thermonitrificans* is the highest among the thermophilic strains reported so far (Table 2.4).

Chapter 3

Purifcation and Characterization of Glucose Isomerase

SUMMARY

A simple procedure involving sonication, heat treatment, ammonium sulfate precipitation and preparative polyacrylamide gel electrophoresis was developed for the purification of glucose isomerase from *S. thermonitrificans*, to homogeneity, with an overall yield of 15%. The molecular weight of the native enzyme, determined by gel filtration, was 1,66,000 and it consisted of 4 identical subunits of M_r 42,000. It is an acidic protein with a pI of 3.8. The optimum pH and temperature were 7.0 and 85°C respectively. The enzyme is a metalloprotein and showed an obligatory requirement of Mg^{2+} and Co^{2+} for its optimal activity. Substrate specificity studies showed that *S. thermonitrificans* glucose isomerase could isomerize only D-xylose and D-glucose. The K_m for D-xylose, D-glucose and D-fructose were 7 mM, 250 mM and 400 mM, respectively. The enzyme was inhibited competitively by D-xylitol, D-sorbitol, D-mannitol, D-galactose, L-arabinose and tris (hydroxymethyl) aminomethane. Moreover, glucose and xylose isomerase activities were competitively inhibited by D-xylose and D-glucose, respectively. Modification of histidine residues of purified glucose isomerase resulted in the loss of glucose and xylose isomerase activities associated with the enzyme. Kinetic and spectral analysis of diethylpyrocarbonate mediated inactivation revealed the involvement of a single histidine residue in the catalytic activity of the enzyme. Furthermore, histidine modification was accompanied by a concomitant loss of both activities associated with the enzyme,

suggesting the presence of a common catalytic site responsible for the isomerization of D-glucose and D-xylose.

INTRODUCTION

Glucose (xylose) isomerases (EC 5.3.1.5) are commercially important enzymes because of their exclusive application in the production of high fructose syrups (Hemmingsen, 1979) Isomerization is a reversible process and significant operating advantages like, less chances of microbial contamination and increased fructose yield can be achieved by carrying out the process at high temperatures (Pedersen, 1993). However, the relatively high pH optima of many of the industrially used glucose isomerases coupled with their high operational temperatures can result in the formation of undesirable coloured by-products (Rehm and Reed, 1987). Therefore, it is often necessary to carry out the reactions below the pH optima of the enzyme. In view of this, it is necesssary to look for thermostable enzymes having pH optima on the acid side. This can be achieved either by screening various organisms to locate such enzymes or by recombinant DNA technology to generate variants of commercially used enzymes with desirable properties (Sicard *et al.*, 1990; Mrabet *et al.*, 1992) . In the preceeding Chapter,we described the medium optimization for the optimal production of glucose isomerase from a thermophilic *S. thermonitrificans*. As a prerequisite for gene manipulation studies, it is essential to study the structure function relationship of the enzyme. Hence, studies were carried out on the purification and extensive

characterization of *S. thermonitrificans* glucose isomerase, the results of which are presented in this Chapter.

MATERIALS AND METHODS

DEAE-cellulose (Bio-Rad, USA); Coomassie Brilliant Blue G-250 and R-250, diethylpyrocarbonate (DEP), N-acetylimidazole (NAI), N-bromosuccinimide (NBS), bovine serum albumin (BSA), D-xylitol, D-sorbitol and D-mannitol, gel filtration and SDS-molecular weight markers (Sigma Chemical Co., USA); Sephadex G-200 (Pharmacia Fine Chemicals, Sweden); Ampholine carrier ampholites (LKB Produktor, Sweden); 2,3,5-triphenyltetrazolium chloride (BDH, England); acrylamide, L-cysteine hydrochloride (Loba-Chemie Indoaustranal Co., India); N-N´methylene-bis acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate and N´N´N´N´-tetramethylenediamine (TEMED) (Sisco Research Laboratories, India); carbazole (Fluka AG, Switzerland) and Tris (hydroxymethyl) aminomethane (Tris) (Qualigens, India) were used. All other chemicals used were of analytical grade.

Enzyme assays

Glucose isomerase activity: This was carried out at pH 7.0 and 70°C by measuring the amount of fructose produced, at 560 nm, as described in Chapter 2 (page 40).

Xylose isomerase activity: This was performed essentially according to Srinivasan *et al.* (1983). The reaction mixture contained 0.5 ml of 200 mM potassium phosphate buffer, pH 7.0, 0.05 ml of 100 mM $MgSO_4.7H_2O$, 0.05 ml of 20 mM $CoCl_2.6H_2O$, 0.1 ml

of 50 mM xylose and 0.1 ml of appropriately diluted enzyme, made upto 1 ml with distilled water. The reaction was initiated by adding the enzyme, followed by incubation at 70°C for 20 min. The reaction was then terminated by the addition of 0.05 ml of 50% (w/v) trichloroacetic acid and the xylulose formed was measured spectrophotometrically, at 540 nm, according to Dische and Borenfreund (1951) as modified by Marshall and Kooi (1957).

One unit of glucose and xylose isomerase activity is defined as the amount of enzyme required to liberate 1 µmole of fructose and xylulose/min respectively, under the assay conditions.

Protein determination

Protein estimation was carried out according to Bradford (1976) using BSA as standard. The blue colour developed, after the addition of Coomassie Brilliant Blue G-250 reagent, was read at 595 nm.

Localization of xylose/glucose isomerase in PAGE

Xylose isomerase : This was performed by a slightly modified method of Yamanaka (1975). After electrophoresis, a vertical strip was removed from the main gel and incubated at 70°C for 5 min with the reaction mixture (5 ml of 200 mM potassium phosphate buffer, pH 7.0, 1 ml of 2 M xylose, 0.5 ml of 100 mM $MgSO_4.7H_2O$, 0.5 ml of 20 mM $CoCl_2.6H_2O$ and 3 ml of distilled water) used for xylose isomerase assay. The gel was washed several times with distilled water to remove the components of the reaction mixture and then incubated with 0.1% (w/v) solution of 2,3,5-

triphenyltetrazolium chloride in 1 N NaOH, at room temperature in dark, for 1 min. Position of xylose isomerase was visualized by a dark pink band of formazan against colourless background of the gel. The darkening of the background can be prevented by washing the gel with 1 N HCl followed by distilled water.

Glucose isomerase : Visualization of glucose isomerase, in polyacrylamide gels, was carried out according to Yip and Dalton (1979). After gel electrophoresis the vertical strip, removed from the main gel, was incubated with the reaction mixture (5 ml of 200 mM potassium phosphate buffer, pH 7.0, 1 ml of 2 M glucose, 0.5 ml of 100 mM $MgSO_4.7H_2O$, 0.5 ml of 20 mM $CoCl_2.6H_2O$ and 3 ml of distilled water) at 70°C for 30 min. The gel was then washed extensively with distilled water, to remove the components of the reaction mixture and incubated with 20 ml of Seliwanoff's reagent (0.05% resorcinol in 6 N HCl) in a boiling water bath for 5 min. The position of the enzyme was visualized by a pinkish brown band against a light brown background.

PURIFICATION OF GLUCOSE ISOMERASE

Cultivation of the organism was carried out as described in Chapter 2 (page 39). Unless otherwise stated, all the operations were carried out at 4°C.

Preparation of crude extract: The cells from 50 ml culture were harvested by centrifugation (9000 g, 20 min) washed twice with distilled water and suspended in 25 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}.

The cells were sonicated using a Ralsonic sonicator at 10,000 Kcyc for 6 min and the cell debris was removed by centrifugation (9000 g, 20 min). The supernatant was used for the next step.

Heat treatment and ammonium sulfate precipitation: The supernatant was then subjected to heat treatment (65°C, 20 min) with constant swirling and chilled immediately on ice. The heat treated enzyme solution was then brought to 0.7 saturation by the addition of solid ammonium sulfate, under constant stirring and left overnight at 4°C. The precipitated protein was collected by centrifugation (9000 g, 20 min) dissolved in minimum volume of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+} and dialyzed extensively against the same buffer to remove the ammonium sulfate. The precipitate obtained after dialysis, if any, was removed by centrifugation (9000 g, 20 min) and the supernatant was concentrated by lyophilization and used for preparative polyacrylamide gel electrophoresis (PAGE).

Preparative PAGE: This was carried out essentially as described by Davis (1964). The lyophilized sample obtained from the above step was subjected to electrophoresis on a 7.5% (w/v) polyacrylamide gel column (5 x 16 cm) at pH 8.8. Electrophoresis was carried out at 4°C with a constant current of 20 mA. The electrode buffer (5 mM Tris-glycine, pH 8.3) was replaced after 16 h. After the completion of electrophoresis (30 - 32 h) a thin vertical gel strip was removed and subjected to staining to locate the enzyme. After locating the enzyme, the corresponding

portion of the main gel was excised, homogenized in a glass homogenizer and eluted with 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}.

DEAE-cellulose and Sephadex G-200 chromatography: The acrylamide impurities present in the enzyme sample were removed by batchwise DEAE-cellulose chromatography at pH 7.0. The bound enzyme was then eluted with 500 mM potassium chloride in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}. The active fractions were pooled, concentrated by lyophilization and desalted on a Sephadex G-200 coloum (1.5 x 100 cm) equilibrated with potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}. Active fractions were pooled and stored at -20°C until further use. No loss of activity was observed when the purified enzyme was stored under these conditions.

Electrophoresis

Native and SDS-polyacrylamide gel electrophoresis was carried out, at pH 8.3, according to Laemmli (1970). After electrophoresis, the native gels were stained with Coomassie Brilliant Blue G-250 (Blakesley and Boezi, 1977) while the SDS gels were stained with Coomassie Brilliant Blue R-250 and destained using methanol:acetic acid:water (30:10:60).

Isoelectric focussing (IEF) in polyacrylamide gels was performed according to Vesterberg (1972) over the pH range 3.0 - 10.0.

Molecular weight determination

Molecular weight determination was done on a Sephadex G-200 column (1.5 x 100 cm) using ß-amylase (M_r 2,00,000), alcohol dehydrogenase (M_r 1,50,000) , bovine serum albumin (M_r 66,000) and carbonic anhydrase (M_r 29,000) as reference proteins. Subunit molecular weight was determined using 12% (w/v) SDS-polyacrylamide gels (pH 8.3) according to Laemmli (1970) with bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000) and soybean trypsin inhibitor (M_r 20,100) as reference proteins.

Amino acid analysis

Amino acid composition of the purified enzyme was carried out on a Shimadzu amino acid analyser having a fluorescent detector. Prior to analysis, the purified protein was dissolved in 50 mM potassium phosphate buffer, pH 8.0, containing 1% (w/v) SDS and incubated at room temperature for 30 min. It was then bubbled with nitrogen and after the addition of DTT (10 mM effective concentration) was incubated, for 2 h, at room temperature. After the incubation period, iodoacetic acid was added to a final concentration of 30 mM and left overnight at room temperature. The solution was bubbled with nitrogen before and after the addition of iodoacetic acid. The treated protein sample was dialysed extensively against deionized (Milli Q) water to remove SDS and other reagents and lyophilized. The sample (346 µg) was hydrolyzed using 200 µl of 6 N boiling HCl containing 0.05% phenol and 0.025% 2-ß-mercaptoethanol, in vacuo, at 110°C

for 24 h. The hydrolysate was then dried on a speed vac concentrator and reconstituted in 200 µl of sodium citrate buffer, pH 2.2. An aliquot of 10 µl was loaded onto the amino acid analyser. The amino acid analysis was carried out on a SHIM-PACK ISC-07/5-1504 sodium type cation exchange resin and the post column derivatization was by o-phthalaldehyde (OPA). Tryptophan content was determined spectrophotometrically, according to Edelhoch (1967).

Atomic absorption spectrophotometry

The magnesium and cobalt content of the enzyme sample was determined by atomic absorption on a Hitachi-Z-8000 Polarized Zeeman atomic absorption spectrophotometer at 285.1 nm and 240.7 nm, respectively. Prior to analysis, the enzyme samples were extensively dialyzed against metal ion free distilled water (Milli-Q) to remove metal ions and buffer salts. The insoluble material, if any, was then removed by centrifugation (9,000 g, 20 min).

CHEMICAL MODIFICATION STUDIES

During chemical modification studies, the residual activity of the modified enzyme was determined using both substrates viz. glucose and xylose.

Modification of histidine residues

Photo-oxidation: This was carried out by exposing (200 µg) of the purified enzyme in 1 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}, in a glass test tube

(1 x 10 cm) containing different concentrations of methylene blue, to 200 W flood light bulb held at a distance of 12 cm for 30 min at 26 ± 1 °C followed by estimation of the residual activities. Enzyme samples treated under identical conditions, in dark, served as control.

Reaction with DEP: Glucose isomerase (100 µg) in 1 ml of potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}, was incubated at 26 ± 1 °C for 20 min, with various concentrations of DEP, freshly diluted with absolute ethanol. Aliquots were withdrawn at suitable intervals and the reaction was arrested by the addition of 10 µl of 10 mM imidazole buffer, pH 7.5. Subsequently, the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of DEP served as control.

The concentration of DEP in the diluted sample was determined by mixing an aliquot of the sample with 3 ml of 10 mM imidazole buffer, pH 7.5, followed by monitoring the increase in the absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of 3000 $M^{-1}cm^{-1}$ (Melchior and Fahrney, 1970). The concentration of the diluted DEP solution was 50 mM. The ethanol concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. The DEP mediated inactivation was also monitored, spectrophotometrically, by measuring the change in the absorbance

at 240 nm, as described by Ovadi *et al.* (1967).

Reaction with hydroxylamine: Decarbethoxylation was carried out according to Miles (1977). The DEP modified enzyme samples were incubated with 200 mM hydroxylamine, pH 7.0, at room temperature for 2 h and the enzyme activities were determined under standard assay conditions.

Modification of tyrosine residues

Glucose isomerase (100 µg) in 1 ml of 50 mM sodium borate buffer, pH 7.5, was incubated with 2 mM N-acetylimidazole for 20 min at 26 ± 1°C followed by estimation of the residual activities under standard assay conditions. The enzyme incubated in the absence of N-acetylimidazole was taken as control. The number of tyrosine residues modified were calculated by using a molar absorption coefficient of 1160 M^{-1} cm^{-1} at 278 nm (Means and Feeney, 1971).

Substrate protection studies

In all the chemical modification reactions, the effect of substrate protection was studied by incubating the enzyme with excess amounts of glucose and xylose followed by treatment with the modifying reagents.

RESULTS AND DISCUSSION

The results of a typical procedure for the purification of *S. thermonitrificans* glucose isomerase, to homogeneity, are summarized in Table 3.1.

Table 3.1: Purification of *S. thermonitrificans* glucose isomerase

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Crude	279.30	252.00	1.11	1.00	100.00
Heat treatment and ammonium sulfate precipitation	220.50	51.20	4.31	3.88	78.95
Preparative PAGE at pH 8.8	80.80	8.70	9.29	8.37	28.93
DEAE-cellulose and Sephadex G-200 chromatography.	42.60	3.70	11.51	10.37	15.15

The enzyme was purified 10-fold with an overall yield of 15%. In the present studies, preparative PAGE method was preferred because our efforts to purify the enzyme on DEAE-cellulose failed, as the enzyme bound very strongly to the matrix, resulting in very poor recoveries. The enzyme obtained after preparative PAGE, though homogeneous, contained acrylamide impurities. However, chromatography on DEAE-cellulose could remove them as acrylamide does not bind to the matrix. Finally, the residual acrylamide impurities and salt were removed by chromatography on Sephadex G-200. The purified enzyme moved as a single band in native, SDS and IEF gels, indicating its homogeneity (Fig. 3.1a, b).

Fig. 3.1a: Electrophoresis of purified glucose isomerase

a. 7.5% (w/v) polyacrylamide gel, Tris-glycine buffer, pH 8.3 current 5 mA per tube, protein loaded 75 µg.

b. SDS-polyacrylamide gel (7.5% w/v), Tris-glycine buffer, pH 8.3, SDS 0.1%, current 5 mA per tube, protein loaded 75 µg.

c. IEF in 7.5% (w/v) polyacrylamide gel pH range 3.0 - 10.0, protein loaded 75 µg.

