STUDIES ON EXTRACELLULAR, SPECIFIC D–XYLOSE ISOMERASE FROM *Chainia* sp

A THESIS

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BY

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Dedicated to my Parents

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DECLARATION

Certified that the work incorporated in thesis: **"STUDIES ON EXTRACELLULAR, SPECIFIC D-XYLOSE ISOMERASE FROM CHAINIA sp."** submitted by Shri Jayant M. Khire was carried out by the candid[illegible]. Such material as has been obt[illegible]s has been duly acknowledged in the thesis.

(H.G. Vartak)
Research Guide

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Khire

(Jayant M. Khire)

Poona

July 1987

LIST OF ABBREVIATIONS

Bis	:	N,N'-Methylen-bis-acrylamide
BSA	:	Bovine serum albumin
CBB	:	Coomassie brilliant blue
CM-cellulose	:	Carboxymethyl cellulose
EDTA	:	Ethylenediaminetetra-acetic acid
Mr	:	Molecular weight
PAGE	:	Polyacrylamide gel electrophoresis
pCMB	:	p-Chloromercuribenzoate
PDA	:	Potato dextrose agar
pI	:	Isoelectric point
SDS	:	Sodium dodecyl sulphate
TCA	:	Trichloroacetic acid
TEMED	:	N,N,N'N'-tetramethylethylene diamine

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CHAPTER I

INTRODUCTION

Enzymatic hydrolysis of starch for the production of 92 - 96% dextrose liquor is being carried out since late 1950's. Development in enzymatic isomerization of glucose to fructose is prominant particularly after 1960. The chemical isomerization of glucose under alkaline conditions was reported by several workers and patents were also awarded (1 - 4). None of the processes using alkaline isomerization were ever commercialized due to difficulties encountered in obtaining more than 40% fructose without forming nondextrose and nonfructose degradation products. This resulted into reduction of sweetness of the dextrose liquor. Moreover, the final product developed a colour as well as off-flavour. In contrast to this, enzymatic isomerization was very specific for conversion of glucose to fructose. Glucose isomerase technology began with the discovery by Marshall (5) in the mid 1950's, which showed that D-xylose isomerase from Pseudomonas hydrophila could isomerize D-glucose to D-fructose. Marshall's work was not actively pursued in the later 1950's, because the organism and processing conditions required for isomerization were not economically feasible. Later, Tsumura and Sato (6) as well as Takasaki (7) from Japan who reported a suitable organism and enzyme system for commercial isomerization of D-glucose to D-fructose.

In the last two decades various workers and industries focused mainly on optimization for the production of

the enzyme, enzyme immobilization, gene transfer and cloning for the hypersecretion of the enzyme.

Importance of D-glucose (xylose) isomerase

Two important commercial uses of D-glucose (xylose) isomerase are: (1) Production of High Fructose Corn Syrup (HFCS), and (2) Production of ethanol, liquid fuel and other pharmaceuticals.

(1) Production of High Fructose Corn Syrup

There has been a growing interest in sweet substances that can replace sucrose. An ever increasing demand for sucrose and its rising price challenged the scientists to find alternative sweetners. Large number of non calorific chemical substances have been tried as alternative sweetners (8). Sweetners like saccharine, cyclamate, acesulpham-K, aspartame and thaumatin are sweeter than sucrose but some of them like cyclamate, saccharine have been banned in most countries on health grounds. Saccharine is bitter, while aspartame is hydrolysed slowly at low pH range. Incorporation of aspartame in soft drinks make them less sweeter on prolonged storage. A protein sweetner, thaumatin, is the sweetest known edible material. Although it is 2000 times sweeter than sucrose it has a distinct 'liquoric' flavour which limits its usefulness as sweetner.

High fructose syrup which is an equilibrium mixture of glucose and fructose (approximately 1:1) is the only

calorific sweetner and can thus be used as an alternative to sucrose.

High fructose syrup is of commercial interest as it is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. It is manufactured from a totally non-sweet substance viz. starch. Secondly it is preferred by the food industry as it does not pose the problem of crystallization unlike sucrose. High fructose syrup is manufactured from starch by the use of several enzymes viz. α - and β-amylases, amyloglucosidase, pullulanase and D-glucose(xylose) isomerase. D-Glucose (xylose) isomerase which converts glucose into an equilibrium mixture (approximately 1:1 ratio) of glucose, fructose and is considered to be the most important enzyme in this series.

Glucose isomerase has an established market for the production of 3 million tonnes HFS syrup, world wide. In U.S.A., Japan, and Europe 30% of the conventional cane sugar has been replaced by High Fructose Syrup.

2. Production of ethanol, liquid fuel and other pharmaceuticals

Biomass has been used as a feedstock in microbial processes for the production of alcoholic beverages for a long time. Recently some broader applications of this material such as production of liquid fuel,

pharmaceuticals, food and chemical feed stocks have been envisaged.

Potential biomass materials include sugar crops, starch and hemicellulosic materials. Industrial processes used only hexose component of cellulose. The pentose sugars from hemicellulosic materials which comprise as much as 40% of the plant materials have not been efficiently used. D-Xylose is the second most abundant sugar in the biosphere comprising 40% of the total dry weight of woody angiosperms. Acid or enzymatic hydrolysis of the hemicellulose yields 80 - 90% of D-xylose along with other hemicellulosic sugars. D-Xylose (or oligomeric xylan) is also present in many waste streams from sulfite and dissolving pulp mills, fire board and hard board manufacturing plants. Thus, use of D-xylose in addition to glucose during production of chemicals or fuel (ethanol) from angiosperm feed stocks could improve the overall process economics. D-Xylose from waste streams could reduce disposal costs and provide alternate by product income to existing processes. In this connection D-xylose isomerase is very important because D-xylulose formed from D-xylose can be fermented to various products. Jeffries (9) presented a flow-sheet for utilization of hardwoods from agricultural and forest residues as shown in Fig. 1.

FIG. 1 : Flow sheet for utilization of hemicellulosic materials

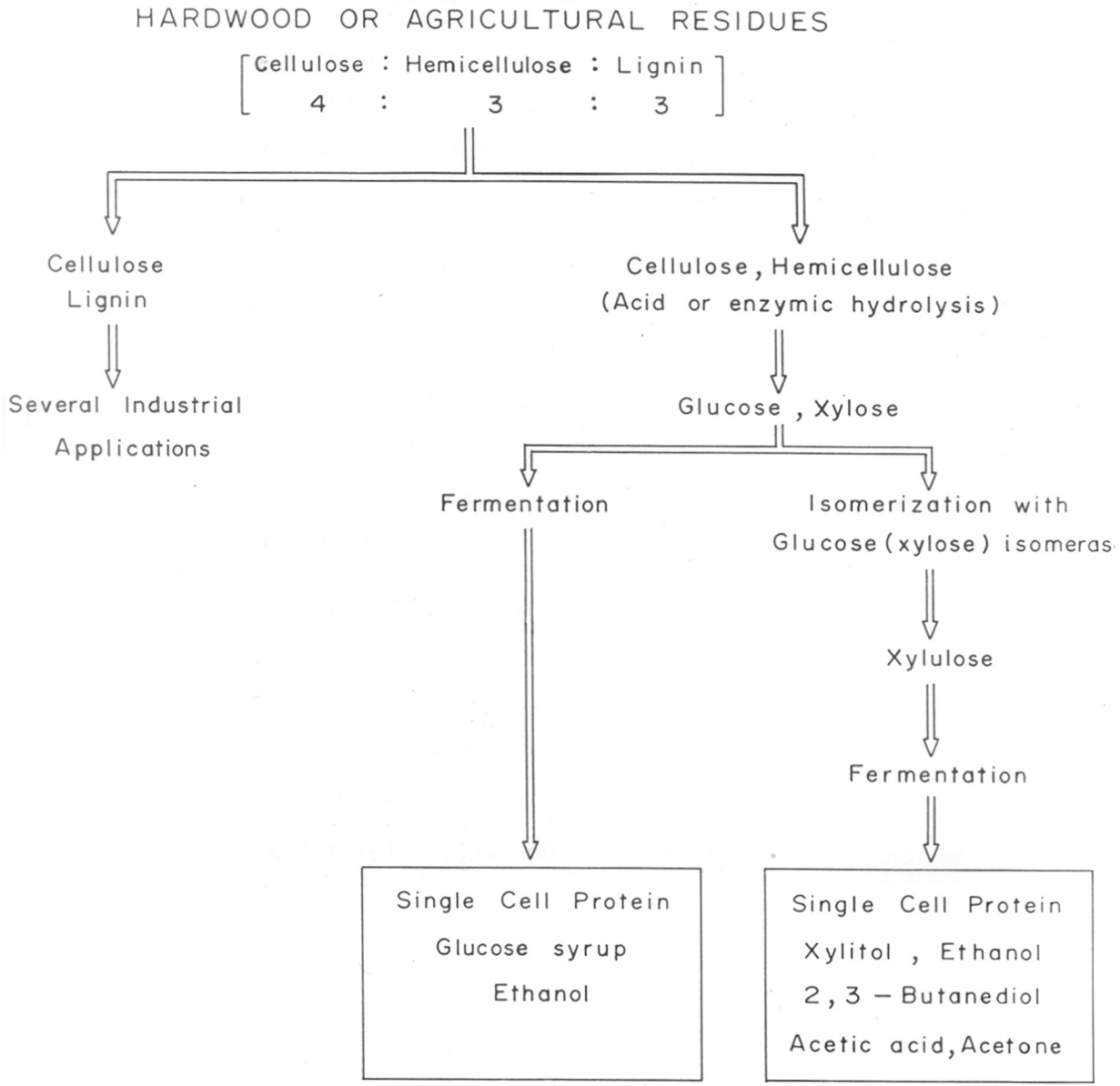

HARDWOOD OR AGRICULTURAL RESIDUES
Cellulose : Hemicellulose : Lignin
4 : 3 : 3
Cellulose
Lignin
Several Industrial
Applications
Cellulose, Hemicellulose
(Acid or enzymic hydrolysis)
Glucose, Xylose
Fermentation
Isomerization with
Glucose (xylose) isomeras
Xylulose
Fermentation
Single Cell Protein
Glucose syrup
Ethanol
Single Cell Protein
Xylitol, Ethanol
2, 3 – Butanediol
Acetic acid, Acetone

FIG. 1.

Types of D-glucose (xylose) isomerases

A microbial enzyme, designated D-glucose (xylose) isomerase catalyzes the isomerization of D-glucose to D-fructose and D-xylose to D-xylulose (Fig. 2). The catalysis takes place in the presence of divalent cations, most commonly Mg^{2+}, Mn^{2+} and Co^{2+}.

The terms D-xylose isomerase and D-glucose isomerase are often used interchangeably in the literature. According to Antrim (10) four separate enzymes are called 'glucose isomerase'. These four enzymes, their Enzyme Commission numbers and major characteristics are listed in Table 1.

1. D-Xylose isomerase (EC 5.3.1.5)- [D-Glucose (xylose) isomerase]

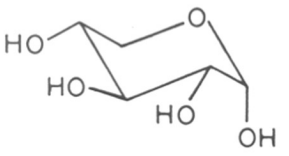
The affinity of xylose isomerase from Ps. hydrophila (11) for glucose was lower than that of xylose (K_m 5 x 10^{-1} M and 3 x 10^{-3} M, for glucose and xylose, respectively). The pH and temperature optima of the enzyme were 8.5 and 42°C, respectively.

2. D-Glucose phosphate isomerase (EC 5.3.1.9)

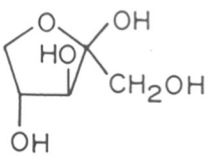
The enzyme which was isolated from Escherichia intermedia is devoid of xylose isomerase activity and did not require xylose as an inducer (12). This enzyme required arsenate to form glucose-arsenate complex, which could act as a substrate analogue for the enzyme (13). The pH and temperature optima of the purified

FIG. 2 : Reactions catalysed by D-glucose (xylose) isomerase (EC 5.3.1.5)

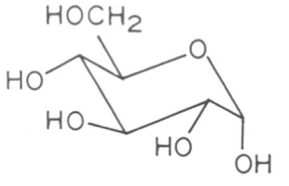
α-XYLOSE



D-XYLULOSE



α-D-GLUCOSE



D-FRUCTOSE

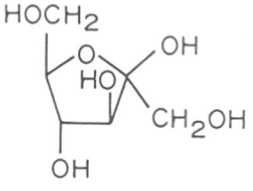


FIG. 2.

Table 1 : Enzyme Nomenclature

	Enzyme	Remarks
1.	Xylose isomerase (D-Xylose ketol-isomerase EC 5.3.1.5) [Also known as D-Glucose(xylose) isomerase]	Absolute dependence on the presence of a source of D-xylose for induction of enzyme. Higher affinity for D-xylose than D-glucose
2.	Glucose phosphate isomerase (D-Glucose-6-phosphate ketol-isomerase) EC 5.3.1.9 Phosphohexose isomerase; Phosphohexomutase; Oxoisomerase; Phosphosaccharomutase	Devoid of xylose isomerase activity
3.	Glucose isomerase (D-Glucose ketol isomerase) EC 5.3.1.18	Specific for D-glucose
4.	Unclassified	Mediates isomerization of both D-glucose and D-mannose to D-fructose

enzyme were 7.0 and 50°C, respectively.

3. D-Glucose isomerase (EC 5.3.1.18)

The enzyme reported from Bacillus megaterium A1 was NAD linked and specific for glucose with pH and temperature optima of 7.8 and 35°C, respectively (14, 15).

4. Unclassified

The unclassified D-glucose is: Palacolobacterum aerogenoides catalyzed the isomerization of both D-glucose and D-mannose to D-fructose in the presence of NAD^+ and Mg^{2+} as cofactors (16). The enzyme exhibited pH and temperature optima of 7.5 and 40°C, respectively.

Although there are four different glucose isomerases, the commercially important one is the first enzyme mentioned above viz. (1) D-Xylose isomerase (EC 5.3.1.5) which is popularly known as D-glucose (xylose) isomerase and is widely used for the production of high fructose corn syrup. As stated above this enzyme is non specific acting on both glucose and xylose.

Since the work in this thesis deals with the finding of a unique specific extracellular xylose isomerase, literature survey is restricted to the above mentioned non specific xylose isomerase which is known as D-glucose (xylose) isomerase. This will be useful in subsequent comparison with the newly isolated, purified and characterized

extracellular specific D-xylose isomerase of the present work.

Occurrence of D-glucose (xylose) isomerase

D-Glucose (xylose) isomerase is produced by most of the microorganisms which are capable of growing on xylose as a carbon source. A list of bacteria producing the enzyme is compiled in Table 2.

Among the yeasts, Candida utilis is capable of producing D-xylose isomerase (75). Recently xylose isomerase activity in yeasts and yeast-like organisms is reported by Zemek et al. (76).

Extraction of the enzyme from plant source i.e. barley malt is reported by Bartfay (77). Later Pubols et al. (78) reported the presence of enzyme from wheat germ.

Induction and regulation of D-glucose (xylose) isomerase

D-Glucose (xylose) isomerase is an inducible enzyme. The enzyme is induced by D-xylose in fermentation media (28, 62, 66, 79, 80 - 82).

Takasaki (7) used corn hulls, corn cobs or wheat bran to replace D-Xylose for the production of D-glucose (xylose) isomerase. Some of the organisms which are being used commercially do not require D-xylose as an inducer eg. Arthrobacter sp. NRRL B-3726, B-3727 and

Table 2 : A list of bacteria producing D-glucose (xylose) isomerase

Bacteria	Reference
Actinomyces olivocinereus	(17)
A. phaeochromogenes	(18)
Actinoplanes missouriensis NRRL B-3342	(19 ,20)
Aerobacter aerogenes	(12)
A. cloacae	(21)
A. levanicum NRRL B-1678	(22)
Arthrobacter spp NRRL B-3724 - B-3728, ATCC 21748	(23 - 25)
Bacillus spp. NRRL B-5350, B-5351	(26)
B. coagulans HN-68,NRRL B-5649 - B-5666	(27, 28)
B. megaterium	(29)
B. stearothermophilus	(30)
Brevibacterium incertum NRRL B-5383	(31)
B. pentoso-aminoacidicum	(32)
Corynebacterium incertum ATCC 31261	(33)
Curtobacterium helvolum HCLB-10352	(34)
Escherichia freundii	(12)
E. intermedia	(13)
Flavobacterium arborescens	(35)
F. devorans NRRL B-5384, ATCC 10829	(31)
Lactobacillus sp. D-80	(36)

Table 2 Continued

Bacteria	Reference
L. brevis, L. buchneri, L. ferment .um	(37)
L. gayoni, L. lycopersici, L. mannitopoeus	(37)
L. xylosus	(38)
Leuconostoc mesenteroides	(37)
Microbispora rosea	(39)
Microellobosporia flavea	(39)
Micromonospora coerulea	(39)
Mycobacterium sp. 279	(40)
Nocardia asteroides	(39)
N. corollina IF-3338	(41)
N. dassonvilleiIMRU-509	(40)
Paracolobactrum aerogenoides	(42)
Pseudomonas hydrophila	(17)
Streptomyces sp. S41-10	(43)
Streptomyces sp.	(44)
Strepto. achromogenes	(45)
Strepto. albus YT-4, YT-5, YT-6,NRRL B-5778	(46 - 49)
Strepto. bikiniensis	(50)
Strepto. bobili	(51, 52)
Strepto. cinnamonensis	(53)
Strepto. flavogriseus	(54)
Strepto. flavoriens IFO 3197	(55)
Strepto. fradiae	(56)
Strepto. galbus	(57)

Table 2 continued

Bacteria	Reference
Strepto. glaucescens ETH 22794	(58)
NRRL B-8071	(59)
Strepto. gracilis	(57)
Strepto. griseolus	(60)
Strepto. griseus	(61)
Strepto. matensis, Strepto. niveus	(57)
Strepto. olivaceus NRRL B-3583, B-3916	(62, 63)
Strepto. olivochromogenes ATCC-15486, 21114, 21713-21715	(31,64, 65)
Strepto. phaeochromogenes SK	(82)
NRRL B-3559	(31)
ATCC 15486	(66)
Strepto. platensis	(57)
Strepto. venezuelae ATCC 21113	(67)
Strepto. wedmorensis ATCC 21230, 21175, 21176	(68, 69)
Streptosporangium album	(70)
Streptomyces kannamyceticus	(71)
Strepto. nigrificans 82/20	(72)
Strepto. violaceus-ruber	(73)
Strepto. olivaceous strain 13	(74)

B-3728 (23, 24, 25), Actinoplanes (20), Streptomyces olivochromogenes (64), and mutant strain of atypical B. cogulance (83). This has been achieved by organism mutation and isolation techniques. A selection scheme for isolation of such mutants has been described by Sanchez and Quinto (84).

Information is sparse on genetic factors responsible for the synthesis of D-glucose (xylose) isomerase in bacteria. David and Wiesmeyer (85) demonstrated that in responsoe to xylose induction, three enzymes were produced in E. coli viz. D-xylose permease, D-xylose isomerase and D-xylulokinase. D-Xylose is isomerised to D-xylulose which in turn is phosphorylated by a kinase to D-xylulose-5-phosphate. D-Xylose permease allowed the transport of D-xylose across a concentration gradient. D-Xylulose-5-phosphate is a normal metabolite of pentose phosphate pathway. According to Shamanna and Sanderson (86, 87) D-xylose utilization in Salmonnella typhimurium is controlled by an operon-like cluster of loci consisting of three structural genes xyl T, xyl B and xyl A, responsible for xylose transport, D-xylulokinase and D-glucose (xylose) isomerase, respectively. Mutants deficient in D-glucose isomerase cannot utilize xylose for growth. They obtained genetic evidence that the three structural genes are under the control of a regulatory gene, xyl R. In the absence of xylose inducer, the xyl R gene product is

postulated to repress the operon. On addition of D-xylose, it combines with xyl R gene product and the resulting complex activates the transcription of the xylose operon. These regulatory relationships are summarized in Fig. 3.

Methods of estimation

Two methods are most commonly employed for D-glucose (xylose) isomerase activity determination: (1) Colorimetric assay and (2) Spectrophotometric assay.

1. Colorimetric assay

The colorimetric assay is widely used in which D-xylulose is determined by cysteine-carbazole method (88). The keto sugars and trioses are determined in this method. A colour reaction of ketohexoses and keto-pentoses, trioses and glycolic aldehyde permits quali-tative as well as quantitative determination in the presence of each other. This method is a modification of the reaction of sugars and aldehydes with carbazole in sulfuric acid (89).

The procedure is as follows: To 1.0 ml of solution containing 1 to 50 µg of ketohexose, 0.2 ml of 1.5% solution of cysteine hydrochloride is added, followed by 6.0 ml of a 70% sulfuric acid. The reaction mixture is shaken after the addition of 0.2 ml of alcoholic carbazole solution (0.12%) and optical density at 540 nm is recorded after 20 min incubation at 35°C.

FIG. 3 : The pathway of D-xylose catabolism and organization of the relevant gene cluster in Salmonella typhimurium (adopted from Shamanna and Sanderson [87]).

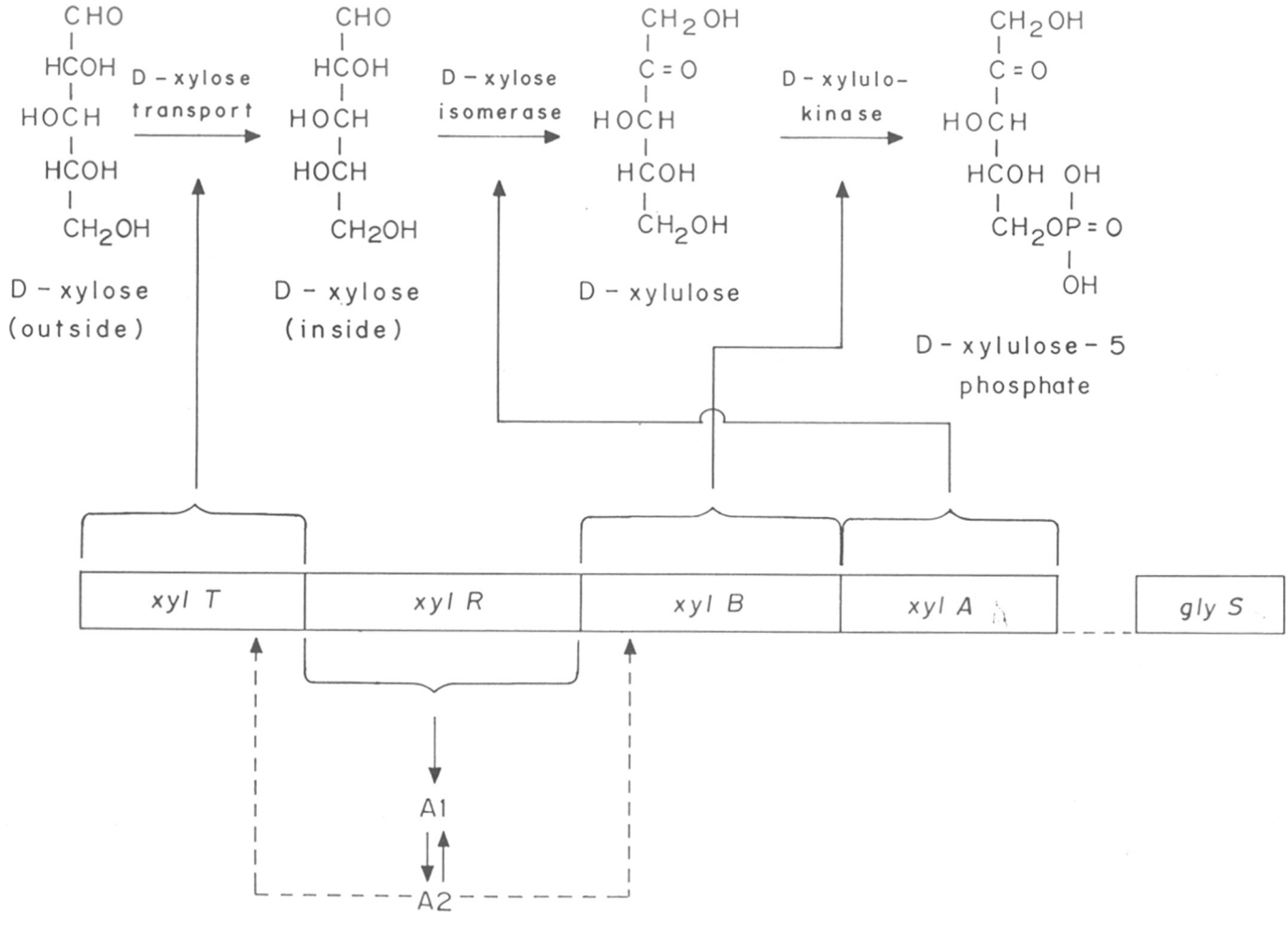

D - xylose transport
D - xylose isomerase
D - xylulo- kinase
D - xylose (outside)
D - xylose (inside)
D - xylulose
D - xylulose - 5 phosphate
xyl T
xyl R
xyl B
xyl A
gly S
A1
A2

FIG. 3.

Colour reaction of various sugars such as fructose, fructose-1-phosphate, fructose-1-6 diphosphate, sorbose and insulin differs considerably. These sugars and sugar derivatives in suitable concentrations, show a purple colour, with a λmax at 560 nm. In the case of fructose optical density was recorded after 10 min incubation at 60°C (11). D-Xylulokinase gives a similar colour with more predominant reddish tint with maximum absorption spectrum of 540 nm. Trioses develop a blue colour which, has maximum absorption spectrum at 650 nm.

2. Spectrophotometric assay

In the spectrophotometric assay of D-xylose isomerase reduction of D-xylulose with excess of D-arabitol dehydrogenase (90) or xylitol dehydrogenase (91) can be followed as the rate of NADH oxidation, at 340 nm. D-Glucose isomerase activity can be determined with crystalline D-mannitol dehydrogenase from Leuconostoc mesenteroides (92) or Lactobacillus brevis (93) as the coupling enzyme.

In case of *A. missouriensis* ATCC 14538 D-glucose isomerase activity is determined by a glucose analyzer (94). Ladisch and Tsao (95) described a method to determine the amount of fructose formed from glucose by liquid chromatography.

Kralova et al. (72) assayed D-glucose isomerase activity by measuring the amount of glucose formed from fructose by the glucose oxidase/peroxidase method.

Callenc et al. (73) assayed D-xylose isomerase activity at 35°C by coupling D-xylose isomerase with D-sorbitol dehydrogenase. Recently, according to Kersters-Hilderson et al. (96), determination of D-xylose isomerase activity by coupling D-xylose isomerase with D-sorbitol dehydrogenase is superior to all the methods because of the following reasons: (a) assay is specific; (b) more sensitive; (c) requires less time (only 3 min); (d) no inhibitory effect from xylitol within experimental period; and (e) reverse reaction is not in existance.

The disadvantage of the above method is that the assay is specific for D-xylose. For D-glucose isomerization, the two-step procedure is required.

Purification of D-glucose (xylose) isomerase

D-Glucose (xylose) isomerase has been purified to homogeneity and characterized from a few microorganisms as shown in Table 3.

A brief account of the purification procedure of D-glucose (xylose) isomerase from different microorganisms is given below. D-Glucose (xylose) isomerase is invariably an intracellular enzyme and thus in the purification procedures the enzyme has to be first extracted from the cells by various techniques such as sonication, treatment with lysozyme, toluene and other organic reagents.

Table 3 : A list of bacteria from which purified D-glucose (xylose) isomerase is reported

Bacteria	Reference
Escherichia intermedia	(97)
Bacillus coagulans	(98,99)
Lactobacillus brevis	(100)
Streptomyces albus YT-5	(101)
Streptomyces bikiniensis	(50)
Streptomyces olivochromogenes	(102)
Bacillus stearothermophilus	(102)
Streptomyces flavogriseus	(54)
Streptomyces nigrificans	(72)

1. Lactobacillus brevis

Purification and crystallization of D-xylose isomerase from the extracts of D-xylose grown cells of L. brevis is reported by Yamanaka in the earlier studies (100). The techniques used for purification were $MnCl_2$ treatment, ammonium sulfate precipitation, heat denaturation of other contaminant proteins, acetone fractionation, DEAE-Sephadex column chromatography and crystallization. The recovery of the enzyme was 21.7% with 11.4 fold purification. Specific activity of the purified enzyme was 64. The pure enzyme showed ribose isomerase activity in addition to xylose and glucose isomerase. The ratio of activites (at saturated substrate concentrations) was 100:85:65 respectively, for D-xylose, D-glucose and D-ribose. Manganese ions were essential for D-xylose isomerase activity and both Mn^{2+} and Co^{2+} were included in the assay mixture for D-glucose and D-ribose isomerase.

From commercial point of view the enzyme appeared to be attractive because of its low pH optima (6 to 7). However, its thermostability was too low for commercial application.

L. xylosus TUA 6 - 9

Enzyme from L. xylosus (38) was purified from the crude extracts to homogeneous state by the following steps: $MnCl_2$ precipitation, ammonium sulfate fractionation, two times DEAE-cellulose and Sephadex G-200 chromatography.

The recovery was 10% and purification was 32 fold. Final specific activity was 4.39.

2. Bacillus coagulans HN-68

D-Glucose isomerizing enzyme from the D-xylose grown cells of B. coagulans, strain HN-68 was purified by Danno (99). Steps involved for purification were, $MnCl_2$ treatment, ammonium sulfate fractionation, DEAE-Sephadex A-50 column chromatography at pH 6.0 and 8.6. The final recovery was 54% with specific activity 141. The purified enzyme was homogeneous on both ultracentrifugation and disc gel electrophoresis. D-Glucose and D-xylose isomerizing activities could not be separated from each other. The pure enzyme showed D-ribose isomerase activity in addition to D-xylose and D-glucose isomerase, which was also found in enzymes from Brevibacterium pentose-aminoacidicum (32) and L. brevis (100).

Bacillus stearothermophilus

Suekane et al. (102) reported the purification of the D-glucose isomerase from B. stearothermophilus. The cells were grown in a medium containing xylose and the enzyme was extracted by the autolysis with 0.0015 % lysozyme and 1% toluene of the cells at 50°C for 24 h. Purification steps involved were heat denaturation of other thermolabile contaminant proteins at 70°C for 15 min, ammonium sulfate fractionation, chromatography on DEAE-Sephadex A-50, BioGel P-200 and on hydroxyapatite

column. Enzyme recovery was 14.3% with 56 fold purification and specific activity was 2.70. The pure enzyme showed D-arabinose and D-ribose isomerase activity in addition to xylose and glucose isomerase.

