AN ABSTRACT OF THE THESIS OF

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Glucose isomerase is an important industrial enzyme used for the production of high fructose corn syrup. An organism that produced glucose isomerase was isolated from soil and identified as a strain of Streptomyces flavogriseus. Two processes were proposed for the production of glucose isomerase from ryegrass straw. In one process, hemicellulose was extracted from straw with NaOH and used as a substrate for enzyme production. The optimum condition for hemicellulose extraction was to extract straw with 4% NaOH for either 3 h at 90°C or 24 h at room temperature. Under these conditions, up to 15% crude hemicellulose (12% as pentosan) could be obtained. The highest level of the enzyme was obtained on 2% straw hemicellulose. The straw residue remaining after removal of hemicellulose could be used as ruminant feed because the digestibility of the residue increased and the feed efficiency for meadow voles was also improved over untreated straw. Moreover, the spent medium contained hemicellulase which could also be recovered for industrial use. The second process consisted of H_2SO_4

hydrolysis of straw followed by cultivation of S. flavo-griseus on the neutralized hydrolysate. A high level of enzyme was also produced on the hydrolysate of 3% straw prepared with 0.1 N H_2SO_4. Glucose isomerase was the sole product from this process.

As a nitrogen source, corn steep liquor produced the best result. The addition of Mg^{2+}, Mn^{2+}, Fe^{2+} or Zn^{2+} to the growth medium significantly enhanced enzyme production. The organism, however, did not require Co^{2+} when Mg^{2+} or Mn^{2+} was present. The time course of hemicellulose fermentation by S. flavogriseus showed that the highest level of extracellular hemicellulase (0.6 unit/ml), intracellular (3.5 units/ml) and extracellular (1.5 units/ml) glucose isomerase was obtained in about 30, 36 and 72 h respectively.

Cells prepared as acetone powder or by lyophilization gave high activity of glucose isomerase. Cationic detergent (cetyltrimethylammonium bromide or cetylpyridinium chloride) treatment extracted almost the same amount of glucose isomerase with a high specific activity from cells as did mechanical disruption (sonication or abrasive grinding). The enzyme was purified by fractionation with $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose and DEAE-Sephadex A-50 columns. The purified enzyme was homogeneous as shown by ultracentrifugation and SDS-polyacrylamide gel electrophoresis. Benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose were effective in the immobilization of partially purified glucose

isomerase.

No appreciable differences in properties were found between purified soluble enzyme, immobilized enzyme (DEAE-cellulose-glucose isomerase) and heat-treated whole cells. Glucose and xylose served as substrates of the enzyme. Whole cells had the highest Km values for glucose and xylose, but the soluble enzyme had the lowest values. The optimum temperature for activity of the soluble and immobilized enzymes was 70°C while 75°C for whole cells. The pH optimum for the three enzyme preparations was 7.5. Magnesium ion or Co^{2+} was required for enzyme activity; an addition effect resulted from the presence of both Mg^{2+} and Co^{2+}. The enzyme activity was inhibited by Hg^{2+}, Ag^{+} or Cu^{2+}. The conversion ratio of the enzyme for isomerization was about 50%. The soluble and immobilized enzymes showed a greater heat stability than whole cells. The soluble enzyme was stable under a slightly wider pH (5.0-9.0) range than the immobilized enzyme and whole cells (5.5-9.0). The molecular weight of the enzyme determined by the sedimentation equilibrium method was 171,000. A tetrameric structure for the enzyme was also found. After operating at 70°C for 5 days, the remaining enzyme activity of the immobilized enzyme and whole cells which were used for the continuous isomerization of glucose in a plug flow type of column in the presence of Mg^{2+} and Co^{2+} was 75 and 55% respectively. Elimination of Co^{2+} decreased their operational stability.

Studies on Glucose Isomerase from *Streptomyces flavogriseus*

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INTRODUCTION

Glucose (or xylose) isomerase (EC 5.3.1.5) catalyzes the reversible isomerization of glucose to fructose (Tsumura and Sato, 1965b). Almost every known glucose isomerase is an intracellular enzyme which also isomerizes xylose. Because of the ever increasing demand for sucrose and its rising price, considerable effort has been made during the past decade to find alternative sweeteners. Producers of starch syrups have been attempting to increase the sweetness of their syrups in order to use them as sucrose substitutes. Fructose has long been recognized as a good alternate source of sugar due to its relatively high sweetness (1.7 times sweeter than sucrose) and other desirable physical and chemical properties. Thus, the use of glucose isomerase to convert glucose in corn syrup to fructose has been in practice for some time (Mermelstein, 1975). The production of high fructose corn syrup (HFCS) in the world in 1978 was 2.4-2.8 billion pounds (Gray et al., 1978). The estimated potential market for HFCS is 5 billion pounds in 1980 and 6.8 billion pounds in 1985. Production of HFCS is concentrated mainly in the United States, but plant capacity in Europe and the Far East is expanding. Glucose isomerase is therefore of world-wide significance. However, the use of

glucose isomerase is limited by its availability (Bucke, 1977).

Since Marshall and Kooi (1957) reported the ability of Pseudomonas hydrophila to convert D-glucose to D-fructose, a large number of microorganisms that are capable of producing glucose isomerase have been found (Table 1). Most of the previous production methods were not economical, because glucose isomerase formation requires expensive xylose as an inducer. Takasaki (1966) isolated an organism from soil, later identified as Streptomyces albus, which could produce glucose isomerase on xylan or xylan-containing materials. S. bikiniensis and S. flavovirens also produced the enzyme when grown on a medium containing xylan (Japan Bureau of Industrial Technics, 1966b; Park and Toma, 1974). These discoveries provided a way for the economical production of glucose isomerase.

In the Willamette valley of Oregon, over one million tons of ryegrass straw are produced annually and disposed of by open field burning. Public objection to the resulting air pollution necessitates finding alternative means of disposal. Ryegrass straw contains about 25% hemicellulose, approximately 65% of which is xylose (Han et al., 1975). Thus, ryegrass straw could be a good substrate for the production of glucose isomerase if an organism capable of growing and producing the enzyme on straw were found.

Therefore, this study was carried out with the follow-

ing goals: (1) to establish physical and chemical treatments of ryegrass straw for glucose isomerase production by microorganisms, (2) to isolate and identify organism capable of producing a high level of the enzyme on ryegrass straw, (3) to find the optimum conditions for enzyme production, (4) to extract, purify and immobilize the enzyme, (5) to determine properties of soluble enzyme, immobilized enzyme (DEAE-cellulose-glucose isomerase complex) and whole cell, (6) to isomerize D-glucose continuously in a column packed with immobilized enzyme or whole cells.

LITERATURE REVIEW

Extraction of hemicellulose from plants

Cell walls of land plants contain large amounts of cellulose microfibrils embedded in a continuous matrix of hemicellulose, lignin and pectin, of which the hemicellulose predominates. The term "hemicellulose" is generally used to designate those polysaccharides that can be extracted from plants by aqueous alkali. It is known that hemicelluloses are not precursors of cellulose and have no part in cellulose biosynthesis but rather represent a distinct and separate group of plant polysaccharides (Whistler and Richards, 1970). Most hemicelluloses are heteroglycans containing two to four and rarely five or six different types of sugar residues such as D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose and L-rhamnose. The predominant polysaccharide in hemicellulose, known as D-xylan, with backbone chains of (1→4) linked β-D-xylopyranosyl residues, occurs in all land plants (Timell, 1962). Annual plants contain about 15 to 30% of these polymers, hardwood 20-25%, and softwood 7 to 12%.

Most of the hemicelluloses examined have been from woods, cereals and grasses. Hemicellulose can be isolated from plants by extraction with an alkaline solution. The hemicellulose A fraction containing mainly L-arabino-D-xylans is precipitated by neutralization of the extract with acetic acid. The B fraction is precipitated by etha-

nol from the remaining solution (O'Dwyer, 1926). Various alkalies including sodium, potassium or calcium hydroxide, and sodium carbonate are used for the extraction of hemicellulose (Norman, 1937). Preece (1931) determined the hemicellulose content of boxwood by actual isolation and weighing of the fraction soluble in 4% NaOH solution. Buston (1934) used a similar method in examining a number of plant materials. Polyuronide hemicellulose of orchard grass was extracted successively with hot water, 0.5% KOH, and 1.5% KOH. Hemicelluloses were obtained from these extracts as precipitates upon acidification, addition of ethanol, and finally by evaporation of the alcoholic solution (Binger and Sullivan, 1954). Shieh and Cheng (1973) reported that sodium hydroxide solution was more effective than lime water in extracting hemicellulose from bagasse pith of sugarcane. The optimum condition for hemicellulose extraction was to extract with 4% NaOH at 100°C for 3 h. In general, the yield of hemicellulose obtained depends on the alkaline strength of the extractant (Wise et al., 1946)

Hemicelluloses are usually extracted from plant tissues after removal of lipid and lignin. Removal of lignin exposes the hemicellulose for easy dissolution in alkali and permits isolation of pure hemicellulose (Rogers et al., 1947). Lipids are removed by extraction with a hot, azeotropic mixture of benzene and ethanol (kurth, 1939). The commonest method used for removing lignin is by treatment of plant

material with chlorous acid at 70 to 75°C. Such delignified plant material is known as holocellulose. The hemicelluloses are then extracted from the holocellulose with alkaline solution. Anhydrous liquid ammonia is also employed for the pretreatment of wheat straw (Adams, 1958). It causes cellulose to swell and the hemicellulose material becomes more accessible to extraction by the usual alkaline solvents.

Alkaline extraction of plant material can result in many changes in the polysaccharide (Whistler and BeMiller, 1958) even under oxygen-free conditions. Nevertheless, elimination of oxygen during alkaline extraction minimizes oxidative degradation of the hemicellulose.

Acid hydrolysis of plant materials

Large amount of cellulosic wastes are disposed of every year by the agricultural and forest products industries. One potential use of these materials is to hydrolyze them and utilize the resulting monomeric sugars. Microorganisms can be grown on these sugars to produce food, fodder or chemicals (Fagan, 1969; Han and Anderson, 1975; Lekaprayoon, 1973; Meller, 1969).

Acid hydrolysis of wood has been extensively studied (Harris and Beglinger, 1946; Saeman, 1945; Shenard and Kresman, 1945). Acids commonly used are hydrochloric acid and sulfuric acid. Dunning and Lathrop (1945) reported the

saccharification of corncobs to produce xylose and glucose. Converse et al. (1973) and Fagan (1969) studied the acid hydrolysis of cellulose in refuse. The Silvichem process was devised to allow the recovery of pentoses and hexoses without any damage of cellulose and without loss of lignin (Funk, 1975). Nee and Yee (1976) reported that bagasse pith of sugarcane was hydrolyzed to pentose with a yield of 80-90% by using dilute sulfuric acid at a concentration of less than 2% by weight and at a temperature lower than 165°C. Grant et al. (1977) studied the sugar yields from sulfuric acid hydrolysis of annual ryegrass straw and the kinetics of sugar production. Xylose, glucose and mannose were the predominant sugars released. The levels of each sugar produced varied with temperature and heating time. The highest yield of total sugar was obtained by heating the straw at 150°C for 10 min.

Production of glucose isomerase

Microorganisms

Glucose isomerase has been found in a large number of bacteria and actinomycetes, but not in molds and yeasts (Table 1).

Pseudomonas hydrophila (Marshall and Kooi, 1957), Aerobacter cloacae (Tsumura and Sato, 1961), A. aerogenes, Escherichia freundii (Natake and Yoshimura, 1963) and

Table 1. Glucose-isomerase producing microganisms.

Microorganism	Reference
Actinomyces olivocinereus	Ananichev et al. (1978)
A. phaeochromogenes	Volkova and Piskunov (1972)
Actinoplanes missouriensis NRRL B-3342	Scallet et al. (1974) Shieh et al. (1974b)
Aerobacter aerogenes	Natake and Yoshimura (1963)
A. cloacae	Tsumura and Sato (1961)
A. levanicum NRRL B-1678	Shieh et al. (1974a)
Arthrobacter spp. NRRL B-3724, 3725, 3726, 3727, 3728, ATCC-21748	Lee et al. (1972) R.J.Reynolds Tobacco Co. (1973) Standard Brands Inc. (1975)
Bacillus spp. NRRL B-5350, 5351	Outtrup (1974)
B. coagulans HN-68, NRRL B-5649—5666	Aunstrup et al. (1972) Yoshimura et al. (1966)
B. megaterium	Takasaki and Tanabe (1962)
B. stearothermophilus	Suekane et al. (1974)
Brevibacterium incertum NRRL B-5383	Coker and Gardner (1976)
B. pentosoaminoacidicum	Ichimura (1965)
Corynebacterium incertum ATCC-31261	Kojima et al. (1976)
Curtobacterium helvolum NCLB-10352	Kelly and Meers (1974)
Escherichia freundii	Natake and Yoshimura (1963)
E. intermedia	Natake and Yoshimura (1964)
Flavobacterium arborescens	Lee (1977)
F. devorans NRRL B-5384, ATCC-10829	Coker and Gardner (1976)
Lactobacillus sp. D-80	Kiangsi Scientific Res. Inst. of Food and Ferment. Industry (1975)
L. brevis	Yamanaka (1963a)

Continued

L. buchneri	Yamanaka (1963a)
L. fermenti	" "
L. gayonii	" "
L. lycopersici	" "
L. mannitopoeus	" "
L. pentoaceticus	" "
Leuconostoc mesenteroides	" "
Microbispora rosea	Horwath et al. (1973)
Microellobospora flavea	" "
Micromonospora coerula	" "
Mycobacterium sp. 279	Szumilo and Szymona (1971)
Norcardia asteroides	Horwath et al. (1973)
N. corallia IF-3338	Sato and Tanaka (1974)
N. dassonvillei IMRU-509	Horwath et al. (1973)
Paracolobactrum aerogenoides	Takasaki and Tanabe (1964)
Pseudomonas hydrophila	Marshall and Kooi (1957)
Streptomyces sp.	Chou et al. (1976)
Streptomyces sp. S41-10	Lai (1976)
S. achinatus	Agency of Industrial Science and Technology (1968)
S. achromogenus	" "
S. albus YT-4, YT-5, YT-6, NRRL B-5778	Sanchez and Smiley (1975) Takasaki (1974) Takasaki and Kambayashi (1970) Takasaki and Tanabe (1969)
S. bikiniensis	Park and Toma (1974)
S. bodiliae	Japan Bureau of Industrial Technics (1966)
S. cinnamonensis	Joseph et al. (1977)
S. echinatus	Agency of Industrial Science and Technology (1968)
S. flavovirens	" "
S. fradiae	Mand et al. (1977)
S. galbus	Buki et al. (1976)

Continued

S. glaucescens NRRL B-8071, ETH-22794	Weber (1974, 1976)
S. gracitus	Buki et al. (1976)
S. griseus	Hsu and Shen (1964)
S. hivens	Buki et al. (1976)
S. matensis	" "
S. nivens	" "
S. olivaceus NRRL B-3583, 3916	Brownewell (1971) Brownewell and Streets (1972)
S. olivochromogenes ATCC-15486, 21114, 21713, 21714, 21715	Armbruster et al. (1973) Coker and Gardner (1976) Cory (1971)
S. phaeochromogenes SK, NRRL B-3559, ATCC-15486	Coker and Gardner (1976) Stranberg and Smiley (1971) Tsumura and Sato (1965b)
S. platensis	Buki et al. (1976)
S. venezuellae ATCC-21113	Iisuka et al. (1972)
S. wedmorensis ATCC-21230, 21175, 21176	Agency of Industrial Science and Technology (1974) Dworschack and Lamm (1971)
Streptosporangium album	Nonomura and Kaji (1974)
S. oulgare	" "

E. intermedia (Natake and Yoshimura, 1964) produced the enzyme which required arsenate for its activity. The need to include arsenate in the reaction mixture for glucose isomerization meant that these enzymes could not be exploited commercially.

Yamanaka (1963a) first discovered that heterolactic acid bacteria could produce glucose isomerase. Among these, L. brevis produced the highest yield of the enzyme. Although this enzyme appears to have attractive properties for economic development, in particular its low pH optimum (Yamanaka, 1968), it is less stable at high temperature than its competitors. Consequently, it has not been developed commercially (Bucke, 1977).

Streptomyces species have been the most extensively studied and used as a source of the enzyme. The first organism discovered that was capable of producing glucose isomerase in this genus was S. phaeochromogenes SK (Tsumura and Sato, 1965b). Since then, at least 23 other species have been reported to have the capability of producing glucose isomerase (Table 1). Cotter et al. (1971) reported that S. albus YT-4 and YT-5 were efficient producers of the enzyme. S. wedmorensis ATCC-21175 was the source of the glucose isomerase used to produce 500,000 tons of high fructose corn syrup in 1974 (Schnyder, 1974). Meanwhile, S. venezuellae ATCC-21113 and S. olivochromogenes ATCC-21114 were studied by Iisuka et al. (1972). Miles Laboratories,

Inc. (1974) produced glucose isomerase using S. olivaceus NRRL B-3588. A mutant of this organism, NRRL B-3916, is the source of the enzyme used on an industrial scale by Miles-Cargill Inc. Weber (1975) isolated a strain of S. glaucescenes ETC-22794 which was distinguished by its ability to produce comparatively large amount of extracellular glucose isomerase. A mutant of S. olivochromogenes ATCC-21114 was also the enzyme source used in the production of Corn products' high fructose corn syrup (invertose) (Godzicki, 1975).

Besides Streptomyces, another organism belonging to actinomycetes, A. missouriensis is also a very potent producer of glucose isomerase (Shieh et al., 1974) and is used commercially by Anheuser-Busch Inc. Microellobospora, Micromonosporд and Norcardia also produce glucose isomerase as demonstrated by Standard Brands Inc. (1975).

Extensive study of glucose isomerase production from B. coagulans HN-68 has been done by Yoshimura et al. (1966). A commercial product made from an atypical variant of B. coagulans was reported by Outtrup (1974). Suekane et al. (1974) isolated a thermostable glucose isomerase from B. stearothermophilus.

Some organisms not previously mentioned also have industrial importance including Arthrobacter sp. NRRL B-3726, 3727, 3728 (Lee et al., 1972; R. J. Reynolds Tabacco Co., 1973) and A. levanicum (Shieh et al., 1974a)

Cultural conditions

As shown in Table 2, most of the organisms require xylose to induce glucose isomerase production. However, pure xylose is too expensive to be used in a commercial fermentation process. Takasaki (1966, 1973, 1974) isolated S. albus YT-4, YT-5 and YT-6 which could produce glucose isomerase on xylan or xylan-containing materials such as wheat bran, corn hulls and corncobs. Streptomyces bikiniensis and S. flavovirens also produce the enzyme when grown on a medium containing xylan (Japan Bureau of Industrial Technics, 1966b; Park and Toma, 1974). In addition to xylose and xylan, lactose, mannose, lactate, mannitol, sorbitol, and glycerol were also reported to be an inducer for enzyme production (Table 2). Dworschack et al. (1971) used acid hydrolysate of corncobs to supply xylose for enzyme induction by S. wedmorensis ATCC-21175. The combination of xylose and glucose, starch or dextrin was used by several organisms to produce glucose isomerase (Table 2). The Agency of Industrial Science and Technology (1974) demonstrated that low levels of xylose, xylobiose or xylose plus xylobiose could be used as the carbon source for enzyme formation. Paracolobactrum aerogenoides could produce glucose isomerase on a medium containing glucose as a sole source of carbon, but the enzyme required NAD for its activity (Takasaki and Tanabe, 1964). Thus, this process has never been exploited commercially. Arthrobacter sp.

Table 2. Cultural conditions for the production of glucose isomerase.

Microorganism	Carbon source	Nitrogen source	Mineral	pH	Temperature (°C)	Time (h)	Reference
Actimoplanes missouriensis NRRL B-3342	Beet molasses	Soy flour or corn steep liquor + $NaNO_3$	$MgSO_4 \cdot 7H_2O$ + KCl + $FeSO_4 \cdot 7H_2O$	7.0	30	72	Shieh et al.(1974b) Shieh (1976, 1977)
Aerobacter aerogenes	Xylose or mannose or lactate or mannitol	$(NH_4)_2HPO_4$	$MgSO_4 \cdot 7H_2O$	--	28	48	Natake and Yoshimura (1963)
A. cloacae	Xylose	$(NH_4)_2HPO_4$	$MgSO_4 \cdot 7H_2O$ + KH_2PO_4	6.8-7.0	30	24	Tsumura and Sato (1961)
A. levaniuum NRRL B-1678	Xylose or birch wood sulfite liquor	Yeast extract	$CaSO_4 \cdot 7H_2O$ + KCl	7.5	23	24	Shieh et al.(1974a)
Arthrobacter NRRL B-3726, 3727, 3728	Glucose	Meat protein + yeast extract + $(NH_4)_2SO_4$	KH_2PO_4 + $MgSO_4 \cdot 7H_2O$	--	--	--	Lee et al.(1972)
Arthrobacter NRRL B-3728	Xylose + glucose	--	Mg^{2+} + Cu^{2+} + Mn^{2+} + Zn^{2+} + Ca^{2+} + PO_4^{-3} + MO_4^{-2}	6.9	30	--	Meers (1976)
Bacillus coagulans HN-68	Xylose	Yeast extract +NH_4Cl	$MgSO_4 \cdot 7H_2O$ + $MnSO_4 \cdot 4H_2O$ + $CaCO_3$	--	40	14-16	Yoshimura et al. (1966)
B. coagulans NRRL-5650 (mutant)	Glucose	Corn steep liquor + $(NH_4)_2SO_4$	$MgSO_4 \cdot 7H_2O$ + $MnSO_4 \cdot 4H_2O$ + K_2HPO_4	6.8	50	13	Novo Industri A/S (1977)
B. coagulans NRRL 5649-66 (mutant)	Xylose	$(NH_4)_2SO_4$	--	--	60	--	Outtrup (1974)
B. stearothermophilus	Xylose	Peptone + corn steep liquor + yeast extract + meat extract	NaCl + $MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$	--	55	--	Suekane (1974)

Continued

Bacillus sp. NRRL B-5350, 5351	Xylose + glucose	Peptone + casein	--	--	38	17	Aunstrup et al. (1972)
Corynebacterium candidus ATCC-31261	Xylose	Peptone	$MgSO_4 \cdot 7H_2O$ + K_2HPO_4 + $Na_2HPO_4 \cdot 10H_2O$	--	30	48	Kojima et al. (1976)
Curtobacterium helvolum NLLB-10352	Xylose	Meat extract + yeast extract + peptone	NaCl + $MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$	--	30	24	Kelly and Meers (1974)
Escherichia intermedia	Xylose	NH_4Cl	$MgSO_4 \cdot 7H_2O$ + K_2HPO_4	--	28	20	Natake and Yoshimura (1964)
Flavobacterium arborescens	Lactose	Protein hydrolysate + yeast extract	K_2HPO_4 + KH_2PO_4	--	30	72	Lee (1977)
Lactobacillus brevis	Xylose	Peptone + yeast extract	Na-acetate + $MgSO_4 \cdot 7H_2O$ + NaCl + $FeSO_4 \cdot 7H_2O$	--	37	16-20	Yamanaka (1963a)
Microbispora rosea nonnitrogenes	Xylose + dextrin	Corn steep liquor + yeast extract + $(NH_4)_2HPO_4$	$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$	--	30	65	Nonomura and Kaji (1974)
Norcardia corallia	Xylose	Meat extract + peptone + NH_4NO_3	$MgSO_4 \cdot 7H_2O$ + KH_2PO_4 + KCl + $CoCl_2 \cdot 6H_2O$ + $MnSO_4 \cdot 4H_2O$	7.0	30	72	Sato and Tanaka (1974)
N. dassovillei	Xylose + glucose + sorbitol	Corn steep liquor	$CoCl_2 \cdot 6H_2O$	--	30	--	Horwath et al. (1973)
Paracolobactrum aerogenoides	Glucose	NH_4Cl	$MgSO_4 \cdot 7H_2O$ + KH_2PO_4 + $Na_2HPO_4 \cdot 12H_2O$ + NaCl	--	30	--	Takasaki and Tanabe (1964)
Streptomyces sp.	Xylose	Tryptone + yeast extract	$MgSO_4 \cdot 7H_2O$	7.0-7.2	30	96	Chou et al. (1976)

Continued

S. albus YT-4	Wheat bran	Corn steep liquor	$CoCl_2 \cdot 6H_2O$	--	30	30	Takasaki (1973)
S. albus YT-5	Wheat bran or xylose or xylan	Corn steep liquor	$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$	--	30	20-24	Takasaki (1966)
S. albus YT-6	Wheat bran	Corn steep liquor	$CoCl_2 \cdot 6H_2O$	--	45	20	Takasaki (1974)
S. bikiniensis	Xylose or xylan	Yeast extract + peptone + beef extract	$MgSO_4 \cdot 7H_2O$ + NaCl	--	--	40	Park and Toma (1974)
S. bodiliae	xylose or sorbitol or glycerol	Polypeptone	$MgSO_4 \cdot 7H_2O$ + K_2HPO_4	--	30	20	Japan Bureau of Industrial Technics (1966a)
S. flavovirens	Xylan	Polypeptone	$MgSO_4 \cdot 7H_2O$ + K_2HPO_4	--	30	--	Japan Bureau of Industrial Technics (1966b)
S. galbus	Sorbitol + glucose + xylose	Corn steep liquor	$CoCl_2 \cdot 6H_2O$	7.0	28	40	Buki et al.(1976)
S. olivaceus NRRL B-3916	Xylose + Corn starch	Peptone + beef extract + yeast extract	$MgSO_4 \cdot 7H_2O$ + NaCl	7.0	--	--	Brownewell and Streets (1972)
S. olivochromogenes ATCC-21114	Xylose + soluble potato starch	Peptone + meat extract + yeast extract	$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$ + NaCl	--	28	48	Iisuka et al. (1971)
S. phaeochromogenes SK	Xylose	Peptone + meat extract + yeast extract	$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$ + NaCl	--	28-30	24	Tsumura et al. (1967b)
S. venezuellae ATCC-21113	Xylose + soluble potato starch	Peptone + meat extract + yeast extract	$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$ + NaCl	--	28	48	Iisuka et al. (1971)
S. wedmorensis ATCC-21175	Acid hydrolysate of corncob	Corn steep liquor	$CoCl_2 \cdot 6H_2O$	7.0	30	40	Dworschack et al. (1971)
Streptosporangium album or S. oulgare	Xylose + dextrin	Corn steep liquor + yeast extract + $(NH_4)_2HPO_4$	$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$	--	30	65	Nonomura and Kaji (1974)

NRRL B-3726, 3727, 3728 were capable of producing the enzyme in the presence of glucose instead of xylose (Lee et al., R. J. Reynolds Tabacco Co., 1973). Similarly, A. missouriensis produced glucose isomerase when supplied with beet molasses and corn steep liquor (Shieh, 1976, 1977). However, the presence of xylose significantly increased the production of the enzyme. The CPC International Inc. (1973) mutants appeared to be the first Streptomyces strain capable of producing the enzyme free from xylose or xylose-containing materials, but it is preferable and more economical to cultivate the organism on a medium containing xylose.

