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Molecular and Industrial Aspects of Glucose Isomerase

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INTRODUCTION

D-Glucose/xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5), commonly referred to as glucose isomerase (GI), is one of the three highest tonnage value enzymes, amylase and protease being the other two. According to Wiseman, GI may be the most important of all industrial enzymes of the future (184). It catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively (Fig. 1). Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that thrive on decaying plant material and also aids in the bioconversion of hemicellulose to ethanol. Isomerization of glucose to fructose is of commercial importance in the production of high-fructose corn syrup (HFCS). Sucrose derived from sugar beet (40%) and sugarcane (60%) was the main sweetener in the world until 1976. The production of HFCS by using glucose isomerase was developed first in Japan and later in the United States. GI gained commercial importance in the United States because of the lack of supply of sucrose after the Cuban revolution in 1958, and it continues to be one of the most important industrial enzymes to this day.

SCOPE OF THIS REVIEW

Because GI is an industrially important enzyme, much of the relevant information on it is documented in the form of patents. However, literature on some of its aspects is available in a few reviews (8, 30, 31, 43, 44, 81, 127, 176, 184). This review aims at presenting updated information on the biochemical and genetic aspects of GI with a view to identifying important problems faced in its commercial application and evolving potential solutions.

HISTORY OF THE ENZYME

The origin of today's successful development of fructose syrup products lies in the discovery of glucose-isomerizing enzymes. Historically, four different types of enzymes have been termed glucose isomerases. The discovery by Marshall and Kooi in 1957 of the glucose-isomerizing capacity of the enzyme from *Pseudomonas hydrophila* was the starting point of the exploitation of this enzyme for the manufacture of HFCS as a substitute for cane sugar (118). Although the affinity of this enzyme was 160 times lower for glucose than for xylose, it was sufficient for the enzyme to be commercially significant. Production of the enzyme required xylose in the growth medium and was enhanced in the presence of arsenate. Later, a xylose isomerase activity, which was independent of xylose, was found in *Escherichia intermedia* (123). The enzyme was a phosphoglucose isomerase (EC 5.3.1.9), which could isomerize the unphosphorylated sugar only in the presence of arsenate. Takasaki and Tanabe isolated from *Bacillus megaterium* AI a glucose isomerase (EC 5.3.1.18) which was NAD linked and was specific for glucose (158, 159). A similar glucose isomerase activity, which catalyzed the isomerization of both glucose and mannose to fructose, was isolated from *Paracolobacterium aerogenoides* (160, 161). The glucose isomerases produced by heterolactic acid bacteria require xylose as an inducer and are relatively unstable at higher temperatures. Of these glucose-isomerizing activities, xylose isomerase (EC 5.3.1.5) is the most suitable for commercial applications. It is heat stable and does not require expensive cofactors such as NAD^+ or ATP for activity. The potential for using sugar substitutes produced from starch was proposed by several workers (48, 133). Enzymatic glucose isomerization was first accomplished on an industrial scale in 1967 by Clinton Corn Processing Co. in the United States. Immobilized GI was commercially available by

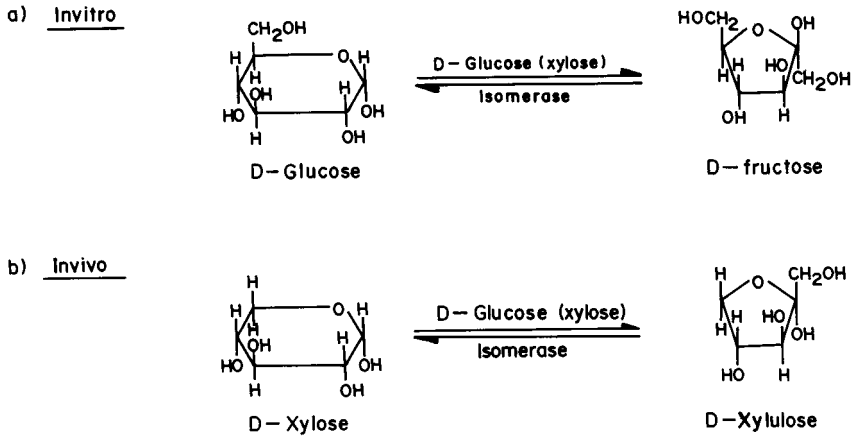

FIG. 1. Reactions catalyzed by GI. (a) In vitro reaction. (b) In vivo reaction.

1974. The demand for HFCS in the food industry increased, and by 1980 practically all major starch-processing companies in the western world were resorting to GI technology. Today, the enzyme commands the biggest market in the food industry.

ENZYMATIC VERSUS CHEMICAL ISOMERIZATION

The chemical conversion of glucose to fructose has been known for the past 100 years and constitutes one of a group of reactions collectively known as the Lobry de Bruyn-Alberda van Ekenstein transformation. These reactions are usually carried out at high pH and temperature. The possibility of producing fructose chemically from glucose has been studied by Barker et al. (12). The reaction is nonspecific and leads to the formation of nonmetabolizable sugars such as psicose and other undesirable colored products. It is difficult to attain a fructose concentration of more than 40% by this method. Moreover, chemically produced fructose has off flavors and reduced sweetness, which cannot be easily remedied. Therefore, it cannot be used commercially. On the other hand, enzymatic conversion of glucose to fructose offers several advantages, such as (i) specificity of the reaction, (ii) requirement of ambient conditions of pH and temperature, and (iii) no formation of side products. Therefore, enzymatic conversion is preferred to chemical isomerization of glucose to fructose, and today the process involving GI has undergone considerable expansion in the industrial market.

IMPORTANCE OF GLUCOSE ISOMERASE

GI serves as an interesting model for studying structure-function relationships by advanced biochemical and genetic engineering techniques. Besides its academic importance, it has received increased attention by industries for its use in producing HFCS and for its potential application in the production of ethanol from hemicelluloses.

Advantages of High-Fructose Corn Syrup as a Sweetener

Increasing demands for refined sugar, coupled with its high cost of production and awareness of the adverse effects of sucrose and invert sugar consumption on human health, have necessitated the search for acceptable sucrose substitutes. A large number of noncalorific and noncarbohydrate artificial sweeteners such as saccharine, cyclamate, acesulfame-K, aspartame, and thaumatin have been discovered and dismissed on the basis of health concerns or other drawbacks. Incorporation of aspartame into soft drinks renders them less sweet after prolonged storage, because aspartame is slowly hydrolyzed at low pH. Thaumatin, an ideal protein sweetener, is 2,000 times sweeter than sucrose but has a distinct, unpleasant flavor. HFCS, an equilibrium mixture of glucose and fructose (1:1) is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. The sweetening capacity of glucose is 70 to 75% that of sucrose, whereas fructose is twice as sweet as sucrose (10). HFCS is manufactured from a totally nonsweet substance, namely, starch. The price of HFCS is 10 to 20% lower than that of sucrose on the basis of its sweetening power. HFCS is preferred by the food industry since it does not pose the problem of crystallization as is the case with sucrose. Moreover, D-fructose plays an important role as a diabetic sweetener because it is only slowly reabsorbed by the stomach and does not influence the glucose level in blood. The major uses of HFCS are in the beverage, baking, canning, and confectionery industries.

Production of High-Fructose Corn Syrup

The market development in HFCS production was marked by a gradual acceptance of HFCS and of the enriched HFCS (55% fructose) as substitutes for sucrose by soft-drink producers. The most common raw material used for the production of HFCS in the United States is the cornstarch manufactured by the wet milling process. The production of HFCS from starch comprises three major processes: (i) liquefaction of starch by α-amylase, (ii) saccharification of starch by the combined action of amyloglucosidase and a debranching enzyme, and (iii) isomerization of glucose by GI. The final product is a corn syrup containing a mixture of glucose and fructose and hence with a greater sweetening capacity than that of sucrose. Other sources of starch such as wheat, tapioca, and rice are used to a minor extent in other parts of the world. The by-products of the corn-milling industry are important in deciding the economics of HFCS production. The annual world consumption of HFCS is estimated to have reached 10 million tons (dry weight) in 1995 (54). At present, HFCS has almost completely replaced sucrose in the United States, and only a moderate (3 to 4%) growth rate in its production is expected on a global basis.

Production of Ethanol

GI catalyzes the isomerization of both glucose and xylose. This property of the enzyme is used in the isomerization of xylose to xylulose, which can be ultimately fermented to ethanol by conventional yeasts. Bioconversion of renewable biomass to fermentable sugars and ethanol is important in view of the rapid depletion of fossil fuels. The biomass consists of cellulose (40%), hemicellulose (30%), and lignin (30%). The economic feasibility of biomass utilization depends on the hydrolysis of cellulose and hemicellulose to glucose and xylose and their subsequent fermentation to ethanol by yeasts. Until recently, the research efforts were focused on the bioconversion of cellulose. Then the awareness that the efficiency of bioconversion of lignocelluloses and agricultural wastes relied mainly on the effective utilization of the hemicellulose component of biomass shifted worldwide attention to hemicellulose fermentation (181). Xylan is a major constituent of hemicellulose and consists of xylose units linked by a β(1,4) linkage. D-Xylose is easily produced by acid or enzymatic hydrolysis of xylan. Industrial yeast strains such as *Saccharomyces cerevisiae* generally ferment hexoses efficiently but D-xylose remains unutilized. A few yeasts such as *Pachysolen tannophilus*, *Pichia stipitis*, *Candida utilis*, and *Candida shehatae* are known to utilize pentoses through the oxidoreductive pathway, but the rates of fermentation are very low (60, 61, 152, 165). Moreover, their low ethanol tolerance and catabolism of ethanol in the presence of oxygen limit their commercial application (58, 59, 113). GI has been used to produce xylulose from xylose, which otherwise represents a major metabolic block in the process of fermentation of xylose to ethanol by conventional yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida tropicalis* (39, 40, 41, 78, 143, 180, 181). Although fermentation rates and product yields for ethanol production from D-xylose are significantly lower than from D-glucose, technology is now emerging to improve the process by transferring the GI gene to the yeast and conducting the isomerization and fermentation of xylose to ethanol simultaneously.

SOURCE ORGANISMS

GI is widely distributed in prokaryotes (Table 1). After its discovery in *Pseudomonas hydrophila*, a large number of bac-

TABLE 1. GI-producing organisms

Species
Actinomyces olivocinereus, *A. phaeochromogenes*
Actinoplanes missouriensis
Aerobacter aerogenes, *A. cloacae*, *A. levanicum*
Arthrobacter spp.
Bacillus stearothermophilus, *B. megabacterium*, *B. coagulans*
Bifidobacterium spp.
Brevibacterium incertum, *B. pentosoaminoacidicum*
Chainia spp.
Corynebacterium spp.
Cortobacterium helvolum
Escherichia freundii, *E. intermedia*, *E. coli*
Flavobacterium arborescens, *F. devorans*
Lactobacillus brevis, *L. buchneri*, *L. fermenti*, *L. mannitopoeus*, *L. gayonii*, *L. fermenti*, *L. plantarum*, *L. lycopersici, L. pentosus*
Leuconostoc mesenteroides
Microbispora rosea
Microellobosporia flavea
Micromonospora coerula
Mycobacterium spp.
Nocardia asteroides, *N. corallia*, *N. dassonvillei*
Paracolobacterium aerogenoides
Pseudonocardia spp.
Pseudomonas hydrophila
Sarcina spp.
Staphylococcus bibila, *S. flavovirens*, *S. echinatus*
Streptococcus achromogenes, *S. phaeochromogenes*, *S. fracliae*, *S. roseochromogenes*, *S. olivaceus*, *S. californicos*, *S. venuceus*, *S. virginial*
Streptomyces olivochromogenes, *S. venezaelie*, *S. wedmorensis*, *S. griseolus*, *S. glaucescens*, *S. bikiniensis*, *S. rubiginosus*, *S. achinatus*, *S. cinnamonensis*, *S. fradiae*, *S. albus*, *S. griseus*, *S. hivens*, *S. matensis*, *S. nivens*, *S. platensis*
Streptosporangium album, *S. oulgare*
Thermopolyspora spp.
Thermus spp.
Xanthomonas spp.
Zymononas mobilis

teria and actinomycetes were found to produce GI that is active in the absence of arsenate. Among the heterolactic acid bacteria, *Lactobacillus brevis* produced the highest yield of enzyme. The enzyme was active at low pH but unstable at high temperature and hence was not suitable for economic exploitation. Reports on extracellular secretion of GI are not common. Extracellular GI has been reported to be produced by *Streptomyces glaucescens* (182) and *S. flavogriseus* (45), for which the release of the enzyme from the cells was attributed to a change in the cell wall permeability and partial lysis of the cells. The extracellular xylose isomerases from *Chainia* sp. (154, 175) and an alkalothermophilic *Bacillus* sp. (42) have been purified to homogeneity by conventional purification techniques such as gel filtration, ion-exchange chromatography, and preparative polyacrylamide gel electrophoresis.