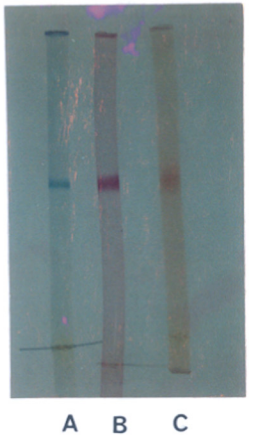

Fig. 3.1b: Electrophoresis of purified glucose isomerase

7.5% (w/v) polyacrylamide gel, Tris-glycine buffer, pH 8.3 current 5 mA per tube, protein loaded 75 µg.

a. Protein staining

b. Activity staining of xylose isomerase

c. Activity staining of glucose isomerase

The M_r of the purified enzyme, determined by gel filtration, was 1,66,000 and it consisted of 4 identical subunits of M_r 42,000 (Fig. 3.2). These values are comparable to those obtained in case of some of the well characterized enzymes from *S. albus* (Hogue-Angeletti, 1975) *S. griseofuscus* (Kasumi *et al.*, 1981b) and *S. violaceus-ruber* (Callens *et al.*, 1985a). The pI of the purified enzyme was 3.8, indicating it to be an acidic protein. The pI of *S. thermonitrificans* enzyme is slightly lower than observed in case of most of the glucose isomerases, pI 4.0 - 5.0 (Pedersen, 1993). Molecular weight determination based on amino acid composition (Table 3.2) gave a value of 42,122 which is in agreement with the value determined by SDS-PAGE. The amino acid analysis showed the preponderance of acidic amino acids like aspartic and glutamic acids and hydrophobic amino acids over basic amino acids. Compared to other glucose isomerases from thermophilic organisms like *Bacillus* TX-3 (Kitada *et al.*, 1989) *B. stearothermophilis* (Suekane *et al.*, 1978) *B. coagulans* (Danno, 1970a) and *S. albus* (Hogue-Angeletti, 1975), glycine and serine content of *S. thermonitrificans* glucose isomerase was higher while that of phenylalanine and lysine was lower. Like glucose isomerases from the aforementioned thermophilic *Bacillus* sp. *S. thermonitrificans* glucose isomerase also showed the absence of cysteine residues. On the contrary, the thermophilic *Streptomyces* sp. namely, *S. albus* showed the presence of a single cysteine residue (Hogue-Angeletti, 1975). Moreover,

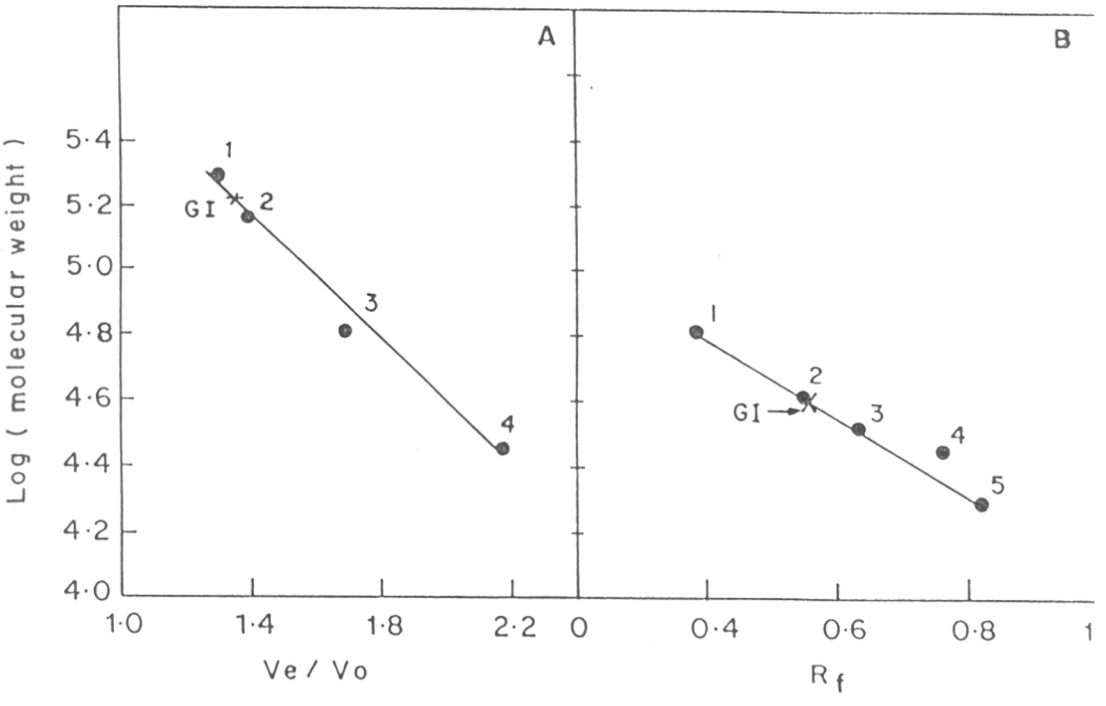

Fig. 3.2: Molecular weight determination of glucose isomerase

a. Gel filtration: Sephadex G-200 column (1.5 x 100 cm was equilibrated with 50 mM potassium phosphate buffe (pH 7.0) containing 5 mM Mg^{2+} and 1 mM Co^{2+}. The colum was calibrated with (i) β-amylase (2,00,000),(ii) alcoho dehydrogenase (1,50,000), (iii) bovine serum albumi (66,000) and (iv) carbonic anhydrase (29,000). V_o is th void volume and V_e the elution volume.

b. SDS-polyacrylamide gel electrophoresis: Relative mobili ties of the reference proteins were plotted against th log molecular weight. The reference proteins used wer (i) bovine serum albumin (66,000), (ii) ovalbumin (45,0((iii) glyceraldehyde-3-phosphate dehydrogenase (36,000) (iv) carbonic anhydrase (29,000) and (v) soybean trypsi inhibitor (20,100).

Table 3.2: **Amino acid composition of *S. thermonitrificans* glucose isomerase**

Amino Acids	No. of residues/monomer
Aspartic acid & Asparagine	61
Threonine	11
Serine	51
Glutamic acid & Glutamine	94
Proline	7
Glycine	48
Alanine	35
Half-cystine	0
Valine	15
Methionine	3
Isoleucine	6
Leucine	19
Tyrosine	3
Phenylalanine	5
Histidine	10
Lysine	4
Arginine	9
Tryptophan*	8

*Determined spectrophotometrically according to Edelhoch (1978).

compared to other thermophilic glucose isomerases (Kitada *et al.*, 1989; Danno, 1970a) *S. thermonitrificans* showed lower content of hydrophobic amino acids.

Optimum pH and pH stability

The pure enzyme had an optimum pH 7.0 and it showed 50% and 77% of its activity at pH 6.0 and 8.0, respectively (Fig. 3.3). *S. thermonitrificans* glucose isomerase also showed comparatively high pH stability and retained significant amount of its activity between pH 5.0 - 9.0 (Fig. 3.4). However, this is inferior as compared to glucose isomerases from *S. griseofuscus* (Kasumi *et al.*, 1981b) and *S. albus* (Takasaki *et al.*, 1969a).

Optimum temperature and temperature stability

The optimum temperature of the purified enzyme was 85°C and showed approximately 70% and 88% of its maximum activity at 80 and 90°C, respectively (Fig. 3.5). The enzyme showed high thermostability and retained its full activity at 70°C for 15 min (Fig. 3.6). The optimum temperature and temperature stability of *S. thermonitrificans* glucose isomerase is comparable to that of the enzyme from *S. griseofuscus* (Kasumi *et al.*, 1981b). However, the thermostability of this enzyme is inferior to those from *Thermus aquaticus* HB-8 (Lehmacher and Bisswanger, 1990) and *Thermotoga maritima* (Brown *et al.*, 1993).

Effect of metal ions

Influence of metal ions on the activity of the enzyme revealed that like most of the *Streptomyces* glucose isomerases

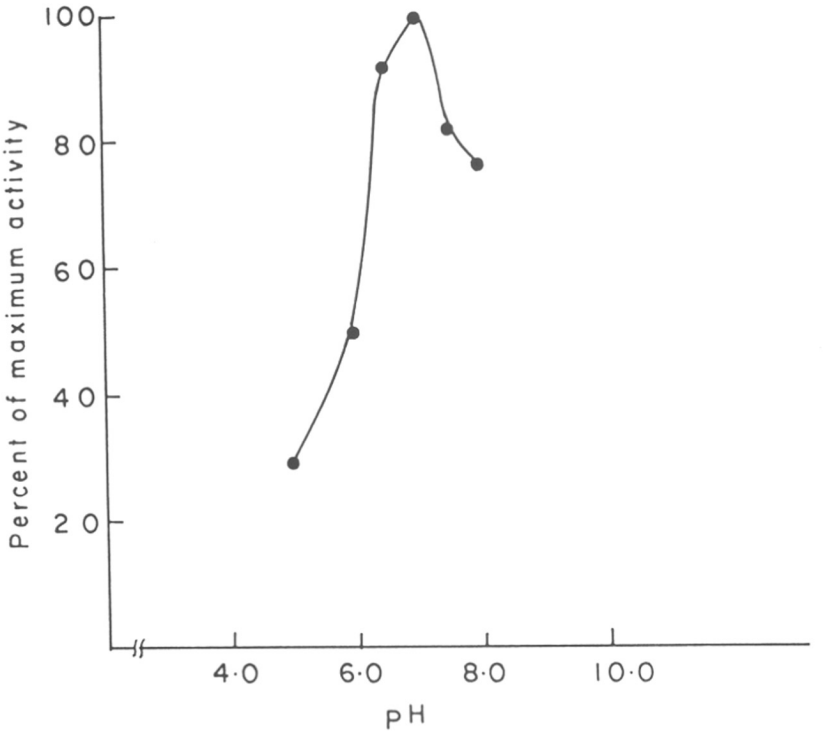

Fig. 3.3: pH activity profile of glucose isomerase.

Purified enzyme (0.2 U) was assayed in a series of pH (5.0 - 8.0) at 70°C as described under Materials and Methods.

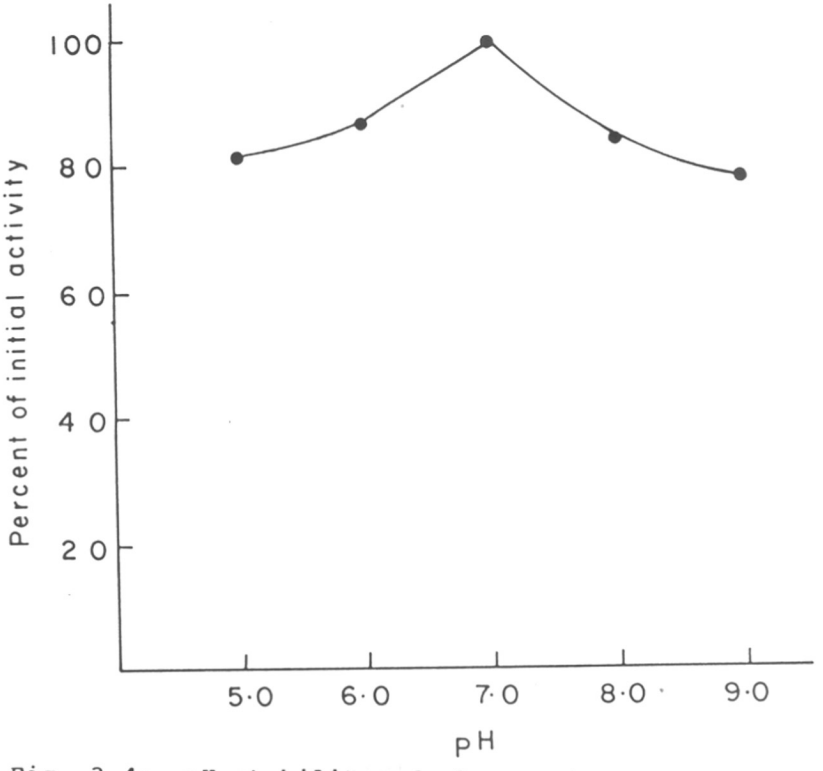

Fig. 3.4: pH stability of glucose isomerase.

Purified enzyme (0.2 U) was preincubated in a series of pH (5.0 - 9.0), at room temperature for 20 min and the residual activity was assayed as described under Materials and Methods.

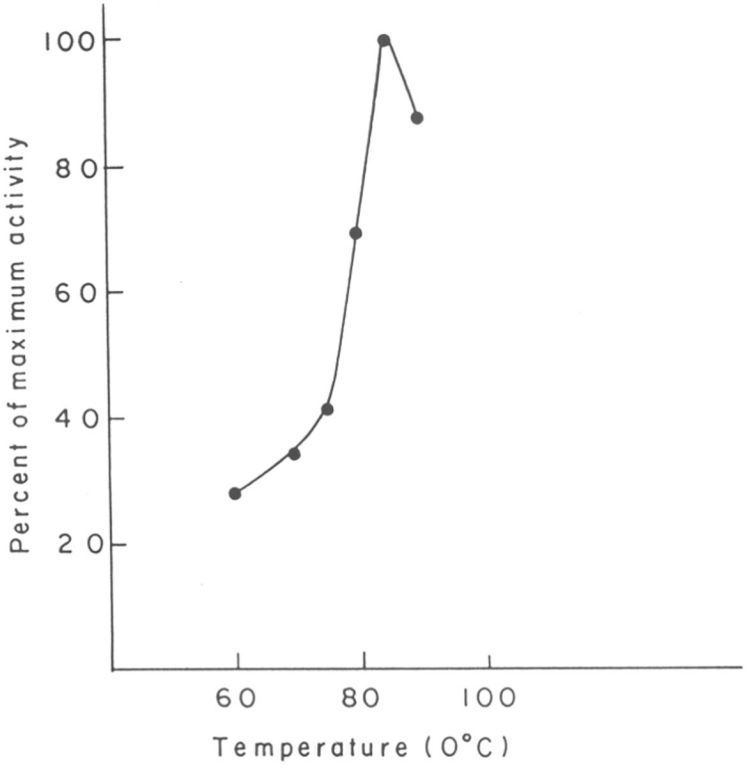

Fig. 3.5: Temperature activity profile of glucose isomerase

Purified enzyme (0.2 U) was incubated in a series of temperatures (60 - 85°C) at pH 7.0 and the activity was determined as described under Materials and Methods.

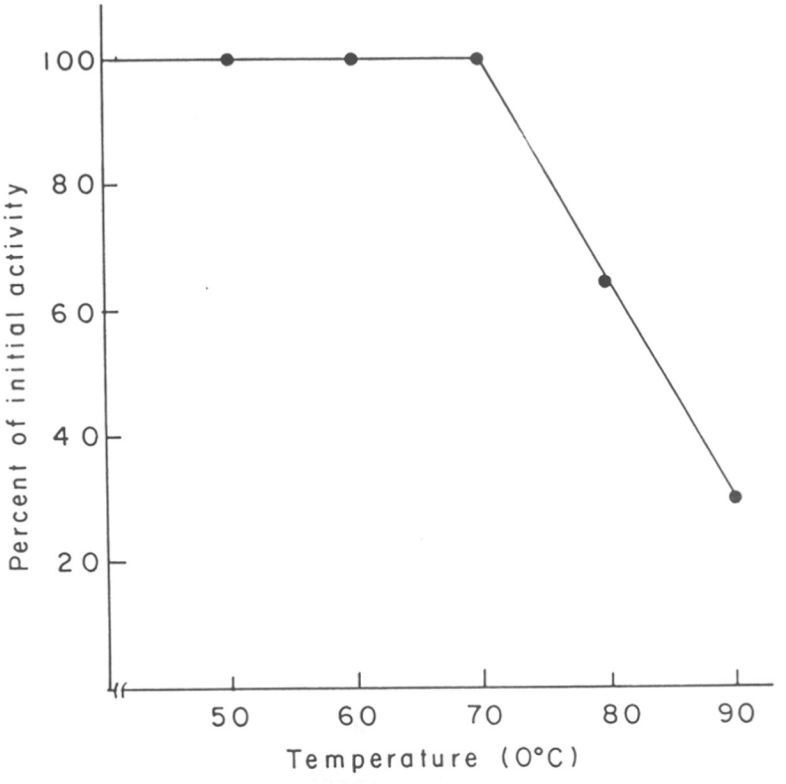

Fig. 3.6: Temperature stability of glucose isomerase

Purified enzyme (0.2 U) was preincubated in a series of temperatures (50 - 90°C), for 15 min at pH 7.0 and the activity was assayed as described under Materials and Methods.

