XYLITOL PRODUCTION FROM D-XYLOSE BY FACULTATIVE ANAEROBIC BACTERIA

by

Sendil Rangaswamy

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

Biological Systems Engineering

_____

Dr. Foster A. Agblevor, Chair

_____ _____

Dr. Jiann-Shin Chen **Dr. Brenda Winkel-Shirley**

_____ _____

Dr. Richard Helm **Dr. Gene Haugh**

Dr. John V. Perumpral, Department Head

January 27, 2003

Blacksburg, Virginia

Keywords: xylitol, D-xylose, *Corynebacterium* sp., xylose reductase, *Candida tropicalis*

XYLITOL PRODUCTION FROM D-XYLOSE BY FACULTATIVE ANAEROBIC BACTERIA

by
Sendil Rangaswamy
Committee Chairman: Foster Agblevor
Biological Systems Engineering

ABSTRACT

Seventeen species of facultative anaerobic bacteria belonging to three genera (*Serratia, Cellulomonas*, *and Corynebacterium*) were screened for the production of xylitol; a sugar alcohol used as a sweetener in the pharmaceutical and food industries. A chromogenic assay of both solid and liquid cultures showed that 10 of the 17 species screened could grow on D-xylose and produce detectable quantities of xylitol during 24-96 h of fermentation. The ten bacterial species were studied for the effect of environmental factors, such as temperature, concentration of D-xylose, and aeration, on xylitol production. Under most conditions, *Corynebacterium* sp. NRRL B 4247 produced the highest amount of xylitol. The xylitol produced by *Corynebacterium* sp. NRRL B 4247 was confirmed by mass spectrometry.

Corynebacterium sp. NRRL B 4247 was studied for the effect of initial D-xylose concentration, glucose, glyceraldehyde, and gluconate, aeration, and growth medium. *Corynebacterium* sp. NRRL B 4247 produced xylitol only in the presence of xylose, and did not produce xylitol when gluconate or glucose was the substrate. The highest yield of xylitol produced in 24 h (0.57 g/g xylose) was using an initial D-xylose concentration of 75 g/l. Under aerobic conditions the highest xylitol yield was 0.55 g/g while under anaerobic conditions the highest yield was 0.2 g/g. Glyceraldehyde in concentrations greater than 1 g/l inhibited *Corynebacterium* sp. B 4247 growth and xylitol production. *Corynebacterium* sp. NRRL B 4247 culture grown in the presence of potassium gluconate (96 g/l) for 48 h and on addition of D-xylose to the media increased accumulation to 10.1 g/l of xylitol after 150 h.

Corynebacterium sp. NRRL B 4247 exhibited both NADH and NADPH-dependent xylose reductase activity in cell-free extracts. The NADPH-dependent activity was substrate dependent. The activity was 2.2-fold higher when DL-glyceraldehyde was used as substrate than with D-xylose. In cell-free extracts the difference in xylose reductase and xylitol dehydrogenase activity was highest at 24 h, whereas for cell cultures that were grown in gluconate and xylose, the difference in the reductase and dehydrogenase activities was highest at 12 h after xylose addition. The NAD^+ dependent xylitol dehydrogenase activity was low compared to the cells grown without gluconate.

The molecular weight of NADPH-dependent xylose reductase protein obtained by gel filtration chromatography was 58 kDa. Initial purification was performed on a DE-52 anion exchange column. Purification using Red Sepharose affinity column resulted in a 58 kDa protein on the SDS PAGE gel and was further purified on a Mono-Q column. The activity stained band on the native gel yielded 58, 49, 39 and 30 kDa bands on the denaturing gel.

The peptides of the 58 kDa protein of *Corynebacterium* sp. B 4247 sequenced by mass spectrometry, identified with E2 and E3 (*Bacillus subtilis*) components of multi-enzyme system consisting of pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex and oxo-acid dehydrogenase complex. A 75% match was shown by the peptide "Q**MSSLVT**R" with E-value of 8e-04 to the keto-aldose reductase Gre3p (*Saccharomyces cerevisiae*) that is induced by osmotic stress and is similar to xylose reductases from other fungi. The peptide "LLNDPQLILMEA**"** had conserved match "LL + DP" over several aldose reductases.

The xylose reductase of the yeast *Candida tropicalis* ATCC 96745 was also purified. The molecular weight of the yeast NADPH-dependent xylose reductase by SDS PAGE was about 37 kDa.

ACKNOWLEDGEMENTS

I thank my major advisor, Dr. Foster A. Agblevor for his advice and guidance. I thank him for the opportunity provided and the facilities provided for this research. In addition I would like to thank him for the valuable hours spent in correcting my writing and for timely help at critical moments. I thank Dr. Jiann-Shin Chen for his suggestions and guidance. I thank Dr. Brenda Winkel-Shirley for the valuable suggestions and inspiration. I thank Dr. Richard Helm and for his time and advice. I thank Dr. Gene Haugh for his guidance and mentorship.

I am sincerely grateful to Dr. Dennis Dean (Fralin Biotechnology Center), Dr. Sriranganathan (CMMID), Dr. Peter Kennelly (Biochemistry Dept.), Dr. Chenming Zhang (Biological Systems Engineering Dept.) and Dr. Kumar Mallikarjunan (Biological Systems Engineering Dept.) for permitting me to use their laboratories and instruments. I am grateful to Harriet Williams, Kim Harich, Dr. Kay Carlson (VMRC), Valerie Cash, Dr. Brian Lower, Ben Potters, Dr. Murat Kasap and Dr. Adnan Ismaiel for their timely assistance and suggestions.

I thank Dr. John Perumpral, Head of the Department, Dr. Saied Mostaghimi, chair graduate studies and Linda Altizer, Joyce Longrie, Terassa Cox, Diane, Dana Partin, Leon Alley, Julie Jordan, Jack Davis, Aubrey Murden of Biological Systems Engineering Department for their time and assistance. I sincerely thank the Biological Systems Engineering Department and Hatch and ASPIRES programs for providing the research funding. Most of all, I thank my parents and God for making it possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ALDH	Aldehyde dehydrogenase
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
Bis	N,N'-methylene-bis-acrylamide
BLAST	Basic local alignment search tool
bp	base pair
BSA	Bovine serum albumin
Da	dalton
DEAE	Diethyl amino ethyl
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethhyl methane sulfonate
EST	Expressed sequence tags
FPLC	Fast performance liquid chromatography
HPLC	High performance liquid chromatography
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
kbp	kilobase pair
kDa	kilodalton
Mb	megabases
Mr	Relative molecular mass
MS	Mass spectrometry
MWCO	Molecular weight cut off
NAD	Nicotineamide adenine dinucleotide
NADH	reduced nicotineamide adenine dinucleotide form
$NADP^+$	Nicotineamide adenine dinucleotide phosphate
NADPH	reduced nicotineamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnological Information
PCR	Polymerase Chain Reaction
PMS	Phenazine methosulfate
pI	Isoelectric point
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SDH	Sorbitol Dehydrogenase
Tris	Tris(hydroxymethyl) aminomethane
UV	Ultraviolet
XR	Xylose reductase
XDH	Xylitol dehydrogenase

LIST OF CULTURES

Bacterial Strains

Cellulomonas cellulans ATCC 12830

Cellulomonas cellulans NRRL B-2381

Cellulomonas cellulans NRRL B-4567

Cellulomonas fimi NRRL B-402

Cellulomonas fimi NRRL B-403

Cellulomonas turbata ATCC 25835

Corynebacterium ammoniagenes NRRL B-4246

Corynebacterium glutamicum NRRL B-2784

Corynebacterium glutamicum NRRL B-3330

Corynebacterium sp. NRRL B-4247

Corynebacterium variabilis NRRL B-4201

Corynebacterium vitaeruminis ATCC 10234

Propionibacterium acnes NRRL B-4224

Serratia grimesii NRRL B-4272

Serratia marcescens NRRL B-3401

Serratia marcescens NRRL B-486

Serratia marcescens NRRL B-284

Yeast strain

Candida tropicalis ATCC 96745

CHAPTER 1
INTRODUCTION

In August 1999, an executive order issued by then president Clinton led to creation of a permanent council for biomass research. The council's aim was to triple the energy produced in the form of fuels, chemicals and electricity from biomass by year 2010. The objective of the council was to coordinate and accelerate the development of biological-based industries that use dedicated biomass feedstocks, and agricultural and forestry wastes.

Agricultural and forestry products contain lignocellulosics as organic materials. Though the composition of lignocellulosics, cellulose, hemicellulose and lignin, vary in different species, the hemicellulose content range is 11-35% (Counsell, 1977). D-xylose and L-arabinose constitute 95% of arabino-xylan hemicelluloses and the pentosans constitute around 19-33% in hardwoods, 10 –12% in softwoods and about 40% in agricultural residues on dry weight basis (Winkelhausen and Kuzmanova, 1998). Seed coats of rice, corn, wheat, oats, or soybeans (Whistler, 1993), rice straw (Roberto *et al*., 1995) and barley straw (du Preez, 1994) are sources of xylose from agricultural residues. The abundance and availability of renewable agricultural residues and forestry products offers advantage to use D- xylose as a major carbon source.

The food industry uses xylitol as an alternative to sucrose, as it has a sweetening power equivalent to sucrose. Xylitol is used as a sweetener for diabetic patients. As xylitol is a value-added product with a growing market potential, production research is warranted. Large-scale commercial production of xylitol is by chemical reduction of D-xylose in the presence of a nickel catalyst at high temperatures. However, the high temperature and pressure requirements, extensive separation and purification procedures, along with usage of catalyst make the chemical route expensive (Meinender *et al.,* 1994). An alternative route for xylitol production is microbial fermentation (Roberto *et al.*, 1995). The microbiological method converts xylose present as xylan in hemicellulose feedstock (Nigam and Singh, 1995) into xylitol. Important feed-stocks used for xylitol production

are corncobs, cottonseed, soybean stalks, popcorn shells, sugarcane bagasse (Horeckar B.L, 1962) and birch wood (Jeffries and Kurtzman, 1994). Microbial conversion utilizes microorganisms such as yeast, fungi and bacteria to convert xylose to xylitol. Yeasts have been extensively studied for xylitol production, but neither bacteria nor other fungi have been explored to that extent. Harnessing facultative bacteria for xylitol production would offer advantages over yeast. In yeast, D-xylose is reduced to xylitol by the enzyme xylose reductase in the presence of NADPH or NADH co-enzymes (Parajo *et al.*, 1998). In some facultative bacteria xylose reduction to xylitol would be possible with the help of reducing equivalents even under oxygen-free conditions by avoiding the NAD/NADH redox system imbalance via an NADH dependent reductase system. Further, facultative anaerobic bacteria would offer a production advantage in oxygen-depleted conditions with a faster reduction to xylitol and a shorter doubling time relative to yeast. This study explores for facultative bacteria that can utilize D-xylose to produce xylitol with the following objectives.

Research Objectives:

- **i.** Screening for facultative bacteria that can utilize D-xylose to produce xylitol.
- **ii.** Select strains yielding high xylitol and utilizing less xylose in different growth conditions.
- **iii.** Perform growth study and optimize conditions for xylitol production on selected strains that use high D-xylose concentrations.
- **iv.** Isolate and purify the xylose reductase involved in xylitol conversion from the highest-yielding strain.
- **v.** Compare high xylitol-yielding bacterial xylose reductase at amino acid level with other known xylose reductases.

CHAPTER 2
REVIEW OF LITERATURE

Xylitol: The sweetener

Xylitol ($C_5H_{12}O_5$) is a pentose sugar alcohol used as sweetener. Emil Fisher and Stahel first synthesized xylitol in 1891 (Fischer and Stahel, 1891). Its chemical structure is shown in Fig. 2-1. Xylitol has a sweetening power similar to sucrose, nearly twice that of sorbitol and approximately three times that of mannitol. The calorific content (17 kJ/kg) is nearly equal to that of sucrose and thus has the potential to replace sucrose in low-calorie products. The physical and chemical properties of xylitol are listed in Table 2-1. The special properties of xylitol find use in food and pharmaceutical industry.

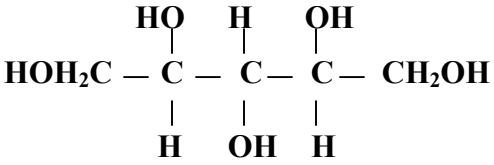


Figure 2-1. Xylitol chemical structure (Beutler, 1984)

Xylitol occurrence

Xylitol occurs in humans and animals as an intermediary product of carbohydrate metabolism. Human adults have been shown to produce about 5 to 15 g xylitol per day (Pepper and Olinger, 1988). It is present in some fruits and vegetables (Emodi, 1978 and Wang *et al.*, 1981) though the xylitol (Table 2-2) present in fruits and vegetables is very low (Parajo *et al.*, 1998).

Xylitol Applications

Food and confectionery

Xylitol does not undergo Maillard reaction, and so it does not darken or reduce the nutritional value of the proteins. Hence in the food industry, xylitol is used in the ingredients and formulations of food to improve storage properties, color and taste

Table 2-1. Physical properties of xylitol (Counsell, 1978; Jaffe, 1978; Bar, 1991)

Property	**xylitol**
Formula	$C_5H_{12}O_5$
Molecular Weight	152.15
Appearance	White, crystalline powder
Odor	None
Solubility at 20 °C	169 g/100 g H_2O
pH in water (1 g/10 mL)	5 – 7
Melting Point (°C)	93 – 94.5
Boiling Point (at 760 mmHg)	216 °C
Density (bulk density) (15 °C)	1.50 g/L
Caloric value	4.06 cal/g (16.88 J/g)
Moisture absorption (%) (4 days, 20-22 °C	
at 60% relative humidity	0.05
at 92% relative humidity	90
Density (specific gravity) of aqueous solution (20 °C)	
10%	1.03
60%	1.23
Heat of solution,	endothermic, 36.61 cal/g (153.76 J/g)
Viscosity (cP) (20 °C)	
10%	1.23
40%	4.18
50%	8.04
60%	20.63
Relative sweetness	Equal to sucrose; greater than sorbitol and mannitol
Specific rotation	optically inactive

Table 2-2. Xylitol presence in fruits and vegetables (Jaffe, 1978)

Product	**Xylitol (mg/100 g dry substance)**
Brewer's Yeast	4.5
Carrot Juice	12
Chestnut	14
Banana	21
Leek	53
Carrot	86.5
Onion	89
Fennel	92
Kohlrabi	94
Lettuce	96.5
Pumpkin	96.5
Spinach	107
White Mushroom	128
Eggplant	180
Endive	258
Raspberry	268
Lamb's Lettuce	273
Cauliflower	300
Strawberry	362
Yellow Plum	935

of food products. Hyvonen and Slotte (1983) reported beneficial effects of xylitol in yogurts either as a sole sweetener or combined with other sweeteners. Xylitol is used for preparation of jams, jellies, marmalades, desserts and relishes (Emodi, 1978). Xylitol is used in confectionary products for infants and adults. It is used solely or in combination with other sugar substitutes in the manufacture of sugarless chocolates, chewing gums, hard caramels, licorice sweets, wafer fillings, chocolate, pastilles, and other confectioneries for diabetics (Bar, 1991).

Pharmaceutical Industry

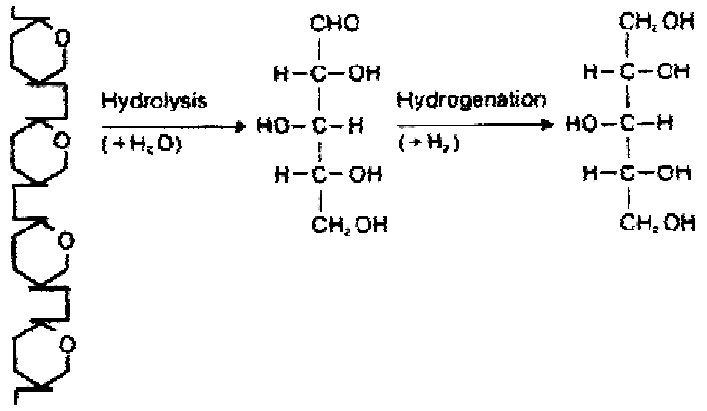
Xylitol can be metabolized in the absence of insulin and can replace sugar on a weight for weight basis (Cao *et al.*, 1994) making it a suitable sweetener for diabetic patients (Emodi, 1978). It is recommended for diabetic patients, as it causes only limited increase in glucose and insulin levels in blood (Hassinger *et al*., 1981). Also xylitol finds use in post-traumatic states or post-operative when efficient glucose utilization is inhibited, due to the induced resistance to insulin by excessively secreted stress hormones (Forster, 1974; Ritzel and Brubacher, 1976). Catabolic disorders can also be corrected due to the anabolic effects produced by xylitol. Xylitol, when used regularly in diet, limits obesity (Parajo *et al.*, 1998). It is used for parenteral nutrition in infusion therapy (Beutler, 1984) as it is inert to amino acids. It is also used in treatments for lipid metabolism disorders (Aguirre-Zero *et al.,* 1993 and Manz *et al.,* 1973). Chewing gums containing xylitol have been shown to prevent ear infection in children (Uhari *et al.*, 1996). Xylitol-coated pharmaceutical, confectionery products and dietary complement preparations (Pepper and Olinger, 1988; Perovitch, 1988) cause a pleasant cool and fresh sensation in oral and nasal cavities similar to vaporization due to its negative heat of dissolution (Forester, 1988).

Xylitol and other sugars are used as a stabilizing agent during protein extractions to prevent denaturation of proteins (Maloney and Amburdkar, 1989). Also xylitol finds use as a stabilizer in fibrinogen pastes for human plasma when used at 80% weight/volume basis. Xylitol has anti-cariogenic properties (Pepper and Olinger, 1988) and reduces

plaque formation (Rala *et al*., 1987) as cariogenic bacteria cannot matabolize xylitol in its metabolism. Xylitol has the capacity for moisture retention and hence is used in toothpastes (Mori and Saraya, 1988).

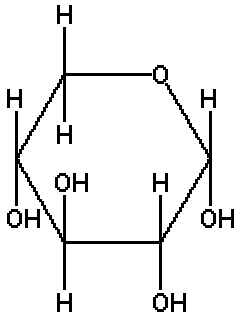
Feedstocks

Arabino-xylan (hemicellulose) is a highly branched polymer (Fig. 2-2) consisting of β,1→4 glycosidic linked xylopyranose (Fig. 2-3) as the structural backbone. The side chains are made of either one or several units of α-L-arabinofuranosyl, D-galactopyranosyl, β-D-glucuronopyranosyl and 4-O-methyl-β–D-glucuronopyranosyl (Whistler, 1993). The xylan is hydrolyzed to its constituent monomer sugars (glucose, xylose, arabinose, galactose, mannose and other oligosaccharides) by using dilute acid, alkali, steam explosion, ammonium explosion (AFEX) (Holtzapple *et al*., 1991) or mixture of enzymes treatments (amylase, xylanase and cellulase) (Hespell *et al.*, 1997, Saha *et al.* 1998). D-xylose is the major pentose sugar found in the hydrolyzate of agricultural residues (Table 2-3). The chemical structure of D-xylose in Haworth and Fischer form is shown in Figure 2-3. Yield of xylose depends on the hemicellulose source (Table 2-3) and on the severity of the hydrolysis process. Production of the hydrolyzate is the first step in the production of xylitol from agricultural residues. This is then followed by purification of the xylose in the hydrolyzate by neutralization or removal of toxics and inhibitory substances in the hydrolyzate. Furfural found in acid hydrolyzates must be removed if microorganisms are used in the subsequent steps (du Preez, 1994). The next step involves hydrogenation of xylose to xylitol by a chemical method using a catalyst (Fig. 2-2) or biological method using microorganisms (Jeffries, 1983 and Parajo *et al*., 1998a) (Fig. 2-4). The final stage involves the purification (Fig. 2-4).

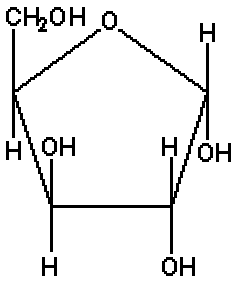


Xylan **Xylose** **Xylitol**

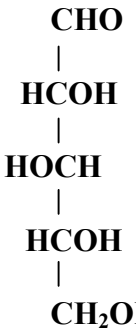
Figure 2-2. Hydrolysis and hydrogenation of xylan to xylitol (Counsell, 1978)



D-Xylopyranose (Hans-Otto Beutler, 1984)
Haworth form



Xylofuranose



Fischer form of D-xylose

Figure 2-3. Chemical structure of D-Xylose

Table 2-3. Percentage sugar in hydrolyzates of agricultural residues

Hydrolyzate	**Xylose (%)**	**Glucose (%)**	**Arabinose (%)**	**Galactose (%)**	**Mannose (%)**	**Reference**
Sugar cane bagasse	58	16	26	-	-	Chen and Gong, 1985
	75	14	11	-	-	Roberto *et al*., 1995
Rice straw	67	21	12	-	-	Roberto *et al*., 1995
Hardwood	70	14	5	5	5	Perego *et al*., 1990
	62	16	4	8	9	Jeffries and Sreenath, 1988
	27	11	5	14	43	Olsson *et al*., 1993
Corn fiber	16	71	11	2	-	Saha *et al*., 1998
	31	41	25	4	-	Hespell *et al*., 1997
Isolated corn fiber xylan	26-60	16-37	24-46	-	-	Hespell *et al*., 1997

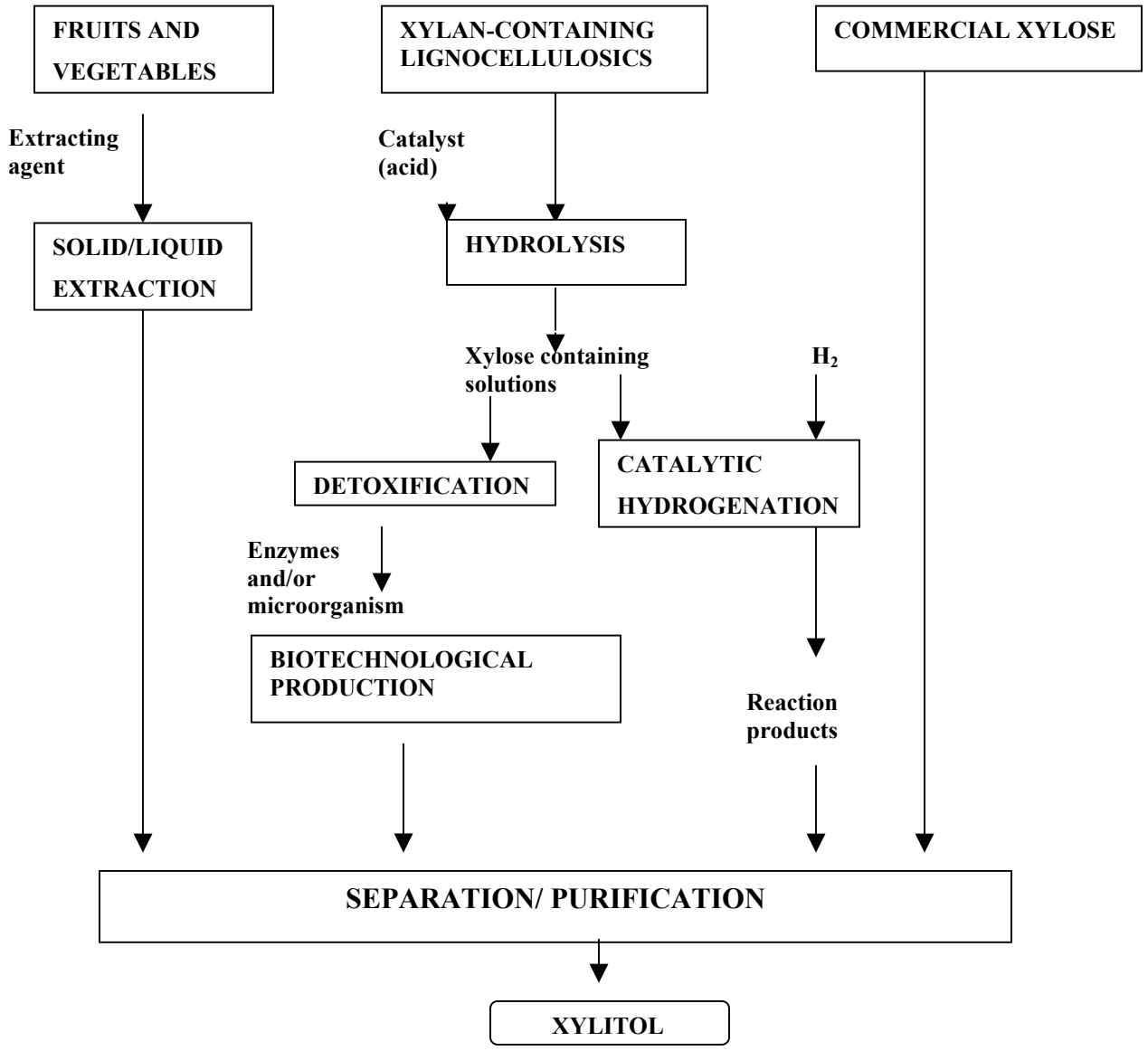


Figure 2-4. Xylitol production methods (Parajo *et al*., 1998a)

Yeasts

In an effort to produce xylitol by microbiological method, yeasts have been screened for xylitol production. Forty-four yeast strains in five genera of *Candida, Hansenula, Kluveromyces, Pichia* and *Pachysolen* were screened for their ability to convert D-xylose to xylitol by Barbosa *et al*. (1988). *Candida guilliermondii* and *Candida tropicalis* were found to be good xylitol producers. *Candida* yeasts fermented about 40% D-xylose in 24-48 h (Onishi and Suzuki *et al.,* 1966). Ten yeast strains were compared for xylose conversion (Gong *et al*., 1981) and *C. tropicalis* HPX2 produced the highest xylitol yield of 0.8 g/g of xylose.

In another experiment Gong *et al.* (1983) screened 20 strains of eleven species of *Candida*, 21 strains of eight species of *Saccharomyces*, and 8 strains of *Schizosaccharomyces pombe.* The *Candida* sp. yielded xylitol (10-15% w/v). *Debaryomyces hansenii* gave a higher yield than several other selected pentose fermenting yeasts utilizing lignocellulosic materials (Amaral-Collaco *et al.*, 1989). Vandeska *et al.* (1995) selected *D. hansenii,* which gave a high yield (0.47-0.48 g/g) than other yeasts. *Candida mogii* gave the highest yield (0.7g/g) in comparison with eleven other yeasts studied by Sirisansaneeyakul *et al*. (1995).

In an experiment by Dominguez *et al*. (1996) comparing six yeast strains, *D. hansenii* gave a yield of 0.71 g/g. A production rate of 2.67 g/l.h xylitol with D-xylose as substrate was obtained using *C. tropicalis* by Horitsu *et al*. (1992). Other Candida species screened were *Candida pelliculosa* (Kitpreechsvanisch *et al.*, 1984 and Nishio *et al.*, 1989), *C. boidinii* (Vongsuvanlert *et al.*, 1989), *C. guilliermondi* (Barbosa *et al.,* 1988; Lee, 1988 and Meyerial *et al.*, 1991), *C. shehatae* (du Preez *et al*., 1986) and *C. tropicalis* (Barbosa *et al.,* 1988). The list of yeast producing xylitol from pure D-xylose as carbon source or mixture of sugars is shown in Table 2- 4.

Table 2-4. Production of xylitol by microorganisms in media made from pure D-xylose or mixture of commercial sugars (Parajo *et al.*, 1998b)

Microorganism	Time (h)	So g/l	Qs g/l.h	%S Cons.	P g/l	Qp g/l.h	Yp/s (g/g)	Yx/s (g/g)	μ_{max} (1/h)	Reference
Candida tropicalis HPX2	24	51	2.08	100	40	1.67	0.8	-	-	Gong *et al.* (1981)
Pachysolen tannophilus IfGB 0101	33.5-41	25-27	0.66-0.75	100	22.9-35.5	0.46-0.56	0.61-0.85	-	-	Debus *et al.* (1983)
P. tannophilus ATCC 32691; *P. tannophilus* PT15; *S. ceresiae* SC138; *Candida* sp; *Candida* sp. XF217	24-72	65-100	0.90-2.71	53.8-100	32-86	0.63-2.17	0.88-0.60	-	-	Gong *et al.* (1983)
C. pelliculosa+ Methanobacterium HU	24	8.5	0.31	88.2	7.8	0.32	1			Kitpreechavanich *et al.* (1984)
Candida sp.B-22	168	250	1.49	100	210	1.25	0.84			Cheng & Gong (1985)
P. tannophilus RL 171	-	50	-	-	13.0	-	-			Wood and Millis (1985)
C. shehatae ATCC 22984	36	92	2.28	89	28.	0.78	0.34			Sreenath *et al.* (1986)
P. tannophilus NRRL 32691	-	15-60	-	18.2	9.4-15	0.09	-			Thonart *et al.* (1984)
P. tannophilus NRRL 2460; *C. guilliermondii* FTI-20037	137.5-62.5	4-4.2	0.008-0.06	12.5-88.1	0.01-2.75	0.002-0.04	0.01-0.73			Lee *et al.* (1988)
C. guilliermondii FTI-20037	78	104	1.33	100	77.2	0.99	0.74	0.048		Barbosa *et al.* (1988)
P. tannophilus NRRL Y-2460; *C. shehatae* Y-492; *Pichia stipitis* CSIR Y-633;	75-190	34-36	0.18-0.40	83.3-100	2-10	0.01-0.05	0.06-0.30	0.012-0.058		Ligthelm *et al.* (1988)
C. boidinii n &z. ousco; 2201	144-120	100	0.53-0.68	77-82	35-48.5	0.24-0,40	0,45 0.59			Vongsuvanlert & Tani (1989)
Schizosaccharomyces pombe Y-164	44	60	1.34	98.3	7	0.16	0.12			Lastick *et al.* (1989)
C. pelliculosa+ Methanobacterium sp.*HU*	33	4.5	0.14	100	2	0.06	0.43			Nishio *et al.* (1989)
Debaryomyces hansenii DTIA 77	28	90	-	-	62.6	2.24	0.70			Girio *et al.* (1989)
	-	10	0.28	44.2	1.37	0.087	0.31	0.32		Furlan *et al.* (1991)
D. hansenii DTIA 77	48	90	1.56	81	40.3	0.84	0.54	0.13		Roserio *et al.* (1991)
C. guilliermondii NRC 5578	406	300	0.74	100	221	0.54	0.75	0.02	0.01	Meyrial *et al.* (1991)
C. shehatae ATCC 22984	120	75	0.63	100	4.8	0.04	0.64	-		Palnitkar & Lachke (1992)
C. tropicalis IFO 0618	-	172	-	-	-	2.67	0.64	-	-	Horitsu *et al.* (1992)
C. guilliermondii NRC 5578	49.0-59.5	50-52	0.84-0.98	92.3-99.8	18.4-27.3	0.31-0.56	0.37-0.57	0.03	-	Peschke *et al.* (1992)
C. guilliermondii FTI 20037	-	54.4	-	-	37	-	0.73	-	-	Felipe *et al.* (1993)

Contd…

Microorganism	Time (h)	So (g/l)	Qs (g/l.h)	%S cons.	P (g/l)	Qp (g/l.h)	Yp/s (g/g)	Yx/s (g/g)	μ_{max} (1/h)	Reference
C. guilliermondii NRC 5578; *Candida parapsilosis* ATCC 28474	168.7-528.6	300-100	1.78-0.19	100	207-74	1.23-0.14	0.69-0.74	-	0.035-0.034	Nolleau *et al.* (1993)
C. tropicalis DSM 7524	70-800	155-300	0.34-0.5	91.5-100	95-220	0.28-1.35	0.50-0.74	-	-	Da Silva & Afschar (1994)
D. hansenii DTIA 77	-	50	0.26-0.33	-	-	0.033-0.372	0.38-1.43	-	0.033-0.201	Girio *et al.* (1994)
Candida sp. B-22	100	260	2.6	100	215	2.15	0.83	-	-	Cao *et al.* (1994)
C. guilliermondii FTI 20037	48	43	0.66	74	23.5	0.49	0.74	-	-	Da Silva *et al.* (1994)
P. tannophilus ATCC 32691; *C. shehatae* NRRL Y17024; *C. parapsilosis* ATCC 28474	200-117	50	0.43	100	3-0	0.03-0.26	0.16-0.61	-	-	Furlan *et al.* (1994)
C.mogii ATCC 18364	-	53	-	-	-	-	0.70	0.12	0.003	Sirisansaneeyakul *et al.* (1995)
C. parapsilosis ATCC 28474; *C. guilliermondii* NRC 5578	-	100-300	0.23-0.67	-	-	0.17-0.44	0.75-0.66	0.05-0.03	0.01-0.025	Nolleau *et al.* (1995)
C. boidinii NRRL-Y17213	245.4-331.9	130-150	0.34-0.5	74.7-92	53.1-58.9	0.16-0.24	0.47-0.48	0.07-0.11	-	Vandeska *et al.* (1995)
Candida sp. L –102	65.5	114	1.74	99.91	100.1	0.46	0.88	0.025	-	Lu *et al.* (1995)
C. shehatae ATCC 22984	120-145	50	0.05-0.4	14-96	8.5-1.4	0.01-0.07	0.18-0.21	0.03	-	Hinfray *et al.* (1995)
C. guilliermondii FTI 20037	-	-	0.70	-	-	0.57	0.82	-	-	Felipe *et al.* (1995)
C. tropicalis IFO 0618	32-42	150	3.59-4.67	100	84.5-104.5	2.01-3.26	0.56-0.70	0.15-0.26	-	Yahashi *et al.* (1996a)
C. tropicalis IFO 0618	36-56	170	-	-	86.2-101.6	1.81-2.71	0.50-0.69	-	-	Yahashi *et al.* (1996b)
C. boidinii NRRL-Y17213	129-242.5	100-130	0.5-0.68	87.7-93	58.2-59.3	0.24-0.46	0.48-0.68	-	0.013-0.020	Vandeska *et al.* (1996)
C. guilliermondii FTI 20037	30	40	1.25	-	21.5	0.66	0.53	0.47	-	Pfeifer *et al.* (1996)
C. entomaea NRRL Y-7785; *Pichia guillermondii* NRRL Y-2075; *C. guilliermondii* FTI 20037	70-96	50-68	0.49-0.93	80.9-100	21.39.1	0.22-0.55	0.41-0.60	-	-	Saha & Bothast (1996)
C. shehatae ATCC 22984	95	119	1.25	99.6	22	0.23	0.19	-	-	Kastner *et al.* (1996a)
C. shehatae ATCC 22984	507	115	-	35.5	-	-	0.21	-	-	Kastner *et al.* (1996b)
C. boidinii NRRL-Y17213	-	100	-	-	-	-	0.2	-	-	Winkelhausen *et al.* (1996)
C. guilliermondii FTI 20037	56-74	19-21	0.28-0.34	100	4-9	0.07-0.16	0.20-0.47	-	-	Lee *et al.* (1996)
P. tannophilus DSM 70352	-	-	-	-	5	0.24	0.26	-	-	Kruse & Schugerl (1996)
D. hansenii NRRL Y-7426	8	14.7	1.72	93.7	5.8	0.67	0.39	-	-	Parajo *et al.* (1996)
C. parapsilosis KFCC 10875	66	300	-	-	210	3.18	0.7	0.1	-	Kim *et al.* (1997)
Xanthophyllomycess dendrorhous ATCC 24228	73	42	0.58	100	22	0.3	0.52	0.17	-	Parajo *et al.* (1997)
D. hansenii NRRL Y-7426	48	279	5.81	100	221	4.6	0.79	-		Dominguez *.et al.* (1997)

Fungi

Production of xylitol has been studied to a lesser extent in fungi and bacteria. Dahiya (1991) studied the xylitol production in *Petromyces albertensis* and reported a yield of 0.4 g of xylitol/g of xylose after 10 days of fermentation.Ueng and Gong (1982) detected low amounts of xylitol in the fermentation of *Mucor* sp. on sugarcane bagasse hemicellulose hydrolyzate. Suihko (1984) reported 1 g/L of xylitol by *Fusarium oxysporum* culture grown in a media containing 50 g/L of xylose in aerobic conditions. *Penicillium, Aspergillus, Rhizopus, Glicoladium, Byssochlamyz, Myrothecium,* and *Neurospora* sp. have been shown to produce small quantities of xylitol in xylose-containing media (Chiang and Knight, 1961).

Bacteria

Screening for xylose utilizing bacteria by Yoshitake J. *et al*. (1973) showed that an *Enterobacter* strain grew on D-xylose and accumulated xylitol extracellularly. Xylitol production by the *Enterobacter* strain No. 553 using D-xylose was through NADPH-dependent D-xylose reductase. This showed that enzymatic conversion was not confined to fungi and yeasts. A productivity of 0.35 g L^{-1} h^{-1} was reported with this strain. *Corynebacterium* species produced xylitol extracellularly only when grown in media having both D-xylose and gluconate (Yoshitake J. *et al.*, 1971 and Yoshitake J. *et al.*, 1973). The author suggested that the NADPH source required for the reduction derived from the oxidation of 6-phosphogluconate by the enzyme phosphogluconate dehydrogenase. An 80% xylitol yield with D-xylose as substrate was reported by Izumori and Tuzaki (1988), using immobilized D-xylose isomerase enzyme and immobilized *Mycobacterium smegmatis*. These workers found that the *M. smegmatis* needed D-xylulose to be present in the media for xylitol production to occur. The authors also concluded that complete transformation of D-xylose to xylitol was not possible, due to the inhibition of D-xylose isomerase by xylitol.