As well as *Streptomyces* spp., several *Bacillus* species are good producers of GI. The occurrence of GI in a few yeasts such as *Candida utilis* (180) and *Candida boidinii* (179) has been documented. *Aspergillus oryzae* is the only fungus which is reported to possess GI activity. The existence of GI in barley malt (13) and wheat germ (129) has been reported. The organisms that are commercially important as GI producers are listed in Table 2. Since GI is a subject of great commercial importance, much of the information on new producer organisms and on developed processes is in the form of patents (25, 79, 80, 88, 112, 122, 126, 147, 182).

TABLE 2. Commercial producers of GI

Organism	Trade name	Manufacturer
Actinoplanes missousriensis	Maxazyme	Gist Brocades and Anheuser-Busch Inc.
Bacillus coagulans	Sweetzyme	Novo-Nordisk
Streptomyces rubiginosus	Optisweet	Miles Kali-Chemie
	Spezyme	Finnsugar
Streptomyces phaeochromogenes	Swetase	Nagase
Arthrobacter sp.		Reynolds Tobacco
Streptomyces olivaceus		Miles Laboratories Inc.

PRODUCTION OF GLUCOSE ISOMERASE

The cost of production of the enzyme is an important factor in evaluation of its suitability for industrial application. Intensive efforts have been made to optimize the fermentation parameters for the production of GI with a view to developing economically feasible technology. Research is focused on three major aspects: (i) improvement of the yields of GI, (ii) optimization of the fermentation medium with special reference to replacement of xylose by a cheaper substitute and elimination of requirement for Co^{2+} ions, and (iii) immobilization of the enzyme.

Improvement of Yield

The yields of GI from various potent producer organisms are listed in Table 3; they range from 1,000 to 35,000 U liter^{-1}. Further improvement in the yield and the properties of the enzyme was achieved by strain improvement, using either conventional mutagenesis or recombinant DNA technology.

Several strains of commercial importance were subjected to mutagenesis to produce elevated levels of enzyme or for constitutive production of enzyme. A 60% increase in the enzyme level was obtained by mutagenizing *Streptomyces wedmorensis* with ethyleneimine and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (20). UV irradiation of *Streptomyces olivochromogenes* resulted in a mutant strain with 70% increased activity (156). A constitutive mutant of *Bacillus coagulans* with twice the activity of the parent was obtained by selecting the mutants on the basis of their resistance to 2-deoxyglucose. Two higher-yielding constitutive mutants showing their highest yields on lactose, with one of them showing higher activity on glucose than on xylose, have been reported by Lee (112). A series of constitutive and high-GI-yielding mutants were isolated by applying multiple UV irradiations to *Streptomyces acidodurans* (26). One of the mutants produced by ethyl methanesulfonate mutagenesis yielded

TABLE 3. Production of GI by various microorganisms

Organism	Yield (U/liter)	Assay		Reference(s)
		Temp (°C)	pH	
Actinoplanes missouriensis	2,500–35,200	75	7.0	7
Bacillus licheniformis	10,500	70	NC[a]	25
Streptomyces wedmorensis	560–2500	70	7.2	59
Streptomyces olivochromogenes	4,800–11,440	60	7.5	9, 87

[a] NC, not clarified.

1,500 U ml^{-1} when grown on only glucose whereas the parent produced 10 U ml^{-1} under similar conditions (79, 80). Strain improvement by genetic manipulation is described in a later section.

Optimization of Fermentation Medium

GI is generally produced by submerged aerated fermentation. Optimization of the fermentation medium has been extensively studied with a view to development of an economically viable fermentation technology for the production of GI. Research efforts were directed mainly toward (i) replacement of xylose by another, inexpensive inducer; (ii) evaluation of the effect of cheaper nitrogen sources on the yield of enzyme; (iii) optimization of pH and temperature for maximum enzyme production; and (iv) substitution of Co^{2+} ions by other divalent metal ions in the fermentation medium. There is no concrete composition of medium for the best production of the enzyme from different microorganisms. Each organism or strain requires its own special conditions for maximum enzyme production (162).

Inducer. Most of the GI-producing organisms have an obligate requirement for D-xylose to induce production of the enzyme. However, xylose is expensive and hence impractical for use on a commercial scale. Starch, glucose, sorbitol, or glycerol could be used in place of 75% of xylose (56). Takasaki and Tanabe showed that *Streptomyces* strain YT-5 was able to grow on xylan or xylan-containing material such as corn cobs or wheat bran (162). This was the first landmark in selecting strains which could grow in a cheaper medium. Today, several strains are capable of producing GI-utilizing glucose instead of xylose. These include *Actinoplanes* strains, mutant strains of *Bacillus coagulans*, and *Streptomyces olivochromogenes*. Another approach to eliminate the requirement of xylose as an inducer is to generate mutants which can produce GI constitutively. One of the wild-type strains of *Actinoplanes missouriensis* is able to produce GI constitutively and is used for commercial production of the enzyme by Gist Brocades (7). Another approach to achieving constitutive enzyme production involves cloning the *xylA* gene in front of a strong *Streptomyces* promoter. The P1-*xylA* gene has been integrated into the chromosome by using the integrative vector pTS55. The resultant strain (CBS1) gave about sevenfold greater activity in the absence of xylose compared with the wild-type strain fully induced by xylose (19).

Nitrogen source. The nitrogen source is a critical factor which needs to be optimized for each source of enzyme. Although complex nitrogen sources are usually used for GI production, the requirement for a specific nitrogen supplement differs from organism to organism. Peptone, yeast extract, or ammonium salts can be used with *Bacillus coagulans*, but urea and nitrate are not suitable (188). Corn steep liquor was found to be a cheap and suitable source of nitrogen by some workers (7, 30, 80), but its use is limited by its seasonal and interbatch variability. Suitable nitrogen sources as substitutes for corn steep liquor are still being evaluated. Soy flour gives a 50% higher yield than corn steep liquor (147). Addition of certain amino acids improves the yield of enzyme in *Streptomyces violaceoruber* (168, 170). A constitutive mutant of *Streptomyces coelicolor* utilizes corn steep liquor better than yeast extract or yeast autolysate (80).

pH and temperature optima. The nature of the nitrogen source affects the pH and consequently the yield of the enzyme. Most GI-producing fermentations are carried out between pH 7.0 and 8.0 without control of pH. *Streptomyces* spp., *Arthrobacter* sp., and *Actinoplanes missouriensis* are grown at around 30°C (7). Thermophilic *Bacillus* spp. are incubated at 50 to 60°C (29, 55). The period of fermentation varies from 6 to 48 h depending on the type of culture used for GI production.

Metal ion requirement. Divalent cations are required in the fermentation medium for optimum production of GI. However, the requirement for specific metal ions depends on the source of enzyme. Co^{2+} was essential for GI production by *Streptomyces* strain YT-5 (162), whereas *Bacillus coagulans* required Mn^{2+} or Mg^{2+} for production of the enzyme (126, 188). Generally, cobalt salts are used in the medium of mesophilic *Streptomyces* species but not thermophilic species. It is important to reduce the addition of Co^{2+} to the medium because of the health hazards related to the human consumption of HFCS containing Co^{2+} and the environmental pollution problem related to the disposal of spent media. Some organisms such as *Arthrobacter* spp. and *Streptomyces olivaceus* (135), as well as some mutants of *Streptomyces olivochromogenes* (7), do not require cobalt for optimal production.

Immobilization of Glucose Isomerase

One of the ways to reduce the cost of production of GI is to recover it efficiently and reuse it several times. Immobilization of GI offers an excellent opportunity for its effective reuse. A plethora of literature on immobilization of GI exists. The largest market for GI is for its immobilized form. Development of immobilized GI has been a subject of great interest (81, 127, 176). The use of GI is expensive because it is an intracellular enzyme and large quantities are needed to compensate for the high K_m for glucose. Therefore, it is important to immobilize GI for its industrial applications. Several methods for immobilizing GI have been described (8). However, only a few are economical and yield enzyme preparations with properties that are suitable for commercial production of HFCS. Table 4 gives a list of commercially used immobilized preparations of GI. Two main methods are used for immobilization of GI: cell-free enzyme immobilization and whole-cell immobilization.

Cell-free immobilization. Soluble enzymes that are immobilized to a support structure have excellent flow characteristics suitable for continuous operations, in contrast to whole-cell immobilized supports, and offer considerable savings in terms of capital equipment. GIs from *Streptomyces phaeochromogenes* and *Lactobacillus brevis* were immobilized on DEAE-cellulose (30). The *Streptomyces* GI immobilized on DEAE-cellulose is being used to produce HFCS in a semicontinuous plant by the Clinton Corn Processing Company. A GI preparation from *Streptomyces* sp. immobilized on porous alumina exhibited a half-life of 49 days and was found to be suitable for continuous use in plug-flow reactors.

The use of enzyme immobilized on controlled-pore alumina in the presence of Co^{2+} had the advantage that the Co^{2+} could be eliminated from the subsequent operations. Monsanto coimmobilized GI on large-pore polyethylene discs by permeating the discs with a solution of polyacrylonitrile in dimethyl sulfoxide and finally fixing it with glutaraldehyde. An elegant procedure involving entrapment of *Streptomyces* GI involving a filament of cellulose acetate was described, and a similar strategy was used to immobilize GI and amyloglucosidase together (30).

Whole-cell immobilization. Because GI is an intracellular enzyme, whole-cell immobilization is the method of choice for most of the commercially available immobilized GIs. Whole cells containing GI were spray-dried and used in the first industrial process to produce HFCS by the Clinton Corn Processing Company. Addition of inorganic salts such as magne-

TABLE 4. Immobilized GIs of commercial importance

Source organism(s)	Trade name	Manufacturer	Immobilization method
Cell-free enzyme			
S. olivochromogenes	G-zyme G-994	CPC (Enzyme Biosystems)	Adsorption on an anion-exchange resin
S. rubiginosus	Spezyme	Genencor International	DEAE-cellulose agglomerated with polystyrene and TiO_2
S. rubiginosus	Optisweet II	Solvay	Adsorption of specific SiO_2 particles followed by cross-linking with glutaraldehyde
S. olivochromogenes	Ketomax 100	UOP	Polyethyleneimine-treated alumina with glutaraldehyde cross-linked GI
Whole cells			
Actinoplanes missouriensis	Maxazyme	IBIS	Cells occluded in gelatin followed by glutaraldehyde
Flavobacterium arborescens	Takasweet	Solvay	Polyamine glutaraldehyde cross-linked cells extruded and granulated
S. griseofuseus	AGIS-600	Godo-Shusei	Chitosan-treated glutaraldehyde cross-linked cells
S. phaeochromogenes	Sweetase	Nagase	Heat-treated cells bound to anion-exchange resin
S. murinus and *Bacillus coagulans*	Sweetzyme T	Novo-Nordisk	Glutaraldehyde cross-linked cells extruded

sium hydroxide to the fermentation broths of *Streptomyces* or *Arthrobacter* species followed by filtration and drying of the cake provided a straightforward method to immobilize cells containing GI (135). Physical entrapment of whole cells in polymeric materials was used as an immobilization method by Novo Industries, whereas chemical entrapment of cells in a membrane followed by cross-linking with glutaraldehyde was used to prepare an immobilized GI to be used on a commercial scale (122). GI from *Streptomyces* sp. strain NCIM 2730 was immobilized on Indion 48-R, leading to an improvement in its pH and temperature stability (66).