Various organic nitrogen sources including corn steep liquor, peptone, polypeptone, tryptone, meat extract, yeast extract, casein, and soy flour have been used for glucose isomerase production by actinomycetes, Arthrobacter, Bacillus and Lactobacillus etc. (Table 2). Among these, corn steep liquor appears to be the most efficient and is used most commonly. Aerobacter, Bacillus, Escherichia and Paracolobactrum could utilize inorganic nitrogen sources such as NH_4Cl, $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ for enzyme production.

Certain mineral salts, $MgSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$ or $MnSO_4 \cdot 4H_2O$ have been utilized very frequently by organisms for the production of glucose isomerase (Table 2). The culture medium usually includes two or more of these mineral salts to achieve a high yield of the enzyme. Several organisms

which do not require cobalt in order to produce glucose isomerase are also shown in Table 2.

Table 2 indicates that the pH of the culture medium used in glucose isomerase production is in the neutral range (6.8-7.2).

Glucose isomerase producing organisms can be classified into mesophiles and thermophiles. Most of the organisms shown in Table 2 are mesophiles. Only very few belong to the thermophiles such as B. coagulans, B. stearothermophilus and S. albus YT-6.

Organisms usually require around 24 h to reach their maximum yield of glucose isomerase (Table 2). As few as 13-16 h is enough for B. coagulans, but some organisms need much longer (72-96 h) for maximum production of the enzyme.

Extraction of glucose isomerase

Microbial enzymes are usually extracted by mechanical disruption (Sonication, abrasive grinding, homogenization, or treatment with a French press) of the cell wall. The commonest method used in laboratories for the extraction of glucose isomerase is sonication (Chou et al., 1976; Stranberg and Smiley, 1971; Takasaki and Tanabe, 1966; Tsumura and Sato, 1965a). Abrasive grinding is also employed for enzyme extraction (Natake and Yoshimura, 1964; Yamanaka, 1968). However, mechanical disruption is time consuming and expensive and is not suited to large-scale production.

Glucose isomerase is easily liberated by autolysis. Cationic detergents such as cetylpyridinium chloride, dimethylbenzylalkylammonium chloride or octadecyltrimethylammonium chloride are utilized in the autolysis of cell suspensions containing glucose isomerase (Cotter et al., 1977; Lloyd and Khaleeluddin, 1976; Takasaki and Kanbayashi, 1969a; Takasaki et al., 1969). Besides detergents, lysozyme, toluene or a combination of lysozyme and toluene is used for the disintegration of the cell walls of enzyme producing organisms (Hirota et al., 1977; Sipos, 1973; Suekane et al., 1974). Cory (1977) reported that 2-propanol could be used for breaking cells of S. olivochromogenes. The nucleic acids were insoluble in 2-propanol and precipitated, but the enzyme was soluble. The enzyme solution obtained was highly stable, retaining 95% of its activity after 30 days storage at 26°C. A stable glucose isomerase solution was prepared by addition of p-hydroxylbenzoate ester, alcohols, Na, K or NH_4 salt of a mineral acid, Na or K salt of polyphosphate, antibiotics, cationic or amphoteric or detergents and adjusted to pH 9.0-12.0 (Okada and Imata, 1977). Bacterial contamination and inactivation of the enzyme were further prevented by addition of NaCl at a pH value of > 9.0.

Purification of glucose isomerase

Although numerous glucose isomerases have been produced

from different organisms, very few of them have been purified to a homogeneous state. The enzyme from L. brevis was partially purified by treatment with $MnCl_2$, fractionation using $(NH_4)_2SO_4$, heat treatment and chromatography on DEAE-Sephadex (Yamanaka, 1963b). Later, Yamanaka (1968) purified and crystallized the enzyme using techniques similar to those employed earlier. The purified enzyme was homogeneous as shown by ultracentrifugation. Purification of glucose isomerase from E. intermedia was done by Natake (1966). The enzyme was purified by treating $MnSO_4$, rivanol and DEAE-Sephadex column chromatography. An enzyme preparation purified about 180-fold was obtained by these procedures. The purified enzyme was proved to be pure by using polyacrylamide gel electrophoresis analysis. Glucose isomerase from B. coagulans HN-80 was purified by $MnSO_4$ treatment, $(NH_4)_2SO_4$ fractionation, chromatography on a DEAE-Sephadex column (Danno et al., 1967). The purified enzyme was homogeneous as determined by polyacrylamide gel electrophoresis and ultracentrifugation. Danno (1970) further purified the enzyme in a crystalline form by using similar procedures. Acetone fractionation, DEAE-cellulose and DEAE-Sephadex column chromatography, and crystallization were used to purify glucose isomerase from S. albus YT-5 (Takasaki et al., 1969). The purified enzyme appeared to be homogeneous on ultracentrifugation and electrophoresis analysis. Glucose isomerases from S. bikiniensis and S.

olivochromogenes were also purified to a homogeneous state by $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex chromatography and gel filtration on Sephadex (Park, 1977; Suekane et al., 1978). Recently, Ananichev et al. (1978) reported that purification of the enzyme from A. olivocinereus by chromatography on DEAE-cellulose alone gave a preparation which was homogeneous as shown by analytical electrophoresis and ultracentrifugation analysis.

Affinity chromatography was reported by Lee et al. (1976a) to be used in the purification of glucose isomerase. Xylitol linked to CNBr-activated Sepharose 4 B by dicyclohexylcarbdiimide condensation was used as the ligand of the affinity chromatography and packed into a column on which glucose isomerase was retained from a buffer solution. The enzyme could be eluted nonspecifically with NaCl. The best purification was 1.5-fold. Glucose isomerase was also purified by a porous anionic exchange resin (trimethylammonium type, SO_4^{2-} form). The resin was packed in a column and the enzyme was eluted with 0.5 N NaCl. The specific activity of the eluted glucose isomerase increased 12.4-fold (Fujita et al., 1976).

Properties of glucose isomerase

Glucose isomerase has been found in various organisms and its enzymatic and physiochemical properties have been extensively studied (Table 3).

Table 3. Summary of properties of glucose isomerases.

Microorganism	Substrate specificity	Km (M)	Temp. optimum (°C)	pH optimum	Metal requirement	Inhibitor	Heat stability (%)	pH stability	$\bar{v}$ (ml/g)	$s_{20,w}$	M. (dalton)	No. of subunit	Reference
Actinoplanes missouriensis	Glucose, xylose, ribose	--	--	7.0	Mg^{2+}, Co^{2+}	--	--	--	--	--	--	--	Scallet et al. (1974)
Aerobacter cloacae	Glucose, xylose	--	50	7.6	As^{3+}, Mg^{2+}	--	--	--	--	--	--	--	Tsumura and Sato (1965a)
Bacillus coagulans* HN-68	Glucose, xylose, ribose	0.09, 0.07, --	75	7.0	Mg^{2+}, Co^{2+}, Mn^{2+}	Cu^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+}	100 (70°C, 10')	--	0.705	10.40	175,000	4	Danno (1970), Danno et al. (1967)
B. stearotnermophilus	Glucose, xylose, ribose, arabinose	--	80	7.5-8.0	Mg^{2+}, Co^{2+}	--	--	--	0.736	9.35	130,000	--	Suekane et al. (1978)
Escherichia intermedia*	Glucose, xylose, 2-deoxy-glucose	--	50	7.0	As^{3+}	--	0 (60°C, 10')	7-9	--	--	--	--	Natake (1966)
Lactobacillus brevis*	Glucose, xylose, ribose	0.92, 0.005, 0.67	--	6.0-7.0	Mn^{2+}, Co^{2+}	Xylitol, arabitol, lyxose	10 (60°C, 30')	--	--	11.46	191,000	--	Yamanaka (1968)
Streptomyces sp.	Glucose, xylose	0.40	80	7.0-8.0	Mg^{2+}	--	50 (70°C, 120h)	--	--	--	--	--	Chou et al. (1976)
S. albus YT-5	Glucose, xylose	0.16, 0.032	80	8.0-8.5	Mg^{2+} or Co^{2+}	Heavy metals (Ag^{+}, Cu^{2+}, Hg^{2+})	90 (70°C, 10')	4-11	0.69	8.00	157,000	4	Takasaki et al. (1969)
S. albus NRRL B-5778	Glucose, xylose, ribose, arabinose, rhamnose, allose	0.086, 0.093, 0.350, -- -- --	70-80	--	Mg^{2+}, Co^{2+}	Sorbitol	--	--	--	--	--	--	Sanchez and Smiley (1975)

Continued

S. bikiniensis*	Glucose, xylose, ribose, rhamnose	--	80	8.0-9.0	Mg^{2+}, Co^{2+}	--	--	--	--	--	52,000	--	Park (1977)
S. olivochromogenes*	Glucose, xylose, ribose, arabinose	--	80	8.0-9.0	Mg^{2+}, Co^{2+}	--	--	--	0.725	7.55	120,000	2	Suekane et al. (1978)
S. phaeochromogenes SK	Glucose, xylose	0.3, --	90	9.3-9.5	Mg^{2+}, Co^{2+}	Tris	96 (80°C, 10')	--	--	--	--	--	Tsumura and Sato (1965b)
S. phaeochromogenes NRRL B-3559	Glucose, xylose	0.25, --	80	8.0	Mg^{2+}	--	40 (70°C, 24h)	--	--	--	--	--	Stranberg and Smiley (1971)

* The enzyme is homogeneous.

Pentoses, hexoses, sugar alcohols, sugar phosphates and deoxy-sugar were examined as substrates for the enzyme. In addition to D-glucose and D-xylose, D-ribose, L-arabinose, L-rhammose, D-allose and 2-deoxy-glucose were also found to be the substrate of the enzyme (Table 3). The Km values for D-glucose, D-xylose and D-ribose, varied from organism to organism, but was in the range, 0.086-0.920, 0.005-0.093, 0.35-0.67 M respectively.

Most of the glucose isomerases listed in Table 3 have a fairly high optimum temperature, around 80°C. The optimum temperature of the enzyme obtained from S. phaeochromogenes increased to 90°C in the presence of Co^{2+}, whereas 80°C without Co^{2+} (Tsumura and Sato, 1965b). The arsenate-requiring glucose isomerases from A. cloacae and E. intermedia have a significantly lower temperature optimum (50°C) than the others.

Glucose isomerase usually functions well in the pH range 7.0 to 9.0 (Table 3). It seems that the pH optimum of the enzyme from Streptomyces is higher than that from other bacteria.

Glucose isomerase in general requires a divalent cation such as Mg^{2+}, Co^{2+}, Mn^{2+} or a combination of these cations for its catalytic activity (Table 3). In some cases, As^{3+} is essential for enzyme action.

The catalytic activity of glucose isomerase is inhibited by Hg^{2+}, Cu^{2+}, Ag^{+}, Zn^{2+}, Ni^{2+} and to some extent by

Ca^{2+} (Table 3). Other inhibitors are xylitol, arabitol, sorbitol, mannitol, lyxose and Tris.

Glucose isomerase from Streptomyces and Bacillus was quite stable at high temperatures (Table 3), but the enzyme from Lactobacillus and Escherichia showed much less thermal stability.

Very few studies on the pH stability of glucose isomerase have been made. As shown in Table 3, the enzyme from E. intermedia and S. albus YT-5 was stable in the pH range 7.0 to 9.0 and 4.0 to 11.0 respectively.

Only small differences exist in the partial specific volumes ($\bar{v}$) of the glucose isomerases shown in Table 3. On the contrary, the sedimentation coefficient ($s_{20,w}$) and molecular weight (M) of the enzyme varied remarkably, from 7.55 to 11.46 s and from 52,000 to as high as 191,000 daltons, depending upon the source of the enzyme.

Table 4 summarizes the conversion ratios of D-glucose to D-fructose catalyzed by glucose isomerases from various organisms. As shown in the table, there is a big difference in conversion ratios which are from 26.4 to 59.0%.

Immobilization of whole cells containing glucose isomerase

The study of immobilized microorganism is a rapidly expanding research area with a vast amount of literature available. It is beyond the scope of this thesis to review extensively all the published works. The task herein then,

Table 4. Conversion ratios of glucose to fructose catalyzed by glucose isomerases.

Microorganism	Whole cell or free enzyme	Glucose conc. (%)	Metal	Assay temp. (°C)	Assay pH	Time (h)	Batch or continuous reaction	Conversion ratio (%)	Reference
Actinoplanes missouriensis	Free enzyme	--	--	70-90	7.0-7.5	--	--	51.0-53.6	Scallet et al. (1974)
Aerobacter aerogenes	Whole cell	--	As^{3+}	39	6.8	--	Batch	30.0	Natake (1963)
A. cloacae	Free enzyme	--	Mn^{2+}, Co^{2+}, Mg^{2+}, As^{3+}	50	7.6	--	Batch	45.0-48.0	Sato and Tsumura (1964)
A. levanicum NRRL B-1678	Whole cell	36	Mg^{2+}, Co^{2+}	55	6.8	72	Batch	50.3	Shieh et al. (1974a)
Arthrobacter sp. ATCC-21748	Whole cell	61	Co^{2+}, Mg^{2+}	65	--	--	Continuous	26.4	Standard Brands Inc. (1975)
Bacillus coagulans HN-68	Free enzyme	2	Co^{2+}	70	--	8	Batch	50.0	Danno et al. (1967)
Brevibacterium pentosoamino-acidinum	Whole cell	--	Co^{2+}	90	8.3	--	--	50.0	Ichimura (1965)
Curtobacterium helvolum NCLB-10352	Whole cell	50	Mg^{2+}	65	--	48	Batch	42.0	Kelly and Meers (1974)
Escherichia intermedia	Free enzyme	36	As^{3+}	40	7.0	24	Batch	45.0	Natake (1966)
Flavobacterium arborescens	--	50	Mg^{2+}	60	8.0	20	Batch	45.0	Lee (1977)
Lactobacillus sp. D-80	--	30-40	Mn^{2+}	60-65	6.5-6.7	--	Batch	40.0	Kiangsi Scientific Res. Inst. of Food and Ferment. Industry (1975)
Microbispora rosea nonnitrogenes	--	--	--	--	--	64	Batch	45.3	Nonomura and Kaji (1974)
Norcardia corallia IF-3338	Free enzyme	40	Co^{2+}, Mn^{2+}, Mg^{2+}	70	7.0	15	Batch	42.4	Sato and Tanaka (1974)

Continued

Pseudomonas hydrophila	Whole cell	--	Mg^{2+}, Mn^{2+}, As^{3+}	42	8.5	--	Batch	33.0	Marshall (1960)
Streptomyces albus	Whole cell	50	Mg^{2+}, Co^{2+}	70	7.0	80	Batch	45.0	Takasaki (1966)
S. cinnamonensis or S. fradiae	Whole cell	27	--	60	--	24	Batch	59.0	Joseph et al. (1977)
S. glaucescens NRRL B-8071	Whole cell	50	Mg^{2+}, Co^{2+}	65	7.0	19	Batch	40.0	Weber (1976)
S. olivaceus NRRL B-3916	--	--	--	60	7.7-7.9	20	Batch	40.2	Brownewell and Streets (1972)
S. phaeochromogenes SK	Whole cell	--	Mg^{2+}, Co^{2+}	60	8.0-9.0	--	--	52.0	Tsumura and Sato (1965b)
Streptosporangium album	--	--	--	65	--	64	Batch	45.0	Nonomura and Kaji (1974)

is to highlight important works related to this study. Methods used to immobilize microorganisms containing glucose isomerase can be divided into five categories: (1) heat treatment, (2) flocculation, (3) entrapment, (4) adsorption, (5) crosslinking.

As mentioned earlier, almost every known glucose isomerase is an intracellular enzyme; in many cases whole microbial cells have been used in laboratories to isomerize glucose (Takasaki et al., 1969; Tsumura et al., 1967a; Yoshimura et al., 1966). In early industrial processes, isomerization of corn syrup at Clinton Corn Processing Co. was catalyzed in batch reactors with a soluble glucose isomerase (Mermelstein, 1975). Takasaki and Kanbayashi (1969b) described an improved method using heat treated (60-85°C, 10 min) S. albus containing the enzyme in a packed bed through which glucose was passed. This method was also applied to cells of S. phaeochromogenes (Ryu et al., 1977). Spray-dried cells of A. missouriensis was used by Anheuser-Busch Inc. in an industrial process (Scallet et al., 1974). Lamm et al. (1974) described the immobilization of Streptomyces by incubating the cells in aqueous solutions of a wide range of inorganic salts in which $CoCl_2$ was the most effective salt. Heat-treated Streptomyces was dipped in a citrate solution and then air dried to immobilize glucose isomerase within cells (Tsumura and Kasumi, 1977). No activity was lost by this treatment.

A method developed by Heady and Jacaway (1974) involved the flocculation of cells with $MgCO_3$ or diatomaceous earth and then drying. This preparation showed some advantages over using cells in batch process. Lloyd et al. (1974) described a similar method in which whole cells of Arthrobacter sp. were trapped in filter aid within a leaf filter and used continuously, with little loss of activity, for 15 days. Polyelectrolyte flocculating agents were also utilized to flocculate the cells of Arthrobacter, B. coagulans and S. albus (Lee and Long, 1974; Matsudaira and Ishige, 1976; R. J. Reynolds Tabacco Co., 1974). A preparation with a half life of 25 days at 65°C was obtained by flocculation of L. brevis with chitosan (Tsumura et al., 1976).

Entrapment of cells in gels or fibers has been widely employed in many organisms containing glucose isomerase. The cells of several Streptomyces species including S. griseus, S. olivaceus and S. phaeochromogenes were reported to be entrapped in polyacrylamide gels and used in a continuous process (Chibata et al., 1974; Ohwaki and Minami, 1975). Kolarik et al. (1974) immobilized whole cells of B. coagulans in cellulose acetate. However, this preparation has the following shortcomings: leakage, a short operational life and a poor diffusion rate. Takahashi (1978a) treated cells of S. albus YT-5 with castor oil and tolylene diisocyanate to immobilize the cells. The immobilized

enzyme did not swell in a glucose solution and had good physical strength and water penetration. Approximately 64% of the initial enzyme activity was present in the immobilized preparation which was stable for 30 days without a significant decrease in enzyme activity. Takahashi (1978b) also reported that the same organism was treated with a drying tung oil to immobilize the cells. The immobilized cell preparation did not swell in hot water, had good water penetration and contained about 36% of the initial enzyme activity.

A stable immobilized cell system was obtained by adsorption of cells of Streptomyces on DEAE-Sephadex A-50 (Shigesada et al., 1975). Eightly percent of the original activity was retained in a column after operating 40 days at 60°C. Kasumi et al. (1975) used a similar method to adsorb cells on DEAE-cellulose or DEAE-Sephadex which was then employed in a continuous isomerization process.

Whole cells containing glucose isomerase have been polymerized by crosslinking with bifunctional or other reagents. Miles Laboratories Inc. (1974) treated cells of S. olivaceus with glutaraldehyde which resulted in a stable immobilized cells. A method developed by Moskowitz (1974) was used to crosslink whole cells of S. phaeochromogenes using diazotized primary diamino compounds. Forty eight percent of the activity was remained after 20 cycles of batchwise operations. The cells of Streptomyces were

crosslinked with diazotized benzidine and the half life of this preparation was 5 days (Lartique and Weetall, 1976).

Several successful techniques were evolved as combinations of the basic methods. Vieth et al. (1973) immobilized cells of S. phaeochromogenes by first heat treating them and then stirring them with a slurry of hide collagen, adjusting the pH to 12, and casting the mixture on a surface. The resulting preparation was tanned using glutaraldehyde or formaldehyde and was used continuously at 70°C for 4 days in a plug-flow type of reactor. Linko et al. (1976) entrapped cells of A. missouriensis in cellulose fibers and then crosslinked them with glutaraldehyde. This preparation was quite stable during 20 days of continuous operation in a column. The half life was about 40 days. Immobilized cells of S. phaeochromogenes with a half life of 60 days were prepared by heating the cells at 70-80°C, lyophilizing, mixing with gelatin or sodium caseinate and then steeping in acetone containing glutaraldehyde to gelatinize (Nakajima et al., 1978). Streptomyces cells possessing glucose isomerase were heat treated and confined within polyester sacs and used in batch or continuous isomerization of enzymically hydrolyzed microcrystalline cellulose. Continuous operation of the column packed with the resulting preparation showed an exponential inactivation of enzyme activity with a half life of 447 h. Inoue et al. (1978) patented a process in

which cells of S. phaeochromogenes were treated with chitosan or partially acylated chitosan solution and then with an organic acid chloride to yield immobilized glucose isomerase with a low swelling rate and high strength.

Miscellaneous methods such as mixing of cells containing glucose isomerase with a photopolymerizable resin and then irradiating the mixture with U. V. light or γ-ray (Fukui et al., 1977; Iida and Hasegawa, 1977). Takasaki (1976) described a novel method in which a glucoamylase produced by a Rhizopus species was covalently linked to the bifunctional reagent treated mycelium pellets of Streptomyces species that produced glucose isomerase. Immobilization of glucoamylase on mycelia containing glucose isomerase enables fructose to be produced directly from starch.

Immobilization of soluble glucose isomerase

Although immobilized cells have the advantage of not requiring enzyme isolation or purification steps when compared to immobilized enzymes, they are usually not as stable as the latter (Abbott, 1977). In addition, enzyme immobilized on the supports selected gave an excellent flow properties and high activity per unit volume (Weetall, 1973). Thus, considerable efforts have been made to find promising methods for the immobilization of soluble enzymes. It was found that more basic methods are available for

immobilizing soluble enzymes than for whole cells mentioned earlier. At the present time there are no good methods for covalently binding cells to solid matrics but this technique is successfully employed for the immobilization of glucose isomerase. The following is a brief review of the methods of glucose isomerase immobilization arranged chronologically.

The first immobilized glucose isomerase was prepared by entrapping the enzyme from S. phaeochromogenes NRRL B-3559 with polyacrylamide (Stranberg and Smiley, 1971), but the polyacrylamide-entrapped enzyme was inactivated in the reaction mixture of glucose isomerization and this sensitivity eventually limited its use. Stranberg and Smiley (1972) also prepared an immobilized glucose isomerase by binding the enzyme from the same organism to diazonium salts of the aminoarylsilane derivative of porous glass beads. These beads are suitable for column application with a half life of 12-14 days.

The enzyme released from cells of S. phaeochromogenes and L. brevis was stirred with DEAE-cellulose and the DEAE-cellulose-enzyme complex could be operated in columns for isomerization of glucose with a half life of 198 h (Sipos, 1972). This process is believed to be used in the Clinton Corn Processing Co. to produce high fructose corn syrup (Isomerose) (Schnyer, 1974).

Emery et al. (1974) prepared an immobilized glucose

isomerase by linking the enzyme from L. brevis to cellulose treated with titanic chloride. However, the density of this immobilized enzyme preparation was too low to be utilized in a continuous reactor.

The entrapment of glucose isomerase from Streptomyces species in a filament of cellulose acetate was demonstrated by Dinelli et al. (1974). The entrapped enzyme retained some activity for 45 days, but it dropped steadily throughout this period.

Porous alumina, Zirconia or titania could be used for the immobilization of glucose isomerase from Streptomyces species (Messing, 1974, 1975). This type of preparation is suitable for continuous use in plug-flow reactors.

Monsanto Co. (1974) immobilized glucose isomerase on large-pore polyethylene discs with polyacrylonitrile and glutaraldehyde, but the activity of the immobilized enzyme was not mentioned.

A stable immobilized glucose isomerase was reported to be prepared by swelling collagen, soaking it in an enzyme solution and drying (Research Corp., 1975).

Partially purified glucose isomerase from Streptomyces sp. was immobilized on ZnO_2-coated porous glass particles (Lee et al., 1976b). Fifty six percent of the free enzyme activity was retained; the half life of the immobilized enzyme was found to exceed 200 days at 50°C.

Stanley et al. (1976) reported the immobilization of

glucose isomerase from S. phaeochromogenes on chitin with glutaraldehyde as a crosslinking agent. The amount of enzyme activity retained by this treatment was about 40%.

Mitsubishi Chemical Industries Co., Ltd. (1976) adsorbed glucose isomerase to a porous anionic (trimethylammonium type) exchange resin resulting in a 91% retention of enzyme activity. Glucose isomerase was also immobilized on $4MgCO_3 \cdot Mg(OH)_2 \cdot 5H_2O$ or $MgO\text{-}Al_2O_3$ (Heady and Jacaway, 1976; Eaton and Messing, 1976).

Matsumoto et al. (1976) demonstrated the immobilization of glucose isomerase onto a crosslinked agar support. Agar was crosslinked with epichlorohydrin and activated by CNBr. The extent of immobilization was 99% and the half life of this preparation was 18 days.

Takasaki (1977) immobilized glucose isomerase on a borate form anionic exchanger e.g. Amberlite IRA-400. Enzyme activity was retained at 50-60% in continuous use for 10 days.

Partially purified glucose isomerase from S. phaeochromogenes was immobilized by adsorption on colloidal silica followed by gelation of the silica by freezing (CPC International Inc., 1977). Columns packed with this immobilized enzyme showed good flow properties.

The immobilization of glucose isomerase from S. phaeochromogenes in several types of asymmetric hollow fibers (Amicon and Romicon) was described by Korus and Olson

(1977). The time course of isomerization and stability for the hollow fiber enzyme approach that of the free enzyme, although polysulfone fibers had to be preconditioned with an inert protein. The hollow fiber enzyme has the advantages of large enzyme loading and ease of use, cleaning and reloading with enzyme.

Kanbayashi and Hasegawa (1977) used 3-aminobenzeneboronic acid derivative of CM-cellulose and DEAE-cellulose to adsorb glucose isomerase. This preparation was packed into column and employed for continuous isomerization of glucose.

A method for glucose isomerase immobilization was described by Yoshida et al. (1977) in which an enzyme solution was adsorbed onto pulverized silica gel, kaolin, activated charcoal, gelatin, starch or cellulose followed by addition of a hydrophilic monomer. Polymerization of the monomeric substances was then achieved by γ-irradiation with ^{60}Co and a water-insoluble enzyme-containing polymer was obtained. This technique could be also applied to immobilize α-amylase and glucoamylase.

DEAE sponge cellulose prepared by Brouillard (1977) was used for immobilizing glucose isomerase and other enzymes. This sponge material had good flow properties when packed in the column.

A solution containing glucose isomerase, MgO, acrylic acid, NaOH, N, N'-methylene(bisacrylamide) was irradiated

with ionizing radiation to immobilize the enzyme (Kawashima and Umeda, 1977). Sixty two percent of the original activity retained by this treatment.

Aminosilochrome was used to immobilize glucose isomerase of A. olivocinereus (Ananichev et al., 1978). The immobilized enzyme had 58% of the activity of soluble enzyme and was more stable than the soluble one.

Glucose isomerase from B. coagulans was partially purified and immobilized by adsorption to anionic exchangers (Huitron and Limon-Lason, 1978). The highest activity was obtained when the enzyme was adsorbed to DEAE-cellulose. A comparison of properties between immobilized and soluble enzyme was also made.