3. Streptomyces nigrificans

A relatively simple method of purification of D-glucose isomerase from the cells of Streptomyces nigrificans was reported by Kralova et al. (72). Protease activity was denatured when the cell extract was heated for 20 min at 60°C in the presence of Mg^{2+} and Co^{2+}. The relatively high concentration of Mg^{2+} (1 M) after heating caused the precipitation of proteins with lower thermostability. Elimination of these proteins from the solution resulted in increased enzyme purity. Before heating, 11 protein fractions were observed on PAGE, while after the heat treatment only 2 fractions were detected. Purification steps included heating, storage at -5°C for three weeks which resulted in precipitation of some proteins which were removed by centrifugation, followed by DEAE-cellulose column chromatography. The enzyme eluted with 0.5 M NaCl in 0.1 M phosphate buffer, pH 7.3 was homogeneous on PAGE. The yield of 25-fold purified D-glucose isomerase was 27% with specific activity 500.8 nkat/mg.

Streptomyces sp. YT-5

Takasaki et al. (101) reported the purification

of D-glucose isomerase from Streptomyces sp. YT-5. Purification steps involved acetone fractionation, DEAE-cellulose column chromatography, DEAE-Sephadex A-50 column chromatography followed by crystallization. The purified enzyme was homogeneous on ultracentrifugation and electrophoresis. Specific activity (7.2) of the sonicated extract was increased to 74 after crystallization. The recovery was 3.4%.

Streptomyces flavogriseus

Homogeneous D-glucose isomerase was obtained by Chen and Anderson (54) from Strepto. flavogriseus. Purification steps were: Fractionation by ammonium sulfate, DEAE-cellulose and DEAE-Sephadex A-50 column chromatography. The purified enzyme was homogeneous as examined by ultracentrifugation and SDS-polyacrylamide gel electrophoresis. Specific activity after DEAE-Sephadex A-50 column was 20.2 as compared to 1.6 of the original extract. The enzyme recovery was 11%.

Streptomyces olivaceous strain 13

Purification of an extracellular and intracellular D-glucose isomerase from Streptomyces olivaceous strain 13 has been reported by Mikhailov et al. (74). The enzymes were similar in their physico-chemical properties. Purification steps for intracellular D-glucose isomerase involved treatment of lytic enzymes to the cells, heat denaturation of other proteins, acetone fractionation,

ion-exchange chromatography (DEAE-Sephadex A-50) and gel chromatography (Sephadex G-200). Purification steps for the extracellular enzyme involved acetone fractionation, ion-exchange chromatography (DEAE-Sephadex A-50) and gel chromatography (Sephadex G-200). The intracellular glucose isomerase recovery was 34% (41.5 fold purification, specific activity 35.7) while the extracellular enzyme recovery was 29% (34.4 fold purification, specific activity 30.7).

Streptomyces olivochromogenes

Suekane et al. (102) reported the purification of D-glucose isomerase from Streptomyces olivochromogenes to homogeneity. Steps involved in purification were ammonium sulfate fractionation, DEAE-Sephadex A-50 and Sephadex G-150 column chromatography followed by crystallization. Specific activity of the sonicated extract was increased to 2.4 from 0.33 after crystallization. A 7.3 fold purified enzyme was obtained with a final recovery of 40%.

Cory (103) reported the purification of D-glucose isomerase from Streptomyces olivochromogenes by digestion with lysozyme to release the enzyme, followed by fractional precipitation of inactive protein and nucleic acids using water miscible organic solvents between 30 to 60% on a weight basis. D-Glucose isomerase was precipitated from the clarified solution by addition of water soluble

magnesium salts (0.02 - 0.3 M) which resulted into the formation of stabilized magnesium enzyme complex. The conventional precipitants like ammonium sulfate, sodium sulfate and NaCl were ineffective. Divalent cations such as Ba^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+} and Mn^{2+} were partially effective.

4. Actinoplanes missouriensis ATCC 14538

A simple procedure of purification of enzyme from Actino. missouriensis ATCC 14538 was reported by Chen et al. (94). Cells were grown in a medium containing corn steep liquor at 30°C for 5 days on a reciprocal shaker. After harvesting, washing and sonication, the cell-free extract was applied on DEAE-cellulose and enzyme was eluted by using linear salt gradient of NaCl. Concentration of eluent was carried out by Amicon filtration. Three fold purification was obtained by this method.

Miscellaneous techniques used for the purification of D-glucose (xylose) isomerase

Miscellaneous techniques used for the purification of D-glucose (xylose) isomerase are available in the literature, however, in most cases the source of micro-organism is not available.

1. Isoelectric precipitation

Jackson and Tsuda (104) reported the enzyme precipitation near isoelectric point (pH 3.5 - 5.0) followed

by ammonium sulfate fractionation.

2. Heat denaturation

Winans (105) reported a purification method in which enzyme was incubated at 40 - 80°C for 5 to 120 min which caused the denaturation and precipitation of inactive protein, resulting in a doubling of the specific activity of the enzyme.

3. Purification by affinity chromatography

Lee et al. (106) reported affinity chromatography technique for the purification of D-glucose isomerase. Xylitol linked to CNBr activated sepharose 4B by dicyclohexyl carbodiimide condensation was used as the ligand of the affinity chromatography on which glucose isomerase was retained. The enzyme could be eluted non specifically with NaCl. Purification was 1.5 fold.

4. Use of anion exchange resin for purification

Fujita et al. (107) reported purification of D-glucose isomerase by porous anion exchange resin (trimethylammonium type, SO_4^{2-} form). The enzyme with 12.4 fold purification was obtained when eluted with 0.5 N NaCl.

Table 4 : The recovery, fold purification and specific activity of D-glucose(xylose) isomerase from various microbial source

Organism	Recovery (%)	Fold purification	Specific activity	Reference
Actinoplanes missouriensis	-	3	-	(94)
Bacillus coagulans HN-68	54	-	141	(98,99)
B. stearothermophilus	14.3	56	2.70	(102)
Lactobacillus brevis	21.7	11.4	64	(100)
Lactobacillus xylosus	10	32	4.39	(38)
Streptomyces flavogriseus	11	-	20.2	(54)
Strepto. nigrificans	27	25	500.8*	(72)
Strepto. olivochromogenes	40	7.3	2.40	(102)
Strepto. olivochromogenes				
(extracellular GI)	34	41.5	35.7	(74)
(intracellular GI)	29	34.4	30.7	(74)
Strepto. sp. YT-5	4	-	-	(101)
Candida utilis	-	-	10.6	(75)

*Expressed in nkat/mg

Physico-chemical and enzymatic properties of D-glucose (xylose) isomerases

1. Molecular weights of D-glucose (xylose) isomerases

Literature survey indicates that only a few D-glucose (xylose) isomerases have been purified to homogeneous state. Moreover, the information of their physico-chemical properties is sparse.

D-Xylose isomerases in general are of high molecular weight ranging from 52,000 to 191,000 (Table 5).

Molecular weights of the enzyme reported for lactic cultures are relatively higher as compared to other organisms, eg. L. xylosus,183,000 with 4 subunits (38) and L. brevis, 191,000 with 4 subunits (100).

Molecular weights reported for Bacillus D-glucose isomerases are also relatively higher eg. B. coagulans HN-68, 175,000 with 4 subunits (98, 99) and B. stearothermophilus, 130,000 (102).

Molecular weights reported from the Streptomyces D-glucose isomerases show a very wide range. Strepto. bikiniensis (50) and Strepto. flavogriseus (54) have molecular weights of 52,000 and 171,000 with 4 subunits each, respectively.

In Actinoplanes missouriensis (94) molecular weight is lower (80,000) as compared to other organisms and has 2 subunits.

Table 5 : Molecular weights of D-glucose (xylose) isomerases from different bacteria

Bacteria	Molecular weight	No. of subunits	Reference
Actinoplanes missouriensis ATCC 14538	80,000	2	(94)
Bacillus coagulans HN-68	175,000	4	(99,100)
Bacillus stearothermophilus	130,000	ND	(102)
Lactobacillus brevis	191,000	4	(100)
Lactobacillus xylosus	183,000	4	(38)
Streptomyces bikiniensis	52,000	ND	(50)
Streptomyces olivochromogenes	120,000	2	(102)
Streptomyces albus YT-5	157,000	2	(101)
Streptomyces flavogriseus	171,000	4	(54)

ND = Not determined

2. Stability of D-glucose (xylose) isomerase to pH and temperature

Most of the D-glucose (xylose) isomerases are stable to a wide range of pH and temperature.

pH stability. D-Glucose (xylose) isomerases are not stable in highly acidic (below pH 4.0) or highly alkaline (above pH 11) solutions. S. albus YT-5 (101) is stable to a wide range of pH (4.0 - 11.0). The purified enzyme preparations of D-glucose (xylose) isomerase enzymes from E. intermedia (97) and Strepto. flavogrisieus (54) were stable in the pH ranges of 7.0 - 9.0 and 5.0 - 9.0, respectively. Repeated freezing and thawing inactivated the crude enzyme from Actinoplanes missouriensis ATCC 14538 (94). The purified enzymes can be protected for several months in a solution of 40% glycerol which does not freeze even at a temperature of -20°C. The data is summarized in Table 6.

Heat stability. Suekane et al. (102) reported that enzymes isolated from Bacillus are more thermostable than from the Streptomyces. The purified enzyme from B. stearothermophilus completely retained its activity at 75°C for 10 min while the Streptomyces olivochromogenes enzyme gradually lost its activity above 55°C. Enzyme from S. albus YT-5 (101) and S. flavogriseus (54) retained 90% and 100% activity, respectively, when heated at 70°C for 10 min. Partially purified enzyme from Streptomyces

sp. (44) retained 50% of the original activity even when it was heated for longer time at 70°C/120 h. D-Glucose (xylose) isomerase from S. phaeochromogenes SK was highly stable at higher temperature (82). The enzyme retained 96% activity when heated at 80°C/10 min. The enzyme from Escherichia intermedia (97) was highly thermolabile, as compared to above mentioned enzymes. When heated at 60°C for 10 min, the enzyme lost its activity. D-Xylose isomerase from L. brevis (100) was also thermolabile. Only 10% of the original activity was recovered when enzyme was heated at 60°C for 30 min. Enzyme from S. phaecohromogenes NRRL B-3559 (66) retained 40% of the original activity when heated at 70°C for 24 h.

D-Xylose isomerase from L. brevis (100) was easily inactivated by heat treatment at 60°C for 2 min at pH 7.4 but the addition of specific cofactors (mg^{2+} or Co^{2+}) to the enzyme, protected it from heat denaturation. Besides cofactors, potassium chloride, bromide or sulfate were also effective for the protection of the enzyme. Takasaki (42) also showed that the heat stability of the enzyme can be increased by the addition of Mg^{2+} or Co^{2+} to the enzyme mixture.

In case of D-xylose isomerase from yeast, Candida utilis (75) Mn^{2+} plays an important role in the thermal

denaturation. The enzyme when heated at 70°C for 1 h without Mn^{2+}, its activity was decreased to 45% while Mn^{2+} added enzyme scarcely lost its activity.

Chen et al. (94) reported that the crude enzyme preparation from A. missouriensis ATCC 14538 was stable when stored in a freezer, but the purified enzyme denatured upon freezing and thawing. The purified enzyme can be stored in a freezer with 40% glycerol. This enzyme solution does not freeze at the storage temperature of -20°C and is stable over several months. Moreover, glycerol does not affect the isomerization reaction.

3. Optimum pH

D-Glucose(xylose) isomerase has generally pH optimum range of 6.5 - 8.5 (19, 28, 66, 101). The data is summarised in Table 6. Lower pH optimum of 6.5 is reported for the enzyme from L. brevis (100) which is favourable for industrial use. Optimum pH in the basic range of 9.0 - 9.5 is also reported for Strepto. phaeochromogenes enzyme (82). This pH optimum of the enzyme could be modified, depending on fermentation medium. The high pH optimum of Strepto. phaeochromogenes could be lowered to pH 7.5 in the presence of Co^{2+} (10^{-3}M) in the fermentation medium (108). Under alkaline conditions, a non-metabolizable sugar, D-psicose, is produced in hot glucose and fructose solution (109).

Low pH optimum prevents D-psicose formation which is an attractive property of the enzyme for industrial application. However, negligible amount of D-psicose is produced when immobilised glucose isomerase is employed in the continuous processes even though the enzyme has high pH optimum. This is due to the shorter reaction time and the pH of the isomerized syrup is rapidly lowered after the reaction (109).

According to Danno (99) the pH optima for different substrates are not necessarily the same. Optimum pH range for the enzyme from B. coagulans HN-68 was 7.0 - 7.5 for D-glucose or D-ribose as substrate and 8.0 - 8.5 for D-xylose as a substrate.

4. Optimum temperature

D-Glucose (xylose) isomerase is generally considered to be a thermostable enzyme. Its optimum temperature is ranging from 45°C for L. brevis (100) to 90°C for A. missouriensis (19). Several investigators (26, 28, 49, 66) have reported that D-xylose isomerase has temperature optima greater than 65°C. Table 6 indicates that many organisms have fairly high optimum temperature of $\approx$ 80°C. It has been demonstrated that Co^{2+} alters the optimum temperature of D-glucose isomerase (82) from Strepto. phaeochromogenes. In the presence and absence of Co^{2+} the enzyme exhibited optimum temperature of 90°C and 80°C, respectively. It has been shown

Table 6 : Influence of pH and temperature on D-glucose (xylose) isomerase

Organism	Temperature optimum °C	pH optimum	Temperature stability %	pH stability	Reference
Actinoplanes missouriensis	ND	7.0	ND	ND	(94)
Aerobacter cloacae	50	7.6	ND	ND	(81)
Bacillus coagulans HN-68	75	7.0	100 70°C,10 min	ND	(99,100)
B. stearothermophilus	80	7.5-8.0	ND	ND	(102)
Escherichia intermedia	50	7.0	0 60°C,10 min	ND	(97)
Lactobacillus brevis		6.0-7.0	10 60°C, 30 min	ND	(100)
L. xylosus		7.5	90 60°C, 30 min	6.5 - 11.0	(38)
Streptomyces sp. S41-10	75	8.5	ND	ND	(43)
Strepto. sp.	80	7.0	50 70°C, 120 h	ND	(44)
Strepto. albus YT-5	80	8.0-8.5	90 70°C, 10 min	4.0 - 11.0	(101)

Table 6 Contd.

Organism	Temperature optimum °C	pH optimum	Temperature stability %	pH stability	Reference
Strepto. albus NRRL B-5778	70 - 80	7.0-9.0	ND	ND	(46)
Strepto. bikiniensis	80	8.0-9.0	ND	ND	(50)
Strepto. flavogriseus	70	7.5	100	5 - 9	(54)
Strepto. flavovirens IFO 3197	85	8.5	ND	ND	(55)
Strepto. olivochromogenes	80	8.0-9.0	ND	ND	(102)
Strepto. phaeochromogenes	80	8.0	40 70°C, 24 h)	ND	(66)
Strepto. phaechromogenes SK	90	9.3-9.5	96 30°C, 10 min	ND	(82)

ND = Not determined

by Hodge (110) that at high temperature the ketose degradation is characterised by a pronounced discoloration of the aqueous sugar solution. Considering this point Chou (44) carried out the isomerization of glucose at 70°C although the optimum temeprature of Streptomyces sp. glucose isomerase was 80°C. The arsenate requiring enzyme from A. cloacae (81) and E. intermedia (97) have significantly lower optimum temperature of 50°C.

5. Cation requirement

D-Glucose (xylose) isomerase requires a divalent cation such as Mg^{2+}, Co^{2+}, Mn^{2+} or a combination of these cations for its catalytic activity. The cation requirement depends on the source of the enzyme. As^{3+} is essential for the enzyme from E. intermedia (97) while Co^{2+} has been demonstrated to activate glucose isomerase as Strepto. phaeochromogenes in the presence of Mg^{2+} and protects the enzyme against thermal denaturaion. From atomic absorption spectroscopic studies the contents of cobalt and magnesium, in case of the D-glucose isomerase from Strepto. albus (101), were found to be 1.4 and 0.3 atoms respectively per mole of the enzyme. Other enzymes which are activated by both Mg^{2+} and Co^{2+} include D-xylose isomerases from Arthrobacter sp. (23 - 25), Streptomyces sp. (61, 82), Aerobacter cloacae (81) and an atypical B. coagulans (111).

Sanchez and Smiley (46) reported that the presence of both Co^{2+} and Mg^{2+} is required for the optimum activity of D-glucose isomerase from Strepto. albus NRRL 5778. More recently it has been reported that maximum D-glucose isomerase production from Strepto. kanamyceticus requires the elements Fe, Mn and Zn at levels of 10, 3 and 3 mg/l, respectively (112). In Actinoplanes enzyme activity was found to be directly related to the concentration of divalent cations. Co^{2+} was necessary for the enzyme activity to reach maximum when Mg^{2+} concentration was 10^{-3}M. When the concentration of Mg^{2+} was increased to 10^{-1}M, the presence of Co^{2+} became unnecessary. The enzyme activity could be maintained at 90% of the maximum activity when 10^{-2} M Mg^{2+} was present without Co^{2+}. This is very important in the industrial production of High Fructose Corn Syrup since Co^{2+} is undesirable for human consumption. Iron has been reported to give some enhancement to Actinoplanes enzyme. According to Aschengern (111) the enzyme requires Mg^{2+} for activity and Co^{2+} is required for the thermal stability. Three classes of D-xylose isomerase according to the metal requirement of the enzyme have been described by Takasaki: (1) those requiring Co^{2+} (98); (2) those requiring Mn^{2+} (113) and (3) those requiring Mg^{2+} (19, 81, 114 - 116).

Danno (117) reported that same enzyme shows different

isomerase activities in the presence of different metal ions. Thus D-glucose and D-ribose isomerizing activity was specifically stimulated by Co^{2+}, while D-xylose isomerase required Mn^{2+} for activity.

The metal ion requirement for the enzymes from various microorganisms is tabulated in Table 7.

6. Enzyme inhibitors

The catalytic activity of D-glucose (xylose) isomerase is known to be inhibited by a few metal ions. These include Cu^{2+}, Zn^{2+}, Ni^{2+} (19, 98), Ag^{+}, Hg^{2+} (101) and Ca^{2+} (111). Inactivation of the enzyme is probably due to metallic ion competition with the required metal ion, as these inhibition studies were usually performed in the presence of optimum concentration of the required metal ion. Inactivation of D-glucose isomerase from Streptomyces sp. YT-5 (101) by Hg^{2+} indicated the presence of thiol group, although other thiol agents such as iodoacetate and p-chloromercuribenzoate were shown to be ineffective. The same effect with sulfhydryl-reacting agents on the enzyme from Pasteurella pestis was demonstrated by Slein (80). The presence of sulfhydryl group at the catalytic site of the enzyme was predicted as cysteine activated the enzyme by 2 - 3 fold. Thus D-xylose isomerase from Pasteurella pestis (Mn-treated, dialyzed preparation) was inhibited by p-chloromercuribenzoate (2.5×10^{-3} M) completely,

and its activity was restored by addition of cysteine hydrochloride to 80% of the original activity. The enzyme was stimulated by cysteine and to a lesser extent by glutathione in the absence of pCMB. Iodoacetate, iodoacetamide, N-ethylmaleimide inhibited the activity of the enzyme.

D-Xylose isomerase activity from Ps. hydrophila was decreased by 20 - 30% on addition of 2,4-DNP or 8-hydroxyquinoline at a final concentration of 10^{-2}M. But the addition of pCMB, iodoacetate, adenine, adenosine, sulfanilamide, fluoroacetate, hydroxylamine, alloxan, citrate, urethane, fluoride and azide showed neither inhibition nor stimulation (79).

L-Arabinose isomerase activity from Lactobacillus (38) was strongly inhibited by EDTA, but D-xylose isomerase activity showed a considerable resistance against the EDTA-inactivation. With L-arabinose isomerase, enzyme activity was fully restored by addition of Mn^{2+}, Co^{2+} or Ca^{2+} after inactivation by EDTA. Mn^{2+} for D-xylose isomerase and Mn^{2+}, Co^{2+} or Si^{2+} for L-arabinose isomerase showed stimulating effect, but Cu^{2+}, Zn^{2+} and Hg^{2+}, strongly inhibited both D-xylose and L-arabinose isomerase activity.

D-Xylose isomeráses are readily inhibited by sugar alcohols in which xylitol is the most potent one (19, 38, 90, 118, 119). Affinity of this inhibitor for

the enzymes is quite high. The reported values of inhibitor constant (Ki) vaired from 1.5 - 7.0 mM. Other sugar alcohol inhibitors of D-xylose isomerases include D-arabitol, 0.13 M; L-arabitol, 0.15 M; D-sorbitol, 0.03 M; and D-mannitol, 0.07 M, respectively (90, 118). Inhibition by these sugar alcohols appear to be competitive in nature (90). In the case of D-xylose isomerase from L. brevis and L. xylosus inhibition by xylitol was found to be competitive but at the same time D-xylose isomerase from L. xylosus showed no inhibitory effect by D-arabitol and D-ribitol (38).

Danno (118) showed that tris (hydroxymethyl) aminomethane to be competitive inhibitor for D-xylose isomerase from B. coagulans HN-68 with a Ki of 3×10^{-4} to 7.5×10^{-4} M which is lower than the Km values for the substrate D-xylose. But Slein (80) reported that tris (hydroxymethyl) aminomethane acted noncompetitively in the case of D-xylose isomerase from Pasteurella pestis. Thus, even though the pH optimum range of D-xylose isomerase is very well covered by tris (hydroxymethyl) aminomethane buffer its use in the enzyme study should be avoided.

7 Substrate specificity

Isomerases are characterised by a broad substrate specificity. Some of the reactions catalysed by these enzymes, are as follows:

D-Xylose ⇌ D-Xylulose

D-Glucose ⇌ D-Fructose

D-Ribose ⇌ D-Ribulose

D-Arabinose ⇌ D-Ribulose

L-Fucose ⇌ L-Fuculose

The knowledge of the substrate specificity helps in understanding the mode of action of the enzyme. Thus when large number of sugars eg. pentoses and deoxy-sugars were examined as substrates for the enzyme reaction only D-glucose, D-xylose, D-ribose, L-arabinose, L-rhamnose, D-allose and 2-deoxyglucose were found to be the substrates (Table 7). According to Sanchez and Smiley (46) with the exception of rhamnsoe and 2-deoxyglucose, these sugars have a Cl conformation and the hydroxyl groups on carbon 2 in the equatorial position. Maximum isomerization is obtained when the hydroxyl groups of carbon 3 and 4 are in the equatorial position as in glucose and xylose.

Although D-xylose isomerase is known to catalyze the reversible conversion of D-glucose to D-fructose and D-xylose to D-xylulose the specificity of the enzyme varies from source to source. The enzyme from Actinoplanes

missouriensis (19), B. coagulans HN-68, B. stearothermophilu (102), L. brevis (100), S. albus NRRL B-5778 (46), S. bikiniensis (50) and S. olivochromogenes (102) can isomerize D-ribose in addition to D-glucose and D-xylose. In addition to ribose, A. missouriensis enzyme acts on galactose also. This specificity is not even genus specific. Streptomyces enzymes are generally considered to be specific for D-xylose and D-glucose, as reported from S. albus YT-5 (101) and S. phaeochromogenes (82). However, the enzyme from S. albus NRRL B-5778 (46) in addition to D-glucose and D-xylose also acts on D-ribose, L-arabinose, L-rhamnose and D-allose. This is the least specific xylose isomerase so far reported.

8. Km and Vmax

The affinities and maximum velocities of D-xylose isomerases for substrates such as D-glucose, D-xylose, D-ribose etc. vary significantly. Km values of 8.6 x 10^{-2} - 9.2 x 10^{-1} M for D-glucose, 5 x 10^{-3} - 9.3 x 10^{-2} M for D-xylose and 3.5 x 10^{-1} - 6.7 x 10^{-1} M for D-ribose have been reported for D-glucose (xylose) isomerases from microbial sources (Table 7). Natural substrate for glucose isomerizing enzymes is assumed to be D-xylose as the enzyme has higher affinity and more rapid turn-over for D-xylose than its other substrates. The apparent km for D-xylose is lower than other sugar

substrates, which reflects a higher affinity for the enzyme. A more facile conversion is reflected by higher Vmax values. Perhaps this is one of the reasons for delayed discovery of the glucose isomerizing activity of D-xylose isomerase (11). It is noticeable that the enzyme from S. albus NRRL 5778 (46) has more affinity for D-glucose than D-xylose (Km for D-glucose 8.6 x 10^{-2} while for D-xylose 9.3 x 10^{-2} M). Scallet (19) reported that the enzyme from A. missouriensis showed higher affinity for D-glucose. Thus enzyme gives higher ketose production per unit volume with D-glucose as substrate when both D-glucose and D-xylose were used.

Besides several experimental conditions the apparent Km for D-xylose varies with Mn^{2+} concentration in case of the enzyme from L. brevis (112). At infinite concentration of Mn^{2+} the Km for D-xylose decreases as much as to 5 mM. With D-ribose and D-glucose, under the same experimental conditions Km values were 0.67 and 0.92 M, respectively. The Km values for enzyme showed marked difference of 1.8 x 10^{-4} and 1.8 x 10^{-3} M with Co^{2+} and Mg^{2+} respectively. At the same time the Km values of the enzyme from S. albus (46) could not be obtained at extrapolated infinite concentrations of metal ions as concentrations of Mg^{2+} and Co^{2+} higher than optimal values inhibited the enzyme.

Table 7 : Various properties of D-glucose (xylose) isomerase

Organism	Metal requirement	Inhibitors	Substrate specificity	Km M	Reference
Actinoplanes missouriensis	Mg^{2+}, Co^{2+}	ND	Glucose, Xylose Ribose, Galactose	ND	(94)
Aerobacter cloacae	As^{3+}, Mg^{2+}	ND	ND	ND	(81)
Bacillus coagulans HN-68	Mg^{2+}, Co^{2+} Mn^{2+}	Cu^{2+}, Zn^{2+} Ni^{2+}, Ca^{2+}	Glucose Xylose Ribose	9.0×10^{-2} 7.0×10^{-2} ND	(99, 100)
Bacillus stearothermophilus	Mg^{2+}, Co^{2+}	ND	Glucose Xylose Ribose Arabinose	2.2×10^{-1} 1.0×10^{-1} ND ND	(102)
Escherichia intermedia	As^{3+}	ND	Glucose, Xylose 2-deoxy-glucose	ND	(97)
Lactobacillus brevis	Mn^{2+}, Co^{2+}	Xylitol Arabitol Lyxose	Glucose Xylose Ribose	9.2×10^{-1} 5.0×10^{-3} 6.7×10^{-1}	(100)
Streptomyces sp. S41-10	Mg^{2+}, Co^{2+}	ND	Glucose Xylose	ND	(43)
Streptomyces sp.	Mg^{2+}	ND	Glucose Xylose	4.0×10^{-1} ND	(44)
Streptomyces albus YT-5	Mg^{2+}	Ag^{+}, Cu^{2+} Hg^{2+}	Glucose Xylose	1.6×10^{-1} 3.2×10^{-2}	(101)

Table 7 Cont.

Organism	Metal requirement	Inhibitors	Substrate specificity	Km M	Reference
Streptomyces albus NRRL B-5778	Mg^{2+}, partially Co^{2+}	Sorbitol (competitive)	Glucose Xylose Ribose Rhamnose Arabinose Allose	8.6×10^{-2} 9.3×10^{-2} 3.5×10^{-1} 3.12×10^{-1} 1.53×10^{-1} ND	(46)
Streptomyces bikiniensis	Mg^{2+}, Co^{2+}	ND	Glucose, Xylose Ribose, Rhamnose	ND	(50)
Streptomyces flavogriseus	Mg^{2+}, Co^{2+}	Ag^{+} Cu^{2+} Hg^{2+}	Glucose Xylose	2.49×10^{-1} 7.8×10^{-2}	(54)
Streptomyces flavovirens IFO 3197	Mg^{2+}, Co^{2+}	Tris	Glucose Xylose	5.0×10^{-1} ND	(55)
Streptomyces olivochromogenes	Mg^{2+}, Co^{2+}	ND	Glucose Xylose Ribose, Arabinose	2.5×10^{-1} 3.2×10^{-2} ND	(102)
Streptomyces phaeochromogenes SK	Mg^{2+}, Co^{2+}	Tris	Glucose Xylose	3.0×10^{-1} ND	(82)
Streptomyces phaeochromogenes NRRL B-3559	Mg^{2+}	ND	Glucose Xylose	2.5×10^{-1} ND	(66)
Lactobacillus xylosus	Mn^{2+}	Xylitol	Glucose Xylose	ND 5.3×10^{-2}	(38)

ND = Not determined

9. Kinetics and equilibrium of D-glucose (xylose) isomerase

D-Glucose (xylose) isomerase follows the simple reversible Michaelis-Menten Kinetic Mechanism (Eq. 1) (120 - 123).

$$E + S \underset{k2}{\overset{k1}{\rightleftharpoons}} ES \underset{K4}{\overset{k3}{\rightleftharpoons}} E + P \quad \text{(Eq.1)}$$

where ES - Enzyme-Substrate complex;
E - Enzyme;
P - Product
S - Substrate
k1, k2, k3 and k4 - rate constants involved in reversible mechanism

Using steady state assumption, this mechanism can be described by rate equation (Eq. 2)

$$v = \frac{E\left[(k3S)/kS - (k2P)/kP\right]}{1 + (S/kS) + (P/kP)} \quad \text{(Eq.2)}$$

where v - rate of product formation
kS - Michaelis constant for substrate
kP - Michaelis constant for product

Study of isomerization of glucose to fructose by the enzyme from Streptomyces sp. ATCC 21175 (123) demonstrated that the enzyme follows reversible Michaelis-Menten Kinetics over a wide range of substrate concentration. Km for glucose and fructose were 2.5×10^{-1} and

2.6×10^{-1} M, respectively at 60°C, Mg^{2+} (5×10^{-3}M) and in absence of Co^{2+}. Rate constant for the forward reaction (k3 in the mechanism) was found to be 0.24 g fructose formed per IGIU (where IGIU is the amount of enzyme which would catalyze the conversion of 1 μmol of glucose to fructose at 60°C, pH 7.0, 2 M glucose). From these data, the turnover number was found to be 7300 min^{-1} - mol^{-1}, which made D-xylose isomerase a relatively fast acting enzyme because enzymes usually have turnover number of 10 - 10^4 min^{-1} - mol^{-1}. Rate constant for the reverse reaction (k2) was found to be 0.028 g glucose formed per h per IGIU. The activation energy of D-xylose isomerase from B. coagulans HN 68 and Streptomyces sp. was determined to be 14,600 and 14,500 cal/mol, respectively (118, 122).