Factors influencing microbial production of xylitol

The microbial conversion of xylose into xylitol is governed by several factors: strain, inoculum age, cell line history, culture conditions, fermentation type, medium composition (synthetic substrates or composite or lignocellulosic hydrolyzates), presence of inhibiting compounds and the influence of other sugars (Watson *et al*., 1984; Parekh *et al*., 1987; Bjorling and Lindman, 1989; Girio *et al*., 1990).

Inoculum age

The age of inoculum plays a major role in affecting the metabolic activity and viability of cells (Sreenath *et al*., 1986; du Preez, 1994). A 24-h old inoculum of *C. shehatae* produced 20 g/l (Sreenath *et al*., 1986) after 22 h with an yield of 0.24 g/g, whereas a 72 h old inoculum produced only 9 g/l xylitol after 65 h with a yield of 0.13 g/g. Productivity and cell growth were favorable when inoculum age was 15 h- 24 h for *C. guilliermondii* (Pfeifer *et al*., 1996).

Cell density

The effect of cell density on xylitol production has been studied in most xylitol producing yeasts. High cell density has been shown to increase the xylitol yield and specific productivity of xylitol in *C. boidinii* (Saha and Bothast, 1997 and Winkelhausen *et al*., 1998). Increase in the initial cell concentration from 0.3 g/l to 3 g/l increased xylitol productivity from 0.68 g/l.h to 2.25 g/l.h in *Debaryomyces hansenii* (Dominguez *et al*., 1997). Cao *et al*. (1994) reported an increase in xylitol production when the initial cell mass was increased from 3.8 to 14 g/l in *Candida* species. Vandeska *et al*. (1995) reported a similar trend in both productivity and xylitol yield. In an initial D-xylose concentration of 50 g/l the xylitol yield and specific production rate doubled when the cell density was increased from 1.3 g/l to 5.1 g/l. At higher cell density *D. hansenii* NRRL Y-7426 grown on wood hydrolyzate was shown to produce more xylitol (Winkelhausen *et al*., 1998). In contrast, 0.67 - 2.41 g/l cell density increase in *C. guilliermondii* FTI 20037 growing on rice straw hydrolyzate did not increase xylitol production (Roberto *et al*., 1996).

Effect of pH

The optimum initial pH for xylitol production depends on the microorganism used. The initial optimum pH for xylitol production for some of the yeasts reported is listed in Table 2-5.

Table 2-5**.** Optimum pH for yeast strains

Yeast	**Initial pH**	**Reference**
C. tropicalis	4	Yahashi*et al*. (1996 a)
C. shehatae	4.5	Kastner *et al*. (1996 a)
C. parapsilosis	4 –5.5	Nolleau *et al*. (1995)
Candida sp.	4-6	Cao *et al*. (1994)
Debaryomyces hansenii	5.5	Dominguez *et al*. (1996)
C guilliermondii	6.0	Nolleau *et al*. (1995)
C. boidinii	7.0	Vandeska *et al*. (1995); Vongsuvanlert and Tani (1989)
P. tannophilus	8.0	Debus *et al*. (1983)

Effect of temperature

Xylitol is produced by most yeast at temperature range of 24 - 45°C, and the optimum temperature range is 28 - 30°C. Xylitol production was uninterrupted in temperature range of 35 - 40°C for *Candida* sp. (Cao *et al*., 1994) and at a temperature range of 28 - 37°C for D. *hansenii* (Dominguez *et al*., 1997). Barbosa *et al*. (1988) reported maximum xylitol concentration and product yield for *C. guilliermondii* at 30 - 35°C range, though maximum growth occurred at 35°C. In *P. tannophilus* a 7°C increase in temperature from the initial 30°C led to a reduction in xylitol production (Barbosa *et al*., 1990).

Inhibition by xylitol

Xylitol consumption has been observed after complete utilization of xylose in some yeast strains (Parajo *et al*., 1998). Accumulation of 50 g/l xylitol caused inhibition in *C. shehatae* fermentation. Da Silva *et al*. (1994) also observed inhibition at 200 g/l xylitol during *C. tropicalis* fermentation. The authors advocated fed batch process type fermentation to remedy the product inhibition caused by xylitol accumulation.

Nitrogen source

The nature and concentration of the nitrogen source in the medium influences the xylitol production and xylose utilization by the microorganism. Organic nitrogen nutrients like yeast extract (Table 2-6) have been shown to increase xylitol production compared to nitrogen salts (Saha and Bothast, 1997 and Horitsu *et al*., 1992). Results of analyzing eight ammonium salts and four organic nitrogen sources used for xylitol production with *P. albertensis* showed that ammonium acetate was most effective among the salts and yeast extract as the most suitable for xylitol formation (Saha and Bothast, 1997). Winkelhausen *et al*., (1998) observed increased xylitol production rate in *C. tropicalis* DSM 7524 when the medium contained 20 g/l yeast extract, while Da Silva and Afschar (1994) observed inhibition for concentrations higher than 15 g/l. In the case of *C. guilliermondii* FTI 20037, the maximum yield of xylitol was obtained when the concentration of yeast extract was 1 g/l (Silva *et al.,* 1997). Sirsansaneeyakul *et al*. (1995) reported improved cell growth, xylitol yield and productivity in *C. mogii* when the fermentation medium contained yeast extract and peptone. Vongsuvanlert and Tani (1989) observed highest productivities with *C. boidinii* when yeast extract was the nitrogen source. Palnitkar and Lachke (1992) observed increased xylose utilization when an organic nitrogen source was in the media. Barbosa *et al*. (1990) also observed higher xylose consumption, but decreased xylitol production, when the medium contained 5 g/l yeast extract with *C. guilliermondi*. Kern *et al*. (1998) observed higher yields of D-xylose reductase and xylitol dehydrogenase in *C. tenuis* CBS 4435 when yeast extract or peptone was used as nitrogen source instead of ammonium salts. Lu *et al*. (1995) studied the influence of aspargine, glycine, traders protein, yeast extract, urea, casein hydrolyzate,

NH_4NO_3, $(NH4)_2SO_4$, NH_4Cl, $NH_4H_2PO_4$ and $NaNO_3$ as nitrogen sources on xylitol production with mutant *Candida* sp. L-102. A maximum xylitol production of 100-g/l xylitol was obtained utilizing 114 g/l xylose when urea (3 g/l) was used as the nitrogen source. Barbosa *et al*. (1988) reported a higher xylitol yield with *C. guilliermondii* when urea was used instead of ammonium sulfate in fermentations. Da Silva and Afschar (1994) observed higher productivities in *C. guilliermondii* but not much difference in xylitol yield when the urea replaced ammonium sulfate or ammonium chloride in the medium. Vandeska *et al*. (1995) observed increased xylitol yields and improved biomass productions by *C. boidinii* when urea was used instead of ammonium sulfate. Thus most studies for xylitol production show organic nitrogen sources such as yeast extract and peptone as better nitrogen sources instead of ammonium salts.

Table 2-6. Composition of yeast extract (Sommer, 1996)

Fraction	**% of total yeast extract**	**Molecular weight**
Free amino acids	35-40	N/A
Peptides	10-15	<600
Oligopeptides	40-45	2000-3000
Other Oligopeptides and Proteins	2 - 5	3000-100,000

Carbon source

It is important to understand the effect of hexose and other pentose sugars on xylose utilization and xylitol production as hydrolyzates contain sugar mixtures of varied compositions (Table 2-3). D-xylose utilization is not inhibited by D-galactose, D-cellobiose and L-arabinose while D-mannose and D-glucose affect xylose utilization (Winkelhausen *et al*., 1998; Lee *et al*., 1996; Lucas *et al*., 1986). Feeding glucose to *C.*

tropicalis growing on xylose in 3-L batch fermentation improved xylitol production (104.5 g/l) as much as 1.3 times (Saha and Bothast, 1997). Saha and Bothast (1997) also observed that glucose was assimilated first in mixed substrates. *C. boidinii* in batch fermentations on a mixture of glucose and xylose showed a faster growth compared to xylose alone in the medium. However, maximum xylitol production (41 g/l) was lower than the xylitol produced (59.3 g/l) with xylose alone.

In the presence of glucose a strong repression of xylose utilization occurred and sequential uptake of sugars was observed in *C. tropicalis* ATCC 96745 (Walther *et al*, 2001). In stirred batch fermentation Silva *et al*. (1996) observed 66% conversion efficiency of xylose to xylitol but this decreased to 45% when glucose was present. *C. guilliermondii* utilized mannose, galactose, L-arabinose and glucose to produce ethanol, arabitol and cell mass but no xylitol was produced (Meyrial *et al*., 1991). *C. guilliermondii* has been shown to consume D-glucose very rapidly but D-mannose, D-xylose, D-galactose and D-fructose were shown to be consumed slowly in that sequence (Lee *et al*. 1996). When more than 10 g/l glucose was present in the medium the xylitol yield decreased and ethanol production was observed (Oh *et al*., 1998). In *C. tropicalis* ATCC 96745 a 50% reduction in yield was observed when the ethanol concentration in the medium was higher than 30 g/l (Walther *et al.*, 2001).

In the case of D-galactose, Lee *et al*. (1996) observed that *C. guilliermondii* NRC 5578 consumed D-galactose before D-xylose, though D-galactose did not repress xylose reductase or xylitol dehydrogenase activity. When the medium contained fructose and xylose, both the sugars were utilized simultaneously at equivalent rates.

Effect of the initial substrate concentration

The composition of the media and the nature of the carbon source influence the production of polyols in yeast (Nobre and da Costa, 1985). Increase in initial xylose concentration usually led to decreased growth rate, unless the aeration rate was increased

(Nolleau *et al*., 1993). Da Silva and Afschar, (1994) observed inhibition in growth due to the high concentrations of substrate.

Optimum initial xylose concentration is essential for growth and xylitol production. Initial xylose concentrations in the range of 20-50 g/l produced the highest specific growth in *C. guilliermondii* (Meyrial *et al*., 1991). Srisansaneeyakul *et al.* (1995) observed maximum specific growth in *C. mogii* when the initial xylose concentration was 5-10 g/1. The optimum initial xylose concentration reported for xylitol production was 60 g/l for *P. tannophilus*, 200 g/l (Gong *et al*., 1981) and 100 g/l (Da Silva and Afschar, 1994) for *C. tropicalis* and 200 g/1(Meyrial *et al*., 1991) for *C. guilliermondii*. A five-fold increase in initial xylose concentration resulted in a 5.5 fold increase in the product yield and an increase in specific substrate consumption and product generation (Sirisansaneeyakul *et al*., 1995). In the case of *Candida* sp. B-22 a 249 g/l initial xylose concentration produced a maximum production rate (0.269 g/g.h) at 84.5% theoretical yield (Chen and Gong, 1985). *C. tropicalis* HXP2 accumulated maximum xylitol (144 g/l) at initial xylose of 200 g/l, while *P. albertensis* and *C. boidinii* accumulated xylitol (39 g/l and 36.8 g/l) at 100 g/l initial D-xylose (Saha and Bothast, 1997). At xylose concentrations greater than 10 g/l *P. tannophilus* accumulated xylitol, but at lower xylose concentrations (5 - 8 g/1) and fed-batch process feeding, ethanol was produced (Woods *and* Millis, 1985). Meyrial *et al*. (1991) obtained an increase in xylitol production when the initial xylose concentration was increased from 10 g/l to 300 g/l, but the increase in xylose affected the specific growth rate and yield. Horitsu *et al*. (1992) observed increased productivity and yield as the initial xylose concentration increased during a *C. tropicalis* fermentation. However in the case of *P. albertensis,* the initial xylose concentration of 150 g/l decreased the xylitol production. Saha and Bothast (1997) observed hyperbolic xylitol formation in *C. mogii* in oxygen-limited fermentations when the initial xylose concentrations was 5-53 g/l. Initial xylose concentration of 3.8 - 26 g/l showed a linear xylitol production rate in fermentations of *Candida* sp. B-22 (Cao *et al*., 1994). In most fermentations improved volumetric and specific xylitol productivity was observed when the initial substrate concentrations were high (Parajo *et al*., 1998b).

Process Strategies

Xylitol production in batch processes using flasks or laboratory reactors has high initial substrate concentrations at the start of the process, but low substrate concentrations and high product concentrations and at the end of the process. The high xylose and xylitol in the media can inhibit xylitol production and reduce productivity (Winkelhausen *et al*., (1998). Continuous culture techniques have shown better productivity and yield in many microorganisms. For continuous cultures to achieve the high production rate a low dilution rate is essential. But the low dilution rate makes the process impractical due to the increase in residence time.

Alternatively, in a fed-batch process (Winkelhausen *et al*., 1998; Horistsu *et al*., 1992) a constant substrate concentration can be maintained during the fermentation. *C. boidinii* NRRL Y-17231 fermentations achieved 75% theoretical xylitol yield in a fed-batch process compared to 53% theoretical yield in batch process (Winkelhausen *et al*., 1998). The maximum productivity (0.46 g/l.h) in the fed-batch process was twice the maximum productivity observed in the batch process.

Effect of vitamins

Vitamins in the medium have been shown to increase productivity and enhance growth in yeasts. When the medium for *C. guilliermondii* was supplemented with 0.05 μg biotin, Lee *et al*. (1988) observed a productivity increase from 0.002 g/1.h to 0.009 g/l.h, while a 0.25 μg/l biotin supplementation increased the productivity to 0.044 g/l.h. They also observed biotin supplementation increased xylitol production in *Pachysolen tannophilus*.

Aeration

The oxygen transfer rates (K_La) effect xylitol production based on the culture. Among *Pichia stipitis, Pachysolen tannophilus*, *C. shehatae* and *C. parapsilosis*, the *C. parapsilosis* culture gave high xylitol yield and higher rates of production at a K_La value of 16.3 h^{-1} (Furlan *et al*., 1994). An oxygen transfer rate of 2.2 mmol/l.h produced a maximum xylitol yield of 0.66 g/g in *C. guilliermondii,* whereas oxygen transfer rate of

0.4 mmol/(l.h) produced a maximum xylitol yield of 0.75 g/g at pH 4.75 in *C. parapsilosis* (Nolleau *et al*., 1995). An aeration rate of 0.46 vvm in a stirred tank bioreactor at 300 rpm (K_La = 10.6/h) produced maximal xylitol of 22.2 g/l during a *C. guilliermondii* FTI 20037 fermentation (Silva *et al.*, 1996). Further K_La increase, increased xylose utilization but not xylitol production. The effect of aeration on xylitol production is shown in Table 2-7. Xylitol production in micro-batch, semi-batch and aerobic batch cultures was studied for *C. parapsilosis* and *C. guilliermondii* (Nolleau *et al.*, 1993). The differences in the optimum xylitol yield between the two cultures were shown to correlate with the degree of aerobic batch nature and initial xylose conditions. For effective xylitol production in *D. hansenii,* semi-anaerobic condition is essential (Saha and Bothast, 1997). In *C. tropicalis* ATCC 96745 the maximum yield was under semi-aerobic conditions (0.62 g/g) but a high productivity of 0.9 g/l.h was achieved under aerobic conditions (Walther *et al*, 2001).

Metabolism

In the first step of xylose metabolism the sugars are transported across the cell membrane into the cell. The sugars are transported across the membrane by passive diffusion or facilitated diffusion (Fig. 2-5). Passive diffusion is driven by the gradient in the concentration of the substrate (Schlegel, 1992) and molecular parameters such as size and lipophilic properties (Nicklin *et al*, 1999). Though facilitated diffusion also occurs down a concentration gradient, it utilizes substrate-specific transport protein carriers and the mechanism is influenced by pH and temperature (Schlegel, 1992; Nicklin *et al*, 1999). Repressed cells of *C. shehate* growing in a medium containing glucose or D-xylose utilized a facilitated diffusion system to transport glucose, D-xylose and mannose but not L-arabinose and galactose (Lucas *et al*., 1986). Carriers were utilized for transportation along with one proton for each molecule of glucose or xylose. Interestingly, the D-xylose and glucose were competitive inhibitors in the system with sequential transport of glucose and D-mannose followed by D-mannose and D-xylose and then by L-arabinose. The substrate binds to the proton and moves across the membrane into the cell where the substrate releases the proton due to a low affinity.

Table 2-7. Aeration effect on xylitol production in yeast (Saha and Bothast, 1997)

Yeast	Xylose (g/l)	Aeration	Xylitol yield (g/g)
Candida tropicalis	100	100 ml/min	0.49
	100	400 ml/min	0.57
	100	500 ml/min	0.45
C. guilliermondii	100	700 ml/min	0.38
	100	Microaerobiosis	0.50
	100	Semiaerobiosis	0.49
	100	Aerobiosis	0.56
C. parapsilosis	100	Microaerobiosis	0.74
	100	Semiaerobiosis	0.61
	100	Aerobiosis	0.50
C. parapsilosis (continuous culture)	10	0.15 vvm	0.31
	10	0.30 vvm	0.27
	10	0.60 vvm	0.08
	10	1.00 vvm	0.04
	10	1.50 vvm	0.02
	10	2.00 vvm	0.04

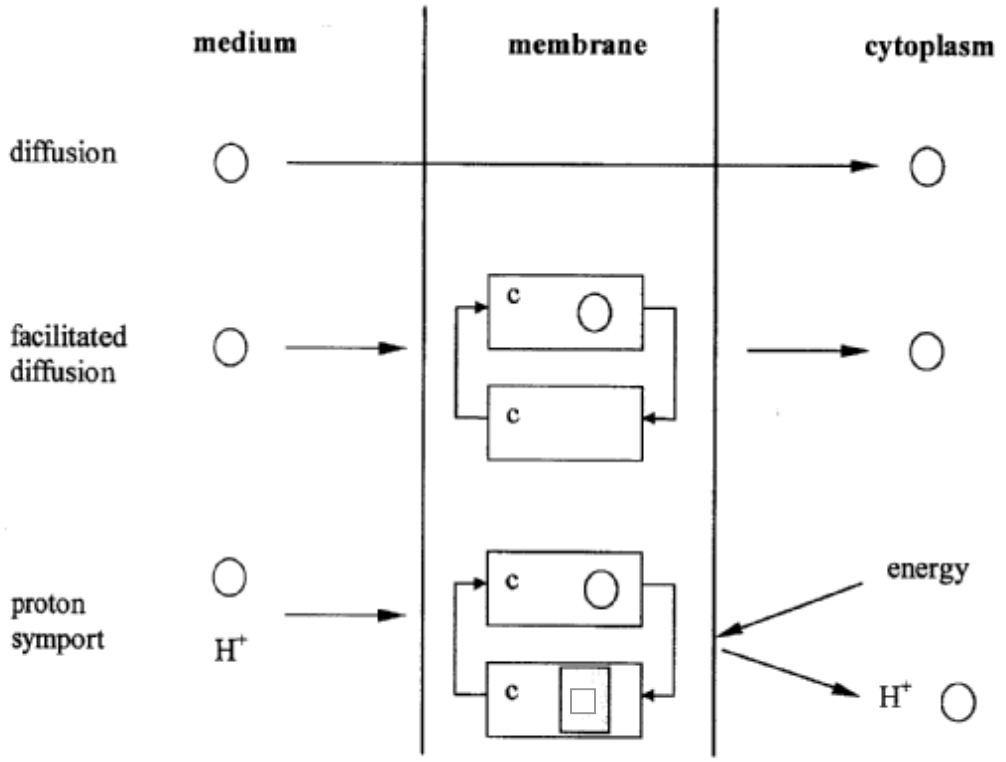


Figure 2-5. Sugar Transport in Cells (Schlegel, 1992)
(sugar, **O**; carrier protein, **c**; energized carrier**,** ▓).

In *Candida mogii* ATCC 18364 a transport uptake by carrier-mediated facilitated diffusion was inferred as the D-xylose utilization rate followed Michaelis-Menten kinetics. Further the kinetic analysis of ^{14}C-xylose transport supports a carrier-mediated facilitated diffusion mechanism (Winkelhausen and Kuzmanova, 1998).

Induction of Enzymes

Yeasts have been shown to induce xylose reductase in the presence of sugars, as this enzyme is needed in the first step for growth on D-xylose. The presence of substrate in the medium has been shown to induce activities of D-xylose reductase and xylitol dehydrogenase. The xylose reductase and xylitol dehydrogenase enzymes were induced in *Pachysolen tannophilus* and *Pichia stipitis* (Bichio *et al.*, 1988) during cell growth in a medium containing xylose, whereas glucose in the medium inhibited induction of the enzymes (Skoog and Hagerdal, 1988). The presence of D-xylose and L-arabinose were found to induce aldose reductase and xylitol dehydrogenase activity compared to other pentose and hexose sugars, while the induction by D-xylose was comparatively higher to that of L-arabinose. Enzyme activity in cells grown on D-glucose, D-mannose, and D-galactose were very low (Winkelhausen *et al*., 1998). Roberto *et al*., (1995b) also observed similar induction results in *C. guilliermondii* FTI-20037.

Metabolic Pathway

Bacteria

The metabolic routes for the utilization of xylose differ in prokaryotes and eukaryotes. In most prokaryotes D-xylose isomerase (EC 5.3.1.4) converts xylose to D-xylulose, which is subsequently phosphorylated by D-xylulose kinase to xylulose 5-phosphate (Gong *et al.*, 1983). However, some strains of *Enterobacter* and *Corynebacterium* species produce xylitol by reduction of xylose (Yoshitake *et al.,* 1973 and Yoshitake *et al.,* 1976). The bacterial strains were able to produce xylitol from D-xylose in the presence of NADPH dependent xylose reductase (Saha and Bothast, 1997).

Yeast and Fungi

Chiang and Knight (1961) reported in filamentous fungi, a two-stage mechanism involving reduction and oxidation that was different from the single step mechanism found in bacteria. In this pathway xylose is first reduced to xylitol by NADH or NADPH dependent xylose reductase (XR) (aldose reductase EC 1.1.1.21), followed by xylitol dehydrogenase (XDH) (EC 1.1.1.9.) linked NAD^+ oxidation of xylitol to xylulose (Fig. 2-7). A higher ratio of XR activity to XDH activity is essential for xylitol accumulation. Table 2-8 lists the XR and XDH activities of yeasts. Yeast does not posses xylose the isomerase usually present in bacteria (Saha and Bothast, 1999). However, xylose isomerase has been shown to be present in *Rhodotorula* and *C. boidinii* no. 2201 (Winkelheusan and Kuzmanova, 1998). The xylulose formed is phosphorylated to xylulose 5-phosphate (Hollman and Touster, 1964) and enters the pentose phosphate pathway. In yeasts the conversion of the pentoses to xylulose-5-phosphate is required for the utilization of the catabolic pathway. The xylulose-5-phosphate enters the oxidative or non-oxidative pentose phosphate pathway. In the oxidative path of the pentose phosphate pathway, glucose-6-phosphate is oxidized to ribulose-6-phosphate (pentose). Twelve NADPH coenzyme molecules are effectively regenerated in the overall cycle of the pentose phosphate pathway. However, when there is repression of fructose-1,6-bisphosphatase activity the pentose phosphate pathway cycle is incomplete and results in only 7 NADPH molecules.

$$\textbf{Glucose- 6- P} + 12\ NAD(P)^+ \longrightarrow 12\ NADPH_2^+ + 12\ H^+ + 6CO_2$$

An alternate path for xylulose-5-phosphate is the conversion to glyceraldehyde-3-phosphate (Fig. 2-6). In the non-oxidative phase of the pentose phosphate pathway, xylulose-5-phosphate produces glyceraldehyde-3-phosphate and fructose-6-phosphate. These are then converted to pyruvate in the Embden-Meyerhof-Parnas pathway. Nucleic acids and aromatic amino acids are synthesized from the intermediary ribose-5-phosphate and erythrose-4-phosphate (Prior and Kottar, 1997).

Table 2-8. Enyzmatic data on xylose reductase (XR) and xylitol dehydrogenase (XDH) activities of xylose fermenting yeasts (Parajo *et al.,*1998b)

Microorganism	XR activity μmol/min.mg		NADH/ NADPH ratio	XDH activity μmol/min.mg		Reference
	NADH	NADPH		NAD^+	$NADP^+$	
P. tannophilus (NRRL Y-2460)	44	151	0.29	114	8	Maleszka *et al*. (1983)
C. utilis (CBS 621)	0	75	0	280	0	Bruinenberg *et al*. (1984)
Pichia stipitis (CBS 5773)	310	600	0.5	720	75	Bruinenberg *et al*. (1984)
P. tannophilus (CBS 4044)	9	220	0.04	910	70	Bruinenberg *et al*. (1984)
C. tenuis (CBS 615)	2	130	0.02	-	-	Bruinenberg *et al*. (1984)
C.tenuis (CBS 2885)	0	100	0	-	-	Bruinenberg *et al*. (1984)
C. tenuis (CBS 4285)	305	670	0.5	-	-	Bruinenberg *et al*. (1984)
C. shehatae (CBS 5813)	210	480	0.4	-	-	Bruinenberg *et al*. (1984)
P. seggobiensis (CBS 6857)	365	640	0.6	-	-	Bruinenberg *et al*. (1984)
C. pelliculosa (1984)	-	1.73	-	-	-	Kitpreechavanich *et al*.
P. tannophilus (ATCC 32691)	-	36^{π}	-	-	36	Gomez (1987)
C. shehatae (ATCC 22984)	0.11	0.21	0.53	0.08	0.011	Girio *et al*. (1989)
P. stipitis (CBS 7126)	180-340	300-570	0.55-0.6	650-770	7-30	du Preez (1989)
C. shehatae (CBS 2779)	280-370	510-580	0.52-0.64	740-1270	25-30	du Preez (1989)
C. tenuis (CBS 2883)	120-200	220-350	0.54-0.57	80-310	7-17	du Preez (1989)
D. hansenii (DTIA 77)	0	60	0	-	-	Girio *et al*. (1990)
C. oleophila (DTIA 111)	0	40	0	-	-	Girio *et al*. (1990)
C. intermedia (DTIA 110)	0	140	0	-	-	Girio *et al* (1990)
P. stipitis (DTIA 81)	720	3460	0.21	-	-	Girio *et al*. (1990)
C. boidinii (NRRL Y171213)	-	-	1.2-2.1	-	-	Vandeska *et al*. (1995)
P. tannophilus (DSM 70352)	0.03	0.23	0.13	0..060	0.135	Kruse & Schugerl (1996)
C. boidinii Kloeckera sp. (no. 2201)	0.288	0.055	5.23	0.272	0.096	Vongsuvanlert & Tani (1989)
C. guilliermondii (NRC 5578)	0.08	0.62	0.129	-	-	Nolleau *et al*. (1993)
C. parapsilosis (ATCC 28474)	0.17	0.42	0.405	-	-	Nolleau *et al*. (1993)
C. guilliermondii (NRC 5578)	0.08	0.62	0.129	1.130	0.011	Nolleau *et al*. (1995)
C. parapsilosis (ATCC 28474)	0.17	0.42	0.405	0.400	0.02	Nolleau *et al*. (1995)
C. mogii (ATCC 18364) (1995)	0.06	0.16	0.375	0.22	-	Srisansaneeyakul *et al*.
C. guilliermondii (FTI-20037, NRC5578)	-	7.91	-	128.0	-	Lee *et al*. (1996)
C. shehatae (ATCC 22984)	0.04-0.07	0.26-0.34	0.15-0.21	0.14-0.18	-	Palnitkar & Lachke (1992)

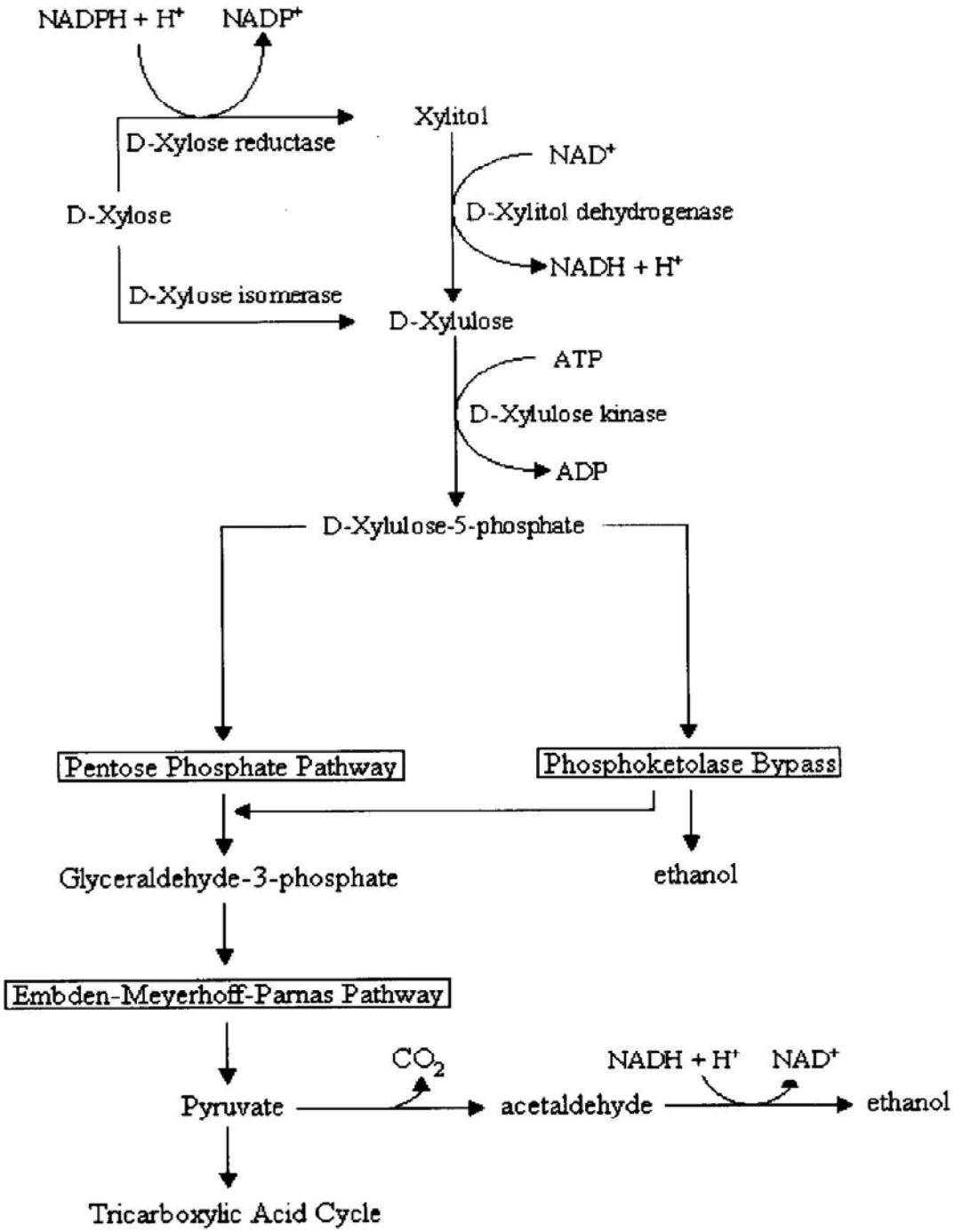


Figure 2-6. Xylose utilization pathway in microorganisms (Saha and Bothast, 1997)

The pyruvate formed in the Embden-Meyerhof-Parnas pathway may either be converted to ethanol or enter the tricarboxylic acid (TCA) cycle (Fig. 2-6). In the fermentation pathway, pyruvate decarboxylase converts pyruvate into acetaldehyde and subsequently alcohol dehydrogenase acts on the acetaldehyde to produce ethanol. In *C. shehatae* alcohol dehydrogenase activity has been shown to increase when the oxygen supply was decreased (Prior and Kottar, 1997) whereas in *P. stipitis* no difference in activity was observed (Skoog and Hagerdal,1990). Alternatively, in the oxidation path pyruvate is oxidized in the tricarboxylic acid cycle and the respiratory chain when there is available oxygen (Fig. 2-7). In the absence of oxygen, yeasts that ferment xylose are not able to grow (Hahn, 1994), as cell growth is coupled to metabolite production and the necessary coenzyme regeneration in the xylose utilization pathway (Winkelhausen and Kuzmanova, 1998).

Co-enzymes

Xylose reductase in some yeasts show a strict requirement for NADPH.

$$\text{Xylose} + \text{NAD(P)H}_2^+ \xrightarrow{\text{Xylose reductase}} \text{xylitol} + \text{NAD(P)}^+ \qquad (2.1)$$

$$\text{Xylitol} + \text{NAD}^+ \xrightarrow{\text{Xylitol dehydrogenase}} \text{xylulose} + \text{NADH}_2^+ \qquad (2.2)$$

Xylitol production in the phosphopentose pathway in yeasts is a controlled process based on NADP+ and NADPH availability (Gong *et al*., 1983). For the economic production of xylitol by xylose catabolism, the regeneration of NADPH is essential and excess generation of NADPH favors xylitol accumulation.

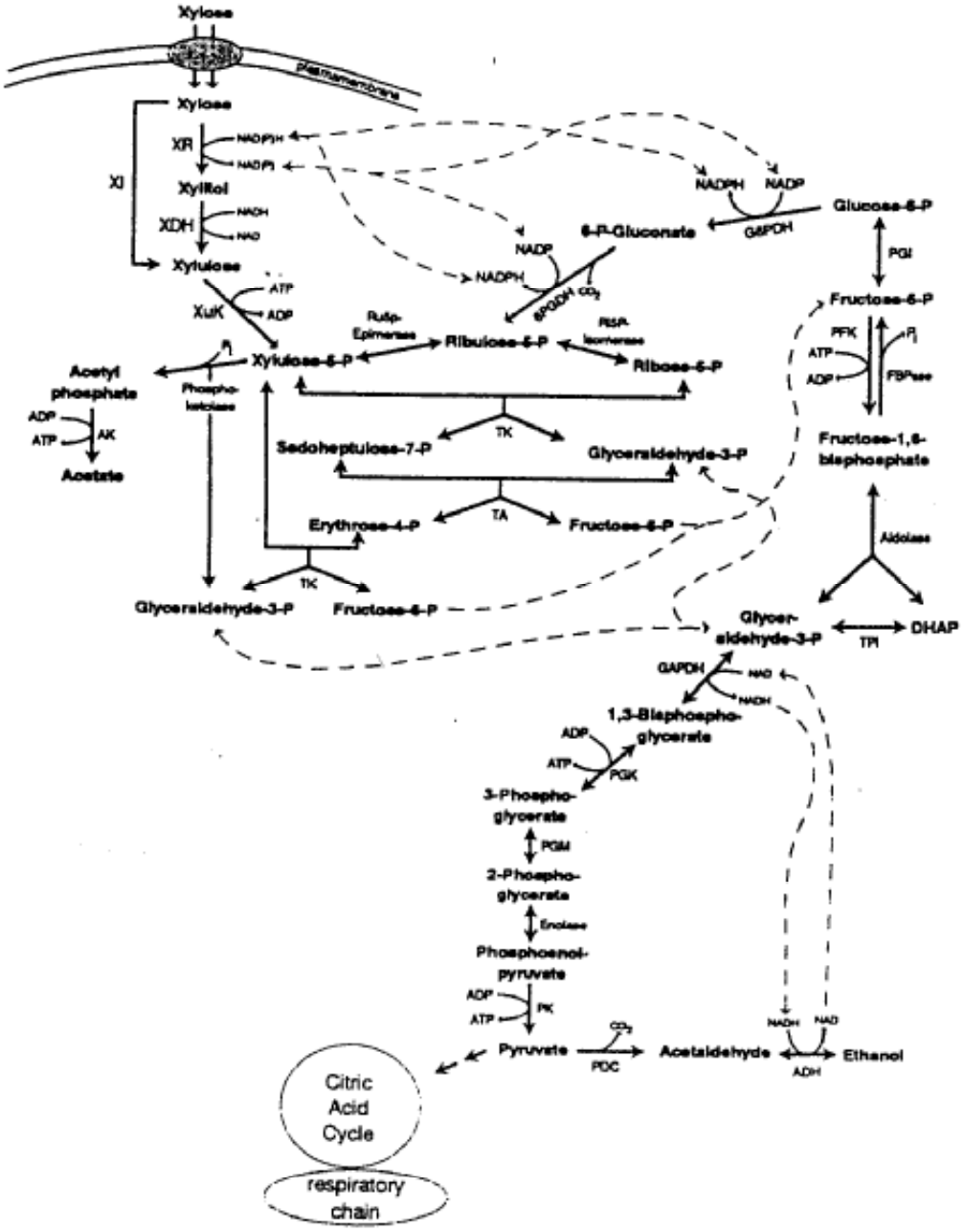
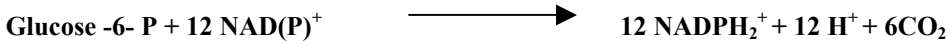


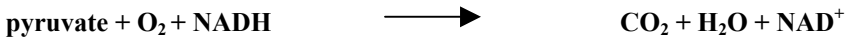
Figure 2-7. Xylose metabolism in yeasts (Prior and Köttar, 1997)

ADH, alcohol dehydrogenase; AK, acetatekinase; FBPase, fructose-1,6-bisphosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceralddiyde-3-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; PGI, phosphoglucose isomerase; PKF, phosphofructokinase; PGK, phosphoglycerate kinase; PDC, pyruvate decarboxylase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TA, transaldolase; TK, transketolase; TPI, triose phosphate isomerase; Ri5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; XI, xylose isomerase; XDH, xylose dehydrogenase; XR, xylose reductase; XuK, xylulokinase.

Regeneration of 12 NADPH molecules occurs when the xylose is converted to glucose 6-phosphate and utilized in the pentose phosphate pathway.