A method was described by Fujita et al. (1978) for immobilization of glucose isomerase on highly porous anionic exchange resins by contact of the enzyme solution with resins (SO_4^{2-}, PO_4^{3-}, OH^-, Cl^- and CH_3COO^- types). Preparations with a high degree of adsorption and high activity yields were obtained.

Kato et al. (1978) described a method in which glucose isomerase solution was infiltrated into filter paper and the paper was dried. The paper was then sprayed with a polyvinyl butyral resin solution at 5-10 μ thickness and air dried to prepare an immobilized enzyme paper. Recently, Hirohara et al. (1978) adsorbed glucose isomerase from S. phaeochromogenes on a macroporous phenol-formalin anionic

exchange resin which was then combined with a cationic or anionic hydrophilic residue. However, in both cases the activities of the immobilized enzymes were not disclosed.

MATERIALS AND METHODS

Materials

D-arabinose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-xylose, sucrose, raffinose, D-glucose-6-phosphate, α-methyl glucoside, glycerol, D-mannitol, D-sorbitol, i-inositol, gluconic acid, maleic acid, sorbic acid, asparagine, cysteine hydrochloride, tyrosine, cetylpyridinium chloride, cetyltrimethylammonium bromide, dimethyl sulfoxide, dioctyl sodium sulfosuccinate, sodium lauryl sulfate, Tween 80, p-chloromercuribenzoate, diethyldithiocarbamate, ethylenediaminetetraacetic acid (EDTA), hydroxylamine, 1, 10-phenathroline, sodium azide, sodium sulfite, Tris(hydroxymethyl) aminomethane (Tris), carbazole, o-dianisidine dihydrochloride, PGO reagent, phenol reagent, phloroglucinol, amino ethyl (AE)-cellulose, benzyl diethylaminoethyl-cellulose, carboxylmethyl (CM)-cellulose, diethylaminoethyl (DEAE)-cellulose, Ecteola cellulose, polyethyleneimine (PEI)-cellulose, and triethylaminoethyl (TEAE)-cellulose were all purchased from Sigma Chemical Co..

Agar, beef extract, casitone, malt extract, peptone, polypeptone, proteose peptone, soluble starch, vitamin-free casamino acids and yeast extract were obtained from Difco laboratories.

CM-Sephadex, DEAE-Sephadex and QAE-Sephadex were purchased from Pharmacia Fine Chemicals Co..

Bovine serum albumin, carbonic anhydrase, lysozyme, ovalbumin, phosphorylase, soybean trypsin inhibitor and sodium dodecyl sulfate were obtained from Bio-Rad Laboratories.

Corn steep liquor was provided by Clinton Corn Processing Co..

Acrylamide, N, N'-methylene bisacrylamide, N, N, N', N'-tetramethylenediamine, ammonium persulfate were from Canalco Inc..

Coomassie brilliant blue G-250 was from Eastman Kodak Co..

Glass beads (0.11-0.12 mm) were obtained from VWR Scientific Co..

Perchloric acid was from Malinckrodt Inc..

Tri-Sil Z was obtained from Pierce Chemical Co..

Culture media

Medium A

The culture medium used for the isolation of actinomycetes had the same composition as that developed by Küster and William (1964) except that it contained straw hemicellulose rather than soluble starch. The medium had the following composition: straw hemicellulose, 10.0 g; vitamin-free casamino acids, 0.3 g; KNO_3, 2.0 g; NaCl, 2.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 20.0 g; distilled water, 1 liter. The pH of the

medium was adjusted to 7.0. After autoclaving and cooling, cycloheximide was added to the medium to a final concentration of 50 μg/ml from a filter sterilized stock solution.

Medium B

The slant agar medium contained straw hemicellulose, 5 g; peptone, 5 g; beef extract, 3 g; $MgSO_4 \cdot 7H_2O$, 1 g; agar, 20 g and distilled water, 1 liter. The pH of the medium was 7.0.

Medium C

The culture medium employed for the screening of actinomycetes capable of producing glucose isomerase had the following composition: straw hemicellulose, 10 g; peptone, 10 g; yeast extract, 5 g; $MgSO_4 \cdot 7H_2O$, 1 g and distilled water, 1 liter. The pH of the medium was 7.0.

Medium D

The culture medium used for the study of time course of glucose isomerase production was composed of straw hemicellulose, 10 g; corn steep liquor, 25 g, $MgSO_4 \cdot 7H_2O$, 1 g and distilled water, 1 liter. The pH of the medium was 7.0.

Media E

Yeast extract-malt extract agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar were used for morphological studies and determination of mycelium color.

Carbon utilization medium was used for the sugar utilization test. Production of melanoid pigments was determined on agar slants of peptone-yeast extract iron agar, tyrosine agar and tryptone yeast broth. The compositions of these media and cultural conditions were described by Shirling and Gottlieb (1966).

Preparation of substrates

Straw hemicellulose

Annual ryegrass (Lolium multiform Lam) was sun-dried and ground with a hammer mill to pass through a 1/4 in. screen. Ten grams of ryegrass straw were mixed with 100 ml of 1 to 24% NaOH and kept at 30 to 120°C for 1 to 48 h. Treated straw was then pressed through eight layers of cheesecloth, and the straw residue was washed twice with distilled water. The final filtrates were combined to make a final volume of 100 ml. The pH of the filtrate was adjusted to 5.0 with HCl. To the filtrate, 150 ml of 95% ethanol was added, and the mixture was allowed to stand at room temperature for 24 h. The precipitate was collected and washed with 70% ethanol to remove lignin and other solubles. The precipitate was dehydrated with 95% ethanol, and the ethanol was removed by filtration and vacuum drying at 50°C. The dry product was used as straw hemicellulose.

H_2SO_4 hydrolysate of straw

One to 6 g of ryegrass straw was mixed with 100 ml of 0.1 or 0.2 N H_2SO_4 and heated for 30 min at 121°C. The mixture was filtered, and the filtrate was neutralized with NaOH and used as H_2SO_4 hydrolysate of straw.

Physical treated straw

A portion of straw was ground with a Wiley mill and passed through a 100-mesh screen, and another portion of straw was ground with a roller type jar ball mill.

Isolation and screening of actinomycetes

Soil samples were collected from the vicinity of Oregon State University, Corvallis. Soil samples were dispersed in sterile water and plated on medium A. After incubation, colonies of typical actinomycetes were isolated and transferred to agar slants (medium B). In order to select the organism which produced the highest level of glucose isomerase, each strain from the slant culture was then grown in 250 ml flasks maintained at 30°C on a rotary shaker in medium C.

Identification of the organism

The organism that produced the highest level of glucose isomerase in medium C was isolated and its morphological and physiological characteristics and color were determined

according to the methods of Shirling and Gottlieb (1966). The identification scheme of Bergey's Manual (1974) was also used.

Electron microscopy

The spore surface of the isolated strain was observed according to the method of Shirling and Gottlieb (1966). Specimen grids coated with collodion were gently pressed to the aerial surface of a 21-day old culture with mature spore chain. These spore chains were adhered to the coated surface of the grids and photographed in a transmission electron microscope (Model EM 300, philips Inc.) without fixing or shadowing at a magnification of 7,600x.

Determination of cell growth

Cell growth was measured by homogenizing the cell suspension in a homogenizer (Brownwill Model MSK) for 1 min, centrifuging the homogenate, and then measuring the protein content of the supernatant.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard. The protein concentration was expressed as mg/ml. The protein concentration of each fraction during column chromatography was estimated by measuring the absorbance at 280 nm and

and expressed as A_{280nm}.

Determination of enzyme activities

Glucose isomerase

The enzyme reaction mixture contained 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.0), 0.2 ml of 1 M D-glucose, 0.1 ml of 0.1 M $MgSO_4 \cdot 7H_2O$, 0.1 ml of 0.01 M $CoCl_2 \cdot 6H_2O$, and 0.2 ml of enzyme solution. The enzyme solution was prepared by breaking the cells with a homogenizer for 1 min and centrifuging the suspension at 12,000 x g for 10 min. The supernatant was collected and used as enzyme solution. The final volume of the enzyme reaction mixture was made up to 2 ml with distilled water. The mixture was incubated at 70°C for 1 h, and the reaction was stopped by adding 2 ml of 0.5 M perchloric acid. The D-fructose produced was determined by the modified method of Dische and Borenfreund (1951). To 1 ml of reaction mixture, was added 0.2 ml of 1.5% cysteine hydrochloride. To this, 6 ml of 75% H_2SO_4 was added, followed immediately by 0.2 ml of 0.12% alcoholic solution of carbazole. The mixture was shaken and allowed to stand at room temperature for 1 h. The absorbance was read at 560 nm on a spectrophotometer (Model PMQ II, Carl Zeiss Co.). A standard curve was constructed using pure D-fructose. One unit of glucose isomerase activity was defined as the amount of the enzyme that produced 1 μ mole

of D-fructose per minute under the assay conditions described.

Hemicellulase

Hemicellulase activity was measured by the method described by Kawaminami and Ijuka (1970) in which 2.5 ml of 0.5% hemicellulose solution, 2.5 ml of 0.05 M acetate buffer (pH 4.0) and 1 ml of enzyme solution was mixed and incubated at 50°C for 30 min. Reducing sugar produced was determined by the method of Petterson and Porath (1966). Three milliliters of dinitrosalicylic acid reagent was added to 2 ml of reaction mixture containing reducing sugar and heated for 15 min in a boiling water bath. After cooling, the absorbance was measured at 640 nm on a spectrophotometer. A standard curve was constructed using pure D-glucose. One unit of hemicellulase activity was equivalent to 1 mg of reducing sugar produced under the assay conditions described.

Analytical procedures

Pentosan

Pentosan was determined according to the modified method of the Association of Official Agricultural Chemists (1970) in which 0.2 g of crude hemicellulose was placed in a 200-ml distillation flask containing 100 ml of 12% HCl and several boiling chips. The flask was connected to a condenser and heated to distill 30 ml in 10 min. During the distillation,

12% HCl was constantly replaced and the total volume of 360 ml of distillate was collected. Forty milliliters of phloroglucinol solution (7.3 g of phloroglucinol in 1 liter of 12% HCl) was added to the distillate. After standing overnight at room temperature, the precipitate was collected on a Gooch crucible, and washed with 150 ml of distilled water. The crucible and precipitate were dried to a constant weight at 100°C. The weight of furfural phloroglucide formed was calculated as pentosan according to the following formula: pentosan = (weight of phloroglucide + 0.0052) x 0.8866.

Xylose

Xylose was determined with a gas chromatography (Model 402, F & M Scientific Corp.) according to the method of Grant et al. (1978). H_2SO_4 hydrolysate of straw was neutralized with NaOH and filtered through Whatman no. 1 filter paper. The filtrate was lyophilized, and 20-30 mg of the lyophilized material was dissolved in 1 ml of Tri-Sil Z. The silylated sugars were injected into a column of General Electric SE 30 silicone on Anakrom diatomaceous earth (Analabs Inc.). The column temperature was 190°C.

Glucose

Glucose was determined by the use of PGO reagent (Sigma Tech. Bull. 510, 1978, Sigma Chemical Co.). PGO reagent is a mixture of peroxidase and gluoose oxidase; it contains

o-dianisidine as a chromogen. To 0.5 ml of D-glucose solution, 5 ml of PGO reagent was added. The mixture was shaken gently and allowed to stand at room temperature for 45 min. At the end of the incubation period, the absorbance was read at 560 nm on a spectrophotometer. Pure D-glucose was used as the standard.

Total nitrogen and crude protein

Total nitrogen was determined by the micro-Kjeldahl method described by Perrin (1953). Crude protein was determined by multiplying a factor of 6.25 to total nitrogen.

Cellulose, hemicellulose, lignin, cell soluble matter and ash

Cellulose, hemicellulose, lignin, cell soluble matter and ash were determined by the methods of Goering and Van Soest (1970).

In vitro rumen digestibility

In vitro rumen digestibility (IVRD) of untreated straw and alkaline treated straw was determined by a modified method of Mellenberger et al. (1970). One half gram of straw and 35 ml of rumen fluid-buffer mixture were added into a 50 ml screw capped tube. Rumen fluid was obtained from a fistulated Holstein steer and mixed with mineral and buffer mixture (McDougall, 1948) at a ratio of 1:1. The tubes were sealed and incubated under CO_2 for 2-3 days at

39°C. The contents of each tube were filtered through a sintered glass crucible (Pyrex, C porosity) and dried overnight at 105°C. The weight loss was reported as percentage IVRD.

Animal feeding trial

Weanling male meadow voles (Microtus canicaudus) were provided by L. G. Forslund who maintains a colony of brown tail meadow voles at Oregon State University. About three weeks old, uniform size male voles were randomly selected from 14 litters. A group of nine animals was subjected to each diet, with one animal per screen bottom cage. The voles were fed from a aluminum cup (10 cm diameter and 5 cm deep). Average daily weight gain and feed consumption rate were measured during 10 days feeding periods after a 2-day adjustment period to the trial diet. The diet consisted of soybean meal, 120 g; corn oil, 50 g; mineral mix (Jones and Foster, 1942), 20 g; ground corn, 510 g and untreated straw or alkaline treated straw, 300 g. The control diet contained 810 g of ground corn and had no straw.

Dehydration and freezing of whole cells

Dehydration in dessicator

The organism was grown at 30°C on medium D. After 36 h, the culture was centrifuged out and the cell mass (mycelium) was washed twice with distilled water. The cell mass

was used throughout the experiment. The cell mass was sprayed on a glass plate and dried at room temperature in dessicator together with sodium hydroxide under reduced pressure.

Lyophilization

A portion of cell mass was lyophilized with a lyophilizer (Virtis Res. Equipment) at -50°C.

Acetone powder

The cell mass was washed twice with cold acetone (-20°C). The cell residue was dried at room temperature under reduced pressure.

Freezing

The cell mass was frozen in a freezer (-20°C).

Extraction of glucose isomerase

Cell mass obtained by the procedure mentioned earlier was then resuspended in 0.05 M sodium phosphate buffer (pH 7.0). The resulting cell suspension was used throughout the experiment. One each of the chemicals shown in Table 10 was added to the cell suspension and the mixture was incubated for 1-22 h at 37°C with or without shaking. Shaking decreased the extraction time somewhat, but did not affect the maximum enzyme level. Twenty milliliters of the cell suspension was mixed with 25 g of glass beads (0.11-0.12 mm).

The mixture was homogenized with a homogenizer at 4°C for 1 min. A portion of the cell suspension was treated with a sonic oscillator (Biosonik IV, VWR Scientific Co.) at 4°C for 10 min. Another portion of the cell suspension was ground with sand at 4°C for 30 min. All treatments were followed by centrifugation at 12,000 x g and 4°C for 10 min to remove cell debris. The supernatant was used for the determination of glucose isomerase activity and protein content.

Purification of glucose isomerase

Extraction

The cell mass was resuspended in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1% cetylpyridinium chloride or cetyltrimethylammonium bromide. The enzyme was extracted for 2 h at 37°C with shaking. The suspension was centrifuged at 12,000 x g for 10 min. The supernatant was used as the enzyme extract. Unless otherwise indicated, all steps of the purification were carried out at 4°C.

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the enzyme extract to give a 70% saturation and the precipitate was discarded. More ammonium sulfate was added to the supernatant to give a 90% saturation. The precipitate was collected, dissolved in 0.05 M sodium phosphate buffer (pH 7.0) and dialyzed over-

night against the same buffer. The dialysate was centrifuged at 12,000 x g for 10 min.

Column chromatography on DEAE-cellulose column

After dialysis and centrifugation, the supernatant was applied to a column of DEAE-cellulose (2.5 x 32 cm) which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl at a flow rate of 50 ml/h. Active fractions (52-70) were collected, dialyzed overnight with the same buffer and centrifuged at 12,000 x g for 10 min.

Column chromatography on DEAE-Sephadex A-50 column

The supernatant was applied to a column of DEAE-Sephadex A-50 (2.2 x 30 cm) which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl at a flow rate of 30 ml/h. Active fractions (92-138) were collected and the enzyme was precipitated by adding solid ammonium sulfate to 90% saturation. The precipitate was dissolved in a minimum volume of 0.05 M sodium phosphate buffer (pH 7.0), dialyzed overnight against the same buffer and centrifuged at 12,000 x g for 10 min. The supernatant was stored at -20^{o}C until further use. The flow diagram for the purification of glucose isomerase is shown

in Figure 1.

Homogeneity of glucose isomerase

The homogeneity of the purified enzyme was determined by ultracentrifugation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

A sedimentation velocity experiment was carried out in a Spinco Model E ultracentrifuge (Spinco Division, Beckman Inc.) equipped with a schlieren optical system. The enzyme was at a concentration of 3.2 mg/ml in 0.05 M sodium phosphate buffer (pH 7.0). The experiment was conducted in a rotor (AN-D) at 4°C at a speed of 54,410 rpm. Photographs were taken at 20 min interval after reaching maximum speed.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was based on the method of Weber *et al*. (1972) with minor modifications suggested by Malencik (1972). Polyacrylamide gels (7.5 and 10%) were used for the study of homogeneity of glucose isomerase and the determination of the molecular weight of the enzyme subunit respectively. The buffer system was 0.01 M Tris-phosphate buffer (pH 6.8).

Electrophoresis was carried out with a Buchler polyanalyst electrophoresis chamber. The power supply (Model 400, Bio-Rad Laboratories) was operated at 4 ma per tube. The running time was about 2 h. After electrophoresis,

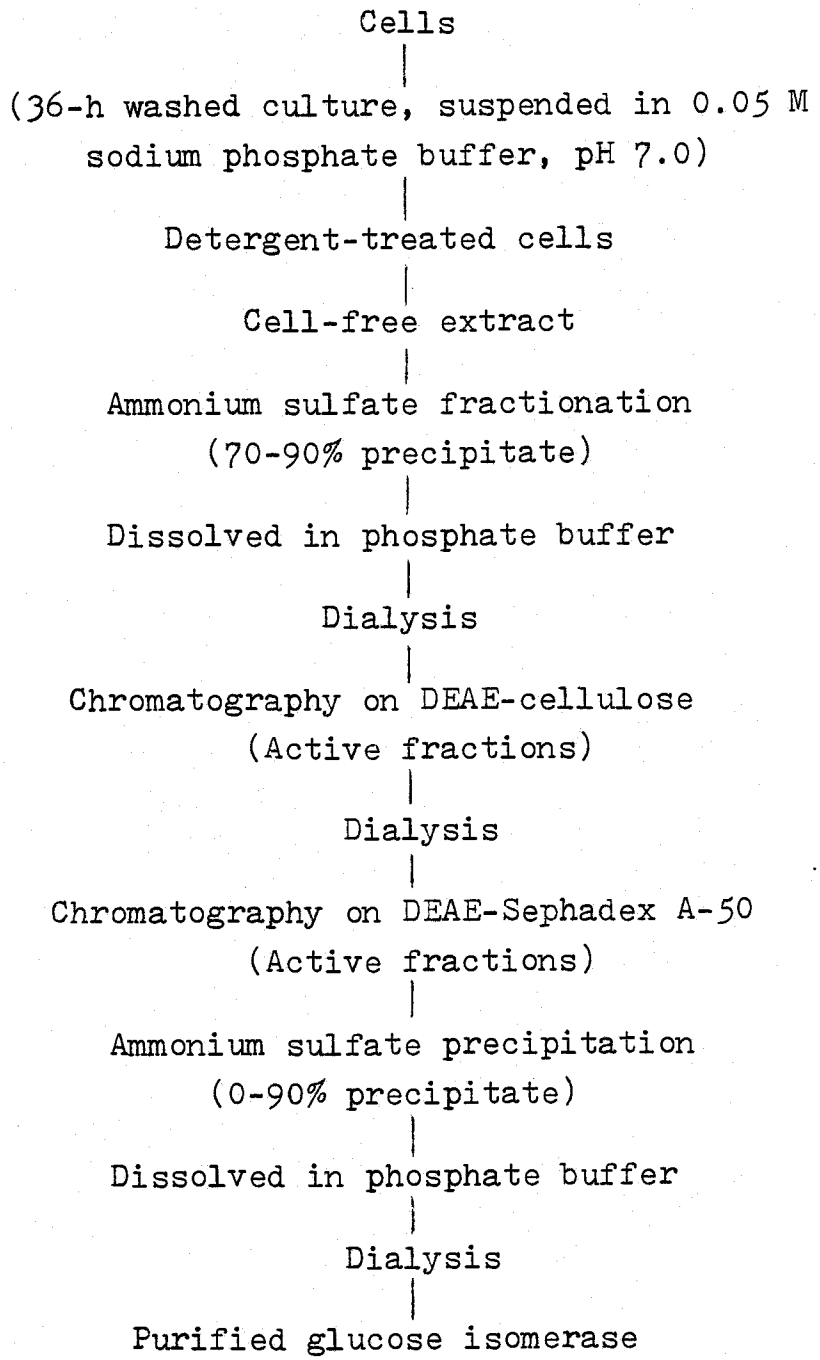

Fig. 1. Flow diagram for the purification of glucose isomerase.

gels were removed from the tubes, and fixed in 12.5% trichloroacetic acid for 10 min, then stained for 2 h with Coomassie Blue G-250. Destaining was conducted in 7.5% acetic acid using quick gel destainer (Canalco Inc.). The mobilities were plotted against the logarithmic values of molecular weights of standard proteins.

Determination of molecular weight

A sedimentation equilibrium experiment was carried out to determine the molecular weight of the enzyme in a Beckman Model E ultracentrifuge (Beckman Inc.) equipped with an absorption optical system. The rotor (AN-D) was allowed to cool to 4°C and the experiment was conducted at 6,803 rpm for 3 days. The enzyme in 0.05 M sodium phosphate buffer (pH 7.0) was at a concentration of 0.62 mg/ml. A series of scan patterns at 280 nm were obtained.

Immobilization of glucose isomerase to ion exchangers

A suspension of 500 mg (dry weight) of various ion exchangers in 0.05 M sodium phosphate buffer (pH 7.0) was stirred gently at 4°C. Glucose isomerase solution (5 ml) which was obtained after precipitation with ammonium sulfate (70-90% saturation) was added and stirred for 30 min at the same temperature. The enzyme-support complex obtained was washed and suspended in the same buffer.

Continuous isomerization of D-glucose

Packed columns of DEAE-cellulose-glucose isomerase or heat-treated whole cells (60°C, 10 min) were continuously operated at 70°C for 5 days. D-glucose was dissolved in 0.02 M sodium phosphate buffer (pH 7.0) to give a concentration of 1 M and the resulting substrate solution was then fortified with mineral salts. The concentrations of mineral salts are shown in Fig. 23. The substrate solution (70°C) was pumped through the column at a flow rate of 12 ml/h. Hot water (70°C) was pumped through water jacket which surrounded the column. The effluent was collected as 12 ml per fraction in tubes.

RESULTS

Extraction of hemicellulose from ryegrass straw

The hemicellulose in ryegrass straw was extracted easily by soaking straw in 4-24% NaOH for 3 h at 100°C. As much as 25% hemicellulose (18% as pentosan) could be obtained by treating straw with 14% NaOH under the same conditions (Figure 2). The level of hemicellulose extracted increased proportionately with increasing NaOH concentration up to about 4%, then the rate of increase slowed down. Almost 60% of the initial amount of straw was solubilized by treating straw with 4% NaOH at 100°C for 3 h.

The extraction of hemicellulose from ryegrass straw was facilitated by the combined application of heat and NaOH. When straw was treated with 4% NaOH for 3 h, the highest yield of hemicellulose was obtained at 90°C (Figure 3). The yield declined as the temperature increased further.

When straw was treated with 4% NaOH at room temperature, hemicellulose was rapidly extracted during the first 12 h and the rate of extraction slowed down thereafter (Figure 4). After 24 h, there was no appreciable quantity of hemicellulose extracted or decrease of straw residue, a final concentration of 15% hemicellulose (12% as pentosan) and 50% straw residue was obtained. The level was almost equivalent to that obtained by heating straw at 90°C for

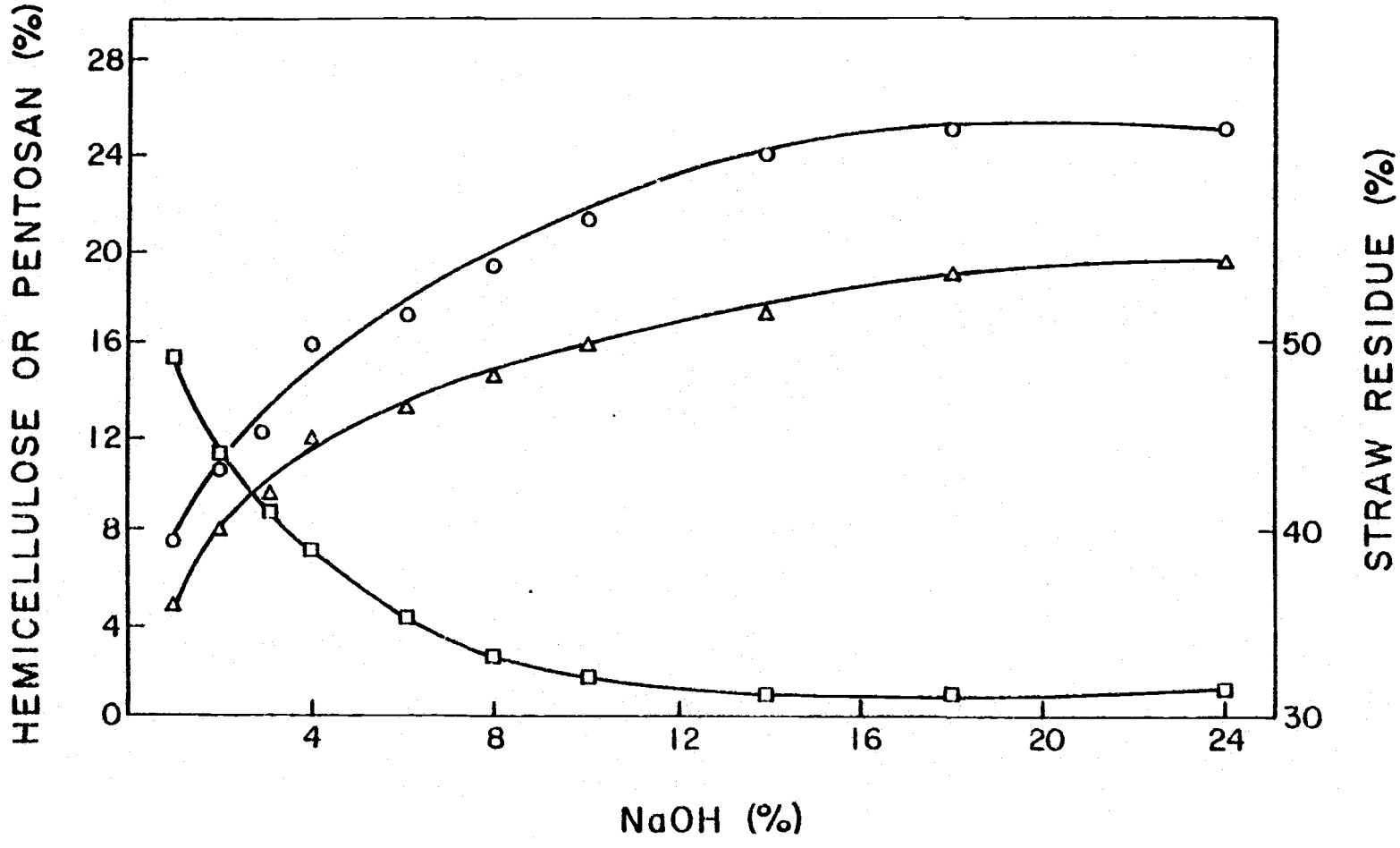

Figure 2. Effect of NaOH concentration on the extraction of hemicellulose from ryegrass straw. One part of ryegrass straw was extracted with ten parts of NaOH at 100°C for 3 h. Symbols: hemicellulose (o); pentosan (△); straw residue (□).