The details of the present technology used by the various manufacturers to produce HFCS are documented in the form of patents (9, 10, 12, 20, 135). In a broader sense, modern technology uses an immobilized GI preparation in a continuous system at a higher temperature (65°C) and higher pH without requiring Co^{2+} ions to be included in the feed syrup.

PURIFICATION OF GLUCOSE ISOMERASE

A number of reports regarding the production of GI from various microorganisms are available. However, few of them describe purification of GI to a homogeneous state. The commercial use of GI involves the immobilized form of the enzyme, which is cheap and effective and does not require the purification and concentration of the enzyme. The purification of GI is important from the academic point of view, since it involves basic studies on chemical modification, structure-function relationships, and properties.

GI is generally an intracellular enzyme, except in a few cases when the enzyme production is extracellular. The enzyme is extracted from microbial cells by mechanical disruption (such as sonication, grinding, or homogenization) or by lysis of the cells with lysozyme, cationic detergents, toluene, etc. (43). Purification of GI from microbial sources by classical purification methods, such as heat treatment, precipitation with ammonium sulfate-acetone-Mg^{2+} or Mn^{2+} salts, ion-exchange chromatography, and/or gel filtration, has been reported (43). Literature on the purification of GI by affinity chromatography methods is also available. An affinity adsorbent xylitol-Sepharose was used to purify the GI from *Streptomyces* spp. (44). Other affinity matrices such as Biogel-P 100 coupled with xylose or mannitol immobilized on silochrome-based adsorbents were also used. Ghatge et al. have reported a single-step, rapid purification of GI from *Streptomyces* sp. strain NCIM 2730 by immunoaffinity chromatography (75).

PROPERTIES OF GLUCOSE ISOMERASE

The enzymatic and physicochemical properties of GI from several organisms have been extensively studied. The knowledge of specific properties of the enzyme, such as its stability, substrate specificity, and metal ion requirement, is important to prevent its inactivation and to assess its suitability for application in HFCS production.

Substrate Specificity

The ability of the enzyme to isomerize a wide variety of substrates such as pentoses, hexoses, sugar alcohols, and sugar phosphates was investigated. Although the substrate specificity of the enzyme from different sources changes, the enzyme was able to utilize D-ribose, L-arabinose, L-rhamnose, D-allose, and 2-deoxyglucose, as well as its most common substrates, D-glucose and D-xylose. Maximum isomerization was obtained with the substrates having hydroxyl groups at carbons 3 and 4 in the equatorial position, as in glucose and xylose. The conversion ratios of D-glucose to D-fructose catalyzed by GI from various organisms in soluble or immobilized form were in the range of 26 to 59%. The K_m values of the enzyme for D-glucose and D-xylose were in the range of 0.086 to 0.920 M, and 0.005 to 0.093 M, respectively (44).

Metal Ion Requirement and Inhibitors

GI requires a divalent cation such as Mg^{2+}, Co^{2+}, or Mn^{2+}, or a combination of these cations, for maximum activity. Although both Mg^{2+} and Co^{2+} are essential for activity, they play differential roles. While Mg^{2+} is superior to Co^{2+} as an activator, Co^{2+} is responsible for stabilization of the enzyme by holding the ordered conformation, especially the quaternary structure of the enzyme (32, 33, 70). Direct metal ion-binding studies were carried out by Danno (50) on GI from *Bacillus coagulans*. Kasumi et al. (93) have reported the presence of four Co^{2+} ions per tetramer of GI from *Streptomyces griseofuscus*.

The catalytic activity of GI was inhibited by metals such as Ag^{+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} and to some extent by Ca^{2+}. Other known inhibitors of GI are xylitol, arabitol, sorbitol, mannitol, lyxose, and Tris (31, 153).

Subunit Structure

The sedimentation constants and molecular weights of GI vary from 7.55 to 11.45 and from 52,000 to 191,000, respec-

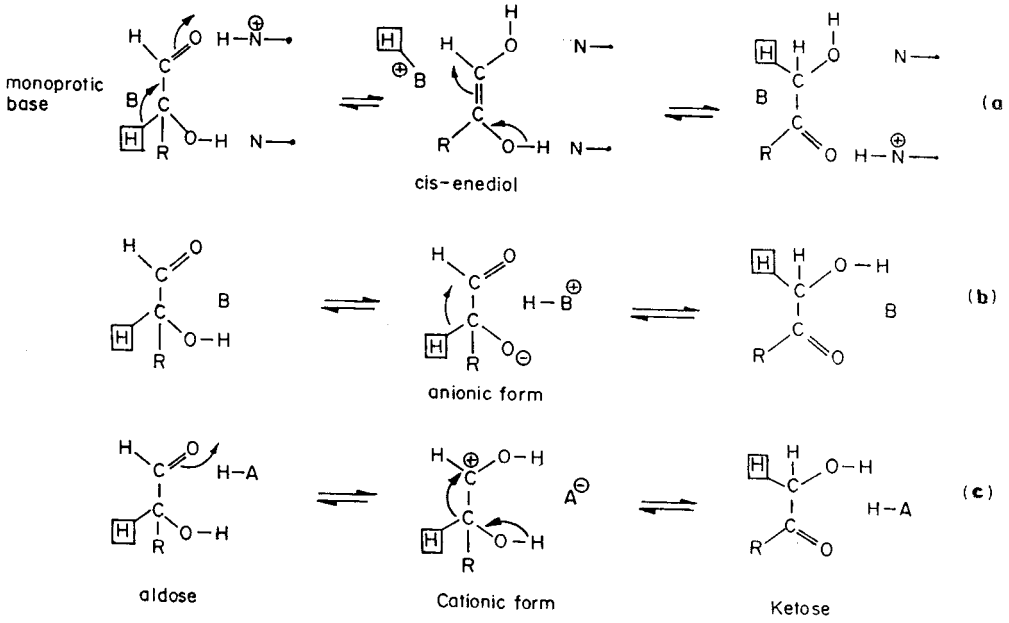

FIG. 2. Mechanism of action of GI. (a) *cis*-Enediol. (b) Proton transfer. (c) Hydride shift. Boxes indicate the hydrogen atoms that are transferred stereospecifically.

tively (44). The subunit structure and amino acid composition of GI reveal that it is a tetramer or a dimer of similar or identical subunits associated with noncovalent bonds and is devoid of interchain disulfide bonds. The extracellular GI from *Bacillus* sp. is a trimer (42). Basuki et al. have reported the existence of isozymes of GI from *Streptomyces phaechromogenes* (14). The isoenzymes differ in their N-terminal amino acids and in the peptide patterns of the digests with trypsin, *Achromobacter* protease I, and cyanogen bromide. Each of the isoenzymes was a tetramer of nonidentical subunits.

The effect of denaturants such as urea, guanidine hydrochloride, sodium dodecyl sulfate, and heat on the activity of GI from *Arthrobacter* and *Streptomyces* spp. was investigated (69, 131). The dissociation and unfolding of the tetrameric GI from *Streptomyces* sp. strain NCIM 2730 revealed that the tetramer and the dimer are the active species whereas the monomer is inactive. The occurrence of a molten-globule-like intermediate in the folding pathway of GI was demonstrated for the first time (76). Intact tertiary rather than secondary structure was shown to be responsible for the biological activity of GI.

Optimum Temperature and pH

The optimum temperature of GI ranges from 60 to 80°C and increases in the presence of Co^{2+}. The optimum pH range of GI is generally between pH 7.0 and 9.0. The enzyme from *Lactobacillus brevis* has a lower pH optimum (between 6 and 7), which is desirable for commercial applications of GI. The enzyme from *Streptomyces* spp., *Bacillus* spp., *Actinoplanes missouriensis*, and *Thermus thermosulfurogenes* is stable at high temperatures, but that from *Lactobacillus* and *Escherichia* spp. is less stable.

Active-Site Studies

The identities of amino acids involved at or near the active site of GI were deciphered with group-specific chemical modifiers and by X-ray crystallography. Evidence for essential histidine and carboxylate residues in GI has been presented (34, 67, 74). The structural environment of functional amino acid residues has been determined by chemical modification and subsequent differential peptide mapping of GI (173). It has long been recognized that GI catalyzes the isomerization of both glucose and xylose. However, whether the reactions occur at the same site or at two different sites was not known. The presence of a single active site for isomerization of both glucose and xylose was demonstrated (68) by using a kinetic method elaborated by Keleti et al. (95).

MECHANISM OF ACTION

Despite its commercial importance, very little information is available about the structural and mechanistic properties of GI. The catalytic mechanism of GI has been a subject of great interest to researchers. Earlier, GI was assumed to function in a manner similar to sugar phosphate isomerases and to follow the ene-diol mechanism (136) (Fig. 2a). Recent studies have attributed the action of GI to a hydride shift mechanism (Fig. 2c). Knowledge of active-site configuration is a prerequisite for studying the structure-function relationship of the enzyme. Different approaches have been used to study the active site of GI and to delineate its mechanism of action. These include (i) chemical modification, (ii) X-ray crystallography, and (iii) isotope exchange. The main features of the mechanism proposed for GI are ring opening of the substrate, isomerization via a hydride shift from C-2 to C-1, and ring closure of the product.

Chemical Modification of Glucose Isomerase

Chemical modification of amino acid residues with specific chemical reagents serves as a simple means of probing the active site of the enzyme. The possible involvement of histidine in the active site of GI was postulated by studying the effect of diethylpyrocarbonate on the inactivation of GI (101). Later, evidence for the presence of an essential histidine residue at the active site of GI from different *Lactobacillus* spp. and *Streptomyces* spp. (67, 171) was provided. Inhibition by diethylpyrocarbonate was remedied by hydroxylamine. Total pro-

tection of enzyme activity was afforded by the substrate and the substrate analog xylitol during chemical modification. Histidine is known to function as a proton-abstracting base and to assist in hydrogen transfers (Fig. 2b). The presence of an aspartate or glutamate residue in GI was documented by its inactivation by Woodward's reagent K or guanidine hydrochloride (74, 172). Involvement of carboxylate residues is implicated in the binding of metal ion cofactors (34). Chemical modification of protected and unprotected GI and subsequent peptide mapping allowed the identification of an active-site region with a consensus sequence consisting of Phe-His-Xaa-Asp-Xaa-Xaa-Pro-Xaa-Gly (173). The results of studies on the chemical modification of GI complement the conclusions drawn on the basis of X-ray crystallographic studies.