When oxygen levels are low, a redox imbalance occurs as the NADH produced in the xylitol dehydrogenase step cannot be re-oxidized back to NAD



This low-oxygen induced imbalance is due to a decreased respiration rate, which limits production of NAD^+. Under these conditions the alternate ethanol route is selected at the end of the pentose phosphate pathway. However, as the pentose phosphate pathway is still active, $NADP^+$ conversion to NADPH continues (Hahn *et al*., 1994). The resulting redox imbalance favors the accumulation of xylitol in the XR/XDH system, as there is a limited supply of NAD^+ for utilization by XDH. Saha and Bothast (1997) found for higher xylitol accumulations in *D. hansenii,* semi-anaerobic conditions were essential to lower the NAD/NADH ratio in the oxidation step of xylitol to xylulose. So non-regeneration of NAD^+ in oxygen-limited conditions results in accumulation of xylitol and its subsequent excretion into the medium. *C. parapsilosis* in oxygen-limited conditions (microaerobiosis) gave a higher yield (0.74 g/g from 100g/L of xylose) compared to *C. guilliermondii* (Taylor *et al.*, 1990). This increase was attributed to the differences in the initial step of xylose metabolism and the NADPH linked xylose reductase activity.

Theoretical Yield

Barbosa *et al*. (1988) proposed a model for xylitol production utilizing xylose when NADPH was the cofactor for XR. To calculate the theoretical yield, they assumed

(i) NADPH served as the cofactor for XR and NAD^+ served as cofactor for XDH.

(ii) the PPP pathway produces NADPH and the respiratory chain generates NAD^+ from NADH

(iii) Absence of transhydrogenase to catalyze the conversion of NADH to NADPH

(iv) NADPH is regenerated from xylitol metabolization when no growth occurs and the excess xylitol is excreted.

6 xylose + 6 $NAD(P)H_2^+$ → 6 xylitol + 6 $NAD(P)^+$ (1)

6 xylitol + 6 NAD^+ → 6 xylulose + 6 $NADH_2^+$ (2)

6 $NADH_2^+$ + 3 O_2 + 18 ADP + 18 Pi → 6 NAD^+ + 18 ATP + 24 H_2O (3)

6 xylulose + 6 ATP → 6 xylulose –5- P + 6 ADP (4)

6 xylulose –5- P + H_2O → 5 glucose –6-P + Pi (5)

5 glucose –6- P + 60 $NAD(P)^+$ + 35 H_2O → 60 $NADH_2^+$ + 30CO_2 + 5Pi (6)

On summing up equations (1 – 6)

6 xylose + 54 $NADP^+$ + 12ADP + 12 Pi + 12 H_2O + 3 O_2 → 54 $NADPH_2^+$ + 12ATP + 30 CO_2 (7)

54 $NADPH_2^+$ + 54 xylose → 54 xylitol + 54 $NADP^+$ (8)

adding equations 7 and 8

60 xylose + 12ADP + 12 Pi + 12 H_2O + 3 O_2 → 54 xylitol + 12 ATP + 30 CO_2 (9)

From equation (9) 0.9 mol xylitol/mol xylose (0.912 g xylitol/g xylose) the theoretical yield was calculated for non-growing conditions. D-xylitol production was found to increase with increased NADPH concentration, which inhibited isomerase enzyme activity thereby reducing the formation of D-xylulose in the case of bacteria (Yoshitake J. *et al.*, 1976).

Yeasts that ferment xylose anaerobically have NADPH and NADH-dependent xylose reductase co-factor specificity (Bruinenberg *et al*., 1984). The NADH-dependent reductase systems were not affected by the NAD^+/NADH redox ratios. Barbosa *et al*. (1988) calculated xylitol yield when $NADH^+$ was the cofactor for XR under aerobic conditions as follows:

6 xylose + 6 $NADH_2^+$ → 6 xylitol + 6 NAD^+ (10)

12 xylitol + 12 NAD^+ → 12 xylulose + 12 $NADH_2^+$ (11)

6 $NADH_2^+$ + 3 O_2 + 18 ADP + 18 Pi → 6 NAD^+ + 18 ATP + 24 H_2O (12)

Adding (10, 11, and 12)

6 xylose + 6 Xylitol + 3 O_2 + 18ADP + 18 Pi → 12 xylulose +18 ATP + 12 H_2O (13)

12 xylulose + 12 ATP → 12 xylulose –5- P + 12 ADP (14)

12 xylulose –5-P + 2 H_2O → 10 glucose –6-P + Pi (15)

10 glucose –6- P + 120 $NAD(P)^+$ + 70 H_2O → 120 $NADPH_2^+$ + 60 CO_2 + 10Pi (16)

On summing up equations (13-16)

6 xylose + 6 Xylitol + 3 O_2 + 6ADP + 6 Pi + 48 H_2O + 120 $NADP^+$ → 120 $NADPH_2^+$ + 6ATP + 60 CO_2 (17)

120 $NADPH_2^+$ + 120 xylose → 120 xylitol + 120 $NADP^+$ (18)

(Adding equations 17 and 18)

126 xylose + 3 0_2 + 6 ADP + 6 Pi + 48 H_2O → 114 xylitol + 6 ATP + 60 CO_2 (19)

The theoretical Yield = 114/126 = 0.905 mol xylitol /mol xylose

In the case of anaerobic conditions when $NADH_2^+$ is the cofactor for xylose reductase:

6 xylose + 6 $NADH_2^+$ → 6 xylitol + 6 NAD^+ (20)

6 xylitol + 6 NAD^+ → 6 xylulose + 6 $NADH_2^+$ (21)

6 xylulose + 6 ATP → 6 Xylulose –5- P + 6ADP (22)

6 xylulose –5- P + 6 H_2O → 5 glucose –6-P + Pi (23)

2 glucose –6- P + 24 $NAD(P)^+$ + 14 H_2O → 60 $NADPH_2^+$ + 12CO_2 + 2Pi (24)

3 glucose –6- P + 18 $NAD(P)^+$ + 3H_2O → $NADPH_2^+$ + 9CO_2 + 3 glyceraldehyde –3- P (25)

3glyceraldehyde –3- P + 3 Pi + 6 ADP → 6 ATP + 3 ethanol +3 CO_2 + 3H_2O (26)

42 $NADPH_2^+$ + 42 xylose → 42 xylitol + 42 $NADP^+$ (27)

On summing up equations (20-27)

48 xylose +15 H_2O → 42 xylitol + 24 CO_2 + 3 ethanol (28)

Yield = 43/48 = 0.875 mol xylitol /mol xylose

Yield = 3/48 = 0.0625 mol ethanol /mol xylose

The XR and XDH activities in xylose fermenting yeasts are listed in Table 2-9. The XDH enzyme of *Debaryomyces hansenii* cells grown in xylose gave a Michaelis constant (Km) of 16.5 mM for xylitol and 0.55mM for NAD^+ (Girio *et al.*, 1996).

Xylose reductase

The aldose reductases (EC 1.1.1.21) present in human tissues, mammals, reptiles, birds, fish, insects and fungi catalyze the reduction of aldehydes and ketones to their respective alcohols (Lee, 1998). The yeast aldose reductase known as xylose reductase is present in the cytoplasm of xylose-fermenting yeasts (Lee, 1998) and is mostly NADPH-dependent. It is monomeric and has a molecular weight ranging from 33 to 40 kDa (Lee, 1998). The functional xylose reductase in *Candida tropicalis* (Yokoyama *et al*., 1995) and in *P. stipitis* (Rizzi *et al.*, 1988) is composed of two identical subunits but the nature is not known. *Candida tropicalis* xylose reductases have isoelectric point (pI) in the range from 4.15 to 4.10 (Yokoyama *et al*., 1995). The xylose reductase of *Neurospora crassa* X1 has two isoenzymes with molecular weight of 30 kDa and 27 kDa with pI of 5.6 and 5.2 respectively (Zhao *et al*., 1998).

A computer-based comparison of *P. stipitis* xylose reductase and the human aldose reductase, rat aldose reductase and bovine aldose reductase showed 42.4%, 40.9% and 40.1% homology respectively (Lee, 1998). The yeast xylose reductase had a homology in the order of 24% with human aldehyde reductase and aldose reductase (Fig. 2-8).

NADPH-dependent D-xylose reductase purified from *C. tropicalis* IFO 0618 by Yokoyama *et al*. (1995) showed activity in the following order: DL-glyceraldehyde, L-arabinose and D-xylose.

Table 2-9. Enzymatic activities for xylitol producing yeasts as a function of the aeration conditions (indicated as type of medium, as percent of dissolved oxygen with respect to saturation conditions or as oxygen transfer rate, OTR) (Parajo *et al*., 1998a)

Microorganism	Reference	Aeration	XRA activity (mU/mg)	XDH activity (mU/mg)	XR/XDH Ratio	NADH XR	NADPH XR	NADH \NADPH ratio
D. hansenii	Girio *et al.*	2.72*	91	47	1.94	-	-	-
	(1994)	4.22*	52	23	2.26	-	-	-
		12.93*	58	51	1.14	-	-	-
C. boidinii	Vandeska	0.17*	-	-	2.1	-	-	-
	al.	0.23*	-	-	1.9	-	-	-
	(1995)	0.3*	-	-	1.8	-	-	-
		0.4*	-	-	1.25	-	-	-
		0.5*	-	-	1.1	-	-	-
C.	Nolleau	Microaerated	-	-	-	0.012	0.194	0.06
guilliermondii	*et al.*	Semi aerated	-	-	-	0.022	0.371	0.06
	(1993)	Aerated	-	-	-	0.050	0.521	0.1
C. parapsilosis	Nolleau	Microaerated	-	-	-	0.161	0.416	0.39
	et al.	Semiaerated	-	-	-	0.012	0.132	0.09
	(1993)	Aerated	-	-	-	0.060	0.086	0.07
C.	Nolleau	Aerated	0.400	1.130	-	0.003	0.400	0.0075
guilliermondii	*et al.*	Anaerobic	0.355	0.455	-	0.001	0.355	0.0028
	(1995)							
C. parapsilosis	Nolleau	Aerated	0.095	0.400	-	0.015	0.095	0.1578
	et al.	Anaerobic	0.130	0.350	-	0 .001	0.130	0.0077
	(1995)	Aerated	-	-	-	-	-	-
C. parapsilosis	Kim *et al.*	0% #	0.45	0.46	0.98	-	-	-
	(1997)	9.7% #	0.47	0.55	0.85	-	-	-
		3% #	0.35	1.02	0.34	-	-	-
P. tannophilus	Hahn-	Anaerobic	-	-	-	0.06	0.05	1.14
	Hagerdal	Oxygen	-	-	-	0.17	0.22	0.76
	et al.	limited						
	(1994)	Aerobic	-	-	-	0.002	0.01	0.14
P. tannophilus	Kruse &							
	Schugerl	0.0083*	-	0.72	-	-	0.06	-
	(1996)							

*OTR, in mmol/l.min

Percent of dissolved oxygen respect to saturation condition.

```
 HAR       - - ASRLL - - - - LNNGAKMPI LGLGTWKSPPGQVTEAVKVAI DVGYRHIDC      44
HUMALR     MAASCVL- - - -LHTGQKMPLI GLGTWKSEPGQVKAAVKYALSVGYRHIDC      46
CTXRO1     MSTTPTI PTI KLNSGYEMPLVGFGCWKVTNATAADQIYNAI KTGYRLFDG       50
CTXRO2     MSTTPTI PTI KLNSGYEMPLVGFGCWKVI NETAADQI YNAI KTGYRLFDG     50
PSXR       M - - - - - -PSI KLNSGYDMPAVGFGCWKVDVDTCSEQI YRAIKTGYRLFDG  44
PTXR       M- - - -TLQYYTLNNGRKIPAI GMGCWKL- -ENAADMVYAAI KEGYRLFDC     44
KLXR       MTYLA- -ETVTKNNGEKMPLVGLGCWKMPNDVCADQIYEAIKIGYRLFDG         48
SCAR       MSSL - - - - - VTLNNGLKMPLVGLGCWKI DKKVCANQIYEAIKLGYRLFDG   45
                          *. .  *  . .  *  . *. *  * *              . .   *..   ***  .*

 HAR       AHVYQNENEVGVAIQEKL – REQVVKREELFIVSKLWCTYHEKGLVKGACQ        93
HUMALR     AAI YG NEPE I GEALKEDVGPGKAVPREELFVTSKLWNTKHHPEDVEPALR      96
CTXRO1     AEDYGNE kEVGEGI NRAI – KEGLVKREELF I TSKLWNNFHDPKNVETALN    99
CTXRO2     AEDYGNE kEVGEGI NRAI – KEGLVKREELF I TSKLWNNFHDPKNVETALN    99
PSXR       AEDYANE kLVGAGVKKAI– DEGI VKREDLFLTSKLWNNYHHPDNVEKALN       93
PTXR       ACDYGNE kEVGEGI NRAI – KDGLVKRKDLFITSKLWNNFHAKENVKKALM      93
KLXR       AQDYANE kEVGQGVNRAI– KEGLVKREDLVVVSKLWNSFHHPDNVPRALE        97
SCAR       ACDYGNE kEVGEGI RKAI –-SEGLVSRKDI FVVSKLWNNFHHPDHVKLALK     94
             *   *  * *    .*  . . .   .   .    * *. . .  . . * * * *  .  *      *   *

 HAR       KTLSDLKLDYLDLYL I HWPTGFK - -  PGKEFFPL -- -- -- -- -- -- -DESGNVVPS   133
HUMALR     KTLADLQLEYLDLYLMHWPYAFE - - RGDNPFPK - -- -- -- - ----  NADGTICYD   136
CTXRO1     KTLSDLNLDYVDLFL I H F P I AFKFVP I EEKYPP GFYTGKED  - - - - NFHYE   145
CTXRO2     KTLSDLNLDYVDLFL I H F P I AFKFVP I EEKYPP GFYTGKED  - - - - NFHYE   145
PSXR       RTLSDLQVDYVDLFL I H F PVTFKFVP LEEKYPP GFYTGKED  -  - - - NFDYE   139
PTXR       KSLSDFNLDYFDLYLMH F P I SFKFVP FEEKYPP GFYTGKED - - --   KF I YE   139
KLXR       RTLSDLQLDYVDI FY I H F PLAFKPVP FDEKYPP GFYTGKEDEAKGH I EEE   147
SCAR       KTLSDMGLDYLDLYYI HF P I AFKYVPFEEKYPP GFYTGKEDEKKGH I TEA   144
             . . * . * .  . . *  * . .  . * . * . * .  .  . . . *

 HAR       DTN ILDTWAAMEELVDEGLVKAI GI SNFNHLQVEM ILNKPGLKYKPAVNQ      183
HUMALR     STHYKETWKALEALVAKGLVQALGLSNFNSRQ I DD I LSVASVR - - PAVLQ    184
CTXRO1     DVPLLDTWKALEKLVEAGK IKS I G I SNFTGAL IYDL I RGATI  -- -KPAVLQ   193
CTXRO2     DVPLLDTWKALEKLVEAGK IKS I G I SNFTGAL IYDL I RGATI  - - KPAVLQ   193
PSXR       DVPI LETWKALEKLVKAGK I RS I GVSNFPGALLLDLL RGAT I  - - KPS VLQ   187
PTXR       DVPI LETWRAMENLVDEGLVKS I GVSNVSGGLLEDLI KAAR I  - - KPAS LQ   187
KLXR       QVPLLDTWRALEKLVDQGK I KSL G I SNFSGALIQDLLRGAR I  - - KPVALQ   195
SCAR       HVP I I DTYRALEECVDEGL  I KS I GVSNFQGSL IQDLLRGCR I  - -KPVALQ   192
             .      . *.  * . *  *    * . . . . * . * *         .   . . .      .      *     *

 HAR        I ECHPYLTQEKL I QYCQSKG  I VVTAYSPLGSPD - - - -RPWAKPEDP SLLE   229
HUMALR     VECHPYLAQNEL I AHCQARG LEVTAY SPLGSSD - - - -RAWRDPDEPVLLE   230
CTXRO1      I EHTPYLQQPKL I EYVQKAG I A I TGYSSFGPQSFLELES K RALNTPTLFE   243
CTXRO2      I EHTPYLQQPKL I EYVQKAG I A I TGYSSFGPQSFLELES K RALNTPTLFE   243
PSXR       VEHHPYLQQPRL  I EFAQSRG  I AVTAYSSFGPQSFVELNQG RALNTSPLFE   237
PTXR        I EHHPYLQQNKLVEYAQLKG I VVTGYSNFGPLSFLELGNE TAKKTQPLYE   237
KLXR        I EHHPYLTQERL I KYVKNAG I QVVAYSSFGPVSFLELENK KALNTPTLFE   245
SCAR       I EHHPYLTQEHLV EFCKLHD  I QVVAYSSFGPQSF IEMDLQ LAKTTPTLFE   242
             . *    * * * *   * .      .     . .  . . . * * . * .  .                  . .    *  *

 HAR       DPR I KA I AAKH-NKTTAQVL I RFPMQRNLVV I PKSVTPER I AENFKVFD -   277
HUMALR     EPVVLALAEKY-GRSPAQ I LLRWQVQRKV I C I PKS I TPSR I LQN I KVFD -   278
CTXRO1     HET I KL I ADKH-GKSPAQVLLRWATQRN I AV I PKSNNPERLAQNLSVVD -   291
CTXRO2     HET I KS I ADKH-GKSPAQVLLRWATQRN I AV I PKSNNPERLAQNLSVVD -   291
PSXR       NET I KA I AAKH-GKSPAQVLLRWSSQRG I A I  I PKSNTVPRLLENKDVNS -   285
PTXR       NKT I TT I AAKH-GKTPFQVLLRWVNQRG I A I  I PKSTFPNTLAVNLHVDE -   285
KLXR       HDT I KS I ASKHKV-TPQQVLLRWATQNG I A I  I PKSSKKERLLDNLR INDA   294
SCAR       NDV I KKVSQNHPGSTTSQVLLRWATQRG I AV I PKSSKKERLLGNLE IEKK      292
             .    .    . .  . . .    . .  * . * . *.      *.  .   * * * * *      .      *    . .
```

```
HAR       FELSSQDMTTLLSYNRNWR---VCALLSC-- -- -- -- -- ---TSHKDYPFHEEE      315
HUMALR    FTFSPEEMKQLNALNKNWRY I VPMLTVDGKRVPRDAGHPLYPFNDPY      325
CTXRO1    FDLTKDDLDN I AKLD IGLRF -  - -  - - - - NDPWDW-- -DNI PI FV-- ----- -      324
CTXRO2    FSLRKDDLDN I AKLD IGLRF -  - -  - - - - NDPWDW-- -DNI PI FV-- ----- -      324
PSXR      FDLDEQDFAD I AKLD INLRF -  - -  - - - - NDPWDW-- -DKI PI FV-- ----- -      318
PTXR      FDLTKEDFFEEI AKLDRHLRF -  - -  - - - - NDPWTW-- -DKI PTFV-- ----- -      318
KLXR      LTLTDDELKQ I SGLNQN I RF -  - -  - - - - NDPWEWLDNEFPTF I -- ----- -      329
SCAR      FTLTEQ ELKD I SALNAN I RF -  - -  - - - - NDPWTWLDGKFPTFA-- ----- -      327
          . . . .  . . .  .  .  .  *  .  .
```

Figure 2-8. Multiple sequence alignment of yeast xylose (aldose) reductases, human aldose reductase and human aldehyde reductase (Lee, 1998)

HAR	human aldose reductase; HUMALR, human aldehyde reductase
SCAR	aldose reductase from *Saccharomyces cerevisiae*
K.LXR	xylose reductase from *Kluyveromyces lactis*
PSXR	xylose reductase from *Pichia stipitis*
CTXROI	first xylose reductase from *Candida tropicalis*
CTXR02	second xylose reductase from *C. tropicalis*
PTXR	xylose reductase from *Pachysolen tannophilus*

'' alignment is perfectly conserved; '.' position is well conserved*

Summary

Xylitol has a potential market and warrants study in its production using microbial methods. Microbial production using bacteria have not been studied extensively as compared to yeasts. Proposed study of xylitol production using facultative bacteria would offer advantages in xylitol production under oxygen-depleted conditions with a shorter doubling time relative to yeast.

Xylitol production in yeast utilizing D-xylose have been shown to depend on the strain, inoculum age, cell line, culture conditions, fermentation type, medium composition, inhibiting compounds and influence of other sugars. Theoretically, xylitol yield is higher in aerobic conditions than in the anaerobic conditions in the pentose phosphate pathway. In yeast the NADPH or NADH-dependent xylose reductase enzyme that reduces D-xylose to xylitol has a molecular weight of 33 to 40 kDa (Lee, 1998).

CHAPTER 3
MATERIALS AND METHODS

Bacterial cultures

Facultative anaerobes *Serratia marcescens* strains NRRL B-284, NRRL B-486, NRRL B-3401, *Serratia grimesii* strain NRRL B-4272, *Cellulomonas fimi* strains NRRL B-402, NRRL B-403, *Cellulomonas cellulans* strains NRRL B-2381, NRRL B-4567, *Corynebacterium glutamicum* strains NRRL B-2784, NRRL B-3330, *Corynebacterium variabilis* strain NRRL B-4201, *Corynebacterium ammoniagenes* strain NRRL B-4246, *Corynebacterium* sp. strain NRRL B-4247, and *Propionibacterium acnes* strain NRRL B-4224 were obtained from the National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois. *Cellulomonas cellulans* strain ATCC 12830, *Cellulomonas turbata* strain ATCC 25835 and *Corynebacterium vitaeruminis* strain ATCC 10234 were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). The cultures were selected based on the species characteristics from *Bergey's manual of systematic bacteriology* (Grimont and Grimont, 1984; Stackebrandt and Keddie, 1984; Collins and Cummins, 1984).

Culture initiation and media composition

Cultures described above were initiated in the following respective culture medium. *Serratia* strains were cultured in *Serratia* ATCC medium 1399 (Atlas 1997), containing 1 g/l pancreatic digest of casein, 1 g/l yeast extract, 1 g/l $(NH_4)_2SO_4$, 7 g/l K_2HPO_4, 3 g/l KH_2PO_4, 0.1 g/l $MgSO_4.7H_2O$ and 5 g/l glucose. The pH was adjusted to 7.0 at 25 °C using 0.5 M NaOH. *Cellulomonas* strains were cultured in *Cellulomonas* PTYG media (Atlas 1997) with the composition 5 g/l peptone, 5 g/l tryptone, 5 g/l yeast extract and 5 g/l glucose. The pH was adjusted to 7.0 with 0.5 M NaOH. *Corynebacterium* strains were cultured in Corynebacterium media (Atlas 1997), containing 10 g/l tryptic digest of casein, 5 g/l NaCl, 5 g/l yeast extract and 5 g/l glucose. The pH was adjusted to 7.2 with 0.5 M NaOH. The microorganisms were subcultured in their respective media containing

20 g/l D-xylose without the presence of glucose. Cultures were maintained in the respective culture medium containing 20 g/l D-xylose and 15 g/l solid agar.

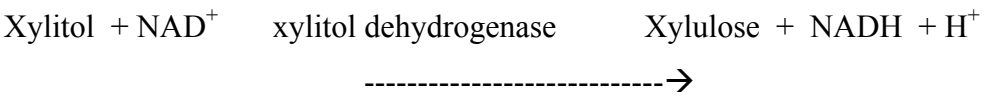
Crude enzyme preparation

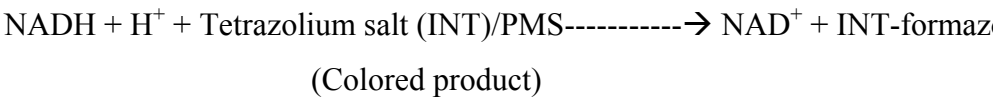
Candida tropicalis ATCC 96745 was grown in 250 ml growth medium containing 3 g/l yeast extract, 3 g/l K_2HPO_4, 1 g/l $MgSO_4.7H_2O$ and 30 g/l D-xylose at pH 5.0 (Yokoyama *et al*. 1995). The yeast cultures were grown at 30 °C at 130 rpm for 48 h. The cells were harvested by centrifuging at 10,000 x g for 15 min and washed with sterile water once and 0.5 M potassium phosphate buffer (pH 7.5) twice. The washed cells were suspended in two volumes of phosphate buffer (pH 7.5) and then ground with sterilized sea-sand in a porcelain pestle and mortar at about 4 °C. The ground crude extract was centrifuged at 15,000 x g for 15 min. The supernatant from the yeast crude cell extract was used as xylitol dehydrogenase source for the assay. SDH was obtained form Sigma Aldrich (St. Louis, Mo.).

Enzyme assay of solid and liquid cultures

Two enzyme sources were used for the assay, whole-cell extract of *Candida tropicalis* ATCC 96745 and standard sorbitol dehydrogenase (SDH) (Sigma). The chromogenic enzyme assay of Beutler (1984) was used to detect bacterial strains that produced extracellular xylitol for cultures grown in Petri dishes and for those grown in liquid media. Solid-agar bacterial cultures were grown in the respective culture medium containing 70 g/l D-xylose and 15 g/l agar on Petri dishes. Sterilized punched filter-paper bits were soaked in a chromogenic reactant mixture containing 100 µl of 1 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), 100 µl of 1 mM phenazine methosulfate (PMS), 50 µl of NAD (Sigma) and 500 µl enzyme (yeast whole-cell extract or SDH). The soaked filter-paper bits were placed on 48-h old cultures in Petri dishes and monitored for12 h for color change. The background color was verified by placing filter-paper bits soaked with the assay mixture on the agar Petri dishes without any culture. In the control assay, no enzyme was added.

Cultures in liquid media were grown for 0-96 h in 22 ml (6-dram) screw-cap tubes in the respective culture medium with100 g/l D-xylose. Salts and sugar solutions were sterilized separately at 121 °C for 20 min. The final medium volume in the tubes was 10 ml in order to simulate aerobic growth. The media were inoculated with 3% (v/v) of the respective cultures grown for 36 h in 20 g/l xylose. After inoculation, the tubes were placed in racks and mounted on Innova 2000 platform shakers (New Brunswick Scientific, NJ) in an environmental chamber (Percival, Boone, Iowa). Fermentation was carried out at 130 rpm, 30 °C. Measurement of cell growth and HPLC analysis were carried out as described in the Analytical methods section. Samples of 0-96 h cultures were centrifuged (15,000 x *g* for 15 min) and 500 µl of the supernatant was added to the chromogenic reactants mixture in a cuvette. The color developed over 75 min was read as absorbance at 650 nm using a Spectronic 1001 spectrophotometer (Milton, Rochester, N.Y.). For the blank, sterile water was added to the assay mixture instead of culture supernatant (substrate). In the control assay, sterile water instead of the enzyme solution was used in the assay.





Influence of culture conditions on screened cultures

The ability of the cultures to produce xylitol under various environmental conditions was studied using substrate concentrations (250 g/l and 350 g/l D-xylose), temperature (45 °C), and anaerobic conditions. Cell growth and the amount of xylitol produced were monitored as detailed in the Analytical methods section.

Effect of high substrate concentrations

Cultures in liquid media were grown in 22 ml (6-dram) screw-cap tubes in the appropriate culture medium of *Serratia*, *Cellulomonas* and *Corynebacterium* with 250 g/l and 350 g/l initial D-xylose in 10 ml working volumes. Salts and sugar solutions were sterilized separately at 121 °C for 20 min. Cultures were incubated at 30 °C for 0-96 h. Cell growth and the amount of xylitol produced were monitored as detailed in the Analytical methods section.

Effect of temperature at 45 °C

Cultures in liquid media were grown in 22 ml (6-dram) screw-cap tubes in the appropriate culture media with 100 g/l initial D-xylose in 10 ml working volumes. Salts and sugar solutions were sterilized separately at 121 °C for 20 min. Cultures were incubated at 45 °C for 0-96 h. Cell growth and the amount of xylitol produced were monitored in the cultures.

Effect of aerobic conditions

Cultures in liquid media were grown in 22 ml (6-dram) screw-cap tubes in the appropriate culture media using 100 g/l initial D-xylose in 15 ml working volumes. Salts and sugar solutions were sterilized separately at 121 °C for 20 min. Cultures were incubated at 30 °C for 0-96 h. Cell growth and the amount of xylitol produced were determined in each culture.

Effect of D-xylose concentration on *Corynebacterium* sp. B-4247

Corynebacterium sp. B-4247 was investigated for growth and xylitol production in Corynebacterium medium containing initial 20 g/l, 75 g/l and 100 g/l D-xylose concentrations. The sugar and salt solutions were sterilized separately at 121 °C for 20 min. Weight loss due to autoclaving was corrected by the addition of sterile water. The medium (200 ml) was inoculated with cultures grown for 36 h, corresponding to an initial optical density (OD) of 0.3 at 660 nm. The cultures were fermented at 30 °C, 130 rpm, for 120 h. Cell growth and xylitol production were measured as described in the Analytical methods.

Effect of gluconate on xylitol production by *Corynebacterium* sp. B-4247

Two media, *Corynebacterium* medium and basal medium were used to study the effect of gluconate on *Corynebacterium* sp. B-4247. The basal medium was composed of 6 g/l $(NH_4)_2SO_4$, 0.2 mg thiamine hydrochloride, 10 g/l KH_2PO_4 , 0.5 g/l $MgSO_4.7H_2O$ and 5 ppm $FeSO_4.7H_2O$ (Yoshitake *et al*. 1971). The pH was adjusted to 7.0 using 0.5 M NaOH. *Corynebacterium* medium and basal medium containing 96 g/l potassium gluconate were also prepared. Sterilization and inoculation of the medium were as described above. Cultures were grown for 48 h (30 °C, 130 rpm) and then D-xylose was added (Yoshitake *et al*. 1971) aseptically to a concentration of 75 g/l. The culture volume after xylose addition was 123 ml (250-ml flasks). Cell growth and xylitol production were monitored for 150 h.

Analytical methods

Bacterial cell growth in the liquid cultures was monitored by measuring the OD of the culture samples at 660 nm in a Spectronic 1001 spectrophotometer. The extracellular xylitol produced was quantified using a Shimadzu HPLC (Shimadzu Scientific, Columbia, MD.). The HPLC setup is shown in Figure 3-1. A calcium carbohydrate column (Supelcogel Ca^{2+}, 30 cm x7.8 mm, Supelco, Bellefonte, PA.) maintained at 80 °C was used for the separation. Samples (0.4 ml) from the culture broth were centrifuged at 26,000 x g for 15 min. The supernatant was decanted, diluted five-fold with deionized water and filtered in 0.2-µm syringe filters; 20 µl of the filtered solution was analyzed. Deionized water was used as mobile phase in isocratic mode with a linear flow rate, beginning at 0.5 ml/min and ending at 0.375 ml/min over 25 min. A refractive index detector (Shimadzu RID 10A) was used. Compounds were identified and quantified by comparison of their retention times with those of authentic standards (D-xylose, xylitol). A calibrated plot of external standards concentration to the peak area was used to deduce the compound concentration in the sample. The xylitol concentration was corrected for the xylitol in the inoculum by subtracting the concentration of xylitol at 0 h culture.

Mass spectrometry

The xylitol produced by *Corynebacterium* sp. B-4247 in *Corynebacterium* medium was confirmed by mass spectrometry. *Corynebacterium* sp. B-4247 culture broth samples were prepared similar to those for the quantification of xylitol. The HPLC conditions were also similar to those for quantification of xylitol, except 100 µl of filtered broth sample was injected. Eluents corresponding to the xylitol peak were collected from the HPLC runs. The fractions were pooled and concentrated in a rotary vacuum evaporator and analyzed in a Shimadzu LCMS QP 8000 (Figure 3-2). Xylitol (1 mg/ml) in deionized water was used as standard. Samples and standards (35 µl) were made in 1.0% formic acid. The mobile phase consisted of 99.5% acetonitrile and 0.5% formic acid; the flow rate was 0.2 ml /min. The samples were ionized using atmospheric pressure chemical ionization (APCI) negative mode and masses were scanned from *m/z* 100 to *m/z* 300. The background noise was subtracted from the averaged spectra using the Shimadzu LCMS workstation class-8000 software.

Biomass and Cell free extract production

Fermentation was carried in 5 l working volumes in a 10 L fermentor (Bio Flo) (Figure 3-3) at 7.2 pH. The *Corynebacterium* sp. B 4247 pre-cultured in 500 ml working volumes (2 l bottles) was transferred aseptically to the 4.5 l sterilized Corynebacterium culture media containing 0.1% antifoam 289 (Sigma Aldrich). The fermentor was run with 1.2 vvm air inflow rate, at 300 rpm, 30 °C, for 24 h. The cells were harvested by centrifuging at 10000 x g for 20 min and washed twice with sterile water and twice with 0.5 M phosphate buffer pH 7.5 to remove the salt and the media. A wet weight of 2.2 – 2.4 g/l was obtained from the fermentor. The washed cell paste were frozen in liquid nitrogen and stored for extraction. During extraction one part wet cell paste was added to two parts by volume of 0.5 M phosphate buffer, (pH 7.5) containing 0.5 mM EDTA and 0.5 mM 2-mercaptoethanol. Lysozyme (2 mg/ml of buffer) was added to the mixture and vortexed. The suspended cells were incubated at 37 °C for 30 min. The supernatant of the digested cells was collected by centrifuging for 20 min at 15,000x g, at 4 °C and used as crude extract. The crude extract was light yellowish color.

Dialysis

Spectrum/Por (Fisher) dialysis bags of MWCO: 10,000 with Spectrum closure were used for dialysis. 100 ml of cell free extract was dialyzed against TEM (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 0.5 mM 2-mercaptoethanol) replacing buffer twice for duration of 10- 12 h at 4°C.

Enzyme assay

The assay was carried in a quartz cuvette in a Spectronic spectrophotometer. To 0.1 ml of the enzyme solution, 0.2 ml of 0.1M phosphate buffer pH 7.0, 0.2 ml of 0.1 M 2-mercaptoethanol, 0.1 ml of 3.4 mM NADPH and 1.2 ml deionized water was added and allowed to stand for one minute to eliminate the endogenous oxidation of NADPH(Yokoyama et al, 1995). The reaction was started by addition of 0.2 ml of 0.5 M substrate (D-xylose) and the absorbance at 340 nm at room temperature was monitored (Yokoyama et al, 1995). The slope was calculated in the linear range of the reaction. Sterile water was used instead of enzyme, as controls and for the assay. A unit of enzyme (U) was expressed as the amount of the enzyme catalyzing oxidation of 1 μmol/min NADPH. The reductase assay was done on the crude extract to check the activity of each harvested batch and to identify the fractions having reductase activity in the purification process. For the dehydrogenase assay the NADPH in the assay was replaced with NAD or NADP and D-xylose was replaced with xylitol in the above reactants.

Protein quantification

Proteins were quantified by the Bradford method, using Coomasie Blue dye (Bio-Rad). Bovine Serum Albumin (BSA) was used as standard for the assay. From the activity calculated by the assay and from the concentration of protein determined by Bradford method the specific activity was calculated as the enzyme units per milligram of protein.

Ion exchange chromatography

A DEAE-cellulose column was used to purify the dialyzed crude extract in a refrigerated chamber (Figure 3-2). A slurry of diethyl aminoethyl (DEAE) cellulose – Grade DE 52 (Whatman) anionic exchange was packed in a column (ϕ 18 x 250 mm) according to the manufacturer's (Whatman) directions. The column was equilibrated with three bed volumes of TEM buffer (20 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM 2-mercaptoethanol), pH 7.5 (Yokoyama et al. 1995). The buffers were degassed in a Ultrasonic FS-9 sonicator (Fischer Scientific) to remove any dissolved air. Crude extract in volumes of 5 ml were applied to the column and washed with 1 bed volume of TEM buffer. The column was eluted with two bed volumes in linear gradient mode 0 to 0.5 M NaCl in TEM buffer at a flow rate of 90 ml/h. Fractions of 2.5 ml were collected using fraction collector (Pharmacia). The protein elution was monitored at 280 nm by a UV-monitor (Pharmacia), and the eluted peak fractions were tested for reductase activity. The active fractions were pooled based on the assay activity. The salt in the active pooled fractions was lowered, by exchanging the buffer twice in Centricon (Millipore) ultra filtration centrifugal concentrators at 10,000 MWCO.

Affinity Chromatography

Hi-Trap Blue column

Hi trap Blue column (Pharmacia) 5 ml volume was used at a flow rate of 30 ml/h at 4 °C. The column was equilibrated with TEM buffer and DEAE-cellulose pooled active fractions were injected. The column was washed with one bed volume of TEM buffer and eluted with linear gradient of 0 to 50 μM $NADP^+$ in the same TEM buffer. Reductase activity was tested in the fractions collected. The active fractions were pooled based on the assay activity. The salt in the active pooled fractions was lowered, by exchanging the buffer twice in Centricon (Millipore) ultra filtration centrifugal concentrators at 10,000 MWCO.

Red Sepharose column

A 2.5 g of Red Sepharose (Sigma Aldrich) was washed in water and suspended in the buffer to swell. This gel was packed in a 10 ml column (Figure 3-5,1) and equilibrated with the TEM buffer (20 mM Tris, 0.5 mM EDTA, 0.5 mM mercaptoethanol at pH 7.5). 1.5 ml desalted DEAE purified active fractions were introduced in the column and washed with one bed volume of buffer at a flow rate of 30 ml/hr. The column was eluted with 0- 50 μM $NADP^+$ linear gradient in the buffer.

FPLC Mono-Q

The protein was further purified by FPLC (Pharmacia Co. Sweden) (Figure 3-4) using Mono-Q HR 5/5 column (Pharmacia Co. Sweden) (Figure 3-5-2). A 0.5 ml of the concentrated protein was injected into equilibrated Mono-Q column with TEM buffer at flow rate of 30 ml/h in a 4 °C chamber. The column was washed with two bed volumes of the buffer and then eluted with 0 to 0.25 M NaCl linear gradient in the same buffer.