As D-glucose (xylose) isomerase catalyzes a reversible reaction, an equilibrium state exists which is described by the equilibrium constant

$$keq = \frac{k1k3}{k2k4}$$

considering commercial importance of D-glucose isomerase detailed study has been carried out (122, 123, 124) and it showed that D-glucose, D-fructose equilibrium depends on temperature. Lloyd and Khaleeluddin (123) reported that fructose concentration at 30°C was 46.5%

while at 85°C it has increased to 54.7%. From this data heat of reaction (ΔH) was calculated to be 1080 cal/mol.

The equilibrium ratio for D-xylose:D-xylulose was shown to be 84 - 86% xylose:14 - 16% xylulose (80, 125, 126). In a steady state condition, the majority of the xylose will not be converted to xylulose. Barker et al. (127) found that for aerobic conditions at pH 7.5, equilibrium was established when the ratio of xylose to xylulose was 84;16 with isomerase from Ps. hydrophila. Addition of borate shifted the equilibrium to 18.5:81.5 in favour of xylulose. This effect of borate is also evident in experiments conducted with isomerase from Lac. pentosus (126). Borate appears to exert its effect through preferential and stronger binding to xylulose, thereby preventing reconversion to xylose.

10. Amino acid analysis

Amino acid composition may not give a very revealing characterization of an enzyme unless one of the common amino acid is absent or present in unusually small or large amounts. Thus the presence of cysteine residue was reported only in the D-xylose isomerase of Strepto. albus (128) but not from Bacillus (99) or from B. stearothermophilus and Strepto. olivochromogenes (102). D-Xylose isomerase is an acidic protein since it has

a substantial preponderance of acidic over basic residues (126).

In D-xylose isomerase from S. albus (128) reported both qualitative and quantitative determination of the NH_2 terminal residue by dansyl chloride method which indicated identity of the polypeptide chains. Data of the enzyme analysis on a protein sequanater corroborate that the protein preparation contains a single, degradable polypeptide chain. Confirmation of these results was given by the column peptide map of the tryptic peptides of S-carboxymethyl xylose isomerase. Of the 44 tryptic peptides anticipated from the amino acid composition on the basis of molecular weight of 41,500 an estimated 41 to 47 were detected. Furthermore only one radioactive peptide containing half-cystine was found.

Ogasahara et al. (129) compared the amino acid compositions of α-amylases from thermophile, B. stearothermophilus and a mesophile, B. subtilis. The data suggested that the lower content of hydrophilic amino acids and the higher content of hydrophobic amino acids are associated with the thermophilic property. The content of cystein in thermophilic α-amylases may be playing a role in thermostability. But such difference in amino acid composition was not detected by Suekane et al. (102) in the glucose isomerase from mesophilic Strepto. oligochromogenes and thermophilic B. stearothermophilus.

11. Enzyme mechanism of D-glucose (xylose) isomerase

In the area of carbohydrate metabolism there are number of enzymes, known as isomerases, that interconvert α-hydroxylaldehydes with α-hydroxylketones. These transformations are in the category of isomerization involving the migration of carbon-oxygen double bonds. They are not important as energy-yielding reactions although their role consists in preparing the substrates for other transformations.

Aldose-ketose isomerases catalyze the interconversion of isomeric aldo- and keto sugars by causing the migration of a carbon-bound hydrogen between C-1 and C-2. These enzymes can be classified into two groups.

1. Acting on free sugars (occuring mainly in microorganisms)
   a. D-Glucose isomerase activity
   b. D-Mannose isomerase, L-Lyxose isomerase
   c. L-Rhamnose isomerase, L-Mannose isomerase
   d. D-Arabinose isomerase, L-Fucose isomerase
   e. L-Arabinose isomerase
   f. D-Xylose isomerase, D-Glucose isomerase
2. Acting on phosphorylated monosacchride substrates (common to all living organisms)
   a. Glucose-6-phosphate isomerase
   b. Mannose-6-phosphate isomerase

c. Glucoseamine-6-phosphate isomerase

d. Ribose-5-phosphate isomerase

e. Arabinose-5-phosphate isomerase

f. Triosephosphate isomerase

Rose et al. (130) have shown through the use of tritium-labeled substrate that the enzyme from L. brevis acted via a mechanism involving a cis-enediol substrate intermediate similar to other members of the class of aldose-ketose isomerases (131, 132). There was complete retention of C-1 proton of ketose (D-xylulose) that is abstracted in the formation of aldose sugar. No proton exchange was observed. They suggested that a simple electrophilic group coordinated the C-1 oxygen polarizing the carbonyl group, while a second basic group removed the C-2 hydrogen to form the cis-enediol intermediate. These authors and others (129,133, 134) suggested that the required metal ion, specifically, in the L. brevis enzyme acted as the electrophilic moiety. Geometric form of the intermediate is critical to the aldose-ketose interconversion mechanism. It thus would follow that a single electrophilic center (acid group or metal ion) is used for the polarization of either carbonyl group as shown in Fig. 4.

In the particular case of D-xylose isomerase, there is evidence from kinetic and magnetic resonance

FIG. 4 : Mechanism of D-glucose (xylose) isomerase [Adopted from Rose *et al*. (130)]

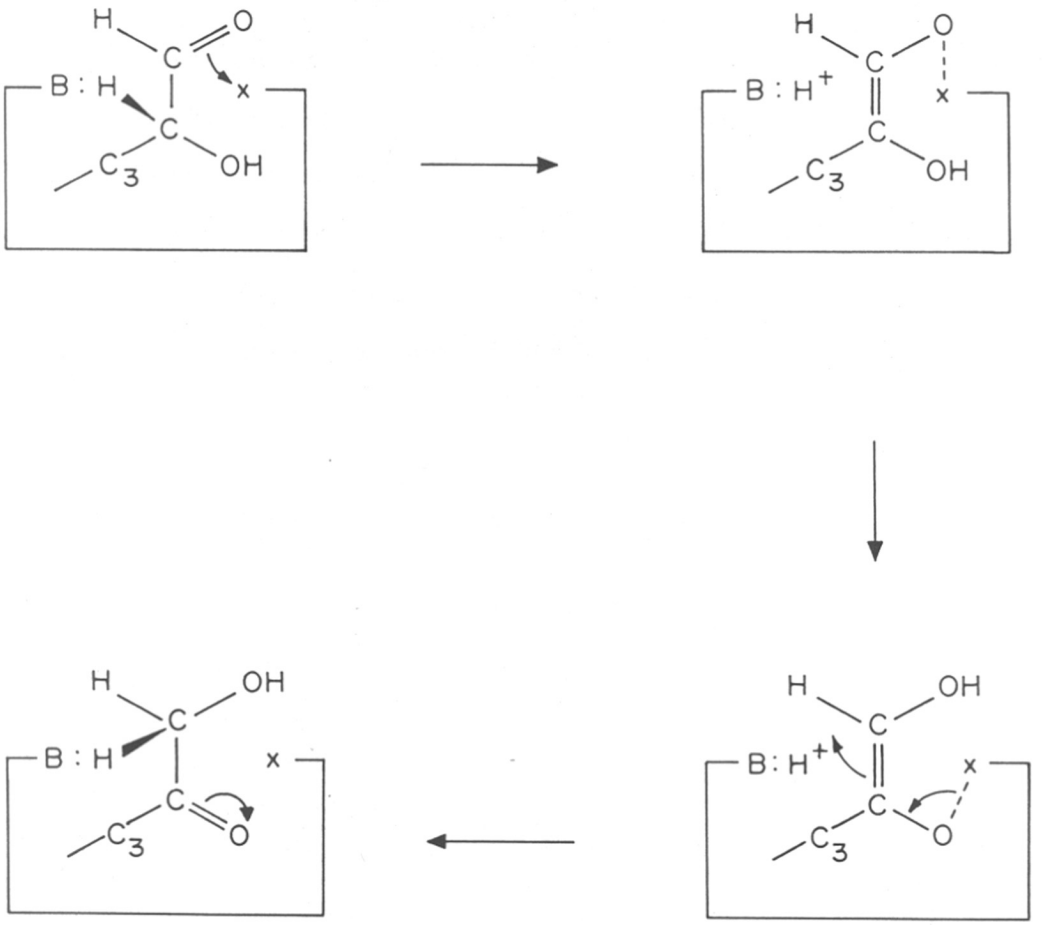


FIG. 4.

studies for a binary enzyme - Mn^{2+} complex for a ternary complex involving the substrate, implicating Mn^{2+} as the functional electrophilic moiety (134). In contrast to the mechanism of other isomerases, however, the hydrogen transfer of xylose isomerase is completely intramolecular, so that there is no exchange of hydrogen with the solvent. Furthermore, this enzyme possesses absolute specificity of the α-anomer of D-xylose and catalyzes only isomerization, not anomerization (124). Suckling (135) has given the following possible mechanisms for the isomerization of α-D-glucose (Fig. 5).

FIG. 5 : A possible mechanism for the isomerization of α-D-glucose [Adopted from Suckling, K.E. (135)]

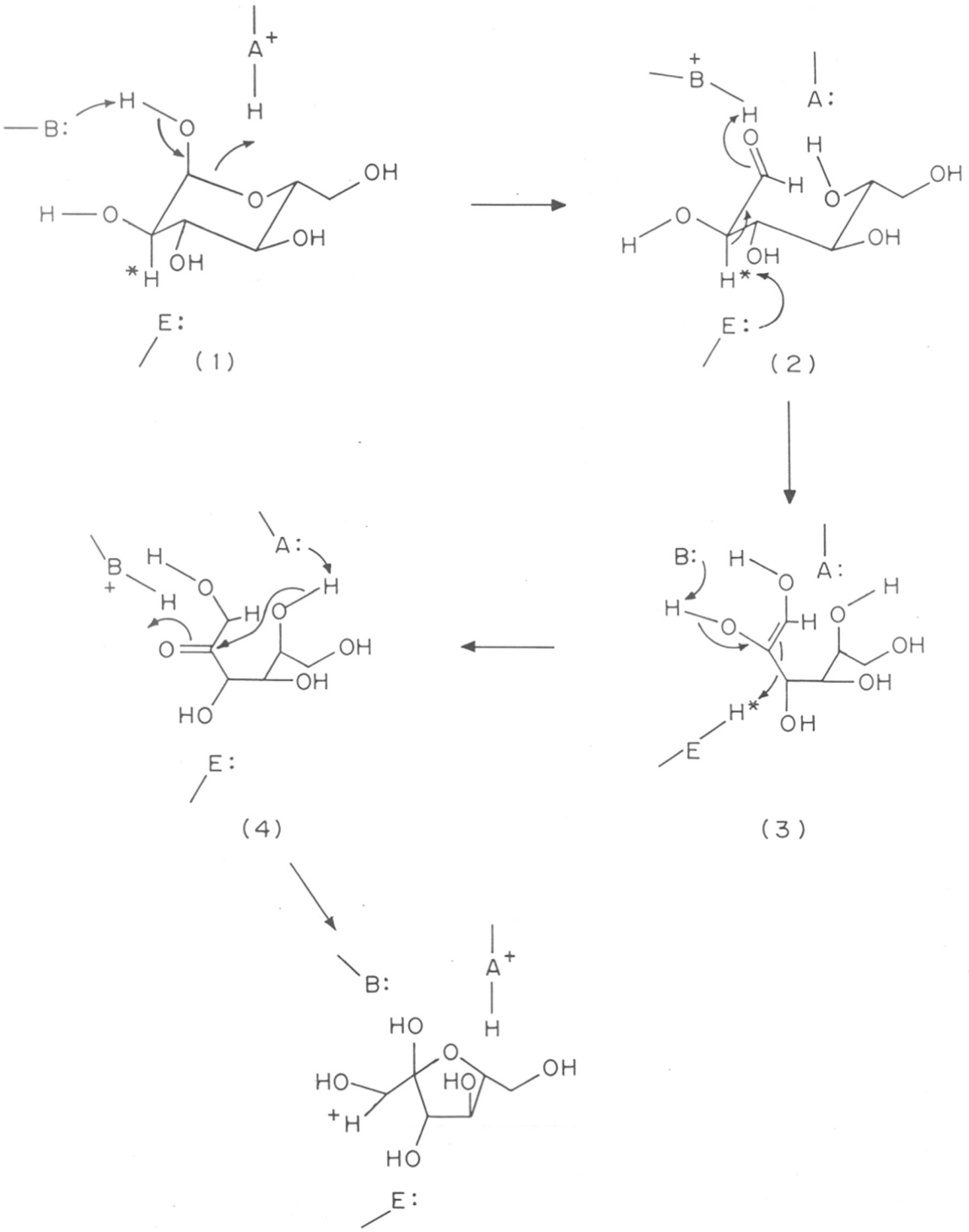

A+
H
B:
H
O
OH
H—O
O
OH
*H
OH
E:
(1)
+
B
H
A:
O
H
H
O
OH
H—O
OH
OH
H*
E:
(2)
B:
H
O
A:
H
O
H
H
O
H
OH
OH
H*
OH
E
(3)
B
+
H
H
O
A:
H
H
O
H
O
OH
OH
HO
E:
(4)
B:
A+
H
HO
O
HO
HO
OH
+H
HO
E:
(5)

FIG. 5.

CHAPTER II

MATERIALS AND METHODS

Materials

Various chemicals used in the present studies have been listed below along with their respective suppliers

Chemicals	Suppliers
DEAE-cellulose (0.92 meq/g medium mesh)	Bio-Rad Laboratories California, U.S.A.
CM-cellulose (0.6 meq/g medium mesh)	Bio-Rad Laboratories California, U.S.A.
Bio-Gel P-150	Bio-Rad Laboratories California, U.S.A.
Sephadex G-200	Pharmacia Fine Chemicals, Uppsala, Sweden
Coomassie Brilliant Blue R-250 and G-250	Sigma Chemical Company, U.S.A.
Cytochrome C (horse spleen)	Sigma Chemical Company U.S.A.
Myoglobin (horse heart)	Sigma Chemical Company, U.S.A.
Ovalbumin	Sigma Chemical Company, U.S.A.
Bovine serum albumin	Sigma Chemical Company, U.S.A.
Standard amino acid mixture	Sigma Chemical Company, U.S.A.
Ammonium persulfate	Sigma Chemical Company, U.S.A.
L-Cysteine hydrochloride	Loba-Chemie Ind. Co. Bombay, India.
Carbazole	Loba-Chemie Ind. Co. Bombay, India.
2:3:5 Triphenyl-tetrazolium chloride	B.D.H. Laboratory Chemicals, Poole, England.

Chemicals	Suppliers
Bacto yeast extract Meat extract	Difco Lab., U.S.A.
Ampholine carrier ampholytes	LKB-Produktor, Bromma, Sweden
Acrylamide	Eastman Kodak Company, U.S.A.
N-N'-methylene-bis acrylamide	Eastman Kodak Company, U.S.A.
N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	Eastman Kodak Company U.S.A.
Sodium dodecyl sulfate (SDS)	Sisco Research Laboratories Pvt. Ltd., Bombay.
UM-10 ultrafiltration membrane	Amicon Corporation, U.S.A.
Rest of the Chemicals	Analytical Grade

Methods

All the reagents and buffers were prepared in glass-distilled water.

Centrifugations were carried out at 0°C - 5°C in the International (Model PRII) and Sorvall (Model RC-5) centrifuges. Ultraviolet and visible absorbance measurements were carried out in a Beckman (Model 25) or Shimadzu spectrophotometer (UV-210A) in cuvettes with 1 cm light path.

All the operations with enzyme solutions were carried out at 0° - 5°C, unless otherwise stated.

Organism

The culture was isolated by dilution poured plate technique from a soil sample collected near Haldighat, Rajasthan, India. Subcultures were maintained on Potato dextrose agar (PDA) by periodic subculture (morphological and physiological properties of the organisms are discussed in Chapter III).

Fermentation

Vegetative inoculum was developed for 48 h on rotary shaker (220 rpm) at 28°C by transferring one week old culture on PDA to 100 ml modified Weber's medium (58) in 500 ml Erlenmeyer flasks. The composition of the medium is given in Table 8.

Table 8 : Composition of modified Weber's medium

Constituents	$g.l^{-1}$
K_2HPO_4	0.5
$CoCl_2.6H_2O$	0.05
$MgSO_4.7H_2O$	0.25
Yeast extract	5.0
Glucose	10.0

pH of the medium was adjusted to 7.0 by 0.1 N NaOH. The medium was autoclaved at 15 lbs (1.08 $kg.cm^{-2}$) for 20 min.

After submerged cultivation for 48 h at 30°C on rotary shaker, D-glucose (xylose) isomerase was induced by the addition of 5 ml solution containing 2 g of D-xylose and 2.5 mg of $CoCl_2.6H_2O$, which was sterilized separately at 10 lbs (0.72 $kg.cm^{-2}$) for 10 mins. Culture was harvested after 72 - 96 h by centrifugation and the clear filtrate was used for enzyme assays.

Enzyme assays

Colorimetric assay was widely used for D-glucose (xylose) isomerase activity, in which D-fructose or D-xylulose is determined by cysteine-carbazole test (88). Since colour development from D-glucose and D-xylose affect the accuracy of these assays, the substrate blank was recorded separately. Blank readings were also recorded for the reagent, substrate, enzyme and enzymic reactions.

Stock solutions for D-glucose and D-xylose isomerase assay: (a) Carbonate-bicarbonate buffer, 0.2 M, pH 9.5; (b) $MgSO_4.7H_2O$, 0.1 M, (c) D-Xylose, 0.05 M and (d) D-Glucose, 1 M.

D-Xylose isomerase assay

The reaction mixture (1.0 ml) contained 0.5 ml of carbonate-bicarbonate buffer, 0.05 ml of $MgSO_4.7H_2O$ and 0.01 - 0.05 ml of the enzyme sample. The reaction was started by the addition of 0.1 ml of D-xylose solution. After incubating the mixture at 35°C for 10 min, the

reaction was terminated by the addition of 0.05 ml of 50% trichloroacetic acid. Colour development by cysteine-carbazole reaction was then carried out for 20 min at 35°C. Optical density of the colour formed was recorded at 540 nm.

D-Glucose isomerase assay

The reaction mixture (2.0 ml) contained 1.0 ml of carbonate-bicarbonate buffer, 0.1 ml of $MgSO_4.7H_2O$ and 0.1 - 0.5 ml of the enzyme sample. After equilibration for 5 min at 70°C, 0.2 ml of D-glucose was added and the mixture was incubated at 70°C for 10 min. The reaction was terminated by the addition of 2.0 ml of 0.5 N perchloric acid. From this 4.0 ml mixture, aliquot of 0.05 ml was removed and the volume adjusted to 1.0 ml with water. The cysteine-carbazole reaction was carried out for 10 min at 60°C for D-fructose determination (11) and the optical density of the violet colour formed was recorded at 560 nm.

Determination of protein

The following methods were used for determination of protein in enzyme samples:

1. Method of Lowry et al. (136) : Protein determination in the latter purification steps were carried out with Folin-Ciocalteu reagent described by Lowry et al. (136). BSA was used as standard protein and the absorbance was

recorded at 750 nm. The concentration of BSA was calculated from its extinction coefficient at 280 nm ($E^{1\ cm}_{1\%}$ = 6.6) according to Cohn et al. (137). Samples, free of ammonium sulfate, tris and containing low concentrations of phosphates, were used to avoid their interference.

2. Spectrophotometric method: Method of Warburg and Christian (138) was used to determine protein concentrations during the purification procedure. Enzyme solutions were diluted, when necessary with the buffer used for dissolving the enzyme and the optical densities at 340 nm and 280 nm were determined. A buffer of the same composition was used as a blank. Correction for ultraviolet absorbing impurities was made by substracting the absorbance at 340 nm.

3. Dye binding method: Protein determination in samples obtained after preparative PAGE was carried out by the method of Bradford (139), since acrylamide impurities do not interfere in this assay. BSA was used as standard protein. The absorbance of Coomassie Brilliant Blue G-250 bound protein was observed at 595 nm.

Concentration of enzyme

The extracellular enzyme was concentrated by ultrafiltration, using UM-10 membrane in 2.5 lit. Amicon filtration unit. The fermented broth was concentrated to 1/10th of the original volume within 8 to 10 h. Only 12% loss of the activity was incurred in this method.

Ammonium sulfate precipitation

Protein precipitation with ammonium sulfate was carried out at 0 - 5°C under constant stirring. The amount of solid ammonium sulfate to be added was calculated according to the following equation (140)

$$x = \frac{50\ (S_2 - S_1)}{1 - 0.28\ (S_2)}$$

where x = g of solid ammonium sulfate to be added for every 100 ml

S_1 = initial concentration of ammonium sulfate

S_2 = required saturation of ammonium sulfate at 0°C.

Ammonium sulfate was added slowly with gentle stirring to avoid frothing. The mixture was allowed to stand overnight at 4°C. Precipitate was collected by centrifugation (7000 rpm, 30 min) and suspended in a small volume of buffer. The suspension was dialysed against 50 - 100 volumes of buffer with changes after 8 - 10 h. Operation was continued for 20 - 30 h.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used extensively both as an analytical technique as well as for preparative work.

Acrylamide gels were prepared according to

Davis (141). Cylindrical gels were 5 - 6 mm in diameter and 7% in acrylamide concentration. The apparatus used for carrying out the electrophoresis was fabricated in this laboratory. It consists of electrode vessels provided with platinum electrode. The upper vessel was provided with holes for attaching the gel tubes. Gel tubes were filled with acrylamide solution, prepared according to the procedure described by Davis (141), leaving about 2 - 4 ml volume space at the top for applying the enzyme samples. The gels were polymerized using ammonium persulfate. After polymerization residual persulfate was removed by passing a current of about 10 mA through the gel tubes for 1/2 h with cathode at the top of the column. Electrophoresis was run at 4°C with 3 - 5 mA current per gel tube for a period of 2.5 to 3 h using 0.0025 M tris-glycine , pH 8.9 as a running buffer. The protein bands were stained with CBB G-250. Relative mobility (R_m) of the protein was calculated as the ratio of the distance travelled by the protein to the distance travelled by the marker.

Enzyme loading

Concentrated enzyme was loaded in all the electrophoresis experiments. Spacer gel was used only in preparative polyacrylamide gel electrophoresis. After fixing the gel tubes, electrode buffer was poured in both the compartments. A current of 10 mA was passed for atleast

1/2 h to remove excess ammonium persulfate from the gel. A solution of 5% sucrose in water was layered on the surface of the gel. A solution of enzyme containing 30% sucrose and 0.001% of tracking dye, bromophenol blue, was loaded in the gel below the sucrose layer using a pipette with a long capillary tube.

Enzyme staining of gels

Enzyme band in polyacrylamide gel was detected by in situ staining method by Yamanaka (113). In this method, after the electrophoresis is completed, gel is removed and D-xylose isomerase activity was detected by incubating the gels with D-xylose at 45°C. To 5 ml of 0.2 M carbonate-bicarbonate buffer, pH 9.5, was added 1 ml of 1 M D-xylose, 0.5 ml of 0.1 M $MgSO_4.7H_2O$ and 3 ml distilled water. The gels were immersed in the above solution at 45°C for 10 min, rinsed with deionized water to remove excess D-xylose, and then were put into 0.1% 2:3:5 triphenyl-tetrazolium chloride in 1N NaOH at 30°C in dark for 1 min. The reaction was stopped by dipping the gels into 2 N HCl and location of D-xylose isomerase was visualized as a red band of formazan.

Preparative polyacrylamide gel electrophoresis (PAGE)

Preparative PAGE method was used for purification of the enzyme. Polyacrylamide gel was prepared in a glass column (15 x 5 cm) with gel height of 12 cm. Spacer gel of 0.5 cm height was layered on the separation gel.

Upper chamber (cathodic) was a glass reservoir (23 x 9 cm) with a B50 male joint which could fit into the glass gel column having a B50 female joint. Glass beaker of 2 lit capacity in which the gel column was immersed served as the lower chamber (Fig. 6).

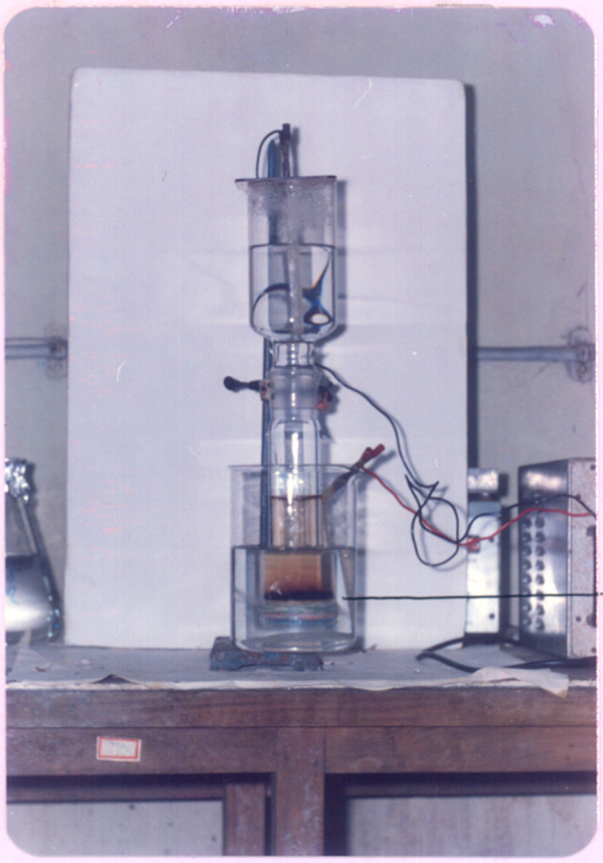
Tris-glycine (0.0025 M) pH 8.9 was used as the bath buffer in each compartment. 0.05 - 0.1 ml of 0.05% bromophenol blue was used as marker in the upper (cathodic) compartment. 5 ml of the enzyme solution (ammonium sulfate precipitated, dialyzed and heated) containing 50 mg protein in 30% sucrose solution was loaded on the spacer gel and electrophoresis was run at 10 mA current (150 Volts). After every 12 - 16 h the bath buffer was replaced with fresh buffer. Electrophoresis was continued till the marker band moved about 10 cms towards anode (72 h).

The gel block is removed and two vertical thin strips of the gel were cut. One was stained with CBB G-250 to reveal total protein pattern of the sample, while other was used to specifically locate the D-xylose isomerase band by in situ staining method.

Ion-exchange chromatography

(a) DEAE-cellulose, equilibrated with 0.01M carbonate bicarbonate buffer, pH 9.5, was used for the removal of non-dialyzable, ionic acrylamide impurities from the specific D-xylose isomerase purified by preparative PAGE.

FIG. 6 : Preparative polyacrylamide gel electrophoresis apparatus



Position of the marker band at the end of the run

(b) The identification of distinct D-xylose and D-glucose isomerases was carried out by ion exchange chromatography using both DEAE-cellulose and CM-cellulose.

DEAE-cellulose was equilibrated with 0.01 M bicarbonate buffer at pH 9.5 while CM-cellulose was equilibrated in 0.01 M sodium phosphate buffer at pH 6.0. Prior to adsorption on DEAE-cellulose or CM-cellulose the pH of the enzyme was adjusted by dialysing at respective buffers for 7 - 8 h.

Molecular weight determination

Gel filtration. The molecular weight of the enzyme was determined using Sephadex G-200 and Bio-Gel P-150 gel filtration chromatography using 0.05 M sodium phosphate, pH 7.5 according to Andrews (142). Sephadex G-200 or Bio-Gel P-150 was suspended in buffer and was allowed to swell by heating in a boiling water bath for 3 h. After cooling, the gel was packed in a column (1.2 x 100 cm) and equilibrated by passing 500 ml of 0.05 M sodium phosphate buffer, pH 7.5 containing 0.005 M $MgSO_4.7H_2O$. The void volume of the column (V_o) was estimated with Blue Dextran 2000 (Molecular weight 2,000,000). Marker proteins (1 $mg.ml^{-1}$) viz. cytochrome C, ovalbumin, bovine serum albumin and aldolase were loaded on the column and eluted with the same buffer (0.05 M sodium phosphate). Fractions, each of 2 ml, were collected at a flow rate of 10 - 12 $ml.h^{-1}$ and

were assayed for the different marker proteins. Blue Dextran (600 nm), cytochrome C (412 nm) and BSA (280 nm) were measured spectrophotometrically. Concentrated enzyme sample was loaded separately. The elution volume (V_e) for each protein was calculated from the midpoint of the peaks. The molecular weight of the enzyme was determined by plotting the graph of V_e/V_o versus logarithm of molecular weight and by extrapolating V_e/V_o for the enzyme sample.

SDS-gel electrophoresis

The method used by Shapiro et al. (143) and Weber and Osborn (144) was followed with slight modifications. It is based on the principle that SDS minimizes the native charge differences and that all proteins migrate as anions as the result of complex formation with SDS. The extensive disruption of hydrogen and hydrophobic bonds by SDS as well as disulfide linkages by 2-mercapto-ethanol results in the quantitative solubilization of many relatively insoluble proteins. The migration rate of SDS-treated proteins is proportional to their molecular weights.

To a 50 µg solution of enzyme in 0.1 ml phosphate buffer, pH 7.5, was added 0.1 ml of a solution containing 2% SDS, 2% 2-mercaptoethanol and 0.2 M sodium phosphate buffer, pH 7.2 and the mixture was incubatedf at 37°C for 3 h. The denatured and reduced solution of the

enzyme was then dialyzed overnight at 25°C against 100 ml of a solution containing 0.1% SDS, 0.1% 2-mercapto-ethanol and 0.01 M sodium phosphate buffer, pH 7.2.

Gels (10 x 0.6 cm) were made using 10% acrylamide 0.26% Bis, 0.1% SDS, 0.05% TEMED, 0.01 M sodium phosphate buffer, pH 7.2 and 0.004% riboflavin. The bath buffer contained 0.1% SDS and 0.1 M sodium phosphate buffer, pH 7.2. For each gel 0.03 ml tracking dye (0.05% BPB) 0.2 ml 20% sucrose and 0.2 ml of the SDS-treated enzyme solution were mixed and loaded. Current used was 8 mA per gel. The anodic compartment was in the lower chamber. Electrophoresis was discontinued after about 6 h when the marker band moved about three fourth of the distance in the gel. Gels were stained in 2.5% CBB R-250 in methanol: acetic acid:water (45:9:46) overnight and destained in ethanol:acetic acid:water (30:10:60) mixture.