(Chen, 1980b), *S. thermonitrificans* enzyme also showed an obligatory requirement for Mg^{2+} and Co^{2+} for its activity. Incubation of the enzyme with 5 mM EDTA, at room temperature, resulted in almost complete loss of its activity but a significant amount of activity (60 - 70%) could be restored by the addition of 5 mM Mg^{2+}. On the contrary, Co^{2+} at this concentration (5 mM) could yield only 20 - 30% activity. However, optimum activation occured when both Mg^{2+} and Co^{2+} ions were present at a concentration of 5 mM and 1 mM, respectively. Other divalent cations like Fe^{2+}, Zn^{2+}, $Mn^{2+}$and Ca^{2+} were not effective (Table 3.3). Furthermore, atomic absorption studies showed that *S. thermonitrificans* glucose isomerase is a metalloenzyme and contains 2.5 and 0.3 moles of Mg^{2+} and Co^{2+} respectively, per monomer of the enzyme. However, unlike *S. albus* enzyme (Barker, 1975) *S. thermonitrificans* enzyme exhibited high Mg^{2+} and low Co^{2+} content.

Inhibitors

Metal ions like Mn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+} and Ca^{2+} are known to inhibit glucose isomerases (Antrim *et al.*, 1979). In the present studies, Hg^{2+}, Fe^{2+}, Zn^{2+} and Ca^{2+} inhibited *S. thermonitrificans* glucose isomerase, when tested in presence of optimum concentrations of Co^{2+} and Mg^{2+} (Table 3.4).

Glucose isomerases are known to be inhibited by D-xylitol, D-sorbitol, D-mannitol, D-arabitol and Tris (Antrim *et al.*, 1979). Moreover, sugars like D-galactose, D-mannose, and

Table 3.3: Metal ion requirement for the activity of glucose isomerase*

Metal ions (5 mM)	Activity (%)
Control	0
Mg^{2+}	60
Co^{2+}	30
Mn^{2+}	0
Ca^{2+}	0
Zn^{2+}	0
Fe^{2+}	0
Cu^{2+}	0
Hg^{2+}	0
Mg^{2+} (5 mM) + Co^{2+} (1 mM)	100

*Control consisted of enzyme (0.2 U) in 50 mM potassium phosphate buffer, pH 7.0, preincubated with 5 mM EDTA for 10 min.

The EDTA treated enzyme (0.2 U) in potassium phosphate buffer, pH 7.0, was incubated with 5 mM concentrations of each metal ion for 10 min and the activity was measured under standard assay conditions.

L-arabinose competitively inhibited glucose isomerase from *S. griseofuscus* S-41 (Kasumi *et al.*, 1981a). In the present studies, all the polyols viz. xylitol, sorbitol and mannitol inhibited the enzyme competitively (Fig. 3.7 - 3.9) and the Ki values were 3.9 mM, 3.8 mM and 3.9 mM. respectively. Like *S. griseofuscus* enzyme, *S. thermonitrificans* glucose isomerase was competitively inhibited by D-galactose, L-arabinose and Tris, and

Table 3.4: Influence of metal ions on the activity of *S. thermonitrificans* glucose isomerase*

Metal ions	Concentration (mM)	Relative activity (%)
Control	-	100
$HgCl_2$	5	0
$FeSO_4$	5	0
$ZnSO_4$	5	0
$CaCl_2$	5	0
$MnCl_2$	5	0

*The purified enzyme (0.15 - 0.2 U)was assayed in presence of different metal ions, at pH 7.0 and 70°C, as described under Materials and Methods

Enzyme samples containing 5 mM Mg^{2+} and 1 mM Co^{2+} served as control.

Ki values were 4.35 mM, 1.95 mM and 16.0 mM, respectively (Fig. 3.10 - 3.12). Glucose isomerase from *L. brevis* is known to be inhibited by lyxose (Yamanaka, 1969) but it had no effect on *S. thermonitrificans* glucose isomerase.

Substrate specificity

S. thermonitrificans glucose isomerase could isomerize only D-glucose and D-xylose (Table 3.5). Similar observations were made in case of enzymes from several *Streptomyces* sp. viz. *S. griseofuscus* S-41, (Kasumi *et al.*, 1981a), *S. violaceous-ruber*

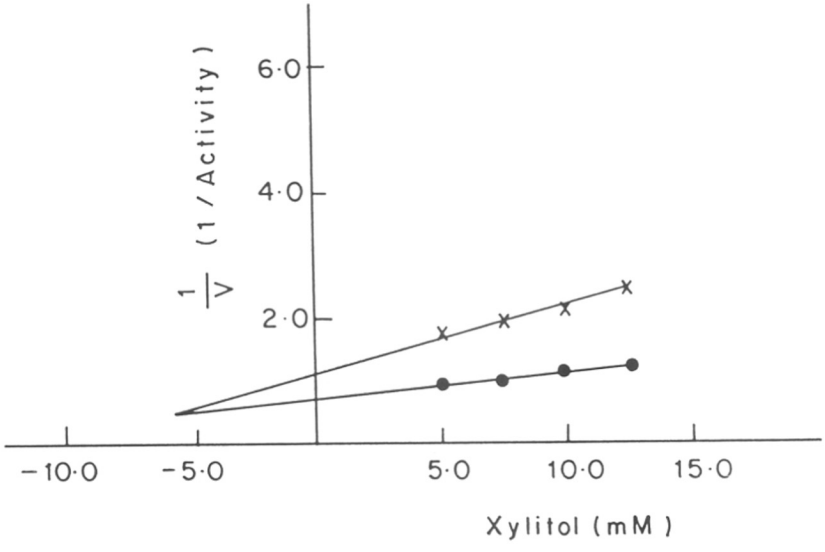

Fig. 3.7: Dixon plots for the inhibition of glucose isomerase by xylitol.

The purified enzyme (0.2 U) was assayed in presence of different concentrations of xylitol (5.0 - 12.5 mM) at fixed concentrations of glucose. Concentrations of glucose were: 100 mM (x) and 200 mM (●).

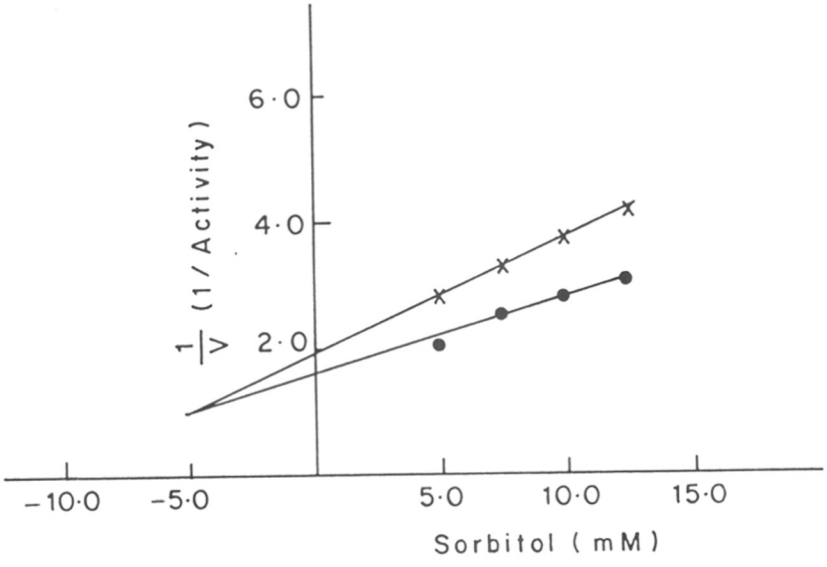

Fig. 3.8: Dixon plots for the inhibition of glucose isomerase by sorbitol.

The purified enzyme (0.2 U) was assayed in presence of different concentrations of sorbitol (5.0 - 12.5 mM) at fixed concentrations of glucose. Concentrations of glucose were: 100 mM (x) and 150 mM (●).

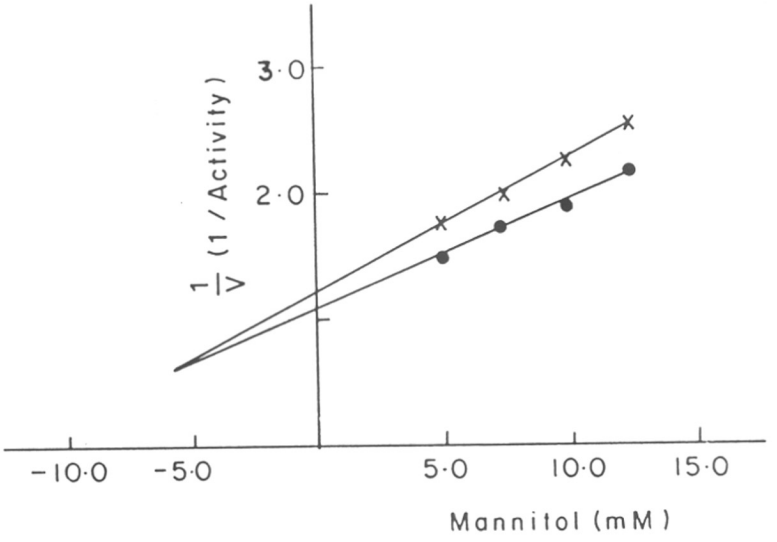

Fig. 3.9: Dixon plots for the inhibition of glucose isomerase by mannitol.

The purified enzyme (0.2 U) was assayed in presence of different concentrations of mannitol (5.0 - 12.5 mM) at fixed concentrations of glucose. Concentrations of glucose were: 100 mM (x) and 150 mM (●).

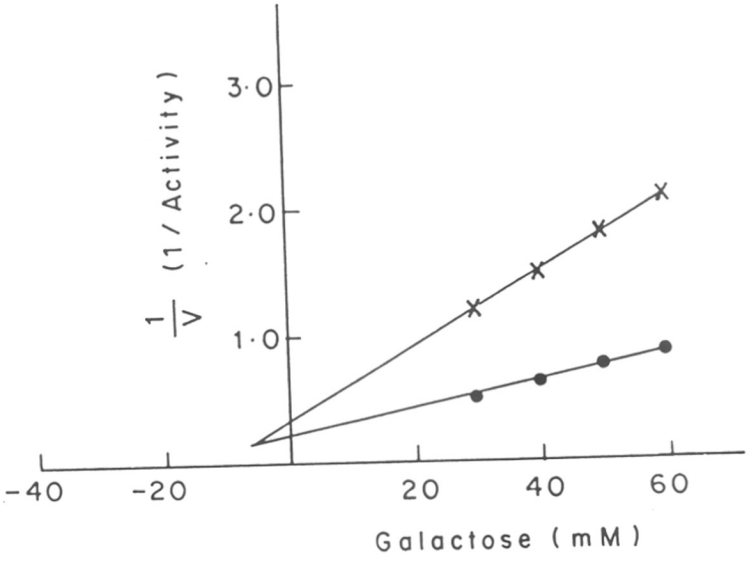

Fig. 3.10: Dixon plots for the inhibition of glucose isomerase by galactose.

The purified enzyme (0.2 U) was assayed in presence of different concentrations of galactose (30 - 60 mM) at fixed concentrations of glucose. Concentrations of glucose were: 100 mM (x) and 200 mM (●).

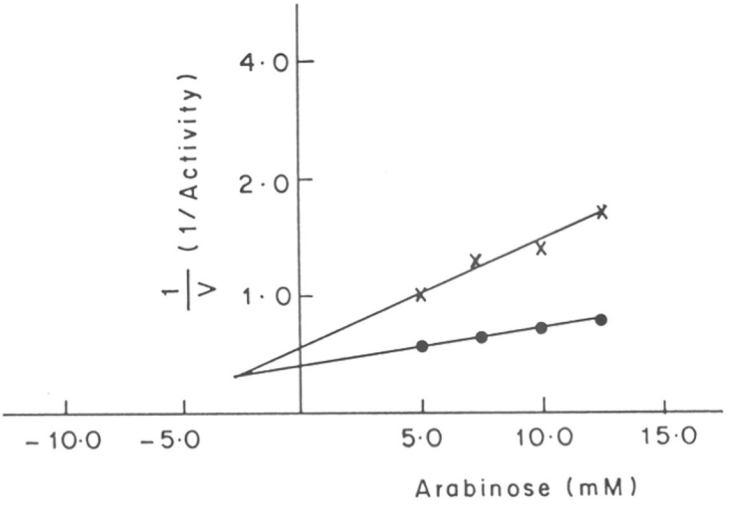

Fig. 3.11: Dixon plots for the inhibition of glucose isomerase by arabinose.

The purified enzyme (0.2 U) was assayed in presence of different concentrations of arabinose (5.0 - 12.5 mM) at fixed concentrations of glucose. Concentrations of glucose were:100 mM (x) and 200 mM (●).

Table 3.5: Substrate specificity of *S. thermonitrificans* glucose isomerase*

Substrate	Activity (%)
D-glucose	100
D-mannose	-
D-galactose	-
D-xylose	27
D-arabinose	-
D-ribose	-

*The purified enzyme (0.2 U) was assayed using 100 mM of different hexoses and 5 mM of pentoses at pH 7.0 and 70°C as described under Materials and Methods.

(Callens *et al.*, 1985b) and from the thermophilic bacterium *T. maritima* (Brown *et al.*, 1993).

Michaelis-Menten constant (K_m) and V_{max} values for D-xylose, D-glucose and D-fructose were 7 mM, 250 mM and 400 mM, and 63.8, 191.7 and 214.5 $\mu mol.min^{-1}.mg^{-1}$ respectively (Fig. 3.13 - 3.15). This suggests that like most of the glucose isomerases, *S. thermonitrificans* enzyme also prefers D-xylose as substrate.

Active site

Histidine has been implicated in the catalytic activity of glucose isomerases from *S. violaceous-ruber* (Vangrysperre *et al.*, 1988; 1989b; 1990), *Streptomyces* sp. NCIM 2730 (Gaikwad *et al.*, 1988), *Chainia* (Pawar *et al.*, 1988) *Lactobacillus xylusus*, and *L.*

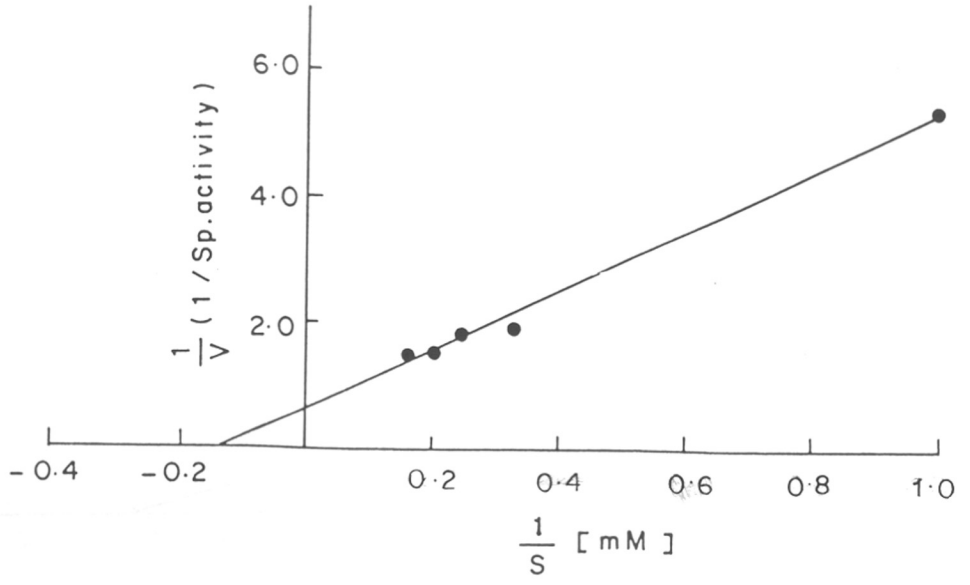

Fig. 3.13: Lineweaver-Burk plot for glucose isomerase with xylose as substrate.

Purified enzyme (0.1 U) was assayed in a series of substrate concentrations (1 - 10 mM) at pH 7.0 and 70°C.