X-Ray Crystallography

X-ray crystallography yields a detailed account of the three-dimensional structure of the protein and allows actual visualization of the complex between the enzyme and its substrate or inhibitor. GI from different bacterial species such as *Actinomyces*, *Arthrobacter*, *Actinoplanes*, and *Bacillus* species has been studied by X-ray crystallography at different levels of resolution, in the presence and absence of inhibitors and metal ions, to understand and explain the mechanism of action. Since GI is a single-substrate–single-product enzyme, it is possible to observe the Michaelis complex directly at a substrate concentration higher than its K_m. The structures of GI from several *Streptomyces* spp. are accurately known. They are all very similar, especially at the active site. The structure of GI from *Streptomyces rubiginosus* as determined at 4-Å (1 Å = 0.1 nm) resolution (37) has shown that the enzyme consists of eight β-strand–α-helix [$(\alpha/\beta)_8$] units as found in triose-phosphate isomerase. The smaller domain forms a loop away from the larger domain but overlaps the larger domain of another subunit, so that a tightly bound dimer is formed. The tetramer is thus considered to be a dimer of active dimers. Resolution of the crystal structure of GI from *Streptomyces olivochromogenes* at 3 Å showed that the GI barrel is 30 Å long and 40 Å in diameter (64). The α/β barrel fold is stable and is useful as scaffolding for the construction of an active site. Characterization of crystals of GI from *Streptomyces violaceoniger* at 2.2-Å resolution revealed a variation in the quaternary structure from that of *S. olivochromogenes* GI in solution (77). The structure of crystalline GI from *Streptomyces rubiginosus* has been determined in the presence of substrate and an active site-directed inhibitor at 1.9-Å resolution. These studies have led to identification of the active-site region and two metal-binding sites. One of the metal ions binds to C-3—O and C-5—O of the substrate, while there is a close contact between histidine and C-1 of the substrate. The results indicate that the mechanism involves an open-chain conformation of substrate and probably a formation of a *cis*-enediol intermediate. Recent studies on X-ray crystallographic structures of the metal activated GI from *S. olivochromogenes* show that the isomerization is catalyzed by two metal cofactors and their bridging through a glutamate residue to promote a hydride shift. Of the two essential magnesium ions per active site, Mg^{2+} was observed to occupy two alternate positions separated by 1.8 Å (35). The observed movement of the metal ions in the presence of substrate was attributed to a step following substrate binding but prior to isomerization (106). The substrates, in their linear extended forms, were observed to interact with the enzyme and the metal cofactor. Carell et al. (36) have shown that GI from *S. rubiginosus* can bind substrates and inhibitors in a variety of binding modes depending on the size of the sugar. D-Threonohydroxamic acid resembles the putative transition state in the isomerization step of xylose by GI and is a potent inhibitor of the enzyme. Studies on the high-resolution X-ray crystallographic structure of a complex between the GI from *S. olivochromogenes* and D-threonohydroxamic acid provides evidence for the metal movement during catalysis on deprotonation, which is followed by the formation of a bridging ligand (4). These results confirm the earlier observations that the protonation of the hydroxyl group occurs after ring opening (3).

The crystal structure of GI from *Arthrobacter* strain B3728 containing the inhibitors xylitol and D-sorbitol has been studied at 2.5- and 2.3-Å resolution, respectively (82). The molecule is a tetramer, and the asymmetric unit of the crystal contains a dimer. Each subunit contains two domains. The main domain is a parallel-stranded αβ barrel. The C-terminal domain is a loop structure consisting of five helical segments and is involved in intermolecular contacts between subunits. The requirement for two metal ions per monomer has also been substantiated by spectroscopic analysis and by electron paramagnetic resonance (EPR) studies (24, 155). The metal ion is complexed at the high-affinity site by four carboxylate side chains of the conserved residues. The inhibitors are bound to the active site in their extended open-chain conformation and complete an octahedral coordination shell for the magnesium cation via their oxygen atoms O-2 and O-4. The active site lies in a deep pocket near the C-terminal ends of the β-strands of the barrel domain and includes residues from a second subunit. Several internal salt linkages that stabilize the tertiary and quaternary structure of the enzyme were detected. Collyer et al. and other investigators (22, 23, 47) have shown further that binding at a second cation site (site 2) is also necessary for catalysis. This site binds Co^{2+} more strongly than site 1 does, and it is octahedrally coordinated to three carboxylate groups, an imidazole and a solvent molecule. During the hydride shift, the C—O-1 and C—O-2 bonds of the substrate are polarized by the close approach of the site 2 cation. After isomerization, ring closure is catalyzed as the reverse of the ring-opening step. The anomerism and stereospecificity of the enzyme are shown to be fully consistent with the proposed hydride shift mechanism (46). Crystallization and characterization of GI from *Bacillus coagulans* (132) and *Actinoplanes missouriensis* (92) is also reported.

Isotopic Exchange

The available crystallographic data for GI rule out a proton transfer mechanism and suggest a hydride shift mechanism. However, structural data alone are insufficient to conclude the mechanism of action of an enzyme. Uncertainty about a proton transfer mechanism in GI was prompted by the absence of solvent exchange during investigations on the incorporation of tritiated water into the product (136). However, the possibility of fast proton transfer in a shielded activity could not be ruled out. Allen et al. (2) have carried out isotope exchange experiments at higher temperature, extreme pHs, and in the presence of guanidine hydrochloride to investigate the possibility of shielded proton transfer. Their nuclear magnetic resonance studies, coupled with the studies on fluorine-substituted substrate analogs, do not support a proton transfer mechanism for GI.

Recent studies of the wild-type and mutant D-xylose isomerases from *Actinoplanes missouriensis* support the crucial role of the water molecule, Trp-690, Asp-255, and the adjacent Glu-186 in proton transfer from 2-OH to O-1 of the open and extended aldose substrate (169).

TRIOSE-PHOSPHATE ISOMERASE VERSUS GLUCOSE ISOMERASE

It was long assumed that the mechanism of action of triosephosphate isomerase (TIM) is similar to that of GI. TIM catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by a proton transfer mechanism involving a single base on the enzyme via formation of an enediol intermediate (1, 134). The turnover rate of xylose isomerase is 5 orders of magnitude lower than that of TIM (21). Initially, the three-dimensional structures of GI and TIM were found to be similar in that both had an eight-stranded parallel β-barrel with connecting α-helices. The active sites of the two enzymes are located in the C-terminal end of the β-barrel. However, the helices and β-strands of GI were significantly longer than those of TIM, and the superposition occurred when the third β-strand of GI is fitted to the first strand of TIM. The substrate for TIM is an open-chain, unhydrated triose phosphate, whereas GI binds to the closed form of the sugars and catalyzes their ring opening. Unlike TIM, GI has an absolute requirement for a divalent metal ion. A strong body of evidence now exists that GI and TIM do not have similar mechanisms of action (63). TIM operates via an enediol mechanism, whereas GI executes a hydride shift mechanism.

GENETIC ENGINEERING OF GLUCOSE ISOMERASE

Recombinant DNA technology provides an excellent means for isolating and manipulating the gene of a desired protein. More than 50% of industrial enzymes are now produced from genetically engineered microorganisms (86). One of the ways to increase the production of GI is to identify the GI gene and clone it on a multicopy vector containing a strong promoter such as *lac*, *tac*, or p_L. The GI gene has been cloned from several microorganisms with the primary aims of (i) overproduction of the enzyme by gene dosage effect, (ii) direct conversion of xylose to ethanol by yeasts, and (iii) engineering of the protein to alter its properties to suit its biotechnological applications. Molecular cloning and expression of GI have been carried out in both homologous and heterologous hosts and in yeasts.

Homologous Hosts

Homologous hosts offer several advantages for the cloning and expression of exogenous DNA. One of them is the easy recognition of the expression signals by the host RNA polymerase. There are a few reports on the homologous cloning of GI from *Escherichia coli* and *Streptomyces* species.

E. coli. The first report on the isolation of the GI gene was from *E. coli* by Ho et al. (84). D-Xylose isomerase and xylulokinase activities were amplified by transformation of a GI-deficient *E. coli* strain with plasmid pMB9 bearing a *Hin*dIII restriction fragment of *E. coli* chromosomal DNA (186). The GI gene from *E. coli* was sequenced and was shown to code for GI by purification of the cloned gene product (142). The molecular cloning, sequencing, and expression of the GI gene in *E. coli* have also been reported by Briggs et al. (28), Lawlis et al. (107), and Ueng et al. (167). GI has been overproduced in *E. coli* by several workers (17, 85). Ho and Stevis observed that hyperexpression of the gene was not accomplished by merely cloning it on a high-copy-number plasmid, probably because the expression of the gene in *E. coli* is highly regulated through its natural promoter. The fusion of the structural gene with strong promoters such as *lac* or *tac* resulted in 20-fold overproduction of the enzyme (85). Ligation of a promoterless DNA fragment containing the *E. coli* gene into a plasmid downstream of a strong p_L promoter followed by the transformation of an *E. coli* strain containing a temperature-sensitive repressor resulted in the overproduction of GI (105). Cloning of the *xylA* gene under the control of the *tac* promoter produced GI, which accounted for 28% of the total cell protein. *E. coli* carrying the gene was encapsulated in calcium alginate beads and used in the column for isomerization of the substrate (17). The properties of the genetically overproduced enzyme were similar to those of the enzyme purified from the parent organism (166).

***Streptomyces* species.** Homologous cloning of GI from *Streptomyces phaeochromogenes* in *Streptomyces lividans* via the *Sst*I site of pIJ702 with thiostrepton resistance and insertional inactivation of melanin pigmentation as markers led to a 50-fold increase in the GI activity of *S. lividans*, which was 2.5 times that of the wild type (96). The GI gene from *Streptomyces violaceoniger* was cloned with pUT 206 as a cloning vector. Subcloning of the cloned fragment permitted localization of the GI gene on a smaller fragment (2.1 kb) (117). The nucleotide sequence and deduced primary sequence of the gene were determined (57).

Another strategy to overexpress the protein was by integrating the *xylA* gene into the chromosome. The *Streptomyces* promoter (P1) has been cloned upstream of the *xylA* gene, leading to strong and constitutive expression. To avoid plasmid instability in the absence of selection pressure, which results in instability of GI expression, the P1-*xylA* gene has been integrated into the chromosome with the integration vector pTS55. Integration into the host chromosome resulted in the CBS1 strain, with about sevenfold-higher GI activity in the absence of xylose as an inducer compared with the wild-type strain that was fully induced by xylose (19).

Heterologous Hosts

GI genes from different organisms have been cloned in *E. coli*. Although cloning of genes in homologous hosts is desirable for an easy recognition of expression signals and efficient secretion of proteins, *E. coli* still remains the most popular host of choice in view of the wealth of information available about this organism. Moreover, several cloning vectors have been constructed for use with *E. coli* as a host to meet various specific requirements. Identification of genes in *E. coli* allows their easy sequencing and manipulation by site-directed mutagenesis to produce tailor-made proteins. A few reports on heterologous cloning with hosts other than *E. coli* are also available.

E. coli. A *Bam*HI restriction DNA fragment coding for GI from *Bacillus subtilis* was isolated by complementation of an isomerase-defective *E. coli* strain. The expression of the gene was shown to be under the control of IS*5*, which is inserted 195 bp upstream from the putative ATG initiation codon of the structural gene for GI (183). The ribosome-binding sequence and two hexamer sequences typical of *Bacillus* promoter regions were located in the DNA fragment. *Eco*RI fragments of chromosomal DNA from *Bacillus licheniformis* were ligated to vector plasmid pBR322 and used to transform a GI-negative mutant of *E. coli* (148). Stable maintenance of the recombinant plasmid containing the GI gene and 20-fold higher activity than with the wild-type host were achieved. The GI gene from a thermophilic *Bacillus* sp. was cloned and expressed in *E. coli*. The GI produced by the recombinant was active at 85°C and was partially purified to yield 49.02 U mg of protein^{-1}, which represents the highest ever recorded specific activity for GI (187). The GI gene from *Streptomyces griseofuscus* S-41 was cloned in *E. coli* NM522 with pUC13 as a vector. The DNA

sequence of the cloned gene was determined, and the amino acid sequence was deduced (97). The GI gene from *Actinoplanes missouriensis* cloned in *E. coli* has GTG as its initiation codon and encodes a GI monomer of 394 amino acids. Most (about 94) of the bases in the third wobble position of the codons are G and C (5). The gene encoding the thermostable GI in *Clostridium thermosulfurogenes* was cloned in *E. coli* by a new plate assay method (110). The expression of the protein in *E. coli* was higher (0.46 U mg^{-1}) than in the parent (0.19 U mg^{-1}) and was constitutive. The GI gene from the thermophile *Clostridium thermohydrosulfuricum* has been cloned in *E. coli* with a *Bacillus* GI gene as a probe for screening of recombinants (52). The cloning, sequencing, and expression of GI genes from *Lactobacillus brevis* (27), *Ampullariella* sp. strain 3876 (139), *Arthrobacter* sp. strain NRRL B3728 (115), *Klebsiella pneumoniae* 1033 (65), and *Thermus thermophilus* (53) in *E. coli* have been reported.