Following standard proteins were used: BSA (67,000), ovalbumin (47,000), myogobin (17,000) and cytochrome C (13,000). Migration of BPB was used as a reference point within each gel. The electrophoretic mobility (R_m, distance of protein migration to distance of dye migration) were plotted against the logarithm of the molecular weights of known polypeptide chain. Extrapolating relative mobility of the enzyme protein its molecular weight was determined.

Polyacrylamide gel electrophoresis (slope method)

The molecular weight of native proteins using gel electrophoresis was determined according to the method described by Hedrick and Smith (145). Separation gels with various concentrations of acrylamide (4 - 9%) were prepared according to Ornstein and David (146), except that the ratio of acrylamide to bis (N,N'-methylene bisacrylamide) was 30:0.8 which was maintained constant in all the gels. Samples (100 µl) in 5 x 10^{-3} M tris-glycine buffer, pH 8.3, containing 50% glycerol and 0.05% BPB were layered on the top of the gels. Electrophoresis was carried out at 2 mA for 30 min and 4 mA for 2 h at 4°C. Under such conditions proteins migrate into the gel as a function of their size, charge and the acrylamide concentration of the gel. At the end of the run, the dye front was marked by inserting 25 gauze copper wire. The staining and destaining were performed as described above. Migration of dye and protein band was measured on a illuminated box using a magnifying glass. Measurements were accurate to ± 0.5 mm. The ratio (R_m) of the distance of the migration of the protein band to that of BPB for various concentrations of acrylamide gel tubes (4 - 9%) was determined. Plots of log relative mobility (100 log R_m x 100) of marker proteins [Pepsin (34,000), Ovalbumin (43,00) and BSA (68,000) relative to BPB versus gel concention gave straight lines with slopes which were linearly

related to the molecular weight of the proteins. The molecular weight of the enzyme was calculated from the slope on the concentration curve.

Determination of the glycoprotein nature of purified D-xylose isomerase

Glycoprotein nature of the enzyme was determined by using gel protein staining method (147). After polyacrylamide gel electrophoresis, gels were washed twice for 2 h in borosilicate tubes with isopropanol-acetic acid - H_2O (25:10:65) to fix the proteins and to remove low molecular weight substances. These washings remove sucrose, which was added in the enzyme solution during loading, and would otherwise react with sulfuric acid to form a furfural derivative. A final wash for 2 h in the same solvent containing 0.2% thymol (w/v) was given for the formation of the stable gel. After washing with thymol, a solution of concentrated sulfuric acid:absolute alcohol (80:20) was added to each gel (10 ml/gel). These tubes were capped and incubated at 35°C for 2.5 h or until the opalescent appearance of the gels just disappeared. Zones containing glycoproteins stained red with a yellow background. This experiment was carried out with positive and negative control.

Determination of isoelectric point (pI)

Isoelectric point (pI) of the purified enzyme was determined using 110 ml capacity electrofocusing column (LKB Produktor, AB, Bromma, Sweden) according to the method

described by Vesterberg and Svensson (148). The density gradient was prepared with glycerol using an automatic gradient mixer. Dense solution, light solution and electrode solutions were prepared according to LKB Instruction Manual. Ampholine carrier ampholytes and enzyme solutions were mixed in light and dense gradient solutions. First a pilot run was taken with Ampholine carrier ampholytes of wide pH range (pH 3.5 - 10.0). Ampholine carrier ampholytes of pH range 3.5 - 5.0 at concentration of 1% (w/v) was then used to determine precise isoelectric point of the enzyme. Anode was at the top of the column. Electrofocusing was carried out at 5°C for 30 h.

At the end of run the voltage was steady at 400 V and current drop was from 20 mA - 1.0 mA which remained constant. After completion of electrofocusing the column was emptied at the rate of 30 - 40 ml/h and fractions of 1 ml were collected. pH of the fractions was recorded and D-xylose isomerase activity was determined.

Amino acid analysis

For amino acid analysis hydrolysis of protein samples (1 mg in 2 ml of 6 M HCl) was carried out over nitrogen atmosphere in sealed test tubes at 110°C for 24, 48 and 72 h. Excess acid was removed by evaporation in vaccuo at room temperature (25 - 28°C) followed by dissolving in water (2 ml) and evaporating in vaccuo to dryness. This was repeated thrice. These samples were analysed

in a Spinco model 120-B automatic amino acid analyzer by the method of Spackman et al. (149).

Hydrolytic destruction of proline, threonine and serine was corrected by extrapolation to zero hydrolysis time from 24, 48 and 72 h hydrolysed samples of the enzyme.

Absorbance of protein dissolved in 0.1 N NaOH at 257.15 nm, 294.4 nm and 280 nm was measured and the amounts of tyrosine and tryptophan were calculated according to the method of Goodwin and Morton (150).

Goodwin and Morton's method. The method is based on the principle that proteins show selective absorption in UV region and the position of absorption maximum varies with pH. Thus, the majority of the constitutents amino acids do not show any absorption in the region 250 - 320 nm. Apparently, phenylalanine, tyrosine, and tryptophan are responsible for the observed UV absorption of protein solutions. In 0.1 N NaOH absorption by tyrosine and tryptophan is much stronger as compared to phenylalanine. Under these conditions the protein solutions may be treated as a two-component system for spectrophotometric analysis. The intensity of absorption at the point where the curves for tyrosine and tryptophan intersect is a direct measure of the total molar solute concentration and will be the same, even though the proportion are varied. At other wave-lengths the intensity of absorption will vary with the relative proportions

of the components. Using 0.1 N NaOH as solvent the two absorption curves intersect at 294.4 nm (ε = 2375) and 275.15 nm (ε = 2748). By determining the absorption of the protein in 0.1 N NaOH at the above two wavelengths and one other wavelength (e.g. at 280 nm) it is possible to determine relative proportions of tyrosine and tryptophan in the protein.

Thus, if X = total mole/l in solution

Y = g mole of tyrosine

X-Y = g mole of tryptophan

At any wavelength other than the point of intersection let tyrosine be A, and tryptophan be B and the observed intensity of absorption for a 1 cm cell be

$$\varepsilon = YA + (X - Y)\ B$$

or

$$Y = \frac{\varepsilon - XB}{A - B}$$

$$X = \frac{\text{Value at an intersection}}{\text{tyr at an intersection}}$$

Partial specific volume (V)

Partial specific volume of the enzyme was calculated from the data on amino acid analysis. The values V for the amino acid residues were taken from Schachman (150)

Cysteic acid determination

Total half-cystine content of the protein (200 - 250 µg) was determined as cysteic acid, after oxidation with performic acid (151), followed by HCl hydrolysis and amino acid analysis. Standard amino acid mixture containing known quantity of half cystine was treated in a similar way and run as a control.

Definition of enzyme unit

One unit of D-glucose isomerase corresponded to the amount of enzyme which produced 1 µ-mole D-fructose. min^{-1} at 70°C, while in the case of D-xylose isomerase it was equivalent to 1 µ-mole of D-xylulose.min^{-1} at 35°C.

CHAPTER III

CULTURE CHARACTERISTICS, PRESERVATION AND ENZYME PRODUCTION BY CHAINIA SP.

> "No other group of microbes, and for that matter no other group of living systems, whether of plant, animal or microbial origin, has been in recent years the focus of so much attention by the investigator, especially the microbiologist, the chemist, and the medical scientist, and by the pharmaceutical manufacturer, as the actinomycetes"
>
> (Waksman, 1959)

Our first knowledge of the actinomycetes dates back to 1875, when Ferdinand Cohn named an organism which he found in the tear duct of the human eye, as Streptothrix foersteri. Since then several actinomycetes have been isolated and described.

Actinomycetes are one of the most abundant bacterial groups in soil and the characteristic smell of damp soil is attributed to the presence of a volatile substance formed by them. In the past fifty years these organisms have acquired great economic importance by virtue of their ability to produce commercially valuable antibiotics belonging to the class of streptomycin, actinomycin, tetracycline and macrolide.

Actinomycetes are widely distributed in nature viz. air, water and soil. Actinomycete colonies are easily distinguishable from fungi and other bacterial cultures. Colonies developed in 2 - 7 days are compact, often leathery, and elevated with a dry surface. When grown in liquid culture, either in a stationary or in

submerged conditions, Streptomyces and Micromonospora grow in the form of flakes or spherical compact masses, leaving the medium clear.

The genus Chainia was proposed by Thirumalachar (153) to accomodate an actinomycete isolated from soil samples in Poona, India and was named in the honour of Professor E.B. Chain. Since then several species of Chainia have been described. C. flava, C. olivacea, C. poonensis, C. purpurogena (154), C. minutisclerotica (155), C. fumigata, C. rosea (156), C. grisea subsp. fusca (157), C. nigra, C. ochracea, C. rubra, C. violens (158). More recently C. kunmingensis (159) has been reported. Various antibiotics produced from Chainia include aburamycin (155) Chainin (160), antibiotics SS-228Y (161) barodamycin (162) and antisarcina activity (163).

Present work

From our laboratory we have reported extracellular specific glucose and xylose isomerases (164, 165) and xylanase (166). A rare actinomycete, Chainia was isolated by the dilution pour plate technique on a medium containing Malt extract-glucose-yeast extract from soil sample near Haldighat, Rajasthan, India. Six different isolates, were isolated and designated as NCL 82-5-1, 82-5-27, 82-5-28, 82-5-3, 82-5-3A and Chainia B. The colonies on isolation plates could be readily distinguished by

their warty appearance due to the development of numerous sclerotial bodies.

Role of sclerotium in morphological identification

The main differentiating feature of the genus Chainia from other actinomycetes is the transformation of the vegetative mycelium into sclerotic granules (154). In the present work this transformation was observed on all the tested media which favoured the growth of the vegetative mycelium and did not require special conditions for their induction. Thirumalachar and Sukapure (154) had reported that two types of sclerotia are produced in Chainia: Simple, i.e. formed singly as in C. flava or compound in which more than two sclerotia are surrounded by a common mycelial sheath e.g. C. antibiotica.

Brief comparative morphological features of seven days old culture grown on 2% PDA of six Chainia strains isolated in the present work are given in the following Table (Table 9).

Morphological and physiological characterization

Growth and colony characteristics were studied on a variety of nutrient media formulations based on those recommended by Shirling and Gottlieb (167) and Waksman (168). Standard biochemical tests were performed according to the S.A.B. Manual of Microbiological Methods.

Table 9 : Comparative morphological features of six *Chainia* strains

Strain	Diffusible pigment on colony reverse	Sclerotia
NCL 82-5-1	Brown	Cerebriform compound
NCL 82-5-3	None	Simple, minute
NCL 82-5-3A	None	Simple
NCL 82-5-27	Yellow	Compound
NCL 82-5-28	None	Compound
Chainia B	Brown	Compound

Description of Chainia sp. (NCL 82-5-1)

Aerobic, Gram-positive organism, colonies on nutrient agar medium are compact with warty centres and little or no aerial mycelium, composed of several multilocular, lobed or cerebrioform sclerotia (Fig. 7) with distinct outer wall and dense granular contents. The sclerotia measure 145 x 120 μ. Patches of aerial mycelium and/or sporophores appearing as open spirals observed on 10 - 14 days' old slant.

Starch, xylan, gelatin, casein, urea are degraded by the organism. Nitrate is reduced; hydrogen sulphide is not produced.

L-arabinose, D-fructose, D-galactose, D-glucose, lactose, D-maltose, D-raffinose, D-mannose and D-xylose are used by the organism as sole carbon source but not cellobiose and melibiose.

It grows at 28°C - 37°C, but not at 55°C. It shows tolerance to sodium chloride (10% w/v) and shows anti-bacterial activity against Gram positive organisms like Staphylococcus aureus and Bacillus subtilis.

Isolated from soil at Haldighat, Rajasthan, India.

Cultural characteristics of Chainia sp. (NCL 82-5-1) on standard media are given in Table 10.

FIG. 7 : Sclerotial morphology

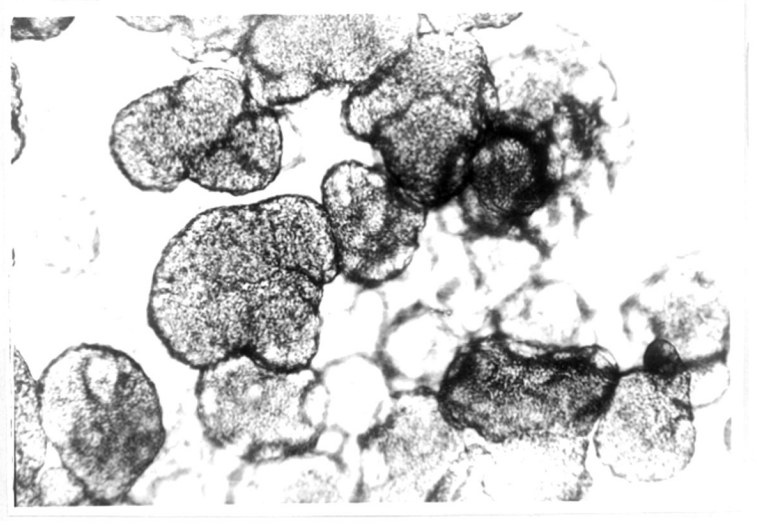


Table 10 : Cultural characteristics of Chainia sp. on standard media

Medium	Growth	Aerial mycelium and sporophore	Sclerotia	Diffusible pigment
Lactose-yeast extract agar	abundant	moderate	abundant	none
Cellobiose agar	good	moderate	abundant	none
Maltose agar	moderate	sparse	moderate	none
Malt extract glucose-yeast extract peptone agar	abundant	abundant	abundant	none
Potato dextrose agar	abundant	sparse	abundant	dark-brown
Glycerol aspargine agar	moderate	sparse	abundant	honey coloured
Starch agar	poor	sparse	moderate	none
Glucose aspargine agar	moderate	moderate	abundant	none
Czapek Dox agar	abundant	moderate	abundant	pale honey coloured
Emerson's agar	abundant	moderate	abundant	Greyish brown
Nutrient agar	moderate	moderate	moderate	none
Glucose peptone agar	abundant	none	abundant	honey coloured

Preservation of Chainia cultures

There are three basic methods (169) used for the maintenance of microorganisms: (1) Storing organisms on agar slants; (2) Drying organisms on soil and (3) Lyophilization.

1. Storing organisms on agar slants

The cultures can be kept in wooden box to protect from dust and to minimise the aerial contamination at room temperature (25 - 28°C).

Mineral oil. Heavily sporulating actinomycete culture can be covered by mineral oil and the cultures preserved under such conditions survive for long periods since they grow at a very reduced rate. The paraffin oil is heated at 170°C for 1 h. The height of the oil level from the top of the slant is usually 1 cm and is fairly critical (170) as the oxygen transmission by layers of mineral oil more than 1 cm becomes less favourable. The method is based on reduced rate of metabolism and prevention of drying (171, 172).

Distilled water culture. Actinomycete culture can be preserved in sterile distilled water in the form of spore suspension (173). Good sporulated culture along with the piece of agar is suspended in 2-3 ml sterile distilled water.

2. Drying organisms (spores) on soil

Soil has been found effective in maintaining

actinomycete cultures. About 2 g of finely ground, loamy soil was placed in each of a number of 10 x 100 mm tubes. Tubes were plugged with cotton and sterilized four times for 30 min at 121°C every alternate day. Culture was grown in a liquid medium. Two ml of culture broth was added into each soil tube, mixed thoroughly and kept at room temperature (28°C) for 2 - 3 weeks for drying.

3. Lyophilization

Lyophilization or freeze drying is a method of preservation of microorganisms in which the vegetative cells or spores (in the case of sporulating cultures) are dried in frozen state under reduced pressure (174). Actinomycete spores dried under these conditions remain dormant for a long time.

Thick spore suspension of the actinomycete is distributed aseptically in ampoules, alongwith the adjuvant like 10% skimmed milk, 12% sucrose or bovine serum albumin. The ampoules are frozen by immersing in -40°C alcohol bath or in a freezing mixture (dry ice and ethyl acetate) and dried in a lyophilizer (primary drying) or in a vaccum desiccator containing a suitable desiccant such as phosphorus pentooxide or silica gel. The ampoule containing the dried material is sealed under vaccum stored at 4 - 8°C in a refrigerator or at -15°C in a deep freeze (175).

Methods of preservation and maintenance described above were used for Chainia sp. The viability and biochemical performance of the preserved culture was checked yearly. The results are compiled in Table 11. It was observed that the preservation methods of lyophilization and distilled water were most suitable and these were used routinely.

Testing of Chainia cultures for D-glucose (xylose) isomerase activity.

Six different Chainia isolates were grown on 2% D-xylose and 1% yeast extract agar plates. Single colonies were screened for D-glucose (xylose) isomerase.

1. Loopful of growth was kept on a test paper (Whatman filter paper No. 1) saturated with 1% glucose in 0.04 M phosphate buffer, pH 7.5 containing 0.005 M $MgSO_4.7H_2O$.

2. The test paper was kept on another new paper and the set of paper was kept in between glass plates and incubated at 60°C for 30 min in an incubator.

3. After incubation the new paper was dried at room temperature and the fructose formed was detected by spraying β-indole acetic acid in trichloroacetic acid. Violet spots appeared on a pink background wherever fructose was formed from glucose. All the six isolates showed violet spots indicating D-glucose (xylose) isomerase activity.

Table 11 : Methods of preservation used for Chainia sp. (NCL 82-5-1)

	1st Year			2nd Year			3rd Year		
	Viability	Sclerotia	Activity XI U/lit	Viability	Sclerotia	Activity XI U/lit	Viability	Sclerotia	Activity XI/u/lit
Routine transfer	+	+	210	+	+	200	+	+	140
Soil culture	+	+	210	+	+	200	-	-	-
Paraffin oil	+	+	200	+	+	200	-	-	-
Lyophilization	+	+	210	+	+	210	+	+	210
Distilled water	+	+	210	+	+	210	+	+	210

Quantitative method for screening of Chainia cultures for D-glucose (xylose) isomerase activity.

Culture. Seven days old culture on 2% PDA were used to inoculate the inoculum flasks.

Inoculum and fermentation medium. Medium containing xylose as a carbon source was used for both inoculum development and fermentation(55).

Fermentation. Vegetative inoculum of six Chainia isolates was developed in 100 ml medium in 500 ml Erlenmeyer flasks for 48 h on a rotary shaker (220 rpm) at 28°C. One week old culture on PDA was inoculated in 100 ml medium in 500 ml Erlenmeyer flasks. The inoculum (10% v/v) was transferred to experimental flasks having the same medium composition and grown for 24 h. Flasks were harvested after 24 h and extracellular D-glucose isomerase activity in the cell-free supernatant was determined after centrifugation. Enzyme activity in the sonicated extract and supernatant liquid was determined at pH 7.0. Table 12 summarizes the results.

The Chainia sp. NCL 82-5-1 was selected for further studies since, only this showed the extracellular isomerase activity. Henceforth it is designated only as Chainia sp.

Table 12 : Intra- and extracellular D-glucose (xylose) isomerase activity of *Chainia* isolates

Isolate	Wet weight of mycelium/ 100 ml medium g	Intracellular GI/litre units	Extracellular GI/litre units
82-5-1	7.55	32	200
82-5-27	9.30	106	0
82-5-28	7.43	76	0
82-5-3	8.01	54	0
82-5-3A	5.94	58	0
Chainia B	4.94	34	0

Enzyme production

Various media were tested for the production of the enzyme.

Media

Wheat bran - yeast extract medium.

Composition

Wheat bran - 5%

Yeast extract 1%

pH 7.0

Sterilised at 121°C for 30 mins.

Culture. Seven days old culture of Chainia sp. grown on PDA at 28°C was used to inoculate the inoculum flask.

Fermentation. Vegetative inoculum was developed for 72 h on a rotary shaker (220 rpm) at 28°C. A 10% (v/v) inoculum was transferred to experimental flasks having the same medium composition and grown similarly for 96 - 120 h. Samples were withdrawn periodically and assayed for extracellular and intracellular glucose isomerase as well as xylose isomerase activity as described in Chapter II. The pH and activity profiles of the extracellular and intracellular D-glucose (xylose) isomerase during fermentation are shown in Table 13. The optimum pH for extracellular enzyme was 9.5 while the intracellular had its optimum pH 7.0.

Table 13 : pH and activity profile of extracellular D-glucose isomerase, D-xylose isomerase and intracellular D-glucose (xylose) isomerase

Time	pH	Extracellular GI U/lit	Extracellular XI U/lit	Intracellular GI (XI) U/lit
24	7.0	764	248	21
48	7.5	1190	444	38
72	8.2	3995	133	149
96	8.3	3555	88	190

Effect of various nitrogen as well as carbon sources was tested on the extracellular enzyme using D-xylose isomerase assay system.

Nitrogen source

Different 'Nitrogen' sources at 1% final concentration of nitrogen were added to wheat bran medium in place of yeast extract. Enzyme activity was measured after 48 h. Results are presented in Table 14.

Effect of various carbon sources on enzyme production

D-Glucose (xylose) isomerase is an inducible enzyme (28, 62, 66, 79 - 82). Enzyme is induced in the presence of D-xylose, xylan or wheat bran. In addition to D-xylose and xylan, straw hemicellulose, D-lactose, mannose, lactate, mannitol, sorbitol and glycerol were also reported to be the inducers for enzyme production (176). The various carbon sources were used to examine enzyme production by Chainia sp. The data indicated that D-xylose, xylan or wheat bran was present as inducer while straw hemicellulose, lactose, mannose, mannitol, sorbitol and glycerol did not induce the D-xylose isomerase activity although a few organisms, have been reported to induce D-xylose isomerase activity in the presence of these inducers. Results are presented in Table 15.

Table 14 : Effect of various nitrogen sources on extracellular D-xylose isomerase activity

Nitrogen source 1%	pH	D-Xylose isomerase U/lit
Yeast extract	7.5	444
Peptone	7.6	600
Tryptone	7.8	622
Casein	7.9	1044
Malt extract	6.2	1080
Corn steep liquor	5.2	422
$(NH_2)HPO_4$	6.7	444
NH_4Cl	4.9	441
NH_4NO_3	4.8	300
$NH_4H_2PO_4$	4.9	466
KNO_3	6.8	311
$NaNO_3$	5.9	88
$Ca(NO_3)_2$	5.7	488

Inoculum was prepared in wheat bran medium containing nitrogen source to be tested for 72 h and then 10% inoculum (v/v) was used to inoculate the fermentation medium having same medium composition as that of the inoculum. D-Xylose isomerase activity was determined after 48 h.

Table 15 : Effect of various carbon sources on extracellular D-xylose isomerase activity

Carbon source 2%	D-Xylose isomerase U/Lit
Glycerol	0
D-Glucose	0
Mannose	0
Mannitol	0
Sorbitol	0
Lactose	0
D-xylose	210
Straw hemicellulose	0
Xylan	185
Wheat bran	130

Large scale enzyme production

Large scale extracellular enzyme production for its subsequent purification modified Weber's medium was used where Co^{2+} was eliminated from the medium.

Fermentation

Vegetative inoculum was developed for 48 h on rotary shaker (220 rpm) at 28°C by transferring seven days old culture on PDA to 100 ml medium in 500 ml Erlenmeyer flask. After the xylose isomerase activity was induced by the addition of 5 ml solution containing 2% xylose and 0.0025% $CoC_2.6H_2O$ after 48 h. The culture filtrate was checked for the enzyme activity over a period of 6 days. The pH and activity profile is given in Fig. 8.

FIG. 8 : pH and activity profile of large scale extracellular D-xylose isomerase production

Symbols: ⊙——⊙ D-Xylose isomerase

△——△ pH

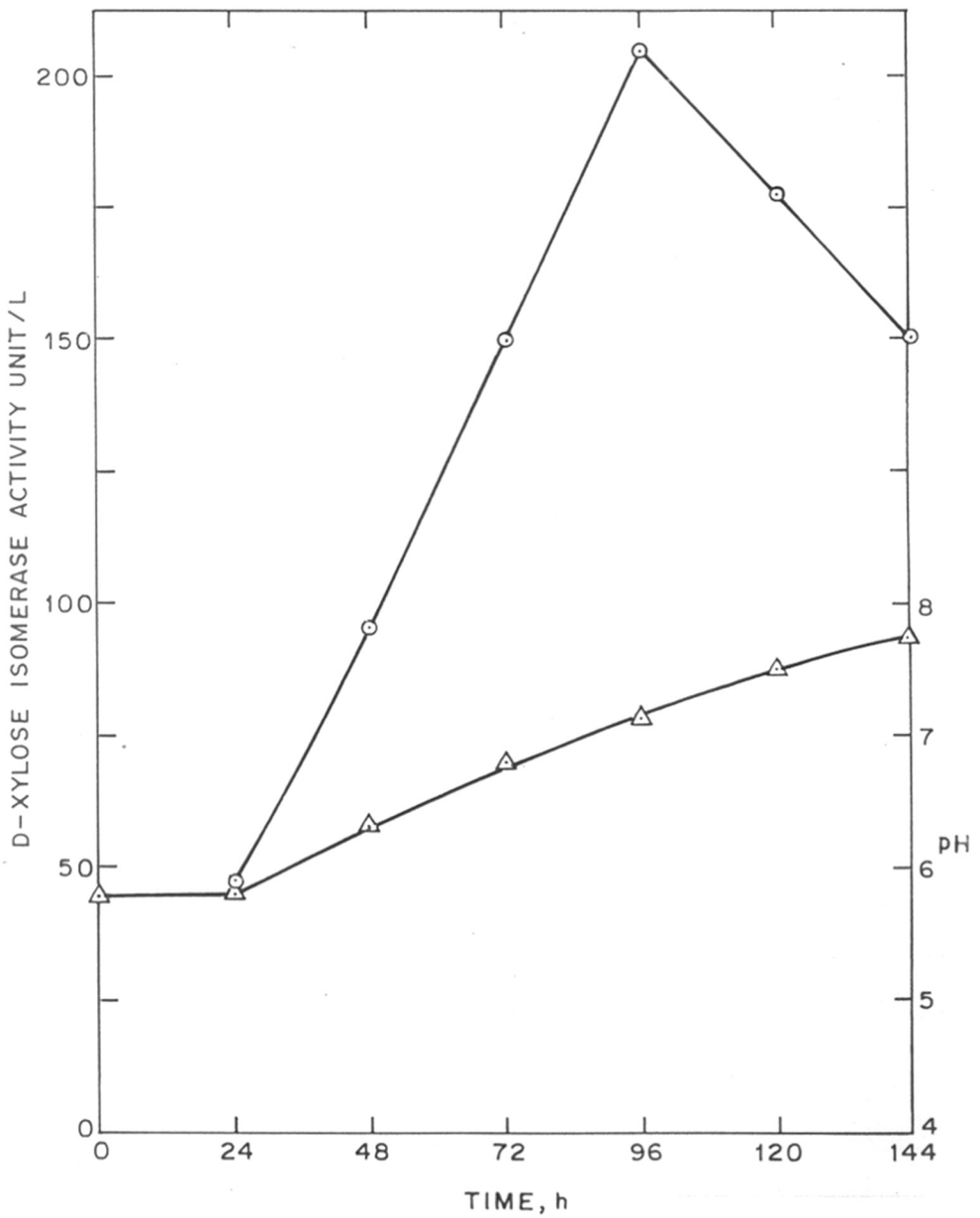

D-XYLOSE ISOMERASE ACTIVITY UNIT/L
200
150
100
50
0
0
24
48
72
96
120
144
TIME, h
8
7
6
5
4
pH

FIG. 8.

CHAPTER IV

PURIFICATION OF EXTRACELLULAR, SPECIFIC D-XYLOSE ISOMERASE

SECTION I

ISOLATION AND SEPARATION OF EXTRACELLULAR D-XYLOSE ISOMERASE FROM D-GLUCOSE (XYLOSE) ISOMERASE

Introduction

D-Glucose (xylose) isomerase (EC 5.3.1.5) catalyses isomerization of D-glucose as well as D-xylose to their ketoisomers. The enzyme is produced by various micro-organisms (Table 2). However, the enzyme from these sources has been shown to have broad specificity acting mainly on D-glucose and D-xylose in addition to D-ribose and D-mannose. Km for D-xylose is significantly lower than that for D-glucose. Danno (99) after unsuccessful attempts to separate these activities concluded that a single enzyme is involved in the isomerization of both the substrates D-glucose and D-xylose. In the present work three different types of isomerase activities were observed in the case of Chainia sp.

(a) A usual intracellular non-specific D-glucose (xylose) isomerase.

(b) Extracellular specific D-xylose isomerase

(c) Extracellular specific D-glucose isomerase.

Separation of both the activities (b and c) was carried out. This was based on their differential adsorption behaviour on DEAE-cellulose and CM-cellulose ion exchange chromatography.

Results

Preparation of the crude extract

Chainia sp. was grown on a medium containing wheat bran as a carbon source for 72 h on a rotary shaker (220 rpm) at 28°C by transferring 7 days old culture on a PDA slant. A 10% (v/v) inoculum was transferred to experimental flasks in the same medium. After 72 - 96 h cultivation the culture broth was centrifuged at 7000 rpm for 30 min and the clear culture filtrate was used for separation and purification of extracellular enzymes.

All the subsequent steps of purification were carried out at 4°C unless otherwise stated (Fig. 9).

Concentration of the enzymes

The culture filtrate was concentrated by precipitation with ethanol. To one litre of culture filtrate kept in ice (4°C), three litres of ethanol (-20°C) was added gently with constant stirring and allowed to stand for 2 h. The filtrate was collected by centrifugation at 3000 rpm for 30 min. Traces of ethanol were removed by drying the precipitate under vaccum in a desiccator. The dried powder was suspended in 25 ml of 0.01 M sodium phosphate buffer, pH 7.5 and dialyzed against the same buffer for 48 h with three changes of the buffer. After dialysis the retentate (contents of the dialysis tubing) was centrifuged at

7000 rpm for 20 min and the clear supernatant was used for further work.

Batchwise DEAE-cellulose ion exchange chromatography

Cell-free culture filtrate showed both D-glucose and D-xylose isomerase activities. To ascertain whether these two activities are associated with a single enzyme or two different enzymes, selective adsorption studies were carried out.

DEAE-cellulose (1 g) was equilibrated with 0.01 M carbonate-bicarbonate buffer, pH 9.5. To 360 mg of DEAE-cellulose, 5 ml of the enzyme containing 12.4 units of D-xylose isomerase and 6.1 units of D-glucose isomerase (previously dialysed against 0.01 M carbonate bicarbonate, pH 9.5) was added and the volume was adjusted to 25 ml with 0.01 M carbonate-bicarbonate buffer pH 9.5. After 1 h and with occassional stirring, the slurry was filtered through Whatman filter paper No. 1 Supernatant was checked for both D-glucose and D-xylose isomerase activities. Only D-glucose isomerase activity was detected in the supernatant.