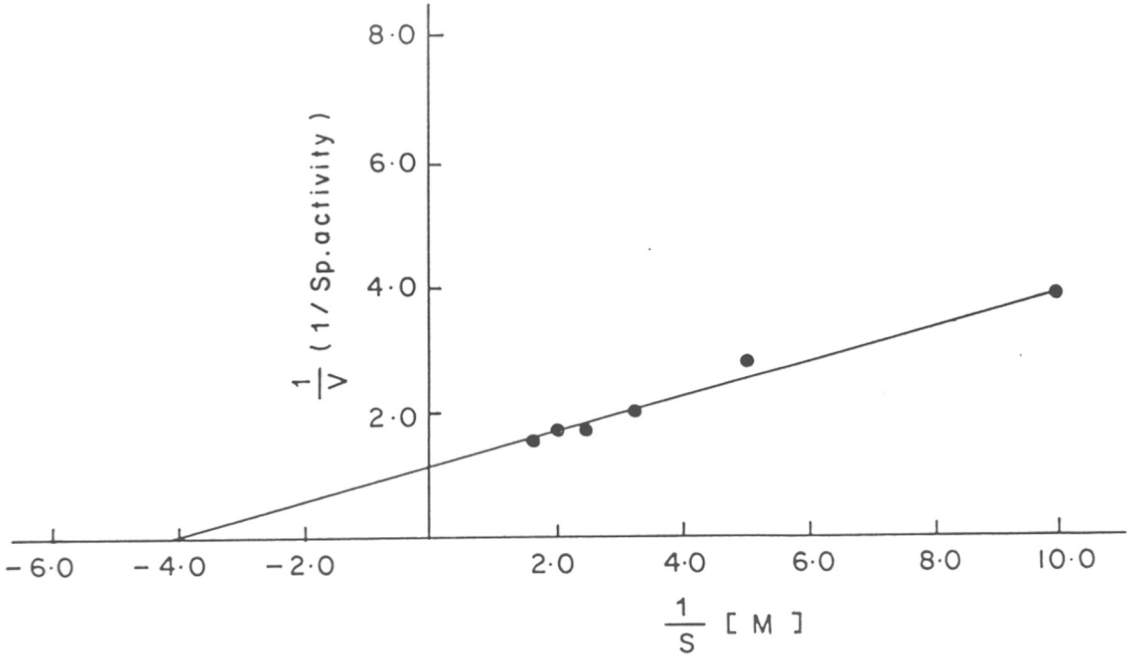

Fig. 3.14: Lineweaver-Burk plot for glucose isomerase with glucose as substrate.

Purified enzyme (0.1 U) was assayed in a series of substrate concentrations (0.1 - 1.0 M) at pH 7.0 and 70°C.

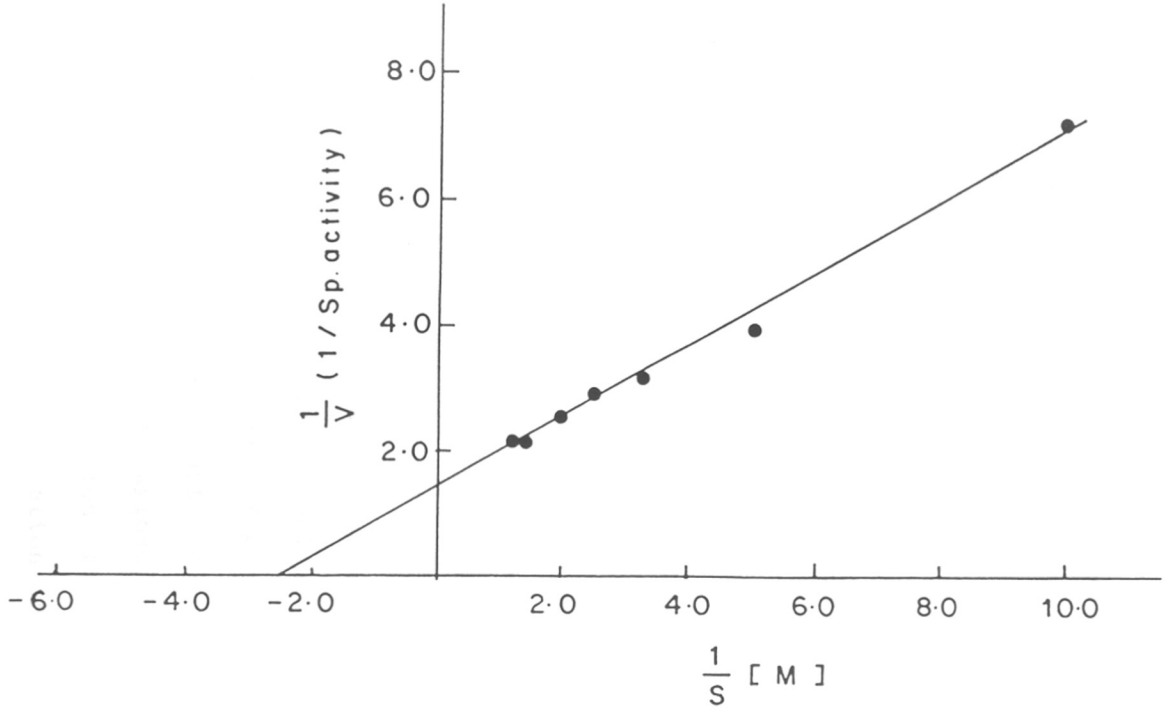

Fig. 3.15: Lineweaver-Burk plot for glucose isomerase with fructose as substrate.

Purified enzyme (0.1 U) was assayed in a series of substrate concentrations (0.1 - 1.0 M) at pH 7.0 and 70°C.

brevis (Vangrysperre *et al.*, 1988), *A. missourensis* (Lambier *et al.*, 1992), and *E. coli* (Vangrysperre *et al.*, 1988; Batt *et al.*, 1990). Hence, modification of histidine was carried out to evaluate its role in the catalytic activity of *S. thermonitrificans* glucose isomerase.

When the purified enzyme was irradiated with 0.2% (w/v) methylene blue, at pH 7.0 and 26 ± 1°C for 30 min, it lost 60-80% of its activity towards D-glucose and D-xylose and the inactivation was dependent on the concentration of the reagent (Fig. 3.16). The inhibition of the activity could be prevented by shielding the enzyme - methylene blue mixture from irradiation, indicating the presence of histidine at or near the active site.

The involvement of histidine was also ascertained by modifying the enzyme with a histidine specific reagent viz. DEP. Carbethoxylation of glucose isomerase, at pH 7.0 for 16 min, resulted in 70 - 80% loss of its initial activity and the inactivation was concentration dependent. No loss of activity was observed in the control samples. The logarithm of percent residual activity plotted as a function of time at various DEP concentrations was linear upto 35% and 25% of the initial activity towards glucose and xylose, respectively (Fig. 3.17). The DEP mediated inactivation followed pseudo first-order kinetics at any fixed concentration of the reagent. The pseudo first-order rate constants were calculated from the slope of plots of log (percent residual activity) versus reaction time and the order was determined from the slope of the plots of log

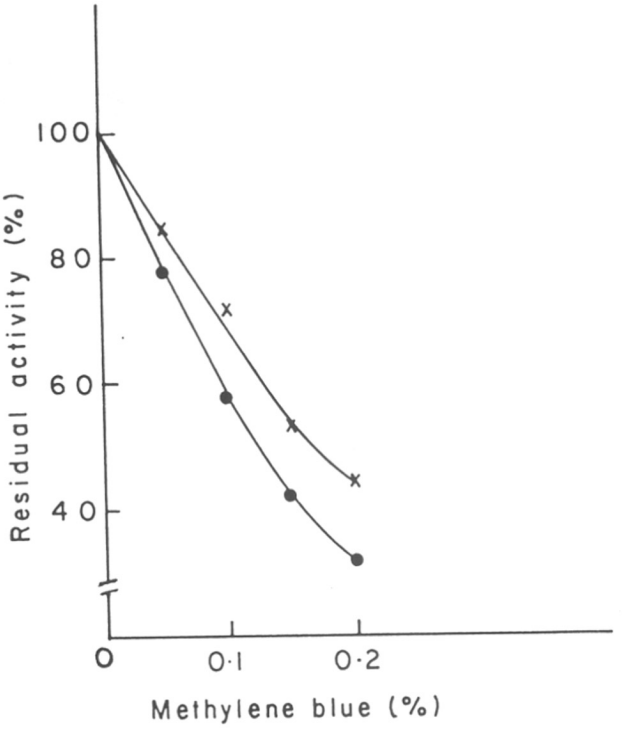

Fig. 3.16: Effect of methylene blue concentration on the activity of glucose isomerase.

Purified enzyme (200 µg) was incubated at pH 7.0 at 26 ± 1°C with various concentrations of methylene blue for 30 min as described under Materials and Methods. Glucose isomerase (●) and xylose isomerase (x).

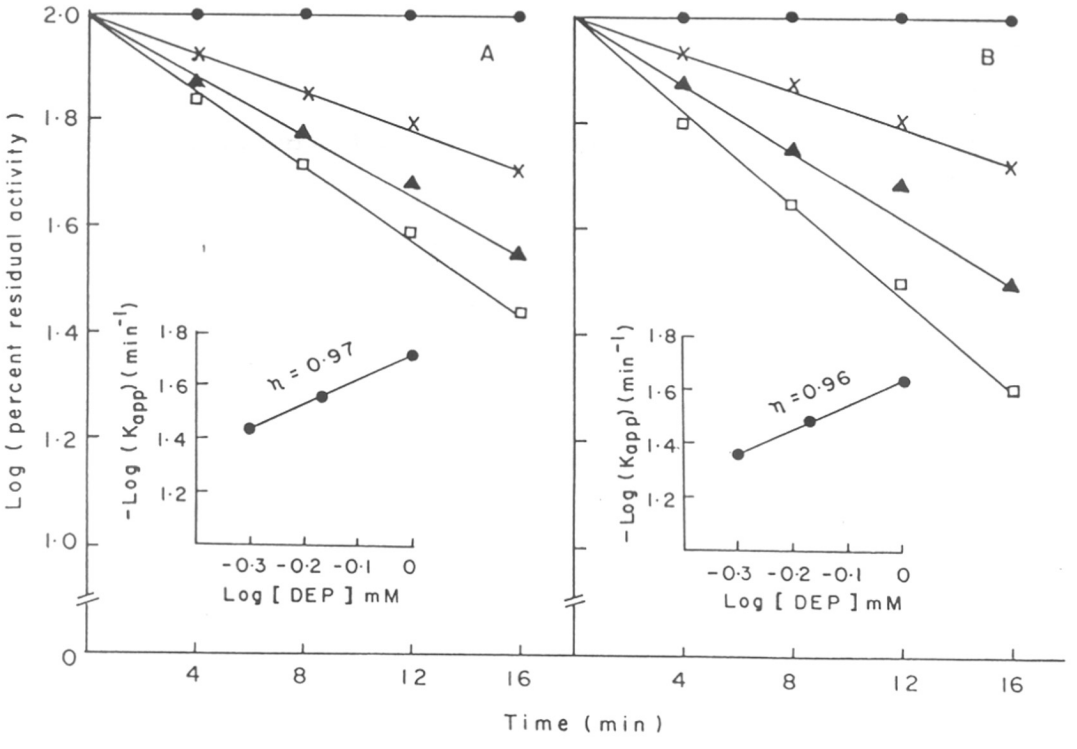

Fig. 3.17: Pseudo first-order plots for the inactivation of glucose isomerase by DEP.

(A) Glucose isomerase (B) Xylose isomerase. Concentrations of DEP were: 0 mM (●), 1.0 mM (x), 1.5 mM (▲) and 2.0 mM (□) . Insets: Second order plots of the pseudo first-order rate constants (K_{app}) (min^{-1}) of inactivation at different concentrations of DEP.

(K_{app}) against log[DEP]. These plots (insets Fig. 3.17) indicated that the loss of enzyme activity towards both substrates occured as a result of modification of a single histidine residue. Moreover, DEP mediated inactivation of the enzyme, was accompanied by an increase in the absorbance of the modified protein at 240 nm, which is characteristic of ethoxycarboxylation of histidine residues. Based on a molar absorption coefficient of carbethoxyhistidine, at 240 nm, to be 3200 M^{-1} cm^{-1} (Ovadi *et al.*, 1967) and the M_r of glucose isomerase to be 1,66,000, the total number of histidine modified was found to be 4.8 residues/molecule of the enzyme. However, modification in presence of substrates viz. glucose and xylose gave a value of 3.70 and 3.50 residues respectively, with significant retention of activity (75 - 80%) suggesting the involvement of a single histidine residue in the catalytic activity of the enzyme. Incubation of the DEP modified enzyme with 200 mM hydroxylamine, at pH 7.0 and 26 ± 1°C for 2 h, restored 80 - 90% of its original activity towards both the substrates, substantiating the role of histidine in the catalytic activity of *S. thermonitrificans* glucose isomerase (Table 3.6).

Though DEP is specific for histidine at or around neutral pH, it also reacts to a lesser extent, with tyrosine, cysteine and lysine residues (Miles, 1977). However, modification of tyrosine residues, of the purified enzyme, with N-acetylimidazole though resulting in the modification of 6 residues out of 12, did not have any significant effect on the enzyme activity,

suggesting that tyrosine may not have a role in the catalytic activity of the enzyme (Table 3.6).

Table 3.6: Effect of different modifying reagents on the activity of glucose isomerase

Modification reaction	No. of residues modified	Residual activity (%)	
		Glucose isomerase	Xylose isomerase
Control	0	100	100
Histidine (DEP)	1	35	25
Decarbethoxylation (Hydroxylamine)	-	85	80
Tyrosine (N-acetylimidazole)	6	85	85

The modification of tyrosine, as a result of DEP treatment, was further ruled out by the observation that there was no significant decrease in the absorbance of the modified protein at 278 nm. Though the above observations support the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine. However, in the present case, the DEP mediated inactivation of the enzyme cannot be attributed to the modification of cysteine residues as amino acid analysis of the purified enzyme revealed the absence of cysteine. Furthermore, the loss of activity of *S. thermonitrificans* glucose isomerase as a result of DEP treatment cannot be correlated to lysine modification, since the DEP modified enzyme could recover

a significant amount of its activity in presence of hydroxylamine. Had the inactivation of the enzyme been due to lysine modification, then hydroxylamine treatment would not have restored its activity.

Studies on substrate protection revealed that methylene blue and DEP mediated inactivation could be prevented to a considerable extent by preincubating the enzyme with excess amount of glucose and xylose (Table 3.7) substantiating the involvement of histidine in the catalytic activity of the enzyme.

Table 3.7 : Influence of histidine modification on the activity of glucose isomerase: Substrate protection studies

Incubation mixture	Residual activity (%)	
	Glucose isomerase	Xylose isomerase
Enzyme	100	100
Enzyme + methylene blue (0.2%)	22	45
Enzyme + glucose (500 mM) + methylene blue	80	-
Enzyme + xylose (7.5 mM) + methylene blue	-	90
Enzyme + DEP (2 mM)	35	25
Enzyme + glucose (500 mM) + DEP	86	-
Enzyme + xylose (7.5 mM) + DEP	-	80

In general, glucose isomerases catalyze the isomerization of glucose and xylose. By competitive inhibition studies, Danno (1970b) showed that the D-glucose, D-xylose and D-ribose isomerizing activities of *Bacillus coagulans* strain HN-68 glucose isomerase is catalyzed by the same active site. Subsequently, Gaikwad *et al.* (1989) demonstrated that the glucose and xylose isomerizing activities associated with *Streptomyces* NCIM 2730 glucose isomerase is also catalyzed by a common catalytic site.

In the present studies, xylose competitively inhibited the glucose isomerizing activity and viceversa (Fig. 3.18, 3.19), suggesting the existence of a common catalytic site for the isomerization of both glucose and xylose. Moreover, the parallel loss of both activities of *S. thermonitrificans* glucose isomerase, on histidine modification, coupled with substrate protection and hydroxylamine treatment data point towards the presence of a common catalytic site responsible for the isomerization of both glucose and xylose.