Gel filtration chromatography

The molecular weight of the protein in the crude extract was determined by gel filtration chromatography using a Sephacryl-S 300 column (Amershem Pharmacia). The column was equilibrated with 50 mM Tris HCl, pH 7.5 containing 0.1 M KCl at 4 °C at a flow rate of 0.5 ml/min. Standard gel filtration molecular weight marker proteins (Sigma Aldrich): cytochrome-C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and blue dextran (2000 kDa) of 1.0 ml volume in equilibration buffer was applied to the column. The elution volumes of the standard proteins were monitored. The ratio of the elution volume (Ve) of the protein to the elution volume of Blue dextran (Vo) was plotted against the molecular weight of standard proteins. *Corynebacterium* sp. B 4247 crude cell extract in 50 mM Tris HCl, pH 7.5 containing 0.1 M KCl was concentrated by ultra filtration using Centricon-YM10 (Millipore) centrifugal membranes and 1.0 ml was injected in the column. Fractions of the sample collected from the column were tested for reductase

activity. The ratio of the volume of the fraction that tested positive for the assay to that of the volume of the Blue dextran was determined, and the molecular weight corresponding to the ratio was determined from the calibration curve.

SDS – PAGE

The molecular weight of the proteins was also determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3-6) (Laemmli, 1970). The 10% Tris-Glycine pre-cast gels (Invitrogen) were used to resolve the proteins. Samples were mixed with SDS sample buffer (Invitrogen) containing mercaptoethanol and denatured at 85 °C for 2 mins and loaded in the wells. The electrophoresis was carried on the manufacturers recommendations at a constant voltage of 125V (starting current 30-40 mA, ending was 8-12 mA) and run approximately for 90 min. The purified protein was compared with standard protein markers of known molecular weights as per the manufacturers (Biorad, Invitrogen) directions. The protein was visualized by staining with 0.25% Coomasie Blue R 250 in 50% methanol and 10% acetic acid. Excess dye was washed with 20% methanol and 10% acetic acid (Rizzi *et al*., 1988).

Native gel Electrophoresis

The samples were prepared as in the above electrophoresis, but without any reducing agents in the sample buffer and loaded in the 10% Tris-Glycine pre-cast gels (Invitrogen) under non-denaturing conditions. The running buffer (Invitrogen) that did not have SDS for native conditions was used. Care was taken to maintain the apparatus temperature by maintaining the electrophoresis around 4°C in a cold chamber while also stirring the running buffer with a magnetic stirrer. The gel was started at 50 V at 10 mA and was run till the sample buffer dye reached the end of the gel.

In Gel staining

The native gel was washed three times in de-ionized water for 5 min. Filter paper (Whatman) strip cut to the size of the gel was laid on the gel. NADPH, D-xylose, INT

and PMS chromogenic mix was applied on the filter paper and incubated in the dark at 37°C, for 6 h. Controls for enzyme specificity was used without the substrate solution (xylose) in the chromogenic reactants.

Mass Spectrometric analysis

Proteomic analysis was done at the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia, Charlottesville, VA. The gel piece corresponding to the band was excised and transferred to a siliconized tube. The gel piece was washed and destained overnight with 50% methanol and dehydrated with acetonitrile. Then 30 µL of 10 mM dithiothreitol (DTT) in 0.1 M ammonium bicarbonate for 30 min was used to rehydrate and reduce the gel piece at room temperature. The DTT solution was removed from the sample and 30 µL of 50 mM iodoacetamide in 0.1 M ammonium bicarbonate was added to alkylate the sample for 30 min at room temperature. The sample was replaced in 100 µL acetonitrile to dehydrate the sample, and then acetonitrile was replaced with 100 µL of 0.1 M ammonium bicarbonate for rehydration. The gel pieces were then dehydrated with 100 µL acetonitrile. The acetonitrile in the sample was discarded and the sample was completely dried by vacuum centrifuging. After drying, the gel pieces were rehydrated for 10 min in 20 ng/µL trypsin containing 50 mM ammonium bicarbonate on ice and care was take to remove the trypsin found in excess. To this sample 20 µl of 50 mM ammonium bicarbonate was added and digested overnight at 37 ^{o}C. The peptides formed during the process were collected using two 30 µl aliquots of 50% acetonitrile with 5% formic acid and the collected aliquots mixed and evaporated to 25 µl volume for analysis by mass spectrometry (MS). A Finnigan LCQ ion trap LC–MS system with Protana nanospray ion source operating at 2.8 kV was used. A Phenomenex Jupiter C18 reversed phase capillary column 8 cm x 75 µm (id) was self-packed and 0.5 – 5 µl volumes from the extract was injected. Acetonitrile in 0.1 M acetic acid gradient was used to elute the peptides from the column at a flow rate of 0.25 µL/min. The double play capability of the instrument was used to analyze the digest. From the full scan mass spectra the peptide molecular weights and production spectra was used for determining the amino acid sequence in sequential scans. The analysis produces approximately 400

CAD spectra of ions ranging over several orders of magnitude some of which are not derived from peptides. Using Sequest search algorithm the data was analyzed. Manual interpretation was done for those peptides that did not match by searching against the EST databases.

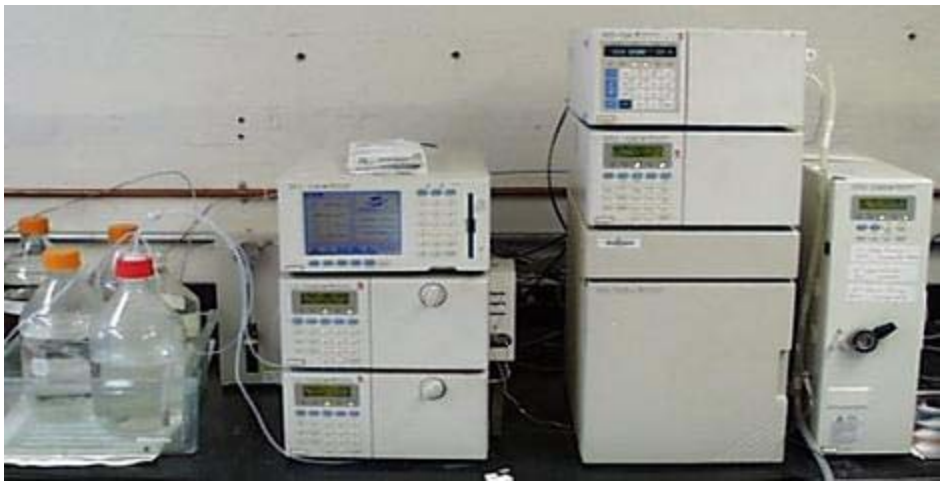


Figure 3-1. High Performance Liquid Chromatography (HPLC) system

1. Oven, Shimadzu
2. Auto Sampler, Shimadzu
3. UV-monitor SP-10AVP(i), Shimadzu
4. Detector –RID-10A, Shimadzu
5. Pump A, Shimadzu
6. Pump B, Shimadzu
7. Monitor, SC-10 AV, Shimadzu
8. Buffers

Figure 3-2. LCMS system

1. HPLC, Shimadzu
2. Mass Spectometer, Shimadzu

Figure 3-3. Fermentor 10 l –Bio Flo

1. Monitor
2. Fermentor Vessel

Figure 3-4. Fast Phase Liquid Chromatography (FPLC) system

1. Two channel Recorder REC-482, Pharmacia
2. LCC 500 Plus controller, Pharmacia
3. Pump A High precision pump P-500, Pharmacia
4. Pump B High precision pump P-500, Pharmacia
5. Single Path UV-monitor UV-1, Pharmacia
6. Buffers to the High precision pump
7. Frac -100 Fraction collector, Pharmacia
8. Peristaltic pump
9. DE-52 anionic exchange column
10. Buffers to the Peristaltic pump
11. 4 °C refrigerated chamber P_H-Unitherm, Puffer Hubbard Refrigerator Division, Grand Haven, Michigan

Figure 3-5. Affinity chromatography columns

1. Red-Sepharose Column
2. Mono-Q column, Pharmacia

Figure 3-6. Electrophoresis Apparatus

1. Xcell sure lock electrophorsis Cell, Invitrogen
2. Power Pack, Biorad
3. Orbital shaker, Invitrogen

CHAPTER 4

SCREENING OF FACULTATIVE ANAEROBIC BACTERIA UTILIZING D-XYLOSE FOR XYLITOL PRODUCTION

Bacterial strain selection

Seventeen facultative bacterial strains belonging to the genera of *Serratia*, *Cellulomonas* and *Corynebacterium* were screened for xylitol production. Serratia strains are gram-negative and can grow in temperature range of 10 –30 °C with a G+C content of 52-60% (Grimont and Grimont, 1984). *S. marcescens* NRRL B 284, NRRL B 486, NRRL B 3401 and *S. grimessii* NRRL B-4272 were obtained from National Center for Agricultural Utilization Research (NCAUR), Peoria, Ill.

Cellulomonas genera are gram positive with a DNA base composition 71 –76 mol% G+C (Tm) and grows at an optimum temperature of 30° C (Stackebrandt and Keddie, 1984). The metabolism is primarily respiratory but also have fermentative metabolism. *Cellulomonas cellulans* strains NRRL B-2381, NRRL B-4567, *Cellulomonas fimi* strains NRRL B-402, NRRL B-403 were obtained from NCAUR collection and *Cellulomonas cellulans* strain ATCC 12830 and *Cellulomonas turbata* strain ATCC 25835 were obtained from American Type Culture Collection (Manassas, VA).

Corynebacterium genera are gram positive, irregular non-sporing bacteria, optimum temperature 30 –37 °C and have a DNA base composition of 51-63 mol% G+C (Collins and Cummins, 1984). *Corynebacterium glutamicum* strains NRRL B-2784, NRRL B-3330, *Corynebacterium variabilis* strain NRRL B-4201, *Corynebacterium ammoniagenes* strain NRRL B-4246, *Corynebacterium* sp. strain NRRL B-4247, and *Propionibacterium acnes* strain NRRL B-4224, were obtained from NCAUR and *Corynebacterium vitaeruminis* strain ATCC 10234 were obtained from American Type Culture Collection (Manassas, VA).

Screening of cultures in solid media

Ten of the 17 cultures of facultative bacteria screened produced xylitol at detectable levels when D-xylose was used as the carbon source. Screening for xylitol was initially conducted using a filter paper assay (Fig. 4-1) of cultures grown on solid agar containing 70 g/l D-xylose in their respective medium in Petri dish (Fig.4-2). The whole-cell extract of *Candida tropicalis* ATCC 96745 and Sorbitol dehydrogenase (SDH) were used as enzyme sources for the assay. The SDH enzyme assay developed color faster than the yeast whole-cell extract assay. The color of the filter paper changed to reddish orange in ten cultures. The ten strains were *C. cellulans* ATCC 12830, NRRL B-4567 and NRRL B-2380, *C. glutamicum* NRRL B-3330 and NRRL B-2784, *C. ammoniagenes* NRRL B-4246, *Corynebacterium* sp. NRRL B-4247, and *S. marcescens* NRRL B-284, B-486 and NRRL B-3401. The intensity of color and the time of development varied between 2 to 10 h. Seven bacterial strains did not change the filter paper color.

Screening of cultures in liquid media

Cultures of Serratia *marcescens* strains B-284, B 486 and B-3401 showed steady growth (Fig. 4-3) and produced xylitol in medium containing 100 g/l D-xylose. Although liquid cultures of *Serratia grimesii* B-4272 grew in D-xylose concentrations of 70 g/l and 100 g/l, they did not produce any color change in the assay.

C. cellulans strains NRRL B-2381, NRRL B-4567 and ATCC 12830 grew in 100 g/l D-xylose (Fig. 4-4) but *C. fimi* NRRL B-402, NRRL B-403 and *C. turbata* ATCC 25835 strains did not grow and did not produce any color change in the assay. Growth of *C. cellulans* ATCC 12830, NRRL 4567 was slower whereas *C. cellulans* B-2381 achieved maximum growth in 24 h.

Corynebacterium glutamicum strains B-3330 and B-2784, *Corynebacterium* sp. B-4247 and *Corynebacterium ammoniagenes* NRRL B-4246 grew in 100 g/l D-xylose (Fig. 4-5) and produced reddish orange color in the assay.

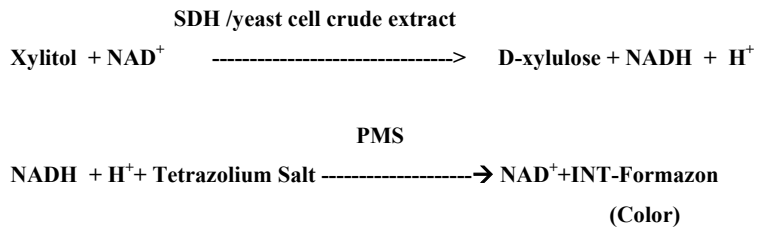


Figure 4-1. Chromogenic assay reaction for xylitol (Beutler, 1984)

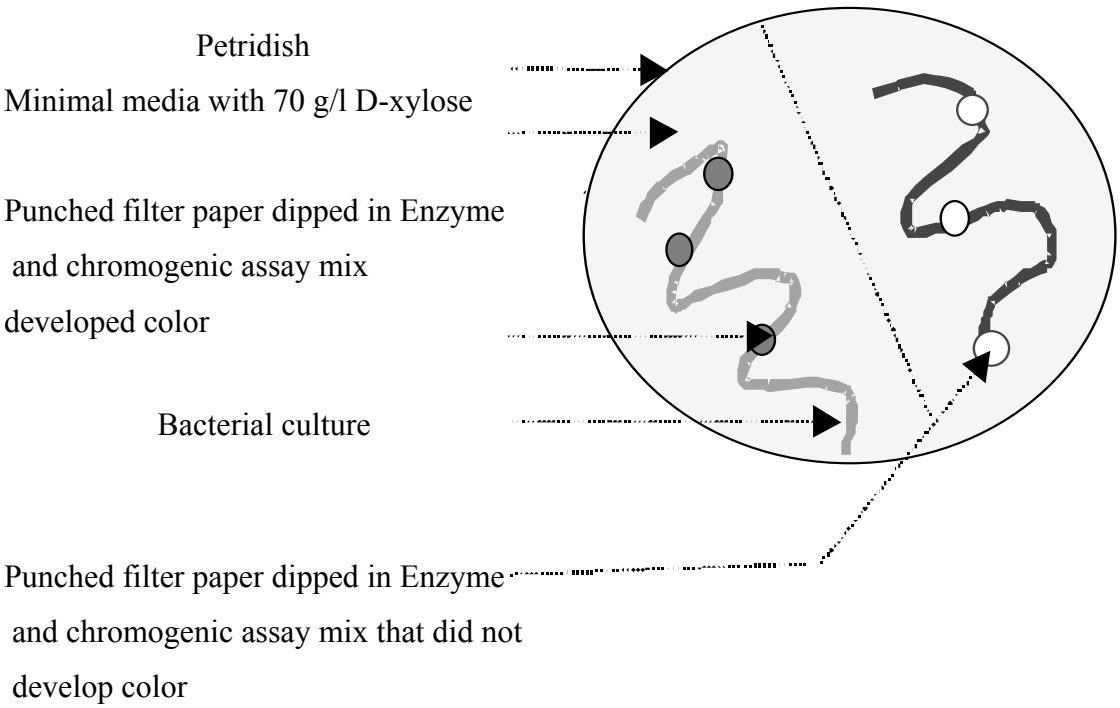


Figure 4-2. Assay on cultures grown in Petri dish

Sterilized punched filter paper bits soaked in $NADP^+$, PMS, Tetrazolium salt and enzyme (SDH or crude cell extract *of Candida tropicalis*) was placed on facultative bacterial cultures grown in media having 70 g/l D-xylose. Filter paper color change to reddish orange (●) in 2-10 h. Filter paper color remained colorless (O).

Corynebacterium variabilis B-4201, *Corynebacterium vitaeruminis* ATCC 10234 and *Propionibacterium acnes* B-4224 did not grow in 100 g/l D-xylose and did not produce color change in the assay. *Corynebacterium glutamicum* NRRL B-2784 grew faster and achieved maximum growth in 24 h compared to other Corynebacterium strains that had a steady slow growth.

Fermented broth of Serratia, Cellulomonas and Corynebacterium cultures assayed for the xylitol presence using Sorbitol dehydrogenase (SDH) is shown in Figures 4-6, 4-7 and 4-8 respectively. The assay developed color within the first two minutes to reddish orange and remained stable. The color development of Serratia, Cellulomonas and Corynebacterium fermented broth when assayed for xylitol with yeast whole-cell extract as the enzyme source is shown in Figures 4-9, 4-10 and 4-11 respectively. The absorbance at 650 nm in the SDH assay was higher than that of the yeast dehydrogenase assay. The cultures that changed the color in the liquid culture assays also changed color in the solid cultures filter-paper assays. In the yeast cell extract assay the absorbance continued to increase after 10 min for the Serratia and Corynebacterium while for the Cellulomonas strains B 2381, B 4567 and ATCC 12830 it started decreasing.

Quantification of xylitol

The xylitol present in the liquid culture broths was quantified using HPLC. The HPLC results are shown in Table 4-1. Similar to the assay results no detectable xylitol was present in the cultures of *S. grimesii* B-4272, *C. fimi* NRRL B-402, NRRL B-403 and *C. turbata* ATCC 25835 strains though these cultures grew in 100 g/l D-xylose concentrations. *C. variabilis* B-4201, *C. vitaeruminis* ATCC 10234 and *P. acnes* B-4224 did not grow in 100 g/l D-xylose and did not produce xylitol. *Corynebacterium* sp. B4247 (0.81 g/l) produced the highest amount of xylitol.

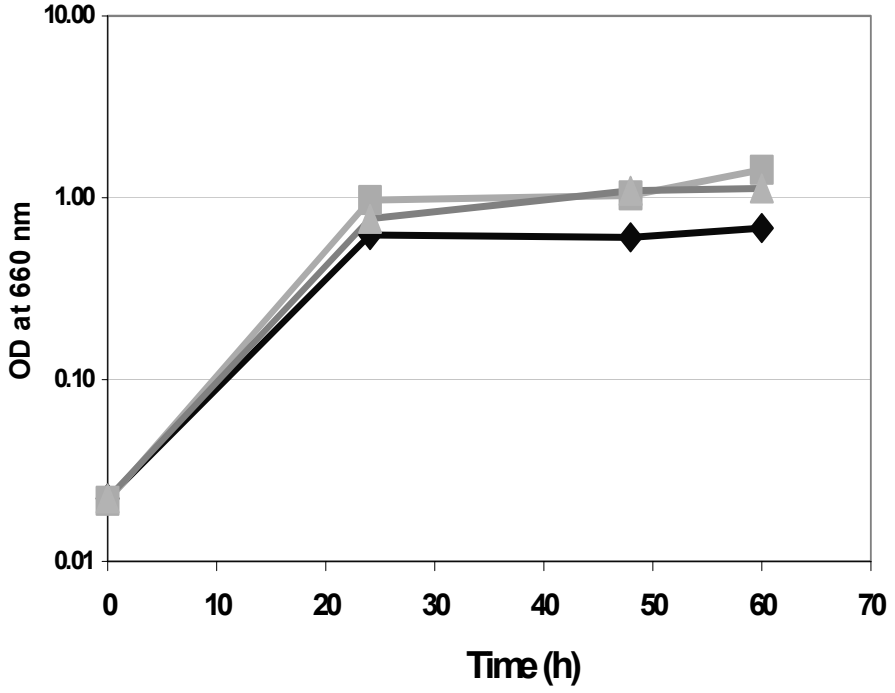


Figure 4-3. *S. marcescens* strains growth in medium containing 100 g/l D-xylose: *S. marcescens* B 284 (♦), B 486 (■), B 3401 (▲)

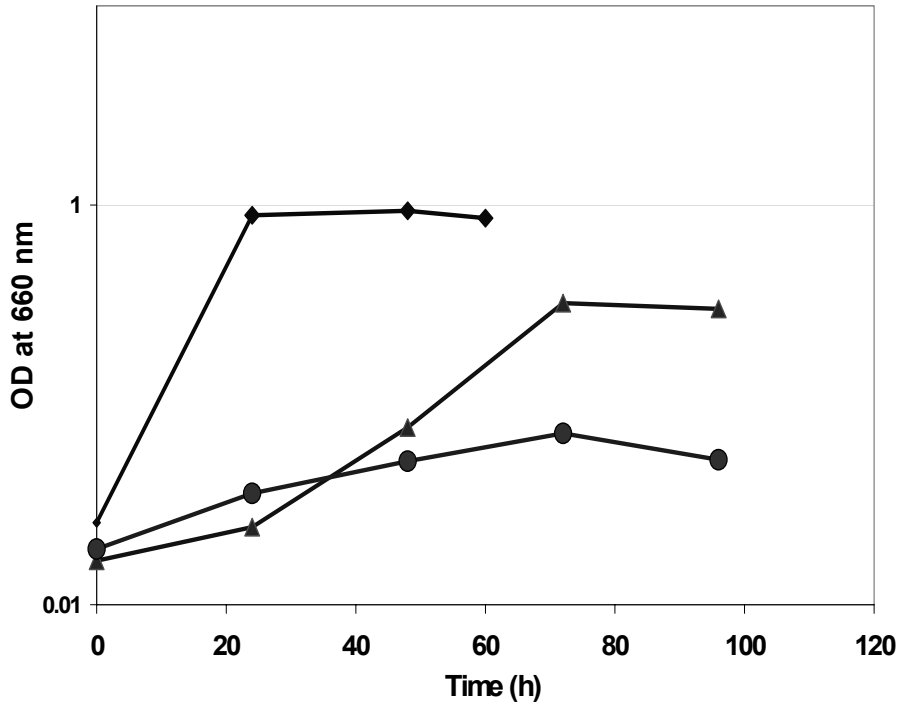


Figure 4-4. Growth of Cellulomonas strains in Cellulomonas medium containing 100 g/l D-xylose: *C. cellulans* ATCC 12830 (●), B 2381* (♦), B 4567(▲)

*B 2381 was grown for 60 h time

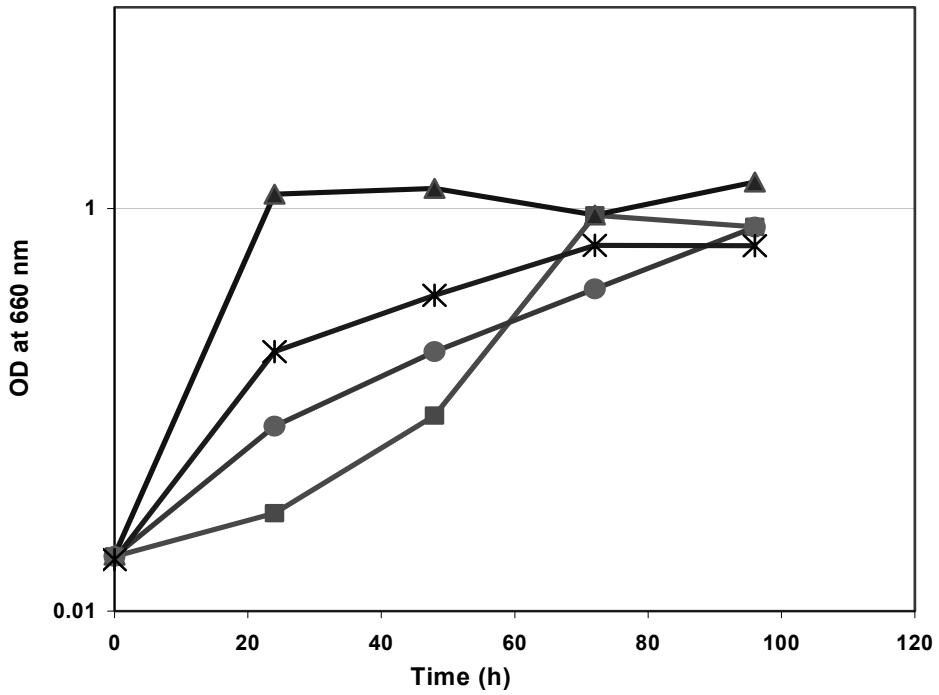
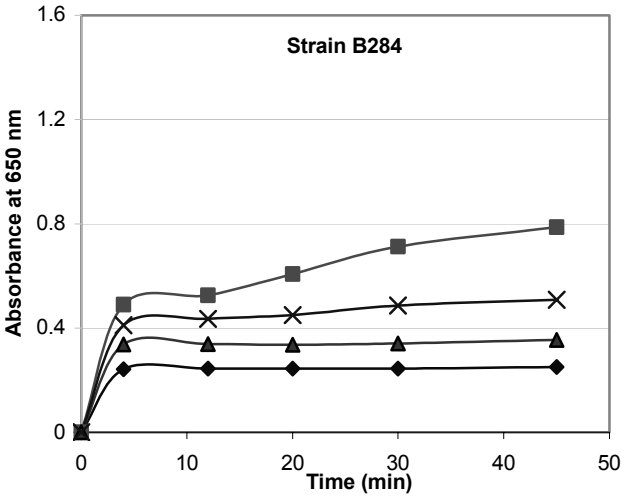
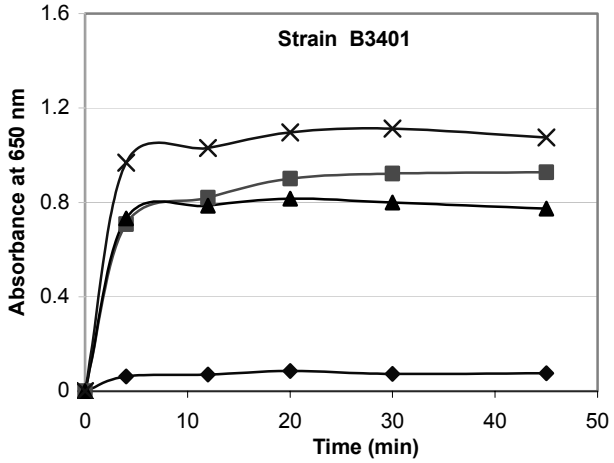


Figure 4-5. Growth of Corynebacterium strains in liquid medium containing 100 g/l D-xylose: *C. glutamicum* NRRL B 2784 (▲), *C. glutamicum* NRRL B 3330 (■), *C. ammoniagenes* B 4246 (●), and *Corynebacterium* sp. B 4247 (*)





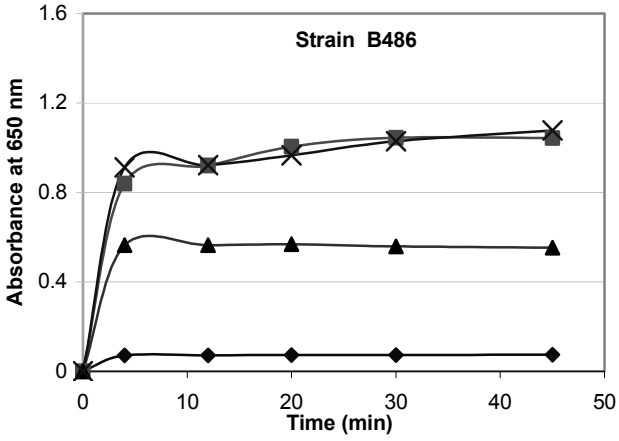


Figure 4-6. Assay for xylitol using sorbitol dehydrogenase for strains of *S. marcescens* B 284, B 486, B 3401 grown for various times: ♦0 h culture, ■24 h culture, ▲48 h culture, x 60 h culture

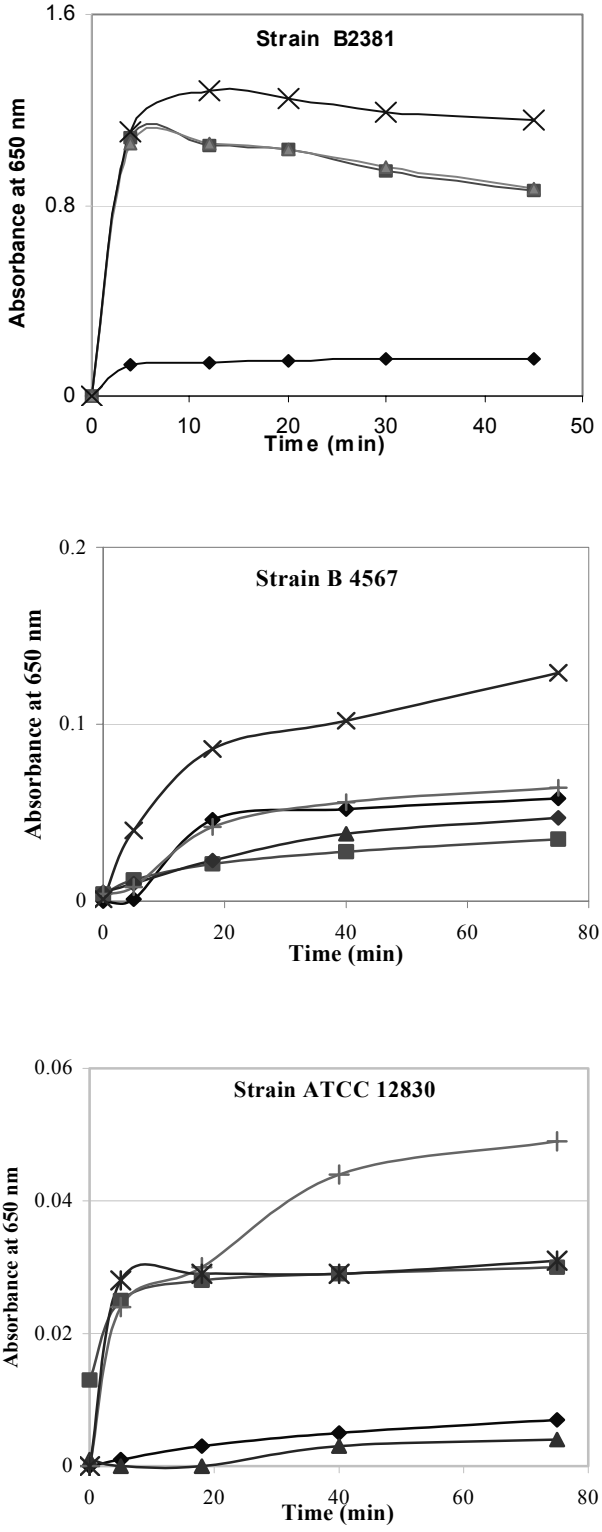


Figure 4-7. Assay for xylitol using sorbitol dehydrogenase for *C. cellulans* B 2381, B 4567 and ATCC 12830 grown for various times: ♦0 h culture, ■24 h culture, ▲48 h culture, + 72 h culture, x 96 h culture

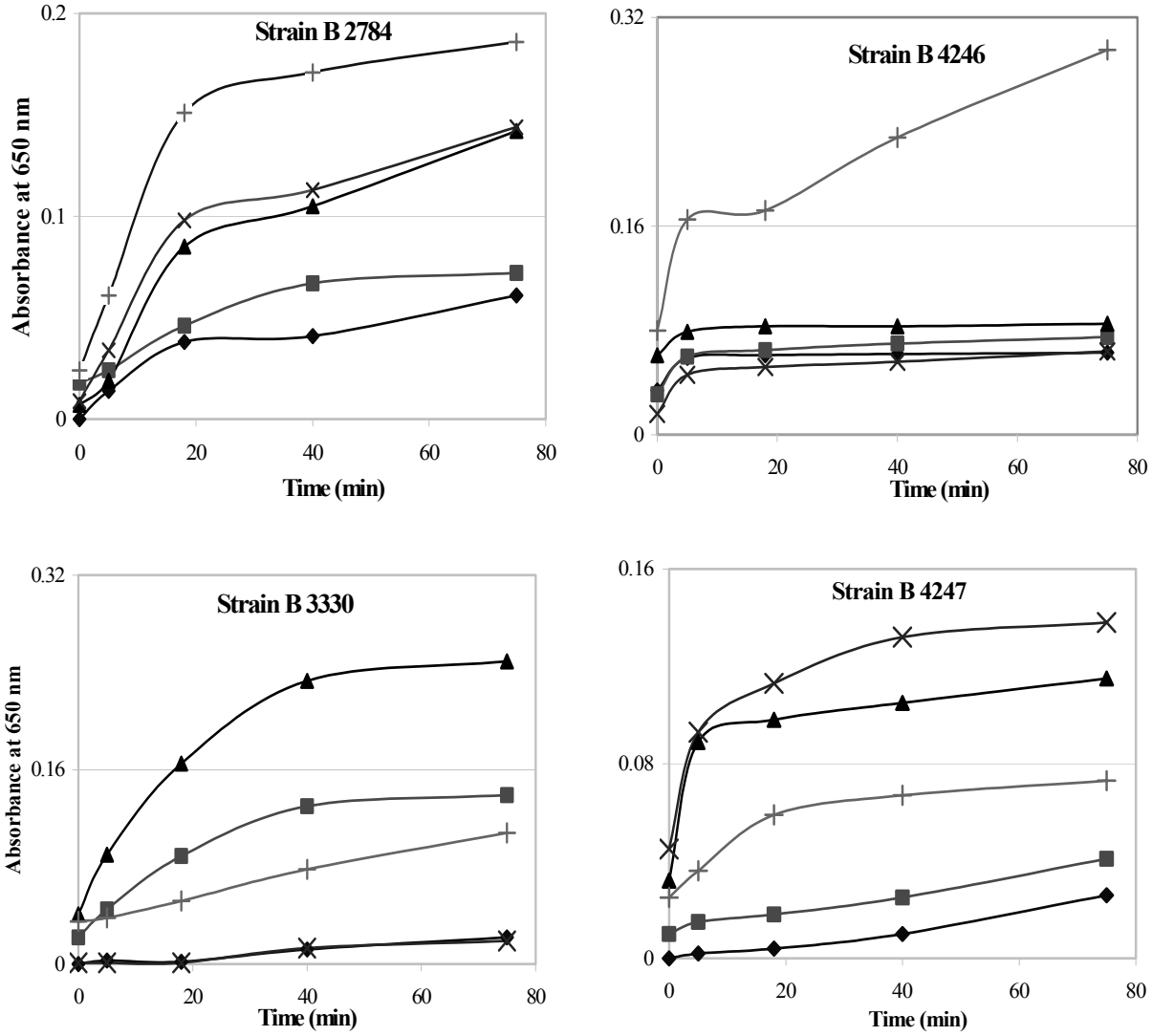
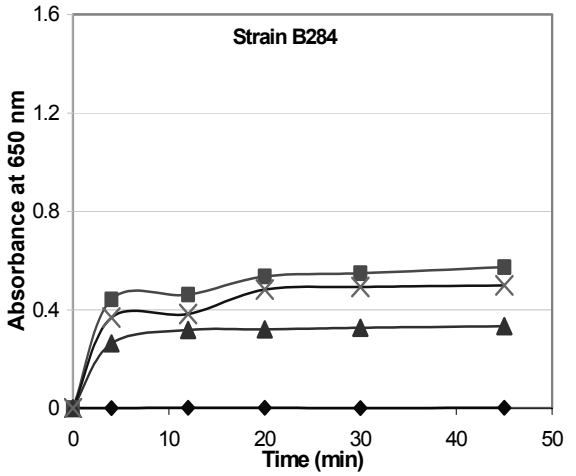
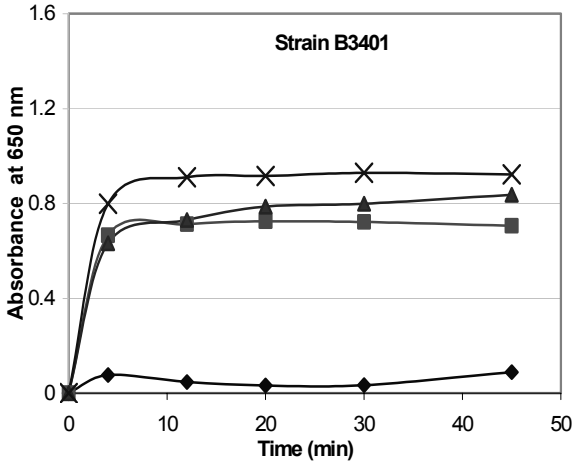


Figure 4-8. Assay for xylitol using sorbitol dehydrogenase for Corynebacterium strains *C. glutamicum* B 2784, B 3330, *C. ammoniagenes* B 4246 and *Corynebacterium* sp. B 4247 grown for various times: ♦0 h culture, ■24 h culture, ▲48 h culture, + 72 h culture, x 96 h culture





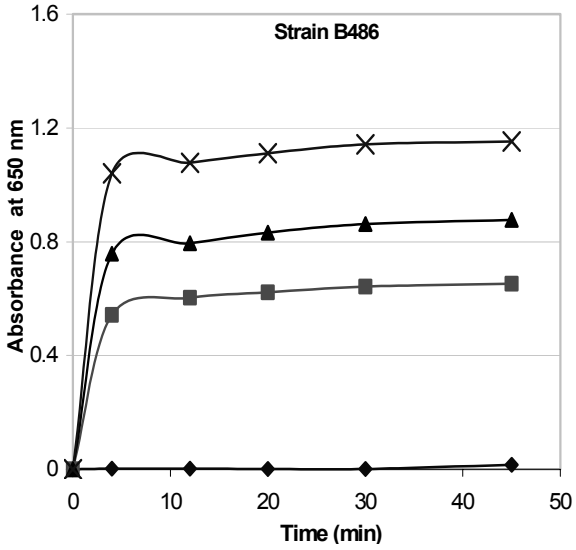


Figure 4-9. Assay for xylitol using crude yeast cell extract for strains of *S. marcescens* B 284, B 486 and B 3401 grown for various times: ♦0 h culture, ■24 h culture, ▲48 h culture, x 60 h culture