Other bacterial hosts. *Bacillus* is generally regarded as a safe microorganism. Therefore, the GI gene from *E. coli* was cloned in *Bacillus* species by using a bifunctional plasmid. However, expression of the gene was not observed. Fusion of the *E. coli* structural gene downstream of the promoter of the penicillinase gene from *Bacillus licheniformis* resulted in functional expression of GI in *Bacillus subtilis* (87). The GI gene from *Clostridium thermosulfurogenes* was cloned in *Bacillus subtilis* with *E. coli-Bacillus* shuttle plasmid pMG1. The expression of the GI gene in *B. subtilis* was constitutive and was higher (1.54 U mg^{-1}) than that produced in *C. thermosulfurogenes* (0.29 U mg^{-1}) (110).

Yeasts. A wide variety of microorganisms can utilize xylose, but none of them can ferment it to ethanol. The main bottleneck lies in the conversion of xylose into xylulose, which is usually performed under aerobic conditions, as in *Candida utilis*. The pentose-utilizing yeasts like *Pachysolen tannophilus* can ferment xylose anaerobically, but the rate of fermentation is formidably low and is accompanied by considerable amounts of side products. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* offer a high fermentation rate, higher end product yield, and increased ethanol tolerance. Transfer of GI genes to these yeasts holds a promise for developing an organism which can ferment xylose directly to ethanol. A 2.4-kb DNA fragment containing the GI gene from *E. coli* was isolated from the Clarke-Carbon gene bank and introduced into *S. pombe* via a shuttle plasmid. The recombinant plasmid showed complementation with GI-deficient *E. coli* and expression of the GI gene in the yeast (41). The transformed *S. pombe* was able to ferment 10% (wt/vol) xylose to produce 3.0% (wt/vol) ethanol. Investigations of the metabolism of D-xylose in the transformed yeast showed that xylitol, which is a by-product of xylose fermentation in yeasts, had no effect on the activity of GI. The low activity of GI in the yeast was due to its proteolytic degradation by the yeast protease and was the limiting step in xylose fermentation by the yeast. The GI genes from *Bacillus subtilis* and *Actinoplanes missouriensis* were isolated by complementation of a GI-negative *E. coli* mutant. The coding region of the GI gene from *B. subtilis* was fused to the yeast pyruvate decarboxylase promoter. The *Saccharomyces cerevisiae* transformants produced xylose isomerase protein to 5% of the total cellular protein, but it was catalytically inactive. The coding region of GI from *A. missouriensis* was fused to the yeast *GALI* promoter. The transformants showed the presence of xylose isomerase-specific mRNA but no enzyme activity (6). Further research inputs are necessary to improve the expression of the GI gene in yeasts and to prevent the enzyme from being degraded by the host proteases.

Plants. The GI gene from *E. coli* has been cloned on a plasmid pBR322 derivative downstream of the nopaline synthetase gene (*nos*) promoter of *Agrobacterium tumefaciens* plasmid pTiC58. This construct was transformed into tobacco leaf discs. The transformants expressed GI in transgenic tobacco, thus indicating that the mRNA was successfully translated by the plant system (128). Cloning of the GI gene from *E. coli* in potato (*Solanum tuberosum*) and in tomato (*Lycopersicum esculentum*) has been achieved and the presence of the *xyl* gene has been confirmed by the expression of GI activity (98, 124).

SEQUENCE HOMOLOGY

To elucidate the structure-function relationship of the D-xylose isomerases, the *xylA* sequences from various organisms have been compared. The nucleotide sequences of a number of GI genes have been determined, and their deduced amino acid sequences are available. D-Xylose isomerases can be separated into two groups on the basis of their amino acid sequences (171). The D-xylose isomerases of *E. coli* and *B. subtilis* constitute one group, and the other group includes the enzymes from *Actinoplanes*, *Ampullariella*, and *Streptomyces* spp. The enzymes in the second group are less similar and lack a stretch of 30 to 40 amino acids which are present in the enzymes from the first group. The enzymes from *E. coli*, *Clostridium thermosulfurogenes*, *Lactobacillus pentosus*, and *B. subtilis* are significantly homologous to each other and consist of approximately 440 amino acids. The GIs from *S. violaceoniger*, *S. griseofuscus*, *A. missouriensis*, and *Ampullariella* spp. are homologous and contain 390 amino acids. The GI from *Thermus thermophilus* is more like the enzymes from the actinomycetes group, while the GI from *Lactobacillus brevis* is similar to that from *L. pentosus*, which is closer to the *E. coli* group (114). Lee et al. have shown the existence of two distinct classes of the enzyme, i.e., the thermolabile GI of the *B. subtilis* and *E. coli* type, and the thermostable GI of the *Streptomyces* type (109). The comparison of GI from mesophilic sources such as actinomycetes with thermostable GI from *T. thermophilus* revealed D → E, Q → H, and G → P substitutions in the latter organism. Q → H substitution reduces the chances of deamidation of glutamine at higher temperature, which is known to be responsible for the irreversible thermoinactivation of the enzyme (177, 178). G → P substitutions provide rigidity to the polypeptide backbone. GI from *S. violaceoniger* shows 86.8% homology with the enzyme from *S. griseofuscus*. Extensive amino acid homology (93.4%) was observed between the GI from *A. missouriensis* and *Ampullariella* sp. The *Streptomyces* enzyme showed significant homology (60%) with the enzyme from the two actinomycetes but poor homology with the enzyme from *E. coli* (24.2%) and *B. subtilis* (26.3%) (97). Meaden et al. (119) have compared the deduced amino acid sequences of the 19 bacterial GIs obtained from the databases (Swiss-PROT version 23) and those described by Scheler et al. (140, 141). Considering the degree of similarity of the proteins and their statistical significance, it was inferred that all the proteins were homologous and made up a single family. On the basis of the G+C content of the DNA and the physicochemical properties of GI, the proteins were divided into two distinct clusters, the proteins from organisms with high G+C DNA content, and those from organisms with low G+C DNA content. The first cluster represents GI from four *Streptomyces* spp., *Thermus thermophilus*, *Actinoplanes missouriensis*, *Ampullariella* spp., and *Arthrobacter* spp. The second cluster includes GI from three *Bacillus* spp., *Staphylococcus xylosus*, two *Lactobacillus* spp., *E. coli*, and *Klebsiella pneumoniae*. The distribution of the pro-

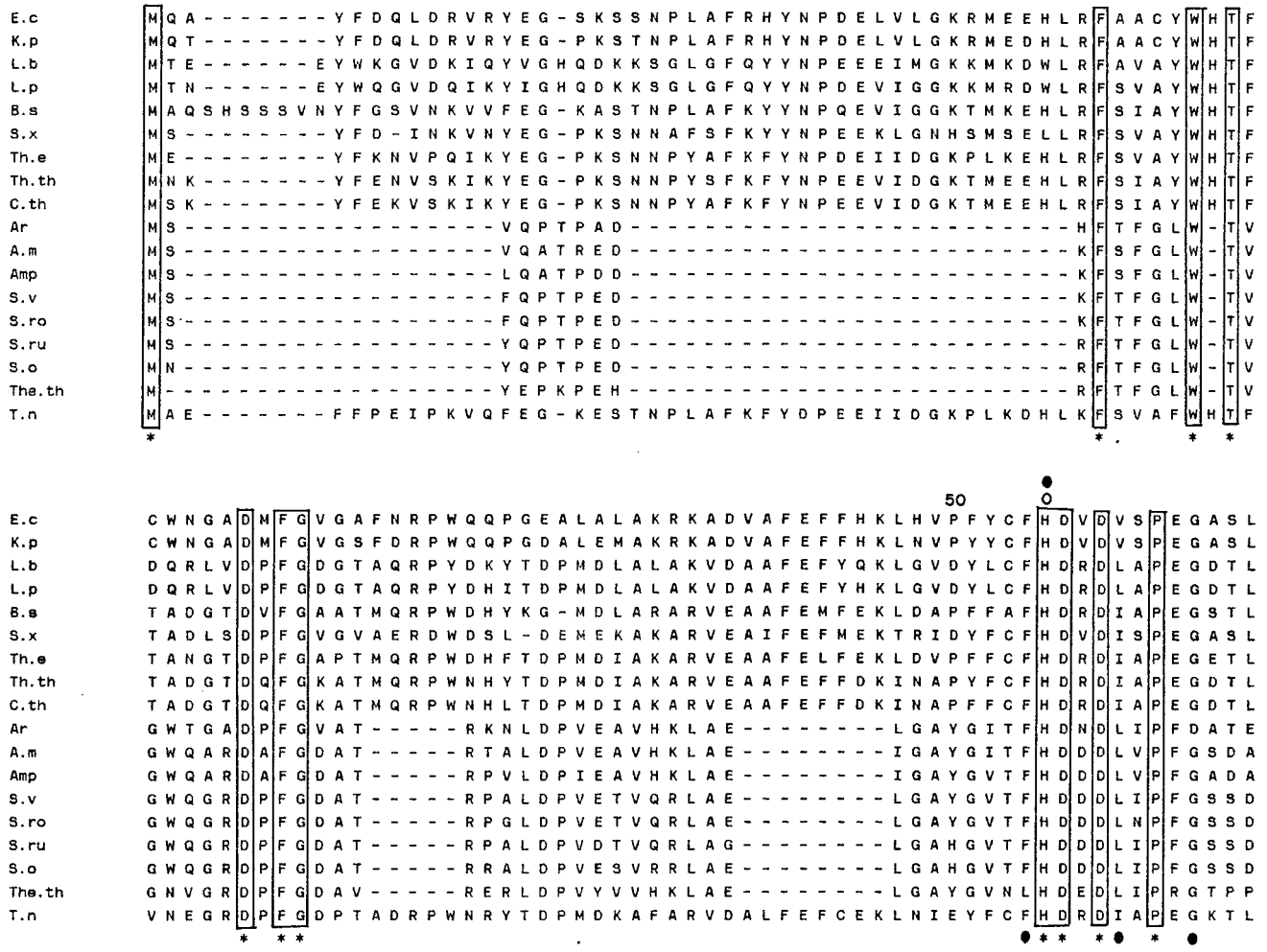

FIG. 3. Homology alignment of the XylA sequences. The XylA protein sequences, deduced from the nucleotide sequences obtained from the EMBL database (updated 1 April 1995), were analyzed with the CLUSTAL V program (82) for multiple alignment. Identical amino acids (*) are boxed, and similar amino acids (.) are indicated below the sequence. Functional amino acid residues involved in catalysis (●), substrate binding (○), metal binding (△), and *cis* peptide linkage (⌐) are indicated above the sequences. The numbering of amino acid residues is based on the sequence of GI from *S. rubiginosus* (35). Abbreviations: E.c, *Escherichia coli*; K.p, *Klebsiella pneumoniae*; L.b, *Lactobacillus brevis*; L.p, *Lactobacillus pentosus*; B.s, *Bacillus subtilis*; S.x, *Staphylococcus xylosus*; Th.e, *Thermoanaerobacter ethanolicus*; Th.th, *Thermoanaerobacter thermosulfurogenes*; Ar, *Arthrobacter* sp.; A.m, *Actinoplanes missouriensis*; Amp, *Ampullariella* spp.; S.v, *Streptomyces violaceoniger*; S.ro, *Streptomyces rochei*; S.ru, *Streptomyces rubiginosus*; S.o, *Streptomyces olivochromogenes*; The.th, *Thermus thermophilus*; T.n, *Thermotoga neopolitana.*

teins between the two clusters relates to the phylogenetic relationships of the organisms. Analysis of the aligned sequences revealed two signature sequences, namely, VXW(GP)GREG (YSTA)E and (LIVM)EPKPX(EQ)P, which recognized all GI and no other proteins in Swiss-PROT (version 23).