CONCLUSION

The characteristics of *S. thermonitrificans* glucose isomerase, in general, are similar to those observed in case of several *Streptomyces* sp. i.e. the enzyme is a metalloprotein and shows an obligatory requirement of Mg^{2+} and Co^{2+} for optimal activity. The enzyme also exhibits limited substrate specificity and catalyzes the isomerization of only glucose and xylose. However, unlike other *Streptomyces* sp. glucose isomerases , *S. thermonitrificans* enzyme does not contain cysteine, a property

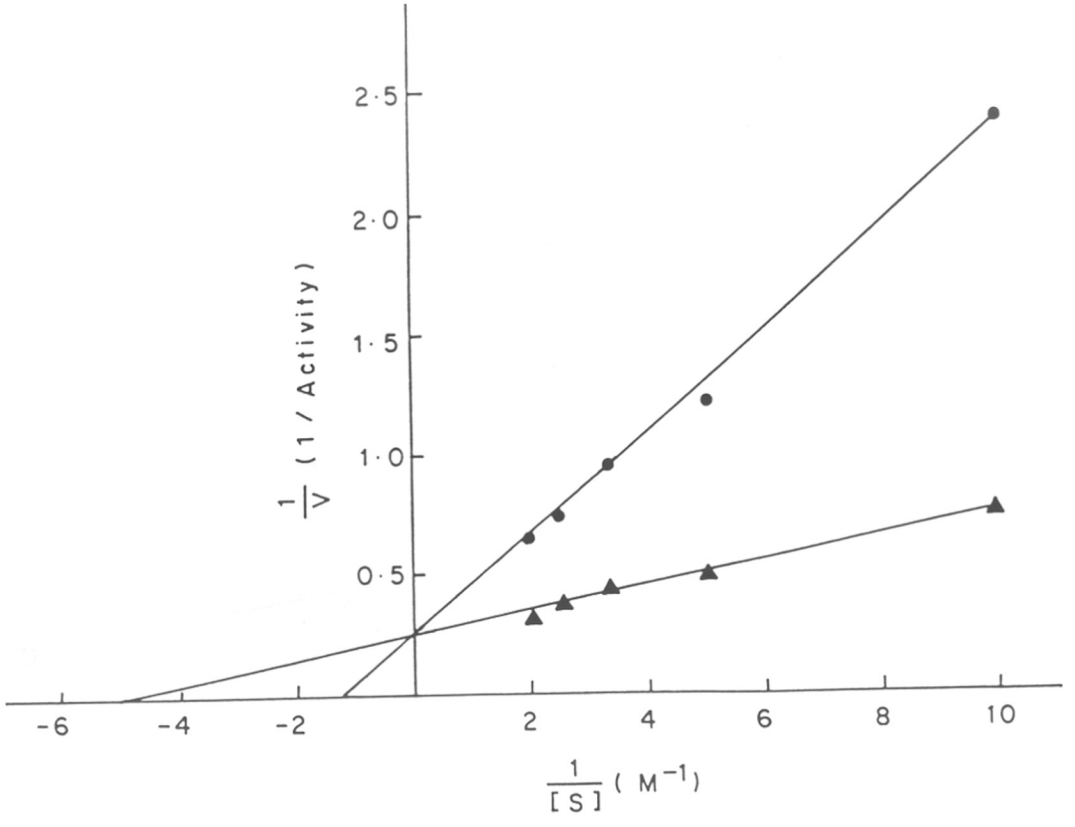

Fig. 3.18: Lineweaver-Burk plots for inhibition of glucose isomerase by xylose.

Purified enzyme (0.4 U) was assayed in a series of substrate concentrations (0.1 - 0.5 M) at pH 7.0 and 70°C.
No inhibitor (▲), 5 mM xylose (●).

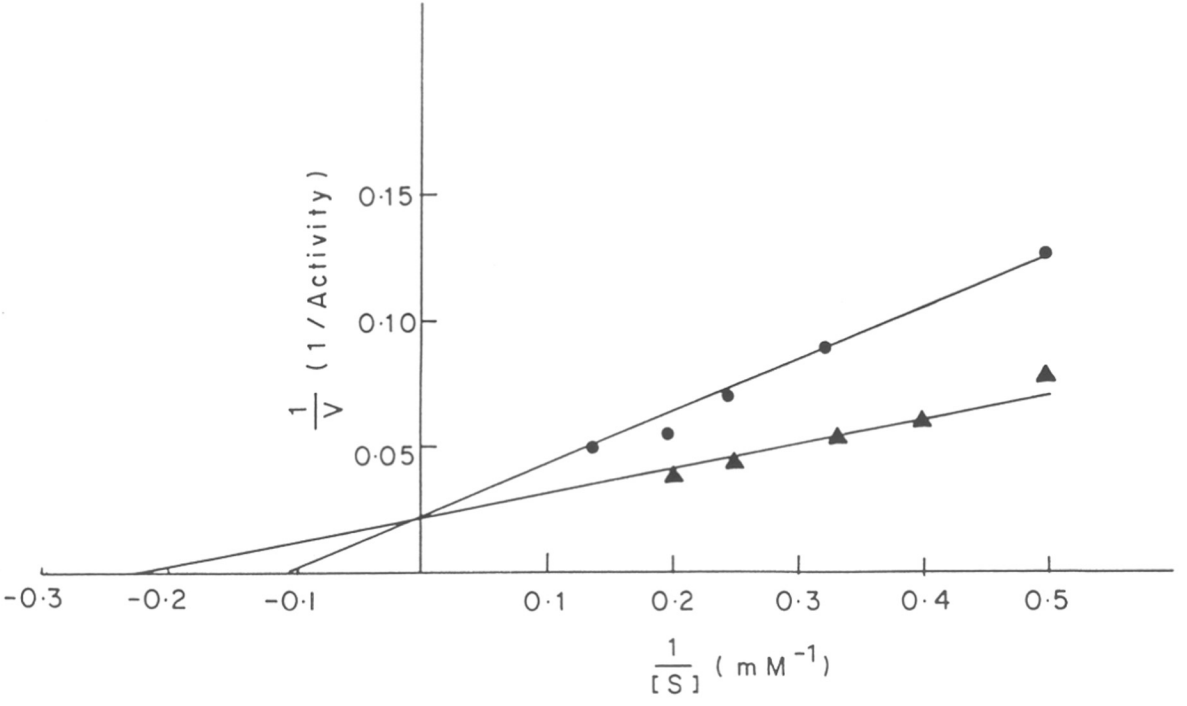

Fig. 3.19: Lineweaver-Burk plots for inhibition of xylose isomerase by glucose.

Purified enzyme (0.2 U) was assayed in a series of substrate concentrations (2.0 - 5.0 mM) at pH 7.0 and 70°C.

No inhibitor (▲), 100 mM glucose (●)

associated with enzymes from *Bacillus* sp. Moreover, the hydrophobic amino acids content of *S. thermonitrificans* glucose isomerase is less compared to that reported in other organisms.

Chapter 4

Immobilization of Glucose Isomerase

SUMMARY

Partially purified glucose isomerase from *Streptomyces thermonitrificans* when coupled to glutaraldehyde activated Indion 48-R, retained 30-40% activity of the soluble enzyme. However, approximately 2-fold increase in the activity could be achieved by binding the enzyme in presence of glucose. Binding the enzyme to matrices presaturated with either glucose or fructose and influence of lysine modification on the activity of the soluble enzyme revealed that the comparatively low activity observed, in case of the enzyme bound in the absence of substrate, is due to the non-specific binding of either substrate or product to the matrix. Immobilization did not affect the pH and temperature optima of the enzyme but it lowered the temperature stability. Immobilization resulted in a marginal increase in the K_m and a 3-fold decrease in the V_{max}. Substrate concentrations as high as 36% glucose could be converted to 18.5% fructose in 5 h, at pH 7.0 and 70°C. The bound enzyme, however, showed inferior stability to repeated use and lost approximately 40% of its initial activity after 5 cycles. Indion 48-R bound glucose isomerase could be stored, in wet state, for 30 days without any apparent loss in its initial activity.

INTRODUCTION

Glucose isomerase (E.C. 5.3.1.5), is a commercially important enzyme used for the industrial production of high fructose syrup from starch. The enzyme itself is considered to be a major cost center and hence considerable attempts have been

made to develop suitable procedures to immobilize the enzyme, as immobilization offers advantages like reusability, greater stability and better process control. Moreover, shifts in the pH optima towards acidic side, as a result of immobilization, can be beneficial as it will prevent the formation of coloured impurities associated with isomerization at high temperature and alkaline pH. Since *S. thermonitrificans* glucose isomerase exhibited good stability at high temperatures it was selected for immobilization studies. If an immobilized enzyme is to be used for the production of food or pharmaceuticals, the matrix and the reagents used for immobilization should be non-toxic. In view of this, Indion 48-R (a cross-linked macroporous anion exchange resin) was selected, as polystyrene resins show good chemical and mechanical stability, are resistant to microbial contamination and can be easily derivatized. Furthermore, binding the enzyme *via* glutaraldehyde was preferred since this method is simple, non-toxic and can be carried out over a wide range of pH. Hence in the present studies, attempts were made to bind glucose isomerase to glutaraldehyde activated Indion 48-R, so as to obtain a highly active and stable immobilized system, suitable for commercial purposes.

MATERIALS AND METHODS

D-glucose (Sarabhai M. Chemicals, India); D-fructose (Loba Chemie Indoaustranal Co., India); glutaraldehyde (50% v/v, Fluka AG, Switzerland); bovine serum albumin and maleic anhydride (Sigma Chemical Co., USA); sodium borohydride (BDH, India) and

formaldehyde (S.D. Fine Chemicals Pvt. Ltd., India) were used. Indion 48-R (a cross-linked macroporous polystyrene anion exchange resin) was from Ion Exchange India Ltd, India. All other chemicals used were of analytical grade.

Maintenance of *S. thermonitrificans* and enzyme production were carried out as described in Chapter 2 (page 39).

Partial purification of the enzyme

This was carried out as described in Chapter 3 (page 56,57). One g (wet weight) of the cells were suspended in 5 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+} and sonicated using a Ralsonic sonicator, at 10,000 Kcyc for 6 min. The cell debris was removed by centrifugation (9000 g, 20 min) and the supernatant was subjected to heat treatment at 65°C for 20 min followed by ammonium sulfate precipitation (0.7 saturation). The precipitated protein was collected by centrifugation (9000 g, 20 min), dissolved in minimum volume of 50 mM potassium phosphate buffer, pH 7.0 (containing 1 mM Co^{2+} and 5 mM Mg^{2+}) and dialyzed extensively against the same buffer to remove the ammonium sulfate. The precipitate obtained after dialysis, if any, was removed by centrifugation and the clear supernatant was used as the source of enzyme. The specific activity of the partially purified enzyme which ranged from 0.9 - 1.0 was used for immobilization studies.

Enzyme assays

Glucose isomerase activity determination was carried out at

pH 7.0 and 70°C, by measuring the fructose formed spectrophotometrically, at 560 nm, as described in Chapter 2 (page 40).

The immobilized enzyme was assayed in a similar manner, by incubating 50 mg (wet weight) of the matrix with 2 ml of the standard reaction mixture, at pH 7.0 and 70°C, in a thermostated shaker water bath (75 - 100 rpm) for 10 min, followed by estimating the fructose formed.

One unit of the enzyme is defined as the amount of enzyme required to liberate 1 µmole of fructose/min under the assay conditions.

Determination of protein

Protein was estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard (Chapter 2, page 41).

Immobilization technique

Indion 48-R was regenerated by alternatively washing with 0.5 M NaOH and 0.5 M HCl. The regenerated resin (1 g wet weight) was activated by incubating with 5 ml of 1% (v/v) glutaraldehyde in 50 mM sodium bicarbonate buffer, pH 9.0, at 10°C for 5 h, under mild agitation. The resin was then washed free of excess glutaraldehyde and used immediately for coupling the enzyme.

In a typical experiment, 15 - 20 U of partially purified glucose isomerase in 5 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+}, 5 mM Mg^{2+} and 100 mM glucose was

incubated with 1 g (wet weight) of 1% (v/v) glutaraldehyde activated resin, at 10°C for 16 h, under mild agitation. The unbound enzyme and protein was removed by decantation and the matrix was washed successively with the coupling buffer, 1 M NaCl in the coupling buffer and finally with the assay buffer (potassium phosphate buffer, pH 7.0) till the washings showed no enzyme activity. The amount of enzyme and protein bound to the matrix was determined by estimating the difference in the enzyme activity and protein before loading on the matrix and after coupling.

Determination of efficiency

Efficiency of the immobilized enzyme was determined by assaying 50 mg (wet weight) of the immobilized preparation, under standard assay conditions, followed by calculating the ratio of measured activity to bound activity. Specific activity of the immobilized preparation is defined as measured activity/mg protein/g matrix.

Chemical modification studies

Reaction with maleic anhydride: The amino groups of purified glucose isomerase were reversibly blocked with maleic anhydride according to Butler *et al.* (1969). Maleic anhydride was dissolved in acetonitrile and the concentration of the reagent was 500 mM. Acetonitrile at this concentration did not have any adverse effect on the activity and stability of the enzyme, during the incubation period. Purified glucose isomerase (100

μg) in 2 ml of 50 mM potassium phosphate buffer, pH 9.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}, was treated at room temperature with a total of 40 μl of maleic anhydride. The reagent was added in 4 instalments (10 μl each) and the pH of the reaction mixture was maintained at 9.0 by the addition of 0.1 M NaOH. After every addition, an aliquot was removed and assayed for the enzyme activity. Enzyme sample incubated in the absence of maleic anhydride served as control.

Reductive methylation: This was carried out according to Means and Feeney (1968). To 1 ml of the purified enzyme (100μg) in 200 mM sodium borate buffer, pH 8.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+}, at 0°C, 0.1 ml of sodium borohydride (0.5 mg/ml) was added, followed by 6 aliquots (5 μl each) of 3.5% (v/v) formaldehyde, at an interval of 10 min. At the end of the reaction, the residual activity of the modified enzyme was determined under standard assay conditions. Enzyme samples incubated in the absence of formaldehyde served as control.

Production of fructose using immobilized glucose isomerase

Isomerization was carried out in shake flasks by incubating 1 g (wet weight) of the immobilized enzyme (5.7 U, measured activity) with 2 ml of 36% (w/v) glucose, in a thermostated shaker water bath (75 - 100 rpm) at 70°C, pH 7.0 and following the conversion by estimating fructose at an interval of 1 h upto 6 h.

RESULTS AND DISCUSSION

Optimization of coupling conditions

In a typical experiment, when 15.6 U of partially purified glucose isomerase was reacted with 1 g (wet weight) of 1% (v/v) glutaraldehyde activated Indion 48-R, at pH 7.0 and 10°C for 16 h, 6.6 U were bound. The effectiveness factor (η) of the immobilized preparation was approximately 0.39, indicating the efficiency of the immobilized system to be 39%. However, when the binding was carried out in presence of glucose (i.e. freezing the enzyme in its native conformation) a significant increase (1.5 - 2.0 fold) in the efficiency occurred. The increase in the efficiency of the enzyme, bound in presence of glucose, may be due to the protection of the active site region of the enzyme during coupling. When an enzyme is bound to an insoluble matrix via glutaraldehyde, primarily ϵ-amino groups of lysine are involved in the binding. The pure enzyme was therefore subjected to maleation and reductive methylation, to evaluate the role of lysine in the catalytic activity of *S. thermonitrificans* enzyme. Modification of lysine residues, however, did not have any adverse effect on the enzyme activity, suggesting that lysine may not have a role in the catalytic activity of the enzyme. This observation also indicates that the increase in the efficiency of the enzyme, bound in the presence of substrate (glucose), can not be correlated to the protection of catalytically active lysine residues during coupling.

In an immobilized system, the expressed activity depends

not only on the rate of diffusion of the substrate but also of the product. In other words, apart from the accessibility of the substrate, the release of the product can also influence the activity of the bound enzyme. Koizumi and Okada (1980) while studying the transformation of sugars using anion exchange resins, noted that glucose binds to the matrix. Since Indion 48-R too, is an anion exchange resin, the non-specific binding of both glucose and fructose was checked by incubating 1 g (wet weight) of Indion 48-R, with 100 mM of either glucose or fructose, at room temperature, for 3 h. The results showed a significant non-specific binding of both the sugars (approximately 10-12 mg/g wet weight resin) to the matrix. This observation suggests that the low activity observed in case of the enzyme bound in the absence of glucose could be due to decreased availability of the substrate (i.e. glucose) or low levels of the product i.e. fructose, as a result of non-specific binding of these sugars onto the matrix. To verify this possibility, the activated matrix was preincubated with 100 mM of either glucose or fructose and after removing the unbound sugars, was used for coupling the enzyme. When the enzyme was bound under the above conditions, a significant (1.5-fold) increase in the efficiency was observed. Comparable results were obtained when the enzyme was bound in presence of fructose. The above results substantiate our view that the low activity observed in case of the enzyme bound in the absence of glucose can be correlated to decreased availability of the substrate, as a

result of non-specific binding of glucose to the matrix. However, the increased activity observed in case of the enzyme bound to the matrix presaturated with either glucose or fructose or when the coupling was carried out in presence of either of these sugars, can be due to a decrease in the non-specific binding of either substrate or product, resulting in higher concentration of fructose in the reaction mixture.