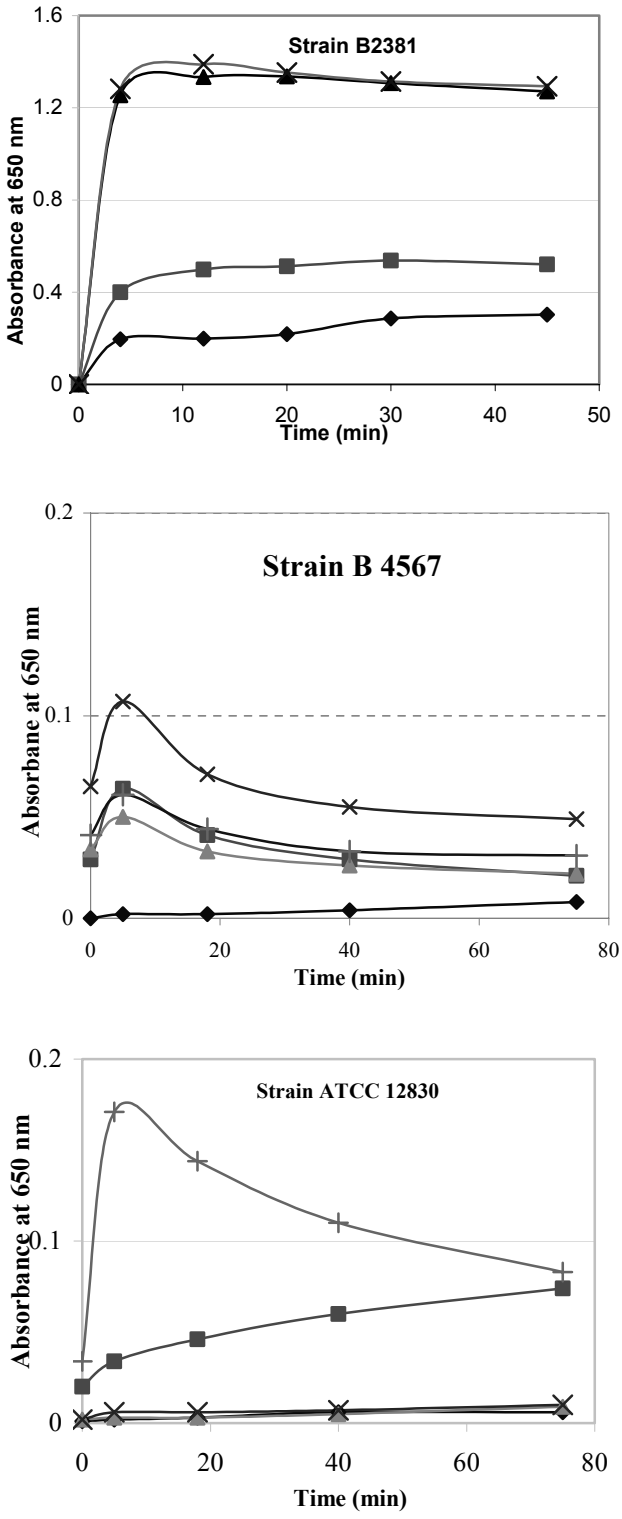


Figure 4-10. Assay for xylitol using crude yeast cell extract for strains of *C. cellulans* B 2381, B 4567 and ATCC 12830 grown for various times: ♦0 h culture, ■24 h culture, ▲ 48 h culture, + 72 h culture, x 96 h culture

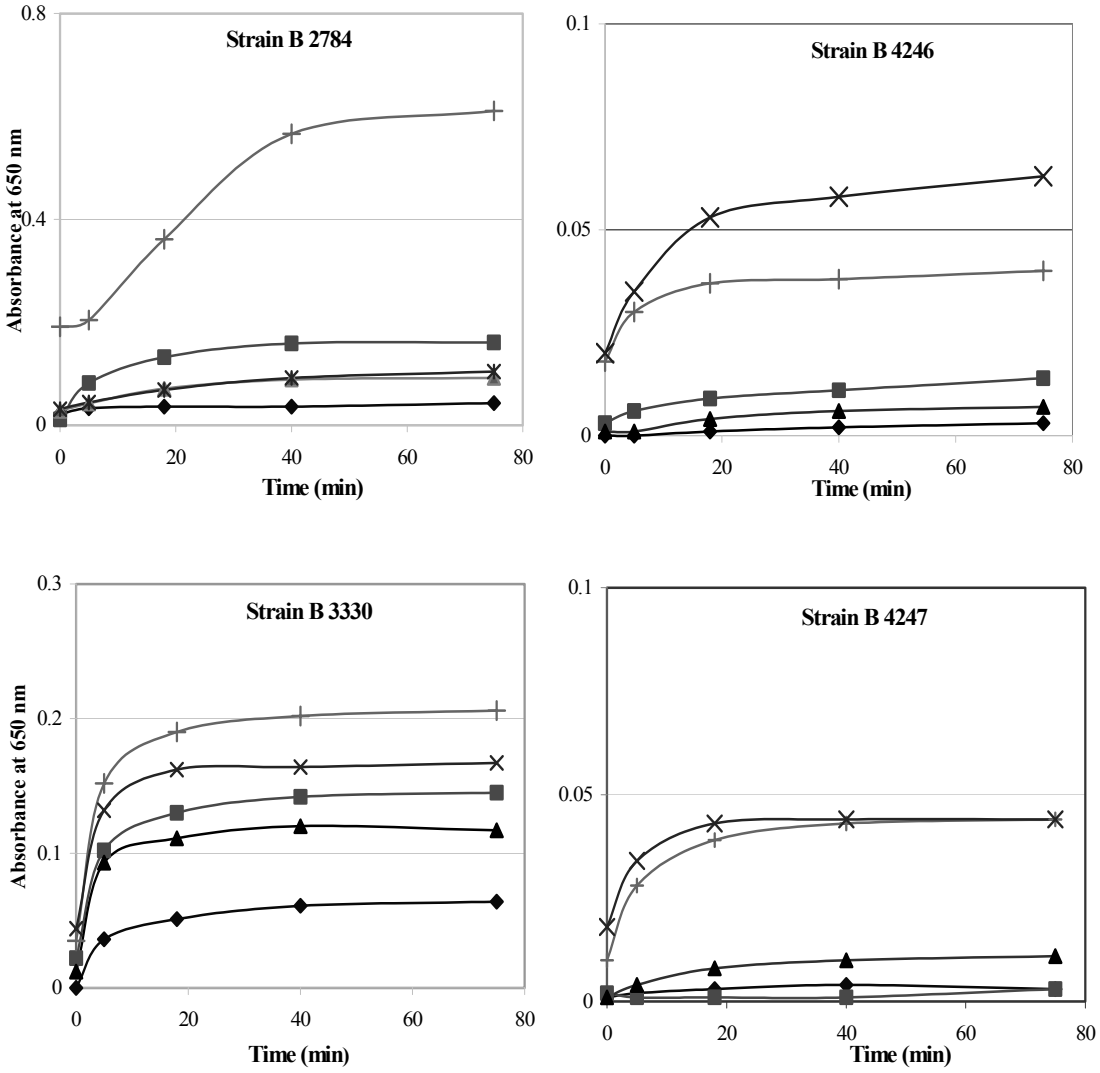


Figure 4-11. Assay for xylitol using crude yeast cell extract for strains of *C. glutamicum* B 2784, B 3330, *C. ammoniagenes* B 4246 and *Corynebacterium* sp. B 4247 grown for various times: ♦0 h culture, ■24 h culture, ▲48 h culture, + 72 h culture, x 96 h culture

Effect of culture conditions on xylitol production in screened cultures

S. marcescens strains B-284, B-486 and B-3401, *C. cellulans* strains B-2381, B-4567 and ATCC 12830 and *Corynebacterium* cultures *C. ammoniagenes* B-4246*, C. glutamicum* strains B-3330, B-2784 and *Corynebacterium* sp. B-4247 were investigated for xylitol production in the presence of high initial substrate concentrations (250 g/l and 350 g/l D-xylose), high temperature (45 °C) and simulated anaerobic conditions. Cultures that grew and accumulated xylitol in the media are listed in Table 4-2.

Serratia marcescens B-3401 showed good growth and produced xylitol in 250 g/l D-xylose (aerobic) condition and under simulated anaerobic condition (100 g/l D-xylose). Growth of this strain decreased after 48 h, but xylitol production continued until 96 h. *S. marcescens* B-284 grew under anaerobic conditions, but no xylitol was produced.

C. cellulans ATCC 12830 produced 2.5 g/l ethanol after 96 h fermentation using 250 g/l D-xylose substrate. Under anaerobic conditions *C. cellulans* ATCC 12830 produced 1.51g/l ethanol after 96 h when the initial xylose concentration was 100 g/l. *Corynebacterium* sp. B-4247 grew in D-xylose concentrations of 250 g/l and 350 g/l but produced xylitol only in 250 g/l D-xylose. The highest xylitol yield under this condition occurred at 48 h. Although growth of this strain was better under anaerobic conditions it did not produce xylitol. It also grew at 45 °C for 48 h but did not produce xylitol. *C. ammoniagenes* B-4246 grew very slowly in 250 g/l D-xylose and produced xylitol (0.68 g/l) and ethanol (1.55 g/l) after 96 h fermentation. The strain grew comparatively better under anaerobic conditions but it did not produce xylitol. *C. glutamicum* B-2784 showed very good growth in both D-xylose concentrations and moderate growth at 45 °C. Under anaerobic conditions, growth slowed down after 48 h. Although *C. glutamicum* B-2784 grew well under all the conditions (250 g/l and 350 g/l initial D-xylose, 45 °C and simulated anaerobic conditions), it did not produce xylitol. *C. glutamicum* B-3330 also grew well under anaerobic conditions but it did not grow in 350 g/l D-xylose. *C. glutamicum* B-3330 produced xylitol (0.22 g/l) only at the lower D-xylose concentration and produced 1.50g/l ethanol at 45 °C at 96 h.

Table 4-1. Xylitol production of bacterial strains screened in 10-ml liquid cultures with an initial D-xylose concentration of 100 g/l. Standard deviation in parentheses.

+ Xylitol yield less than 0.01 g/(g xylose), ++ xylitol yield between 0.01 and 0.015 g/(g xylose), +++ xylitol yield greater than 0.015 g/(g xylose)

Bacterial culture	Bacterial strain	Growth time (h)	Xylitol (g/l)	Xylitol yield
Cellulomonas cellulans	ATCC 12830	72	0.19 (0.01)	++
		96	0.06 (0.00)	+
	NRRL B-4567	96	0.19 (0.00)	+++
	NRRL B-2381	24	0.09 (0.00)	+
		48	0.38 (0.01)	++
		60	0.51 (0.06)	+++
Corynebacterium glutamicum	NRRL B-2784	72	0.15 (0.04)	+
		96	0.24 (0.04)	++
	NRRL B-3330	72	0.12 (0.00)	+
		96	0.12 (0.06)	+
Corynebacterium ammoniagenes	NRRL B-4246	72	0.11 (0.00)	+
		96	0.13 (0.00)	+
Corynebacterium sp.	NRRL B-4247	48	0.42 (0.00)	+++
		72	0.47 (0.06)	+++
		96	0.81 (0.20)	+++
Serratia marcescens	NRRL B-284	24	0.25 (0.00)	++
		60	0.24 (0.00)	+
	NRRL B-486	60	0.14 (0.00)	+
	NRRL B-3401	60	0.35 (0.12)	+

Table 4-2. Effect of culture conditions on xylitol production in liquid cultures. Standard deviation in parentheses. + Xylitol yield less than 0.01 g /(g xylose), ++ xylitol yield between 0.01 and 0.015 g /(g xylose), +++ xylitol yield greater than 0.015 g /(g xylose)

Culture condition	Bacterial culture	Growth time (h)	Xylitol (g/l)	Xylitol yield
Aerobic: 250 g xylose/l	*S.marcesens* B-3401	48	0.24 (0.00)	+
		96	0.71 (0.15)	+++
	C.cellulans B-4567	96	1.76 (0.00)	+++
	C.cellulans ATCC 12830	96	1.39 (0.22)	+++
	C.glutamicum B-3330	96	0.22 (0.04)	++
	Corynebacterium sp. B-4247	48	0.45 (0.22)	+++
		96	0.71 (0.52)	++
	C.ammoniagenes B-4246	96	0.68 (0.03)	+++
Anaerobic condition: 100 g xylose/l	*S.marcesens* B-3401	48	0.21 (0.13)	+
		96	0.29 (0.00)	+++

Discussion

Selection of *Corynebacterium* sp. B 4247

The assay used in the present study offers a fast and reliable method to screen cultures for xylitol production. Results from the solid cultures were confirmed spectrophotometrically in liquid cultures. Furthermore, the xylitol produced in the small-volume liquid cultures was quantified by HPLC.

The 24 h culture of the *S. marcescens* B 284 showed higher absorbance than the 60 h grown cultures in the SDH assay and yeast crude cell extract assay. This result was confirmed in HPLC as the xylitol produced in the 24 h culture was more than the 60 h culture. This suggests that the xylitol was probably utilized for growth. This is consistent with Grimont and Grimont (1984) that some *S. marcescens* strains are capable of utilizing xylitol for growth. In the case of *S. marcescens* B 3401 the xylitol accumulation difference in the 24 h, 48 h and 60 h was small in both assays. Though in *S. marcescens* B 486, the 24 h and 60 h grown cultures showed xylitol presence in the assay the xylitol was only quantifiable in the HPLC for the 60 h culture. The xylitol from the 24 h culture was below the detection limit of the HPLC.

The variations observed between the repeated screenings could have been due to agitation difference in the low volumes in the screw-cap tubes. The results obtained using the two enzyme assays complemented one another with respect to overall color development in the cultures, but varied with respect to color intensity over time. This variation between the two assays could be due to the various cellular components present in addition to the dehydrogenase in the yeast crude cell extract. This is evident especially in the case of the *Cellulomonas* strains B 4567, B 2381 and ATCC 12830 where there was a marked decrease in the absorbance using yeast crude cell extract assay while the decrease is not evident in the SDH assay. The cellular components could have been involved with the color development or reacted in the assay. However, yeast whole-cell extract offers an economical enzyme source for the assay.

Corynebacterium sp. B-4247 showed the highest levels of xylitol production as well as sustained production in high xylose concentrations. Moreover, it grew under simulated anaerobic conditions (100 g/l D-xylose) and at 45 °C for 48 h making it a suitable candidate.

CHAPTER 5

CORYNEBACTERIUM SP. B-4247 CULTURE CONDITIONS

To maximize xylitol production, the growth conditions for *Corynebacterium* sp. B 4247 have to be optimized. Initial substrate concentration, aeration, media, inoculum density, temperature, pH, xylose induction, glucose effect, mixed substrates and nitrogen source are major environmental factors that affect cell growth and xylitol production (Parajo *et al*. 1998b).

Initial screening experiments of *Corynebacterium* sp. B 4247 were conducted at 30º C and 45º C. Initial substrate concentration, aeration, media composition and D-xylose induction are important parameters that could influence the metabolism and the substrate utilization pattern of the organism for xylitol production.

Initial substrate concentration

Corynebacterium sp. B-4247 was studied for initial substrate concentration effect at 20 g/l, 75 g/l and 100 g/l D-xylose in Corynebacterium medium. *Corynebacterium* sp. B 4247 produced xylitol at all D-xylose initial concentrations (Fig. 5-1, 5-2, 5-3). Cell growth and xylose consumption at initial substrate concentrations of 20 g/l, 75 g/l (Fig. 5-1, 5-2) and 100 g/l (Fig. 5-3) were similar. Xylitol accumulation increased with increasing initial D-xylose concentration. The influence of initial substrate concentration on xylitol yield is shown in Figure 5-4. The maximum yields of xylitol were obtained at 24 h for all the three initial concentrations and the highest yield (0.57 g/g) was obtained with an initial xylose concentration of 75 g/l.

Aeration

The effect of aeration on *Corynebacterium* sp. B 4247 was investigated in cultures grown in 100 ml, 200 ml, 350 ml and 450 ml volumes containing 75 g/l D-xylose in Corynebacterium medium in 500 ml flasks. Under aerobic conditions

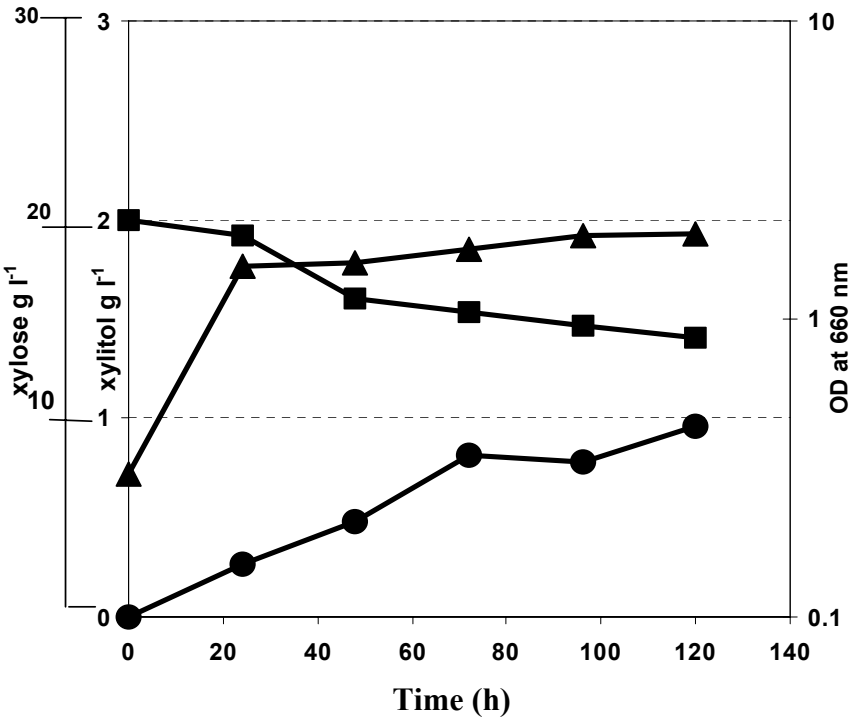


Figure 5-1. Xylitol production by *Corynebacterium* sp. B 4247 in medium containing 20 g/l initial D-xylose: D-xylose g/l (■), xylitol g/l (●), OD at 660 nm (▲)

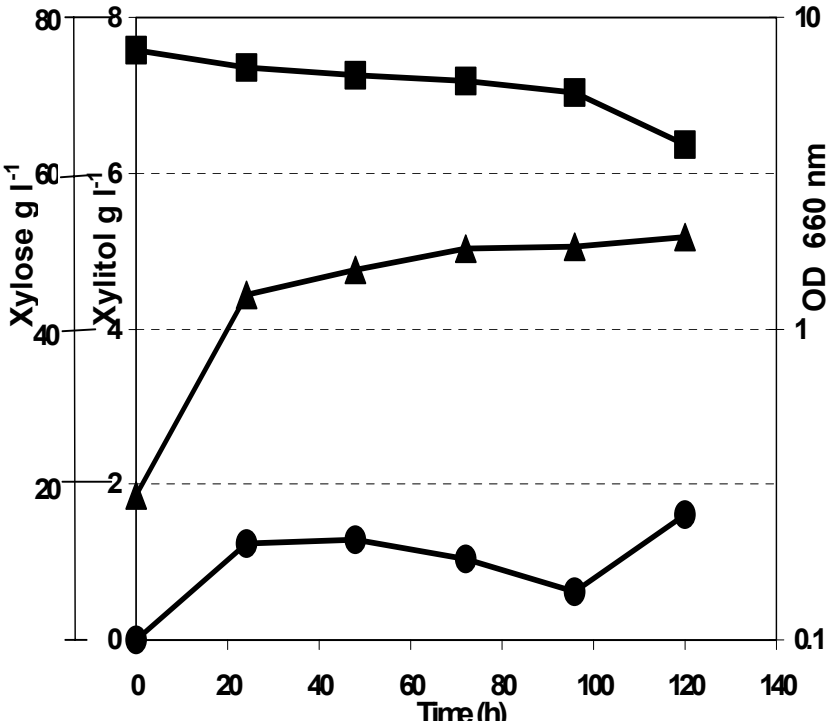


Figure 5-2. Xylitol production by *Corynebacterium* sp. B 4247 in medium containing 75 g/l initial D-xylose: D-xylose g/l (■), xylitol g/l (●), OD at 660 nm (▲)

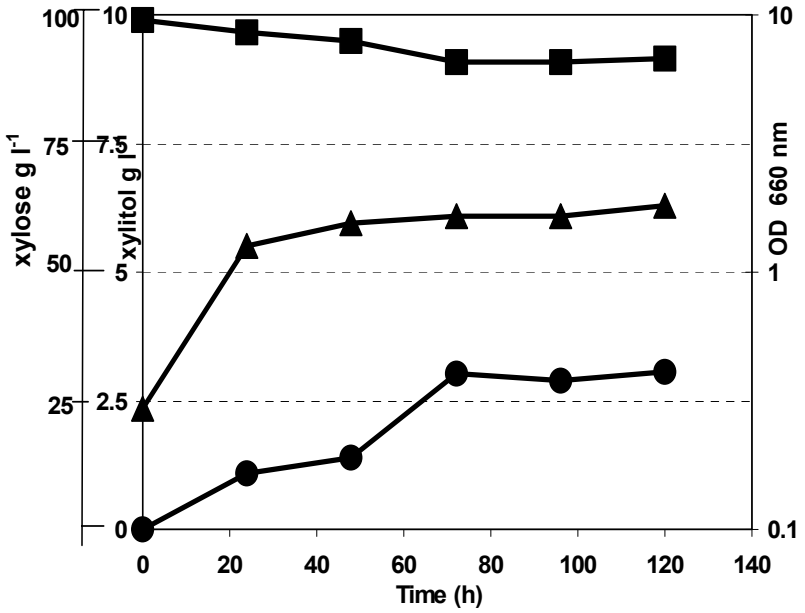


Figure 5-3. Xylitol production by *Corynebacterium* sp. B 4247 in medium containing100 g/l initial D-xylose: D-xylose g/l (■), xylitol g/l (●), OD at 660 nm (▲)

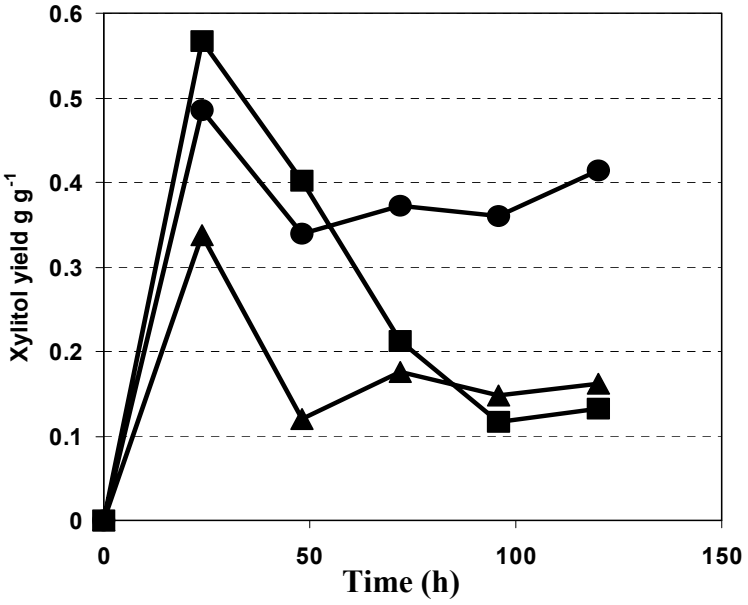


Figure 5-4. Xylitol yield in Corynebacterium medium: 20 g/l initial D-xylose concentration (▲), 75 g/l initial D-xylose concentration (■), 100 g/l initial D-xylose concentration (●)

(100 ml and 200 ml volumes) (Fig. 5-5) the xylose consumption and xylitol production increased with increase in cell growth during the initial 60 h. After 72 h and 96 h for the 100 ml and 200 ml respectively, cell growth leveled off and there was no further xylitol accumulation. The highest xylitol yield of 0.55 g/g was obtained in 100 ml volume in 24 h, when the rate of growth was highest. However the highest accumulation of xylitol was in the 200 ml volume at 96 h (3.2 g/l). Thus it appears xylitol accumulation in *Corynebacterium* sp. B 4247 is growth associated.

Under micro- anaerobic conditions (350 ml and 450 ml) (Fig. 5-6), the highest growth attained was 41.5% (350 ml) and 32.3% (450 ml) the growth attained in aerobic cultures. The cultures continued to grow slowly along with slow increase in xylitol accumulation. In the 350 ml volume the highest xylitol yield (0.2 g/g) was attained at 120 h (Fig. 5-7) when the growth was maximum whereas in the 450 ml volume the highest yield was attained at 96 h (0.14 g/g) when the growth was still increasing.

Effect of gluconate on xylitol production by *Corynebacterium* sp. B-4247

The influence of potassium gluconate on the production of xylitol by *Corynebacterium* sp. B-4247 was investigated (Fig. 5-8). When 75 g/l D-xylose was added to the medium after 48 h growth in a medium containing 96 g/l potassium gluconate the highest xylitol yield (0.39 g/g) (Fig. 5-9), was 22% less than the medium without gluconate. Furthermore, when the *Corynebacterium* medium was replaced by basal medium containing 96 g/l potassium gluconate, the yield of xylitol (0.24 g/g) decreased by over 50% relative to the amount produced in *Corynebacterium* medium without gluconate.

The xylitol accumulation increased in both the Corynebacterium medium (10.1g/l) and basal medium (5.5 g/l) in the presence of gluconate compared to the medium without gluconate. Addition of xylose in the start of the culture (instead of adding after 48 h growth in gluconate) did not produce this effect.

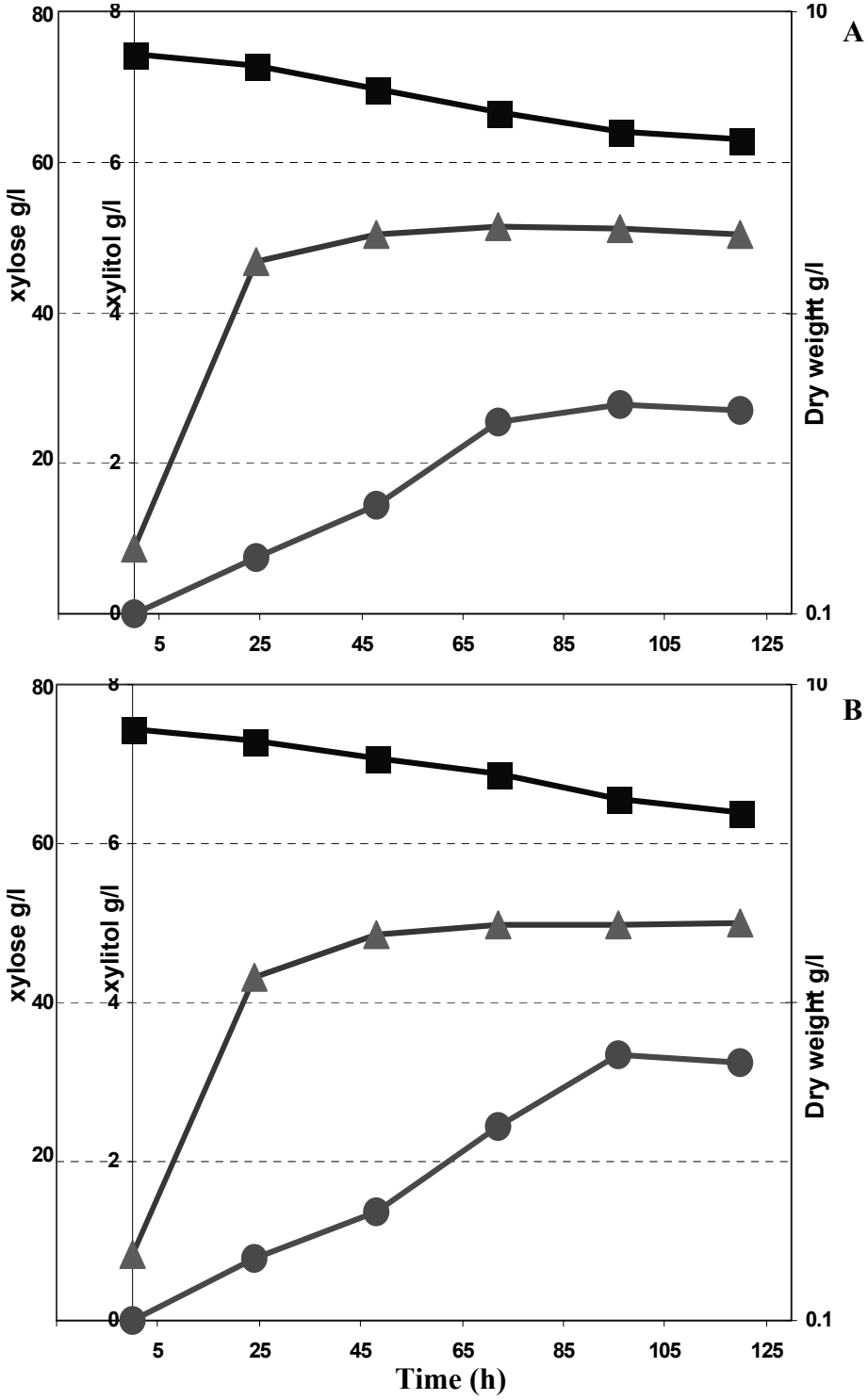


Figure 5-5. Xylitol production in medium containing 75 g/l initial D-xylose in 100 ml (A) volume and 200 ml (B) volume by *Corynebacterium* sp. B 4247: D-xylose g/l (■), xylitol g/l (●), OD at 660 nm (▲)

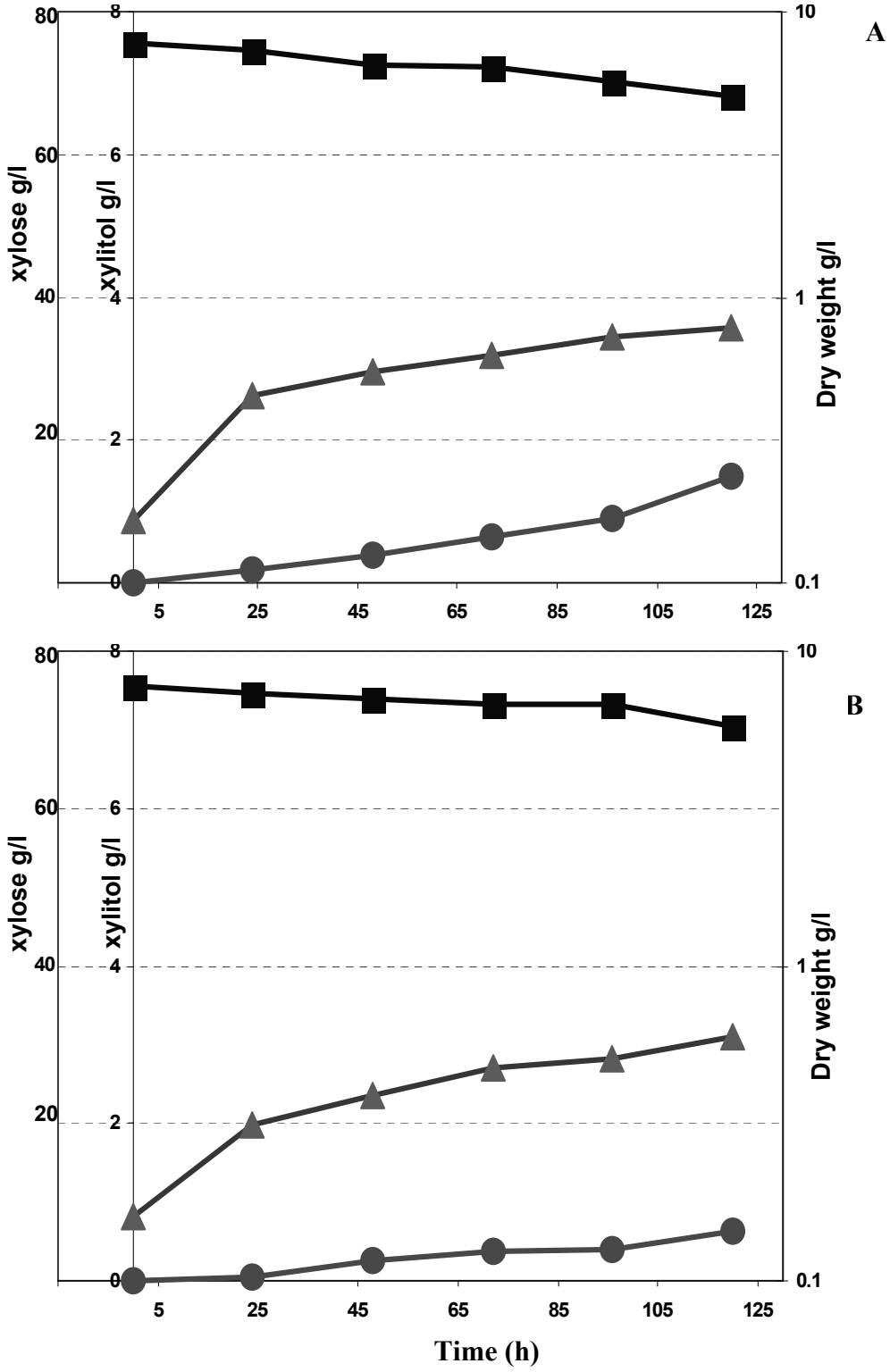


Figure 5-6. Xylitol production in medium containing 75 g/l initial D-xylose in 350 ml (A) volume and 450 ml (B) volume by *Corynebacterium* sp. B 4247: D-xylose g/l (■), xylitol g/l (●), OD at 660 nm (▲)

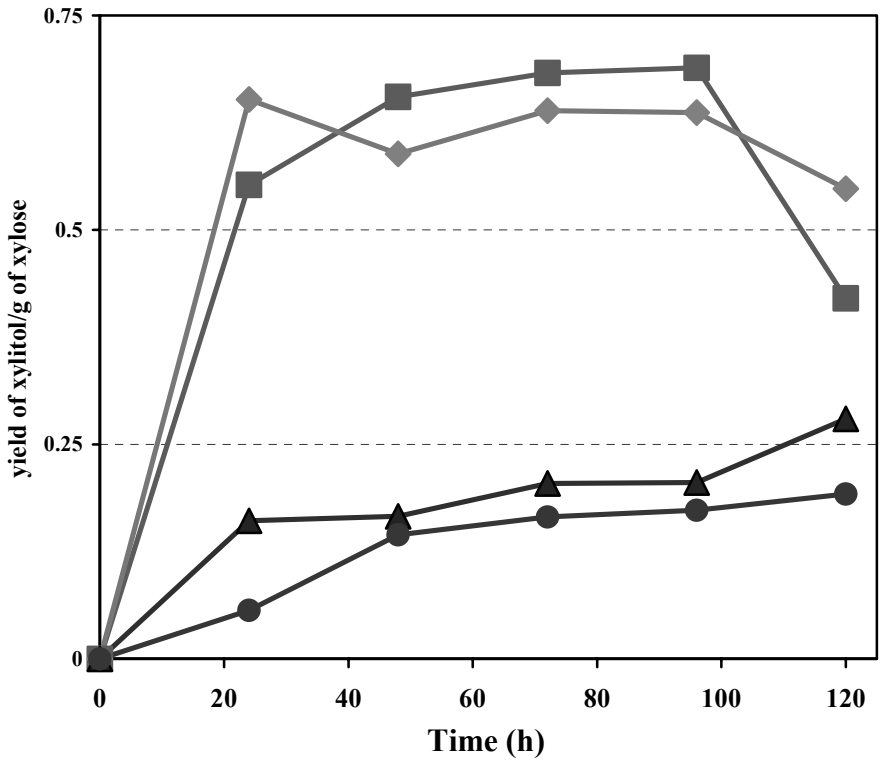


Figure 5-7. Xylitol yield in 75 g/l initial D-xylose in Corynebacterium medium volumes: 100 ml volume (■), 200 ml volume (♦), 350 ml volume (▲), 450 ml volume (●)

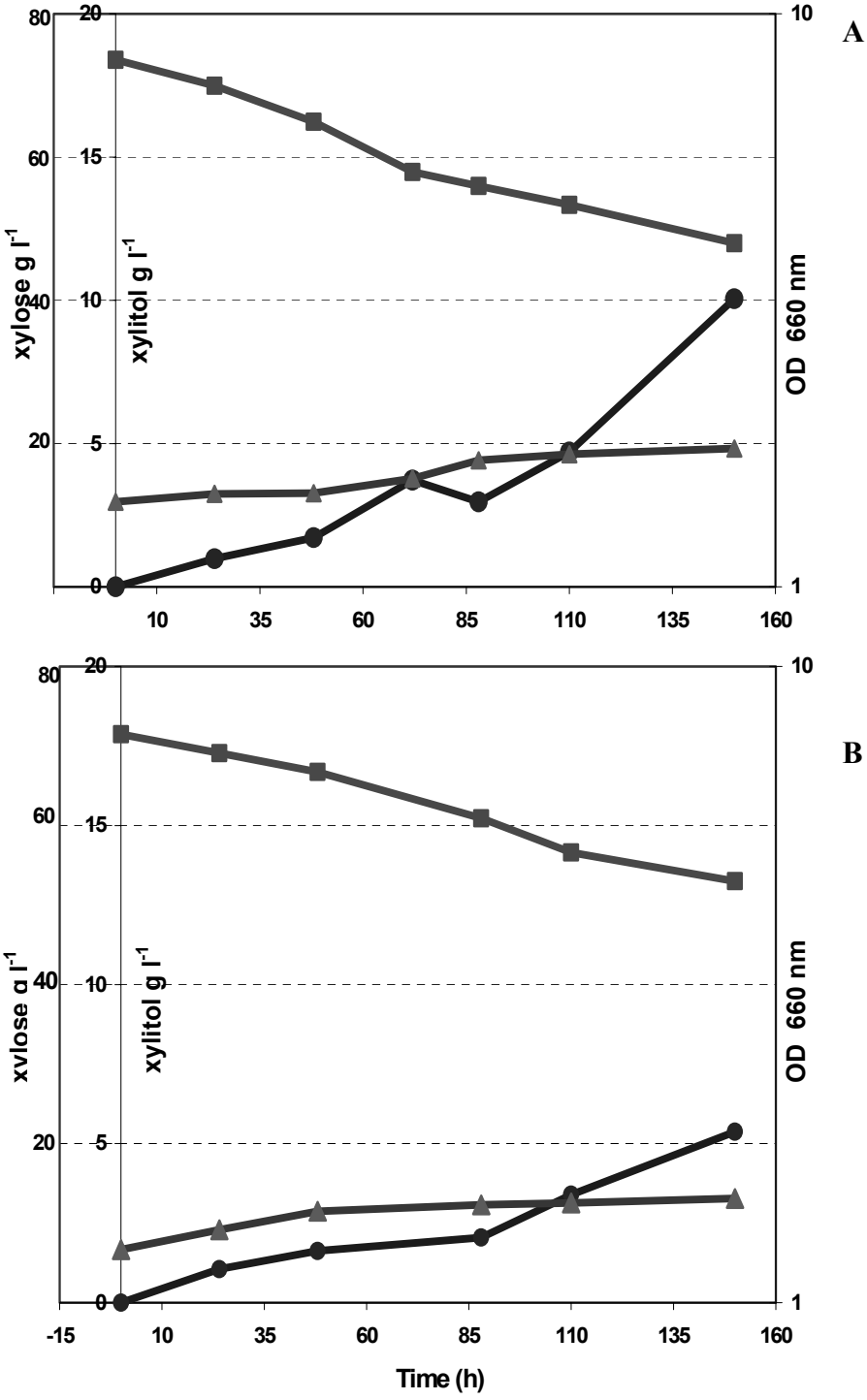


Figure 5-8. Xylitol production by *Corynebacterium* sp. B 4247 in the presence of gluconate: (A) Corynebacterium medium (75 g/l xylose); (B) Basal medium (75 g/l xylose): D-xylose (■), xylitol (●), OD at 660nm (▲)

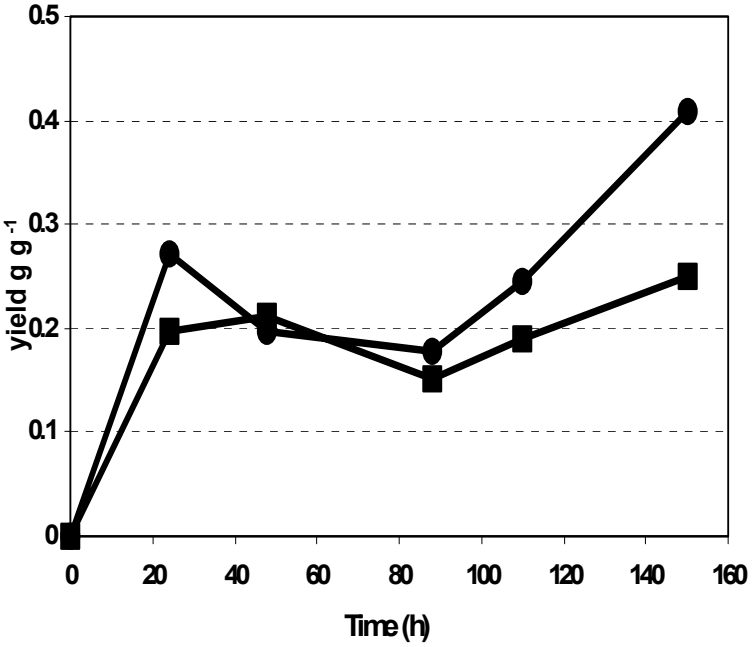


Figure 5-9. Comparison of xylitol yield in medium containing 75 g/l initial D-xylose: Corynebacterium medium (●), Basal medium (■)

Effect of glucose on *Corynebacterium* sp. B4247 xylitol production

In the production of xylitol using substrates such as crude hemicellulose hydrolyzates, glucose will always be present in the medium. Thus it is important to know what effect, if any, the sugar will have on xylitol production. Therefore the utilization of glucose by *Corynebacterium* sp. B4247 was investigated in the presence of glucose.