We have compiled and performed a multiple alignment of *xylA* sequences from 18 bacterial sources by using the program Clustal V (83) (Fig. 3). In spite of the low homology between the *Streptomyces* and *E. coli* or *Bacillus* enzymes, the amino acids involved in the substrate and metal ion binding, as well as in catalysis, are completely conserved. The *cis* peptide linkage between the adjacent glutamic acid and the proline which is responsible for the formation of the rigid structure at the active site is also well conserved in all the GIs studied. Thus, the essential structure at the catalytic center of GI appears to be analogous in all the enzymes that were compared. The information on the conserved and homologous regions in the *xylA* sequences will be a valuable tool for isolating novel GIs with desirable catalytic properties.

GENETIC REGULATION OF GLUCOSE ISOMERASE BIOSYNTHESIS

D-Xylose, though not as common a sugar as glucose, is a major component of plant hemicelluloses. The microorganisms that survive on decaying plant materials have evolved efficient biochemical pathways to assimilate D-xylose. D-Xylose as an energy source is utilized by bacteria through a pathway involving transport across the cytoplasmic membrane and isomerization to D-xylulose. The pentulose residue is phosphorylated by xylulokinase to yield D-xylulose-5-phosphate, which is further metabolized via the pentose phosphate and Embden-Meyerhoff pathways. A xylose-H^+ proton symporter and a binding protein-dependent system are responsible for the transport of xylose into *E. coli* K-12 (164). Investigations on the organization of genes involved in the xylose metabolism pathway are useful in understanding the molecular mechanism of gene regulation. Considerable information on the biochemical and ge-

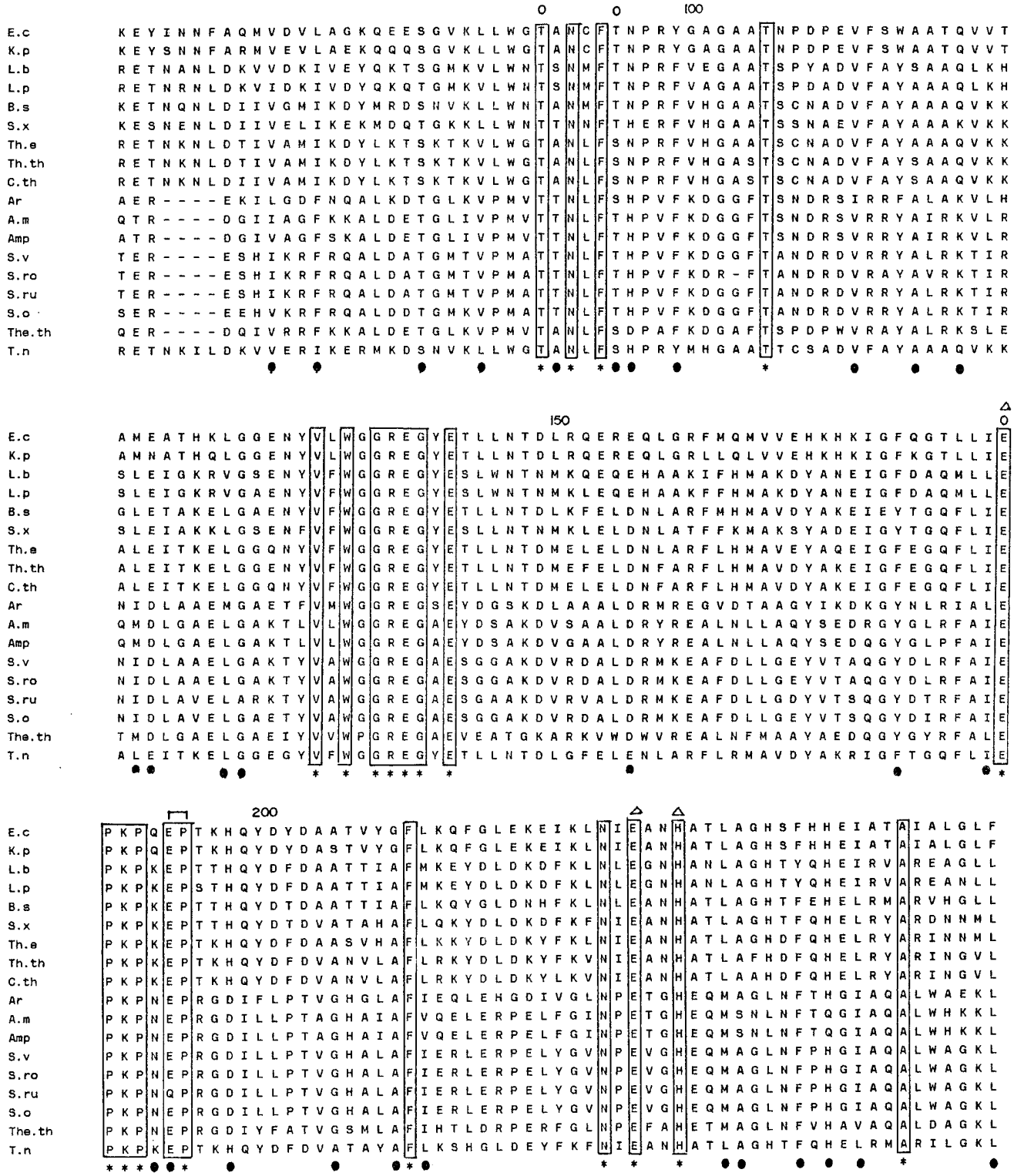

FIG. 3—*Continued.*

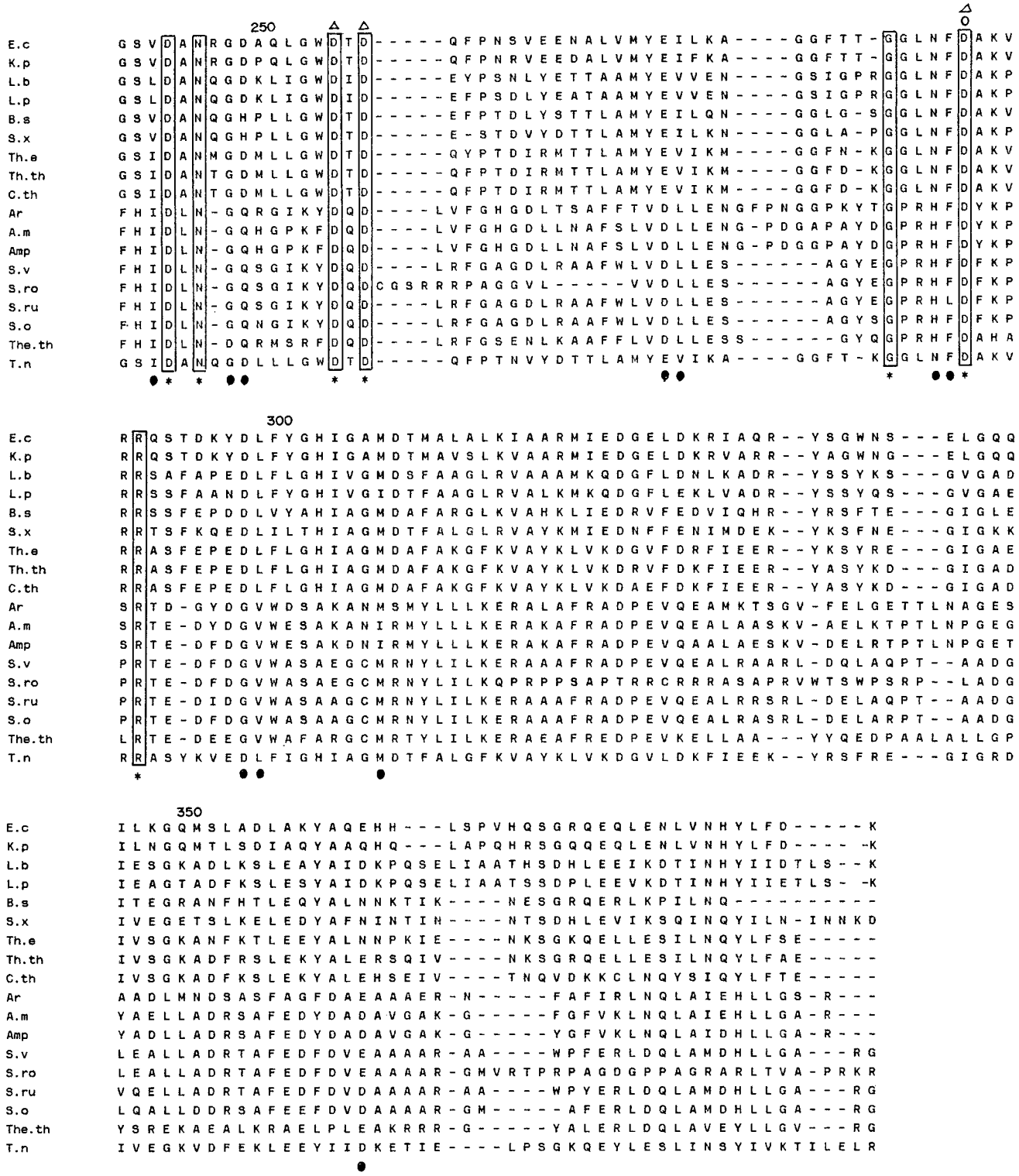

FIG. 3—*Continued.*

netic aspects of xylose utilization in various microorganisms has emerged in the recent past.

Genetic Organization of *xyl* Genes

***Salmonella typhimurium* and *E. coli*.** Genetic studies on *Salmonella typhimurium* provided evidence for the existence of four clustered genes (*xyl* operon) that are responsible for xylose catabolism; these are *xylT*, a gene specifying the transport of xylose across the cell membrane; *xylA*, the glucose/xylose isomerase gene; *xylB*, the xylulokinase gene; and *xylR*, a regulatory element essential for transcription of *xyl* genes (145, 146). The transduction analysis of *S. typhimurium* genes indicated the order to be *xylT-xylR-xylB-xylA*. Studies on the *E. coli* genome revealed an analogous genetic organization and similar xylose utilization pathway (116). Isolation of *E. coli* mutants with mutations in *xylA*, *xylB*, and *xylR*(*T*), coupled with the complementation data, suggests the order of genes to be *xylR*(*T*)*-xylA-xylB* (138). These results strongly support a repressor-operator mechanism for the regulation of *xylAB* expression and postulate a model for coordinate (positive) control of the *xylA*, *xylB*, *xylT* genes by the *xylR* gene product (51, 138). In the absence of xylose, the *xylR* product acts as a repressor, while it acts as an activator in the presence of xylose, which is analogous to the action of the *araC* gene product of the arabinose regulon (125).

The regulation of the xylose operon in *E. coli* was also studied by fusion with Mud (Ap^r *lac*) phage (15). The β-galactosidase activity of the fusion product was induced by xylose but not by arabinose and was repressed by glucose.

***Klebsiella pneumoniae*.** Feldmann et al. have suggested the presence of a regulatory gene in the 5′ upstream region of the *xylA* gene of *Klebsiella pneumoniae*, which is responsible for the *xyl*-negative phenotype in recombinant *E. coli* mutants (65).