Optimization of coupling pH on the efficiency of the immobilized preparation showed that the most active preparations are obtained when the coupling is carried out at pH 7.0. The decrease in the efficiency of the enzyme bound at higher pH (>7.0) was due to the overcrowding of the enzyme on the matrix, as there was a decrease in the specific activity of the bound enzyme (Fig. 4.1). Influence of glutaraldehyde concentration showed that the enzyme bound to 1% (v/v) glutaraldehyde activated Indion 48-R to be most active (Fig. 4.2). Effect of matrix to enzyme ratio on the activity of the bound enzyme revealed that after an initial increase, the efficiency decreased with increase in the enzyme load and most active preparations are obtained when 15-20 U of partially purified enzyme is reacted with 1 g (wet weight) of 1% (v/v) glutaraldehyde activated Indion 48-R at pH 7.0. The decrease in the efficiency at higher enzyme load (>20 U) can be attributed to the overcrowding of the enzyme on the matrix, as there was a decrease in the specific activity of the bound enzyme (Fig. 4.3). The results of a typical procedure for the immobilization of partially purified glucose isomerase from

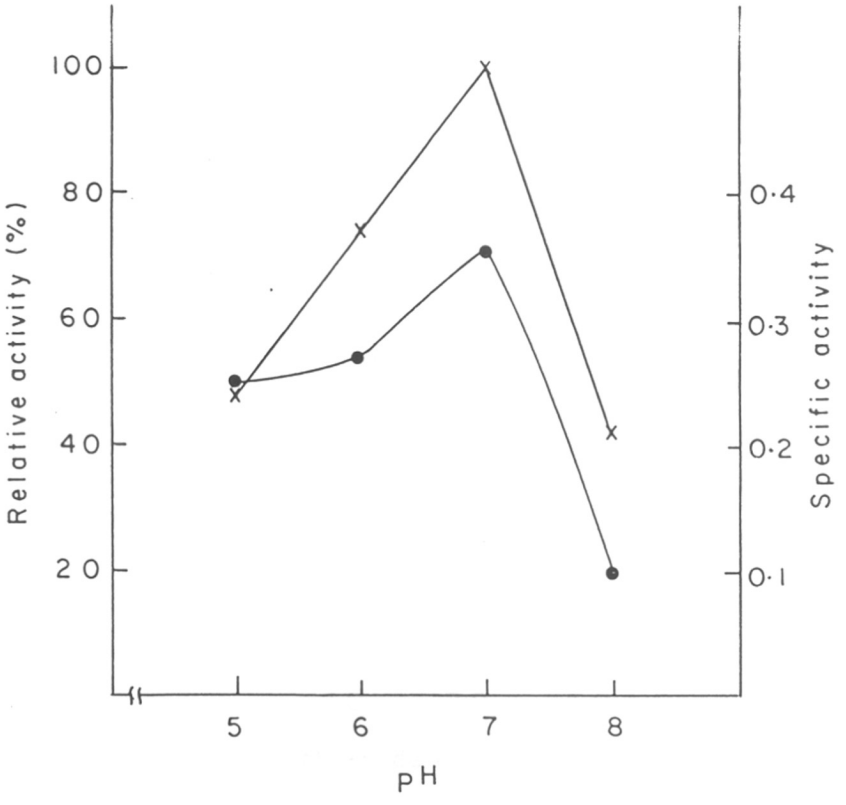

Fig. 4.1: Effect of coupling pH on the activity of Indion 48-R bound glucose isomerase. Relative activity (x) and specific activity (●)

1 gm (wet weight) of 1% (v/v) glutaraldehyde activated matrix was incubated with 12.5 U of partially purified glucose isomerase at different pH (5.0 - 8.0) and 10°C for 16 h and efficiency of the immobilized enzyme was determind as described under Materials and Methods.

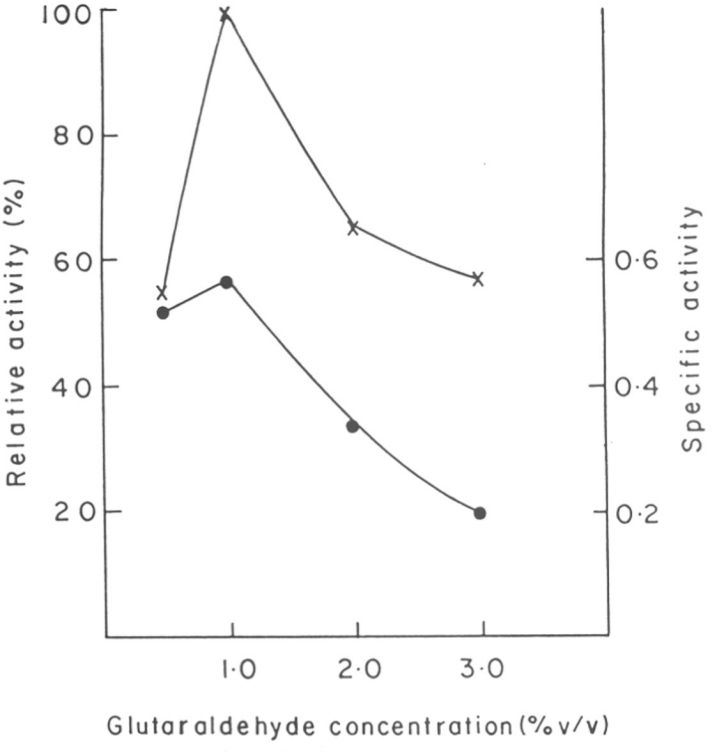

Fig. 4.2: Effect of glutaraldehyde concentration on the activity of Indion 48-R bound glucose isomerase. Relative activity (x) and specific activity (●).

1 g (wet weight) of the matrix was activated with varying concentrations of glutaraldehyde (0.5 - 3% v/v) at pH 9.0 for 5 h. Coupling of glucose isomerase to the activated matrix was carried out by incubating 16 U of the enzyme with the activated matrix at pH 7.0, followed by determining the efficiency of the bound enzyme under standard assay conditions.

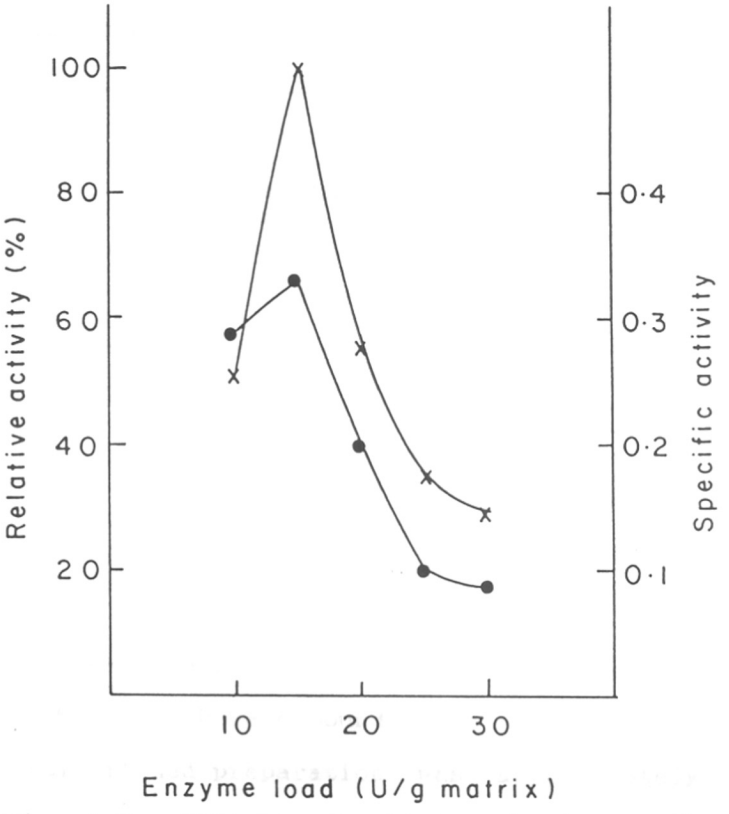

Fig. 4.3: Effect of enzyme load on the activity of Indion 48-R bound glucose isomerase. Relative activity (x) and specific activity (●).

1 g (wet weight) of 1% (v/v) of glutaraldehyde activated matrix was incubated with varying amounts of the enzyme (10 - 30 U) at pH 7.0 and 10°C for 16 h and efficiency of the immobilized system was determined as described under Materials and Methods.

S. thermonitrificans on Indion 48-R, under optimized conditions, are given in Table 4.1.

Table 4.1: Immobilization of glucose isomerase on glutaraldehyde activated Indion 48-R.

Enzyme loaded		Enzyme bound		Activity of the complex (U expressed)	Efficiency* (%)
U	Protein (mg)	U	protein (mg)		
17.4	18.1	8.3	8.9	5.7	68.7

$$*\text{Efficiency} = \frac{\text{Activity of the complex}}{\text{Bound activity}} \times 100$$

When 17.4 U of partially purified glucose isomerase was reacted with 1 g (wet weight) of 1% (v/v) glutaraldehyde activated Indion 48-R, in presence of 100 mM glucose, at pH 7.0 and 10°C for 16 h, 8.3 U were bound. The effectiveness factor (η) of the immobilized preparation was approximately 0.69, indicating the efficiency of the immobilized preparation to be 69%.

Optimum pH, temperature and kinetic parameters

Comparison of the pH activity profiles of soluble and immobilized enzyme showed no change in the optimum pH (Fig. 4.4), suggesting the absence of partitioning effects in the micro-environment of the immobilized enzyme. Similarly, no change was

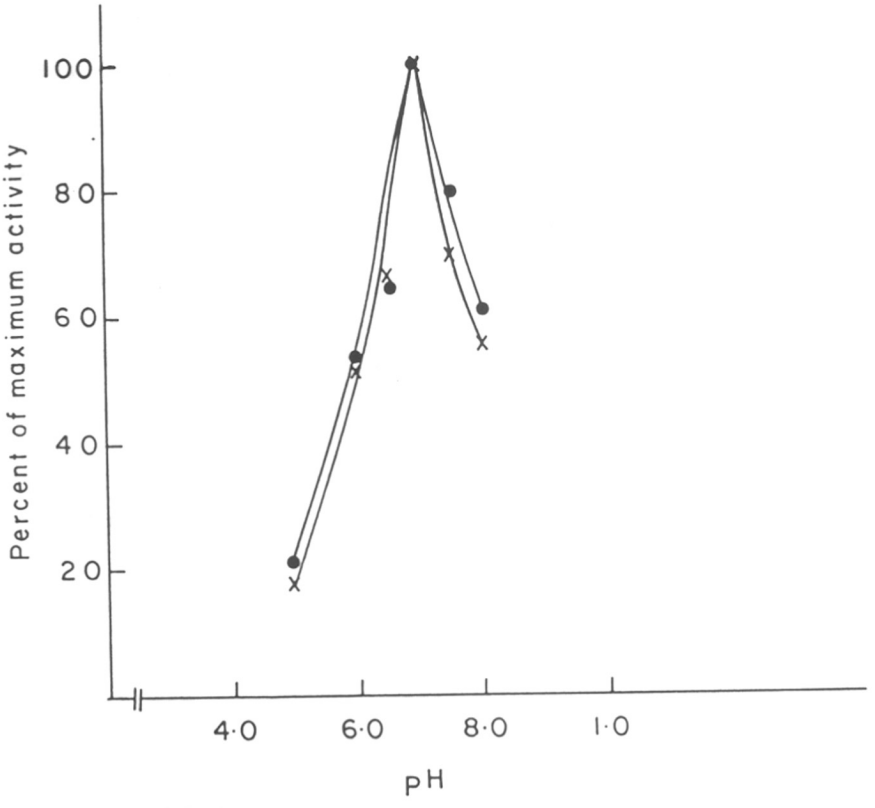

Fig. 4.4: pH activity profiles of soluble (•) and immobilized (x) glucose isomerase.

Both soluble (0.65 U) and immobilized (0.16 U) enzymes were assayed in a series of pH (5.0 - 8.0) at 70°C as described under Materials and Methods.

observed in the optimum temperature of the enzyme as a result of immobilization (Fig. 4.5). Evaluation of kinetic parameters of Indion 48-R bound enzyme showed that it followed Michealis-Menten kinetics and there was a marginal increase in the K_m and approximately 3-fold decrease in the V_{max} (Table 4.2, Fig. 4.6). The marginal increase in the K_m points toward the absence of significant diffusional barriers whereas, the decrease in the V_{max} can be correlated to blocking or masking of some of the active sites during coupling.

Table 4.2: Kinetic data of soluble and immobilized glucose isomerase

State of the enzyme	K_m (mM)	V_{max} (μmol/min/mg)
Soluble	266	200
Immobilized	330	66.6

Stability of the enzyme

In batch operations, Indion 48-R bound enzyme could convert high concentrations of glucose (36 %) to 18.5% fructose, in 5 h at pH 7.0 and 70°C, with a conversion efficiency of 51% (Table 4.3). The bound enzyme, however, showed inferior temperature stability compared to its soluble counterpart, as indicated by the inactivation pattern. While the soluble enzyme completely retained its activity at 80°C, the bound enzyme lost more than

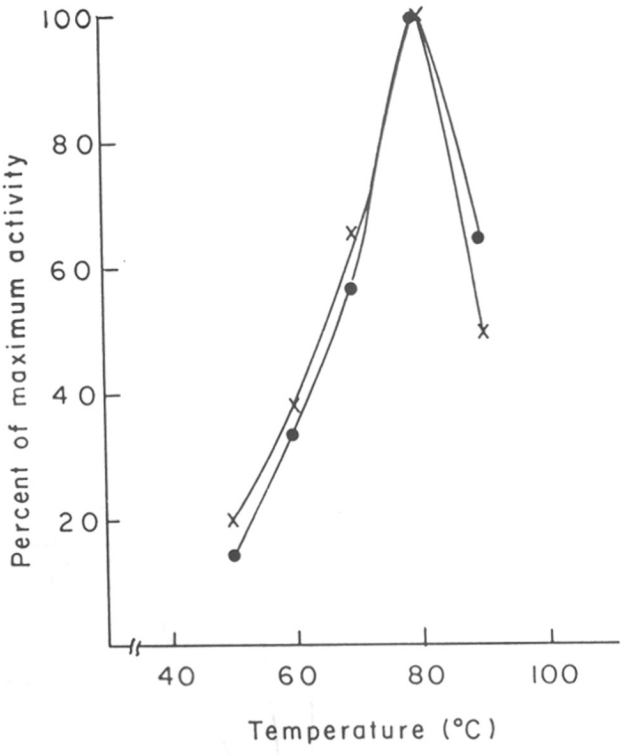

Fig. 4.5: Temperature activity profiles of soluble (●) and immobilized (x) glucose isomerase.

Appropriate amounts of soluble (0.6 - 0.65 U) and immobilized (0.15 - 0.2 U) enzymes were incubated in a series of temperatures (50 - 90°C) at pH 7.0 and the activities were determined as described under Materials and Methods.