Corynebacterium sp. B4247 grew in glucose containing medium independent of whether the inoculum was pre-cultured in glucose or xylose. In medium containing 10 g/l glucose the cultures grew rapidly but the cells pre-cultured in glucose achieved a higher growth than those pre-cultured in xylose (Fig. 5-10 A). No xylitol was detected in the media with 10 g/l glucose. When the strain was cultured aerobically with 20-100 g/l of glucose and in the absence of xylose, no detectable xylitol was produced. However, the culture grew and produced ethanol within 48 h.

Effect of glyceraldehyde on *Corynebacterium* sp. B4247 xylitol production

When the *Corynebacterium* sp. B 4247 was grown in the presence of glyceraldehyde concentration greater than 1 g/l, the cultures neither grew nor produced xylitol. When the concentration was less than 1 g/l there was reduced growth and reduced xylitol compared to the culture that did not contain any glyceraldehyde.

Effect of inoculum

When the strain was cultured aerobically with 10 g/l D-xylose containing media the cultures had an initial lag of 4 h and a lesser growth when the inoculum was taken from a 5 g/l glucose grown culture than when the inoculum was from a 20 g/l xylose grown cultures (Figure. 5-10 B). Xylitol production was observed after 24 h cultures.

Confirmation of xylitol production by Mass spectrometry

The authenticity of the xylitol produced by *Corynebacterium* sp. B-4247 was further verified by mass spectrometry. The mass spectra of a xylitol standard in water and a concentrated xylitol fraction of *Corynebacterium* sp. B-4247 culture medium are shown in Fig. 5-11. Peak intensities at *m/z* 151, *m/z* 181 and *m/z* 197 were similar for the sample and the standard (Fig. 5-11). In the mass spectra of *Corynebacterium* sp. B 4247 cultured in medium with gluconate, additional peaks due to the gluconate and other compounds were present.

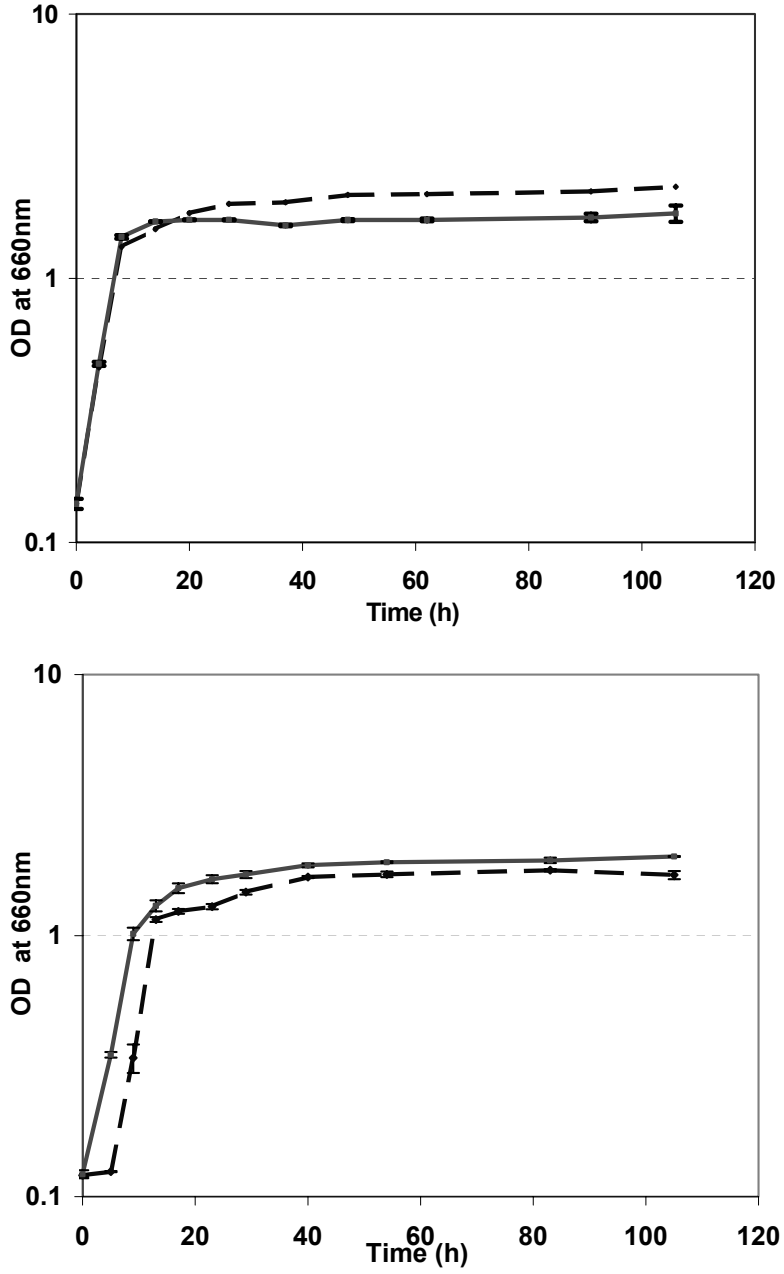


Figure 5-10. *Corynebacterium* sp. B-4247 growth in medium containing 10 g/l Glucose (A) and 10 g/l D-xylose (B) from two different inoculum: inoculum from 5 g/l glucose cultures (…), inoculum from 20 g/l D-xylose cultures (▬).

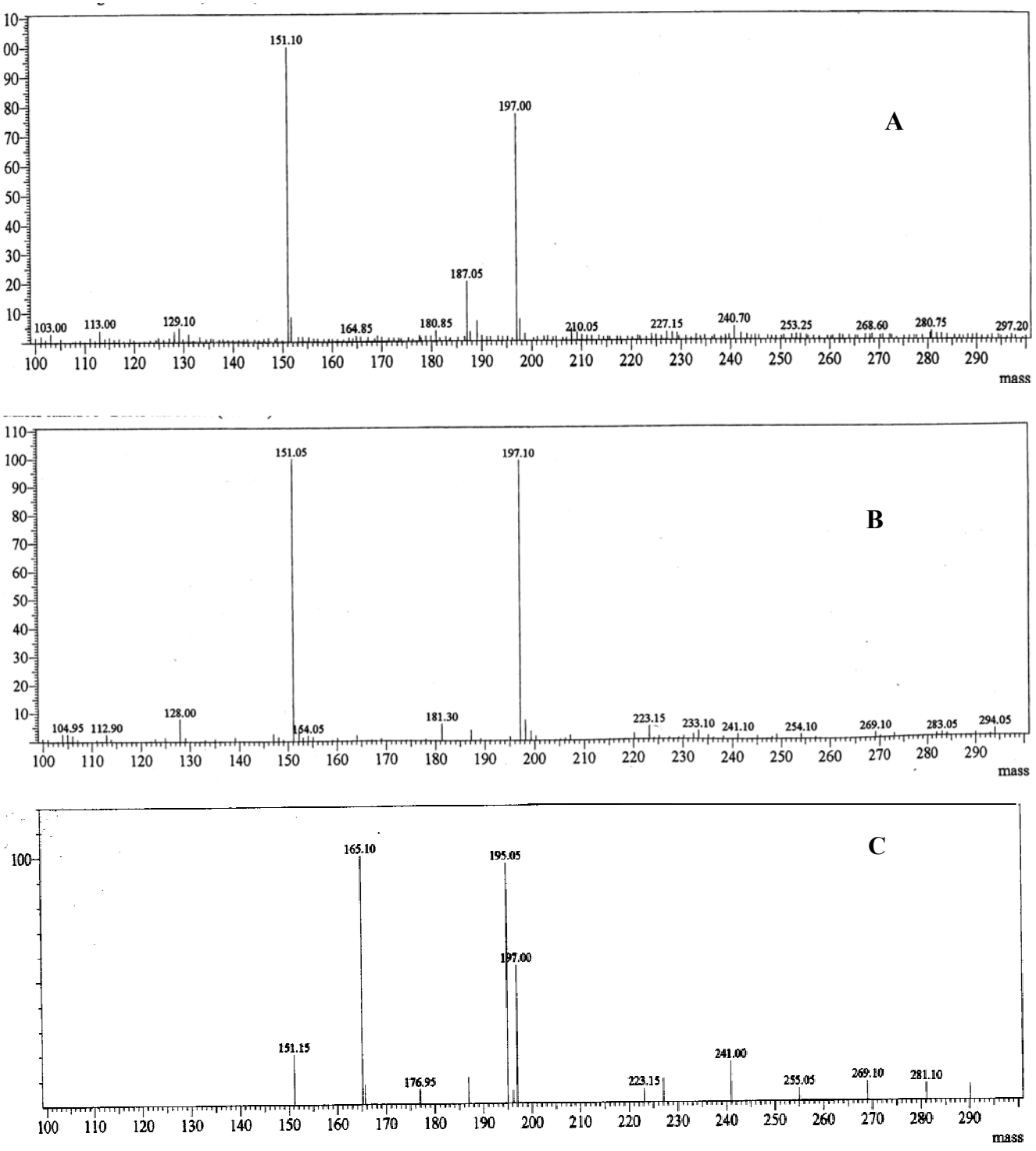


Figure 5-11. Mass spectra: standard xylitol in water (A), HPLC xylitol fraction from C*orynebacterium* sp. B 4247 fermentation broth (B), HPLC xylitol fraction from *Corynebacterium* sp. B 4247 fermentation broth grown in the presence of gluconate (C)

Discussion

In yeasts (Barbosa *et al*. 1988) the production and accumulation of xylitol in the medium increases with time until the substrate is depleted. In *Corynebacterium* sp. B-4247, xylitol production appears to be growth-associated at all three initial D-xylose concentrations. Once the stationary phase was reached, the xylitol concentration was constant although there was continuous utilization of D-xylose. As a result, the xylitol yield decreased with time. The xylitol yield was highest at 24-48 h. The maximum xylitol yields were 0.33 g/g, 0.48 g/g and 0.57 g/g for 20 g/l, 75 g/l and 100 g/l initial D-xylose concentration, respectively.

Increase in aeration increased the growth and xylitol yield in *Corynebacterium* sp. B 4247. The highest growth (1.92 g/l dry weight basis) and maximum xylitol yield (0.55 g/g) occurred in 100 ml volumes under aerobic conditions whereas growth, xylitol yield and xylitol accumulation decreased in culture volumes of 350 ml and 450 ml under anaerobic conditions in the 500 ml flasks. The xylitol yield at 24 h and 48 h in 100 ml, 200 ml, 350 ml and 450 ml cultures correlated 93% and 92%, with their respective cell growth at 24 h and 48 h. Thus the xylose metabolism and xylitol yield was coupled to higher aeration. A higher aeration increases the xylose metabolism and also a higher NADPH availability. In the aeration conditions the availability of NADPH is due to the recycling of NADPH in the pentose phosphate pathway (Hahn *et al*., 1994). Yoshitake *et al.* (1976) observed increased concentrations of NADPH increased xylitol formation by also reducing the isomerase activity.

Xylitol accumulation in *Corynebacterium* medium was 1.7 g/l (75 g/l initial D-xylose) after 24 h and remained the same (1.8 g/l) at the end of 120 h. However, when potassium gluconate was added to the medium, xylitol accumulation increased to 10 g/l after 150 h. Rapid cell growth occurred in the presence of gluconate and also after the addition of xylose to the medium after 48 h. Xylitol accumulation started after maximum cell growth has been attained. This is in contrast to the above-described growth-associated effect. The xylose in the medium was utilized by the cells at a faster rate in the presence of gluconate than in the absence of gluconate. This was observed in both *Corynebacterium* and basal

medium. The results agree with the findings of Yoshitake *et al.* (1976), for *Corynebacterium* sp. no. 208, a soil isolate that produces xylitol (66 g/l in 14 days) only in the presence of gluconate in basal medium. This suggests that in the presence of gluconate the xylitol utilizing rate is lower than the xylitol production rate. In contrast, when there is no gluconate, the rate of xylitol production was more or less equal to the rate of utilization after 24 h. The induction of xylitol dehydrogenase activity was slower than the induction of xylose reductase activity.

Cell growth and xylitol production in Corynebacterium medium were almost double those in basal medium under the same conditions. This suggests that media that produces more growth in the presence of xylose also accumulates more xylitol.

The presence of glyceraldehyde in the medium acts as an inhibitor to growth and xylitol production. Glyceraldehyde concentrations less than 1 g/l were sufficient to reduce the growth and xylitol production in the *Corynebacterium* sp. B 4247.

Even though *Corynebacterium* sp. B 4247 grows well in media containing glucose (Fig. 5-10 A) or gluconate (Fig. 5-8), it is not able to produce xylitol using these substrates. In the absence of xylose, xylitol was not produced under aerobic or anaerobic conditions. This clearly shows that the xylitol detected in the medium was derived only from xylose. In media containing xylose when the *Corynebacterium* sp. B 4247 inoculum was obtained from glucose cultures it showed a longer lag phase, slower xylitol accumulation and a slight diauxic growth pattern compared to the inoculums from xylose cultures.

CHAPTER 6

ISOLATION AND PURIFICATION OF XYLOSE REDUCTASE FROM *CORYNEBACTERIUM* SP. B 4247

In-vitro xylose reductase and xylitol dehydrogenase activities were studied during the growth of *Corynebacterium* sp. B 4247 to determine the appropriate time during the growth for harvesting the cells for enzyme isolation. Activities were measured as the change in the absorbance due to the reduction of NAD^+ or oxidation of NADPH at 340 nm in spectrophotometric assays of cell-free extracts. Cell-free extracts prepared by three different methods, sonication, grinding with sea sand in a pestle and mortar and lysing with lysozyme, were tested for xylose reductase activity. Cell-free extracts prepared by sonication or by grinding with sea sand in a pestle and mortar had lower activity compared to cell-free extracts prepared by lysing with lysozyme. The foaming during sonication affected the concentration and xylose reductase activity of the cell free extract.

Co-enzyme specificity

Corynebacterium sp. B 4247 cells grown in Corynebacterium medium containing 75 g/l D-xylose were harvested periodically. The cells were lysed using lysozyme (2 mg/ml of buffer) and the cell-free extracts were assayed for xylose reductase and xylitol dehydrogenase activity. In the cell-free extracts assay with NADPH as co-enzyme and D-xylose as substrate, the specific activity of reductase increased sharply within 12 h and reached a maximum (0.19 U/mg) in 24 h (Fig. 6-1). After 24 h the specific activity started decreasing and reached a minimum at 72 h (0.04 U/mg).

In cell-free extract assayed for reductase activity with NADH as co-enzyme and D-xylose as substrate (Fig. 6-1), the specific activity for reductase (0.24 U/mg) was maximum at 24 h and it was greater than the NADPH dependent reductase activity. The specific activity was lowest (0.062 U/mg) at 48 h (Fig. 6-1).

In cell-free extracts assayed for dehydrogenase activity with NAD^+ as co-enzyme and xylitol as the substrate, the specific dehydrogenase activity reached a maximum at 36 h (0.12 U/mg), and decreased until 96 h (Fig. 6-1). The NADP dependent xylitol dehydrogenase activity was not detected throughout the period. The difference in the specific activities of NAD^+ dependent dehydrogenase and NADH or NADPH dependent reductase was highest when the culture was 24 h old.

Substrate specificity

The NADPH-dependent reductase activity was found to be higher when glyceraldehyde was used as substrate instead of xylose. The reductase activity was 217% with DL-glyceraldehyde and 13% with glucose when the reductase activity of D-xylose is taken as 100%.

Enzyme activity of cells grown in the presence of gluconate

In-vitro reductase activity in *Corynebacterium* sp. B 4247 cells grown in the presence of gluconate with D-xylose addition after 48 h was studied in order to find the effect of gluconate on the NADPH dependent reductase activity. In a cell-free-extract assay with NADPH as co-enzyme and D-xylose as substrate (Fig. 6-2), the specific activity of NADPH-dependent xylose reductase attained the maximum (0.23 U/g) in the first 12 h after addition of D-xylose. After 12 h the specific activity decreased and leveled off after 72 h.

Cell-free extract obtained from cells grown in the presence of gluconate and xylose added after 48 h of growth was assayed for xylitol dehydrogenase activity. The dehydrogenase activity was not detectable when assayed with NAD^+ as co-enzyme and xylitol as substrate (Fig. 6-2).

Gel filtration chromatography

The molecular weight of xylose reductase protein was determined using gel filtration in Sephacryl S-300 column. Cell-free extracts of *Corynebacterium* sp. B 4247 and active DE-52 were concentrated using centrifugal ultra filtration and loaded on to the column. The eluted fractions were tested for xylose reductase activity. From the calibrated plot of void volume ratio to the molecular weight of standards (Fig. 6-3) the molecular weight of the active fraction was determined to be 58 kDa.

Enzyme Purification

Yeast xylose reductase purification

The NADPH dependent xylose reductase of *Candida tropicalis* ATCC 96745 was first purified according to the method of Yokoyama *et al.* (1995) (Figure. 6-4). A purification of 21-fold was obtained and the stepwise purification is summarized in Table 6-1. A similar process was adopted for purifying the NADPH dependent xylose reductase of *Corynebacterium* sp. B 4247.

Corynebacterium sp. B 4247 xylose reductase purification

DEAE-cellulose ion-exchange chromatography

A wet cell paste of 108 g was obtained from 45 l of 24 h grown culture. The cells were lysed in two buffer volumes per gram of wet cell weight. DEAE-cellulose column containing DE-52 (Whatman) was used in the initial purification of the crude extract. DEAE-cellulose fractions eluted with 0-0.5 M NaCl gradient was monitored for the protein activity by change in the absorbance at 280 nm (Fig. 6-5). The peak containing fractions were assayed for NADPH-dependent xylose reductase activity. The specific activity for NADPH-dependent reductase activity was highest in the fractions eluting at 0.23 M – 0.28 M NaCl concentrations in the elution buffer.

.

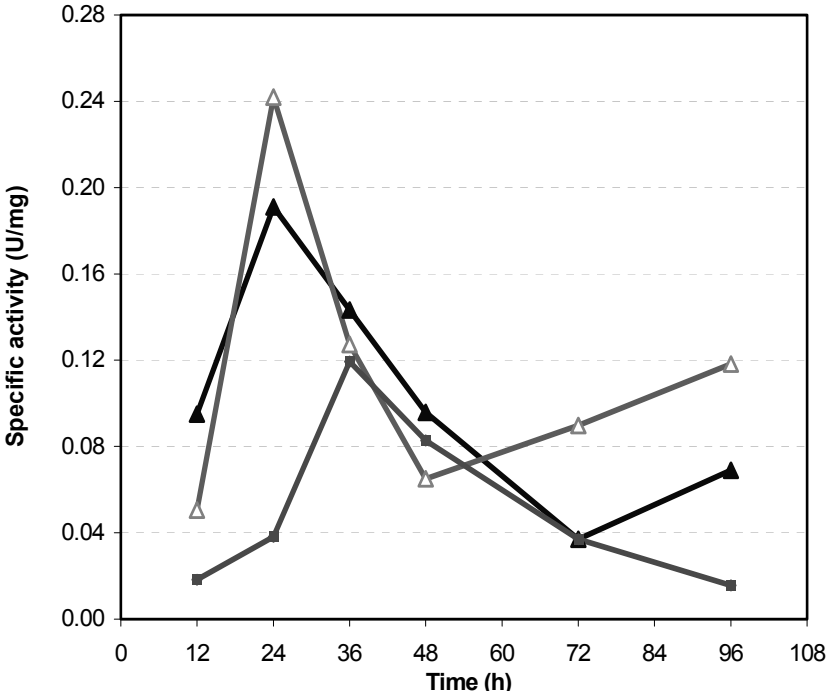


Figure 6-1.Changes in specific activities of reductase and dehydrogenase enzymes of *Corynebacterium* sp. B 4247 during growth in Corynebacterium medium containing 75 g/l D-xylose: NADPH dependent xylose reductase activity (▲), NADH dependent xylose reductase activity (Δ), NAD^+ dependent xylitol dehydrogenase activity (■)

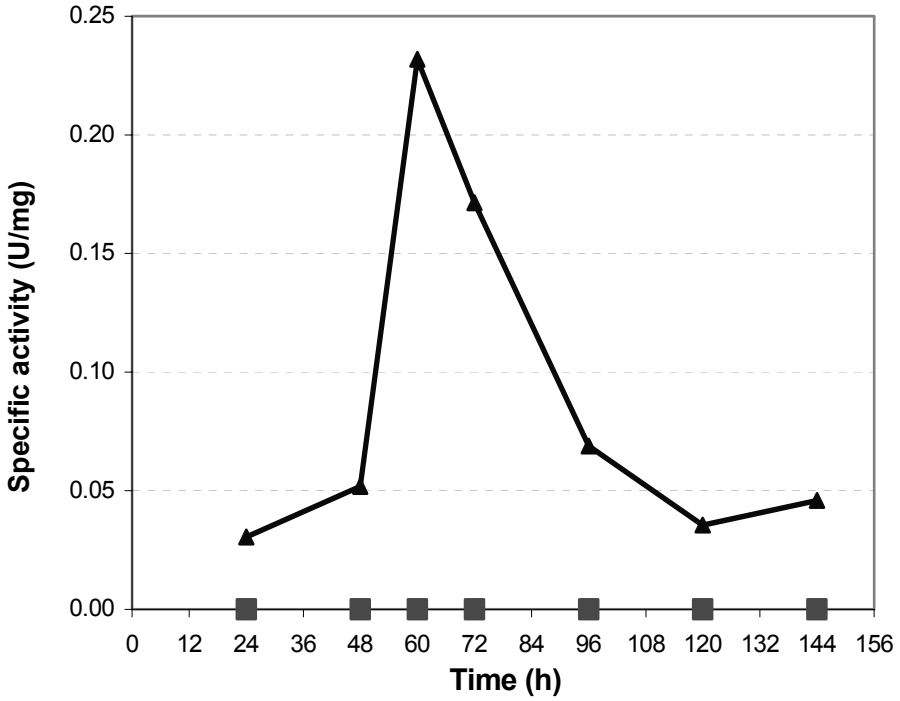


Figure 6-2. Changes in specific activities of reductase and dehydrogenase enzymes of *Corynebacterium* sp. B 4247 during growth in Corynebacterium medium when the cultures were initially grown in the presence of gluconate and 75 g/l D-xylose added after 48 h: NADPH dependent D-xylose reductase activity (▲), NAD^+ dependent xylitol dehydrogenase activity (■)

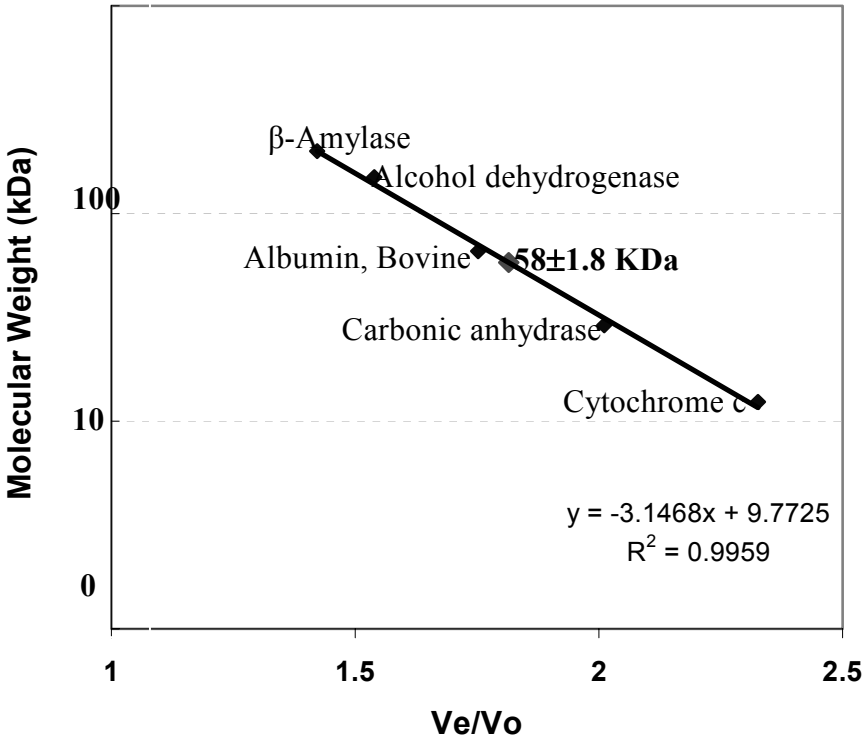


Figure 6-3. Molecular weight of NADPH dependent xylose reductase activity containing fraction by gel filtration

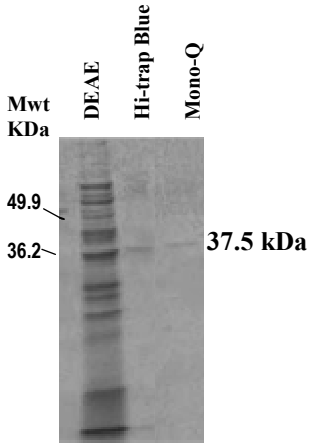


Figure 6-4. Purification of NADPH dependent xylose reductase of *Candida tropicalis* ATCC 96745: DEAE- DE-52 active fractions, Hi-trap Blue- active fraction from Hi-trap Blue, Mono-Q- active fraction from Mono-Q FPLC

Table 6-1.Purification steps of xylose reductase in yeast *Candida tropicalis* ATCC 96745

Step	**Total Protein (mg)**	**Total activity (U)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Cell-free extract	29.8	32.1	1.1	1.0	100.0
DE-52	5.4	17.6	3.3	3.0	54.9
Hi-Trap Blue	0.34	7.8	22.9	20.8	24.3
Mono-Q FPLC	0.14	3.6	26.3	23.9	11.1

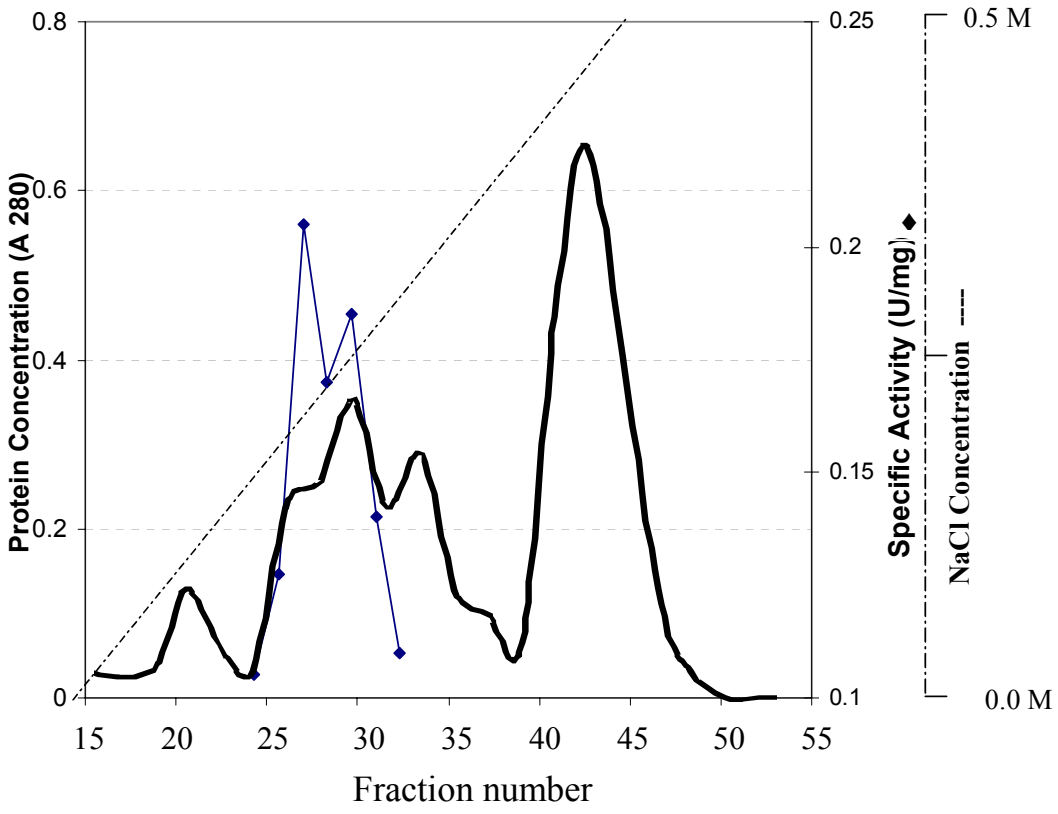


Figure 6-5. Elution pattern of fractions from DEAE cellulose column: Eluted with 0 –0.5 M NaCl in the buffer (- --): protein concentration at A280 (—); specific activity for NADPH- dependent xylose reductase (♦)

Affinity chromatography

Separation in Hi-trap blue column

The DEAE-cellulose column active fractions were pooled and salt content reduced by exchanging buffer in centrifugal ultra-filtration filters. The fractions were then loaded on a Hi-trap blue column (Pharmacia) for further separation of proteinsThe Hi-trap column was effective for the purification of xylose reductase of yeast *Candida tropicalis* ATCC 96745. However, when the Hi-Trap column was used for purification of *Corynebacterium* sp. B 4247 DEAE fraction, the NADPH-dependent reductase activity was found in the wash fractions (non-binding) and in the fractions eluted with 0 – 100 μM NADP-containing buffer (binding). The fractions did not have any increased specific activity over DEAE purification. The fractions were examined in the SDS-PAGE for purity (Fig. 6-6).

Separation using Red Sepharose column

The Red Sepharose column was used to purify the DEAE-cellulose fractions of *Corynebacterium* sp. B 4247. The DEAE-cellulose active fractions were pooled and the salt content reduced by exchanging buffer in centrifugal ultra-filtration filters. The fractions were then injected into the Red Sepharose column. NADP (0- 50 μM) gradient was used to elute the fractions (Fig. 6-7). The fractions eluted as a single peak and the fractions eluting at 6-11 μM of NADP in the buffer contained higher specific activity for xylose reductase. The active fractions were pooled and purity of the fractions analyzed in the SDS-PAGE (Fig. 6-8). The column was also eluted with 0.25 M NaCl and the fractions having higher xylose reductase activity were pooled and analyzed by SDS-PAGE. A 11.4 fold purification resulted in this step (Table 6-2).

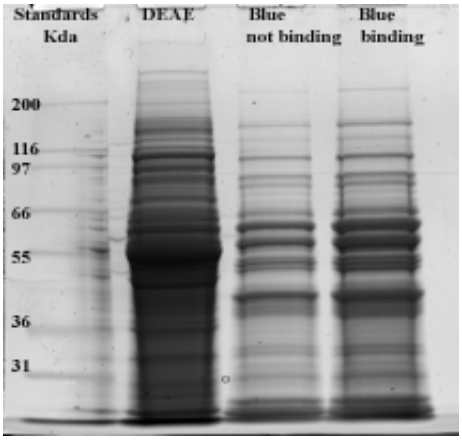


Figure 6-6. Hi-trap Blue fractions when run in SDS-PAGE

Blue not binding: washed fractions of Hi-trap column;

Blue binding: eluted fractions in the Hi-trap column

DEAE: DEAE cellulose column fraction

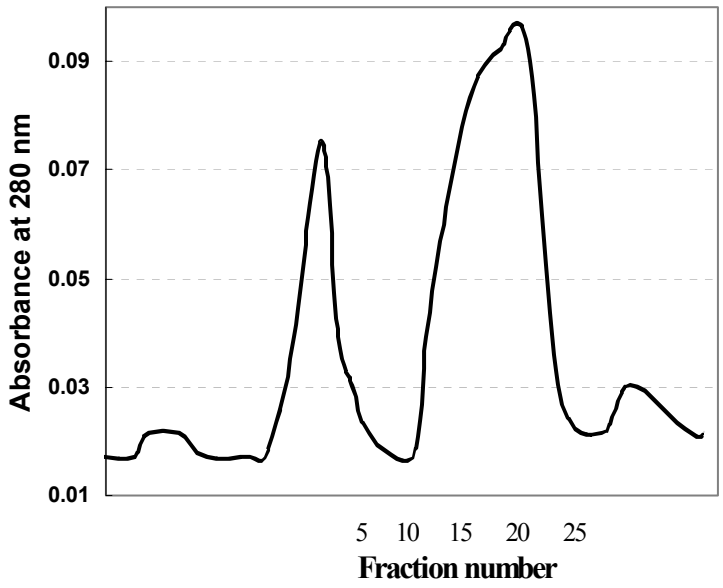


Figure 6-7. Elution profile of the proteins from the Red Sepharose affinity column

Separation in Mono-Q column

Red Sepharose active fractions were exchanged buffer using the centrifugal ultra-filtration filters. The concentrated active fractions were injected into Mono-Q column connected to FPLC. The column was eluted with 0-2.5 M NaCl gradient in the elution buffer and the change in protein concentration was monitored (Fig. 6-9). Fractions having reductase activity eluted at 0.14 – 0.18 M NaCl. The fractions were concentrated and the purity examined by SDS-PAGE (Fig. 6-10). A 19 fold purification resulted from this purification step (Table 6-2).