Batchwise CM-cellulose ion exchange chromatography

CM-cellulose (1 g) was equilibrated with 0.01 M sodium phosphate buffer, pH 6.0. To 180 mg of CM-cellulose in 0.01 M phosphate buffer, pH 6.0, 5 ml of the enzyme containing D-xylose isomerase (5.8 units) and 3.8 units

of D-glucose isomerase (previously dialysed against 0.01 M phosphate buffer, pH 6.0) was added and the volume was adjusted to 25 ml with 0.01 M phosphate buffer, pH 6.0. After 1 h and with occassional stirring the slurry was filtered through Whatman filter paper No. 1. Supernatant was adjusted to pH 9.5 and checked for D-glucose and D-xylose isomerase activities. Only D-xylose isomerase activity was detected in the supernatant.

A brief outline for the separation of the extracellular specific D-glucose and D-xylose isomerases is shown in Fig. 9.

Effect of various additives on extracellular specific D-glucose isomerase and D-xylose isomerase

The characterization of both extracellular D-glucose isomerase and D-xylose isomerase was carried out by observing the effect of various additives such as arsenate, NAD, D-ribose, D-xylose and D-glucose. Table 16 summarizes the comparative activities of D-glucose isomerase and D-xylose isomerase in the presence of various additives at different concentrations.

Table 16 : Effect of additives on D-xylose and D-glucose isomerase activities[a]

Additions	D-Xylose isomerase		D-Glucose isomerase	
	Units	% Inhibition	Units	% Inhibition
Control	4.5	-	8.9	-
Arsenate (10^{-1} M)	4.5	0	4.1	46
Arsenate (3×10^{-1} M)	1.9	42	0.0	100
NAD (1.4×10^{-4} M)	4.5	0	4.9	55
NAD (4.2×10^{-4} M)	3.1	69	0.0	98
D-Ribose (1.3×10^{-4} M)	4.5	0	4.1	47
D-ribose (4.0×10^{-4} M)	3.5	78	0	95
D-Xylose (1.3×10^{-4} M)		NA	0	100
D-Glucose (2.0×10^{-4} M)	4.5	0		NA
D-Glucose (5.0×10^{-4} M)	4.5	0		NA
D-Glucose (10.0×10^{-4} M)	4.5	0		NA
D-Glucose-6-phosphate* (20.0×10^{-4} M)		NA		Fructose formation Nil

a. Conditions of assay as in Materials and Methods

NA = Not applicable

*The reaction mixture contained D-glucose-6-phosphate instead of D-glucose

FIG. 9 : Isolation and separation of extracellular D-xylose isomerase from D-glucose (xylose) isomerase.

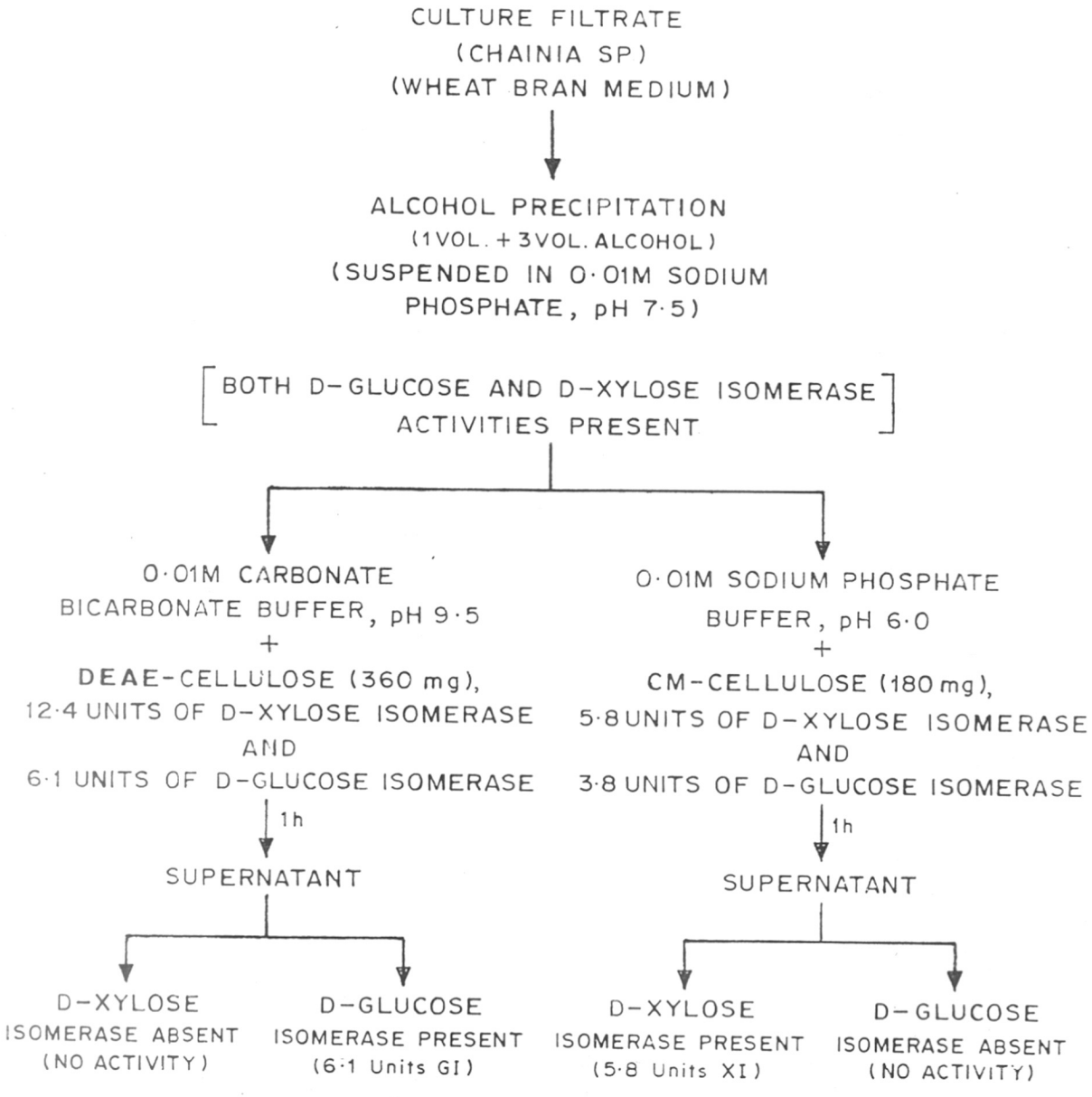

CULTURE FILTRATE
(CHAINIA SP)
(WHEAT BRAN MEDIUM)
ALCOHOL PRECIPITATION
(1VOL. + 3VOL. ALCOHOL)
(SUSPENDED IN 0·01M SODIUM PHOSPHATE, pH 7·5)
[BOTH D-GLUCOSE AND D-XYLOSE ISOMERASE ACTIVITIES PRESENT]
0·01M CARBONATE BICARBONATE BUFFER, pH 9·5
+
DEAE-CELLULOSE (360 mg),
12·4 UNITS OF D-XYLOSE ISOMERASE
AND
6·1 UNITS OF D-GLUCOSE ISOMERASE
1h
SUPERNATANT
D-XYLOSE ISOMERASE ABSENT (NO ACTIVITY)
D-GLUCOSE ISOMERASE PRESENT (6·1 Units GI)
0·01M SODIUM PHOSPHATE BUFFER, pH 6·0
+
CM-CELLULOSE (180 mg),
5·8 UNITS OF D-XYLOSE ISOMERASE
AND
3·8 UNITS OF D-GLUCOSE ISOMERASE
1h
SUPERNATANT
D-XYLOSE ISOMERASE PRESENT (5·8 Units XI)
D-GLUCOSE ISOMERASE ABSENT (NO ACTIVITY)

FIG. 9.

Conclusion

Isomerization of D-glucose and D-xylose is supposed to be catalyzed by one and the same enzyme i.e. D-Glucose (xylose) isomerase (EC 5.3.1.5).

Danno (99) made an unsuccessful attempt to separate enzyme activities towards D-glucose, D-xylose and D-ribose using a non-specific crystalline D-glucose (xylose) isomerizing enzyme from Bacillus coagulans, strain HN-68. Separation of the extracellular D-xylose and D-glucose isomerases was carried out using their differential adsorption behaviour on ion exchangers. D-Xylose isomerase being an acidic protein and D-glucose isomerase being a basic protein get adsorbed on DEAE-cellulose and CM-cellulose respectively. Extracellular specific D-glucose isomerase and D-xylose isomerase showed different behaviour when effect of various additives was tested. At lower concentrations of arsenate (1×10^{-1} M) NAD (1.4×10^{-4} M) and D-ribose (1.3×10^{-4} M) partial inhibition (46 - 55%) of D-glucose isomerase was observed while D-xylose isomerase was not inhibited at these concentrations. However, at higher concentrations of arsenate (3×10^{-1} M), NAD (4.2×10^{-4} M) and D-ribose (4×10^{-4}M); D-glucose isomerase was completely inhibited while D-xylose isomerase was partially inhibited (42 - 78%). The retention of D-xylose isomerase

to the extent of 22 - 50% is significant. When D-glucose-6-phosphate was used as a substrate in place of D-glucose, extracellular glucose isomerase did not produce D-fructose which indicated that the enzyme was different from D-glucose-6-phosphate isomerase. NAD was not required as co-factor but had an inhibitory effect on the enzyme indicating that this enzyme is not a NAD linked D-glucose isomerase. This inhibition due to NAD might be due to the D-ribose moiety of NAD.

The extracellular isomerase system in Chainia sp. offers a wide scope to resolve the nature of enzymes having isomerase activities towards D-glucose and D-xylose. The significant differences observed in adsorption behaviour on DEAE-cellulose and CM-cellulose, as well as the differential responses to NAD arsenate and pentose sugars, seem to imply that the two enzymes are distinct.

Summary

Chainia sp. when grown in a liquid medium containing wheat bran or xylose as a carbon source secretes D-glucose and D-xylose isomerases into the medium. Specific D-glucose and D-xylose isomerases are identified as two distinct enzymes and this observation can be considered as a first report in the literature. The enzymes are separated from each other by DEAE-cellulose ion exchange chromatography in 0.01 M carbonate-bicarbonate buffer, pH 9.5. Treatment with DEAE-cellulose selectively adsorbs D-xylose isomerase activity leaving the unadsorbed D-glucose isomerase activity in the supernatant. While treatment with CM-cellulose in 0.01 M sodium phosphate buffer, pH 6.0 selectively adsorbs glucose isomerase activity leaving the unadsorbed D-xylose isomerase activity in the supernatant.

Results presented in this Section have been published (163).

SECTION II

PURIFICATION OF EXTRACELLUAR SPECIFIC D-XYLOSE ISOMERASE

Introduction

Production of D-glucose (xylose) isomerase (EC 5.3.1.5) which is generally an intracellular enzyme, has been reported from large number of microorganisms, especially bacteria and more particularly from genus Streptomyces (50, 54, 72, 101, 102). Purification of the enzyme to homogeneous state has been reported from a few organisms (Table 3). The only report on extracellular secretion of the enzyme is from Streptomyces glaucescens ETH-22794 which is patented (58). However, purification and further characterization of the enzyme is not yet reported. Chainia sp. when grown in a medium containing wheat bran, xylan or D-xylose as a carbon source secretes specific extracellular D-glucose and D-xylose isomerases along with the conventional intracellular D-glucose (xylose) isomerase activity.

Results

Preparation of crude extract

Chainia sp. was grown on the modified Weber's medium with glucose as a carbon source for 48 h. D-Glucose (xylose) isomerase activity was induced by the addition of 2% xylose and 0.0025% $CoCl_2$ as described in Chapter III. The extracellular D-glucose and D-xylose isomerase activity secreted was observed periodically. Enzyme activity was maximum at 96 h. During this period pH of the culture filtrate increased to 8.2 from 7.0. The culture was harvested after 96 h by centrifugation and the clear culture filtrate was used for purification of the enzyme.

Purification

Extracellular specific D-xylose isomerase was purified from about two litre of the culture filtrates of *Chainia* sp. All operations were carried out at 0 - 4°C. Unless otherwise mentioned. Analytical polyacrylamide gel electrophoresis was used to follow the increase in enzyme purity at various stages.

Step I : Concentration of fermented broth by Amicon filtration:

Fermented broth was concentrated by ultrafiltration, using UM-10 membrane in 2.5 lit Amicon filtration unit. Enzyme was concentrated to 1/10th of its original volume within 8 - 10 h under air pressure to 50 psi. No enzyme

activity was detected in the filtrate. About 92% of the original enzyme activity was recovered.

Step II : Ammonium sulfate precipitation

The culture filtrate was further concentrated by precipitating the proteins with solid ammonium sulfate to 90% saturation, as described in Chapter I. Several harvests were concentrated by ultrafiltration and ammonium sulfate precipitation. The precipitate was suspended in a small volume of 0.01 M sodium phosphate, pH 7.5 and stored at -15°C until use. This suspension was dialyzed against the same buffer at 4°C with changes of the buffer after every 8 - 10 h for 48 h till it was free of ammonium sulfate. The recovery of the enzyme was 84.7% and 1.57% fold purification (specific activity 0.193).

Step III : Thermal denaturation of other proteins

D-Xylose isomerase was found to be thermostable, hence other thermolabile proteins from the concentrated enzyme preparation from Step II, were removed by heating the broth at 60°C for 30 min. The proteins denatured due to heat treatment were coagulated and were removed by centrifugation at 10,000 rpm for 30 min. Enzyme recovery was 80.9% with 2.51 fold purification (specific activity 0.309).

Step IV : Preparative polyacrylamide gel electrophoresis

After Step III, the enzyme showed 4-5 bands on analytical polyacrylamide gel electrophoresis (Fig. 10). Further purification was carried out by preparative polyacrylamide gel electrophoresis. The enzyme was visualised as a red band on polyacrylamide gel by the use of *in situ* staining method to detect D-xylose isomerase.

Purification by preparative polyacrylamide gel electrophoresis was performed in an apparatus as described in Chapter II. About 50 mg of enzyme preparation (15.4 units) was loaded each time at the cathodic end of polyacrylamide gel column (5 x 12 cm). A constant current of 10 mA at 150 volts was applied and the electrophoresis was carried out at 4 - 6°C for 75 - 80 h. After completion of the run two verticle strips of the gels were cut as described in Chapter II. One strip was stained with CBB G-250 to reveal the protein pattern of the loaded sample. The other strip was kept near gel block for comparison and corresponding D-xylose isomerase enzyme containing portion of the block was removed by cutting with sharp knife. Enzyme was eluted from the gel into the buffer (0.05 M sodium phosphate, pH 7.5, containing $MgSO_4.7H_2O$, 5 x 10^{-3} M). Recovery of the activity was 4.4 units and protein 4.6 mg (29%). The eluate was filtered to remove impurities of polyacrylamide gel.

FIG. 10 : In situ staining method for D-xylose isomerase.

Protein staining by CBB G-250

In situ staining of D-xI

Position of the marker band

Position of the marker band

The soluble impurities from polyacrylamide gel were removed by dialysis. The fractions containing D-xylose isomerase activity were pooled from eleven such runs and processed further.

Step V : DEAE-cellulose ion exchange chromatography

The enzyme obtained in Step IV was further purified by batchwise treatment with DEAE-cellulose. The anion exchanger was equilibrated with 0.01 M sodium phosphate buffer, pH 7.5, containing $MgSO_4.7H_2O$, 5×10^{-3} M. DEAE-cellulose (240 mg) was added to 10 ml of the enzyme (protein 63 mg, activity 60.5 units XI). Volume was made upto 25 ml with sodium phosphate buffer, pH 7.5. Final molarity of the buffer was 0.01 M. After 2 h of occassional stirring it was filtered through Whatman filter paper No. 1. D-Xylose isomerase was adsorbed on DEAE-cellulose. The DEAE-cake was washed with 50 ml of 0.15 M phosphate buffer, pH 7.5, followed by 50 ml of 0.15 M phosphate buffer. The enzyme was eluted with 10 ml of 0.25 M sodium phosphate, pH 7.5. This preparation was concentrated by lyophilization. Final recovery of the enzyme was 23%. The specific activity of the enzyme was 10.24.

Step VI : Gel filtration on Sephadex G-200

D-Xylose isomerase was further purified by gel filtration on Sephadex G-200 column. The enzyme (4 mg)

from Step V was loaded on Sephadex G-200 column (1.2 x 100 cm) previously equilibrated with 0.05 M sodium phosphate, pH 7.5, containing $MgSO_4.7H_2O$, 5 x 10^{-3} M and eluted with the same buffer. Fractions of 2 ml were collected at a flow rate of 12 ml/h and were analysed for protein contents and D-xylose isomerase activity. The elution profile is shown in Fig. 11

Fractions (35 - 45) with specific activity of 1.37 were pooled, concentrated by lyophilization, dissolved in 0.01 M $MgSO_4.7H_2O$ and dialysed for 8 - 10 h against the same buffer.

Yield and specific activity of D-xylose isomerase at different stages of purification is summarized in Table 17.

Criteria of purity

The purified extracellular specific D-xylose isomerase preparation was homogeneous as revealed by disc gel electrophoresis at pH 7.5 and 8.3 (Fig. 12 a, b) A strict coelution of protein and D-xylose isomerase activity was observed on Sephadex G-200 (Fig. 11). The enzyme preparation showed only one protein band in SDS-polyacrylamide gel electrophorsis (Fig. 12c).

FIG. 11 : Protein and activity profile of pure extracellular D-xylose isomerase on Sephadex G-200.

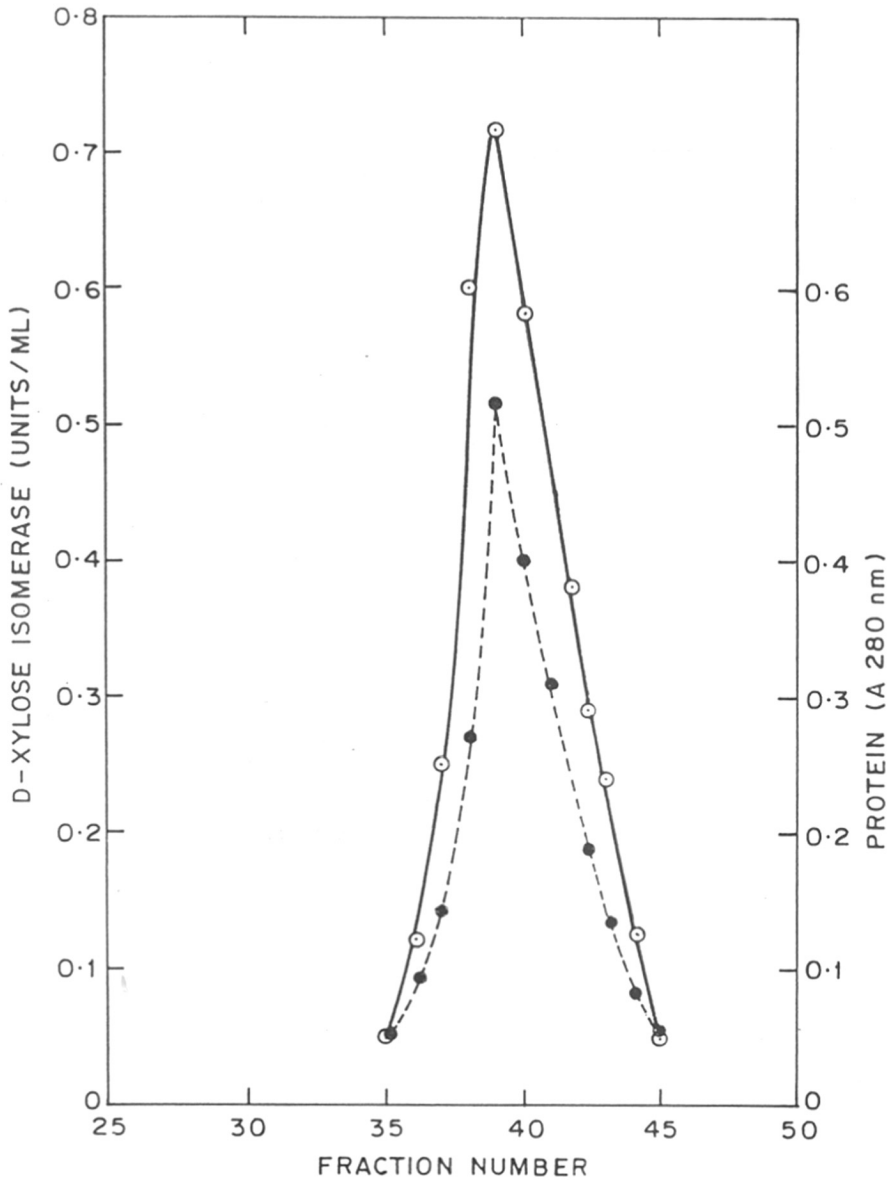

0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
D-XYLOSE ISOMERASE (UNITS/ML)
0·6
0·5
0·4
0·3
0·2
0·1
0
PROTEIN (A 280 nm)
25
30
35
40
45
50
FRACTION NUMBER

FIG. 11

Table 17 : Purification of extracellular specific xylose isomerase from Chainia sp.

	Step	Total protein mg	Total units	Specific activity	Purification (fold)	Recovery (%)
	Fermented broth	1700	210	0.123	-	100
I.	Amicon filtration	1533	193	0.126	1.02	91.9
II.	Ammonium sulfate precipitation	920	178	0.193	1.57	84.7
III.	Thermal denaturation	550	170	0.309	2.51	80.9
IV.	Preparative PAGE	63	60.5	0.960	7.80	28.8
V.	DEAE-Cellulose chromatography	38.35	48.4	1.260	10.24	23.0
VI.	Sephadex G-200	17.45	24.2	1.380	11.21	11.5

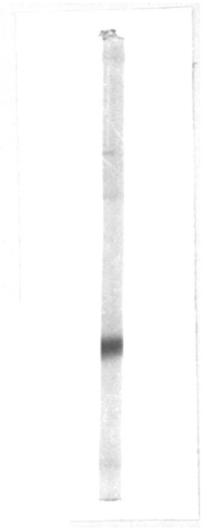
*Total 11 batches of preparative were carried out. About 50 mg protein was used in each batch.

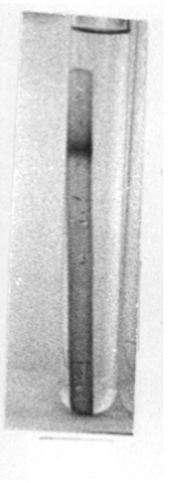
FIG. 12 : Polyacrylamide gel electrophoresis of D-xylose isomerase

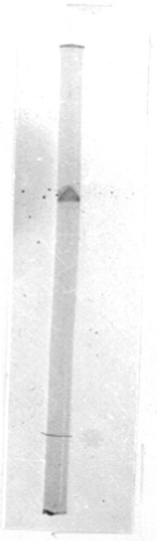
(a) Disc gel electrophoresis at pH 8.9

(b) Disc gel electrophoresis at pH 7.5

(c) SDS-gel electrophoresis when enzyme was treated with 1% 2-mercaptoethanol and 0.5% iodoacetamide.







Summary

Presence of specific D-glucose and D-xylose isomerases has been demonstrated in the cell-free culture filtrates of Chainia sp. The extracellular specific D-xylose isomerase was purified to homogeneity and the purification steps were as follows:

i) Concentration of culture filtrate by ultrafiltration (UM-10 membrane)

ii) Ammonium sulfate precipitation of the concentrated broth (0 - 90% saturation).

iii) Heat treatment to dialyzed concentrated preparation from Step (ii) to denature other proteins

iv) Preparative polyacrylamide gel electrophoresis

v) DEAE-cellulose chromatography (batchwise)

vi) Gel filtration on Sephadex G-200.

The purified enzyme was homogeneous on polyacrylamide gel when electrophoresis was carried out at two different pH values (pH 7.5 and 8.3). Homogeneity was also confirmed by carrying out SDS-gel electrophoresis and gel filtration on Sephadex G-200.

CHAPTER V

PHYSICO-CHEMICAL AND ENZYMIC PROPERTIES OF THE EXTRACELLULAR SPECIFIC D-XYLOSE ISOMERASE

Introduction

In the present Chapter physico-chemical and enzymatic properties of extracellular specific D-xylose isomerase from Chainia sp are described and compared with those of conventional intracellular non-specific D-glucose (xylose) isomerases from other microbial sources. Various physico-chemical properties such as molecular weight, subunit determination, isoelectric point, glycoprotein nature and amino acid composition. Enzymatic properties include effect of pH and temperature, various metal ions and other compounds such as sugar alcohols on enzyme activity. Moreover, the substrate specificity, kinetics and active site group specific reagents on D-xylose isomerase have also been studied.

Molecular weight determination

(1) Gel filtration. The relative molecular mass of the enzyme was estimated by using both, Bio-gel P-150 and Sephadex G-200 columns (1.2 x 100 cm). The elution volume of the enzyme was compared with those of marker proteins of known molecular weights, as described in Chapter II. A plot of Ve/Vo versus log molecular weight, according to the procedure of Andrews (142) indicated the relative molecular weight of 70,800 and 70,200 respectively (Fig. 14a, b).

(2) SDS-gel electrophoresis. An estimate of relative molecular mass of extracellular, specific D-xylose isomerase and its possible subunit nature was made by observing its migration in the SDS-polyacrylamide gel (143, 144) as described in Chapter II. A plot of log molecular weights versus relative mobilities of marker proteins yielded, a straight line (Fig. 15). An estimate of molecular weight of 71,200 was calculated from the plot for purified D-xylose isomerase. The enzyme on treatment with 1% SDS in combination with 1% 2-mercaptoethanol and 0.5% iodoacetamide showed only one protein band in SDS-gel electrophoresis with molecular weight corresponding to the native protein. This indicated that the enzyme is composed of one polypeptide chain. D-Glucose isomerase obtained from other microbial sources has shown to contain either two subunits as in Strepto. olivochromogenes (102),

FIG. 14a : Molecular weight determination of purified enzyme by gel filtration (142)
A Bio-Gel P-150 column (1.2 x 100 cm) was calibrated with:
(1) Myoglobin (Mr 17,500)
(2) Ovalbumin (Mr 43,000)
(3) Bovine serum albumin (Mr 68,000)
(4) Transferrin (Mr 74,000)
The column was equilibrated with 0.05M sodium phosphate buffer, pH 7.5
Vo - Void volume Ve - elution volume.