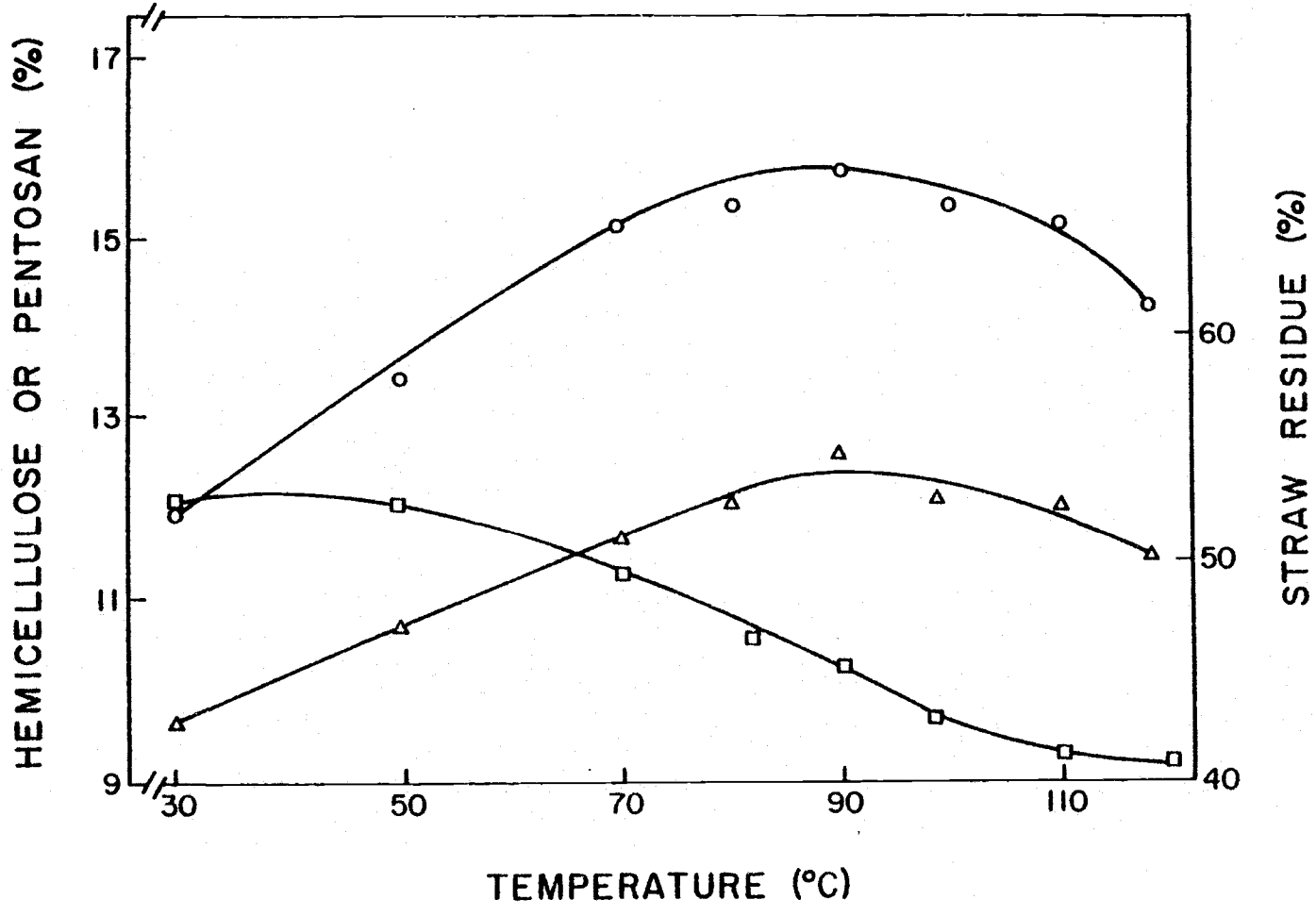

Figure 3. Effect of temperature on the extraction of hemicellulose from ryegrass straw. One part of ryegrass straw was extracted with ten parts of 4% NaOH at different temperatures for 3 h. Symbols: hemicellulose (o); pentosan (Δ); straw residue (□).

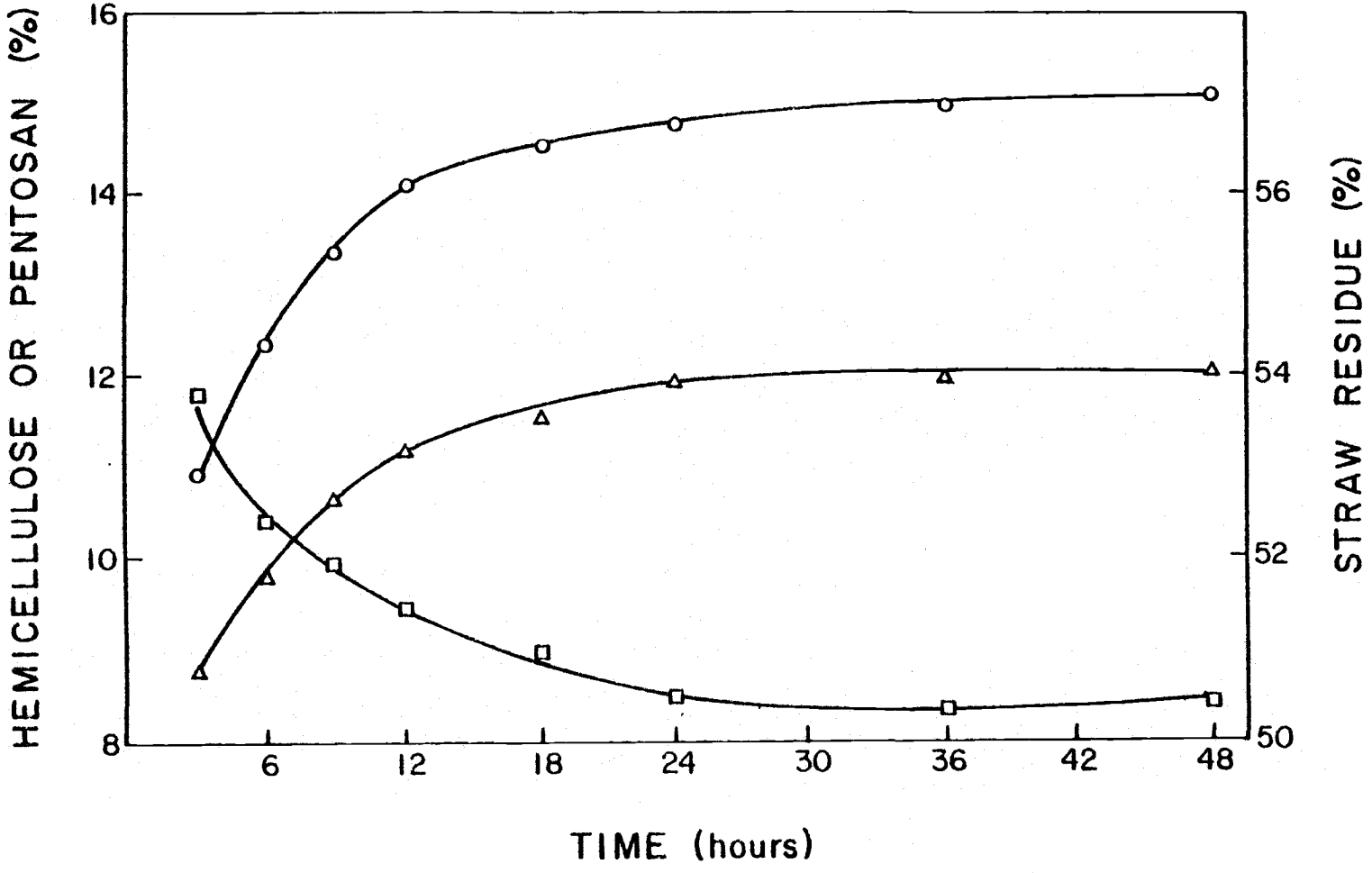

Figure 4. Time course of hemicellulose extraction from ryegrass straw. One part of ryegrass straw was extracted with ten parts of 4% NaOH at room temperature. Symbols: hemicellulose (o); pentosan (Δ); straw residue (□).

3 h.

Isolation and identification of the organism

Over 200 strains of actinomycetes were isolated and tested for their ability to produce glucose isomerase. The one that produced the highest level of glucose isomerase was characterized morphologically and physiologically as well as for color.

The isolate grew in the form of a many-branched mycelium with a typical aerial mycelium and had a smell of damp soil, characteristics of Streptomyces. When grown on yeast extract-malt extract agar, oatmeal agar, salts-starch agar, or glycerol-asparagine agar, the isolate produced smooth-surfaced rectiflexible spores (Figure 5) having more than 10 spores per mature chain. The aerial mycelium color on the medium matched the gray color series of Tresner and Backus (1963). The reverse side of the colony was brown yellow on yeast extract-malt extract agar, yellow on oatmeal agar, and green yellow on salts-starch agar; reverse mycelium pigment was not a pH indicator. Melanoid pigments were not found in peptone-yeast extract iron agar, tyrosine agar, or tryptone yeast broth. No pigment or only a trace of yellow was found in yeast extract-malt extract agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar.

The isolate utilized D-glucose, L-arabinose, D-xylose, D-mannitol, D-fructose and L-rhamnose. No growth or only a

Figure 5. Electron micrograph of the spores (top) and light micrograph of aerial mycelium (bottom) of Streptomyces flavogriseus.

trace was observed with i-inositol, sucrose and raffinose. The characteristics of the isolate were identical to those of Streptomyces flavogriseus described by Shirling and Gottlieb (1969).

Optimum conditions for the production of glucose isomerase

Carbon sources

Table 5 shows the level of glucose isomerase produced by S. flavogriseus grown on various carbon sources. The organism produced a high level of glucose isomerase when grown on straw hemicellulose, xylan, xylose or H_2SO_4 hydrolysate of straw. Optimum substrate concentration for the enzyme production was 2% straw hemicellulose, from which 3.04 units/ml of the enzyme was produced in 48 h. Specific activity of the enzyme was also highest at this concentration. Optimum concentration for xylan and xylose was 3.0% and 1.25% respectively. The level of the enzyme produced on NaOH extract of straw or other carbon sources listed in the table was significantly lower than that on xylose-containing compounds.

The organism also produced a high level of glucose isomerase on H_2SO_4 hydrolysate of 3% straw which contained 0.2% xylose (Figure 6). The level of enzyme production decreased when the straw concentration increased beyond 3%. It was also observed that more enzyme was obtained on hydrolysate prepared from straw hydrolyzed with 0.1 N H_2SO_4 than

Table 5. Effect of carbon sources on the production of glucose isomerase by S. flavogriseus after 48 h of cultivation.

Carbon source (%)	Cell growth (mg of protein/ml of culture)	Enzyme activity (units/ml of culture)	Relative activity[a] (%)	Specific activity (units/mg of protein)
Straw hemicellulose				
0.5	1.51	0.67	22	0.44
1.0	2.02	2.17	71	0.98
1.5	2.02	2.28	75	1.13
2.0	2.02	3.04	100	1.50
3.0	2.02	2.48	82	1.23
Xylan				
0.5	1.51	0.93	31	0.62
1.0	1.55	2.20	72	1.41
1.5	1.68	2.35	77	1.40
2.0	1.51	2.67	88	1.77
3.0	1.82	2.89	95	1.59
Xylose				
0.50	1.74	1.85	61	1.06
1.00	1.76	2.35	77	1.26
1.25	2.24	2.89	95	1.29
1.50	2.24	2.81	92	1.25
H_2SO_4 hydrolysate of straw (3.0)	1.38	2.76	91	2.00
NaOH extract of straw[b] (2.5)	1.00	0.69	23	0.69
Ball milled straw (1.0)	0.36	0.26	9	0.72
Ground straw, 100 mesh (1.0)	0.25	0.22	7	0.88
Galactose (1.0)	0.81	0.67	22	0.83
Glycerol (1.0)	0.90	0.50	16	0.56
Mannose (1.0)	1.06	0.41	13	0.39
Glucose (1.0)	0.65	0.33	11	0.51
Arabinose (1.0)	0.50	0.33	11	0.66

a/ Relative activity expressed as percentage of enzyme activity produced on 2.0% straw hemicellulose.

b/ Ryegrass straw (10%) extracted with NaOH (4%) at room temperature for 24 h and filtered. One volume of filtrate diluted with 3 volumes of distilled water and neutralized to pH 7.0.

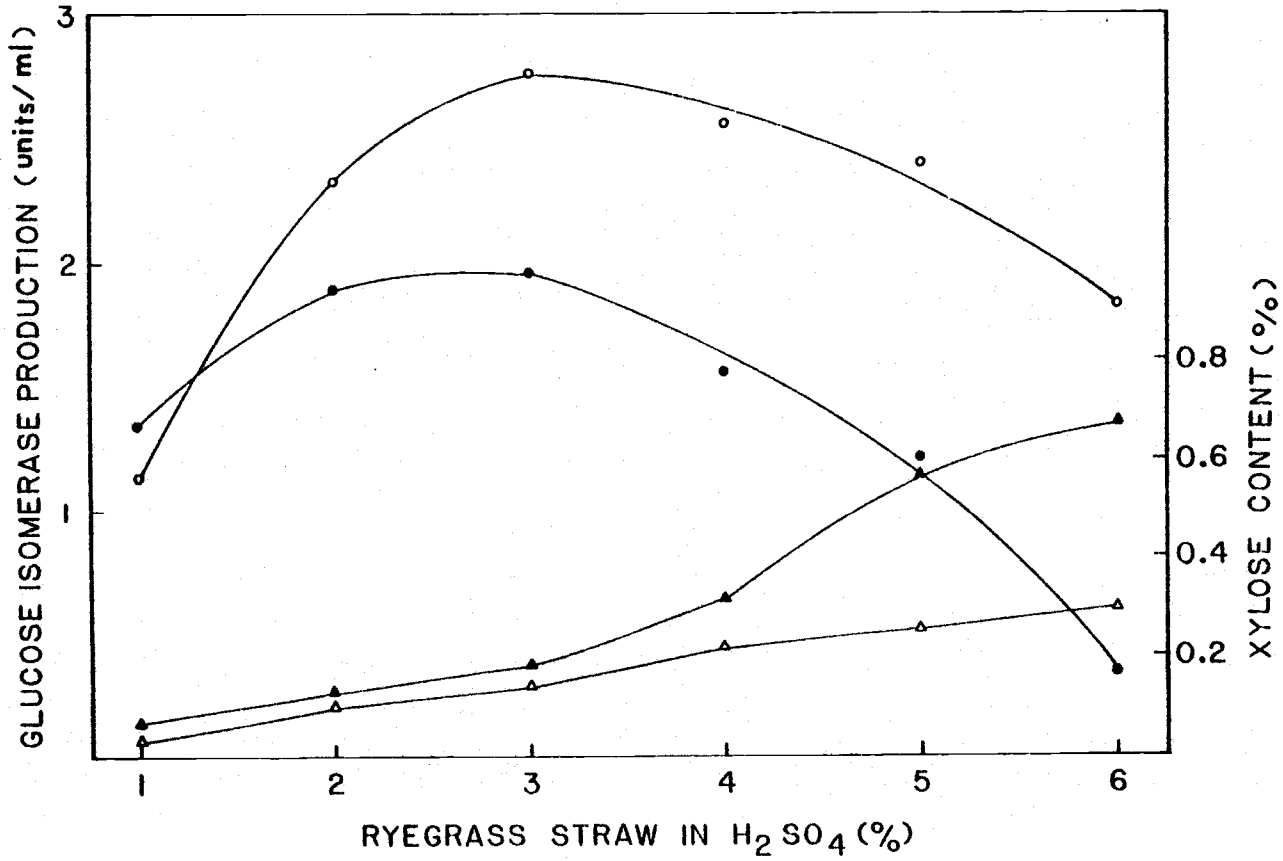

Figure 6. Effect of ryegrass straw and H_2SO_4 concentrations on the production of glucose isomerase. Symbols: glucose isomerase produced on 0.1 N H_2SO_4 hydrolysate (o); xylose content in 0.1 N H_2SO_4 hydrolysate (△); glucose isomerase produced on 0.2 N H_2SO_4 hydrolysate (●); xylose content in 0.2 N H_2SO_4 hydrolysate (▲).

with 0.2 N H_2SO_4, whereas the reverse was true on increasing xylose content.

Nitrogen sources

The addition of 2.5% corn steep liquor produced the highest yield of glucose isomerase (Table 6). Soy flour extract, yeast extract and various peptones were also effective in increasing the production of glucose isomerase. Inorganic nitrogen sources with the exception of dibasic ammonium phosphate (0.69 units/ml) were ineffective.

Mineral salts

The addition of Mg^{2+}, Mn^{2+}, Fe^{2+}, or Zn^{2+} significantly enhanced the enzyme production (Table 7). A relatively higher concentration of $MgSO_4 \cdot 7H_2O$ (0.1%) than that of the other minerals was required to induce enzyme formation. The highest levels of the enzyme were obtained by the addition of 0.03% $MnSO_4 \cdot 4H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$ or 0.01% $FeSO_4 \cdot 7H_2O$; less effective were $ZnSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $Fe_2(SO_4)_3$ and $CoCl_2 \cdot 6H_2O$, while 0.1% $CoCl_2 \cdot 6H_2O$ and 0.01% $HgCl_2$ completely inhibited the cell growth. The addition of the combination of $MgSO_4 \cdot 7H_2O$ (0.1%) and $MnSO_4 \cdot 4H_2O$ (0.03%), $MgSO_4 \cdot 7H_2O$ (0.1%) and $CoCl_2 \cdot 6H_2O$ (0.03%), or $MnSO_4 \cdot 4H_2O$ (0.03%) and $CoCl_2 \cdot 6H_2O$ (0.03%) resulted in the production of less enzyme than did the addition of $MgSO_4 \cdot 7H_2O$ (0.1%) or $MnSO_4 \cdot 4H_2O$ (0.03%) alone.

Table 6. Effect of nitrogen sources on the production of glucose isomerase by S. flavogriseus after 48 h of cultivation.

Nitrogen source (%)	Cell growth (mg of protein/ ml of culture)	Enzyme activity (units/ml of culture)	Relative activity[a] (%)	Specific activity (units/mg of protein)
Corn steep liquor				
1.0	0.80	0.92	32	1.15
1.5	0.96	1.76	61	1.83
2.0	1.42	2.48	86	1.75
2.5	1.42	2.89	100	2.03
3.0	1.55	2.63	91	1.70
4.0	1.48	2.52	87	1.70
Soy flour extract[b] (2.0)	1.30	2.17	75	1.67
Yeast extract (1.0)	1.32	2.00	69	1.52
Polypeptone (1.0)	1.35	1.85	64	1.37
Proteose peptone (1.0)	1.06	1.85	64	1.75
Peptone (1.0)	1.01	1.80	62	1.75
Tryptone (1.0)	1.62	1.76	61	1.09
Casitone (1.0)	0.96	1.44	50	1.50
Beef extract (1.0)	1.00	0.78	27	0.78
Urea (1.0)	0.39	0.26	9	0.67
$(NH_4)_2HPO_4$ (0.5)	0.72	0.69	24	0.96
NH_4Cl (0.5)	0	0	0	0
$(NH_4)_2SO_4$ (0.5)	0	0	0	0
NH_4NO_3 (0.5)	0	0	0	0
Ammonium acetate (0.5)	0	0	0	0
Ammonium tratrate (0.5)	0	0	0	0

a/ Relative activity expressed as percentage of enzyme activity produced on 2.5% corn steep liquor.

b/ Soy flour (2.0%) was extracted with distilled water at 100°C for 2 h and then filtered.

Table 7. Effect of mineral salts on the production of glucose isomerase by S. flavogriseus after 48 h of cultivation.

Salt (%)	Cell growth (mg of protein/ ml of culture)	Enzyme activity (units/ml of culture)	Relative activity[a] (%)	Specific activity (units/mg of protein)
None	1.43	0.98	100	0.69
$MnSO_4 \cdot 4H_2O$				
0.01	1.37	2.33	238	1.70
0.03	1.42	2.74	280	1.93
0.10	1.32	2.67	272	2.02
$MgSO_4 \cdot 7H_2O$				
0.03	1.48	1.81	185	1.22
0.10	1.42	2.74	280	1.93
0.50	1.42	2.48	253	1.75
$CoCl_2 \cdot 6H_2O$				
0.01	1.34	1.26	129	0.94
0.03	1.43	1.72	176	1.20
0.10	0	0	0	0
$FeSO_4 \cdot 7H_2O$ (0.01)	1.32	2.74	280	2.08
$ZnSO_4 \cdot 7H_2O$ (0.01)	1.03	2.33	238	2.26
$CaCl_2 \cdot 2H_2O$ (0.01)	1.45	1.85	189	1.28
$Fe_2(SO_4)_3$ (0.01)	1.42	1.76	180	1.24
NaCl (0.01)	1.42	1.24	127	0.87
$BaCl_2 \cdot 2H_2O$ (0.01)	1.48	1.24	127	0.84
$NiSO_4 \cdot 7H_2O$ (0.01)	1.29	1.11	113	0.86
$CuSO_4 \cdot 5H_2O$ (0.01)	0.71	0.74	76	1.04
$HgCl_2$ (0.01)	0	0	0	0
$MnSO_4 \cdot 4H_2O$ (0.03) + $MgSO_4 \cdot 7H_2O$ (0.10)	1.48	1.89	193	1.28
$MgSO_4 \cdot 7H_2O$ (0.10) + $CoCl_2 \cdot 6H_2O$ (0.03)	1.22	1.43	146	1.17
$MnSO_4 \cdot 4H_2O$ (0.03) + $CoCl_2 \cdot 6H_2O$ (0.03)	1.35	1.08	110	0.80

a/ Relative activity expressed as percentage of enzyme activity produced on salt-free medium.

pH

Figure 7 shows the effect of the initial pH of the medium on cell growth and enzyme production. The organism grew at pH 5.5 to 9.0, and the maximum cell growth after 48 h of incubation was reached at pH 6.0 to 8.5. Glucose isomerase was produced at pH 5.0 to 10.0, and the maximum enzyme was obtained at pH 6.0 to 8.5. Thus, an optimum of pH 7.0 was selected for enzyme production in further experiments. The pH of the spent media was in the alkaline range, a characteristic of cultures of Streptomyces.

Time course

Cell growth reached a maximum at around 36 h of cultivation (Figure 8). In the early stage of cultivation, extracellular hemicellulase was produced and reached its highest level (0.6 units/ml) at about 30 h. The pH of the spent medium rose slowly during cultivation and reached 9.0 after 4 days. During the logarithmic phase of growth, hemicellulose degradation and intracellular glucose isomerase formation were rapid, and the rate of enzyme formation was almost linear with cell growth. After 48 h of cultivation, there was no appreciable further degradation of hemicellulose. The highest level of intracellular glucose isomerase (3.5 units/ml) was obtained at about 36 h, thereafter the enzyme level decreased sharply. Extracellular glucose isomerase was highest (1.5 units/ml) at 72 h.

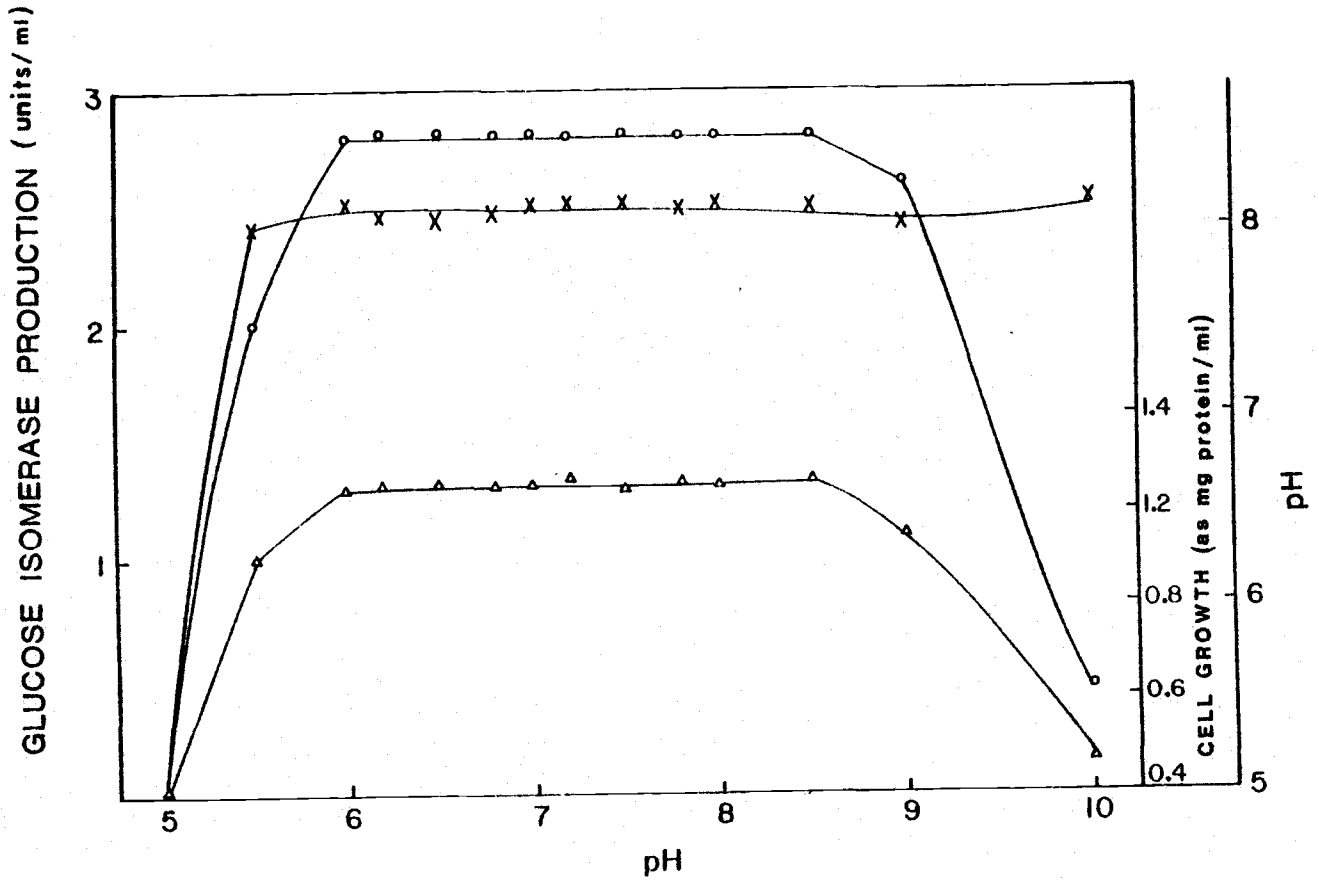

Figure 7. Effect of pH on the production of glucose isomerase (o), cell growth (Δ); and pH of the spent medium (x).