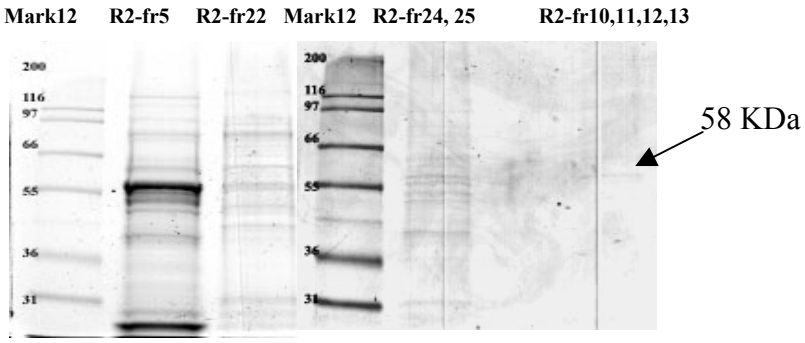


Figure 6-8. Red Sepharose fractions in SDS-PAGE. Mark12-Standard protein marker, R2- fr5, R2fr22, R2- fr24,25 - fractions eluted with 0-50 µM NADP, R2- fr10,11,12,13– fractions when eluted with 0.25 M NaCl

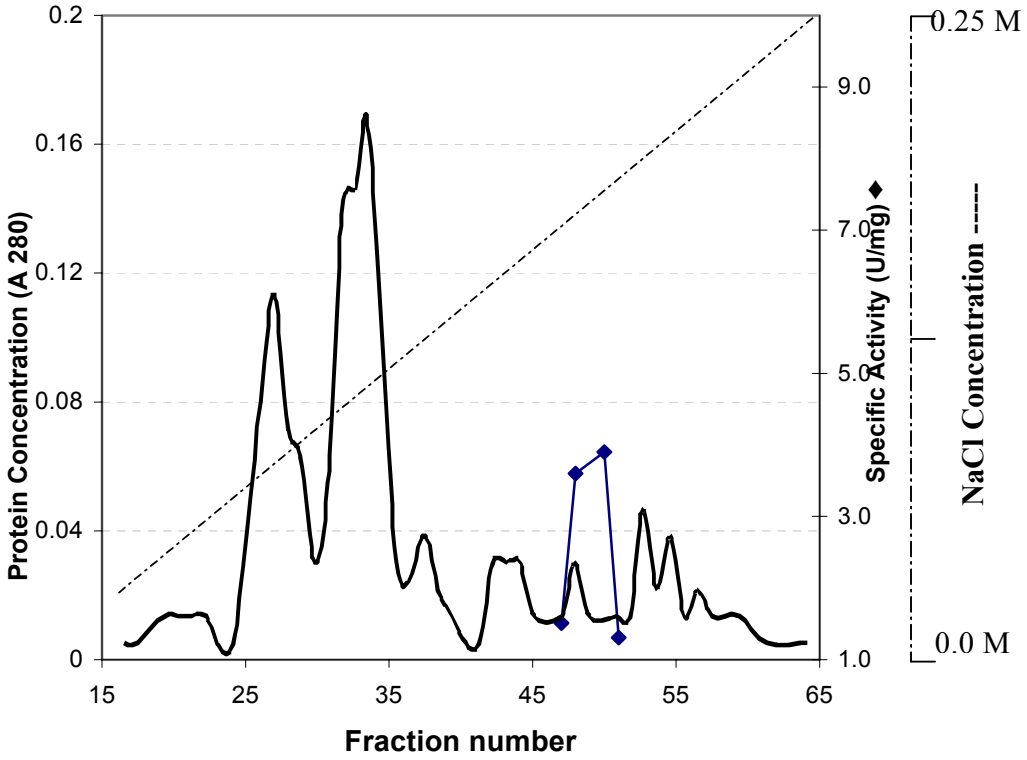


Figure 6-9. Elution pattern of fractions from Mono-Q FPLC column:
Eluted with 0 –0.25 M NaCl in the buffer (- --): protein concentration at A280 (▬); specific activity for NADPH-dependent xylose reductase (♦)

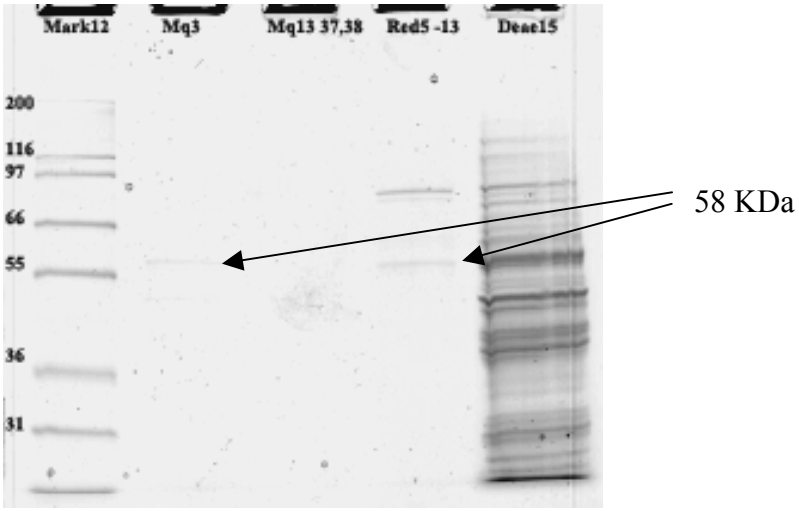


Figure 6-10. SDS-PAGE of the active xylose reductase fractions in the purification steps

Mark12: Standard protein marker

Mq3: Mono-Q fraction concentrated by ultra filtration

Mq13, 37, 38: pooled fractions form the Mono- Q column

Red5-13: fraction from red Sepharose

DEAE 15: fraction from DEAE column

Table 6-2. Purification steps of xylose reductase in *Corynebacterium* sp. B 4247

Step	**Total Protein (mg)**	**Total activity (U)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Cell-free extract	194.6	43.2	0.22	1.0	100.0
DE-52	44.2	22.1	0.50	2.3	51.0
Red Sepharose	3.41	8.6	2.51	11.4	19.8
Mono-Q FPLC	0.74	3.1	4.19	19.1	7.2

Activity staining in native gel

Crude extract of *Corynebacterium* sp. B 4247 was concentrated using centrifugal ultra filtration filters and was fractionated by PAGE under native conditions. The gel was used for in-gel assay. NADPH, D-xylose and chromogenic mix solution was spread on a filter paper laid on the gel. Similarly, a control was used with sterile water instead of D-xylose.

The gel was stained reddish orange with dark bands at two regions (Fig.6-11). These bands were cut and run in SDS-PAGE. Band 1 in the native gel corresponded to 43.6 kDa band in the SDS-PAGE gel. Band 2 in the native gel corresponded to 58.3, 49.3, 39.4 and 30.2 kDa bands (Fig. 6-12). The 49.3 kDa fragment appeared to be sheared.

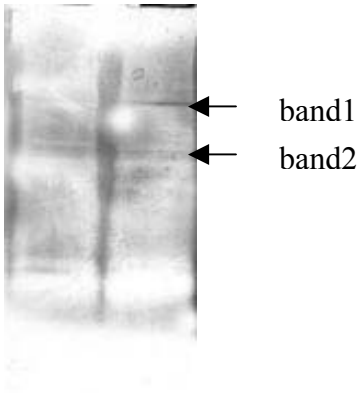


Figure 6-11. Zymogram of in-situ reductase assay in the native gel

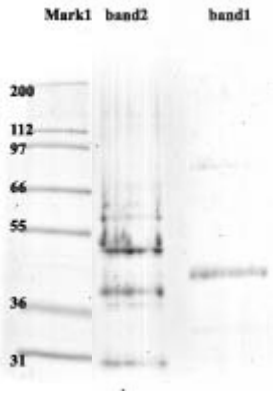


Figure 6-12. Band 1 and band 2 from in-gel assay run in the SDS denaturing gel.

Mark12: – Standard protein marker, band2: band 2 from the in-situ assayed native gel, band1: band 1 from the in-situ assayed native gel

Discussion

The specific activity trend of NADPH dependent-xylose reductase (Figure 6-1) in *Corynebacterium* sp. B 4247 grown in 75 g/l initial D-xylose concentration was similar to its xylitol yield trend (Figure 5-4). The NADPH-dependent xylose reductase activity and the xylitol yield were maximum at 24 h. After 24 h, the NADPH dependent specific activity followed a similar trend in the xylitol yield. The NAD^+ dependent dehydrogenase activity at 24 h (Figure 6-1) was low when the xylitol yield at 24 h was maximum but increased to its maximum at 36 h. The large difference in NADPH reductase activity and NAD^+ dependent dehydrogenase activity in the first 24 h accounted for the high xylitol yield. After 24 h, the activity of NAD^+ increased due to the utilization of xylitol and therefore xylitol yield decreased (Fig. 5-4).

The NADPH-dependent xylose reductase activity is growth dependent, as shown by the increase in activity during the growth phase whereas in the stationary phase the NADPH dependent reductase activity decreased continuously with corresponding decrease in xylitol yields. Thus xylitol is a primary metabolic product for *Corynebacterium* sp. B 4247. Xylitol utilization as a primary metabolite is further evidenced by the continued increase in the activity of NAD^+ dependent xylitol dehydrogenase when NADPH-dependent xylose reductase activity increased during the log phase. Further NAD^+ dependent xylitol dehydrogenase activity decreased only after the decrease in NADPH dependent reductase.

Corynebacterium sp. B 4247 grown in the presence of gluconate with D-xylose addition after 48 h exhibited very low NAD^+ dependent dehydrogenase activity (Figure 6-2) compared to the medium without gluconate (Figure 6-1). This reduced NAD^+ activity could be one of the reasons for increased accumulation of the xylitol in the medium. This explains the continued increase in xylitol levels when *Corynebacterium* sp. B 4247 was grown in the presence of gluconate (Figure 5-9) and is absent in the cultures grown without gluconate. When grown in the presence of gluconate, the maximum NADPH dependent reductase activity occurs at 12 h after xylose addition. D-xylose was added

after 48 h when the culture was at the end of the growth phase; the growth due to D-xylose is less. Since the gluconate is used not D-xylose in primary metabolism the NADPH dependent D-xylose reductase activity is slow and results in low xylitol yields. But the absence of NAD^+ dependent dehydrogenase activity over a longer duration accumulates the xylitol as an overflow metabolite in the media.

Corynebacterium sp. B 4247 cell-free extract has both NADH dependent xylose reductase activity and NADPH reductase activity similar to *Pichia stipitis* (Bruinenberg et al., 1984), *Pachysolen tannophilus* CBS 4044 (Verduyn *et al.*, 1985), *Neurospora crassa* (Zhao *et al.*,1998) and *C. shehatae* (Ho *et al*., 1990). The NADH activity was about 0.54 (12 h) and about 1.28 (24 h) times of the NADPH D-xylose reductase activity in crude cell extracts of *Corynebacterium* sp. B 4247 (Figure 6-1). The NADH to NADPH activity ratios of *C. boidinii* (*Kloeckara* sp.), *C. parapsilosis*, *C. shehatae, P. stipitis* were 5.23, 0.41, 0.55 – 0.6, and 0.5 respectively (Table 2-8). Ho *et al*. (1990) suggested that higher NADH/NADPH activity ratio could lead to more effective xylose fermentation. The choice of NADPH or NADH as co-enzyme for xylose reduction could be dependent on the concentrations of the co-enzymes as suggested by Verduyn *et al*. (1985) as in the case of *P. stipitis*. As reasoned by Parajo *et al*. (1998) similar to *P. stipitis* and *C. shehatae* the dual co-factor specificity of xylose reductase could avoid the imbalance of the NAD^+ /NADH redox system in anaerobic conditions.

Cell free extracts of *Corynebacterium* sp. B 4247 and *C. tropicalis* ATCC 96745 had 2.2 times and 2.9 times the substrate-specific reductase activity to glyceraldehyde than to D-xylose. In *C. tropicalis* IFO 0618, Yokoyama *et al.* (1995) reported similar substrate specificity of 2, 2.68 and 1.43 times for glyceraldehyde than to that of D-xylose by three xylose reductase isomers XR1, XR2 and XR3. However, in *Corynebacterium* sp. B 4247, glyceraldehyde presence greater than 1 g/l in the media was found to be inhibitory to growth and xylitol production. Hence, glyceraldehyde as a favorable substrate for the enzyme may limit the utilization of D-xylose and consequently inhibit growth and production.

The Hi-trap blue column was effective in the purification of *Candida tropicalis* NADPH dependent xylose reductase but was ineffective for bacterial reductase under similar conditions. When Red Sepharose column was used instead of Hi-trap blue column for purification, *Corynebacterium* sp. B 4247 reductase activity was found in the eluted fractions of (0-50 μM) NADP in the buffer. The reductase activity was also found in fractions eluted with 0.25 M of NaCl. This is consistent with Ho *et al.* (1990) who showed that the reactive Red Sepharose had a higher recovery of reductase enzyme in *C. shehatae* whereas the Cibacron Blue was not effective. This suggests that the *C. tropicalis* and *Corynebacterium* sp. B 4247 xylose reductase have different binding properties to the blue sepharose ligand. In the anion exchange columns the elution of the *C. tropicalis* and *Corynebacterium* sp. B 4247 reductase activity containing fractions eluted at closer NaCl concentrations in the buffer suggesting the pI should be closer.

Multiple forms of aldose reductase have been shown to occur (Suzuki and Onishi, 1975) In the red Sepharose purification step the fractions (R2-fr24, 25 in Fig. 6-9) that eluted at concentrations higher than 35 μM NADP had a prominent 39 kDa protein in the SDS-PAGE (Fig.6-9), although the fractions had lower specific activity. The 39 kDa protein could also be aldose reductase or a subunit of the 58 kDa protein, as a 39 kDa protein of higher intensity was present in band 2 of the in-gel assay (Figure. 6-12).

The molecular weight of the native xylose reductase protein of *Corynebacterium* sp. B 4247 determined by gel-filtration (58 kDa) agrees with the size of native xylose reductase proteins characterized in other microorganisms (Table 6-3). The 58 kDa protein showed a higher specific activity when purified using Red Sepharose eluted with NADP (0-50 μM) and in the fraction eluted with 0.25 M NaCl. Further purification on a Mono-Q column yielded a 19.2 fold purification of the 58 kDa protein. Further, the 58 kDa protein corresponded to a resulting band from the band 2 of the in-gel assay (Fig. 6-12).

Table 6-3 Xylose reductase from other organisms (Yokoyama *et al*., 1995)

Strain			**M.W.**		**pI**
			(native)	**(subunit)**	
C. *tropicalis*	IFO 0618	XRl	58,000	36,498	4.15
		XR2	58,000	36,540	4.10
		XR3	58,000	-	-
C. *utilis*	ATCC 9950		70,000	-	-
P. stipitis	CBS 5773		65,000±4,000	34,000±2,000	-
P. quercuum	IFO 0949	I	160,000	-	-
		IIa	61,000	-	-
		IIb	61,000	-	-
Rhodotorula sp.			62,000	-	5.05

CHAPTER 7

ANALYSIS OF THE 58 KDA PROTEIN OF *CORYNEBACTERIUM* SP. B-4247 BY LIQUID CHROMATOGRAPHY -TANDEM MASS SPECTROMETRY

The proteomic analysis of the isolated 58 kDa protein of *Corynebacterium* sp. B 4247 that had xylose reductase activity was performed at Biomolecular Research Facility of the University of Virginia. The protein was digested using recombinant trypsin and the digest was separated and analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis. The double play capability of the instrument allowed for acquisition of the full scan mass spectra for individual peptide molecular weights, as well as the product ion spectra to determine the amino acid sequences of each peptide. The data was analyzed using the Sequest search algorithm. Twelve peptides of the 58 kDa protein of the *Corynebacterium* sp. B 4247 that matched to the *Bacillus subtilis* (protein 1) dihydrolipoamide dehydrogenase E3 subunit of both pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (are shown in Sequest format in Figure 7-1). Two peptides that matched *B. subtilis* (protein 2) dihydrolipoamide acetyltransferase E2 subunit are shown in Sequest format in Figure7-2. The additional peptide "GNVEIHTSAM" was deduced by de nova sequencing (manual interpretation).

The individual peptides identified from the 58 kDa *Corynebacterium* sp. B 4247 protein were used in BLAST (Basic Local Alignment Search Tool) searches of the non-redundant database of NCBI (National Center for Biotechnological Information) for "short nearly exact matches", limiting the search to known aldose reductases. The peptides, the matches and bit scores are displayed in Table7-1. The peptide "QMSSLVTR" showed 75% identity to the keto-aldose reductase Gre3p (*Saccharomyces cerevisiae*). This protein is reported to be induced by osmotic stress, and is similar to xylose reductases from other fungi that reduce xylose to xylitol. The peptide "LLNDPQLILMEA**"** contains an "LLXDP" conserved sequence that is found in several aldose reductases (Table 7-2). The 15 peptides were linked and searched using FASTS option in FASTA search program of VIGEN (Virginia Tech. Center for Genomics) against NCBI and Swiss-Prot library. The matching proteins with the scores and E-values are listed in Table 7-3.

>gi|16078525|ref|NP_389344.1| (NC_000964) dihydrolipoamide dehydrogenase E3 subunit of both pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes [*Bacillus subtilis*]□gi|118672|sp|P21880|DLD1_BACSU Dihydrolipoamide dehydrogenase (E3 component of pyruvate complex) (S complex, 50 kDa subunit)□gi|98440|pir||E36718 dihydrolipoamide dehydrogenase (EC 1.8.1.4) - *Bacillus subtilis*□gi|143380|gb|AAA62684.1| (M57435) dihydrolipoamide dehydrogenase E3 subunit [*Bacillus subtilis*]□gi|2633832|emb|CAB13334.1| (Z99111) dihydrolipoamide dehydrogenase E3 subunit of both pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes [*Bacillus subtilis*]□gi|3282145|gb|AAC24935.1| (AF012285) dihydrolipoamide dehydrogenase E3 [*Bacillus subtilis*] [MASS=49733]

MVVGDFPIET DTLVIGAGPG GYVAAIRAAQ LGQKVTVVEK **ATLGGVCLNV GCIPSK**ALIN
AGHRYENAK**H SDDMGITAEN VTVDFTK**VQE WKASVVNK**LT GGVAGLLK**GN KVDVVKGEAY
FVDSNSVR**VM DENSAQTYTF KNAIIATGSR PIELPNFK**YS ER**VLNSTGAL ALK**EIPKKLV
VIGGGYIGTE LGTAYANFGT ELVILEGGDE ILPGFEK**QMS SLVTR**RLKKK GNVEIHTNAM
AKGVEERPDG VTVTFEVKGE EK**TVDADYVL ITVGRRPNTD ELGLEQVGIE MTDR**GIVKTD
KQCRTNVPNI YAIGDIIEGP PLAHKASYEG KIAAEAIAGE PAEIDYLGIP AVVFSEPELA
SVGYTEAQAK EEGLDIVAAK FPFAANGR**AL SLNETDGFMK** LITRKEDGLV IGAQIAGASA
SDMISELSLA IEGGMTAEDI AMTIHAHPTL GEITMEAAEV AIGSPIHIVK
>monoisotopic mass = **49683**

position	sequence
142- 150	NAIIATGSR
218- 225	QMSSLVTR
129- 141	VMDENSAQTYTFK
163- 173	VLNSTGALALK
99- 108	LTGGVAGLLK
70- 87	HSDDMGITAENVTVDFTK
276- 294	RPNTDELGLEQVGIEMTDR
389- 400	ALSLNETDGFMK
151- 158	PIELPNFK
41- 56	ATLGGVCLNVGCIPSK
263- 275	TVDADYVLITVGR
142- 158	NAIIATGSRPIELPNFK

Figure 7-1. Peptides of the 58 kDa protein of *Corynebacterium* sp. B 4247 identified in *Bacillus subtilis* (protein1)

>gi|16078524|ref|NP_389343.1| (NC_000964) pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit) [*Bacillus subtilis*]□gi|129054|sp|P21883|ODP2_BACSU Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (E2) (S complex, 48 kDa subunit)□gi|98439|pir||D36718 dihydrolipoamide S-acetyltransferase (EC 2.3.1.12) precursor - *Bacillus subtilis*□gi|143379|gb|AAA62683.1| (M57435) dihydrolipoamide acetyltransferase E2 subunit [*Bacillus subtilis*]□gi|2633831|emb|CAB13333.1| (Z99111) pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit) [*Bacillus subtilis*]□gi|3282144|gb|AAC24934.1| (AF012285) dihydrolipoamide acetyltransferase E2 [*Bacillus subtilis*] [MASS=47539]

MAFEFKLPDI GEGIHEGEIV KWFVKPNDEV DEDDVLAEVQ NDKAVVEIPS PVKGKVLELK
VEEGTVATVG QTIITFDAPG YEDLQFKGSD ESDDAKTEAQ VQSTAEAGQD VAKEEQAQEP
AKATGAGQQD QAEVDPNKRV IAMPSVRKYA REKGVDIRKV TGSGNNGRVV KEDIDSFVNG
GAQEAAPQET AAPQETAAKP AAAPAPEGEF PETREKMSGI RKAIAKAMVN SKHTAPHVTL
MDEVDVTNLV AHRKQFK**QVA ADQGIK**LTYL PYVVKALTSA LKKFPVLNTS IDDKTDEVIQ
KHYFNIGIAA DTEKGLLVPV VKNADRKSVF EISDEINGLA TKAREGKLAP AEMKGASCTI
TNIGSAGGQW FTPVINHPEV AILGIGRIAE KAIVRDGEIV AAPVLALSLS FDHRMIDGAT
AQNALNHIKR **LLNDPQLILM EA**
>monoisotopic mass = **47491**

position	sequence
258- 266	QVAADQGIK
431- 442	LLNDPQLILMEA

Protein Coverage: 21/442 = **4.8%** by amino acid count, 2261/47491 = **4.8%** by mass

Figure 7-2. Peptides of the 58 kDa protein of *Corynebacterium* sp. B 4247 identified in *Bacillus subtilis* (protein2)

Table 7-1. Search for "short nearly exact matches" of sequenced peptides with xylose reductases and aldose reductases in non-redundant (nr) database of NCBI. (PAM30 matrix was used for the search and homology is based on the number of amino acids matches to the total amino acids in the peptide.)

Peptide	Matching sequence (accession number)	Name of the sequence and the species	Match	Homology (%)	Bit Score
NAIIATGSR	gi|17562292	Aldo keto reductase family member (34.7 kD) [*Caenorhabditis elegans*]	AT SR 253 IPKS**AT**P**SR** 261	(4/9) 44.44%	12.1
QMSSLVTR	gi|6321896	keto-aldose reductase; Gre3p [*S. cerevisiae*]	MSSLVT _ **MSSLVT** L 7	(6/8) 75%	21.8
VMDENSAQ TYTFK	gi|15800149 gi|15829727 gi|25316196 gi|25316252 gi|13359930	putative NAD(P)H-dependent xylose reductase [*E. coli* O157:H7 EDL933] putative NAD(P)H-dependent xylose reductase [*E. coli* O157:H7] probable NAD(P)H-dependent xylose reductase [imported] - *E. coli* (strain O157:H7, substrain RIMD 0509952) probable NAD(P)H-dependent xylose reductase yajO [imported] - *E. coli* (strain O157:H7, substrain EDL933) putative NAD(P)H-dependent xylose reductase [*E. coli* O157:H7]	DEN AQ 242 ES**DEN**D**AQ**IAERL 254	(5/13) 38.46%	17.6
	gi|16128404|] gi|1786621	putative NAD(P)H-dependent xylose reductase [*E. coli* K12] putative NAD(PH-de)pendent xylose reductase [*E. coli* K12]	DEN AQ 266 ES**DEN**D**AQ**IAERL 278	(5/13) 38.46%	17.6
	gi|24111797 gi|24050585	putative NAD(P)H-dependent xylose reductase [*Shigella flexneri* 2astr. 301] putative NAD(P)H-dependent xylose reductase [*Shigella flexneri* 2astr. 301]	DEN AQ 242 2ES**DEN**D**AQ**IAERL 254	(5/13) 38.46%	17.6
VLNSTGALA LK	gi|17538386 gi|15408884 gi|17550248 gi|6321896	Aldo keto reductase family family member [*C. elegans*] putative aldose reductase [*Oryza sativa* (japonica cultivar-group)] Aldose reductase family member (35.2 kD) [*C. elegans*] Induced by osmotic stress; similar to xylose reductase from otherfungi. Reduces xylose to xylitol.; Gre3p [*S. cerevisiae*]	LALK 47 ENEKDLG **LALK** 57 LALK 50 NNEKDLG**LALK** 60 ALAL 157 NDQISR**ALAL**G 167 LALK 84 HHPDHVK **LALK** 94	(4/11) 36.36% (4/11) 36.36% (4/11) 36.36% (4/11) 36.36%	14.6 14.6 14.2 14.6

LTGGVAGLLK	gi|17550248	Aldose reductase family member (35.2 kD) [*C. elegans*]	GG .. LK 92LE**GG**LRES**LK** 101	(4/10) 40%	9.5
HSDDMGITAENV TVDFTK	gi|17551208	Aldo keto reductase family precursor family member [*C. elegans*]	SDDM 89 F**SDDM** SQKIESSVEDIWR 106	(4/18) 22.2%	17.2
	gi|17566692	Aldo keto reductase family family member [*C. elegans*]	+MGI T EN+ + DF 254 ATVE**MGI**IVIPKTT NPER MK**EN**INIF**DF** 281	(8/25) 32%	15.5
	gi|1351442 gi|1364169	NAD(P)H-dependent xylose reductase D-xylose 1-dehydrogenase (NADP) (EC 1.1.1.179) - (*Kluyveromyces marxianus* var. *lactis*)	M. AE -VT - -- -- --MTYL **AE**T**VT** LNNGE 14	(5/18) 27.77%	14.2
RPNTDELGLEQVGI EMTDR	gi|17538107	Aldo keto reductase [*C. elegans*]	DELG 233 KNYC**DELG** ILTMGYCPLAK 251	(4/19) 21.05%	15.5
ALSLNETDGFMK	gi|5917775 gi|11558038	aldose-reductase-like protein MVDP/AKR1-B7 [*Rattus norvegicus*] rhoB-crystallin [*Lepidodactylus lugubris*]	LSLN 289 I**LSLN**RNWRACG 300 LSLN 292 L**LSLN** KNMRVYS 303	(4/12) 33.33% (4/12) 33.33%	15.1 15.1
PIELPNFK	gi|17553316	Aldo keto reductase family [*C. elegans*]	EL NF 348 NL**ELSNF**S 355	(4/8) 50%	15.9
ATLGGVCLNVGC IPSK	gi|17551208 gi|17550248 gi|17566692	Aldo keto reductase family precursor family member [*C. elegans*] Aldose reductase family member (35.2 kD) [*C. elegans*] Aldo keto reductase family family member [*C. elegans*]	ATLGG 45 **ATLGG**QQAWTNELAPG 60 ATLG 202 **ATLG**SPGRVNFTLPTG 217 ATLG 202 **ATLG**SPGRMSVVGSNG 217	(5/16) 31.25% (4/16) 25% (4/16) 25%	16.8 14.2 14.2
	gi|17561300	Aldo/keto reductase family family member (32.5 kD) [*C. elegans*]	VG IP 227 LLSWATSQK**VGIIP**KS 242	(4/16) 25%	12.9
TVDADYVLITVGR	gi|13925690 gi|17564128	aldose reductase [*Coccidioides posadasii*] Reductase 1 family member (37.3 kD) [*C. elegans*]	TVD DYV 25 **TVD**I**DYV**DTYKAM 37 DA Y LI 37 AL**DAGYRLI**DTAH 49	(6/13) 46.15% (6/13) 46.15%	21.0 15.1

NAIIATGSRPIELPNFK	gi|12513275 gi|25316252 gi|25316196 gi|13359930	putative NAD(P)H-dependent xylose reductase [*E. coli* O157:H7 EDL933] probable NAD(P)H-dependent xylose reductase yajO [imported] - *E. coli* (strain O157:H7, substrain EDL933) probable NAD(P)H-dependent xylose reductase [imported] - *E. coli* (strain O157:H7, substrain RIMD 0509952) putative NAD(P)H-dependent xylose reductase [*E. coli* O157:H7]	SRPI 23 W TLPEES**SRPII**KRALE 49	(4/17) 23.52 %	15.9
	gi|24050585	putative NAD(P)H-dependent xylose reductase [*Shigella flexneri* 2astr. 301]	SRPI 33 WTLPEES**SRPI** IKRALE 49	(4/17) 23.5%	15.9
	gi|17553316	Aldo keto reductase family [*C. elegans*]	EL NF 339 MDSVQQVLDNL**ELSNF**S 355	(4/17) 23.5%	15.9
	gi|9255883	xylose reductase [*Candida shehatae*]	PIE 115 HF PIAFKFV**PIE** EKYPP 131	(3/17) 17.65 %	13.4
	gi|1786621	putative NAD(P)H-dependent xylose reductase [*E. coli* K12]	SRPI 56 AWTLPES**SRPI** IKRAL 72	(4/17) 23.5%	15.9
	gi|17553316	Aldo keto *reductase family* [*C. elegans*]	EL NF 339 MDSVQQVLDNLE **ELSNF**S 355	(4/17) 23.5%	15.9
	gi|1790225	aldose reductase [*Candida boidinii*]	PIE PNF 112 HFPIAQKFV**PIE**KKYP**PNF**Y 132	(6/17) 35.3%	15.9
QVAADQGIK	gi|9255883	xylose reductase [*Candida shehatae*]	AADQ IK 30 ST**AADQ**VYNA**IK** 41	(6/9) 66.67 %	15.5
	gi|15241032 gi|7327826	At5g01670.1 [*Arabidopsis thaliana*] aldose reductase-like protein [*A. thaliana*]	QGIK 63 QREVG**QGIK** 71	(4/9) 44.44 %	15.5
LLNDPQLILMEA	gi|15408884	putative aldose reductase [*Oryza sativa* (japonica cultivar-group)]	LND QL 95 T**LND**L**QL**EYLD 107	(5/12) 41.67 %	16.3
GNVEIHTSAM	gi|17552492	Aldo keto reductase family family member (32.8 kD) [*C. elegans*]	2 NVEI 5 NVEI 222 Y**NVEI**PVLLL 231	(4/10) 40%	16.3
	gi|6321896	Induced by osmotic stress; similar to xylose reductase from other fungi. Reduces xylose to xylitol.; Gre3p [*S. cerevisiae*]	GN+EI 285 **GNLEI** EKKFT 294	(4/10) 40%	15.1

Table 7-2. Peptide "**LLNDPQLILMEA"** matches to aldose reductase

(Homology based on the number of matches to the number of the amino acids in the peptide)

Subject ID	Name of the sequence	Matching Sequence	Identity%	Bit score
gi|4539944	aldose reductase ALDRXV4 [*Xerophyta viscosa*]	LL+DP 223 **LL**S**DP**TVLKIAN 234	(4/12) 33.33	15.91
gi|515112	Chain C,A,B,D Aldose Reductase (E.C.1.1.1.21) [*Sus scrofa*]	LL DP 226 **LL**E**DP**RIKAIAA 237	(4/12) 33.33	15.06
gi|27712942	similar to Aldose reductase (AR) (Aldehyde reductase) [*Rattus norvegicus*]	LL DP 228 **LL**E**DP**RIKEIAA 239	(4/12) 33.33	15.06
gi|20345865	similar to aldo-keto reductase family 1, member B1 (aldose reductase) [*Mus musculus*]	LL DP 228 **LL**E**DP**RIKAIAA 239	(4/12) 33.33	15.06
gi|20348989	similar to Aldose reductase (AR) (Aldehyde reductase) [*Mus musculus*]	LL DP 129 **LL**E**DP**RIKAIAA 140	(4/12) 33.33	15.06
gi|3150035	aldose reductase-like peptide [*Homo sapiens*]	LL DP 228 **LL**E**DP**KIKEIAA 239	(4/12) 33.33	15.06
gi|786001	aldose reductase [*Mus musculus*]	LL DP 228 **LL**E**DP**RIKAIAA 239	(4/12) 33.33	15.06
gi|20985906	similar to Aldose reductase (AR) (Aldehyde reductase) [*Mus musculus*]	LL DP 154 **LL**E**DP**RIKAIAA 165	(4/12) 33.33	15.06
gi|1184820	Aldose reductase [*Sus scrofa*]	LL DP 228 **LL**E**DP**RIKAIAA 239	(4/12) 33.33	15.06
gi|584742	Aldose reductase (AR) (Aldehyde reductase) [*Sus scrofa*]	LL DP 228 **LL**E**DP**RIKAIAA 239	4/12) 33.33	15.06
gi|493797	Aldose Reductase (E.C.1.1.1.21) Mutant With Cys 298 Replaced By Ser(C298s) Complex With Nadph [*Homo sapiens*]	LL DP 227 **LL**E**DP**RIKAIAA 238	(4/12) 33.33	15.06
gi|109150	aldehyde reductase (EC 1.1.1.21) - rabbit (fragment)	LL DP 214 **LL**E**DP**RIKAIAD 225	(4/12) 33.33	15.06
gi|4894207	aldose reductase [*Mus musculus*]	LL DP 228 **LL**E**DP**RIKAIAA 239	(4/12) 33.33	15.06
gi|13529257	aldo-keto reductase family 1, member B1 (aldose reductase) [*Homo sapiens*]	LL DP 228 **LL**E**DP**RIKAIAA 239	(4/12) 33.33	15.06
gi|13435726	aldo-keto reductase family 1, member B1 (aldose reductase) [*Mus musculus*]	LL DP 228 **LL**E**DP**RIKAIAA 239	(4/12) 33.33	15.06

gi|27717029	similar to Aldose reductase (AR) (Aldehyde reductase) [*Rattus norvegicus*]	LL DP 210 **LLEDP**RIKAAAV 221	(4/12) 33.33	15.06
gi|3114350	Pig Aldose Reductase, Holo Form	LL DP 227 **LLEDP**RIKAIAA 238	(4/12) 33.33	15.06
gi|178489	aldose reductase [*Homo sapiens*]	LL DP 228 **LLEDP**RIKAIAA 239	(4/12) 33.33	15.06
gi|28647	aldose reductase (AA 1-316) [*Homo sapiens*]	LL DP 228 **LLEDP**RIKAIAA 239	(4/12) 33.33	15.06
gi|559768	aldose reductase [*Oryctolagus cuniculus*] (rabbit)	LL DP 228 **LLEDP**RIKAIAA 239	(4/12) 33.33	15.06
gi|463377	aldose reductase-related protein [*Mus musculus*]	LL DP 228 **LLEDP**KIKEIAA 239	(4/12) 33.33	15.06
gi|576365	Aldose Reductase (E.C.1.1.1.21) Mutant With Tyr 48 Replaced By His(Y48h) Complexed With Nadp+ And Citrate [*Homo sapiens*]	LL DP 227 **LLEDP**RIKAIAA 238	(4/12) 33.33	15.06
gi|2981775	Alrestatin Bound To C298aW219Y mutant human ALDOSE Reductase	LL DP 227 **LLEDP**RIKAIAA 238	(4/12) 33.33	15.06
gi|20857829	similar to aldose reductase-related protein 2 (aldehyde reductase) (FR-1 PROTEIN) [*Mus musculus*]	LL DP 115 **LLEDP**KIKEIAA 126	(4/12) 33.33	15.06
gi|442618	Aldose Reductase (E.C.1.1.1.21) Complex With Nadph [*Homo sapiens*]	LL DP 227 **LLEDP**RIKAIAA 238	(4/12) 33.33	15.06
gi|3046247	aldose reductase [*Mus musculus*]	LL DP 228 **LLEDP**RIKAIAA 239	(4/12) 33.33	15.06
gi|27482412	similar to aldo-keto reductase family 1, member B1 (aldose reductase) [*Homo sapiens*]	LL DP 135 **LLEDP**RIEAITA 146	(4/12) 33.33	15.06
gi|55759	aldose reductase [*Rattus norvegicus*]	LL DP 228 **LLEDP**RIKEIAA 239	(4/12) 33.33	15.06
gi|3114346	Pig Aldose Reductase Complexed With Sorbinil	LL DP 228 **LLEDP**RIKAIAA 239	(4/12) 33.33	15.06
gi|27709582	aldose reductase-like protein [*Rattus norvegicus*]	LL DP 228 **LLEDP**KIKEIAA 239	(4/12) 33.33	14.64

Table 7-3. List of matching proteins to the 15 peptides by FASTS search using FASTA (VIGEN) against NCBI/Blast Swiss-Prot library

Matching sequence (accession number)	Name of the sequence	Bit s	E-value
gi|118672	Dihydrolipoamide dehydrogenase	306	6.4e-83
gi|11135078	Probable soluble pyridine nucleotide transhydrogenase	48	3.9e-05
gi|266529	MERA_STRLI MERCURIC REDUCTASE HG(II) RE	44	0.00048
gi|129053	Dihydrolipoamide acetyltransferase	44	0.00077
gi|731029	Trypanothione reductase (TR)	43	0.0012
gi|18271667	Probable pyridine nucleotide-disulfide ox	42	0.0022
gi|1170041	Glutathione reductase (GR)	40	0.0074
gi|21431593	Mercuric reductase	37	0.048
gi|416801	CHMU_YEAST Chorismate mutase (CM)	34	0.28
gi|21431846	STS1_ARATH Strictosidine synthase 1 pr	34	0.32
gi|134588	SNF1_YEAST CARBON CATABOLITE DEREPRESSIN	34	0.62
gi|126667	M49_STRPY M protein, serotype 49 precursor	33	0.85
gi|1174667	THD1_HAEIN Threonine dehydratase biosyn	33	1.8
gi|730714	SASB_ANAPL FATTY ACYL-COA HYDROLASE PREC	33	2
gi|6093521	NODI_RHIS3 Nodulation ATP-binding prote	32	2.2
gi|2492587	YD34_MYCPN Hypothetical ABC transporter	31	3.5
gi|18266778	NODI_RHIME Nodulation ATP-binding prot	31	6.5

Discussion

An attempt to obtain the 58 kDa protein sequence of *Corynebacterium* sp. B-4247 by Edman degradation was not possible because of insufficient quantity of sample. Hence, the protein was purified again and analysed by liquid chromatography-tandem mass spectrometry. In the analysis of the 58 kDa protein of *Corynebacterium* sp. B 4247 by mass spectrometry, the Sequest algorithm search match was based on the NCBI non-redundant database since the *Corynebacterium* sp. genome sequence is not available. Thus, the peptides obtained may not provide full information on the peptides released by tryptic digestion. Future efforts to genetic engineer the bacterial xylose reductase for xylitol production will need more accurate amino acid sequence information. Obtaining the 58 kDa protein sequence by polymerase chain reaction (PCR) with primers synthesized according to the peptide sequences may not be sufficient as the peptides matched more than one protein of the *B. subtilis* genome and PCR amplification may produce sequences related to dihydrolipoamide dehydrogenase components and other proteins. Hence it is essential to obtain a more definite amino acid sequence of xylose reductase from *Corynebacterium* sp. B 4247.