FIG. 14b : Molecular weight determination of purified enzyme by gel filtration (142)
A Sephadex G-200 column (1.2 x 100cm) was calibrated with:
(1) Cytochrome C (Mr 12,500)
(2) Ovalbumin (Mr 43,000)
(3) Bovine serum albumin (Mr 68,000)
(4) Aldose (Mr 1,58,000)

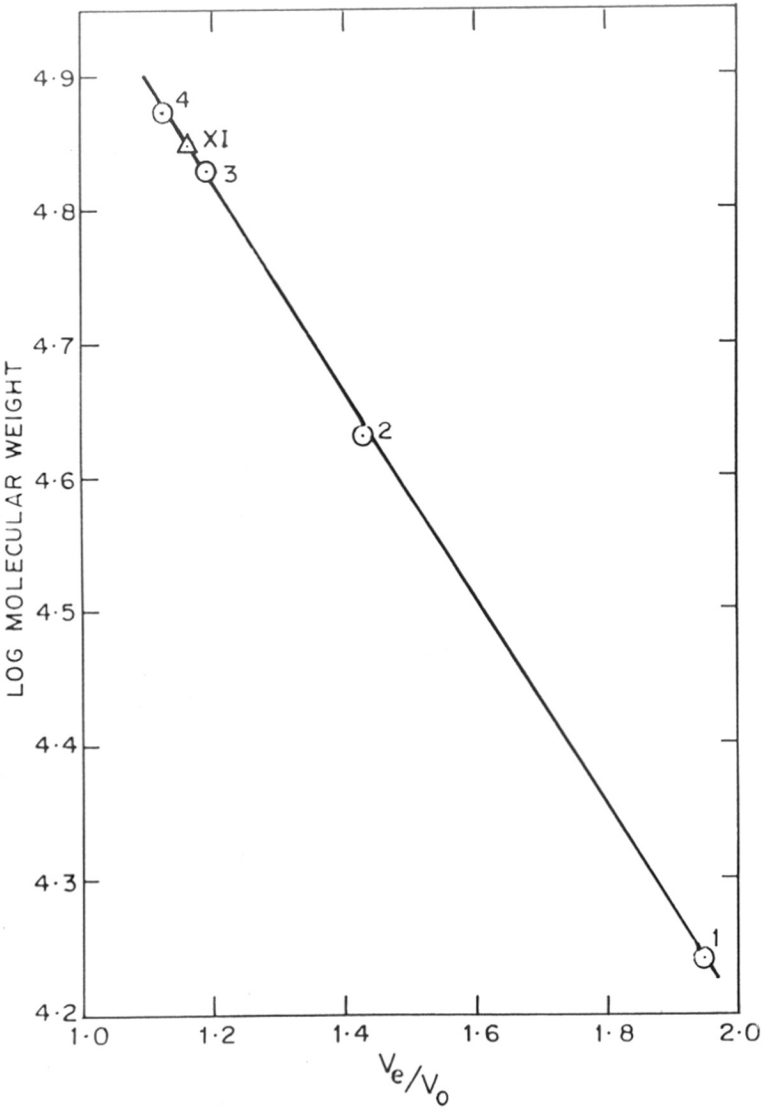


FIG. 14 a

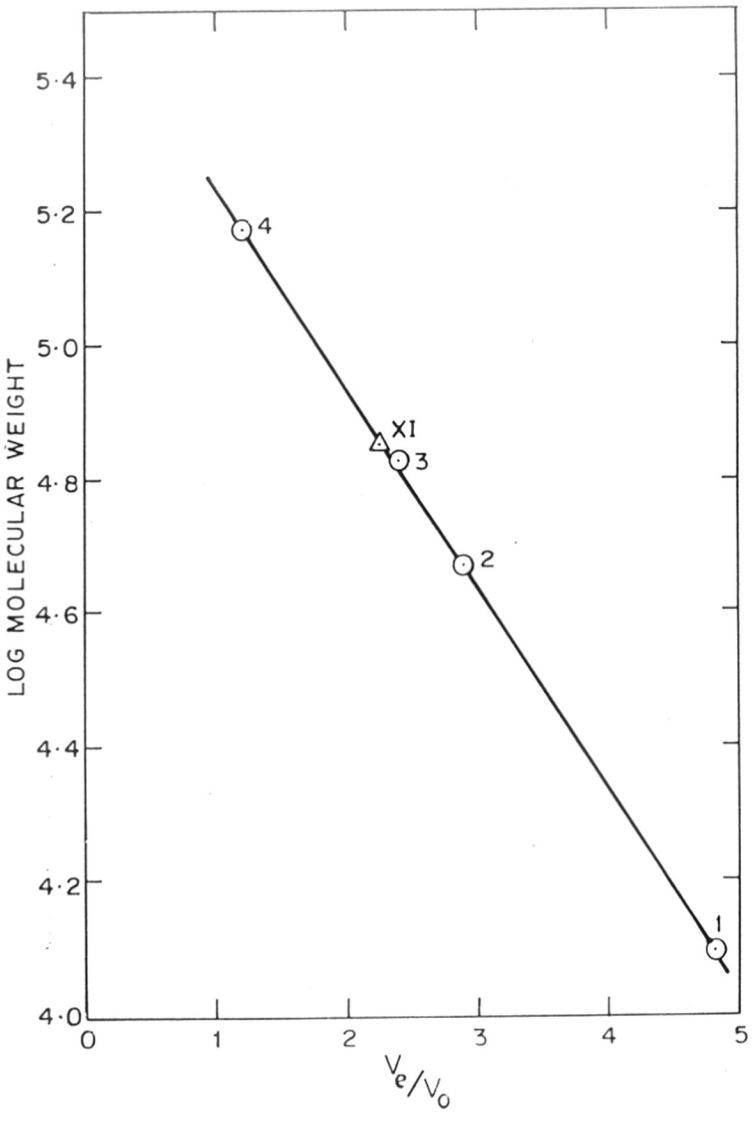


FIG. 14 b

FIG. 15 : Molecular weight determination of the purified enzyme by SDS-gel electrophoresis (143,144). Relative mobilities were plotted against log molecular weight of marker proteins

(1) Cytochrome C (Mr 12,500)

(2) Myoglobin (Mr 17,500)

(3) Ovalbumin (Mr 43,000)

(4) BSA Monomer (Mr 68,000)

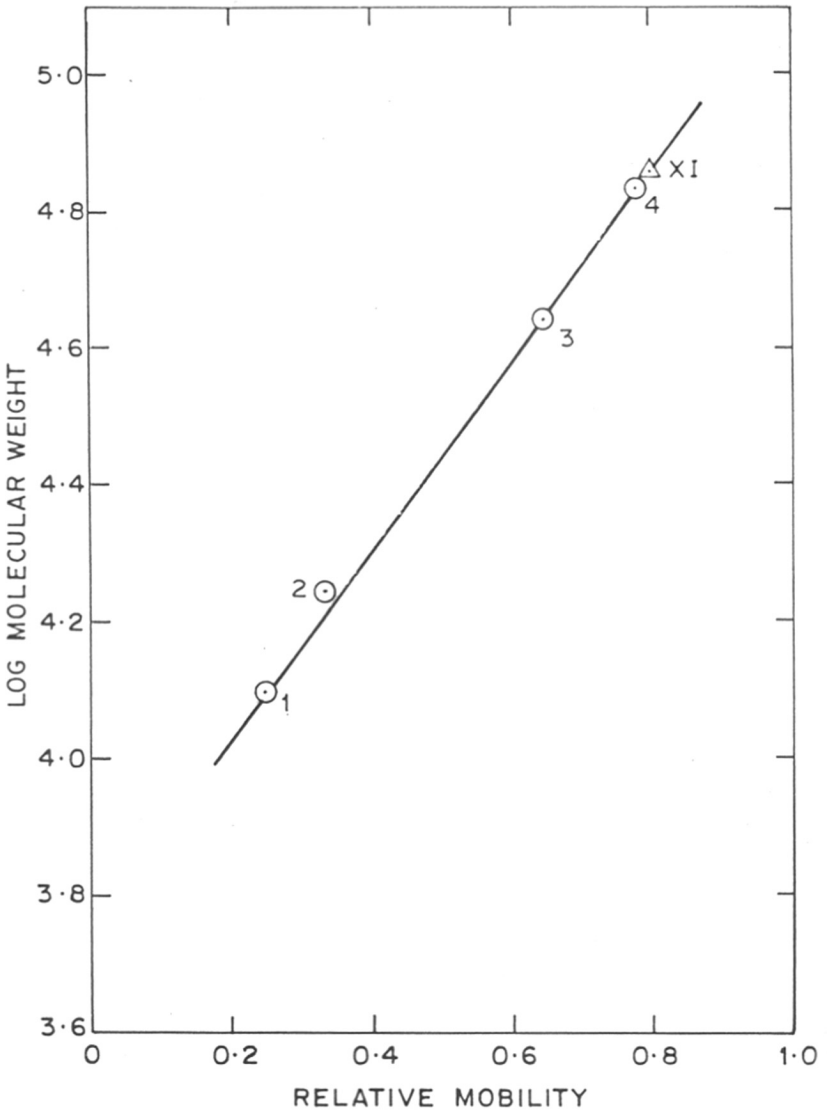

5·0
4·8
4·6
4·4
4·2
4·0
3·8
3·6
LOG MOLECULAR WEIGHT
0
0·2
0·4
0·6
0·8
1·0
RELATIVE MOBILITY
1
2
3
4
XI

FIG. 15

Strepto. albus YT-5 (101) and Actinoplanes missouriensis (94) or four subunits as in Lac . xylosus (38), Strepto. flavogriseus (54), Lac. brevis. (100) and Bacillus coagulans (99, 100).

(3) Gel electrophoresis (Slope method). The relative molecular mass of 73,000 for extracellular, specific D-xylose isomerase was determined by the slope method of Hedrick and Smith (145). This agrees with the values obtained by other methods. The procedure is described in Chapter II. The migration of protein band of D-xylose isomerase in disc gel electrophoresis at different concentrations (4-9%) of acrylamide (w/v) is shown in Figs. 13, 17.

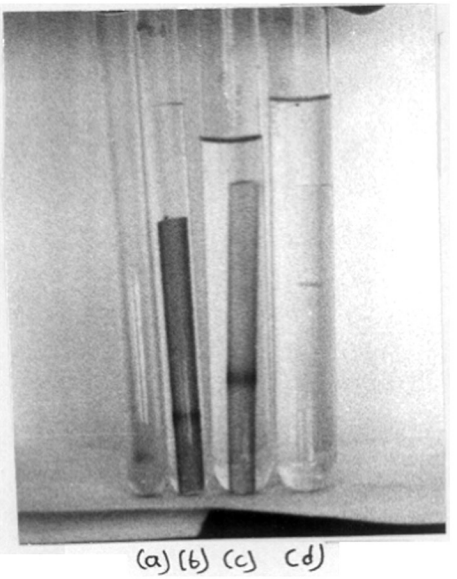
Isoelectric point (pI). From isoelectric focusing experiments with the purified enzyme using 1% Ampholine carrier ampholytes, (pH range 3.5 - 5.0 and 3.0 - 10.0), the isoelectric point, pI was determined to be 3.55 (Fig. 16). Thus D-xylose isomerase is an acidic protein. The isoelectric points for purified microbial D-glucose (xylose) isomerases have not been reported except from a Bacillus source (10) which has a pI of 4.9.

Glycoprotein nature. After electrophoresis, gels were washed with isopropanol-acetic acid:water (25:10:65) to fix the protein and to remove low molecular weight substances. Final wash was given in the same solvent

FIG. 13 : Migration of D-xylose isomerase in polyacrylamide gel electrophoresis with different concentrations of acrylamide (w/v)

(a) 5 % (b) 6 %

(c) 7 % (d) 9 %



(a) (b) (c) (d)

FIG. 17 : Molecular weight determination of the purified enzyme by slope method (145)

(a) Plots of 100 log (Rm x 100) of marker proteins against acrylamide gel concentrations (%)

The standard proteins used were

(A) Pepsin (Mr 34,000)

(B) Ovalbumin (Mr 43,000)

(C) Bovine serum albumin (Mr 68,000)

(b) The plot of 100 log (Rm x 100) of D-xylose isomerase against acrylamide gel concentrations

(c) The plot of the negative slope of each marker protein against the molecular weight.

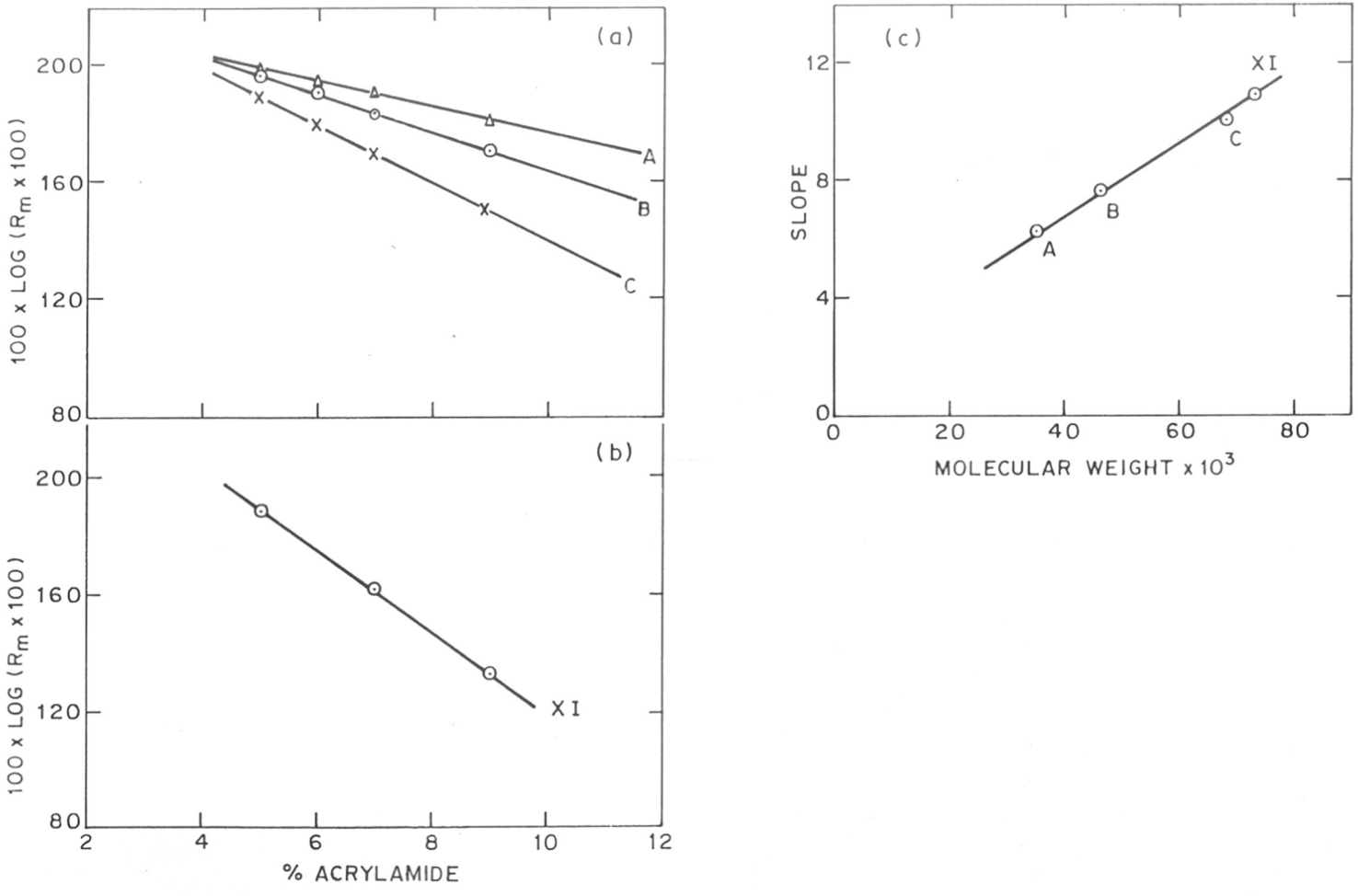

(a)
200
160
120
80
100 x LOG (R_m x 100)
A
B
C
(b)
XI
2
4
6
8
10
12
% ACRYLAMIDE
(c)
12
8
4
0
SLOPE
XI
C
B
A
0
20
40
60
80
MOLECULAR WEIGHT x 10^3

FIG. 17

FIG. 16 : Isoelectric focusing profile of D-xylose isomerase.

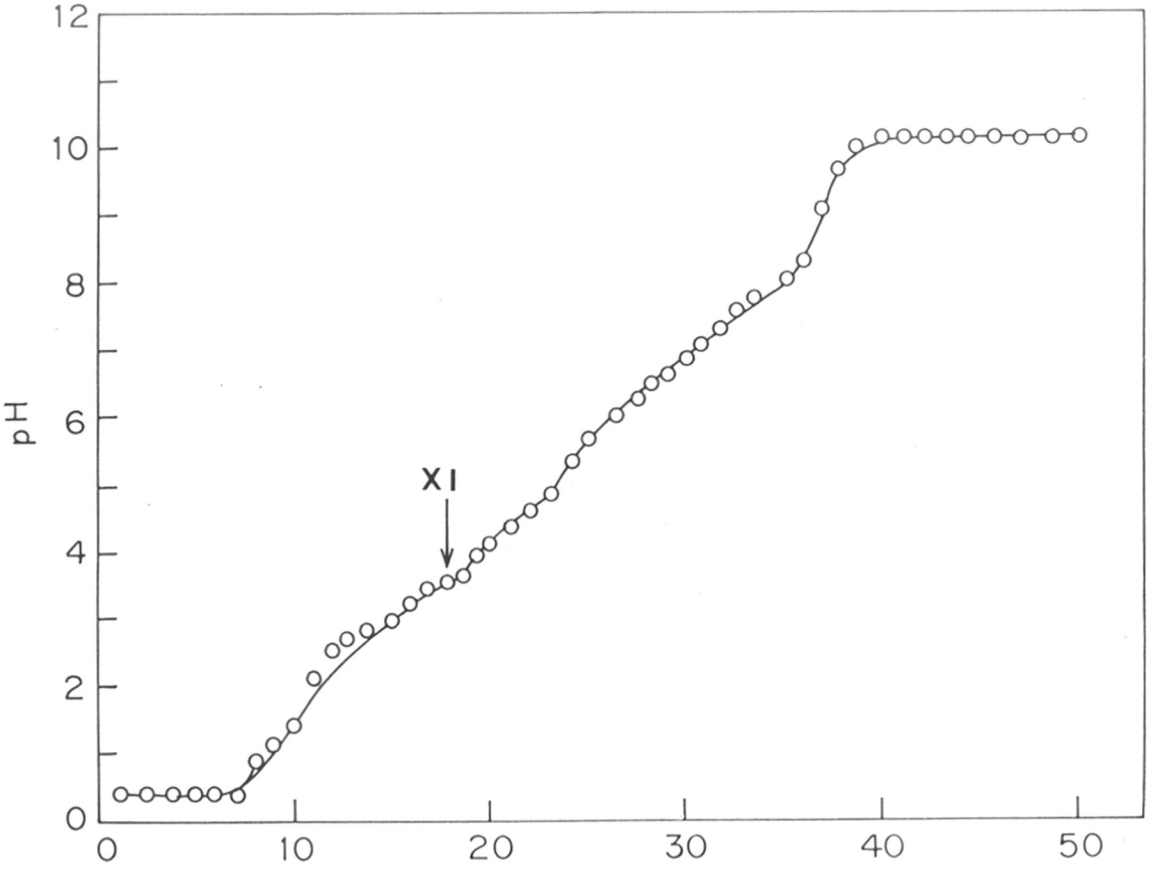


FIG. 16

containing 0.2% thymol (w/v). Gels were stained by gel protein staining method for glycoprotein (147). There was no purple band of glycoprotein corresponding to protein band stained by usual CBB G-250 dye, indicating the absence of carbohydrate moiety in the enzyme.

No other D-glucose (xylose) isomerase reported in the literature containing carbohydrate moiety attached to the enzyme.

Amino acid analysis

The hydrolysates of extracellular, D-xylose isomerase were analyzed for amino acid analysis in a Spinco model 120-B automatic amino acid analyzer by the method of Spackman et al. (149) (Table 18a, b).

Tyrosine and Tryptophan

Tyrosine and tryptophan were determined spectrophotometrically by the method of Goodwin and Morton (150). The purified D-xylose isomerase (0.496 mg) were taken separately in 1.0 ml of 0.1 N NaOH and absorbance of the enzyme at 294.4 nm and 280 nm was recorded in a Beckman Spectrophotometer Model 25. In 0.1 N NaOH as a solvent, the curves for tyrosine and tryptophan intersect at 294.4 nm (ε = 2375 $M^{-1}.cm^{-1}$) and 275.15 nm (ε = 2748 $M^{-1}.cm^{-1}$). By determining the absorption of the protein in 0.1 N NaOH at one of these two wavelengths and at 280 nm, the relative proportions of tyrosine and tryptophan were determined. It was calculated that D-xylose isomerase

contains 4 residues each of tyrosine and tryptophan per mole of the enzyme.

The results of amino acid analysis of D-xylose isomerase representing closest integer values was multiplied by a proportionality factor to bring the total composition to 100%. The total number of amino acid residues per mole of enzyme was determined to be 536. The amino acid profiles of homogenous D-xylose isomerase is compared with the profiles of homogenous D-glucose (xylose) isomerase reported from other organisms (Table 18 b)

Amino acid composition

D-Glucose (xylose) isomerase generally do not contain half-cystine [except from Strepto. albus (101)]. Chainia D-xylose isomerase is devoid of half-cystine residue. Glycine content (molar %) in D-glucose (xylose) isomerase purified from Actinomyces olivacinereus (17), Strepto. albus (101), B. coagulans (99, 100) and B. stearothermophilus (30) varies in the range of 7.4 - 13.7%. D-Xylose isomerase from Chainia sp. contain higher amounts of glycine viz. 14.9%. Aspartic acid residues are generally in the range of 10.3 - 12.7%. The aspartic acid residues of D-xylose isomerase are slightly lower (8.2%) as compared to other sources (10.4 - 12.7%). Similarly, molar % of methionine, leucine, tyrosine, phenylalanine are slightly lower than D-glucose (xylose) isomerase from other microorganisms.

Table 18a : Amino acid composition of extracellular, D-xylose isomerase from Chainia sp.

Amino acid	Number of residue/ molecule	Molar percent of amino acid
Aspartic acid	44	8.2
Threonine*	34	6.3
Serine*	36	6.7
Glutamic acid	60	11.2
Proline	38	7.1
Glycine	80	14.9
Alanine	50	9.3
Valine**	18	3.3
Methionine	2	0.4
Isoleucine**	16	3.0
Leucine	40	7.5
Tyrosine @	4	0.8
Phenylalanine	8	1.5
Histidine	30	5.6
Lysine	38	7.1
Arginine	34	6.3
Tryptophan @	4	0.8
Half-cystine	-	-

Conditions are described in the text

* Values for Threonine and Serine are extrapolated to zero time

** Values for valine, Isoleucine are those for 72 h hydrolysis

@ Tryptophan and Tyrosine are determined spectrophotometrically

Table 18b: Molar percent of constituent amino acid of homogeneous D-glucose (xylose) isomerases from other microorganisms

Amino acid	*Chainia* sp. Present work	*Actinomyces olivacinereus* (17)	*Streptomyces albus* (101)	*Bacillus coagulans* (99, 100)	*Streptomyces olivaceous* (30)	*Bacillus stearothermophilus* (30)
Aspartic aicd	8.2	11.8	12.1	12.7	11.4	10.4
Threonine	6.3	4.4	3.8	6.1	5.2	5.7
Serine	6.7	5.3	2.4	4.9	2.7	4.3
Glutamic acid	11.2	12.6	10.3	9.5	10.0	11.6
Proline	7.1	2.6	4.9	3.1	5.2	2.9
Glycine	14.9	13.7	9.5	7.4	9.5	8.3
Alanine	9.3	10.9	12.2	9.8	12.9	8.9
Half-cystine	-	-	0.3	-	-	-
Valine	3.3	7.8	4.9	3.7	5.3	5.6
Methionine	0.4	1.9	2.2	1.9	1.8	2.0
Isoleucine	3.0	3.9	2.7	3.6	3.9	4.0

Table 18b contd.

Amino acid	Chainia sp Present work	Actinomyces olivacinereus (17)	Streptomyces albus (101)	Bacillus coagulans (99,100)	Styreptomyces olivaceous (30)	Bacillus stearothermophi (30)
Leucine	7.5	8.4	9.7	9.5	9.9	9.5
Tyrosine	0.8	2.0	2.4	4.1	2.1	4.7
Phenylalanine	1.5	3.5	6.2	6.7	5.3	6.4
Histidine	5.6	1.9	2.7	3.4	2.4	3.0
Lysine	7.1	4.9	2.7	8.4	2.8	6.5
Arginine	6.3	3.1	8.9	4.3	8.2	4.5
Tryptophan	0.8	1.1	2.1	0.9	1.5	1.7
Residues	536	1327	1480	1428	886	964
Molecular weight	70,000	1,60,000	1,65,000	1,73,000	1,20,000	1,30,000

Enzymic properties

The purified extracellular, specific D-xylose isomerase was stable when stored at -15°C at pH 7.5 with 5 x 10^{-3} M $MgSO_4.7H_2O$. No significant loss of activity was observed over a months period. In the absence of $MgSO_4.7H_2O$, the enzyme loses its activity rapidly and within three weeks the enzyme was completely inactivated. Repeated freezing and thawing decreases the enzyme activity as reported with D-glucose isomerase from A. missouriensis (94). The rate of D-xylose isomerization to D-xylulose was linear upto 5 µg protein under assay conditions (35°C, 10 min, pH 9.5).

Influence of pH on stability

For determining the influence of pH on stability of extracellular, D-xylose isomerase, the enzyme was incubated at 60°C for 30 min in different buffers with pH range of 6 - 10. The pH ranges of the buffers used were: sodium phosphate (0.05 M, pH 6 - 8), carbonate-bicarbonate (0.05 M, pH 8.5 - 10.0). After incubating the enzyme samples for 30 min at 60°C the residual enzyme activity was determined under the standard assay conditions. The observed activities were compared with those of reference samples maintained at 4°C at pH 7.5, for the duration of the experiment. The enzyme was most stable at pH 7.5 - 8.0 (Fig. 18). Rapid loss of enzyme activity was observed above pH 8.0. Most

FIG. 18 : pH Stability of extracellular D-xylose isomerase.

D-Xylose isomerase (5 µg) was incubated at 60°C for 30 min in 0.05 M buffer in the pH range 6.0 - 10.0. The residual activity (%) in each sample compared with that of control kept at 4°C, pH 7.5.

FIG. 19 : Thermal stability

Extracellular D-xylose isomerase (3 µg) in 0.05 ml of 0.05 M sodium phosphate buffer, pH 7.5, was heated at different temperatures for 10 min and then cooled. The residual activity (%) in each sample compared with that of control kept at 4°C, pH 7.5.

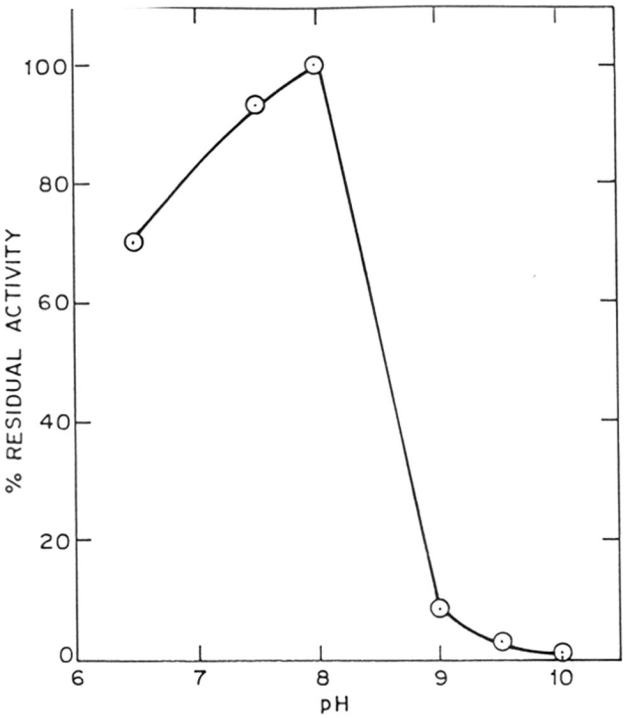

100
80
60
40
20
0
% RESIDUAL ACTIVITY
6
7
8
9
10
pH

FIG. 18

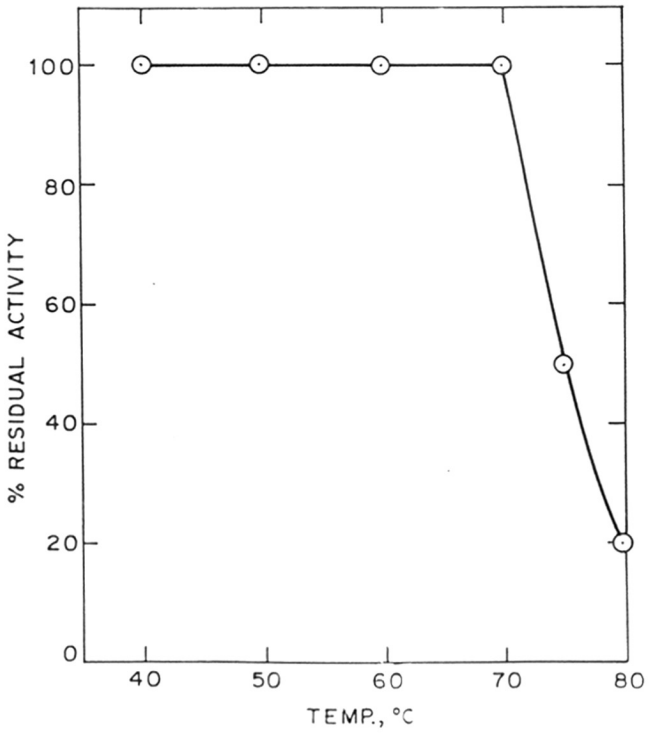

100
80
60
40
20
0
% RESIDUAL ACTIVITY
40
50
60
70
80
TEMP., °C

FIG. 19

of the D-glucose (xylose) isomerases reported from other microbial sources are stable in the same pH range. Only Strepto. albus YT-5 (101) D-glucose (xylose) isomerase was reported to be stable over a wide range of 4.0 - 11.0 pH.

Thermostability

Thermostability of purified, extracellular D-xylose isomerase was determined by incubating the enzyme sample (30 µg in 1.0 ml of 0.05 M sodium phosphate buffer, pH 7.5), free from metal ion, at various temperatures covering a range of 40° - 80°C. After 10 min of incubation, 0.1 ml aliquot was estimated for the residual D-xylose isomerase activity. The heat stability curve of the enzyme is shown in Fig. 19. Enzyme was stable upto 70°C when incubated for 10 min. Beyond 70°C, enzyme loses its activity rapidly. At 75°C it retains 50% of the original activity while at 80°C only 20% of the original activity was observed. Most of the D-glucose (xylose) isomerases reported in the literature are thermostable (Table 7). D-Glucose (xylose) isomerase from Strepto. phaeochromogenes SK is highly stable and retained 96% activity when heated at 80°C for 10 min (82). While an enzyme from E. intermedia lost its activity when heated at 60°C for 10 min (97).

Table 19 : Effect of metal ions on the thermal stability of D-xylose isomerase

Metal ion (1×10^{-3} M)	Residual activity %
Control	100
$MgSO_4.7H_2O$	49
$CoCl_2.6H_2O$	100
$ZnSO_4.7H_2O$	0
$CuSO_4.7H_2O$	0
$CaCl_2.2H_2O$	0
$FeSO_4.7H_2O$	0

The enzyme solution in 0.05 M sodium phosphate buffer, pH 7.5 was incubated at 80°C for 10 min with the metal indicated, and then immediately cooled. The residual D-xylose isomerase activity was estimated as described in Chapter II.

Effect of metal ions on thermal stability of D-xylose isomerase

The enzyme solution (free of metal ions) was heated with and without metal ions at 80°C for 10 min. Aliquots were removed after different time intervals and after cooling immediately, 0.1 ml aliquot from each sample was tested for residual D-xylose isomerase activity (Fig. 20). Mg^{2+} and Co^{2+} were effective against thermal denaturation of the enzyme. Co^{2+} was found to be most effective (100% activity was retained). Other metal ions like Mn^{2+}, Zn^{2+}, Cu^{2+} were not effective. Results are tabulated in Table 19. Takasaki (41) also showed that the heat stability of the enzyme can be increased by the addition of Mg^{2+} or Co^{2+} to the enzyme mixture. Krolava et al (72) used this property of D-glucose (xylose) isomerase and eliminated the thermolabile protease from crude enzyme solution from Strepto. nigrifica 82/20. In the case of Candida utilis, Mn^{2+} was required for thermostability (75).

Optimum pH and temperature

With D-xylose as substrate, maximum activity was observed at pH 9.5 (Fig. 21). Buffers used to cover the pH range were tris-HCl 7.2 - 9.0, sodium phosphate 6 - 8, glycine-NaOH 8.5 - 10.0 and carbonate-bicarbonate 8.5 - 10.0. Most of the intracellular D-glucose (xylose) isomerases reported in the literature have optimum

FIG. 20 : Influence of metal ions on thermal stability of D-xylose isomerase.

D-Xylose isomerase (25 µg) was incubated at 80°C in 0.05 M sodium phosphate buffer, pH 7.5 for different time intervals with:

(A) 1×10^{-3} M $CoCl_2.6H_2O$

(B) 1×10^{-3} M $MgSO_4.7H_2O$

(C) Without any metal ions

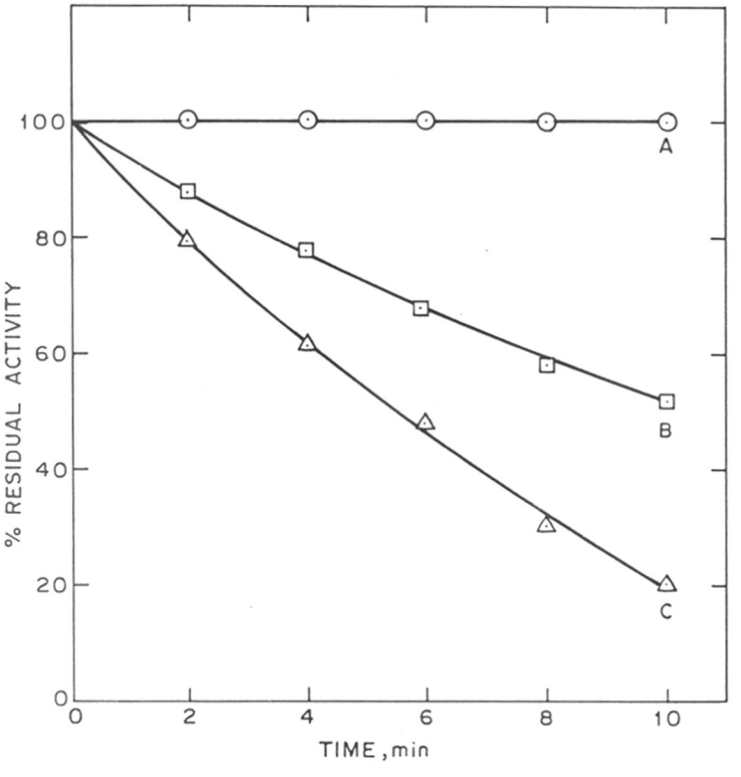


FIG. 20

FIG. 21 : Optimum pH

Activity of D-xylose isomerase (3 µg) towards D-xylose.