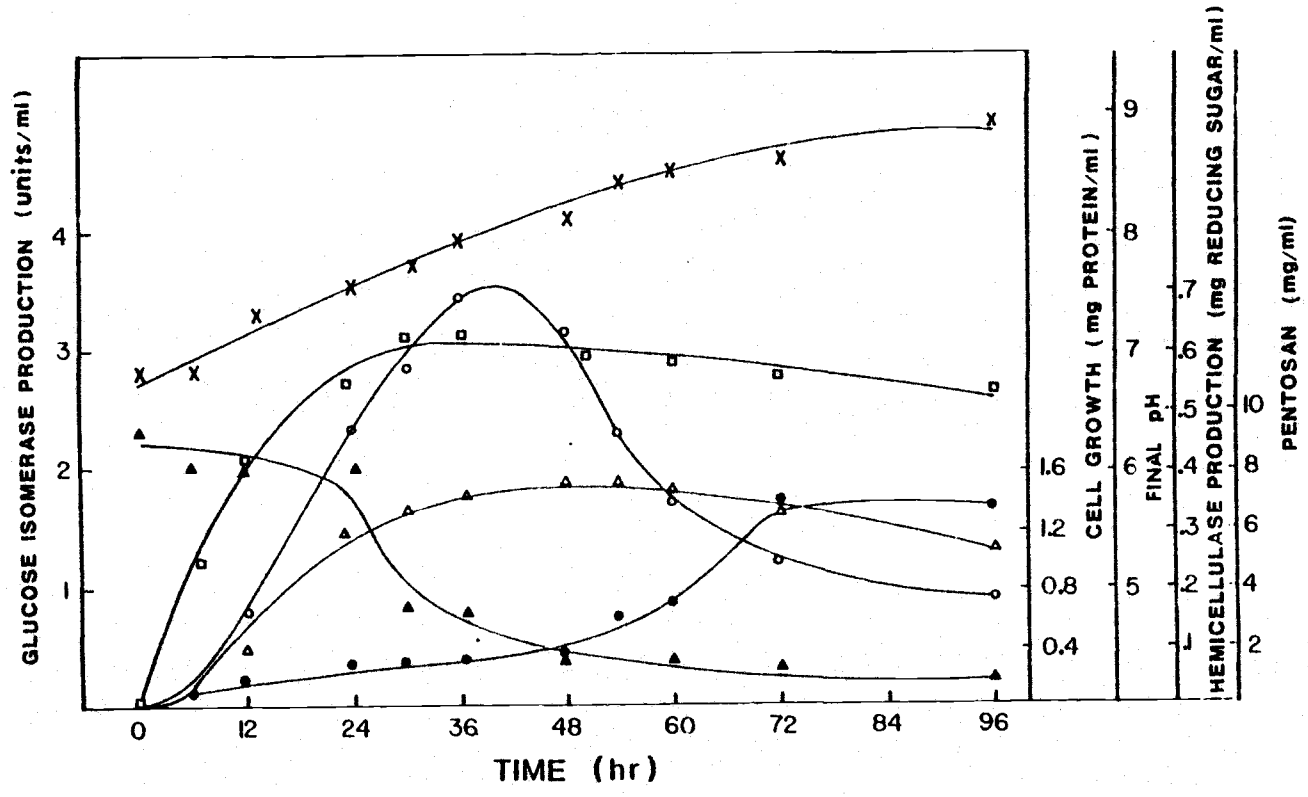

Figure 8. Time course of hemicellulose fermentation by S. flavogriseus in the production of glucose isomerase as indicated by intracellular glucose isomerase (o), extracellular glucose isomerase (●), hemicellulase production (□), cell growth (△), pH of the spent medium (x), and pentosan content (▲).

Chemical composition, IVRD and animal feeding trial of untreated and NaOH-treated straw

NaOH treatment removed about half of the hemicellulose present in ryegrass straw (Table 8). Hemicellulose, cell soluble matter, protein and IVRD in NaOH-free straw residue (NFSR) were significantly less than those in partially washed straw residue (PWSR). The IVRD of PWSR and NFSR was 78.6 and 56.2% respectively which was much higher than that of untreated straw (44.9%).

Animal feeding trials were carried out with a diet containing untreated straw, PWSR or NFSR (Table 9). Weight gain, feed intake and feed efficiency were significantly increased by NaOH treatment and almost equivalent to those of control diet. This effect, however, was diminished when the treated straw residue was thoroughly washed. The pH of PWSR was 12.0, but it did not adversely affect the palatability of the diet for weanling meadow voles.

Proposed processes for glucose isomerase production from ryegrass straw

Figure 9 shows the flow chart of the two processes for glucose isomerase production from ryegrass straw. The initial treatment in each of these was grinding. This increased the surface area, facilitating chemical action.

Chemical treatments released hemicellulose or xylose which was used as substrate for enzyme formation by the

Table 8. Chemical composition and *in vitro* rumen digestibility (IVRD) of untreated straw and NaOH-treated straw residues (% dry matter).

Composition and IVRD	Untreated straw (%)	Partially washed straw residue[a] (%)	NaOH-free straw residue[b] (%)
Cellulose	41.9	48.6	66.6
Cell soluble matter	28.2	23.4	15.9
Hemicellulose	26.3	14.2	11.0
Lignin	6.9	5.4	7.1
Protein	3.8	2.0	1.4
Ash	1.0	0.7	0.6
IVRD	44.9	78.6	56.2

a/ The NaOH-treated straw residue was partially washed with water. The pH of the substrate was 12.0.

b/ The NaOH-treated straw residue was washed with water until free from NaOH. The pH of the substrate was 7.0.

Table 9. Response of weanling meadow voles for diet containing untreated straw or NaOH-treated straw residues.

Diet	Weight gain[a] (g)	Feed intake[a] (g)	Feed efficiency (g of feed/ g of wt. gain)
Control	0.241	3.56	14.8
Untreated straw	0.222	4.15	18.7
Partially washed straw residue[b]	0.241	3.78	15.7
NaOH-free straw residue[c]	0.194	3.69	19.0

a/ Average per day of 9 animals for 10-day feeding period.

b/ The NaOH-treated straw residue was partially washed with water. The pH of the substrate was 12.0.

c/ The NaOH-treated straw residue was washed with water until free from NaOH. The pH of the substrate was 7.0.

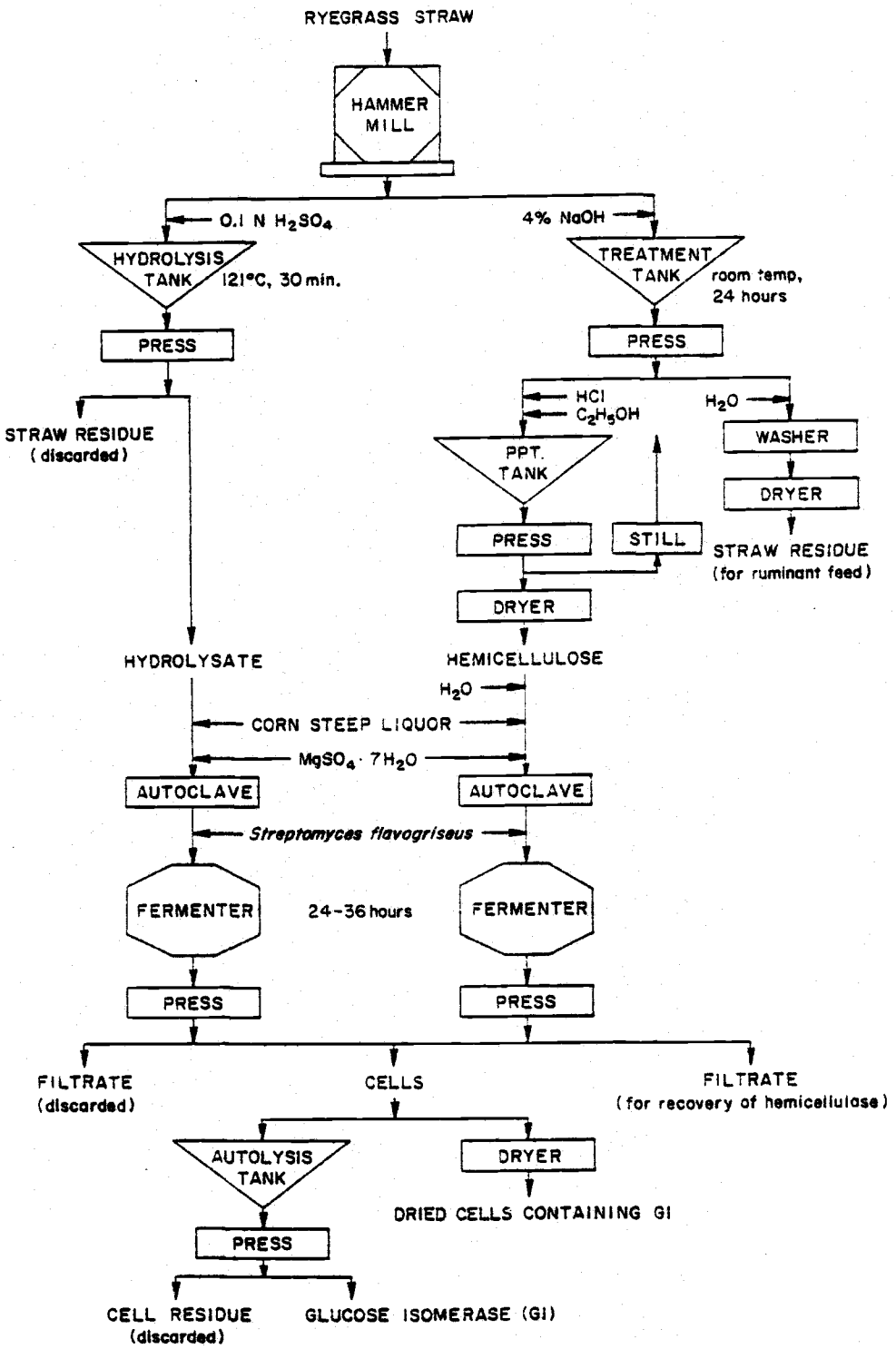

Figure 9. Flow chart of processes for the production of glucose isomerase from ryegrass straw.

organism. In the first process, the ground straw was extracted with 4% NaOH at room temperature for 24 h at a liquid to solid ratio of 10:1. The NaOH extract obtained was then neutralized with HCl and precipitated with ethanol. Ethanol can be recovered by distilling the filtrate after removal of hemicellulose. About 15% hemicellulose (12% as pentosan) was obtained by this treatment. The straw residue after removal of hemicellulose was partially washed with water and could be used as ruminant feed.

The second process involved hydrolyzing the ground straw at 121°C for 30 min with 0.1 N H_2SO_4 using a liquid to solid ratio of 100:3. Under these conditions, 0.2% of the weight of straw is solubilized to xylose. The resulting hydrolysate was neutralized with NaOH.

The straw hemicellulose or H_2SO_4 hydrolysate of straw was then fortified with corn steep liquor and magnesium sulfate, sterilized, and inoculated with S. flavogriseus. The fermentation was carried out at 30°C for 24-36 h. When the enzyme level reached maximum, the cells were harvested and utilized either as whole cells or the soluble enzyme extracted by autolysis of cells.

The spent medium of hemicellulose fermentation contained hemicellulase which could also be recovered for industrial use.

Dehydration and freezing of whole cells

The enzyme activity of the cells remaining after dehydration and freezing is shown in Table 10. Cells prepared as acetone powder or by lyophilization gave the highest activity of glucose isomerase. The cells obtained by other methods showed a much lower activity than the former.

Extraction of glucose isomerase

The cells suspended in 0.1% cetyltrimethylammonium bromide (CTAB) produced a maximum 3.02 units/ml of enzyme in 3 h (Table 11), and those in 0.1% cetylpyridinium chloride (CPC) produced 2.92 units/ml of the enzyme in 2 h. Similar levels of the enzyme were produced on cell-free extracts prepared by sonication and abrasive grinding. The level of enzyme obtained from the supernatant of the homogenized cell suspension was significantly lower than those mentioned above, indicating that homogenization is not a very effective method of enzyme extraction. However, addition of 0.1% CTAB to the homogenized cell suspension before centrifugation increased the enzyme yield to the same level of those above. The whole cells without any treatment gave a somewhat lower level of enzyme (2.65 units/ml), compared to those treated with cationic detergents. It may be that the cell wall itself serves as a diffusion barrier. Anionic (sodium lauryl sulfate and dioctyl sodium sulfosuccinate)

Table 10. Activity changes of whole cells of S. flavogriseus by different treatments.

Treatments	Activity remained (% of control)
Acetone powder	92.5
Lyophilization	91.3
Air drying	62.5
Freezing	56.3

Table 11. Activity and specific activity of glucose isomerase extracted from cells of S. flavogriseus.

Treatment (%)	Enzyme activity (units/ml)				Specific activity (units/mg of protein)			
	1 h	2 h	3 h	22 h	1 h	2 h	3 h	22 h
Whole cells	2.65				--			
Chemical extraction								
Cetylpyridinium chloride (0.1)	2.38	2.92	2.90	2.96	2.27	2.37	2.35	2.41
Cetyltrimethyl-ammonium bromide (0.1)	2.14	2.62	3.02	3.00	1.90	2.12	2.42	2.36
Lysozyme (0.1) + toluene (1.0)	1.47	1.86	2.04	2.91	0.96	1.18	1.24	1.82
Toluene (1.0)	0.96	1.63	1.98	2.87	0.94	1.12	1.27	1.79
Tween 80 (0.1)	0.31	0.60	0.65	1.53	2.53	3.08	2.98	2.87
Dimethyl sulfoxide (0.1)	0.42	0.52	0.67	1.45	3.62	3.25	3.58	3.21
Sodium lauryl sulfate (0.1)	0.36	0.55	0.72	0.89	0.31	0.45	0.52	0.96
Dioctyl sodium sulfosuccinate (0.1)	0.41	0.49	0.83	0.91	0.42	0.50	0.72	0.84
Mechanical disruption								
Sonication	2.97				1.94			
Abrasive grinding	2.87				1.91			
Homogenization	2.12				1.41			
Homogenization + cetyltrimethylammonium bromide (0.1)	3.01				2.01			

and neutral detergents (Tween 80) were ineffective, lysozyme and toluene or toluene alone were relatively less effective than cationic detergents (CTAB and CPC) in extracting the enzyme.

The specific activity of glucose isomerase extracted by different treatments varied (Table 11). The specific activity of the enzyme extracted with cationic detergents was higher than that liberated by sonication or abrasive grinding, but was the highest with dimethyl sulfoxide and Tween 80. However, because of low yields, extraction with the latter two chemicals is not practical.

Purification of glucose isomerase

Fractionation with ammonium sulfate gave a high degree of purification of the enzyme (Table 12). The majority of the activity was found in the 70-90% fraction. This fraction contained about 50% of the original activity and a purification of around 4.9-fold.

After dialysis, the 70-90% fraction was further subjected to chromatography on a DEAE-cellulose column (Figure 10). The enzyme activity was eluted at a low concentration of NaCl (0.13-0.16 M) and was found in the fractions 54-70.

The enzyme solution from the preceding step was applied to a column of DEAE-Sephadex A-50. As shown in Figure 11, the enzyme was eluted at 0.22 to 0.35 M NaCl and was found in the fractions 92-138.

Table 12. Ammonium sulfate fractionation of glucose isomerase of S. flavogriseus.

Saturation of $(NH_4)_2SO_4$ (%)	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Yield (%)
Extract	648	405	1.6	100.0
0-30	15	75	0.2	2.3
30-40	20	60	0.3	3.1
40-50	35	70	0.5	5.4
50-60	58	83	0.7	9.0
60-70	65	35	1.9	10.0
70-80	231	29	8.0	35.7
80-90	131	17	7.6	20.2

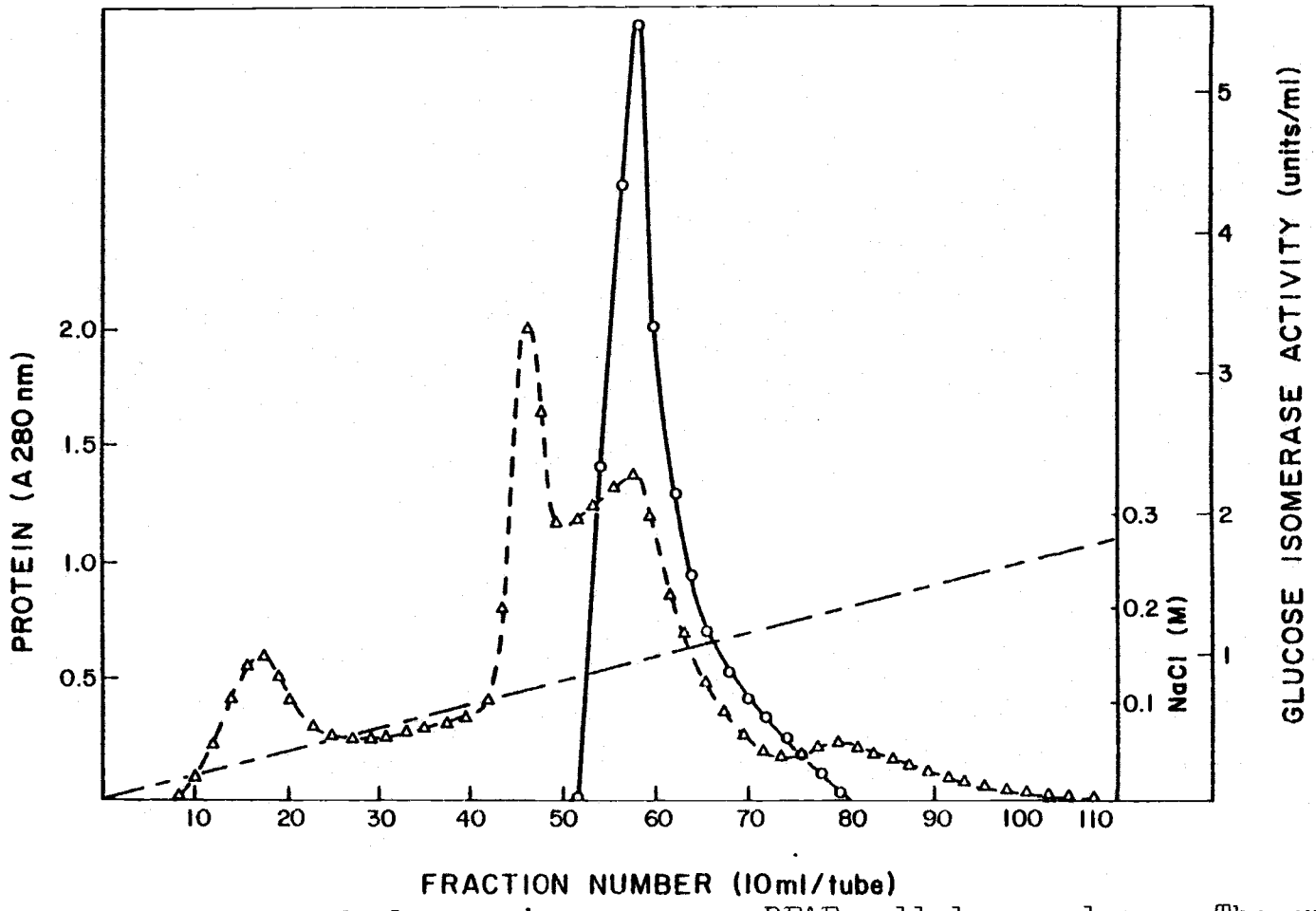

Figure 10. Chromatography of glucose isomerase on DEAE-cellulose column. The enzyme solution was applied to a DEAE-cellulose column (2.5 x 32 cm) which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl at a flow rate of 50 ml/h. Symbols: glucose isomerase activity (o); protein (Δ); NaCl (———).

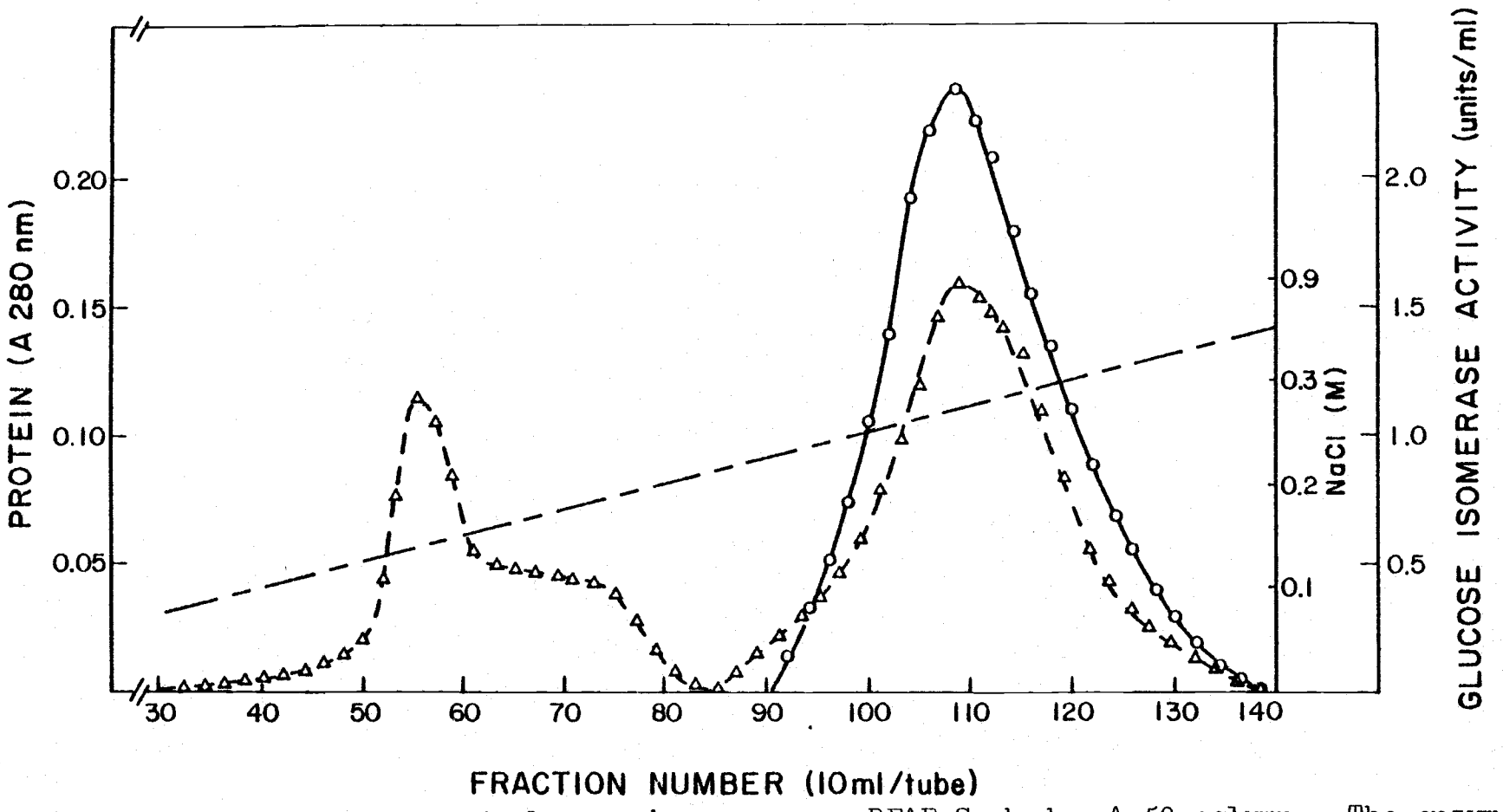

Figure 11. Chromatography of glucose isomerase on DEAE-Sephadex A-50 column. The enzyme solution was applied to a DEAE-Sephadex A-50 column (2.2 x 30 cm) which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl at a flow rate of 30 ml/h. Symbols: glucose isomerase activity (o); protein (Δ); NaCl (----).

The results of the overall purification procedure are summarized in Table 13. The enzyme was purified about 12.6-fold over the extract with an overall yield of 11%.

Homogeneity of the purified glucose isomerase

Homogeneity of the purified enzyme was determined by ultracentrifugation and SDS-polyacrylamide gel electrophoresis. The purified enzyme proved to be homogeneous as evidenced by a single symmetrical moving peak on ultracentrifugation analysis (Figure 12) and a single band of protein on SDS-polyacrylamide gel at pH 6.8 (Figure 13).

Immobilization of glucose isomerase

The results for the immobilization of glucose isomerase on different ion exchangers are given in Table 14. Anionic exchangers were effective in adsorbing glucose isomerase; whereas cationic exchangers were not. Benzyl DEAE-cellulose and TEAE-cellulose retained more enzyme activity and protein than other ion exchangers. However, these two supports are much more expensive than DEAE-cellulose. In order to determine the maximum protein and enzyme activity that DEAE-cellulose could adsorb, different concentrations of protein were added to DEAE-cellulose suspensions. The maximum values for protein and enzyme activity that could be bound occurred when 191 mg of protein/g of support were added, being 105 mg of protein/g of support and 1052 units/g of support respectively. The specific activity of immobilized

Table 13. Purification of glucose isomerase of S. flavogriseus.