***Bacillus* species.** The regulation of the *xyl* operon in *Bacillus* spp. has been studied by constructing a *xyl-lacZ* fusion gene and integrating it into the *amy* gene of *B. subtilis* 168 (71). Increased expression of the *xyl-lacZ* fusion product, when titrated with the *xyl* regulatory DNA in *trans*, suggested a negative regulation of the *xyl* operon in *B. subtilis*, in contrast to the positive control mechanism described for the *xyl* operon of *E. coli* and *S. typhimurium*, suggesting that regulation occurs at the level of transcription. A 25-bp sequence, located 10 bp downstream of the *xyl* promoter, was identified as a *xyl* operator in *B. subtilis* 23 (99). The outer 10 bp of the *xyl* operator exhibits palindromic symmetry. The penultimate base pair near the end of the central 5-bp nonpalindromic sequence was shown to be important for binding to the repressor. *xyl* repressor binds to the palindromic *xyl* operator but not to a half-site, suggesting that the active repressor may be at least a dimer (71). A computer analysis of the *xylR* sequences from *B. subtilis* revealed that operator recognition may be mediated by an α-helix–turn–α-helix motif located between amino acids 29 and 48 of the primary structure (140). Kauder et al. have shown that an operator binding-negative mutation of the *xyl* repressor from *B. subtilis* is *trans* dominant in *Bacillus megaterium* (94). This arrangement of two operators is also present in *xyl* regulatory sequences of GI from *B. subtilis*, *Staphylococcus xylosus* (149), and *Lactobacillus pentosus* (114) and is assumed to be responsible for efficient regulation in these bacteria. Recently, the binding sites of the *xyl* repressor in the *xyl* regulatory region have been characterized (49) by protection and interference of binding. These studies have suggested the presence of tandem overlapping *xyl* operators separated by 4 bp. Mutational inactivation of one or both operators, coupled with elegant DNA retardation experiments, have confirmed the existence of two

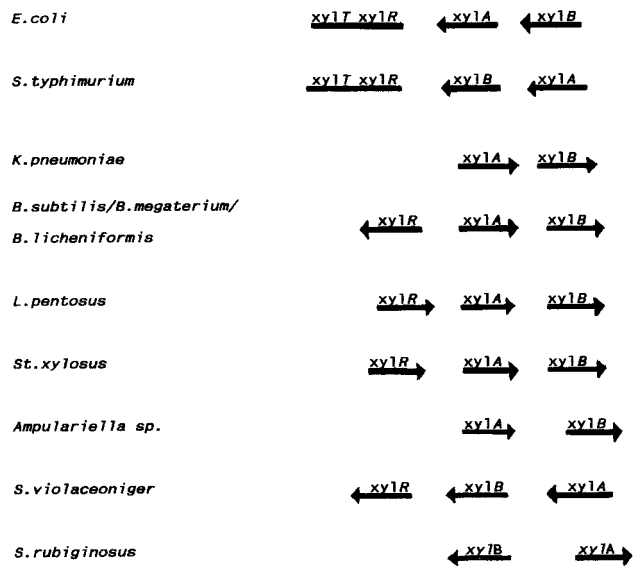

FIG. 4. Genetic organization of the *xyl* genes. The orientation of transcription of the individual genes (*xylA*, *xylB*, *xylR*) of the *xyl* operons of different microorganisms is compared. The 5′ → 3′ orientation is indicated wherever it is known.

binding sites which can be occupied simultaneously. A tandem overlapping arrangement of two operators is also present in the *xyl* regulatory sequences of *B. megaterium*, *S. xylosus*, and *L. pentosus* and is assumed to be responsible for efficient regulation in these bacteria (140).

***Staphylococcus* species.** *xyl* genes from *S. xylosus* were cloned in *S. carnosus* by complementation to xylose utilization (149). The *xylR*, *xylA*, and *xylB* open reading frames were located with the same polarity. Primer extension analysis revealed that the transcription of *xylR* was constitutive while that of *xylA* was xylose inducible. The two transcriptional units, *xylR* and *xylA*, were separated by a transcriptional terminator between the genes, and the presence of promoter-like sequences was observed upstream of both transcriptional start sites. *xylA* and *xylB* are separated by only 5 nucleotides between the stop and start codons, respectively. This observation, together with the absence of terminator-like structures between *xylA* and *xylB*, strongly suggests that they are cotranscribed. Cotranslation of these two genes is also indicated by the presence of a potential Shine-Dalgarno sequence for *xylB* (AAGGA) which overlaps with the last codons of *xylA*. These results strongly support a repressor-operator mechanism for the regulation of *xylAB* expression (151).

A novel approach involving fusion of the *luxA* and *luxB* genes of the gram-negative marine bacterium *Vibrio harveyi* MAV to the *xyl* operon from *S. xylosus* was used for quantifying the induction and catabolite repression of the *xyl* operon in *S. carnosus* TM 300 (150).

***Streptomyces* species.** Mutants of *Streptomyces violaceoniger* that are deficient in either xylose isomerase and/or xylulose kinase were isolated (116). Chromosomal fragments with the ability to complement all three different classes of *xyl*-negative mutants were cloned on a plasmid. Localization of the genes indicated that the putative xylulose kinase gene resides near the glucose isomerase gene, which is consistent with the organization of the locus in *Salmonella typhimurium* (73), *E. coli* (186), and *Bacillus subtilis* (183) (Fig. 4).

Divergent Promoters

Studies on the *xylA* gene of *Streptomyces violaceoniger* have indicated that *xylA* and *xylB* promote transcription in opposite directions (164). The existence of divergent promoters in *Streptomyces* spp. and other prokaryotes was reported previously (18). Sequence analysis has indicated the presence of a third reading frame, which encodes a regulatory protein. The two genes are separated by a short region (195 bp), which revealed the presence of an element with palindromic symmetry typical of bacterial operators. It is suggested that a regulatory molecule may act within the divergent transcription unit to control the expression of opposite genes and also regulates its own synthesis.

Wong et al. have conducted a detailed analysis of the genetic organization and regulation of the xylose degradation genes in *Streptomyces rubiginosus* (185). The study reveals that the genetic organization of *xylA* and *xylB* genes in this organism differs considerably from that in the other bacteria. The two genes are transcribed divergently from within a 114-bp sequence separating the two coding regions, in contrast to the earlier observation that *xylA* and *xylB* genes are part of an operon. The transcription initiation sites are 40 and 20 bp upstream of the translation initiation sites of *xylA* and *xylB*, respectively. The promoters of the genes share a 33-bp overlapping sequence in the untranscribed region between the two genes. The transcription of *xyl* genes in *S. rubiginosus* is induced by xylose and repressed by glucose. It was believed that the 114-bp nucleotide intergenic region provides the binding site(s) for the regulatory proteins.

Catabolite Repression

The expression of the *xyl* operons in *Salmonella typhimurium* and *E. coli* seems to be regulated by a positive control mechanism (146) and by catabolite repression exerted by glucose (51). In addition, a regulatory effect of the xylose isomerase itself has been described for *E. coli* (15). In *E. coli*, catabolite repression is mediated via transcriptional activation by the catabolite gene activator protein and cyclic AMP (cAMP).

In *Bacillus subtilis*, the *xyl* operon is negatively regulated by the *xyl* repressor and inducible by xylose (100). The cAMP-cAMP receptor-mediated mechanism as observed in *E. coli* is not functional in *B. subtilis*, as evidenced by observations such as (i) the cAMP concentration is unaffected by the strength of catabolite repression, (ii) the cAMP receptor protein has not been detected in gram-positive bacteria, and (iii) catabolite repression in *B. subtilis* is negatively regulated at the transcriptional level (99). Jacob et al. have demonstrated that glucose repression occurs at the level of transcription, is independent of a functional repressor gene for xylose induction, and depends on a *cis* sequence in the translated reading frame of *xylA* (89). A 34-bp *cis*-acting element has been located 125 bp downstream from the transcriptional start site in the xylose isomerase reading frame in *B. subtilis* W23. Studies by Kraus et al. indicate that glucose shows an additional inducer exclusion type of repression of *xylA* which is independent of the *cis*-acting element but requires a functional *xylR* and is dependent on the concentrations of glucose and the inducer (xylose) (99).

In conclusion, the organization of *xylA* and *xylB* seems to be highly conserved in all bacteria. These two genes are always adjacent to each other, but a closer inspection reveals marked differences in their organization, as shown in Fig. 4. In *Bacillus subtilis*, the *xylR* gene has a polarity opposite to that of the *xylA* gene, unlike in *Staphylococcus xylosus* and *Lactobacillus* spp. In *Streptomyces* spp., the *xylA* and *xylB* genes are transcribed divergently on different strands, whereas in *E. coli*, *Lactobacillus* spp., and *Bacillus* spp., they are transcribed from the same strand. The analysis of *xyl* genes from a variety of organisms will help to form a consensus opinion about the genetic organization and regulation of *xyl* genes.

GENETIC IMPROVEMENT OF GLUCOSE ISOMERASE BY SITE-DIRECTED MUTAGENESIS

Advances in recombinant DNA techniques allow successful isolation of genes of almost any protein. Engineering proteins by manipulation of their genes is at present a viable approach which complements structure-function studies performed by preexisting methods and allows production of tailor-made proteins with desirable properties to give a complete insight into the mechanism of the enzyme. These studies lead to a hypothesis which can be verified by protein engineering. Site-directed mutagenesis (SDM) of GI has been carried out with several objectives of academic and industrial importance, such as (i) increasing the thermal stability, (ii) lowering of the pH optimum, (iii) changing of the substrate preference, (iv) deducing the functional role of essential amino acid residues, and (v) studying the subunit interactions. These studies have contributed substantially to our knowledge about the molecular mechanism of GI and have created new possibilities of producing an enzyme with properties that are better suited for biotechnological applications. A few examples of how SDM has helped to increase our knowledge about the mechanism of action of the enzyme and has produced an enzyme with improved properties are noted below.

Thermal Stabilization

Most of the commercial preparations of GI have a temperature optimum of 60 to 65°C. The activity of GI declines as a result of its thermal inactivation. This confers a limitation on the operating time of the reactor. Several mechanisms are known to be involved in the irreversible inactivation of GI, such as irreversible unfolding, glycation, and/or deamidation of Asn or Gln (177).

Under practical conditions, GI is exposed to high sugar concentrations (3 M), which may lead to nonenzymatic glycation of lysine residues and subsequent inactivation of GI. Elegantly designed protein engineering experiments on GI from *Actinoplanes missouriensis* have shown that a GI mutant containing a substitution of arginine for lysine at position 253 at the dimer-dimer interface increases the half-life of the enzyme by 30% (130). The largest stability gain was achieved in a triple mutant (G70S/A73S/G74T) of the enzyme, for both soluble and immobilized preparations. The hydrophobic interaction among the aromatic amino acid residues present in the active site of GI is postulated to be one of the important factors that help to maintain the association of monomers into active dimers. An increase in thermostability may therefore be achieved by strengthening the interactions at the interface of the active dimers. Enhancement of the thermostability of GI from *Thermoanaerobacterium thermosulfurigenes* was obtained as a consequence of the reduction of the water-accessible hydrophobic surface by site-directed mutagenesis of aromatic amino acids in the active site. Replacement of W139 with F, M, or A resulted in increased catalytic efficiency proportional to the decrease in hydrophobicity of the side chain of the substituted amino acid (120).

The effect of changing the residues at the subunit interfaces on the activity and thermostability of GI from *Arthrobacter* spp. was studied by Varsani et al. (174). Introduction of one or two disulfide linkages or salt bridges at the subunit interfaces does

not result in any change in enzyme activity or stability. An analysis of the results indicates that subunit dissociation is not on the pathway of thermal inactivation but that movements of active-site groups may trigger conformational changes which may be responsible for the initiation of the unfolding of the protein. Attempts were made to study the effect of altering the metal ion at the M-2 site on the thermostability of the D-xylose isomerase of *S. rubiginosus*. Studies on SDM-generated positional analogs of His-220 mutants of *S. rubiginosus* have confirmed the role of the geometry and the binding affinity of the metal ion at site 2 in the stability of D-xylose isomerase. Even a subtle difference in the coordination of the M-2 site metal ion affects the catalytic activity in the case of His-220 mutants, indicating the possible role of site 2 in isomerization (38).