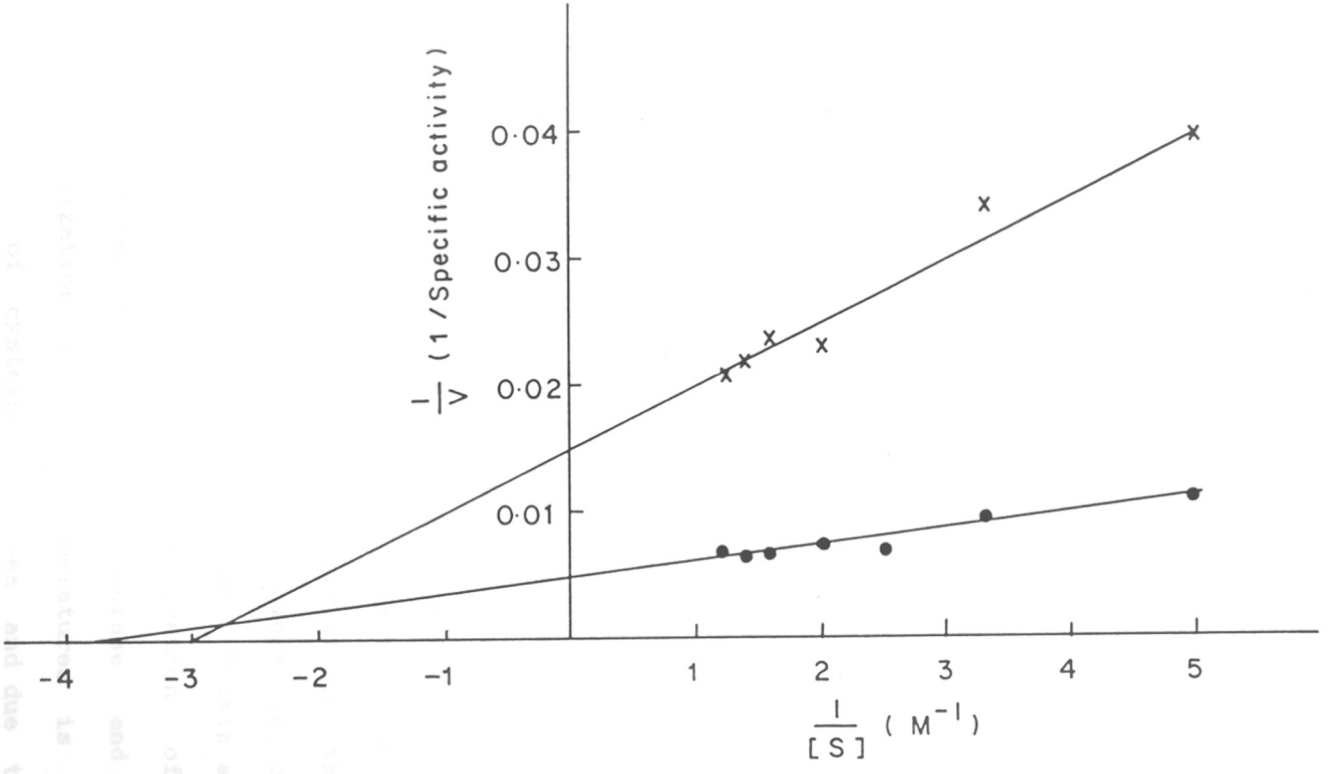

Fig. 4.6: Lineweaver-Burk plots for soluble (•) and immobilized (×) glucose isomerase.

Soluble (0.32 U) and immobilized (0.19 U) enzymes were assayed in a series of substrate concentrations (0.1 - 1.0 M) at pH 7.0 and 70°C.

Table 4.3: Isomerization of high concentration of glucose to fructose*

Time (h)	Conversion (%)
1	12.6
2	20.0
3	26.0
4	38.0
5	51.0

*The experiment was carried out by incubating 1 g (wet weight) of the immobilized enzyme (5.7 U/g) with 2 ml of 36% (w/v) glucose at pH 7.0 and 70°C.

70% of its initial activity (Fig. 4.7). The inferior temperature stability of the immobilized glucose isomerase indicates the lack of rigidity of the enzyme structure in the bound form. The bound enzyme also showed less stability to repeated use and lost approximately 40% of its initial activity after 5 cycles (Fig. 4.8). In the absence of any detectable leaching (as evidenced by the absence of enzyme activity in the supernatant) the decrease in the activity, after every use, can be correlated to slight inactivation of the enzyme. Volkin and Klibanov (1989) investigated the thermoinactivation of immobilized *S. olivochromogenes* glucose isomerase and noted that the destabilization at high temperatures is probably caused by oxidation of cysteine residues and due to the presence of degradation products of glucose and fructose in the isomerized

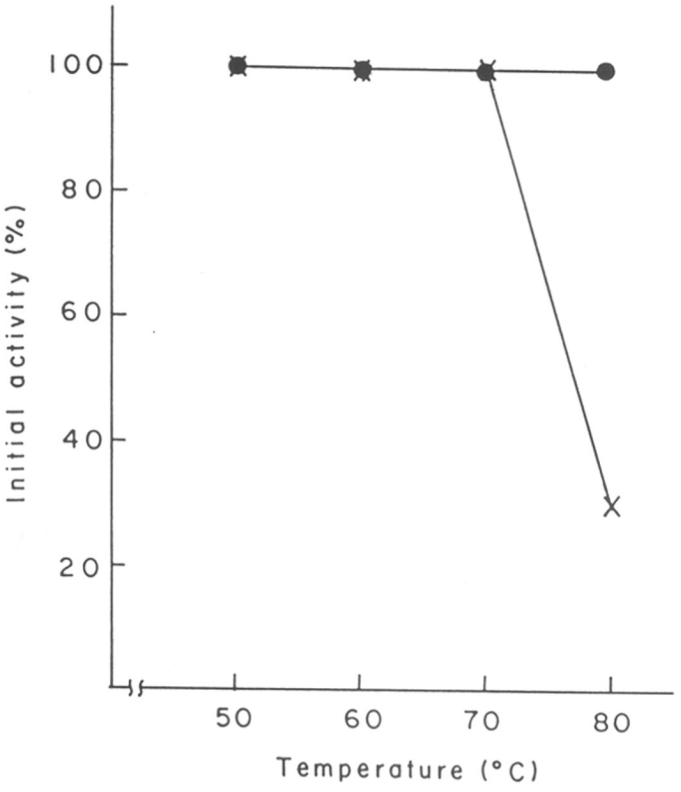

Fig. 4.7: Temperature stability of soluble (●) and immobilized (x) glucose isomerase.

Soluble and immobilized enzymes (0.2 U) were preincubated at different temperatures (50 - 80°C) for 1 h in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+} and their residual activities were determined as described under Materials and Methods.

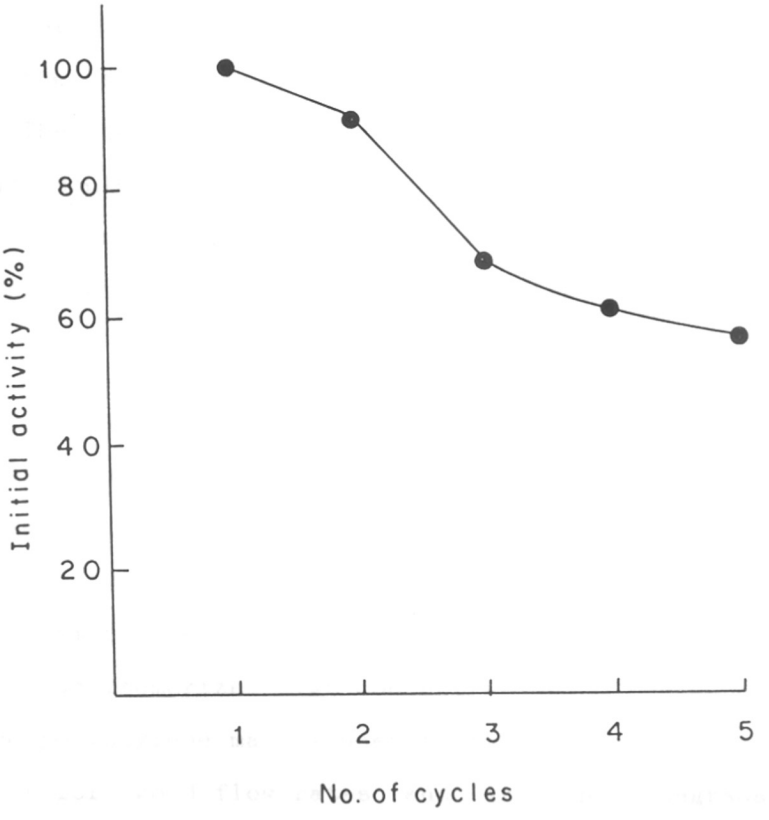

Fig. 4.8: Effect of number of assay cycles on the activity of immobilized glucose isomerase.

750 mg wet weight (4.26 U) of the Indion 48-R bound glucose isomerase was assayed at pH 7.0 and 70°C using 1 M glucose as the substrate. After every cycle (4 h) the bound enzyme was washed free of substrate and products and used for the next assay.

syrup. However, in the present studies, the inferior temperature stability of Indian 48-R bound *S. thermonitrificans* glucose isomerase cannot be attributed to the oxidation of cysteine residues as the enzyme does not contain cysteine (Chapter 3, page 67). The bound enzyme, however, showed good storage stability and could be stored in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM Co^{2+} and 5 mM Mg^{2+} for 30 days, without any apparent loss in its initial activity.

CONCLUSION

With commercial application in mind, we have attempted to develop a simple protocol for the preparation of a highly active and stable immobilized glucose isomerase, suitable for large scale operations. Our observations have commercial significance because, the immobilized system shows very high retention of activity and can isomerize high concentrations of glucose to fructose. The polystyrene matrix used in the present studies is stable, can afford good flow rates and is non-biodegradable. The immobilized system however, showed poor operational stability. Hence, efforts in this direction are required if Indion 48-R bound glucose isomerase were to find successful industrial application.

References

Amotz, S. and Thieren, N. O. (1973) German Patent DP2,317,680.

Ananichev, A. V., Ulezlo, I. V., Rezchikov, A. A., Bezborodov, A. M., Egorov, A. M. and Berezin, I. V. (1978) *Biokhimiya* **7**, 1023-1029.

Anheuser-Busch Inc. (1975) U.K. Patent 1,399,408. (*Chem. abstr.* **81**, 24191m)

Antrim, R. L., Colilla, W. and Schnyder, B. J. (1979) Glucose isomerase production of high-fructose syrups. In: *Applied Biochemistry & Bioengineering* (Wingard, L. B. Jr., Katchalski-Katzir, E. and Goldstein, L. eds.), Vol. 2, Academic Press, New York, pp. 97- 155.

Antrim, R. L. and Auterinen, A. J. (1986) *Stärke* **38**, 132.

Armbruster, F. C., Heady, R. E. and Cory, R. P. (1974) U.S. Patent 3,813,318. (*Chem. abstr.* **79**, 3843u)

Aschengreen, N. H. (1975) *Process Biochem.* **10**, 17-19.

Banerjee, M., Debnath, S. and Majumdar, S. K. (1993) *J. Microb. Biotechnol.* **8**, 44-50.

Barker, S. A. (1975) *Process Biochem.* **12**, 39-40.

Barker, S. A. (1976) *Process Biochem.* **10**, 20-25.

Barker, S. A., Pelmore, H. and Somers, P. J. (1983) *Enzyme Microb. Technol.* **5**, 121-124.

Bartfay, J. (1960) *Nature*. **185**, 924-925.

Basuki, W., Iizuka, M., Ito, K., Furuichi, K. and Minamiura, N. (1992) *Biosci. Biotech.* Biochem. **56**, 180-185.

Batt, C. A., Jamieson, A. C. and Vandeyar, M. A. (1990) *Proc. Natl. Acad. Sci*. **87**, 618-622.

Baxter Laboratories Inc. (1972) British Patent 1,274,158.

Bengston, B. L. and Lamm, W. R. (1973) French Patent 2,172,882.

Bhatt, R. R., Joshi, S. and Kothari, R. M. (1979) *Enzyme Microb. Technol*. **1**, 113-116.

Blakesley, R. W. and Boezi, J. A. (1977) *Anal. Biochem*. 82, 580-582.

Bok, S. H., Seidman, M. and Wopat, P. W. (1984) *Appl. Environ. Microbiol*. **47**, 1213-1215.

Bradford, M. M. (1976) *Anal. Biochem*. **72**, 248-255.

Brown, S. H., Sjoholm, C. and Kelly, R. M. (1993) *Biotech. Bioeng*. **41**, 878-886.

Bucke, C. (1977) Industrial glucose isomerase. In: *Topics in Enzyme and Fermentation Biotechnology* (Wiseman, A. ed.) Vol. 1, Ellis Horwood, Chichester, U.K. pp. 147-171.

Butler, P. J. G., Harris, J. I., Hartley, B. S. and Leberman, R. (1969) *Biochem. J*. **112**, 679-689.

Callens, M., Kersters-Hilderson, H., Van Opstal, O. and De Bruyne, C. K. (1985a) *Biotechnol. Letts.*. 7, 597-600.

Callens, M., Kersters-Hilderson, H., Vandekerckhove, J., Van Opstal, O. and De Bruyne, C. K. (1985b) *Biochem. Int.*11, 467-475.

Callens, M., Kersters-Hilderson, H., Vandekerckhove, J., Van Opstal, O. and De Bruyne, C. K. (1986) *Enzyme Microb. Technol*. 8, 696-700.

Carrell, H. L., Glusker, J. P., Burger, V., Manfre, F., Trisch, D. and Biellmann, J. F. (1989) *Proc. Natl. Acad. Sci*. 86 4440-4444.

Carrell, H. L., Hoier, H. and Glusker, J. P. (1994) *Acta Cryst*. D50, 113-123.

Chen, W. P., Anderson, A. W. and Han, Y. W. (1979) *Appl. Environ. Microbiol*. 37, 324-331.

Chen, W. P. (1980a) *Process Biochem*. 15, 30-35.

Chen, W. P. (1980b) *Process Biochem*. 15, 36-40.

Chou, C. C., Ladisch, M. R. and Tsao, G. T. (1976) *Appl. Environ. Microbiol*. 32, 489-493.

Collyer, C. A., Henrick, K. and Blow, D. M. (1990) *J. Mol. Biol.* 212, 211-235.

Cory, R. P. (1978) U.S. Patent 4,077,842 (British Patent 1,527,972).

CPC International, Inc. (1975) British Patent 1,411,763. (*Chem. abstr.* **79**, 3843u)

CPC International, Inc. (1977) Netherlands Patent 7,703,672. (*Chem. abstr.* **88**, 168515n)

Crueger, A. and Crueger, W. (1984) In: *Biotechnology* (Rehm, H. J. and Reeds, G. eds.) Vol. 6a, pp. 421-457.

Danno, G., Yoshimura, S. and Natake, M. (1967) *Agric. Biol. Chem.* **31**, 284-292.

Danno, G. (1970a) *Agric. Biol. Chem.* **34**, 1795-1804.

Danno, G. (1970b) *Agric. Biol. Chem.* **34**, 1805-1814.

Dauter, Z., Dauter, M., Hemker, J., Witzel, H. and Wilson, K. S. (1989) *Biotechnol. Letts.* **247**, 1-8.

Davis, B. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427.

Dekker, K., Yamagata, H., Sakaguchi, K. and Udaka, S. (1991) *Agric. Biol. Chem.* **55**, 221-227.

Diers, I. (1976) In: *Continuous Culture 6* (Dean, A. C. R., Ellwood, D. C., Evans, C. G. T. and Melling, J. eds.) pp. 208-225.

Dische, Z. and Bornenfreund, E. (1951) *J. Biol.Chem.* **192**, 583-587.

Drocourt, D., Bejar, S. Calmels, T., Reynes, J. P. and Tiraby, G. (1988) *Nucleic Acids Res.* **16**, 9337.

Dworschack, R. G., Chen. S. C., Lamm, W. R. and Davis, L. G. (1972) U.K. Patent 1,284,218. (*Chem. abstr.* **76**, 57748y)

Edelhoch, H. (1967) *Biochemistry* **6**, 1948-1954.

Farber, G. K., GlasFeld, A. Tiraby, G., Ringe, D. and Petsko, G. A. (1989) *Biochemistry* **28**, 7289-7297.

Fujita, Y., Matsumoto, A., Miyachi, I., Imai, J., Kawakami, J., Hishida, T. and Kamata, A. (1980) U.S. Patent 4,205,127.

Gaikwad, S. M., More, M. W., Vartak, H. G. and Deshpande, V. V. (1988) *Biochem. Biophys. Res. Commun.* **155**, 270-277.

Gaikwad, S. M., Pawar, H. S., Vartak, H. G. and Deshpande, V. V. (1989) *Biochem. Biophys. Res. Commun.* **159**, 457-463.