Fraction	Protein (mg)	Enzyme activity (units)	Specific activity (units/mg of protein)	Yield (%)
Extract	2860	4756	1.6	100
$(NH_4)_2SO_4$ fractionation (70-90%)	287	2242	7.8	49
Dialysis	229	1876	8.2	41
DEAE-cellulose	131	1464	11.2	32
DEAE-Sephadex A-50	25	503	20.2	11

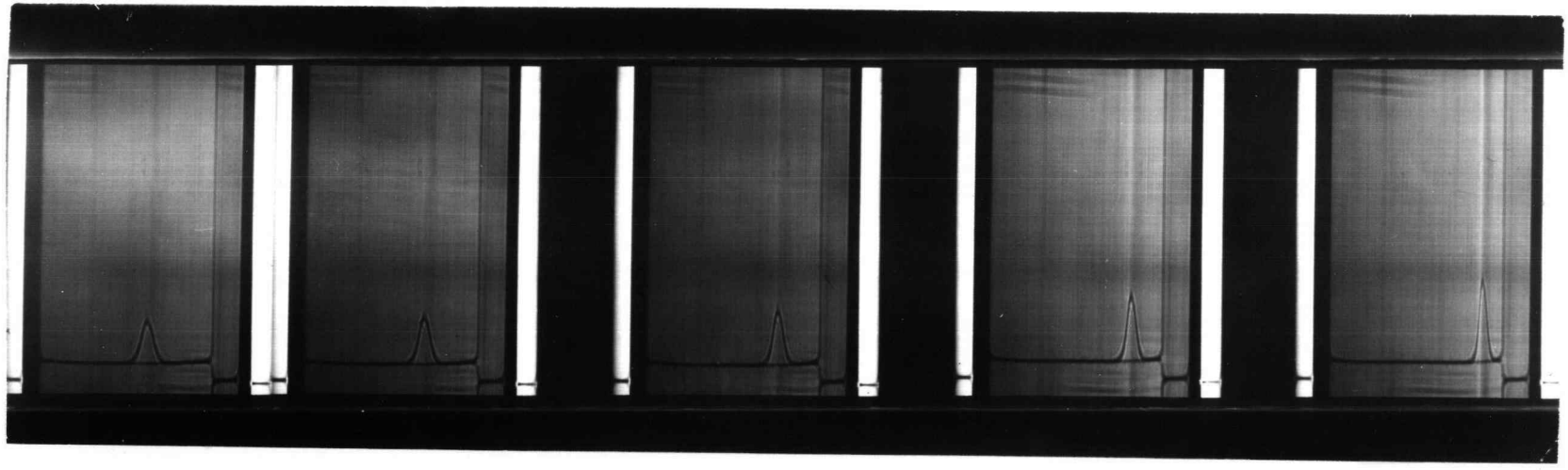

Figure 12. Sedimentation patterns of purified glucose isomerase.

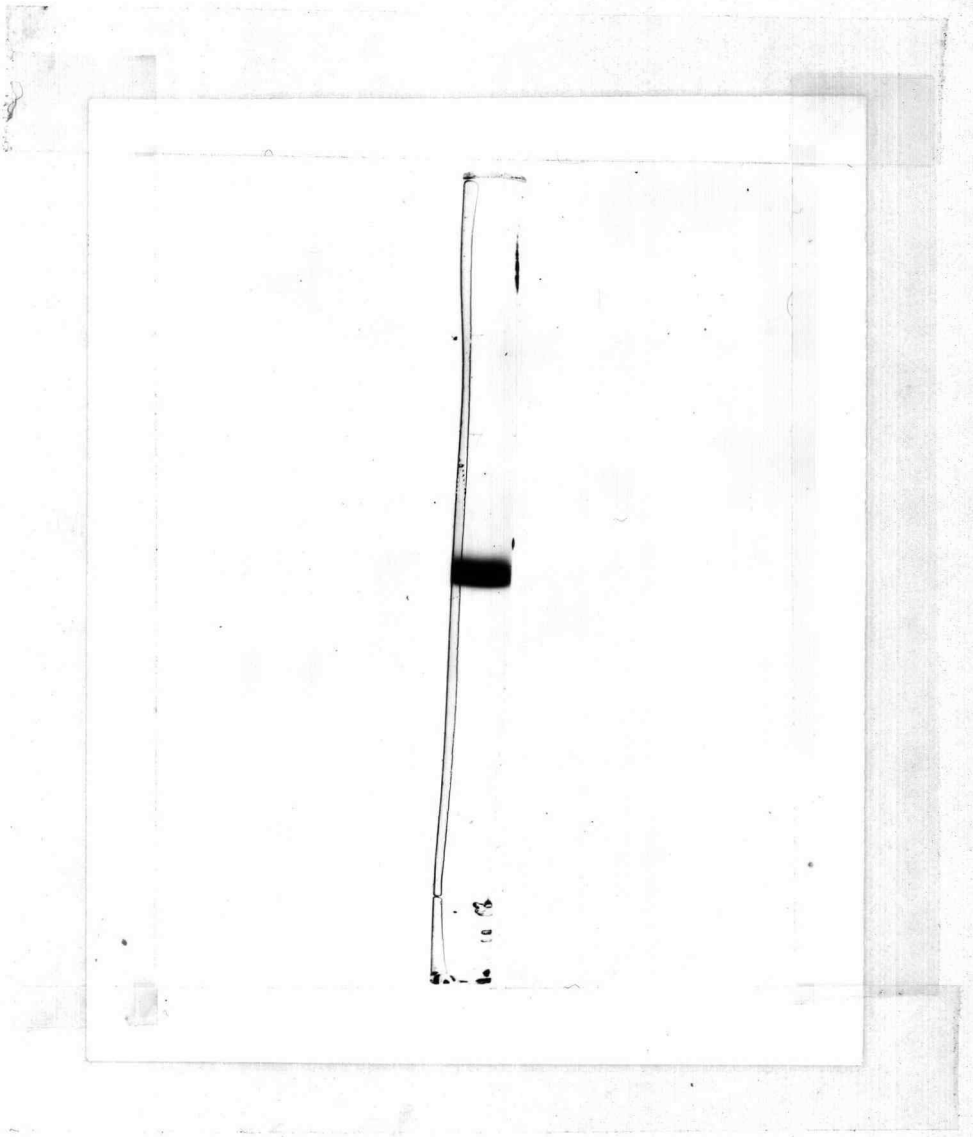

Figure 13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis performed on the purified glucose isomerase.

Table 14. Preparation of immobilized glucose isomerase of S. flavogriseus on ion exchangers.

Support	Protein (mg/g of support) added	Protein (mg/g of support) bound	Activity (units/g of support) added	Activity (units/g of support) bound	Activity retained (%)	Specific activity (units/mg of protein) added	Specific activity (units/mg of protein) bound
Benzyl DEAE-cellulose	127	81	953	762	80	7.5	9.4
TEAE-cellulose	127	79	953	734	77	7.5	9.3
DEAE-cellulose	64	32	480	312	65	7.5	9.8
	95	52	713	506	71	7.5	9.7
	127	73	953	705	74	7.5	9.6
	191	105	1433	1032	72	7.5	9.8
	254	106	1908	1052	55	7.5	9.9
DEAE-Sephadex A-50	127	70	953	534	56	7.5	7.6
DEAE-Sephadex A-25	127	68	953	515	54	7.5	7.6
PEI-cellulose	127	62	953	410	43	7.5	6.6
QAE-Sephadex	127	53	953	334	35	7.5	6.3
Amino ethyl cellulose	127	8	953	57	6	7.5	7.1
CM-Sephadex	127	10	953	48	5	7.5	4.8
CM-cellulose	127	2	953	19	2	7.5	1.0
Ecteola cellulose	127	4	953	19	2	7.5	0.5

enzymes prepared on benzyl DEAE-cellulose, TEAE-cellulose and DEAE-cellulose was higher than that of the soluble enzyme. Therefore, the immobilized enzyme prepared by adsorption on DEAE-cellulose was used for further experiments.

Properties of glucose isomerase

Substrate specificity

Purified soluble enzyme, immobilized enzyme (DEAE-cellulose-glucose isomerase complex) and heat-treated whole cells (60°C, 10 min) were used as enzyme preparations for the following experiments.

As shown in Table 15, all three enzyme preparations isomerized D-xylose and D-glucose to their respective ketoses, but they did not isomerize other pentoses, hexoses, sugar alcohols, α-methyl glucoside and D-glucose-6-phosphate.

Lineweaver-Burk plots of glucose isomerase action (Figure 14) were used to calculate Km values for D-xylose and D-glucose. The Km value of the soluble enzyme for D-xylose and D-glucose was 0.078 and 0.249 M respectively. The apparent Km value of the immobilized enzyme for D-xylose and D-glucose was 0.104 and 0.297 M respectively. The apparent Km value of the whole cells for D-xylose and D-glucose was 0.120 and 0.376 M respectively.

Effect of temperature

The optimum temperature for activity of the soluble

Table 15. Substrate specificity of glucose isomerase.

Substrate (0.1 M)	Enzyme activity (μ mole of ketose formed)		
	Soluble enzyme	Immobilized enzyme	Whole cell
D-xylose	5.50	2.80	6.30
D-glucose	0.80	0.55	0.85
D-glucose-6-phosphate	0	0	0
α-methy-glucoside	0	0	0
D-arabinose	0	0	0
D-galactose	0	0	0
D-mannose	0	0	0
L-rhamnose	0	0	0
D-mannitol	0	0	0
D-sorbitol	0	0	0

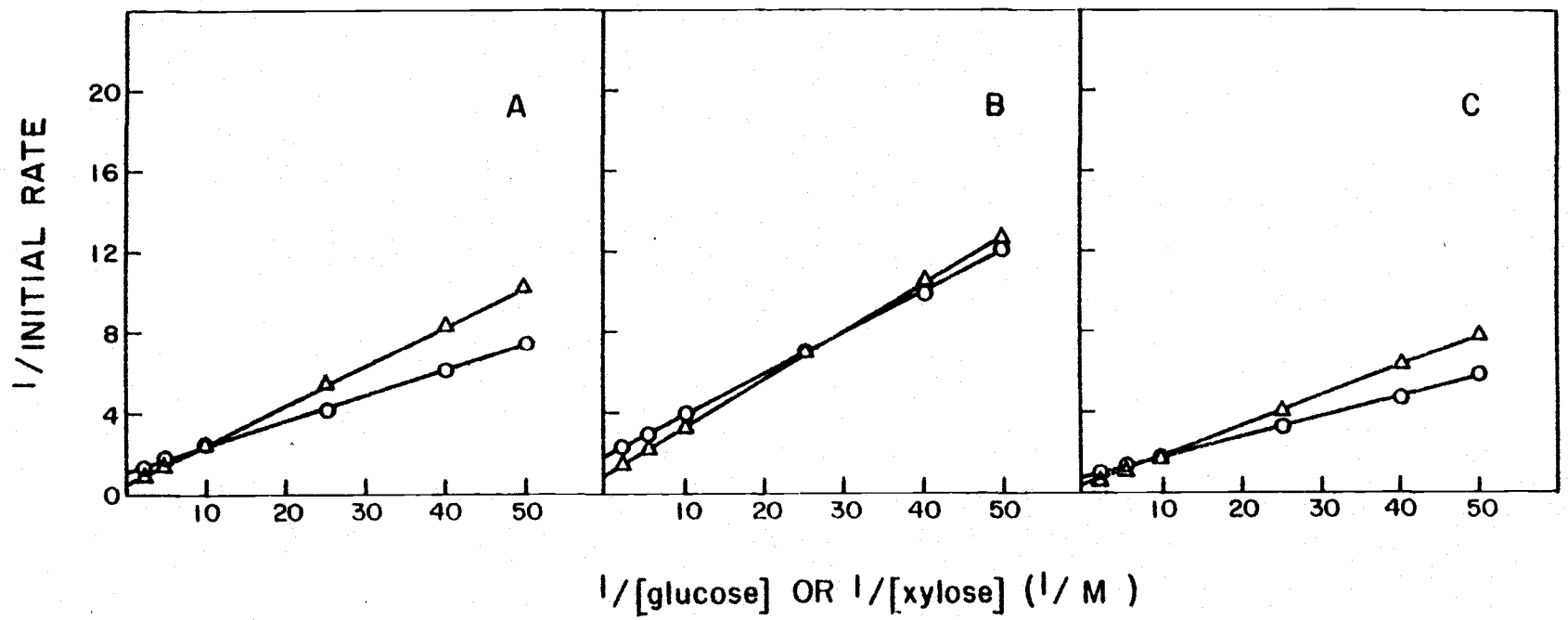

Figure 14. Effect of concentrations of D-glucose and D-xylose on glucose isomerase activity. Symbols: soluble enzyme (A); immobilized enzyme (B); whole cell (C); D-glucose (o); D-xylose (Δ).

and immobilized enzyme was 70°C when the incubation time was 1 h (Figure 15). The enzyme activity using whole cells functioned optimally at 75°C.

Effect of pH

Figure 16 shows the effect of pH on the enzyme activity. The pH optimum for activities of three enzyme preparations was 7.5 when the enzyme was incubated for 1 h at 70°C.

Effect of metal ions

Magnesium ion or Co^{2+} stimulated activities of three enzyme preparations (Table 16). Manganese ion, Ni^{2+}, Ca^{2+} or Zn^{2+} showed a slight effect, but others were ineffective. An addition effect resulted from the presence of both Mg^{2+} and Co^{2+}. The optimum concentration of Mg^{2+} for enzyme activation was 5×10^{-3} M (Figure 17).

Inhibitors

The enzyme activity was inhibited by heavy metals, such as Hg^{2+}, Ag^{+} and Cu^{2+} at a concentration of 10^{-3} M (Table 17). However, other chemicals shown in the table did not inhibit the enzyme action.

Conversion ratio for isomerization

The isomerization of D-glucose to D-fructose was reversible (Figure 18). At 70°C, the conversion ratio of three enzyme preparations for isomerization was the same, about

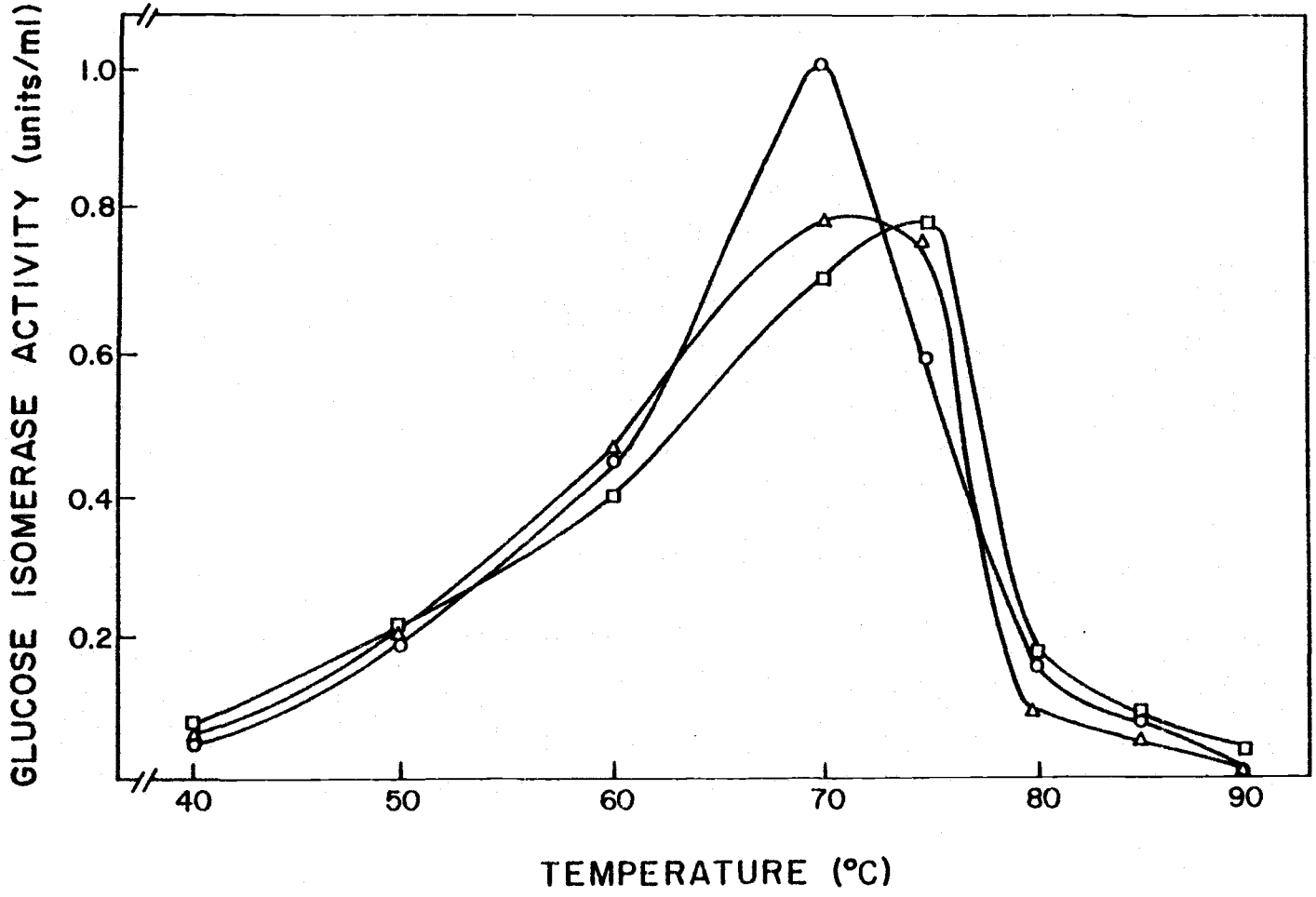

Figure 15. Effect of temperature on glucose isomerase activity. Symbols: soluble enzyme (o); immobilized enzyme (Δ); whole cell (□).

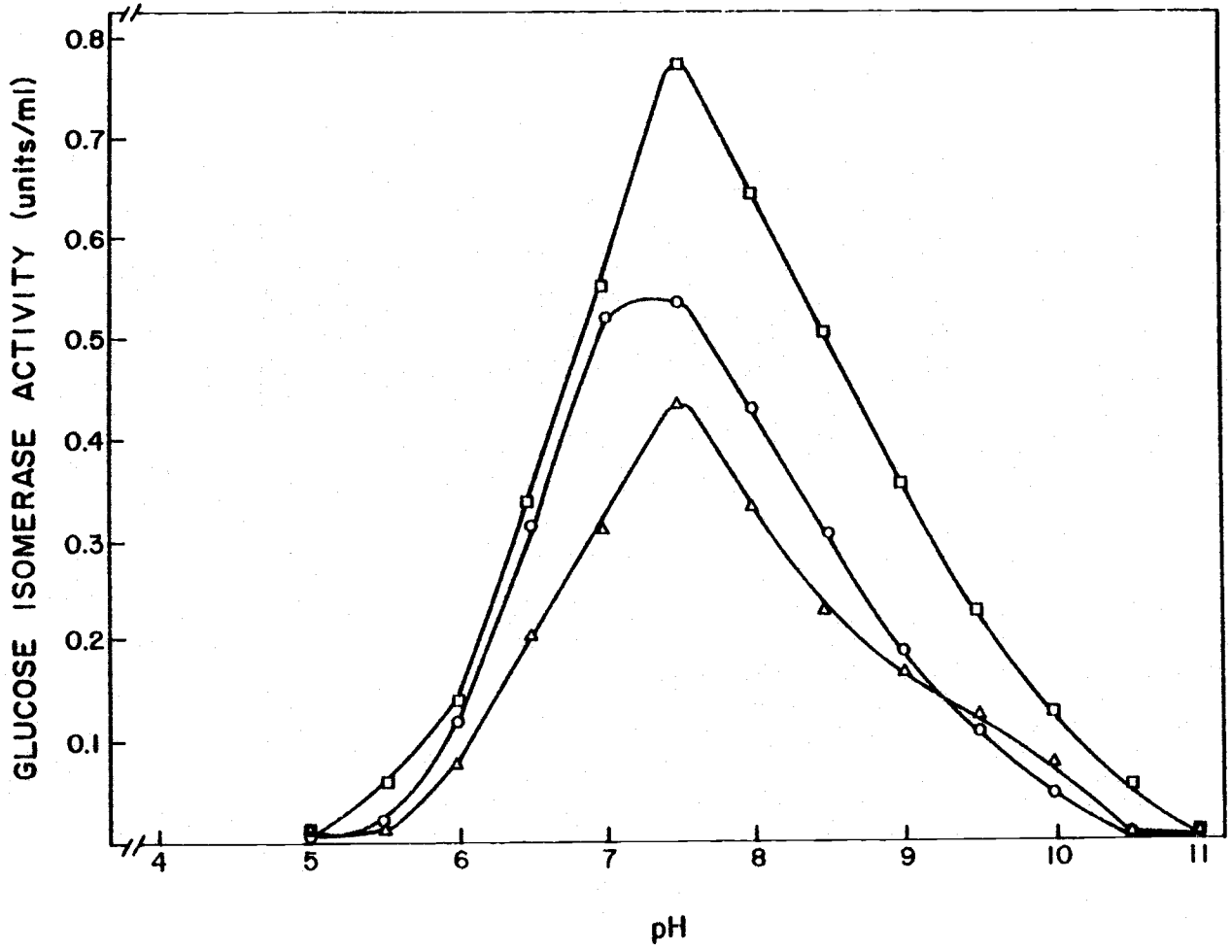

Figure 16. Effect of pH on glucose isomerase activity. Acetate buffers (0.05 M) were used for pH values of 4.0 to 5.0; sodium phosphate buffers (0.05 M) for pH 6.0-7.5; Tris buffers (0.05 M) for pH 8.0-9.0 and carbonate-bicarbonate buffers (0.05 M) for pH 9.5-11.0. Symbols: soluble enzyme (o); immobilized enzyme (Δ); whole cell (□).

Table 16. Effect of mineral salts on glucose isomerase activity.

Mineral salt	Concentration (M)	Relative activity (%)		
		Soluble enzyme	Immobilized enzyme	Whole cell
None	0	0	0	0
$MgSO_4 \cdot 7H_2O$	5×10^{-3}	100	100	100
$CoCl_2 \cdot 6H_2O$	"	71	82	76
$MnSO_4 \cdot 4H_2O$	"	21	28	19
$NiSO_4 \cdot 6H_2O$	"	17	28	38
$CaCl_2 \cdot 2H_2O$	"	15	6	19
$ZnSO_4 \cdot 7H_2O$	"	8	19	14
$BaCl_2 \cdot 2H_2O$	"	0	0	0
$CuSO_4 \cdot 5H_2O$	"	0	0	0
$FeSO_4 \cdot 7H_2O$	"	0	0	0
$Fe_2(SO_4)_3$	"	0	0	0
$HgCl_2$	"	0	0	0
$MgSO_4 \cdot 7H_2O$ + $CoCl_2 \cdot 6H_2O$	5×10^{-3} + 5×10^{-3}	283	300	252

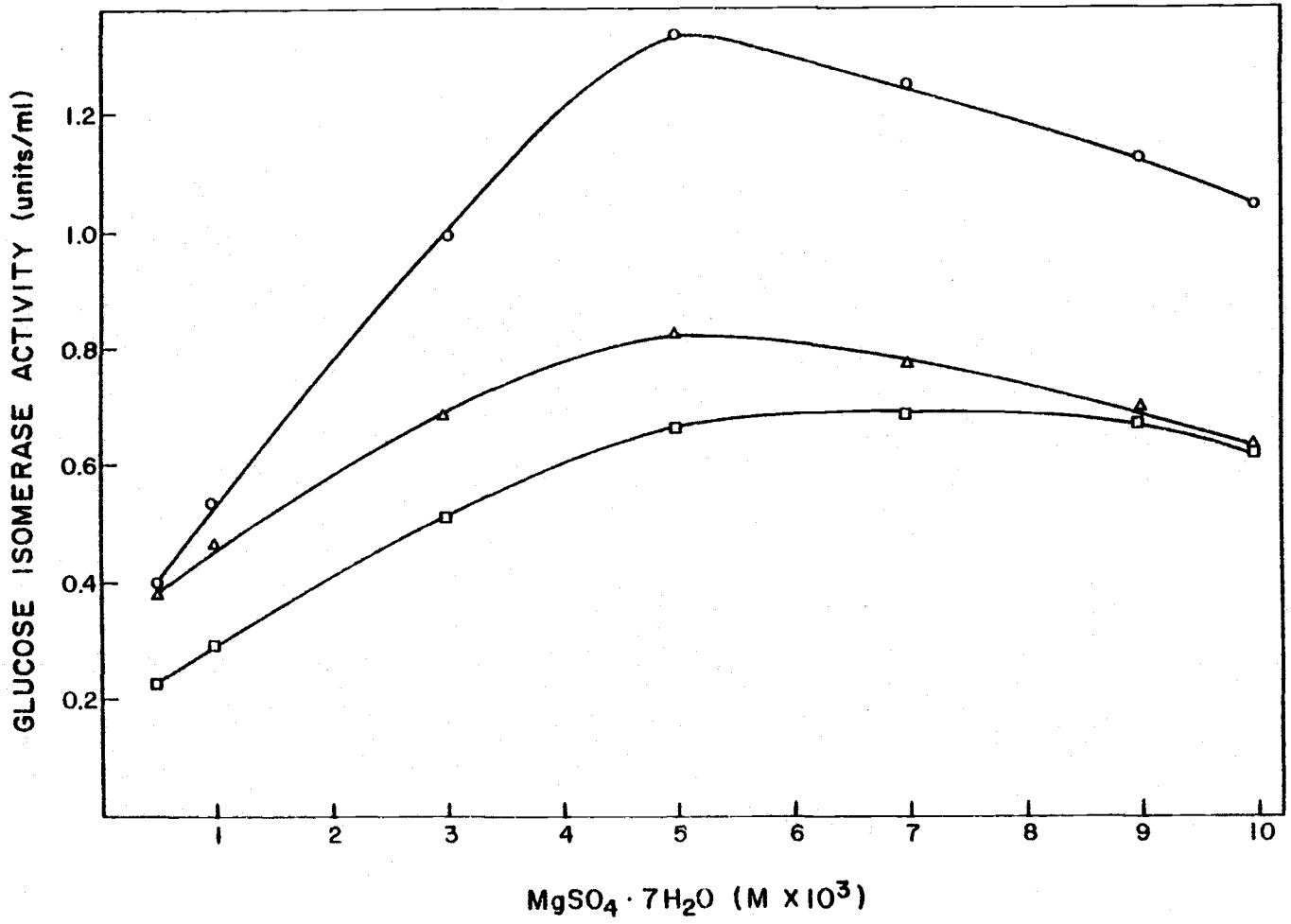

Figure 17. Effect of concentrations of magnesium sulfate on glucose isomerase activity. Symbols: soluble enzyme (o); immobilized enzyme (Δ); whole cell (□).

Table 17. Effect of various chemicals on glucose isomerase activity[a].

Chemical (10^{-3} M)	% Inhibition		
	Soluble enzyme	Immobilized enzyme	Whole cell
None	0	0	0
$HgCl_2$	100	87	81
$AgNO_3$	84	90	66
$CuSO_4 \cdot 5H_2O$	48	69	56
p-chloromercuri-benzoate	0	0	0
Maleic acid	0	0	0
Sorbic acid	0	0	0
Sodium azide	0	0	0
Sodium sulfite	0	0	0
EDTA	0	0	0
1,10-Phenathroline	0	0	0
Diethyldithio-carbamate	0	0	0
Hydroxylamine	0	0	0
Tris	0	0	0
D-gluconic acid	0	0	0
D-sorbitol	0	0	0
D-mannitol	0	0	0

a/ The enzyme was incubated at 35°C for 30 min in the presence of chemical as indicated and 5×10^{-2} M Mg^{2+} and 5×10^{-3} M Co^{2+}. The residual enzyme activity was measured.