Buffers: pH 6.0 - 8.0, phosphate buffer, pH 8.5 - 10.0 carbonate bicarbonate buffer

FIG. 22 : Optimum temperature

Activity of D-xylose isomerase (3 µg) towards D-xylose at different temperatures

FIG. 23 : Arrhenius plot showing effect of temperature on the rate of D-xylose isomerization activity by D-xylose isomerase (pH 9.5)

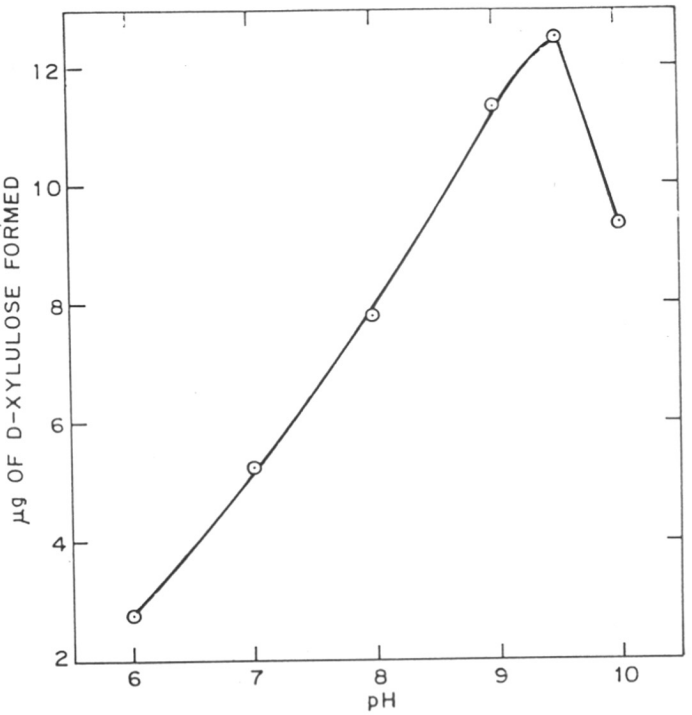


FIG. 21

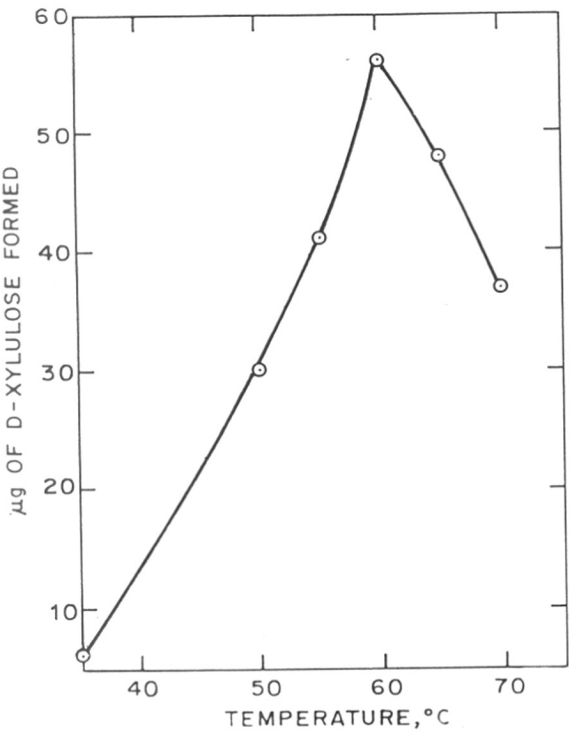


FIG. 22

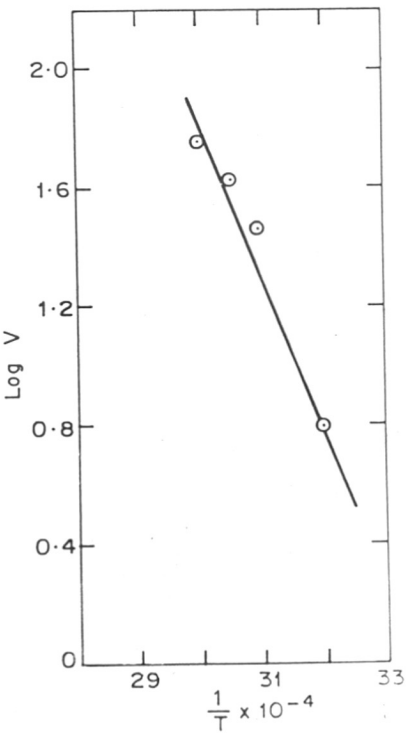


FIG. 23

pH range of 6.5 - 7.5. Only Strepto. phaeochromogenes (82) has optimum pH range of 9.0 - 9.5.

Effect of temperature on D-xylose isomerase activity was determined using the standard assay system in which the temperature of the assay was varied from 35°C - 80°C. Optimum temperature of the enzyme with D-xylose as subsdtrate was found to be 60°C (Fig. 22). A straight line was obtained when the data was plotted according to Arrhenius (Fig. 23). The energy of activation (Ea) from the plot was calculated to be 172.9 KJ. D-Glucose (xylose) isomerase obtained from other microbial sources has shown a wide range of optimum temperature varying from 50°C in E. intermedia (81) to 90°C in Strepto. phaeochromogenes (82). Activation energy of pure D-glucose isomerizing enzyme from B. coagulans HN-68 was reported to be 14,600 cal per mol (99).

Effect of various metal ions

For determining the effect of different metal ions (5×10^{-3} M, final concentration) on D-xylose isomerase, the enzyme (3 µg) was pre-incubated with the additive at 35°C, pH 9.5 for 10 min. Activity of the preincubated enzyme, with and without the metal ion, was determined. The effect of metal ion on D-xylose isomerase activity is shown in Table 20. Maximum enzyme activity was obtained in the presence of Mg^{2+}.

Table 20 : Effect of metal ions on D-xylose isomerase activity

Metal ion (5×10^{-3} M)	Relative activity %
None	0
$MgSO_4.7H_2O$	100
$CoCl_2.6H_2O$	80
$MnSO_4.H_2O$	30
$CaCl_2.2H_2O$	0
$Li_2SO_4.H_2O$	0
$FeSO_4.7H_2O$	0
$CuSO_4.5H_2O$	0
$ZnSO_4.7H_2O$	0
KCl	0
NaCl	0

The enzyme (3 μg) was incubated with different metal ions (5×10^{-3} M, final concentration), and D-xylose isomerase activity was calculated as described in Chapter II.

With Co^{2+} and Mn^{2+}, 80% and 30% activity respectively, was obtained. No enzyme activity was observed in the absence of the metal ion. D-Glucose (xylose) isomerases from various microbial sources also require the presence of specific metal ion such as Co^{2+}, Mn^{2+}, Mg^{2+} or Cr^{2+} for their catalytic activity (10). These metal ions alone or in combination are required for the maximum enzyme activity.

Effect of various concentrations of Mg^{2+}

As shown in Table 21, the enzyme required Mg^{2+} for D-xylose isomerizing activity. Various concentrations of Mg^{2+} were used to perform enzyme assay. Maximum enzyme activity was obtained when Mg^{2+} concentration was 5×10^{-3} M. Beyond this limit Mg^{2+} inhibited the enzyme activity. This is in agreement with D-glucose (xylose) isomerase from Strepto. albus where Mg^{2+} and Co^{2+} higher than optimal concentration in the reaction mixture inhibited the enzyme activity (101).

Effect of other metal ions together with Mg^{2+}

Effect of other metal ions was studied by adding 8×10^{-3} M metal ion to be tested to 3 µg of the enzyme at pH 9.5, containing 5×10^{-3} M Mg^{2+}, and incubating at 35°C for 10 min. The reaction was started by the addition of xylose. Results are summarized in Table 22. In the presence of Co^{2+} (5×10^{-3} M), a three fold increase in the activity was observed. Metal ions

Table 21 : Effect of various concentrations of Mg^{2+}

$MgSO_4.7H_2O$ (M)	Relative activity (%)
0	0
2×10^{-3}	67
5×10^{-3}	100
1×10^{-2}	83
2.5×10^{-2}	26
1×10^{-1}	0

The enzyme (3 µg) was incubated with various concentrations of Mg^{2+} at 35°C for 10 min and then D-xylose isomerase activity was determined as described in Chapter II.

Table 22 : Effect of metal ions, EDTA, cysteine monohydrochloride and other compounds on D-xylose isomerase activity

Compound	Fold activity
None	1.0
$CoCl_2.6H_2O$	3.0
$MnCl_2.6H_2O$	1.0
$CaCl_2.2H_2O$	0.1
$FeSO_4.7H_2O$	0.34
$CuSO_4.5H_2O$	1.0
$HgCl_2$	0.2
$SnCl_2$	0.5
$ZnSO_4.7H_2O$	1.0
$BaCl_2$	1.0
KCl	0.8
Cysteine HCl	1.36
Urea	1.0
EDTA	1.0

The enzyme (3 µg) was incubated with 5×10^{-3} M $MgSO_4.7H_2O$ at pH 9.5 and additive 5×10^{-3} M at 35°C for 10 min, and the D-xylose isomerase activity was determined as described in Chapter II.

like Mn^{2+}, Ba^{2+}, Zn^{2+} and Cu^{2+} have no effect on enzyme activity. Metal ions like Sn^{2+} and Ca^{2+} inhibit the enzyme activity by 50% and 90%, respectively.

EDTA above 5 x 10^{-3}M concentration in the reaction mixture was inhibitory to the enzyme activity. At 4 x 10^{-2} M concentration 93% inhibition was observed. Similarly pCMB and 8-hydroxyquinoline in above 5 x 10^{-3} M concentration were inhibitory. Results are summarized in Table 23.

D-Glucose (xylose) isomerase from various microbial sources requires both Mg^{2+} and Co^{2+} for maximum enzyme activity e.g. L. brevis (100), A. missouriensis (94) and B. coagulans (99).

Effect of sugar alcohols on D-xylose isomerase activity

D-Glucose (xylose) isomerases are readily inhibited by sugar alcohols like sorbitol, mannitol and xylitol (19, 38, 90, 118, 119). To detect the effect of these sugar alcohols on D-xylose isomerase the enzyme (3 µg) was mixed with various concentrations of these alcohols at 35°C for 10 min. The reaction was started with the addition of D-xylose. Results are summarized in Table 24. Among these xylitol is the most potent inhibitor. At 10^{-2} M concentration only 10% of the original activity was shown by the enzyme. In the presence of 10^{-1} M D-sorbitol, mannitol or dulcitol

TABLE 23 : Effect of various concentrations of EDTA, 8-hydroxyquinoline and pCMB on D-xylose isomerase activity

Concentration (M)	Relative activity		
	EDTA	8-hydroxyquinoline	pCMB
Control	100	100	100
5×10^{-3}	100	100	100
1×10^{-2}	63	95	-
2×10^{-2}	49	63	-
4×10^{-2}	7	46	-

The enzyme (3 µg) was incubated with various concentrations of inhibitor, at 35°C for 10 min. Reaction was started by the addition of $MgSO_4.7H_2O$ (5×10^{-3} M) and D-xylose for 10 min.

Table 24 : Inhibition of D-xylose isomerase by sugar alcohols

Sugar alcohol concentration (M)	Relative activity %			
	Sorbitol	Mannitol	Dulcitol	Xylitol
Control	100	100	100	100
1×10^{-2}	58	100	80	10
2×10^{-2}	44	88	76	0
5×10^{-2}	30	69	65	0
1×10^{-1}	18	58	44	0

The enzyme (3 μg) was incubated with various concentrations of sugar alcohols at 35°C for 10 min and then reaction was proceeded by the addition of D-xylose as described in Chapter II.

in the reaction mixture 18%, 58% and 44% of the original activity of the enzyme was recovered respectively. Ki (inhibitor constant) for D-sorbitol is 9 mM (Fig. 24) while Ki for xylitol is 2.1 mM (Fig. 25). Ki for xylitol from D-glucose (xylose) isomerase from various sources varied from 1.5 - 7.0 mM while for D-sorbitol it was reported to be 29 mM (10).

Substrate specificity

The substrate specificity of the purified extracellular, D-xylose isomerase was studied. As shown in Table 25 ketose i.e. D-xylulose was produced only from D-xylose. The enzyme had no action of D-glucose, D-ribose, L-arabinose, L-rhamnose, D-mannose and D-galactose.

D-Glucose (xylose) isomerase is known to act on both D-glucose and D-xylose. The enzyme from A. missouriensis (94), B. stearothermophilus (102), L. brevis (100), Strepto. albus NRRL B-5778 (46), Strepto. bikiniensis (50) and Strepto. olivochromogenes (102) can isomerize D-ribose in addition to D-glucose and D-xylose. Enzyme from Strepto. albus NRRL B-5778 in addition to D-glucose, D-xylose and D-ribose also acts on L-arabinose, L-rhamnose and D-allose and is the least specific D-glucose (xylose) isomerase so far reported. Extracellular D-xylose isomerase from Chainia sp is the only reported enzyme which acts on D-xylose but not on D-glucose.

FIG. 24 : Inhibition of D-xylose isomerase activity by D-sorbitol.
Enzyme was incubated with 0 - 0.05 M concentration of D-sorbitol and 0.0025M(A) and 0.005 M (B) concentrations of D-xylose.

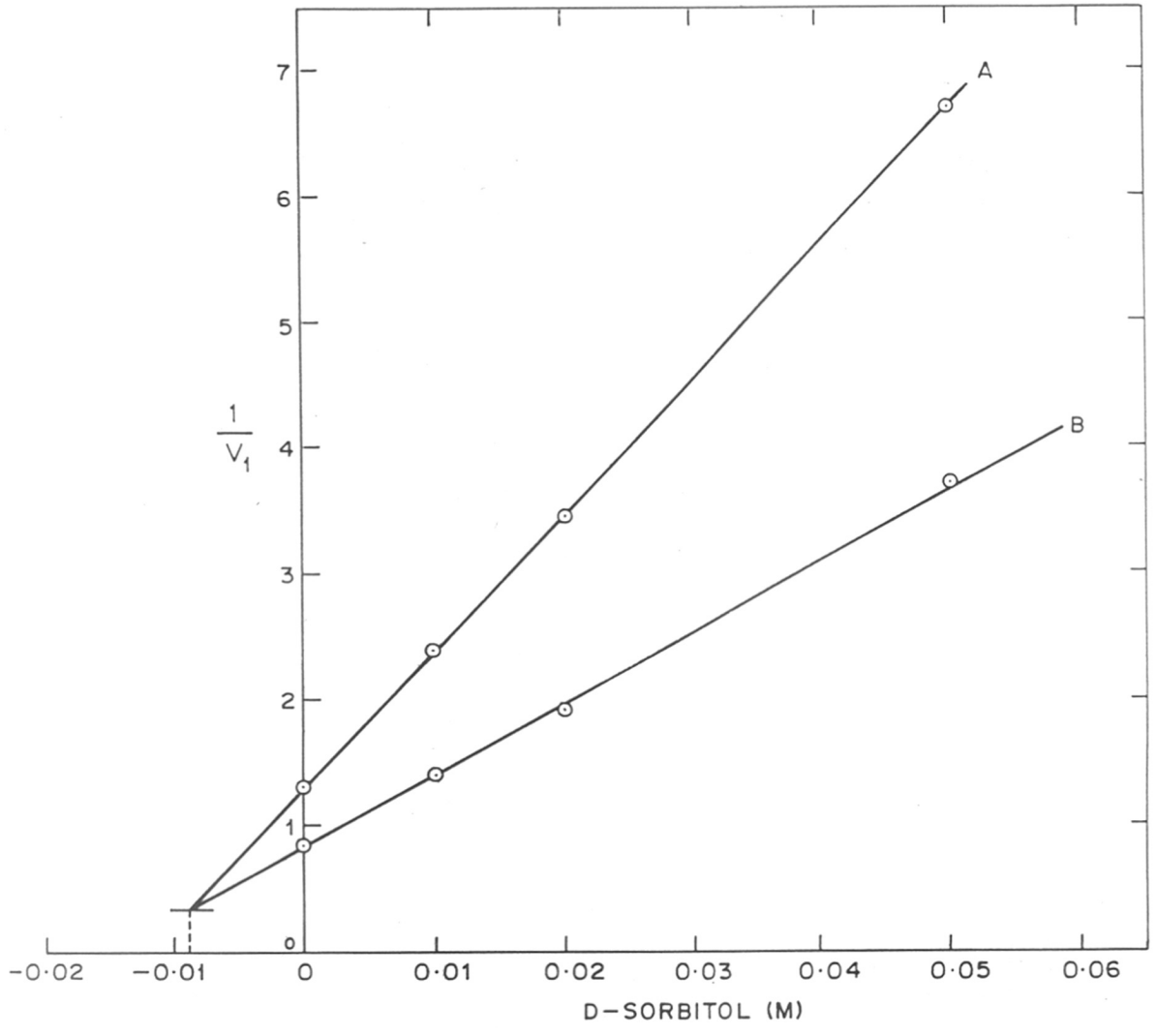


FIG. 24

FIG. 25 : Inhibition of D-xylose isomerase by xylitol.

Enzyme was incubated with xylitol (0 - 0.005 M) and 0.0025 M (A) and 0.005 M (B) concentrations of D-xylose.

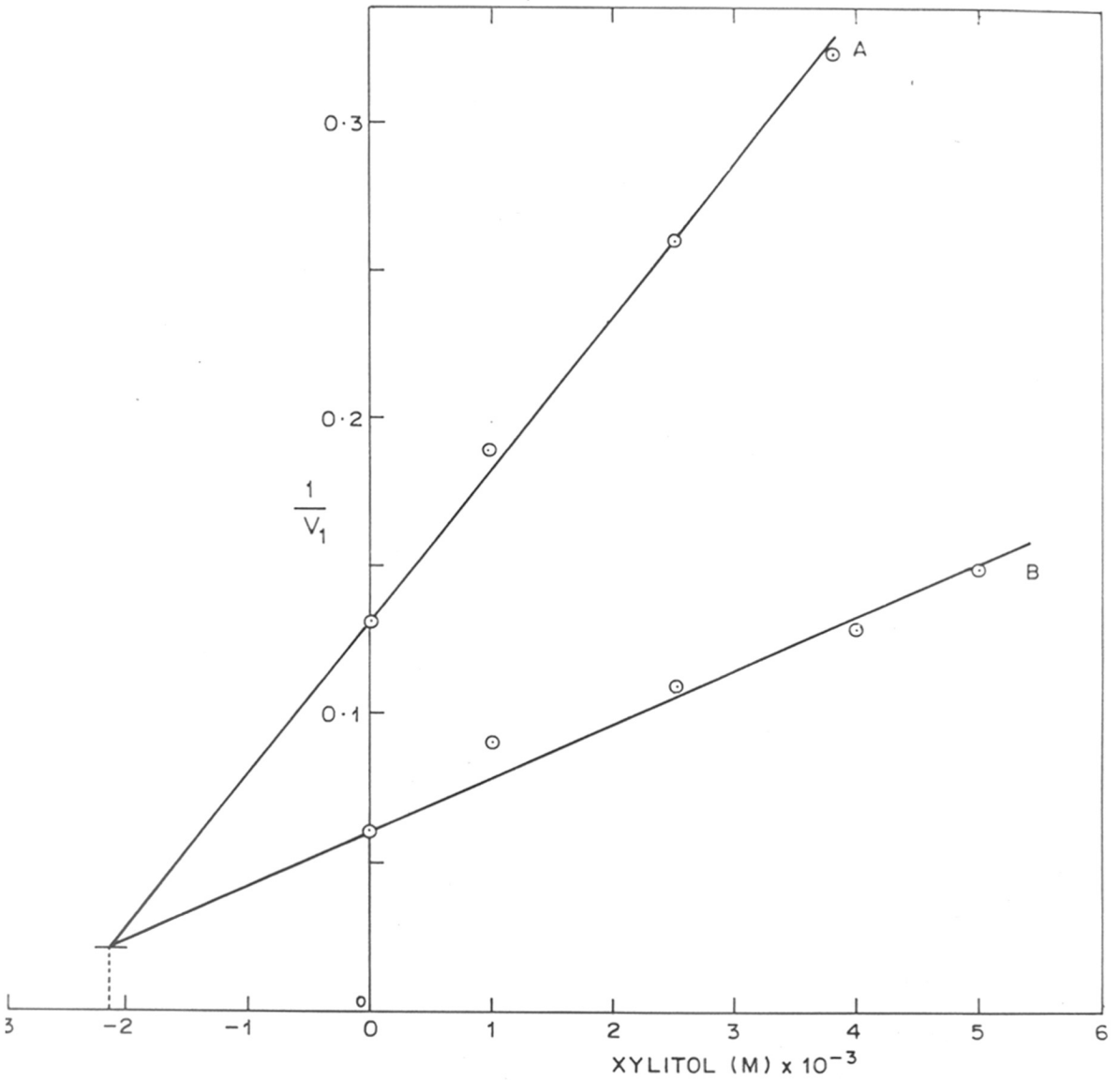


FIG. 25

Table 25 : Substrate specificity of D-xylose isomerase

Substrate (5×10^{-3} M)	Ketose formed (μg)
D-Xylose	18
L-Arabinose	0
D-Glucose	0
D-Ribose	0
L-Rhamnose	0
D-Mannose	0
D-Galactose	0

Enzyme (3 μg) was used for each assay, except for D-glucose (30 μg). Reaction mixture with pentose was incubated at 35°C for 10 min. Reaction mixture with hexose was incubated at 60°C for 1 h. Ketose in the resepctive mixture was determined by the cysteine-carbazole method.

Km and Vmax

Isomerization of D-xylose to D-xylulose with pure extracellular, specific D-xylose isomerase under standard assay conditions but at different substrate concentrations yielded a linear Lineweaver-Burk plot with a km value of 7×10^{-3} M at pH 9.5, 35°C (Fig. 26a, b). The Vmax calculated from Lineweaver-Burk plot, was 4.9 mg/min.

Km value for D-xylose for the D-glucose (xylose) isomerase from various microorganisms ranges from 5×10^{-3} M - 9.3×10^{-2} M while for D-glucose it ranges from 8.6×10^{-2} M - 9.2×10^{-1} M. Thus the apparent Km for D-xylose is lower than D-glucose, which reflects a higher affinity for D-xylose. Only in the case of D-glucose (xylose) isomerase from S. albus NRRL 5778 (46) Km for D-glucose (8.6×10^{-2} M) is lower than that for D-xylose (9.3×10^{-2} M).

In the present work on pure extracellular, D-xylose isomerase from Chainia sp, as it is specific only for D-xylose, Km for D-glucose cannot be determined. Its Km value of 7×10^{-3} M for D-xylose is comparable with some of the non-specific D-glucose (xylose) isomerases showing higher affinity towards D-xylose.

Time course and equilibrium concentration

The apparent equilibrium concentration of the reaction was determined by incubating D-xylose isomerase. The amount of D-xylulose formed was determined in the

FIG. 26a,b: Determination of Km of D-xylose isomerase acting on D-xylose under standard conditions (pH 9.5, 35°C)

(a) Lineweaver and Burk plot

(b) Eadie and Hofstee

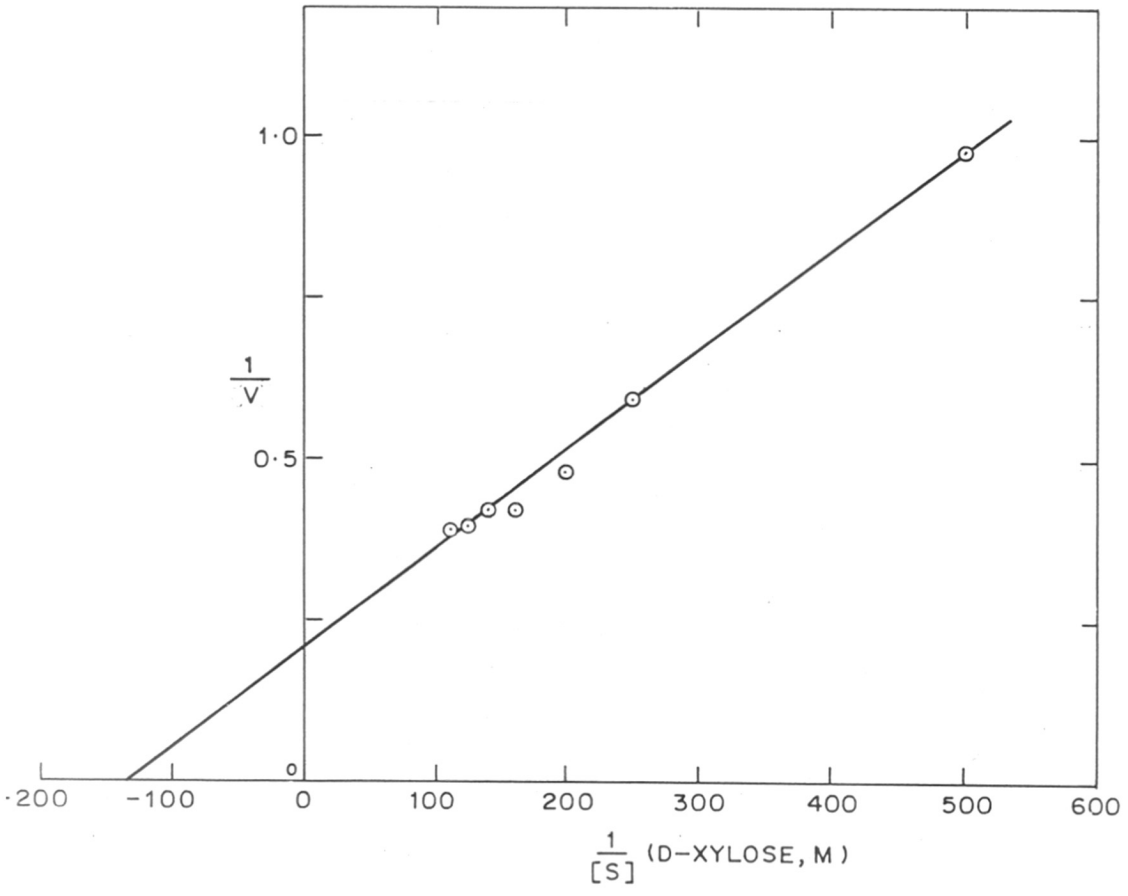


FIG. 26a

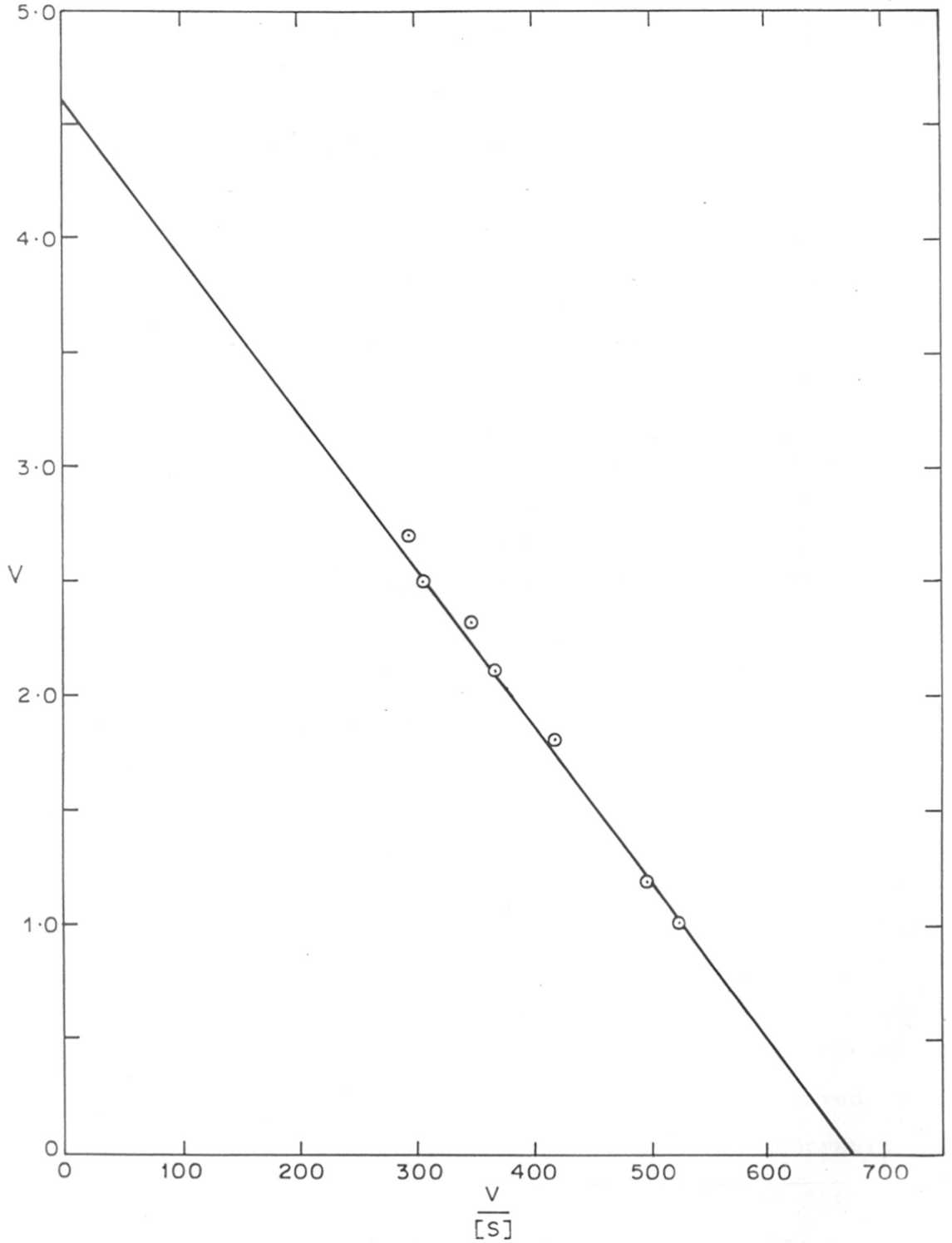


FIG. 26b

reaction mixtures which were incubated at 35°C and at 60°C for different time intervals. Results are shown in Table 26. At 35°C equilibrium of 89.5 µg D-xylose:10.5 µg D-xylulose was attained in 30 min while at 60°C the equilibrium was shifted to 74 µg D-xylose. 26 µg D-Xylulose in 15 min. Thus it was observed that D-xylose: D-xylulose equilibrium depends on temperature. At higher temperature equilibrium shifted towards the formation of D-xylulose.