Deciphering the Role of Metal Ions

Dissecting the role of metal ions with respect to thermostability and catalysis is difficult. The biochemical properties of GI from *A. missouriensis* were investigated after the side chains involved in metal binding were substituted by SDM (92). The results demonstrate that the two metal ions play an essential role in binding and stabilizing the open forms of the substrate and in catalyzing hydride transfer between the C-1 and C-2 positions. The distinct role of two magnesium ions essential for the GI activity of *Streptomyces olivochromogenes* was determined by neutron activation analysis and SDM (3). One of the metal-binding sites, M-1, was removed by substitution of Glu-180 by Lys. Ring-opening assays with the mutant E180K and with 1 thioglucose as the substrate showed that Glu-180 is essential for isomerization but not for ring opening. The wild type and the mutant show no other significant structural differences.

Alteration of Substrate Specificity

GI displays higher affinity for xylose than for glucose. However, increased affinity toward glucose is desirable in view of its application in the production of HFCS. Attempts to alter the substrate preference of the thermophilic GI from *Clostridium thermosulfurogenes* were made by redesigning the amino acids situated in the substrate-binding pocket (121). The W-139 → F substitution reduced the K_m and increased the K_{cat} of the mutant toward glucose, while the reverse effect toward xylose was observed. Double mutants (W-139 → F/V-186 → T and W-139 → F/V-186 → S) had five- and twofold-higher catalytic efficiency, respectively, than did the wild type.

These results provide evidence that the substrate specificity can be altered by reducing the steric constraints and enhancing the hydrogen-binding capacity for glucose in the substrate-binding pocket of the active site.

Functional Role of Essential Amino Acid Residues

The essential active-site histidine residue in the GI from *Clostridium thermosulfurogenes* was identified by substituting histidine residues at four different positions. Substitution of His-101 by phenylalanine abolished the enzyme activity, whereas substitution of other histidine residues had no effect (109).

His-101 and His-271 were shown to be essential components of the active site of GI from *E. coli* by selective substitution of each amino acid (16). It was speculated that His-101 is the catalytic base mediating the reaction whereas His-271 behaves as a ligand for one of the metal ions in the active site of GI.

SDM was used to assess the structural and functional roles of specific amino acid residues in the GI from *Actinoplanes missouriensis*. His-220 and His-54 were important but not essential for catalysis (102). His-54 was implied to govern the anomeric specificity. Lys-183 was assumed to play a crucial role in the isomerization step by assisting the proton shuttle. Lys-294 is indirectly involved in binding the activating cations, whereas Trp-16 and Trp-137 contribute to maintenance of the general architecture of the substrate-binding site.

SDM of the conserved tryptophan residues in the *E. coli* enzyme (Trp-49 and Trp-188) reveals that fluorescence quenching of these residues occurs during the binding of xylose by the wild-type enzyme. Additional active-site substitutions at His-101, which result in inactivation of enzyme, show altered spectral characteristics (90).

TABLE 5. Effect of temperature on the concentration of fructose

Temp (°C)	Fructose concn (%)
25	43.5
30	46.5
40	47.9
45	48.2
55	50
65	51.5
70	52
75	53.1
80	53.9
85	54.7
90	55.6

Alteration in pH Optimum

Commercial application of GI demands an acidic pH optimum to enable starch liquefaction and glucose isomerization to be carried out in a single step. Glu-186 is a conserved residue which is situated near the active site of GI from *A. missouriensis* but does not participate in the substrate or metal ion binding. The negative charge from this group was removed by its mutation to glutamine, which resulted in lowering its pH optimum to 6.25 and in changing its preference from Mg^{2+} to Mn^{2+} (163). This study adds new information on the catalytic mechanism of aldose-ketose isomerization by GI and demonstrates that a single amino acid substitution is able to shift the pH optimum by more than 1 pH unit.

IDENTIFICATION OF IMPORTANT PROBLEMS AND POTENTIAL FRUITFUL SOLUTIONS

Introduction of enzymatic glucose isomerization for the production of HFCS is beset by several problems. Among the major problems are the inactivation of GI at higher temperatures, the high pH optima of many of the GI preparations, the requirement of Co^{2+} for enzyme activity, the lower affinity of GI for glucose than xylose, and the suboptimal concentrations of the product. Intensive research into ways of overcoming these problems has resulted in the development of substantially improved processes. Nevertheless, there is scope for further improvement in all the above-mentioned areas to evolve an economically feasible commercial process to substitute glucose totally by HFCS. Some of the important problems faced in the industrial applications of GI and the plausible solutions thereof are discussed below.

Enhancement of Thermostability

The equilibrium conversion of glucose to fructose under industrial process conditions is around 50%, and the enthalpy of the reaction is 5 kJ/mol. The commercial application of

TABLE 6. Relation between the feed syrup and temperature in HFCS production

Concn of glucose in feed syrup (%)	Temp (°C)[a]
96	115
98	110
100	105

[a] Temperature required to obtain a 3% increase in fructose content over the equilibrium concentration.

HFCS requires the use of high fructose concentrations. The concentration of fructose desired for many applications in industry is higher than 50%. Higher isomerization yields may be achieved by increasing the reaction temperature. The effect of temperature on the concentration of fructose at equilibrium is shown in Table 5. Moreover, the temperature required for conversion depends on the glucose content of the feed syrup (Table 6). Use of higher concentrations of feed syrup and increased temperatures of operation keep the reaction times required for the isomerization processes from becoming excessive. The isomerization temperature is normally 55 to 60°C. Lower temperatures lead to an increased risk of microbial infection. Higher temperatures increase the isomerization rate but reduce enzyme and monosaccharide stability. Losses of enzyme activity encountered during operation are attributed to the heat denaturation. The enzyme exhibits an exponential decay as a function of time. A thermostable enzyme, stable at acidic pHs, may increase the efficiency of the process and reduce the possibilities of by-product formation. Reports on thermostable GI from a few microorganisms are available (127).

Enrichment of Fructose

The major application of HFCS is in the sweetening of soft drinks. A 55% HFCS concentration matches the sweetness of sucrose and allows 100% substitution. Its price is 10 to 20% lower than the price of sucrose, based on sweetening power. A 42% HFCS concentration is used in the baking, dairy, and confectionery industries and for preparing canned food, jam, jelly, and ketchup. However, its application in these industries is limited by some of the drawbacks inherent to HFCS, namely, its hygroscopic and viscous nature, browning tendency, and inability to crystallize. In the commercial processes, 42% fructose is generally produced in the equilibrium mixture; this needs to be enriched for its major applications. The earliest method to enrich fructose involved the complexation of fructose by the addition of borate compounds during isomerization (157). The degree of enrichment depended on the glucose concentration and the amount of borate added. This method resulted in the production of syrups containing 80% fructose. However, the cost of removal and recovery of borate prevented the economic success of this process. The most straightforward complete conversion of glucose to fructose has forever been the dream of corn-milling and -refining industries.

Another route to increase the fructose yield by using D-glucose was to produce a transient overshoot equilibrium concentration of products as described by Schray and Rose (144). Another approach to make 55% fructose is to increase the isomerization temperature (8). Increasing the temperature to more than 70°C leads to an increase in the HFCS concentration by 50% or more. Resinous molecular exclusions have been used to increase the fructose concentration. A syrup containing more than 90% fructose was obtained by forming fructose-oxyanion complexes with germanate (11). Modern chromatographic techniques with ion-exchange resins are the best for separating fructose from glucose. A syrup containing 95% fructose is on the market in France and is sold in crystalline form.

Lowering of Isomerization pH

The optimum pH for isomerization is between 7.0 and 9.0. The activity of the enzyme decreases rapidly at lower pH values. Low pH is preferable for the sake of monosaccharide stability and for the compatibility of the process with saccharification of starch by α-amylase. The most common raw material used for HFCS production is cornstarch manufactured by wet milling of corn. Liquefaction and saccharification of the starch involve participation of α-amylase, glucoamylase, and debranching enzyme, all of which have pH optima in the range of 4.5 to 6.2, whereas that for isomerase is between pH 7.0 and 8.0. A big saving in cost will be possible if the two processes can be carried out simultaneously at the same pH in a single reactor. Isomerization at low pH is advantageous, because it reduces the formation of the colored carbonyl compounds at higher temperatures and may lead to lower costs of ion-exchange and carbon purification. The GI from *Thermus aquaticus* (108) is reported to be active at pH 3.5 and to be fully active at pH 5.5. The term "uni-pH process" implies a process in which liquefaction, saccharification, and isomerization are carried out at the same pH, preferably at pH 4.5 to 5.0, which is the pH optimum for amylase and amyloglucosidase. The presence of Ca^{2+} is a prerequisite for the action of amylase, whereas Ca^{2+} is inhibitory to GI. Acid-stable glucose isomerases which are resistant to inhibition by Ca^{2+} are useful in a uni-pH process. A GI from a *Thermoanaerobacter* sp. was characterized with a view to developing a single-step process for sweetener production (111).

The combination of saccharification and isomerization is an ideal development in the progress of HFCS production, and it is likely to be in operation once an acid-stable, thermostable, and Ca^{2+}-tolerant GI is discovered. Such GIs will be found either by screening or by protein engineering of the existing enzymes used for commercial production of HFCS.

Simultaneous Isomerization and Fermentation of Xylose

The current shortage of petroleum and natural gas has prompted renewed interest in the microbial conversion of pentose-containing renewable biomass resources to ethanol and other useful feedstocks (137). Many yeasts can grow on xylose, but they are inefficient in fermenting the sugar anaerobically and have a very low ethanol tolerance (91). *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Candida tropicalis* are able to ferment xylulose derived from isomerization of xylose with GI in totally anaerobic fermentations (104). Simultaneous isomerization and fermentation of xylose (SIFX) is preferred to isomerization prior to fermentation, because the ratio of xylulose to xylose (1:5) is low at equilibrium. Removal of xylulose from the mixture facilitates conversion of xylose to xylulose, which is simultaneously converted to ethanol by the yeast. The optimum pH for fermentation is 5.0, whereas GI is most stable at neutral pH. Both isomerization and fermentation can occur (103) at a compromise pH of 5.5 or 6.0. Despite the difference in the rates of fermentation of glucose and xylose, final yields of ethanol in SIFX were impressive. Low enzyme levels or inhibition of the enzymes by xylose, xylulose, or ethanol may be responsible for the inefficiency of SIFX. Nevertheless, SIFX provides a significant improvement over existing systems for fermentation of xylose to ethanol. Use of immobilized GI and yeasts may lower the cost of SIFX and the

use of acid-stable GI will contribute to the greater efficiency of SIFX.

FUTURE SCOPE

The ideal GI should possess a lower pH optimum, a higher temperature optimum, a resistance to inhibition by Ca^{2+}, and a higher affinity for glucose than do presently used enzymes. Introduction of all these properties into a single protein is a Herculean task, which has been an obstacle in the development of an economically feasible commercial process for enzymatic isomerization of glucose to fructose. Advances in recombinant DNA technology and protein engineering have opened new and encouraging possibilities of combining the desirable properties in a single organism to produce a tailor-made protein. Reduction of enzyme cost by amplification of the GI gene may cause an increase in fermentation productivity. Isolation of a mutant for the constitutive production of GI and elimination of the requirement of metal ions will contribute significantly to the improvement of the existing processes for HFCS production. Combination of saccharification of starch with isomerization will result in shortening of reaction time and lead to a major saving in terms of equipment cost. However, the major drawback in the development of the uni-pH process is that the wide difference in optimum reaction conditions for the two enzymes tends to lower the efficiency of a simultaneous system. Efforts to produce thermostable and acid-stable GI with higher affinity for glucose by SDM of the GI gene are already under way, with a view to evolving a GI preparation suitable for biotechnological applications.

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