Gaikwad, S. M. and Deshpande, V. V. (1992) *Enzyme Microb. Technol.* **14**, 855-858.

Gong, C. S., Chen, L. F. and Tsao, G. T. (1980) *Biotech. Bioeng.* **22**, 833-845.

Hafner, E. W. (1985) US Patent 4,551,430 (*Chem. Abstr.* **104**, 33063s)

Hasal, P., Cejkova, A. and Vojtisek, V. (1990) (*Chem. Abstr.* **118**, 35139j)

Hasal, P., Cejkova, A. and Vojtisek, V. (1992) *Folia Microbial.* (Prague) **37**, 365-371.

Hemmingsen, S. H. (1979) Development of an immobilized glucose isomerase for industrial application. In: *Applied Biochemistry and Bioengineering* (Wingard, L. B. Jr., Katchalski-Katzir, E. and Goldstein, L., eds.), Academic Press, New York, Vol. 2, pp. 157-183.

Henrick, K., Collyer, C. A. and Blow, D. M. (1989) *J. Mol. Biol.* **208**, 129-157.

Hochster, R. M. and Watson, R. W. (1954) *Arch. Biochem. Biophys.* **48**, 120-129.

Hogue-Angeletti, R. A. (1975) *J. Biol. Chem.* **250**, 7814-7818.

Horitsu, H., Kawai, Y., Konishi, H. and Kawai, K. (1992) *Biosci. Biotech. Biochem.* **56**, 165-166.

Hupkes, J. V. and van Tilburg, R. (1976) *Stärke*, **28**, 356.

Iisuka, S., Ayukawa, T., Suekane, M. and Kanno, M. (1971) US Patent 3,622,463.

Jackson, D. M. and Tsuda, Y. (1980) US Patent 4,237,231.

Japan Bureau of Industrial Technics (1966) Japanese Patent

667,431.

Jørgensen, O. B., Kaisel, L. G., Nielsen, N. B., Pederson, S. and Rugh, S. (1988) *Stärke* **40**, 307-313.

Jensen, V. J. and Rugh, S. (1987) *Methods Enzymol.* **136**, 356-370.

Kasumi, T., Hayashi, K. and Tsumura, N. (1981a) *Agric. Biol. Chem.* **45**, 619-627.

Kasumi, T., Hayashi, K. and Tsumura, N. (1981b) *Agric. Biol. Chem.* **45**, 1097-1103.

Katwa, L. C. and Rao, M. R. R. (1983) *Biotechnol. Letts.* **5**, 191-196.

Katz, E., Ehrenthal, I. and Scallet, B. L. (1972) US Patent 3,690,948.

Kent, C. A. and Emery, A. N. (1973) *J. Appl. Chem. Biotechnol.* **23**, 689-703.

Khire, J. M., Lachke, A. H., Srinivasan, M. C. and Vartak, H. G. (1990) *Appl. Biochem. Biotechnol.* **23**, 41-52.

Kitada, M., Dobashi, Y. and Horikoshi, K. (1989) *Agric. Biol. Chem.* **53**, 1461-1468.

Koizumi, K. and Okada, Y. (1980). (*Chem. Abstr.* **93**, 8398b)

Kolarik, M. J., Chen, B. J., Emery, Jr. and Lim, H. C. (1974) Glucose isomerase cells entrapped in cellulose acetates. In

Immobilized enzymes in food and microbial processes (Olson, A.C. and Cooney, C.L. eds.), Plenum Press, pp. 71-83.

Kralova, B., Demnerova, K. and Skrivan, P. (1985) *Biotech. Letts.* **7**, 593-596.

Kumakura, M., Yoshida, M. and Kaetsu, I. (1979) *Appl. Environ. Microbial* **37**, 310-315.

Kwon, H. J., Kitada, M. and Horikoshi, K. (1987) *Agric. Biol. Chem.* **51**, 1983-1989.

Lantero, O. J., Jr. (1986) US Patent 4,760,024.

Laemmli, U. K. (1970) *Nature* **227**, 670-685.

Lai, C. L. (1975) Report of Taiwan Sugar Res. Inst. **70**, 27.

Lambeir, A. M., Lauwereys, M., Stanssens, P., Mrabet, N. T., Snauwaert, J., Tilbeurgh, H., Matthyssens, G., Lasters, I., Maeyer, M., Wodak, J. J., Jenkins, J., Chidami, M. and Jannin, J. (1992) *Biochemistry* **31**, 5459-5466.

Lee, C. K., Hayes, L. C. and Long, M. E. (1972) US Patent 3,645,848.

Lee, C. K. (1977) US Patent 4,061,539 (British Patent 1,527,972). (*Chem. abstr.* **88**, 61137p)

Lee, C. K. (1981) US Patent 4,283,496.

Lee, C., Saha, B. C. and Zeikus, J. G. (1990) *Appl. Environ.*

Microbiol. **56**, 2895-2901.

Lehmacher, A. and Bisswanger, H. (1990) *J. Gen. Microb.* **136**, 679-686.

Linko, Y. Y., Pohjola, L. and Linko, P. (1977) *Process Biochem.* **77**, 14-32.

Lloyd, N. E. (1974) US Patent 3,821,086.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.

Lu, W. X., Zhang, S. Z., Wang, Z. Q., Jin, Q. S. and Wang, X. D. (1992) Beijing, China Conference, Oct. 12-16.

Luiten, R. G. M., Quax, W. J., Schuurhuizen, P. W. and Mrabet, N. T. (1989) European Patent Application EP 9351029.

Marshall, R. O. and Kooi, E. R. (1957) *Science* **125**, 648-649.

Means, G. E. and Feeney, R. E. (1968) *Biochemistry*, **7**, 2192-2201.

Means, G. E. and Feeney, R. E. (1971) In: *Chemical Modification of Proteins* (Means, G. E. and Feeney, R. E. eds.) Holden-Day Inc., SanFrancisco, pp. 214-230.

Meers, J. L, (1977) UK Patent 1,492,258. (*Chem. abstr.* **84**, 162913m)

Melchoior, W. B. Jr. and Fahrney, B. (1970) *Biochemistry* **9**, 251-258.

Messing, R. A. (1974) US Patent No. 3,850,751. (*Chem. abstr.* 81, 165577d)

Messing, R. A. (1975) US Patent No. 3,868,304. (*Chem. abstr.* 82, 41873y)

Miles, E. W. (1977) *Methods Enzymol.*, **47**, 431-442.

Miles Laboratories (1974) UK Patent 1,376,787.(*Chem. abstr.* 78, 14508u)

Mikhailov, A. M., Mosichev, M. S., Rezchikov, A. A. Egorov, A. M. Oreschenko, L. T. and Kuznetsov, V. D. (1986) *Appl. Biochem. Microbiol.* **22**, 150-154.

Mitsuhashi, S. and Lampen, J. O. (1953) *J. Biol. Chem.* **204**, 1011-1018.

Mrabet, N. T., Van den Broeck, A., Van den Brande, I., Stanssen, P., Laroche, Y., Lambeir, A. M., Matthijssens, G., Jenkins, J., Chidami, M., van Tilderugh, H., Rey, F., Jannin, J., Quax, W. M., Lasters, I., De Maeyer, M. and Wodak, S. J. (1992) *Biochemistry* **31**, 2239-2253.

Natake, M. and Yoshimura, S. (1963) *Agric. Biol. Chem.* **27**, 342-348.

Natake, M. and Yoshimura, S. (1964) *Agric. Biol. Chem.* **28**, 505-509.

Nonomura, H. and Kaji, K. (1974) Japanese Patent 74,14,684. (*Chem. abstr.* 81, 48560g)

Outtrup, H. (1974) German Patent 2,400,323. (*Chem. abstr.* 81, 118544f)

Ovadi, J., Libor, S. and Elodit, P. (1967) *Acta Biochim. Biophys.* (Budapest) 2, 455-458. (Chemical Abstract 68, 112072K).

Park, Y. K. and Toma, M. (1974) *J. Gen. Appl. Microbiol.* 20, 67-69.

Park, Y. H., Chung, J. W. and Han, M. H. (1980) *Enzyme Microb. Technol.* 2, 227-233.

Parrish, F. W. (1970) US Patent 3,514,327.

Pawar, H.S., Kannan, K., Srinivasan, M.C. and Vartak, H.G. (1988) *Biochem. Biophys. Res. Commun.* 155, 411-417.

Pedersen, S. (1993) Industrial aspects of immobilized glucose isomerase. In: *Bioprocess Technol.* 16, 185-208.

Pubols, M. H., Zahnley, J. C. and Axelrad, B. (1963) *Plant Physiol.* 38, 457-461.

Quax, W. J., Mrabet, N. T., Luiten, R. G. M., Schuurhuizen, P. D., Stanssens, P. and Lasters, I. (1991) *Bio/Technol.* 9, 738-742.

Rasmussen, H., Cour, T., Nyborg, J. and Schulein, M. (1994a) *Acta*

Cryst. **D50**, 124-131.

Rasmussen, H., Cour, T., Nyborg, J. and Schulein, M. (1994b) *Acta Cryst.* **D50**, 231-233.

Rohrbach, R. P. (1981) US Patent 4,268,419.

Rehm, H. J. and Reed, G. (1987). Production of enzymes by fermentation. In: *Biotechnology* Vol. 7a (Kennedy, J. F. ed.) Weinhein, Verlag Chemie. pp. 172-211.

Rey, F., Jenkins, J., Jannin, J., Laster, I., Alard, P., Claessens, M., Matthyssens, G. and Wodak, S. (1988) *Proteins Struct. Func. Genet.* **4**, 165-172.

Reynolds, R. J. (1973) British Patent 1,328,980.

Rose, I. A., O'Connell, E. L. and Mortlock, R. P. (1969) *Biochim. Biophys. Acta* **178**, 376-379.

Sailaja, K. and Joseph, R. (1993) *Stärke* **45**, 306-309.

Sakai, S. and Tamanaka, K. (1968) *Biochim. Biophys. Acta* **151**, 684-686.

Scallet, B. L., Shieh, K., Ehrenthan, I. and Slapshak, L. (1974) *Stärke* **26**, 405.

Shieh, K. K. (1976) US Patent 3,992,262. (*Chem. abstr.* 86, 28502q)

Shieh, K. K. (1977) US Patent 4,003,793. (*Chem. abstr.* 86, 87685z)

Shukla, J. L. and Prabhu, K. A. (1985) *Enzyme Microb. Technol.* 7, 499-502.

Sicard, P. J., Leleu, J. B., Lestrem, G. and Tiraby, G. (1990) *Stärke*, **42**, 23-27.

Siddiqui, K. S., Loviny-Anderton, T. L., Rangarajan, M. and Hartley, B. S. (1993) *Biochem. J.* **296**, 685-691.

Sipos, T. (1973) British Patent 1,274,158.

Slein, M. W. (1955) *J. Am. Chem. Soc.* **77**, 1663-1667.

Smith, M. G. (1962) *Biochem. J.* **83**, 135-144.

Smith, C. A., Rangarajan, M. and Hartley, B. S. (1991) *Biochem. J.* **277**, 255-261.

Srinivasan, M. C., Vartak, H. G., Powar, V. K. and Khire, J. M. (1983) *Biotechnol. Letts.* **5**, 611-614.

Storey, K. B. and Chakrabarti, A. C. (1990) *Appl. Biochem. Biotechnol.* **23**, 139-154.

Strandberg, G. W. and Smiley, K. L. (1971) *Appl. Microbiol.* 21, 588-593.

Suekane, M., Tomura, M. and Tomimura, C. (1978) *Agric. Biol. Chem.* **42**, 909-917.

Takasaki, Y. and Tanabe, O. J. (1962) *Agric. Chem. Soc.* Japan, **36**, 1010.

Takasaki, Y. and Tanabe, O. J. (1966) *Agric. Biol. Chem.* **30**, 220-225.

Takasaki, Y. (1966) *Agric. Biol. Chem.* **30**, 1247-1253.

Takasaki, Y., Kosugi, Y. and Kanbayashi, A. (1969a) *Agric. Biol. Chem.* **33**, 1527-1534.

Takasaki, Y., Kosugi, Y. and Kanbayashi, A, (1969b) *Streptomyces* Glucose Isomerase. In: *Fermentation Advances* (Perlman, D. ed.) Academic Press, New York, pp. 561-589.

Takasaki, Y. (1974) Japanese Patent 7,442,555 (*Chem. abstr.* **81**, 123360c)

Tomoyeda, M. and Horitsu, H. (1964) *Agric. Biol. Chem.*, **28**, 139-143.

Tsao, G. T., Reid, T. H., Hiller, F. L. and Hubbard, L. H. (1969) US Patent 3,432,345.

Tsumura, N. and Sato, T. (1961) *Agric. Biol. Chem.* **25**, 616-619.

Tsumura, N., Hagi, M. and Sato, T. (1967) *Agric. Biol. Chem.* **31**, 902-907.

Vandamme, E. J., De Laporte, A., De Vocht, M. and Van Hoe, L. (1981) *Abh. Akad. Wiss. DDR. Abt. Math. Natur. Wiss. Tech.*

(3N,Mikrob Enzymprod.) pp 193-208.

Vangrysperre, W., Callens, M., Kersters-Hilderson, H. and De Bruyne, C. K. (1988) *Biochem. J.* **250**, 153-160.

Vangrysperre, W., Kersters-Hilderson, H., Callens, M. and De Bruyne, C.K. (1989a) *Biochem. J.* **260**, 163-169.

Vangrysperre, W., Amp, C., Kersters-Hilderson, H. and Tempst, P. (1989b) *Biochem. J.* **263**, 195-199.

Vangrysperre, W., Vandamme, J., Vandekerckhove, J., De Bruyne, C. K., Cornelis, R. and Kersters-Hilderson, H. (1990) *Biochem. J.* **265**, 699-705.

Verhoff, F. H., Boguslawski, G., Lantero, O. J., Schlager, S. T. and Jao, Y.C. (1985) Glucose isomerase. In: *Comprehensive Biotechnology*, (MooYoung, M. ed.) Pergman Press, New York, Vol. 3, pp. 837-859.

Vesterberg, O. (1972) *Biochim. Biophys. Acta* **257**, 11-19.

Vieth, W. R., Mead, B., Wang, S. S. and Saini, R. (1973) *Biotechnol. Bioeng.* **15**, 565.

Vieth, W. R., Venkatsubramanian, K., Constantinider, A. and Davidson, B. (1976) In: *Applied Biochemistry & Bioengineering* (Wingard, L. B. Jr. Katchalski-Katzir, E. and Goldstein, L. eds.) Vol. 1, Academic Press, New York, pp 222-327.

Volkin, D. B. and Klibanov, A. M. (1989) *Biotech. Bioeng.* **33**, 1104-1111.

Walon, R. G. P. and Stouffs, R. H. M. (1980) European Patent Application 0036662A2.

Weber, P. (1974) German Patent 2,408,708. (*Chem. abstr.* **82**, 2613u)

Wei, K., Zhang, X., Lai, G. M., Zhou, H., Li, W. and Shen, J. C. (1994) *Macromol. rapid commun.* **15**, 405-409.

Weidenbach, G., Bonse, D. and Ritchter, G. (1983) *Stärke* **36**, 412.

Whitted, B. E. and Boguslawski, G. (1985) US Patent 4,510,247.

Wovcha, M. G. and Brooks, K. E. (1980) UK Patent 2,031,905.

Yamanaka, K. (1963) *Agric. Biol. Chem.* **27**, 265-270.

Yamanaka, K. (1968) *Biochim. Biophys. Acta* **151**, 670-680.

Yamanaka, K. (1969) *Arch. Biochem. Biophys.* **131**, 502-506.

Yamanaka, K. (1975) *Methods Enzymol.* **41**, 466-471.

Yamanaka, K. and Takahara, N. (1977) *Agric. Biol. Chem.* **41**, 1909-1911.

Yip, M. T. and Dalton, D. R. (1979). In: *Organic Chemistry in the Laboratory*, D.Van Nostrand Company, New York, pp. 150-151.

Yoshimura, S., Danno, G. and Natake, M. (1966) *Agric. Biol. Chem.* **30**, 1015-1023

Zemek, J., Kadlecikova, B., Kuniak, L., Kucar, S. and Kackova-Kratochvilova, A. (1986) *Acta Aliment.*, **15**, 111.