Similar observations were reported in the case of D-glucose:D-fructose equilibrium (127, 128). On the other hand in the case of D-glucose (xylose) isomerase from B. coagulans HN-68 (99), at higher temperature (70°C) there was no shift in equilibrium constant for the isomerization of D-glucose to D-fructose. Only slight increase in the initial reaction rate was observed. The equilibrium ratio for D-xylose:D-xylulose was shown to be 84-86% D-Xylose: 14-16% D-xylulose (74, 129, 130).

Effect of borate on equilibrium concentration

Effect of borate on equilibrium concentration of D-xylose:D-xylulose enzyme reaction was observed by performing enzyme reaction with different concentrations of borate ions at 35°C (Table 27). It was observed that addition of borate shifted the equilibrium from 79:21 to 20:80 in favour of D-xylulose formation.

Table 26 : Equilibrium concentration of D-xylulose at different temperature and time.

Temperature (°C)	D-Xylulose concentration in µg			
	10'	15'	30'	60'
35	10.5	15	21	21
60	22	26	26	26

D-Xylose (100 µg) was incubated at indicated temperatures for different time with 3 µg of pure enzyme and assayed for D-xylose isomerase enzyme activity as described in Chapter II.

Table 27 : Effect of borate on equilibrium concentration of D-xylulose

D-Xylose µg	:	Borate µg	Equilibrium concentration D-Xylose µg	:	D-Xylulose µg
100	:	0	79	:	21
100	:	20	58	:	43
100	:	50	51	:	49
100	:	100	33	:	67
100	:	120	27	:	73
100	:	150	23	:	77
100	:	200	20	:	80
100	:	250	20	:	80

D-Xylose (100 µg) was incubated at 35°C with 3 µg of enzyme with different concentrations of borate. Amount of D-xylulose formed was determined by cysteine-carbazole method.

Similar observations were noted in the case of preparation from Ps. hydrophila (131) and from Lac. pentosus (130). Borate appears to exert its effect through preferential and stronger binding to D-xylulose, thereby affecting reconversion to D-xylose.

Effect of active site group specific reagents on the extracellular, specific D-xylose isomerase

Effect of various active site group specific reagents on D-xylose isomerase activity was studied. Sample of enzyme (50 µg) was incubated at indicated reagent concentration and time interval (Table 28, 29). Aliquots (0.1 ml) from reaction mixture along with enzyme and inhibitor blanks were taken separately and assayed for enzyme activity. Controls were also run under similar conditions in the presence and absence of modifying reagent.

Inactivation of D-xylose isomerase by arginine modifying reagents

α-Dicarbonyl reagents like phenylglyoxal and 2,3-butanedione are known to react with arginine residues in proteins (177 - 181). Phenylglyoxal reacts with the guanidino group of arginine residue (182). The reaction also occurs with ε-amino group of lysine residue when proteins are treated with high concentrations of phenylglyoxal for a long period. The reaction is

carried out in dark to avoid possible photochemical effects.

In the present study D-xylose isomerase was completely inactivated by phenylglyoxal at 10 mM concentration of the reagent. The activity of the enzyme decreased following a pseudo first order kinetics (Fig. 27). Kinetic analysis by the method of Levy et al (183) with a plot of log K (pseudo-first order rate constant) versus log (Phenylglyoxal) resulted in a straight line with a slope of 0.90 (Fig. 27, inset) indicating that one mole of phenylglyoxal per mole of enzyme is required to produce inactivation.

The enzyme was completely inactivated by 2,3-butanedione at 50 mM concentration which confirms the presence of arginine in the active site.

Inactivation of D-xylose isomerase by diethyl pyrocarbonate

Diethyl pyrocarbonate is a specific reagent for histidine residue in the pH range of 6.0 - 7.5 and has been used widely for the modification of histidine residue in various proteins (184 - 187). Treatment of D-xylose isomerase with diethyl pyrocarbonate (5 mM) in 0.02 M potassium phosphate buffer, pH 7.5 resulted in a rapid and complete inactivation of the enzyme (Fig. 28), suggesting a role of histidine in the active site of the enzyme. The rate of inactivation of the enzyme was a function of reagent concentration although

FIG. 27 ; Kinetics of the inactivation of D-xylose isomerase by phenylglyoxal.

The enzyme (50 µg/ml) was incubated with various concentrations of phenylglyoxal in 0.02 M carbonate-bicarbonate buffer, pH 9.0 at 28°C. Control was incubated under identical conditions. Aliquots were removed at indicated time intervals for measurement of enzyme activity. The phenylglyoxal concentrations were:

0.02 mM (A), 0.05 mM (B) and 1 mM (C)

Inset : plot of the log of pseudo-first order rate constant (k) of phenylglyoxal inactivation reaction versus log phenylglyoxal concentration.

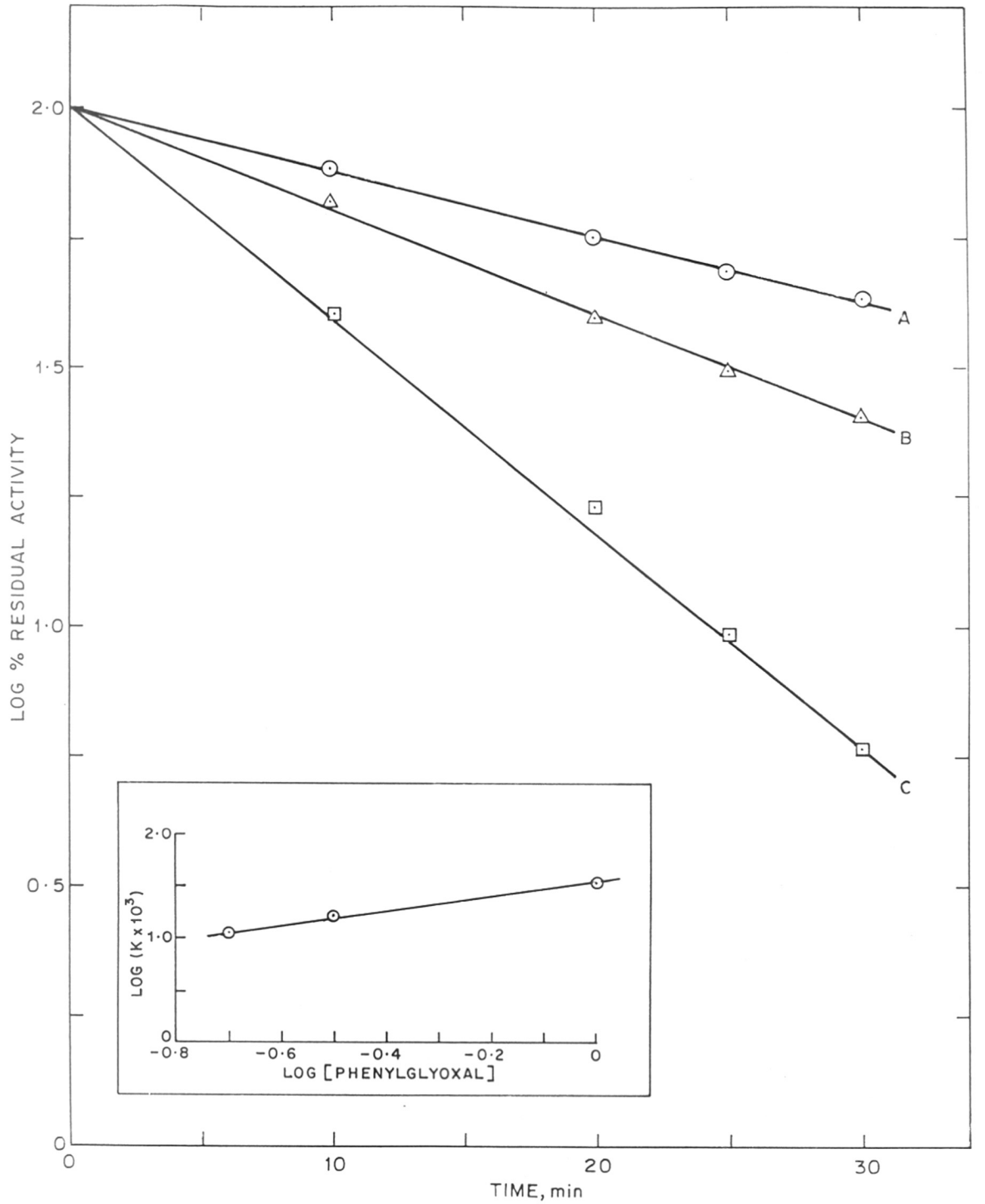

2·0
1·5
1·0
0·5
0
LOG % RESIDUAL ACTIVITY
0
10
20
30
TIME, min
A
B
C
2·0
1·0
0
LOG (K x 10³)
-0·8
-0·6
-0·4
-0·2
0
LOG [PHENYLGLYOXAL]

FIG. 27

FIG. 28 : Kinetics of the inactivation of D-xylose isomerase by diethylpyrocarbonate. The enzyme (50 µg/ml) was incubated with various concentrations of diethylpyrocarbonate in 0.02 M potassium phosphate buffer, pH 7.5 at 28°C. Control was incubated under identical conditions. Aliquots were removed at indicated time intervals for measurement of enzyme activity. The diethylpyrocarbonate concentrations were:
1.0 mM (A), 2.0 mM (B) and 5.0 mM (C)
Inset: plot of the log of pseudo-first order rate constant (K) of diethylpyrocarbonate inactivation reaction versus log of diethylpyrocarbonate concentration.

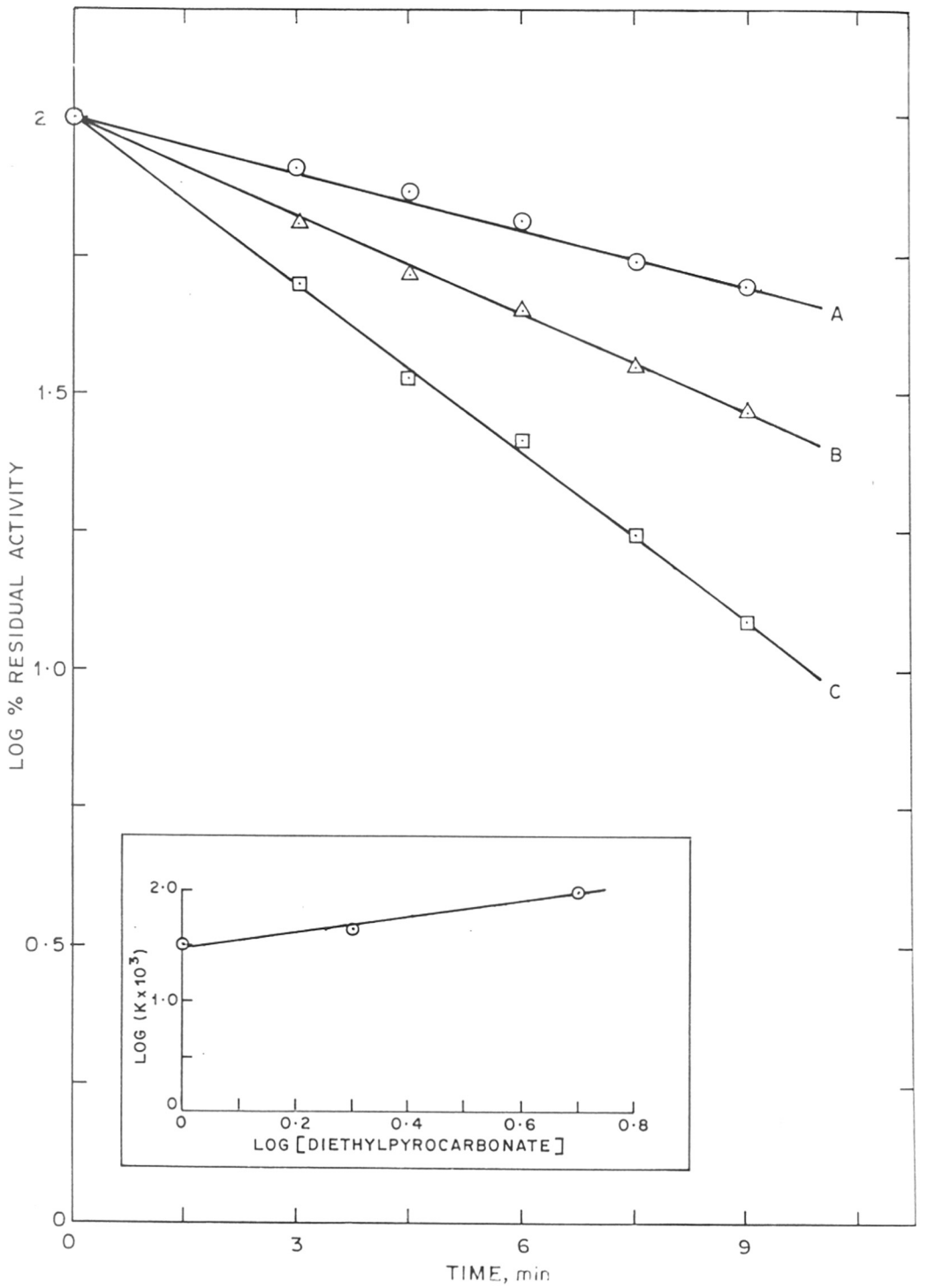

LOG % RESIDUAL ACTIVITY
2
1·5
1·0
0·5
0
0
3
6
9
TIME, min
A
B
C
LOG (K x 10³)
2·0
1·0
0
0·2
0·4
0·6
0·8
LOG [DIETHYLPYROCARBONATE]

FIG. 28

although at any particular concentration of the reagent, the reaction followed a pseudo first order kinetics.

Table 28 : Effect of active site group specific reagents

Group specific reagent	Concentration (mM)	Incubation time (min)	Activity %
Cysteine			
(a) Control	-	-	100
(b) Chloromercuribenzoate	0.05	30	
(c) Iodoacetate	1.0	30	100
Aspartic/Glutamic acid			
(a) Control	-	-	100
(b) EDAC (1-Ethyl-3-(3-Dimethyl aminopropyl carbodiimide HCl)	10	30	100
Arginine			
(a) Control	-	-	100
(b) Phenylglyoxal	10	30	0
(c) 2,3-Butanedione	50	30	0
Histidine			
(a) Control	-	-	100
(b) Diethypyrocarbonate	5	10	0

Table 28 contd.

Group specific reagent	Concentration (mM)	Incubation time (min)	Activity %
Serine			
(a) Control	-	-	100
(b) Phenyl methane sulfonyl fluoride (PMSF)	2	30	100
Tyrosine			
(a) Control	-	-	100
(b) N-Acetylimidazole	10	30	100
Lysine			
(a) Control	-	-	100
(b) Trinitrobenzene sulphonic acid	1	120	100
Tryptophan			
(a) Control	-	-	100
(b) N-Bromosuccinimide (NβS)		30	100

Table 29 : Amino acid modifying reagent

Amino acid	Modifying reagent	Type of reaction	Condition of reaction
Cysteine	i) p-Chloromercuribenzoate	Merceptide formation	pH 7.0 sodium phosphate
	ii) Iodoacetate	Carboxyamidomethylation	pH 9.0 carbonate bicarbonate
Aspartic/ Glutamic acid	EDAC (1-Ethyl-3-(3-Dimethyl aminopropyl carbodiimide HCl)	N-acylurea formation	pH 5.0 sodium phosphate
Arginine	i) phenylglyoxal		pH 9.5 carbonate-
	ii) 2,3-Butanedione		bicarbonate buffer
Histidine	i) Diethylpyrocarbonate	Carbethoxylation	pH 7.5 potassium phosphate
Serine	i) Phenylmethane sulfonyl fluoride	Sulfonylation	pH 9.5 carbonate bicarbonate
Tyrosine	i) N-Acetylimidazole	Acylation	pH 7.5 sodium phosphate
Lysine	i) 2,4,6-trinitrobenzene sulphonic acid (TNBS)	Displacement of sulfite from TNBS	pH 9.5 carbonate bicarbonate
Tryptophan	i) N-Bromosuccinimide	Oxidation	pH 5.0 Acetate buffer

RESULTS AND DISCUSSION

D-Glucose (xylose) isomerase, an industrially important enzyme has been reported from a large number of microorganisms. It is used in the production of High Fructose Corn Syrup (HFCS) and manufacture of liquid fuels. In most of the cases, the enzyme is intracellular.

In the present work isolation, purification as well as physico-chemical and enzymatic properties of a purified extracellular, specific D-xylose isomerase from a Chainia sp. are presented. Isolation and methods of preservation of Chainia sp. are described. Some of the cultural and biochemical characteristics of Chainia on standard media have also been studied. Results of these studies are summarized and discussed below.

1. Organism and culture characteristics

Six strains of Chainia were isolated from the soil and screened for D-glucose (xylose) isomerase activity. The strain NCL 82-5-1 was selected because it showed a unique property of secreting extracellular D-glucose and D-xylose isomerases in addition to conventional intracellular D-glucose (xylose) isomerase. Reports on extracellular secretion of the enzyme are very rare (58). Recently Mikhailov et al. (79) have reported extracellular and intracellular D-glucose (xylose) isomerase from Streptomyces olivaceous strain 13 with identical physico-chemical properties. However, it is not clear whether

the two enzymes are distinct or whether the extracellular enzyme is the result of leakage of intracellular enzyme.

The Chainia sp. produces lobed, multilocular, cerebriform sclerotia measuring 145 x 120 μ. It is a typical characteristic of the genus. Growth characteristics of the culture on various media indicated that numerous sclerotia are formed only in the media which support good growth. Aerial mycelium was more predominent at the tip of the medium in the slant after 15 days of incubation at 28°C. Organism is mesophilic, grows well and produces abundant sclerotia at 28°C. Most of the D-glucose (xylose) isomerase producing organisms are mesophilic although a few thermophilic organisms like B. stearothermophilus (30) are reported in the literature. Among the preservation methods used, lyophilization and sterile distilled water methods were found to be the best because revival of these cultures after three years showed same extracellular D-xylose isomerase activity (210 u/lit) and sclerotia formation remained unaffected. Routine repeated subculture showed loss of sclerotia formation, which is a typical morphological feature of the organism, and decreased extracellular D-xylose isomerase activity (140 u/lit). Loss of sclerotia formation is reported as a result of dual phenomenon as described by Thirumalachar (154).

Validity of the genus Chainia has been questioned from time to time. According to Goodfellow et al. (188) although Chainia shows the transient ability to produce sclerotia, they show a few similarities with genus Streptomyces such as their DNA is rich in guanine plus cytosine (189 - 191), contain streptomycete-like fatty acids (190), share a high DNA homology with Streptomyces (191 - 193), susceptible to polyvalent streptomycete phages (192, 194 - 196), menaquinones (197, 198) and polar lipids (199), cluster with Streptomyces species in numerical phenetic surveys and have a cell wall chemotype I (200).

2. Isolation and separation of extracellular specific D-xylose isomerase from D-glucose (xylose) isomerase

Uptill now, the enzymic isomerization reaction of D-glucose and D-xylose was considered to be catalyzed by a single enzyme. During the characterization of extracellular enzyme complex from Chainia it was observed that, there were two distinct components with specific activities towards D-xylose and D-glucose. D-Xylose isomerase was completely adsorbed on DEAE-cellulose

at 0.01 M carbonate-bicarbonate buffer, pH 9.5 while D-glucose isomerase was completely adsorbed on CM-cellulose at 0.01 M sodium phosphate, pH 6.0 (165). These differences in adsorption behaviour on DEAE-cellulose and CM-cellulose chromatography suggested that the extracellular D-xylose isomerase and D-glucose isomerase are distinct enzymes. Later on it was observed that D-xylose isomerase gets completely adsorbed on DEAE-cellulose at 0.01 M sodium phosphate buffer, pH 7.5. This is the pH at which the enzyme is most stable and practically no loss in enzyme activity incurred in this step of purification.

Danno (99) in earlier studies made unsuccessful attempts to separate D-glucose isomerase, D-xylose isomerase and D-ribose isomerase activities from B. coagulans HN-68. He concluded that these activities cannot be separated from each other indicating a wide substrate specificity exhibited by a single enzyme. Although extracellular D-glucose and D-xylose isomerase from Chainia sp. were separated from each other, it was observed that the conventional intracellular D-glucose (xylose) isomerase from the same organism cannot be separated.

3. Purification

The extracellular, specific D-xylose isomerase was purified to homogeneity by following steps:

(1) Amicon filtration (UM-10 membrane), (2) Ammonium sulfate precipitation (0 - 90%), (3) heat denaturation of other proteins (60°C, 30 min), (4) preparative polyacrylamide gel electrophoresis (pH 8.9), (5) ion exchange chromatography (pH 7.5) on DEAE-cellulose and (6) gel filtration (Sephadex G-200). In the literature preparative PAGE has not yet been used for the purification of the D-xylose isomerase. During the present work large scale PAGE was carried out to purify the D-xylose isomerase after heat denaturation of other proteins from the ammonium sulphate precipitated enzyme preparation. A quick *in situ* location of the enzyme on gel facilitated purification of the enzyme by PAGE. By this procedure fairly purified enzyme preparation was obtained from which polyacrylamide impurities were removed by subsequent purification steps.

4. Physico-chemical properties

4.1 Molecular weight. An average molecular weight of pure D-xylose isomerase by gel filtration on Bio-Gel P-150, Sephadex G-200 and by slope method is 71,000 Most of the D-glucose (xylose) isomerases reported in the literature have molecular weight higher than 100,000 (54, 100 - 102). Only in *A. missouriensis* (80,000) (94) and in *Strepto. bikiniensis* (52,000) (50)

low molecular weight D-glucose (xylose) isomerases are reported.

4.2 Subunit structure. Pure extracellular D-xylose isomerase when treated with 2% SDS and 1% 2-mercapto-ethanol has shown a single band on SDS-gel electrophoresis with a molecular weight of 71,200 which corresponds with that of the native enzyme suggesting that the enzyme consists of a single polypeptide chain. Most of the D-glucose (xylose) isomerases reported in the literature contained either 2 subunits (94, 101, 102) or 4 subunits (38, 54, 99, 100).

4.3 Isoelectric point (pI). Isoelectric point of D-xylose isomerase is 3.55. pI for Bacillus enzyme is 4.9 (10). Although D-glucose (xylose) isomerase is purified from many microorganisms, pI of the enzyme in most of the cases is not known.

4.4 Glycoprotein nature. D-Xylose isomerase does not contain carbohydrate moiety attached to the enzyme. D-Glucose (xylose) isomerases reported in the literature are also devoid of carbohydrate moiety.

4.5 Amino acid composition. D-Xylose isomerase from Chainia sp. contains higher amount of glutamic acid and glycine, 11.9 and 14.9% on molar % basis respectively as compared to other constituent amino acids. D-Glucose (xylose) isomerases from other microbial sources contain

7.4 - 13.7% glycine. Most of the D-glucose (xylose) isomerases from microbial sources do not contain half-cystine, except Strepto. albus (101). D-Xylose isomerase of Chainia is also devoid of half-cystine residue. Molar % of methionine, leucine, tyrosine, and phenylalanine are slightly lower than D-glucose(xylose) isomerases from other microorganisms. The enzyme consists of 536 amino acid residues.

5. Enzymic properties

5.1 pH stability. D-Xylose isomerase from Chainia sp. is stable in the pH range of 7.5 - 8.0 when heated at 60°C for 10 min in 0.05 M sodium phosphate buffer. The purified enzyme preparations of D-glucose (xylose) isomerase from E. intermedia (97) and Strepto. flavogriseus (54) are stable in the pH range of 7.0 - 9.0 and 5.0 - 9.0, respectively. Wide range of pH stability (4.0 - 11.0) is reported in the case of D-glucose (xylose) isomerase from Strepto. albus YT-5 (101).

5.2 Heat stability. Pure D-xylose isomerase is stable at 70°C for 10 min in the absence of metal ions. In the presence of Mg^{2+} (1×10^{-3} M) the enzyme retained 50% of its activity when heated at 80°C for 10 min whereas in the presence of Co^{2+} (1×10^{-3} M) under similar conditions no loss in activity was observed. Heat stability of the enzyme is very important from

commercial point of view as most of the immobilized D-glucose (xylose) isomerase columns are operated at 60° - 70°C to avoid contamination. Thus although L. brevis enzyme has low optimum pH of 6.5, its use in industry is limited due to its instability above 40°C. D-Xylose isomerase from Chainia sp. can be immobilized for the production of D-xylulose as it is stable at higher temperatures.

5.3 Optimum pH. Optimum pH of the D-xylose isomerase from Chainia sp. is 9.5. It is more towards alkaline side as compared to other D-glucose (xylose) isomerases from other microorganisms. Only in the case of Strepto. phaeochromogenes it is reported to be 9.5 (82).

5.4 Optimum temperature. Optimum temperature of D-xylose isomerase is 60°C. Most of the D-glucose (xylose) isomerases reported in the literature have fairly high optimum temperatures around 80°C (Table 7).

5.5 Cation requirement. D-Xylose isomerase requires Mg^{2+} (5×10^{-3} M, final concentration) for its activity as well as for stability. Enzyme activity is increased 300 times when in addition to Mg^{2+} in the reaction mixture, Co^{2+} (5×10^{-3} M, final concentration) is added. All the D-glucose (xylose) isomerases in the literature require metal ions for its maximum activity. D-Xylose isomerase from Chainia sp. has no action

on D-glucose hence it cannnot be used for syrup manufacture. It can be used only for isomerization of D-xylose to D-xylulose, which can be fermented to ethanol by yeasts.

5.6 Enzyme inhibitors. D-Xylose isomerase from Chainia has been found to be inhibited by Ca^{2+} and Hg^{2+}. Since the enzyme from Chainia is devoid of thiol groups the inhibition by Hg^{2+} can be explained on the basis of the structural changes caused by the heavy metal ions. D-Xylose isomerase from Chainia sp. has also been found to be inhibited by sugar alcohols like sorbitol (inhibitor constant, Ki 9 mM) and xylitol (2.1 mM). Low Ki for xylitol in the case of D-xylose isomerase from Chainia sp. and other D-glucose (xylose) isomerases reported in the literature indicate high affinity of this inhibitor for the enzyme.

5.7 Substrate specificity. D-Xylose isomerase from Chainia sp. acts only on D-xylose. This is a unique property of the enzyme. Most of the D-glucose (xylose) isomerases reported in the literature act on D-glucose as well as D-xylose and thus the enzyme is designated as D-glucose (xylose) isomerase. In some organisms it can isomerize D-ribose to D-ribulose (19, 46, 50, 90, 100, 102). Isomerization of galactose has also been reported in the case of D-glucose (xylose) isomerase

A. missouriensis (94). Broad specific D-glucose (xylose) isomerase has been reported from S. albus NRRL B-5778 (46) which in addition to D-glucose also acts on D-ribose, L-arabinose, L-rhamnose and D-allose.

5.8 Km and Vmax. Km and Vmax values for extracellular D-xylose isomerase are 7 x 10^{-3} M and 4.9 mg/min at pH 9.5, 35°C, respectively. Since the enzyme is specific for D-xylose Km for D-glucose cannot be determined. Extracellular ammonium sulfate precipitated and dialysed enzyme preparation showed both D-glucose and D-xylose isomerase activities. Km for D-glucose was 2.44 x 10^{-1} M (pH 9.5, 70°C). Thus extracellular D-glucose (xylose) isomerase showed significantly lower Km of 7 x 10^{-3} M with D-xylose as compared to D-glucose. Km for most of the D-glucose (xylose) isomerases for substrates such as D-glucose, D-xylose and D-ribose vary significantly. Km values has been reported to be 8.6 x 10^{-2} - 9.2 x 10^{-1} M for D-glucose, 5 x 10^{-3} - 9.3 x 10^{-2} M for D-xylose and 3.5 x 10^{-1} - 6.7 x 10^{-1} M for D-ribose (Table 8). Km for D-xylose is lower as compared with its other substrates suggesting its stronger affinity with the enzyme. Only the enzyme from Strepto. albus NRRL B-5778 (46) exhibits marginally more affinity for D-glucose than D-xylose (km for D-glucose 8.6 x 10^{-2} M while for D-xylose 9.3 x 10^{-2} M). Similarly in the case of D-glucose (xylose) isomerase

from A. missouriensis (94), the enzyme has higher affinity for D-glucose. Such organisms are very useful in the production of HFCS.

5.9 Equilibrium between D-xylose and D-xylulose.

The apparent equilibrium concentrations of D-xylose and D-xylulose in the reaction catalysed by D-xylose isomerase from Chainia sp. varies with temperature (Table 26). At 35°C equilibrium of 89.5 D-xylose:10.5 D-xylulose was attained while at 60°C the equilibrium was shifted to 74 D-xylose:26 D-xylulose. It has been observed that addition of borate shifted the equilibrium from 79:21 to 20:80 in favour of D-xylulose. Thus, D-xylose isomerase showed a similar trend that have been reported for D-glucose (xylose) isomerase from other microorganisms (127, 128, 130, 131).

5.10 Effect of active site groups specific reagents

Effect of various active site group specific reagents on D-xylose isomerase activity was studied. It was observed that arginine and histidine are involved in the active site of the enzyme. Chemical modification studies for the determination of active site are not reported in literature in the case of conventional intracellular D-glucose (xylose) isomerases from various microorganisms.

The occurrence of extracellular specific D-glucose and D-xylose isomerases in Chainia sp. with the conventional intracellular non-specific D-glucose (xylose) isomerase poses many interesting problems both at protein and genetic levels. At protein level it can be studied whether the specific D-glucose and D-xylose isomerases arise due to the post-translational modifications of intracellular non-specific D-glucose (xylose) isomerase. However, this will be possible only if the intracellular enzyme has two distinct binding sites for glucose and xylose. At DNA level it would be interesting to study whether the same gene codes for the intracellular glucose (xylose) isomerase and extracellular specific glucose and xylose isomerases.

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