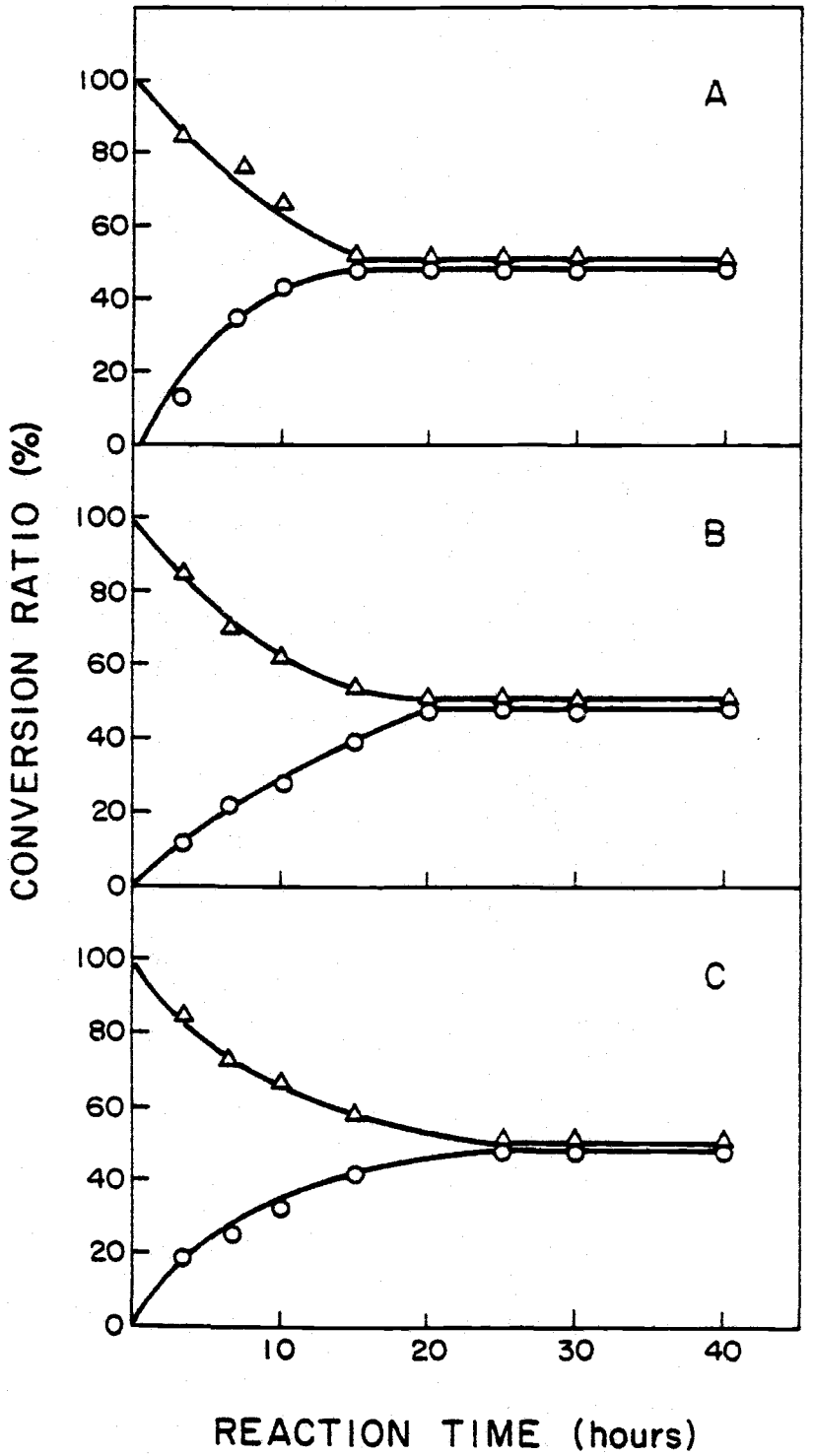

Figure 18. Time course of isomerization catalyzed by glucose isomerase at 70°C. Symbols: started from 0.1 M glucose (o); started from 0.1 M xylose (Δ); soluble enzyme (A); immobilized enzyme (B); whole cell (C).

50%.

Heat stability

The heat stability of the three enzyme preparations for various temperatures in the presence of Mg^{2+} and Co^{2+} is shown in Figure 19. There was almost no difference in heat stability between the soluble and immobilized enzymes for 10 min incubation period, but less heat stability was observed for whole cells.

pH stability

Figure 20 shows the pH stability of the three enzyme preparations. The enzyme preparations were kept at room temperature for 24 h. The soluble enzyme was stable under a slightly wider pH range (5.0-9.0) than the immobilized enzyme and whole cells (5.5-9.0).

Molecular weight

The purified enzyme migrated on SDS-polyacrylamide gel at a position corresponding to a molecular weight of 43,000 (Figure 21).

A sedimentation equilibrium experiment was carried out to determine the molecular weight of the enzyme in a ultracentrifuge. The molecular weight of the purified enzyme was calculated from the following equation:

$$M = \frac{2RT}{\omega^2(1-\bar{v}\rho)} \cdot \frac{\ln c(r)/c(a)}{r^2 - a^2}$$

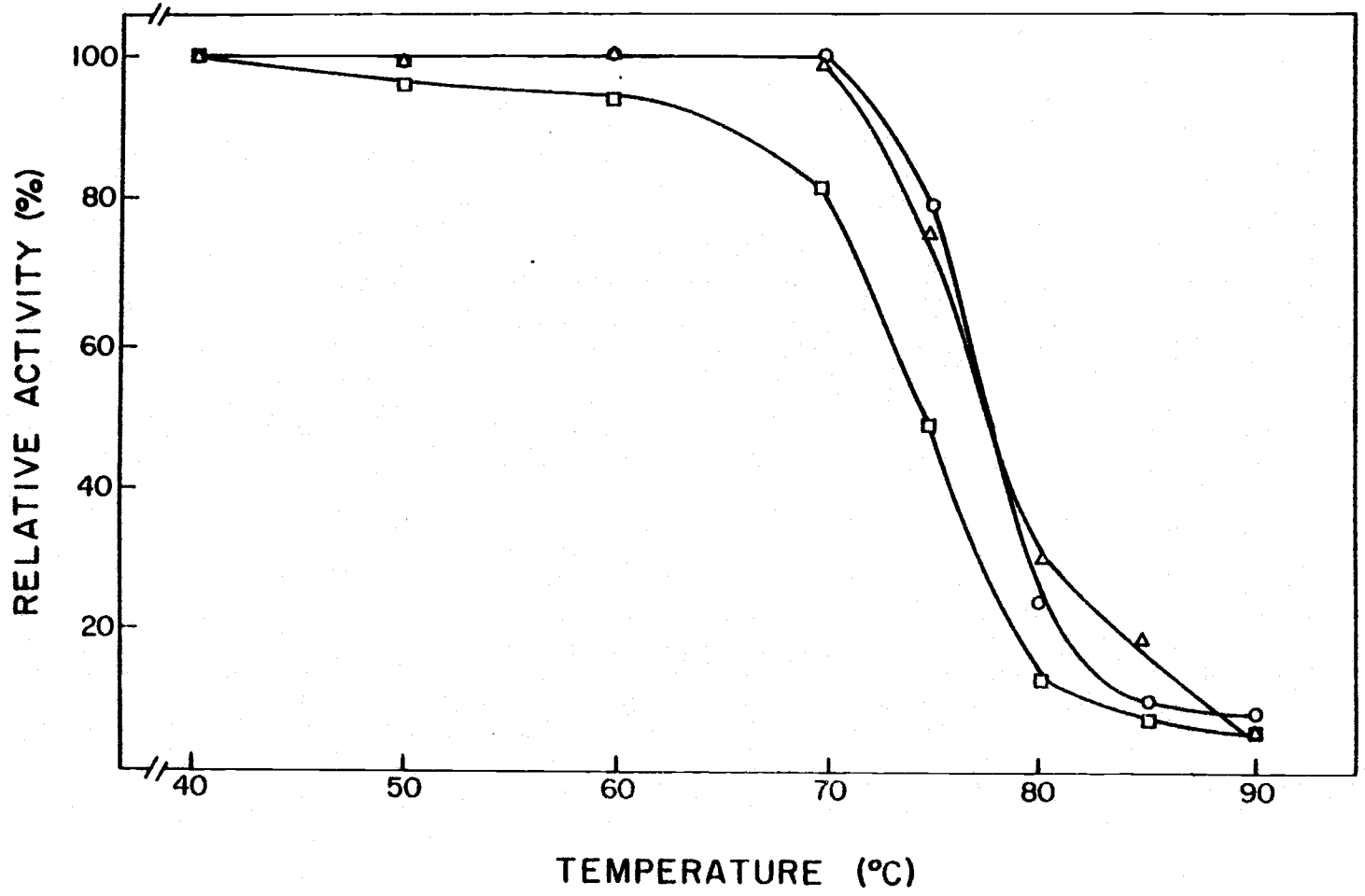

Figure 19. Heat stability of glucose isomerase. The aqueous enzyme solution, immobilized enzyme and cell suspensions were heated for 10 min at various temperatures as indicated and the residual activity was measured. Symbols: soluble enzyme (o); immobilized enzyme (△); whole cell (□).

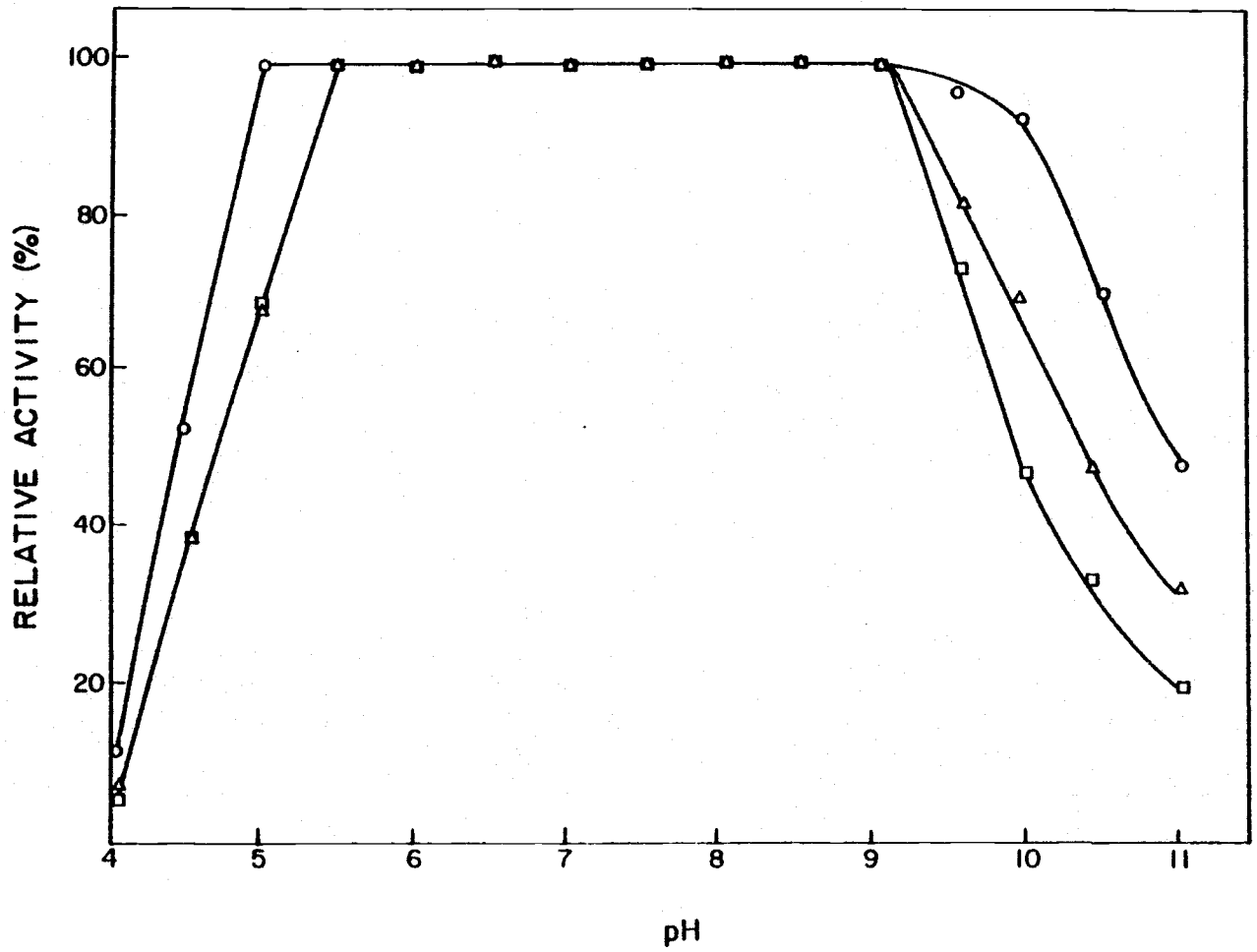

Figure 20. pH stability of glucose isomerase. The buffer systems were same as those indicated in Figure 16. The enzyme was incubated at different pH values and room temperature for 24 h and then neutralized to pH 7.0. The residual activity was measured. Symbols: soluble enzyme (o); immobilized enzyme (△); whole cells(□).

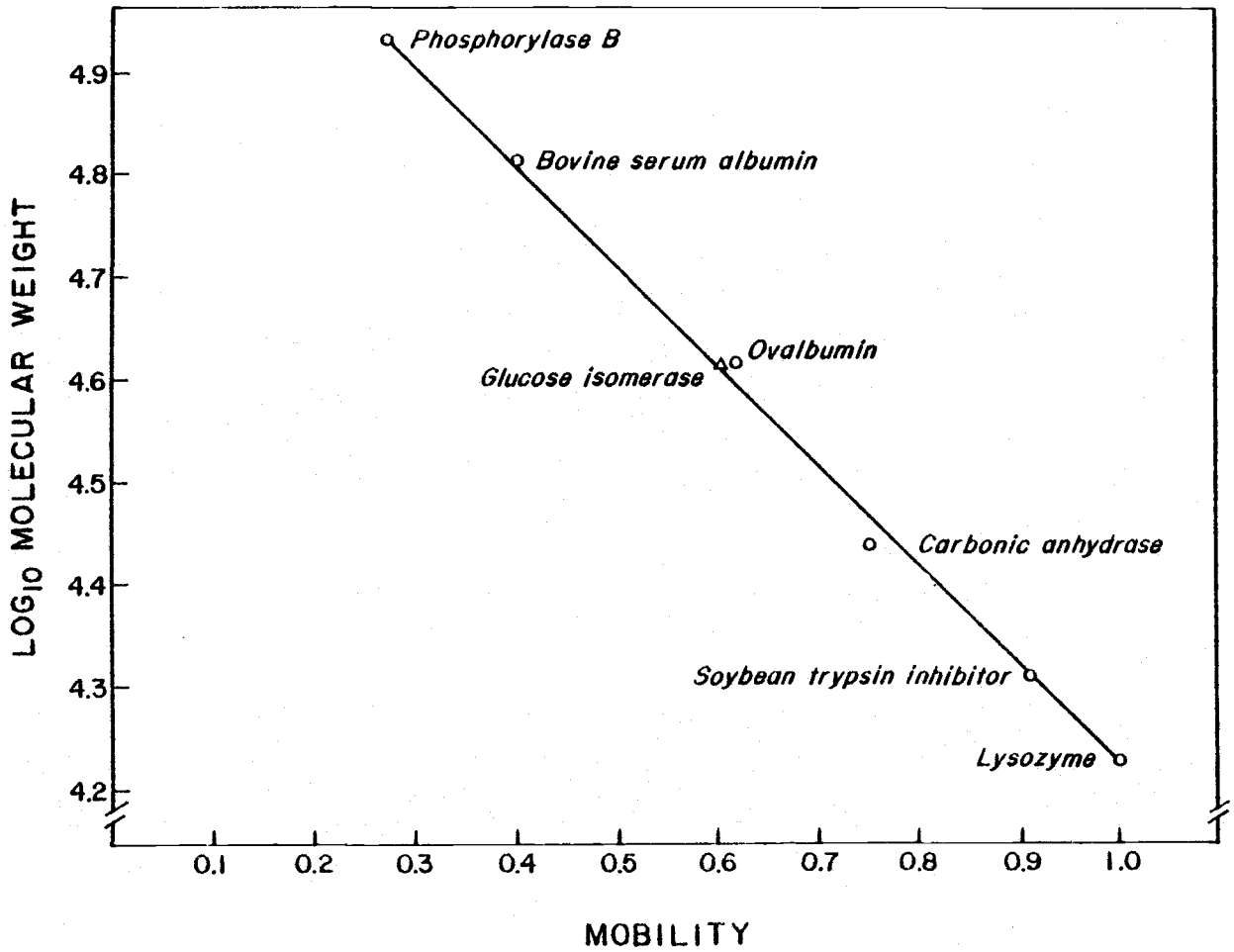

Figure 21. Comparison of molecular weight of standard proteins (lysozyme, 14,300; soybean trypsin inhibitor, 21,000; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 68,000; phosphorylase B, 94,000) and glucose isomerase by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

in which M is the molecular weight, R is the gas constant (8.314×10^7 ergs/$^\circ$K, mole), T is the absolute temperature in Kelvin (277°K), ω is the rotor velocity (712.4 radians/sec), $\bar{v}$ is the partial specific volume (0.69 ml/g, value from Takasaki et al., 1969), ρ is the density of the solvent (0.999973 g/ml at 4°C), c(r) and c(a) are the concentrations of the protein at a distance of r and a (meniscus) from the axis of rotation (expressed as absorbance at 280 nm). A plot of $\ln c(r)/c(a)$ versus $r^2 - a^2$ is shown in Figure 22. The slope of the straight line which represented $\ln c(r)/c(a) / r^2 - a^2$ was 0.584. The molecular weight of the purified enzyme calculated from the equation was 171,000. These data suggested a tetrameric structure for the enzyme, composed of four polypeptide chains of equal molecular weight.

Properties of glucose isomerase from S. flavogriseus are summarized in Table 18.

Continuous isomerization of D-glucose

Figure 23 shows the stability of the immobilized enzyme and whole cells when used for the continuous isomerization of D-glucose in a plug flow column. The immobilized enzyme was more stable than whole cells. The addition of both Mg^{2+} and Co^{2+} enhanced the stability of the immobilized enzyme and whole cells. After operating at 70°C for 5 days, the remaining enzyme activity for the immobilized enzyme and whole cells in the presence of Mg^{2+} and Co^{2+} was 75 and 55% respec-

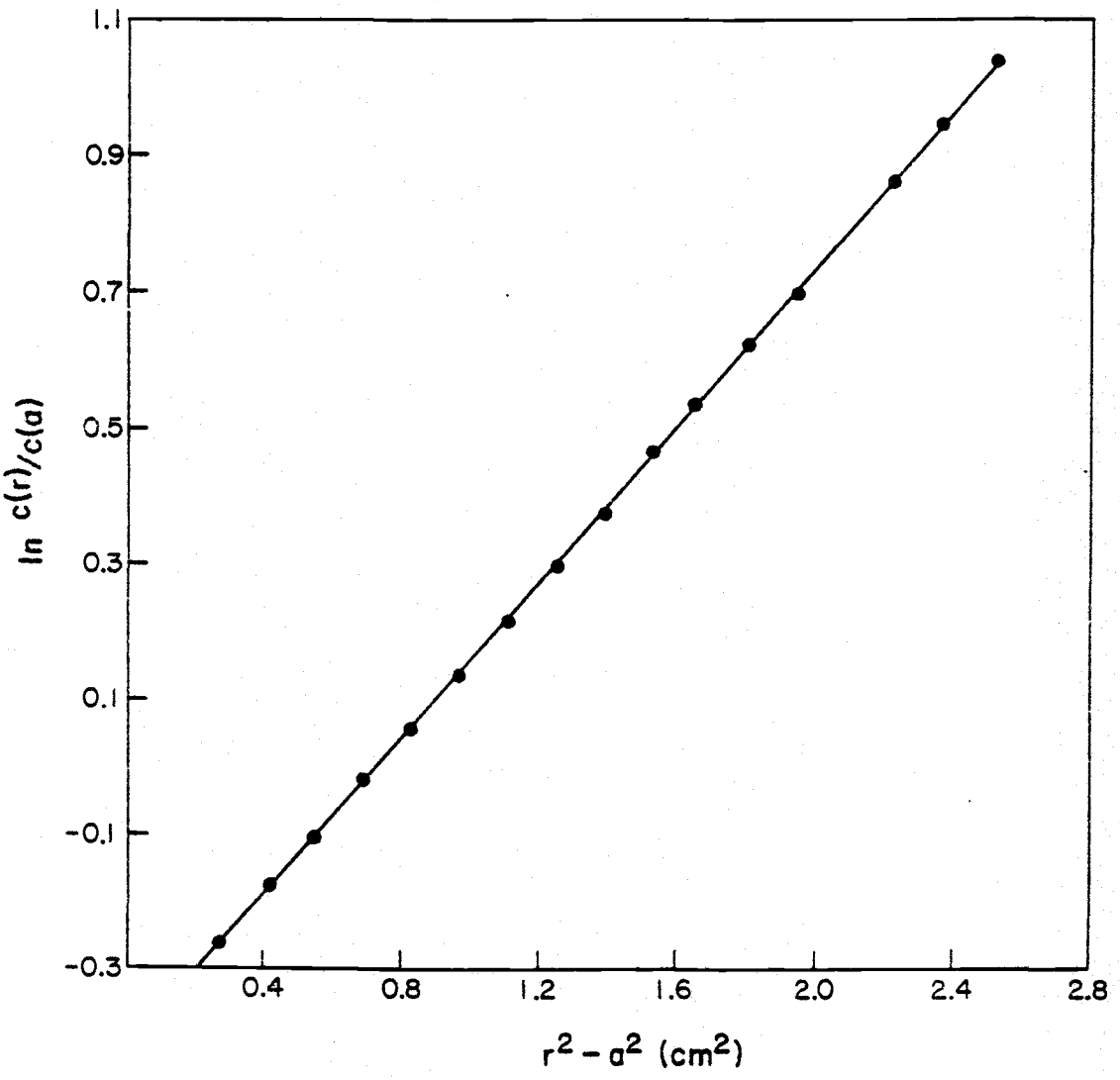

Figure 22. Sedimentation equilibrium of glucose isomerase as observed by absorption optical method.

Table 18. Properties of glucose isomerase of S. flavogriseus.

Property	Soluble enzyme	Immobilized enzyme	Whole cell
Substrate specificity	D-glucose, D-xylose	D-glucose, D-xylose	D-glucose, D-xylose
Km for D-glucose (M)	0.249	0.297	0.376
Km for D-xylose (M)	0.078	0.104	0.120
Optimum temperature (^{o}C)	70	70	75
Optimum pH	7.5	7.5	7.5
Metal requirement	Mg^{2+} or Co^{2+}	Mg^{2+} or Co^{2+}	Mg^{2+} or Co^{2+}
Inhibitor	Hg^{2+}, Ag^{+}, Cu^{2+}	Hg^{2+}, Ag^{+}, Cu^{2+}	Hg^{2+}, Ag^{+}, Cu^{2+}
Conversion ratio at 70^{o}C (%)	50	50	50
Heat stability (70^{o}C, 10 min) (%)	100	98	80
pH stability	5.0-9.0	5.5-9.0	5.5-9.0
Molecular weight of subunit	43,000	--	--
Molecular weight	171,000	--	--
Number of subunit	4	--	--

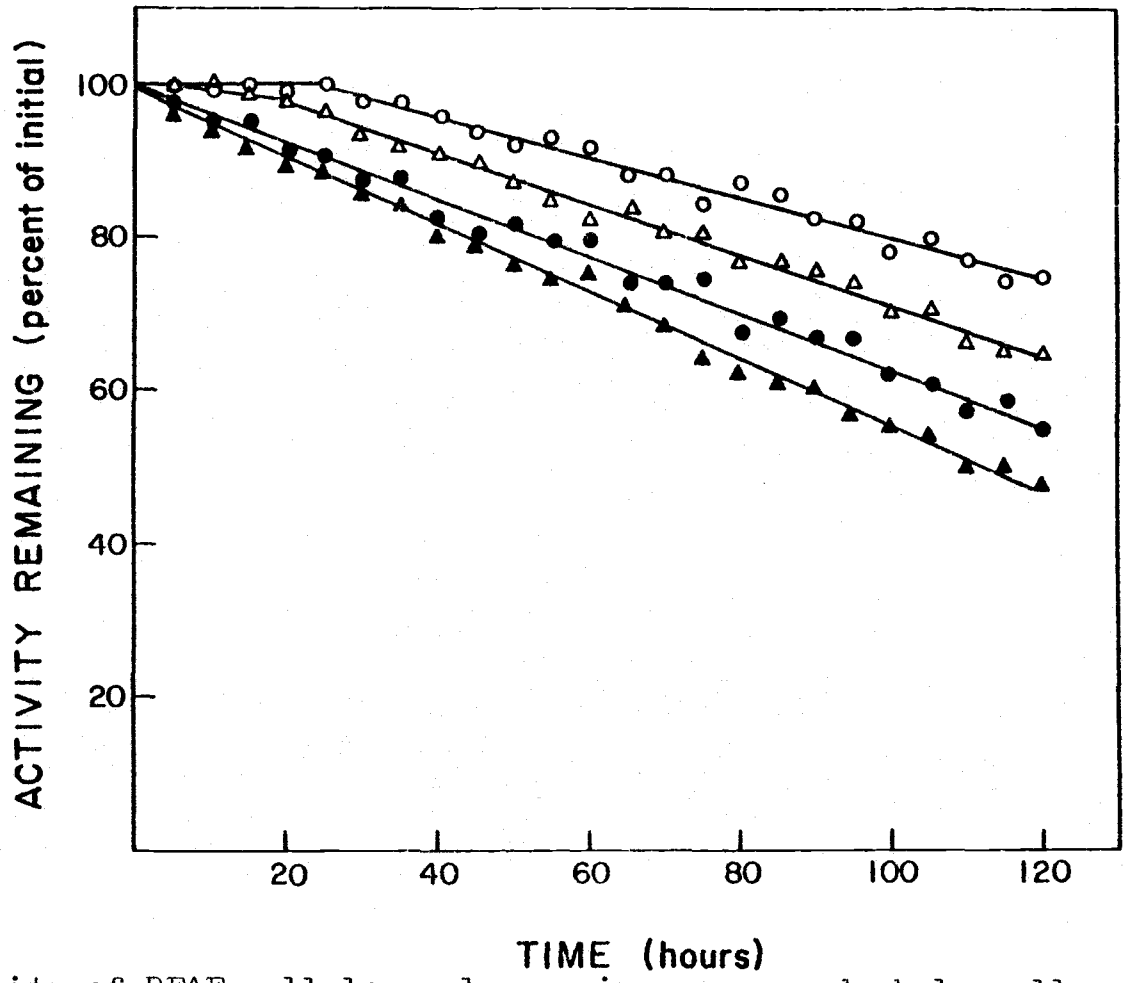

Figure 23. Stability of DEAE-cellulose-glucose isomerase and whole cells used for continuous isomerization of 1 M D-glucose in a plug flow column. Symbols: column (2.5 x 5 cm) packed with DEAE-cellulose-glucose isomerase providing 5 mM Mg^{2+}, & 1 mM Co^{2+}(o); column (2.5 x 9 cm) packed with whole cells providing 5 mM Mg^{2+} & 1 mM Co^{2+}(△); column (2.5 x 5 cm) packed with DEAE-cellulose-glucose isomerase providing 5 mM Mg^{2+} (●); column (2.5 x 9 cm) packed with whole cells providing 5 mM Mg^{2+} (▲).

tively. However, the remaining enzyme activity in the presence of Mg^{2+} alone decreased to 65 and 47% respectively. In all cases, the conversion ratio of D-glucose to D-fructose in the very beginning of operations was around 40% at a flow rate of 12 ml/h.

DISCUSSION

Recent worldwide interest in commercial utilization of many cellulosic plant materials e.g. sugarcane bagasse, wheat straw, corncobs and stalks—has inspired investigations of their hemicelluloses. Even though the chemical composition of ryegrass straw has been extensively studied (Han et al., 1975), the extraction of hemicellulose from straw was not done in the previous work. In this study, a procedure for economical extraction of hemicellulose from straw was developed. The optimum conditions for extraction were with 4% NaOH for either 3 h at 90°C or 24 h at room temperature. However, the alternate choice depends on the economy or convenience of using high temperature for a short time (90°C, 3h) or low temperature for a long time (room temperature, 24h). For preparation of large amount of hemicellulose, extraction of straw at room temperature for 24 h is considered to be more practical.

The extraction of hemicellulose from straw with NaOH was enhanced by heat, but the yield declined if the temperature was higher than 90°C, probably due to the degradation of hemicellulose. Ross and Thompson (1965) reported that extraction of hemicellulose with alkali at high temperature could result in loss of the O-methyl group from 4-O-methyl-D-glucuronic acid residues of hemicellulose. The degradation can be prevented if the hemicellulose is reduced with borohydride. D-xylans were found to be degraded slowly

when treated with cold, dilute alkali under the usual extraction conditions (Aspinall et al., 1961). Moreover, elimination of oxygen during alkaline extraction minimizes oxidative degradation of the hemicellulose (Whistler and Bemiller, 1958). Obviously, these treatments are impractical on a large scale.

The straw hemicellulose was employed as a sole source of carbon in the medium used to isolate the organisms that were capable of degrading straw hemicellulose and producing glucose isomerase. The medium used for the isolation of soil actinomycetes had the similar composition as that developed by Kuster and William (1964). The growth rate of actinomycetes is generally lower than that of bacteria and fungi. Thus, the medium was designed to inhibit development of bacteria and fungi and to allow actinomycetes colonies to appear.

Several workers selected glucose isomerase producing organisms using pure culture directly from culture collections (Stranberg and Smiley, 1971; Yamanaka, 1963a) while others isolated organisms from soil by means of enrichment techniques (Natake and Yoshimura, 1963, 1964; Tsumura and Sato, 1961; Yoshimura et al., 1966). In these studies, enrichment culture media containing xylose or mannitol as a carbon source were inoculated with soil and incubated for several days, and then cultivated on plates with agar medium. The colonies formed were tranferred to agar slants.

Sanchez and Smiley (1975) used the direct plating technique on an inorganic-agar medium containing xylose as a sole source of carbon. Direct plating technique was also employed in this study except that xylose was replaced by straw hemicellulose. It appears that the direct plating technique is more efficient and also involves the use of less laboratory materials than the enrichment technique.

The organisms isolated by either direct plating or enrichment technique only show that they can grow on the medium used. In order to test for glucose isomerase production, each organism must be grown on a suitable liquid medium and then the cells must be harvested for the enzyme essay. The procedure is tedious and time consuming. Nevertheless, good screening tests are presently not available. The further development of a powerful selective medium on agar plates would enable us to select glucose isomerase producing organisms efficiently.

Although Streptomyces species have been the most extensively studied and used as a source of glucose isomerase, no reports were found concerning the production of glucose isomerase from S. flavogriseus which was employed throughout this study. Chemical treatments of ryegrass straw appeared to be much more effective in enhancing glucose isomerase production than physical treatment. The organism produced a high yield on straw hemicellulose, H_2SO_4 hydrolysate of straw, xylose, or xylan. The highest level was obtained on

2% straw hemicellulose. The optimum concentrations of xylose and xylan for enzyme formation were found to be higher than those reported by Takasaki (1966). Among various H_2SO_4 hydrolysates of straw, 3% straw prepared with 0.1 N H_2SO_4 was the most effective. The levels of glucose isomerase and xylose in the hydrolysates did not correlate, probably due to toxic substances, such as furfural and other resious materials, which often inhibit microbial growth and enzyme synthesis (Israilides, 1978) produced during acid hydrolysis. It is interesting to note that the level of the enzyme produced on the straw hydrolysate containing 0.2% xylose was equivalent to a medium containing 1.25% xylose. Grant et al. (1977) studied the acid hydrolysis of ryegrass straw by using conditions similar to this study. They indicated that acid hydrolysate contains glucose, mannose and other monosaccharides in addition to xylose. The results suggested that low levels of xylose are enough to induce high level of glucose isomerase formation if other carbon sources such as glucose are also present in the medium. Venkatasubramanian (1977) investigated the glucose isomerase induction and repression in S. venezuellae. In the presence of both glucose and xylose, a typical diauxic growth was observed. Glucose was rapidly utilized first, xylose consumption started only after glucose was completely exhausted and enzyme synthesis began only when xylose was taken up by the cells. The above results also suggested that optimal pro-

duction of cells and enzyme could be obtained by growing the cells in an inexpensive glucose medium and providing a low level of xylose to induce glucose isomerase production. The production cost could be reduced considerably by this modification.

A medium containing NaOH extract of straw was only 23% as effective for enzyme production as 2% straw hemicellulose, probably due to the presence of high concentration of salt after neutralization and other inhibitory substances. The preparation of NaOH extract of straw does not require an ethanol precipitation and distillation as well as a drying processes which are essential for preparation of straw hemicellulose. NaOH extract of straw could be a cheap and effective carbon source for enzyme production if additional treatments such as desalting or partial purification which might improve enzyme production were made.

The results in this study showed that organic nitrogen sources including corn steep liquor, soy flour extract, yeast extract and various peptones were much more effective in production of glucose isomerase than inorganic nitrogen sources such as $(NH_4)_2HPO_4$, probably the organic nitrogen sources contain unknown growth factors which favor cell growth. In this study, it was unnecessary to remove the sludge from corn steep liquor in order to achieve a high yield of enzyme production (data not shown), but was essential for enzyme production by A. missouriensis (Shieh et al., 1974).

It was found that Mg^{2+}, Mn^{2+}, Fe^{2+} or Zn^{2+} significantly enhanced enzyme production indicating that these metal ions play a role either in the biosynthesis of the enzyme or as active enzyme cofactors. In general, Mg^{2+}, Mn^{2+} or Co^{2+} is usually required by organisms for enzyme production (Table 2). The addition of Zn^{2+} brought about an obvious repression on the growth and enzyme production, this effect was also noted for B. coagulans and E. intermedia (Natake and Yoshimura, 1964; Yoshimura et al., 1966). In this study, combination of Mg^{2+} and Mn^{2+}, Mg^{2+} and Co^{2+}, or Mn^{2+} and Co^{2+} resulted in the production of less enzyme than did the addition of Mg^{2+} or Mn^{2+} alone. The result was different from that reported by Takasaki (1966) in which the addition of Co^{2+} to the Mg^{2+}-containing medium remarkably stimulated enzyme formation. The present study also indicated that Co^{2+} was not required for enzyme production of S. flavogriseus when Mg^{2+} or Mn^{2+} was present. The results were different from others (Giovenco et al., 1973; Stranberg and Smiley, 1971; Takasaki et al., 1969; Tsumura et al., 1967a) which required Co^{2+} to stimulate the formation of glucose isomerase. The elimination of Co^{2+} in the culture medium is important in commercial production because Co^{2+} is an environmental hazard (Jacobziner and Raybin, 1961; Somers, 1974).

The optimum pH for growth and glucose isomerase production by S. flavogriseus fell between 6.0 to 8.5. No

information was found in the literature concerning the effect of pH on glucose isomerase formation, however, neutral pH (6.8-7.2) ranges are generally chosen for enzyme production (Table 2). The pH of the spent medium was alkaline, a characteristic results in cultures of Streptomyces, probably due to the release of ammonia during fermentation. The opposite was observed in cultures of B. coagulans in which the pH decreased from neutral to acidic (Yoshimura et al., 1966).

Bacillus coagulans, L. brevis, S. albus and S. bikiniensis have been used to study the time course of cultivation and formation of glucose isomerase (Park and Toma, 1974; Takasaki, 1966; Yamanaka, 1963b; Yoshimura et al., 1966). These studies showed that B. coagulans and L. brevis required less time to reach the maximum yield of glucose isomerase than did S. albus and S. bikiniensis. For S. flavogriseus, the results were similar to that of the latter. In the early stage of growth, extracellular hemicellulase was produced which degraded straw hemicellulose into hexoses and pentoses for growth and induction of glucose isomerase. It is important to harvest the cells at the appropriate time as the level of intracellular enzyme decreased sharply after 36 h. Extracellular glucose isomerase, which perhaps was released by autolysis, was highest at 72 h of culture. It was not suprising that S. glaucescens produced extracellular glucose isomerase (Weber, 1975), since this capability could

be also demonstrated for S. flavogriseus if the cultivation time were extended.

Sodium hydroxide has been most commonly used to improve the digestibility of cellulosic crop residues (Anderson and Ralston, 1973; Beckman, 1922; Chandra and Jackson, 1971). By treating cellulosics with NaOH, the fiber structure is believed to be swollen and water holding capacity and penetrability by microorganisms and enzymes are increased (Han, 1978). In this study, ryegrass straw residue obtained after removal of hemicellulose increased the in vitro rumen digestibility from 44.9 to 78.6%, an increase almost equivalent to that obtained by NaOH treatment before removal of hemicellulose (Han et al., 1978). Thus, removal of hemicellulose did not adversely affect the digestibility of straw, however, washing with water to remove residual NaOH reduced digestibility of treated straw residue to 56.2%. Thus, if the residue is to be used as feed the washing NaOH-treated straw should be adjusted to balance the maximum recovery of hemicellulose and maximum increase in digestibility.

In studies on forage quality, the meadow vole has proven to be a useful bioassay animal for determination of nutritive quality and toxic constituents of plants (Keys and Van Soest, 1970; Shenk, 1976; Shenk et al., 1974). The vole is a herbivorous animal with a digestive system capable of gastric and cecal digestion of fiber. Because of their size, feeding

studies can be easily conducted with voles, and the result is useful for predicting the response of ruminant animals to NaOH-treated straw. Weight gain, feed intake and feed efficiency were studied in voles and found to be significantly increased by NaOH treatment. This effect, however, was diminished when the treated straw residue was thoroughly washed. The high pH (12.0) of the partially washed straw residue did not adversely affect the palatability of the diet for meadow voles. In Beckman's (1922) process large volumes of water were required resulting in heavy losses of solubilized nutrients. Wilson and Pigden (1964) described a dry process in which wheat straw was treated with a reduced volume of 20% NaOH solution and then left in situ. Feeding trials showed that treated straw containing 2% residual NaOH was rapidly accepted by sheep when mixed with corn silage or ground alfafa hay. Chandra and Jackson (1971) suggested that until a 6% NaOH treatment was reached, the residual NaOH was not high enough to require washing. Anderson and Ralston (1973) also found that the general health of the sheep consuming NaOH-treated ryegrass straw was not impaired. These observations were similar to the results of this study indicating that NaOH-treated ryegrass straw residue could be used as a ruminant feed.

Two processes were developed for the production of glucose isomerase from ryegrass straw. In one process, ryegrass straw was treated with NaOH and hemicellulose

obtained from NaOH extract of straw was used as the carbon source for cultivation of S. flavogriseus. Glucose isomerase activity can be detected in some organisms such as S. albus, S. bikiniensis and S. flavovirens grown on a medium containing xylose or xylan (Japan Bureau of Industrial Technics, 1966b; Park and Toma, 1974; Takasaki, 1966). Streptomyces flavogriseus not only produced the enzyme on xylose or xylan, but it also produced a higher level of the enzyme than those organisms mentioned above on straw hemicellulose. Thus, it should have a considerable economic advantage over the previous processes as straw hemicellulose is less expensive to produce than pure xylose or xylan and straw residue remaining after removal of hemicellulose can be used as ruminant feed. Moreover, the spent medium contains hemicellulase which can be recovered for industrial use (Aunstrup, 1977).

The second process consisted of dilute H_2SO_4 hydrolysis followed by cultivation of S. flavogriseus on the neutralized hydrolysate. Dworschack et al. (1971) used acid hydrolysate of corncobs to supply xylose for enzyme induction by S. wedmorensis ATCC-21175. Streptomyces flavogriseus produced almost as much glucose isomerase on acid-treated straw as it did on alkali-treated straw. A lesser amount of chemicals was required for acid hydrolysis than for alkaline treatment. However, H_2SO_4 treatment required a high temperature (121°C) while NaOH treatment was effective at room temperature. Furthermore, acid hydrolyzed straw was not

suitable for ruminant feeding because of its low digestibility (Israilides et al., 1978). Also, the spent medium did not contain any recoverable hemicellulase. For the above reasons, NaOH treatment was considered to be a more favorable process in the production of glucose isomerase.

In the future, further enhancement of glucose isomerase production could be done by a combination of genetic engineering techniques and the more classical methods such as mutation and enviromental manipulations which are usually employed for improving enzyme production (Skinner, 1975).

The cells which were harvested from the culture could be utilized as whole cells or soluble enzyme extracted from autolyzed cells. The activity of the cells remaining after dehydration or freezing were studied. Cells prepared as acetone powder or by lyophilization gave higher glucose isomerase activity than other methods. However, Tsumura et al. (1967b) indicated that cells of S. phaeochromogenes dehydrated on a clay plate at room temperature showed the best result and did not lose enzyme activity during dehydration while acetone powder and lyophilized cells lost some activity.

In this investigation, cationic detergent (cetyltrimethylammonium bromide or cetylpyridinium chloride) treatment extracted almost the same amount of glucose isomerase with high specific activity from cells of S. flavogriseus as did mechanical disruption (sonication or abrasive grind-

ing). Lysozyme and toluene or toluene alone were relatively less effective than cationic detergents in extracting the enzyme. Takasaki and Kanbayashi (1969a) reported that the liberation of glucose isomerase was remarkably accelerated when a cationic detergent (cetylpyridinium ohloride) was added to a cell suspension of Streptomyces. The yield of glucose isomerase from this treatment was almost complete, compared with that by sonication. Treatment of cell suspensions with lysozyme and toluene also proven to be a promising method for enzyme extraction (Hirota et al., 1977; Sipos, 1973; Suekane et al., 1974). The results were similar to those obtained from this study; however, this study indicated detailed quantitatively data on yields and release kinetics. Also, it quantitatively compared the effectiveness of alternate chemical treatments and mechanical disruption and such comparisons were not made in the previous work. The extraction of glucose isomerase by cationic detergents is simple, inexpensive and suitable for large-scale extraction of the enzyme.

As mentioned earlier, numerous methods are available for immobilization of glucose isomerase. Among them, attachment of the enzyme to ion-exchange materials is considered to be a simple, inexpensive and promising method (Weetall, 1973). In this study, various anionic and cationic exchangers were used to immobilize partially purified glucose isomerase. It was found that anionic exchangers were effec-

tive in adsorbing glucose isomerase; whereas cationic exchangers were ineffective. Benzyl DEAE-cellulose and TEAE-cellulose retained more enzyme activity and protein than other ion exchangers. A buffer system of pH 7.0 was used in the preparation of the immobilized enzyme. The data suggested that the isoelectric point (pI) of the glucose isomerase was below 7.0. DEAE-cellulose has been used for immobilization of glucose isomerase by several investigators (Huitron and Limon-Lason, 1978; Lloyd and Khaleeluddin, 1976; Park and Toma, 1975; Sipos, 1972), the process is now used commercially by Clinton Corn Processing Co. (Schnyder, 1974). However, no reports were found concerning the effectiveness of benzyl DEAE-cellulose and TEAE-cellulose in the immobilization of glucose isomerase, possibly because these two materials are too expensive for practical use. Therefore, DEAE-cellulose was selected in this study for further investigation of the maximum enzyme that DEAE-cellulose could adsorb. The retention of activity after adsorption to DEAE-cellulose appeared to be lower than that reported for the enzyme of B. coagulans adsorbed to DEAE-cellulose (Huitron and Limon-Lason, 1978). They also examined the adsorption of the enzyme to AE-cellulose, DEAE-Sephadex A-25 and A-50. The retained activity of the DEAE-cellulose-glucose isomerase complex was higher than that obtained with other absorbents. In both studies, immobilization of glucose isomerase showed an increase in specific activity because

impurities in the crude enzyme preparations were not retained.

Glucose isomerase is usually prepared in the form of heat-treated whole cells or immobilized enzyme for industrial application by taking advantage of the fact that these preparations are relatively stable and capable of being reused under a continuous operation. Therefore, a comparison of properties between purified soluble enzyme, heat-treated (60°C, 10 min) whole cells and immobilized enzyme (DEAE-cellulose-glucose isomerase complex) was made in this study to provide information for their practical applications.

Glucose and xylose served as substrates for the enzyme of S. flavogriseus, but the enzyme did not isomerize other pentoses, hexoses, sugar alcohols, glucoside and sugar phosphate showed in Table 15. Besides glucose and xylose, some glucose isomerases from other microbial sources also catalyze the isomerization of ribose, arabinose, rhamnose, allose and deoxy-glucose (Table 3). Sanchez and Smiley (1975) indicated that steric correlation was found among these carbohydrates with the exception of rhamnose and 2-deoxy-glucose, since these sugars have a C 1 conformation and the hydroxyl groups on carbon 2 are in the equatorial position. When the hydroxyl groups of carbon 3 and 4 are in the equatorial position as in glucose and xylose, maximum isomerization is obtained.

Immobilization of an enzyme on the surface of a carrier

usually changes the Km value of that enzyme (Weetall, 1973). Some apparent Km values are large than the values of the soluble enzymes, while others are smaller. Kinetics of immobilized enzymes are generally affected by carrier and substrate charges, pore diameter of the carrier, bulk and pore diffusion rates, and many other parameters. In this study, it was found that the immobilization of glucose isomerase on DEAE-cellulose increased the apparent Km values for both glucose and xylose. The apparent Km values of whole cells were even higher than those of the immobilized enzyme. Surprisingly, Lloyd and Khaleeluddin (1976) reported that both the soluble and immobilized form (DEAE-cellulose-glucose isomerase complex) of the enzyme gave the same Km value for glucose in batch reactions. Later, Huitron and Limon-Lason (1978) observed a slight increase in apparent Km value for glucose on adsorption of glucose isomerase to DEAE-cellulose. They believed that this was due to microenvironmental effects. It is interesting to note that the Km value of the enzyme from either S. flavogriseus or other organisms showed higher affinity for xylose than glucose except for the enzyme from S. albus NRRL B-5778 (Sanchez and Smiley, 1975).

The optimum temperature for activity of the enzyme was 70°C, which was lower than that (80°C) reported by other investigators (Table 3). Chou et al. (1976) indicated that degradation of ketoses occurs at the higher temperatures

shown by a pronounced discoloration of an aqueous sugar solution. Thus, the temperature of 70°C was used to isomerize glucose even though the optimum temperature was 80°C. In this case, the use of the enzyme from S. flavogriseus is not hampered by its relatively low optimum temperature.

The pH optimum for activity of the enzyme was 7.5 which was apparently lower than that of enzyme from other Streptomyces species (Table 3). The glucose isomerases from A. missouriensis, B. coagulans and L. brevis also have a low pH optimum (Danno, 1970; Scallet et al., 1974; Yamanaka, 1968). Under alkaline conditions, a non-metabolizable sugar, D-psicose, is produced in hot glucose and fructose solution (Bucke, 1977). Therefore, low pH optimum is an attractive property for enzyme application because the use of neutral or lower pH in the isomerization of glucose prevents the formation of D-psicose. In this study, the soluble enzyme, immobilized enzyme and whole cells had the same pH optimum. The pH optimum of DEAE-cellulose-glucose isomerase complex, however, shifted slightly toward acidic side in comparison to the soluble enzyme (Huitron and Limon-Lason, 1978). A big shift from 7.0 for soluble glucose isomerase, to 8.5 for immobilized cell debris of B. coagulans was reported by Poulsen and Zittan (1975).

As in most investigations, glucose isomerase of S. flavogriseus required Mg^{2+} or Co^{2+} for its activity. The metal requirements for enzyme activity appeared to be

different from that for enzyme production. It was found that an addition effect resulted from the presence of both Mg^{2+} and Co^{2+}. Tsumura and Sato (1965b) also demonstrated that Co^{2+} activated glucose isomerase of S. phaeochromogenes in the presence of Mg^{2+} and protected the enzyme against heat denaturation. For the enzyme from S. albus, the requirements for Mg^{2+} and Co^{2+} were similar to those described above and the crystalline enzyme contained 1.4 atoms of Co^{2+} and 0.3 atom of Mg^{2+} per molecular of the enzyme (Takasaki et al., 1969). Both Mg^{2+} and Co^{2+} were required for high activity of glucose isomerase from A. missouriensis; Magnesium ion was essential for activity while Co^{2+} enhanced the activity (Scallet et al., 1974). An important feature for the enzyme of Arthrobacter sp. was that it did not require Co^{2+} for activity or stability (Lee et al., 1972). In this study, Mg^{2+} was more effective than Co^{2+}, but the reverse was true for glucose isomerase of B. coagulans (Danno et al., 1967). Like the glucose isomerase from L. brevis (Yamanaka, 1968), the enzyme from B. coagulans required Co^{2+} to isomerize glucose and ribose but Mn^{2+} to isomerize xylose (Danno, 1970). He concluded that the active form of the enzyme was that which contained Co^{2+} or Mn^{2+}. Danno (1971) proposed a possible function of metal ions for the isomerization and for substrate specificity of the enzyme. Binding Co^{2+} and Mn^{2+} caused suitable conformation charges in the catalytic site of the enzyme for

glucose and xylose isomerization respectively.

Various chemicals were tested as inhibitors of glucose isomerase of S. flavogriseus. Only heavy metals such as Hg^{2+}, Ag^{+} and Cu^{2+} were found to have inhibitory effect on activity. Sorbitol, mannitol and Tris did not inhibit the enzyme although these chemicals were reported to be inhibitors for glucose isomerase by several investigators (Sanchez and Smiley, 1975; Takasaki et al., 1969; Tsumura and Sato, 1965b). Chemicals including p-chloromercuribenzoate, maleic acid and sorbic acid which can react with sulfhydryl groups of enzymes did not inhibit enzyme activity. The results suggested that a imdazole, carbcxyl or peptide group which can combine with heavy metals was probably involved in the active site of the enzyme rather than the sulfhydryl group. As mentioned earlier, the enzyme required Mg^{2+} or Co^{2+} for its activity, but the cation-chelating reagent, EDTA, did not inhibit enzyme activity, perhaps because either the concentration (10^{-3}M) of EDTA which is not high enough to inhibit enzyme activity or the stability of the cation-enzyme complex which is greater than that of the complex formed between the cation and EDTA (Whitaker, 1972).

The purified soluble enzyme from S. flavogriseus was quite stable, since it retained full activity after incubation at 70°C for 10 min. The thermal stability of glucose isomerase from B. coagulans was reported to be as good as that of the enzyme from S. albus (Danno, 1970; Takasaki et

al., 1969). The enzyme from A. missouriensis appeared to be the most stable of all the known glucose isomerase because it retained activity at 90°C for 20 min. In this study, it was also found that almost no difference existed in thermal stability between soluble and immobilized enzymes, but less stability was observed for whole cells. In many enzyme systems immobilization increases the stability under conditions of increased temperature (Weetall, 1973). Some immobilized enzymes have less stability at high temperature even in the presence of substrates which usually increase the stability. At present, it is difficult to predict which enzymes will show increased or decreased stability on immobilization. The heat stability of soluble and immobilized glucose isomerase (adsorbed on DEAE-cellulose) for various temperatures has also been studied by Huitron and Limon-Lason (1978). For 15 min periods studied, they did not find any differences between the preparations. However, Park and Toma (1975) reported that DEAE-cellulose bound enzyme was relatively less heat resistant than semipurified soluble enzyme.

In this investigation, only a slight difference was observed in pH stability between soluble enzyme (5.0-9.0), immobilized enzyme (5.5-9.0) and whole cells (5.5-9.0). The enzyme from S. albus was stable in a wider pH (4.0-11.0) range (Takasaki et al., 1969) but the pH stability of the enzyme from L. brevis was apparently narrow, 7.0-9.0

(Yamanaka, 1968).

The sedimentation equilibrium method was used for the determination of molecular weight of glucose isomerase. When compared to other methods, this method has the advantages of having both a firm theoretical basis and a very high precision of measurement (error < 1%) (Freifelder, 1976). Moreover, it only requires a small amount of material and it is also a preferred technique for determination of the homogeneity of proteins. Only a minor disadvantage is the length of time required to reach equilibrium. The partial specific volume of glucose isomerase from S. albus (Takasaki et al., 1969) was used in this study for the calculation of molecular weight. The molecular weight of glucose isomerase determined by this method was 171,000. Eventually, this will increase the error of measurement. The plot of $\ln^{c(r)/c(a)}$ versus $r^2 - a^2$ obtained from the experiment which can be used as another criterion of enzyme purity was fairly linear, indicating that the purified enzyme was homogeneous. SDS-polyacrylamide gel electrophoresis, the most common way of estimating the molecular weight of protein subunits (Freifelder, 1976), was also utilized. The purified glucose isomerase migrated on the gel at a position corresponding to a molecular weight of 43,000. The results suggested a tetrameric structure for the enzyme.

The operational stability is an important property for immobilized enzymes or whole cells since the length of

operation determines their usefulness. The operational stability of the immobilized enzyme used for the continuous isomerization of glucose in a column was fairly high, while it was lower for whole cells. Huitron and Limon-Lason (1978) demonstrated a greater operational stability of DEAE-cellulose-glucose isomerase complex than this study. They also found that the operational half life of the immobilized enzyme was a function of the Mg^{2+} concentration and also depended on the geometry of the columns. The operational stability of the immobilized enzyme and whole cells employed in this study could be improved if the optimum conditions were found. The conversion ratios of glucose to fructose catalyzed by the immobilized enzyme and whole cells in the very beginning were the same, around 40%. No doubt, decreasing the flow rate will increase the conversion ratio to a higher level, because the conversion ratio depends on the reciprocal of flow rate (Huitron and Limon-Lason, 1975).

For production of glucose isomerase, most organisms require xylose as an inducer. Pure xylose is too expensive as a substrate for commercial production of the enzyme. The isolated strain of S. flavogriseus produced hemicellulase and glucose isomerase when grown on hemicellulose medium. These characteristics are especially important in the economical production of glucose isomerase, because straw hemicellulose is inexpensively produced by alkaline extraction of ryegrass straw and the resulting residue could be used as ruminant

feed. The organism also produced a high level of the enzyme on H_2SO_4 hydrolysate of straw. In addition, this enzyme has several attractive properties such as low pH optimum for activity, high thermal stability and unusual metal requirements making it a potentially useful industrial enzyme.

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