The peptides of the 58 kDa protein of *Corynebacterium* sp. B 4247 identified with sequence 1 and sequences 2 (*B. subtilis*) are from E2 and E3 components of multi-enzyme system consisting of pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex and oxo-acid dehydrogenase complex. The pyruvate dehydrogenase complex serves as the key enzyme linking the glycolytic pathway and the TCA cycle and regulates the metabolic pathway. This multi-enzyme regulatory mechanism could explain the growth associated aerobic nature of xylitol production in *Corynebacterium* sp. B 4247. Further study is essential, as the binding mechanisms proposed by Lee (1998) for xylose reductase could not be applied.

The FASTA search of the NCBI non-redundant protein database with the15 identified peptides using the FASTS produced matches (Table. 7-3) to E3 (dihydrolipoamide dehydrogenase) and E2 (dihydrolipoamide acetyltransferase) component of pyruvate

dehydrogenase, trypanothione reductase (TR), glutathione reductase (GR) and mercuric reductase of class I and class II oxido reductases. Because the peptide identifications were based on the *B. subtilis* E2 and E3 components of multi-enzyme system consisting of pyruvate dehydrogenase complex the resulting matches were from the family of class I and class II oxidoreductases.

The bacterial putative NAD(P)H-dependent xylose reductase sequences of *E. coli* (gi|1786621|gb|AAC73522.1| AE000148), *Yersinia pestis* KIM [gi:22125622] and *Salmonella typhimurium* LT2 [gi:16763801] have not been shown to produce xylitol. Whereas *Corynebacterium* sp. B 4247 has been shown to produce xylitol and the 58 kDa protein of *Corynebacterium* sp. B 4247 has been shown to have both NADH and NADPH –dependent xylose reductase activities. Hence, the aldose reductase activity present in the 58 kDa protein of *Corynebacterium* sp. B 4247 may not be similar to putative reductases of the *E. coli* (gi|1786621|gb|AAC73522.1| AE000148), *Yersinia pestis* KIM [gi:22125622] and *Salmonella typhimurium* LT2 [gi:16763801] as the peptides did not show any match to them.

The 58 kDa protein of *Corynebacterium* sp. B 4247 is closer to the molecular weight of the native xylose reductase protein of some yeasts. The peptide "Q**MSSLVT**R" produced a 75% match (Table 7-1) with E-value of 8e-04 to the *S. cerevisiae* keto-aldose reductase (Gre3p) protein that is induced by osmotic stress, and is similar to xylose reductases from other fungi that reduce xylose to xylitol. Though some *S. cerevisiae* mutants are capable of reducing xylose to xylitol (Gong *et al*., 1983), they are not efficient xylitol producers (Parajo *et al*., 1998b).

The peptides of the 58 kDa match to several plants, human, bacteria and yeast aldose reductases (Table 7-1). Ten of the peptides showed match to the aldo-keto reductase family of *C. elegans*, a nematode (Table 7-1). The peptide "LLNDPQLILMEA**"** had conserved match "LL + DP" over several aldose reductases (Table 7-2). These findings are consistent with the conclusion of Jez *et al*. (1997) that aldose reductase family represents a divergent evolution from a multifunctional ancestral protein.

CHAPTER 8
CONCLUSIONS

The bacterial strains that utilized D-xylose for growth were investigated for xylitol production. Ten of the 17 bacteria screened from three genera of facultative bacteria (*Serratia, Cellulomonas*, *and Corynebacterium*) could grow on D-xylose and produce detectable quantities of xylitol during 24-96 h fermentation. The strains are *C. cellulans* ATCC 12830, NRRL B-4567 and NRRL B-2380, *C. glutamicum* NRRL B-3330 and NRRL B-2784, *C. ammoniagenes* NRRL B-4246, *Corynebacterium* sp. NRRL B-4247, and *S. marcescens* NRRL B-284, B-486 and NRRL B-3401. *Corynebacterium* sp. B 4247 produced the highest amount of xylitol.

Corynebacterium sp. B 4247 gave the highest xylitol yield (0.57 g/g) in 75 g/l initial D-xylose concentration under high aeration conditions (100 ml volume in 500 ml flask). D-xylose in the media induced xylitol production. Glyceraldehyde concentrations greater than 1 g/l in the media inhibited growth and xylitol production. In cell-free extracts the NADPH dependent reductase activity with DL-glyceraldehyde as substrate was 2.17 times that to D-xylose as substrate.

Addition of D-xylose to cultures grown in gluconate-containing media for 48 h, increased accumulation of xylitol but reduced yield. One reason for the accumulation of xylitol in the presence gluconate was the low NAD^+ dependent xylitol dehydrogenase activity. Maximum difference between the specific activity of xylose reductase and xylitol dehydrogenase in cell-free extracts occurred at 24 h. For cell cultures that were grown in gluconate for 48 h, the maximum difference in the reductase and dehydrogenase activities occurred at 12h after xylose addition. The co-factor specific activity for reductase activity in cell-free extracts was slightly higher with NADH compared to that of NADPH.

The native enzyme of *Corynebacterium* sp. B 4247 that reduces D-xylose has a molecular weight of 58 kDa. The bands that had in-gel activity for xylose in the native gel corresponded to 58, 49, 39 and 30 kDa in the SDS-PAGE. The purified fraction that had

highest specific activity from the Red Sepharose column corresponded to a 58 kDa protein.

The peptides of the 58 kDa protein of *Corynebacterium* sp. B 4247 identified with E2 and E3 (*B. subtilis*) components of a multi-enzyme system consisting of pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex and oxo-acid dehydrogenase complex. A 75% match was shown by the peptide "Q**MSSLVT**R" with E-value of 8e-04 to the *S. cerevisiae* keto-aldose reductase (Gre3p) that is induced by osmotic stress, and is similar to xylose reductases from other fungi that reduce xylose to xylitol. The peptide "LLNDPQLILMEA**"** had match to "LL + DP" which is conserved among several aldose reductases.

Yeast xylose reductase of *C. tropicalis* ATCC 96745 was purified. The molecular weight of NADPH dependent xylose reductase by SDS-PAGE was about 37 kDa.

RECOMMENDATIONS

1. Mixed growth studies with glucose, D-xylose and gluconate are essential to better understand xylose metabolism in *Corynebacterium* sp. B 4247.

2. Genetic engineering of the 58 kDa protein of *Corynebacterium* sp. B 4247 will require additional preliminary work. Two routes are possible. First, protein sequence information of the 58 kDa protein has to be obtained by Edman degradation. The alternate way of using the peptide sequence to synthesize PCR primers and elucidate the 58 kDa protein sequence by PCR is not possible as the peptides in the 58 kDa protein were identified based on two proteins of the *B. subtilis* genome.

   Secondly, de nova sequencing of the data obtained by mass spectral analysis can reveal peptides unique to the xylose reductase, which, after confirmation with synthetic peptides, can be used to generate PCR primers.

References

Aguirre-Zero, O., D.T. Zero, and H.M. Proskin. 1993. *Caries Research* 27**:**55-59.

Amaral-Collaco, M.T., G.M Girio, and M.A. Peito. 1989. In *Enzyme Systems for lignocellulosic degradation*, ed. Coughlan M.P. pp 221-230. London: Elsevier.

Atlas, R.M. 1997. In *Handbook of microbiological media*, ed. L.C. Parks. New York: CRC.

Bar, A.1991. Xylitol. In *Alternative sweetener*, ed. Nabors, L. O. and Gelardi, R. C. 2nd. edition, 349-379. N.Y., Basel, Hong-Kong: Marcel Dekker Inc.

Barbosa, M.F.S., H. Lee, H. Schneider, and C.W. Forsberg. 1990. Temperature mediated changes of D-xylose metabolism in the yeast *Pachyoslen tannophilus*. *FEMS Microbiol. Letters*. 72:35-40.

Barbosa, M.F.S., M.B. de Medeiros, I.M. de Mancilha, H. Schneider, and H. Lee1988. Screening of yeasts for production of xylitol from D-xylose and some factors which affect xylitol yield in *Candida guilliermondii. J. Indust. Microbiol*. 3: 241-251.

Beutler, H.O 1984. Xylitol. In *Methods of enzymatic analysis*, ed. Hans Ulrich Bergmeyer, 3rd edn, vol 6, pp 484-490. Weinheim: Verlag Chemie.

Bichio, P.A., P.L. Runnals, J. D. Cunningham, and R. Lee.1988. Induction of xylose reductase and xylitol dehydrogenase activities in *Pachysolen tannophilus* and *Pichia stiptis* on mixed sugars. *Appl. Environ. Microbiol.* 54:50-54.

Bjorling, T. and B. Lindman. 1989. Evaluation of xylose fermenting yeasts for ethanol production from spent sulfite liquor. *Enzyme Microbiology Technology*. 11:240-246.

Bruinenberg, P.M., P.H.M. de Bot, J.P. van Dijken, and W.A. Scheffers. 1984. NADH linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. *Appl. Microbiol. Biotechnol.* 19: 256-260.

Cao, N.J., R. Tang, C.S. Gong, and L.F. Chen. 1994. The effect of cell density on the production of xylitol from D-xylose by yeast. *Applied Biochemistry and Biotechnology*. 45/46: 515-519.

Chen, L.- F., C.- S. Gong. 1985. Fermentation of sugarcane bagasse hemicellulose hydrolyzate to xylitol by a hydrolyzate-acclimatized yeast. *J. Food Science* 50:226-228.

Chiang, C., and S.G. Knight. 1961. Metabolism of D-xylose by moulds. *Nature.*188:79-81.

Collins, M.D., and C.S. Cummins. 1984. Genus *Corynebacterium* Lehmann and Neumann 1896, 350^{AL}. In *Bergey's manual of Systematic Bacteriology*. Vol.2, ed. Sneath, P.H.A., (associate eds.) N.S. Mair and M.E. Sharpe, (ed-in-chief.) J. G. Holt. pp. 1265 –1276. Baltimore: Williams and Wilkins.

Counsell, J. M.1977. Xylitol. *International Symposium by Roche Products Ltd and Xyrofin Ltd*. London.

Counsell, J.N. 1978. Xylitol. London: Applied Science Publishers.

Da Silva, S.S., and A.S. Afschar. 1994. Microbial production of xylitol from D-xylose using *Candida tropicalis. Bioprocess Engineering* 11:29-134.

Dahiya, J.S., 1991. Xylitol production by *Petromyces albertensis* grown on medium containing D-xylose. *Can. J. Microbiol.* 37:14-18.

Debus, D., H. Methner, D. Schulze, and H. Dellweg. 1983. Fermentation of xylose with the yeast *Pachysolen tannophilus*. *European Journal of Applied Microbiology and Biotechnology*. 17: 287-291.

Dominguez, J.M., N.J. Cao, C.S. Gong, and G.T. Tsao. 1996. Pretreatment of sugar cane bagasse hemicellulose hydrolysate for xylitol production by yeast. *Applied Biochemistry and Biotechnology* 57/8: 49-56.

Dominguez, J.M., N.J. Cao, C.S. Gong, and G.T. Tsao. 1997. Dilute acid hemicellulose hydrolysates from corncobs for xylitol production of yeast. *Bioresource Technology* 6: (1) 85-90.

Du Preez, J.C., D. van Driessel, and B.A. Prior. 1989. Effect of Aerobiosis on fermentation and key enzyme levels during growth of *Pichia stipitis*, *Candida shehatae* and *Candida tenius* on D-xylose. *Arch. Microbiol*. 152:143-147.

Du Preez, J.C., M. Bosch, and B.A. Prior. 1986. The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stiptis*. *Appl. Microbiol. Biotechnol.* 23:228-233.

Du Preez, J.C. 1994. Process parameters and environmental factors affecting D-xylose fermentation by yeasts. *Enzyme Microb. Technol.* 16: 944-956.

Emodi, A.1978. Xylitol: Its properties and food applications. *Food Technol.* 32: 20-32.

Felipe, M.G., I.M. Manciha, M. Vitolo, I. C. Roberto, and S. Rosa. 1993. Preparation of xylitol by fermentation of a hydrolyzates of hemicellulose obtained from sugar cane bagasse. *Arquivos de Biologia E Technologia*. 36:103-114.

Felipe, M.G., D.C. Vieira, M.Vitolo, S.S. Silva, and I.C. Roberto, and I.M. Mancilha. 1995. Effect of acetic-acid on xylose fermentation to xylitol by *Candida guilliermondii Journal of Basic Microbiology*. 35:171-177.

Fischer.E., and R. Stahel. 1891. Zur Kenntnis der Xylose, *Berichte Dtsch. Chem. Gsellschaft* 24: 528-539.

Forester, M. 1988 US Pat. 4762719. H

Forster, H. 1974. In *Sugars in Nutrition*, ed. H.L. Sipple, and K. McNutt. pp 259. New York: Academic Press.

Furlan, S.A., P. Boutlloud, P. Strehaiano, and J.P. Riba. 1991. Study of xylitol formation from xylose under oxygen limiting conditions. *Biotechnol. Lett.* 13:203-206.

Furlan, S.A., P. Boutlloud, and H.F Decastro. 1994. Influence of oxygen on ethanol and xylitol production by xylose fermenting yeasts. *Process Biochemistry* 29: (8) 657-662.

Girio, F.M., M.A. Peito, and M.T. Amaral-Collaco. 1989. Enzymatic and physiological study of D-xylose metabolism by *Candida shehatae. Appl. Microbiol. Biotechnol*. 32: 199-204.

Girio, F.M., M.A. Peito, and M.T. Amaral-Collaco. 1990. In Biomass for Energy and Industry, Vol. 2, eds Grassi, G., Gosse, G. and dos Santos, G. : Elsevier Applied Science.

Girio, F.M., J.C. Roseiro, P. Sa-Machado, A.R. Duarte-Reis, and M.T. Amaral-Collaco. 1994 . Effect of oxygen transfer rate on levels of key enzymes of xylose metabolism in *Debaryomyces hansenii. Enzyme Microb. Technol*.16:1074-1078.

Girio, F.M., F. Pelica, and M.T. Amaral Collaco.1996. Characterization of xylitol dehydrogenase from *Debaryomyces hansenii. Applied Biochemistry and Biotechnology* 56:(1) 79-87

Gomez, G.I. 1987. *Ph.D. dissertation.* Faculte des Sciences Agronomiques de l'Etat Gembloux, Belgium.

Gong, C.H., L.F. Chen, and G.T. Tsao. 1981. Quantitative production of xylitol from D-xylose by a high xylitol producing yeast mutant *Candida tropicalis* HXP2. *Biotechnol. Lett*. 3: 130-135.

Gong, C.S., T.A. Glaypool, L.D. McCraken, C.M. Maun, P.P. Ueng, and G.T. Tsao. 1983. Conversion of pentoses by yeasts. *Biotech. Bioeng*. 25: 85-102.

Grimont. P.A.D., and F. Grimont. 1984. Genus VIII. *Serratia* Bizio 1823, 288^{AL}. In *Bergey's manual of Systematic Bacteriology*. Vol.1, ed. Krieg, N.R., (ed-in-chief.) J. G. Holt. pp. 477 – 484. Baltimore: Williams and Wilkins.

Hahn-Hagerdal, B., H. Jeppson, K. Skoog, B. and A. Prior. 1994. Biochemistry and physiology of xylose fermentation by yeast. *Enzyme Microb. Techol.* 16: 933-943

Hassinger, W., G. Sauer, U., Cordes, U. Kraise, J. Beyer, and K. Baessler, 1981. *Diabetologia*. 21: 37-39.

Hespell, R B., P. J. Q'Bryan, M. Moniruzzaman, and R. J. Bothast. 1997. Hydrolysis by commercial enzyme mixtures of AFEX-treated corn fiber and isolated xylans. *Appl. Biochem. Biotechnol.* 62: 87-96.

Hinfray, C., T. Jouenne, L. Mignot, and G.-A Jooter. 1995. Influence of the oxygenation level on D-xylose fermentation by free and agar-entrapped cultures of C. *shehatae. Appl. Microbiol. Biotechnol.* 42: 682-687.

Hollman, S., and O.Touster. 1964. *Non-Glycolic pathways of Metabolism of Glucose*. pp.126. Newyork and London: Academic Press Inc.

Holtzapple, M.T., J.-H. Jun, G. Ashok, S.L. Patibandla, and B.E. Dale. 1991. The ammonia freeze explosion (AFEX) process. A practical lignocellulose pretreatment. *Applied Biochemistry and Biotechnology* 28/29: 59-74.

Horeckar, B.L 1962. *Pentose metabolism in Bacteria.* pp.19. New York: Whiley.

Ho, N.W.Y., F.P. Lin, S. Huang, P.C. Andrews, and G.T. Tsao. 1990. Purification and charecterization, and amino terminal sequence of xylose reductase from *Candia shehatae*. Enzyme Micorbiol Technol. 12:33-39.

Horitsu, H.,Y.Yahashi, K. Takamizawa, K. Kawai, T.Suzuki, and N. Watanabe. 1992. Production ofxylitol from D-xylose by *Candida tropicalis:* Optimization of production rate. *Biotechnology and Bioengineering.* 40:1085-1091.

Hyvonen, L. and M. Slotte. 1983. Alternative Sweetening of yoghurt. *Journal of Food Technology*. 18: 97-112.

Izumori, K. and K. Tuzaki. 1988. Production of xylitol from D-xylose by *Mycobacterium smegmatis. J. Ferment. Technol*. 66: 33-36.

Jaffe, G.M. 1978. Xylitol – A Specialty Sweetner. *Sugar y Azucar*. 93: 36-42.

Jeffries, T.W., and H.K. Sreenath. 1988. Fermentation of Hemicellulosic sugars and sugar mixtures by *Candida shehatae. Biotechnology and Bioengineering* 31: 502-506.

Jeffries, T.W. 1983. Utilization of xylose by bacteria, yeast and fungi. *Advances Biochem. Biotechnol*. 27: 2-27.

Jeffries, T.W. and C.P. Kurtzman. 1994. Taxonomy of xylose-fermenting yeasts. *Enzyme Microb. Technol.* 6: 922-932.

Jez, M.J., J. Melanie, J. Bennett, B.P. Schlegel, M. Lewis, and T.M. Penning. 1997. Comparative anatomy of the aldo-keto reductase superfamily. *Biochem. J.* 326:625-636.

Kastner, J.R., R.S. Roberts, and W.J. Jones. 1996a. Effect of pH on cell viability and product yields in D-xylose fermentations by *Candida shehatae. Applied Microbiology Biotechnology* 45: 224-228

Kastner, J.R., R.S. Roberts, and W.J. Jones. 1996b. Effect of xylitol on the metabolism and viability of *Candida shehatae. Biotechnology Letters.* 18: 31-34.

Kern, M., B. Nidetzky, K. Kulbe, and D. Hiltrich.1998. Effect of nitrogen sources on the levels of aldose reductase and xylitol dehydrogenase activities in xylose fermenting yeast *Candida tenuis. Journal of Fermenting and Bioengineering* 85: 196-202

Kim, S., J.H. Kim, and D.K. Oh. 1997. Improvement of Xylitol Production by Controlling Oxygen Supply in *Candida parapsilosis Journal of Fermentation Bioengineering*, 83: 267-270.

Kitpreechsvanich, V., M. Hayashi, N. Nishio. and S. Nagai. 1984. Conversion of D-xylose into xylitol by xylose reductase from *Candida pelliculosa* coupled with oxido-reductase system of methanogen strain. *Biotechnol. Lett*. 6: 651-656.

Kruse, B. and K. Schugerl.1996. Investigation of Ethanol Formation *by Pachysolen tannophilus* from Xylose and Glucose/Xylose Co-substrates. *Process Biochemistry*. 31: 389-407.

Laemmli, U.K 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227: 680-685.

Lastick, S. M., M.Y. Tucker, J. R. Beyette, G. R. Noll, and K. Grohmann. 1989. Simultaneous fermentation and isomerization of xylose. *Applied Microbiology and Biotechnology*. 30:574-579.

Lee, H. 1998. The structures and function of yeast xylose (aldose) reductase. *Yeast* 14: 977-984.

Lee, H., A.L. Atkin, M.F.S. Barbosa, D.R. Dorsheid, and H. Schneider. 1988. Effect of biotin limitation on the conversion of xylose to ethanol and xylitol by *Pachysolen tannophilus* and *Candida guilliermondii*. *Enzy. Microb. Technol.* 10:81-84.

Lee, H., C. R. Sopher, and Y.F. Yau. 1996. Induction of xylose reductase and xylitol dehydrogenase activities on mixed sugars in *Candida guilliermondii*. *J. Chem. Tech. Biotechnol.* 66:375-379.

Lighthelm, M.E., B.A .Prior, and du Preez.1988. The oxygen requirement of yeasts for the fermentation of D- xylose and D-glucose to ethanol. *Appl. Microbiol. Biotechnol.* 28:63-68.

Lu. J., L.B. Tsai, C.S. Gong, and G.T. Tsao. 1995. Effect of nitrogen sources on xylitol production from D-xylose by *Candida* sp. L-102. *Biotechnology Letters* 17-2: 67-170

Lucas, C., and N.van Uden. 1986. Transport of hemicellulose monomers in the

xylosefermenting yeast *Candida shehatae. Appl. Microbiol. Biotechnol.* 23: 491-495.

Maleszka, R.L.G., A.P. Neirinck, H. James, Rutten, and H. Schneider.1983. Xylitol dehydrogenase mutants of *Pachysolen tannophilus* and the role of xylitol in D-xylose catabolism. *FEMS Microbiology Letters*.17:227-229.

Maloney, P.C. and S.V. Amburdkar.1989. Functional reconstitution of prokaryote and eukaryote membrane-proteins. *Archives of Biochemistry and Biophysics*. 269:1-10.

Manz, U., E. Vanninen, and F. Voirol. 1973. In *Food R.A. Symp. Sugar and Replacements.* 10 Oct., London.

Meinander, N., B. Hahn-Hagerdal, M. Linko, P. Linko, and H. Ojamo. 1994. Fed-batch xylitol production with recombinant XYL-I-expressing *Saccharomyces cerevisiae* using ethanol as a co-substrate. *Appl. Microbiol. Biotechnol*. 42:334-339.

Meyerial, C., J.P. Delegenes, R. Moletta, and J.M. Navarro. 1991. Xylitol production from D-xylose by *Candida guilliermondii:* Fermentation behavior. *Biotechnol. Lett*. 13: 281-286.

Mori, S. and J. Saraya. 1988. *Journal of Japanese Kokai Tokkyo Koho lP*, 63:135-322.

Nicklin, I., K. Graeme-Cook, T. Paget, and R.A. Killington. 1999. *Notes in microbiology*. New York, Berlin, Heidelberg: Springer Verlag.

Nigam, P., and D. Singh. 1995. Process for fermentative production of xylitol- a sugar substitute. *Process Biochem.* 30:117-124.

Nishio N., K. Sugawa, N. Hayase and S. Nagai. 1989. Conversion of D-xylose into xylitol by immobilized cells *Candida pelliculosa* and *Methanobacteium sp*. HU. *J.Fermentation. Bioeng*. 67: 356-360.

Nobre, M.F., and M. da Costa. 1985. Factors favoring the accumulation of arabinitol in the yeast *Debaryomyces hansenii*. *Canadian Journal of Mirobiology*. 31:467-471.

Nolleau, Y., L. Preziosi-Belloy, and J.M. Navarro. 1995. The reduction of xylose to xylitol by *Candida guilliermondii* and *Candida parapsilosis:* Incidence of oxygen and pH. *Biotechnology Letters*. 17-4: 417-422

Nolleau, V., K. Preziosibelloy, J.P. Delgenes, and J.M. Navarro. 1993. Xylitol production from xylose by two yeast strains- Sugar tolerance. *Current Microbiol*. 27: (4) 191-197.

Oh, D.-K., S.-Y. Kim, and J.-H. Kim.1998. Increase of xylitol production rate by controlling redox potential in *Candida parapsilosis*. *Biotechnology and Bioengineering*. 58:440-444.

Olsson, L., and B. Hahn-Hagerdal. 1993. Fermentative performance of bacteria and yeasts in lignocellulose hydrolyzates. *Process Biochemistry*. 28: 249-257.

Onishi, H., and T. Suzuki. 1966. The production of xylitol, L-arabinose and ribitol by yeasts. *Agric. Biol. Chem.* 30:1139-1144.

Palnitkar, S. and A. Lachke. 1992. Effect of nitrogen sources on oxidoreductive enzymes and ethanol production during xylose fermentation by *Candida shehatae*. *Canadian Journal of Microbiology*. 38:258-260.

Parajo, J.C., H. Dominguez, and J.M. Dominguez. 1996. Production of xylitol from concentrated wood hydrolysates by *Debaryomyces hansenii:* effect of the initial cell concentration. *Biotechnol. Lett*. 18:593-598.

Parajo, J.C., V. Santos, and M. Vazquez.1997. Co-production of carotenoids and xylitol by Xanthophyllomyces dendrorhous (*Phaffia rhodozyma*). *Biotechnology Letters*. 19:139-141.

Parajo, J.C., H. Dominguez and J.M. Dominguez. 1998a. Biotechnological production

of xylitol. Part1: Interest of xylitol and fundamentals of its biosynthesis. *Bioresource Technology*. 65:(3)191-201.

Parajo, J.C., H. Dominguez, and J.M. Dominguez. 1998b. Biotechnological production of xylitol. Part 2: Operation in culture media made with commercial sugars. *Bioresource Technology*. 65:203-212.

Parajo, J.C., H. Dominguez, and J.M. Dominguez. 1998c. Biotechnological production of xylitol. Part 3: Operation in culture media made with lignocellulose hydrolyzates. *Bioresource Technology*. 66:25-40.

Parekh, S.R., R.S. Parekh, and M.Wayman, 1987. Fermentation of wood-derived acid hydrolyzates in a batch bioreactor and in continuous dynamic immobilized cell bioreactor by *Pichia stipitis* R. *Process Biochem*. 22: 85-91.

Pepper, T., and P.M. Olinger, 1988. Xylitol in sugar-free confections. *Food Technol*. 10: 98-106.

Perego, P., A. Convery, E. Palazzi, M.D. Borghi, G. Ferraiolo. 1990. Fermentation of hardwood hemicellulose hydrolyzate by *Pachysolen Tannophilus, Candida Shehatae* and *Pichia stipitis. Journal of Industrial Microbiology* 6:157-164.

Perovitch, P. 1988 . Fr. Pat. 2,605,854.

Peschke, N., E. Roca, and M.J. Nunez. 1992. IV National Congress and I Spanish-Portuguese Congress on Biotechnology, Biotec-92. Santiago de Compostela, Spain.

Pfeifer, M.J., S.S. Silva, M.G.A. Felipe, I.C. Roberto, and I.M. Mancilha.1996. Effect of culture conditions on xylitol production by *Candida guilliermondii* FTI 20037. *Appl. Biochem. Biotechnol*. 57-58:423-430.

Prior, B.A., S.G. Kilian, and J.C. Du Preez. 1989. Fermentation of D-xylose by the yeasts *Candida shehatae* and *Pichia stipitis* - prospects and problems. Process Biochemistry, 24, 21-32.

Prior, B.A., and P. Kotter. 1997. Pentose utilization in yeasts. in: *Yeast sugar metabolism*. Zimmermann, F. K., Entian, K.-D:435-457.

Rala, G., A . Scheie, and S. Assev. 1987 . *Deutsche Zahnarztul?,* 42, 39-41.

Ritzel, G. and G. Brubacher. 1976. In Monosaccharides and Polyalcohols in Nutrition, Therapy and Dietetics.Huber, Bern.

Rizzi, M., P. Erlemann, N.A. Bui-Thanj and H. Dellweg. 1988. Xylose fermentation by yeasts. 4. Purification and kinetic studies of xylose reductase from *Pichia stipitis*. Appl. *Microbiol. Biotechnol*. 29:2-3.

Roberto, I.C., S. Sato, I.M.de Mancilha. 1996. Effect of inoculum level on xylitol production from rice straw hemicellulose hydrolyzates by *Candida guilliermondii*. *Journal of Industrial Microbiology*. 16:348-350.

Roberto, I.C., M.G.A. Felipe, I.M. de Mancilha, M.Vitolo,S. Sato, and S.S. da Silva.1995. Xylitol production by *Candida guilliermondii* as an approach for the utilization of agroindustrial residues. *Bioresource Technology* 51: 255-257.

Roberto, I.C., S. Sato, I.M. de Mancilha, and M.E.S. Taqueda. 1995b. Influence of media composition on xylitol fermentation by *Candida guilliermondii* using response surface methodology. *Biotechnol Lett.* 17-11:1223-1228.

Saha, B.C. and R.J. Bothast.1996. Production of L-arabitol from L-arabinose by *Candida entomaea* and *Pichia guilliermondii. Applied Microbiology and Biotechnology*. 45: 299-306.

Saha, B.C., and R.J. Bothast, 1997. In *Fuels and chemicals from biomass,* American chemical society, ACS symposium series. 666:307-319

Saha, B.S., B.S. Dien, and R.J. Bothast, 1998. Fuel ethanol production from corn fiber: Current status and technical prospects. *Applied Biochemistry and Biotechnology* 70-72: 115-125.

Saha, B.C. and Bothast, R.J. 1999. Pretreatment and enzymatic saccharification of corn fiber. *Applied Biochemistry and Biotechnology* 76:65-77.

Schlegel, H.G. 1992. *Allgemeine Mikrobiologie.* Thieme Verlag Stuttgart, New York, 7. Auflage.

Silva, S. S., I.C. Roberto, M.G.A. Felipe, and I. M.Mancilha.1996. Batch fermentation of xylose for xylitol production in stirred tank bioreactor. *Process Biochem*. 31:549-553

Silva, S. S., I.C. Roberto, M.G.A. Felipe, and I. M.Mancilha.1998. Factors that affect the biosynthesis of xylitol by xylose-fermenting yeasts. *Applied Biochemistry and Biotechnology* 70-72: 331-339

Silva, S.S., A. Quesada-Chanto, and M. Vitolo.1997. Upstream parameters affecting the cell growth and xylitol production by *Candida guilliermondii* FTI 20037, *Zeitschriftfiir Naturforschung*. 52c:359-363.

Sirisansaneeyakul, S., M. Staniszewski, and M. Rizzi. 1995. Screening of Yeasts for Production of Xylitol from D-Xylose. *Journal of Fermentation Bioengineering*. 80:565-570.

Skoog, K., and B.Hahn-Hagerdal. 1988. Xylose fermentation. *Enzyme Microb. Technol*. 10:66-80.

Skoog, K., and B. Hahn-Hagerdal. 1990. Effect of oxygenation on xylose fermentation by *Pichia stipitis*. *Applied and Environmental Microbiology* 56: 3389-3394

Sommer, R. 1996. Production of Yeast Extracts. *Presented at 9th International Symposium on Yeasts*, Sydney, August.

Sreenath, H.K., T.W. Chapman, and T.W. Jeffries. 1986. Ethanol production from D-xylose in batch fermentations with *Candida shehatae:* Process variables. *Appl. Microbiol. Biotechnol.* 24: 294-299.

Sirisansaneeyakul, S., M. Staniszewski, and M. Rizzi.1995. Screening of yeasts for production of xylitol from D-xylose. *J. Ferment. Bioeng*. 6:564-570.

Stackebrandt, E., and R. M. Keddie. 1984. Genus *Cellulomonas* Bergey *et al*. 1923, emund. mut. char. Clark 1952, 50^{AL}. In *Bergey's manual of Systematic Bacteriology*. Vol.2, ed. Sneath, P.H.A., (associate eds.) N.S. Mair and M.E. Sharpe, (ed-in-chief.) J. G. Holt. pp. 1325 –1329. Baltimore: Williams and Wilkins.

Suihko, M.L. 1984. Ph.D Thesis. University of Helsinki.

Suzuki, T., and H. Onishi. 1975. Agric. Biol. Chem. 39:2389.

Taylor, K.B., M.J. Beck, D.H. Huang, and T.T. Sakai. 1990. The fermentation of xylose - studies by c-13 nuclear-magnetic-resonance spectroscopy. *J. Industrial Microbiol* 6:29-41.

Thonart, P., X. Gomez, J. Dideelez, and M. Paquot. 1984. Biological conversion of xylose to xylitol by *Pachsolen tannophilus*. In *Anaerobic digestion and carbohydrate hydrolysis of waste,* ed. G.L. Ferrero, M.P. Ferranti, and H. Naveau, pp. 429-431 Commission of the European Communities, Luxemburg.

Ueng, P.P., and C.S. Gong. 1982. Ethanol production from pentoses and sugarcane bagasse hemicellulose hydrolysate by *Mucor sp*. and *Fusarium sp. Enzyme Microb. Technol* 4:169-171

Uhari, M., T. Kontiokari, M. Koskela, and M. Niemela. 1996. Xylitol chewing gum in prevention of acute otitis media: double blind randomized trial. *British Medical Journal*. 313: 1180-1184.

Verduyn, C., J. Frank, J.P. van Dijken, and W.A. Scheffers. 1985. Multiple forms of

xylose reductase in *Pachysolen tannophilus* CBS 4044. *FEMS Microbiol. Lett*. 30:313-317.

Vandeska, E., S. Amartey, S. Kuzmanova, and T.W. Jeffries. 1996. Fed-batch culture for xylitol production by *Candida boidinii. Process Biochem*. 31, 265-270 .

Vandeska, E., S. Kuzmanova and T.W. Jeffries. 1995. Xylitol formation and key enzyme activities in *Candida boidinii* under different oxygen transfer rates. J. *Ferment. Bioeng*. 80:513-516.

Vongsuvanlert. V., and Y. Tani 1989. Xylitol production by a methanol yeast, *Candida boidinii* (*Kloeckera sp*.). No.2201. *J. Ferment. Bioeng*. 67: 35-39.

Walther, T. 1999. The influence of hemicellulosic sugars on xylitol production by *Candida tropicalis.* Technical University Dresden, Department of Mechanical Engineering, final thesis.

Walther, T., P. Hensirisak, and F.A. Agbelvor. 2001. The influence of aeration and hemicellulosic sugars on xylitol production by *Candida tropicalis*. *Bioresource Technol*. 76:213-220.

Wang, Y.-M and J.Eys. Vans. 1981. Nutritional significance of fructose and sugar alcohols. 1: 437-475.

Watson, N. E., B. A. Prior, and P.M. Lategan. 1984.Oxygen requirements for D-xylose fermentation to ethanol and polyols by *Pachysolen tannophilus*. *Enzyme Microbiology Technology*. 6: 447-450

Whistler, R.L.1993. Hemicelluloses. In *Industrial gums-Polysaccharides and their derivates,* ed. R L. Whistler, and J.N. BeMiller Third edition, pp. 295-308, San Diego: Academic press.

Winkelhausen, E., P. Pittman, S. Kuzmanova, and T.W. Jeffries. 1996. Xylitol formation in oxygen limited chemostat culture. *Biotechnology Letters*, 18. 753-758.

Winkelhausen. E and S. Kuzmanova. 1998. Microbial conversion of D-xylose to xylitol. *Journal of Fermentation and Bioengineering*. 86 (1): 1-14.

Woods, M. A. and N.F. Millis. 1985. *Biotechnology Letters.* Effect of slow feeding of xylose on ethanol yield of *Pachysolen tannophilus*.7: 679-682.

Yahashi, Y., H. Horitsu, K. Kawai, T. Suzuki, and K.Takamizawa. 1996. Production of xylitol from D-xylose by *Candida tropicalis:* the effect of D-xylose feeding. *J. Ferm. Technol.* 81:148-152.

Yokoyama, S., T. Suzuki, K. Kawai, H. Horitsu, and K. Takamizawa. 1995. Purification, characterization and structure analysis of NADPH-dependent D-xylose reductases from *Candida tropicalis*. *J. Ferment. Bioeng*. 79: 217-223

Yoshitake, J., H. Ishizaki, M. Shimamura, and T. Imai. 1973a. Xylitol production by an *Enterobacter* species. *Agric. Biol Chem*. 37: 2261-2267

Yoshitake, J., H. Ohiwa, and M. Shimamura. 1971. Production of polyalcohol by a *Corynebacterium* sp. Part I. Production of pentol from aldopentose. *Agric. Biol Chem*. 35: 905-911.

Yoshitake, J., M. Shimamura, and T. Imai. 1973b. Xylitol production by an *Corynebacterium* species. *Agric. Biol Chem*. 37: 2251-2259

Yoshitake, J., M. Shimamura, H. Ishizaki, and Y. Irie 1976. Xylitol production by an *Enterobacter liquefaciens. Agric. Biol Chem*. 40(8): 1493-1503.

Zhao, X., P. Gao, and Z. Wang. 1998. The production and properties of a new xylose reductase from fungus *Neurospora crassa*. *Appl Biochem Biotechnol*.70-72: 405-414.

Appendix A

One unit (U) of enzyme was defined as the rate of decrease of 1μmol of NADPH per min

$$\text{The catalytic Activity (b)} = \frac{\Delta A \times V \times 1000}{\Delta t \times \xi \times d \times v} \ \mu\text{mol /min.l}$$

$$\text{The catalytic Activity (b)} = \frac{\Delta A \times V \times 1000}{\Delta t \times \xi \times d \times v} \ \text{U/l}$$

$$\text{The catalytic Activity (b)} = \frac{\Delta A \times V}{\Delta t \times \xi \times d \times v} \ \text{U/ml}$$

The specific Activity = b/m U/mg

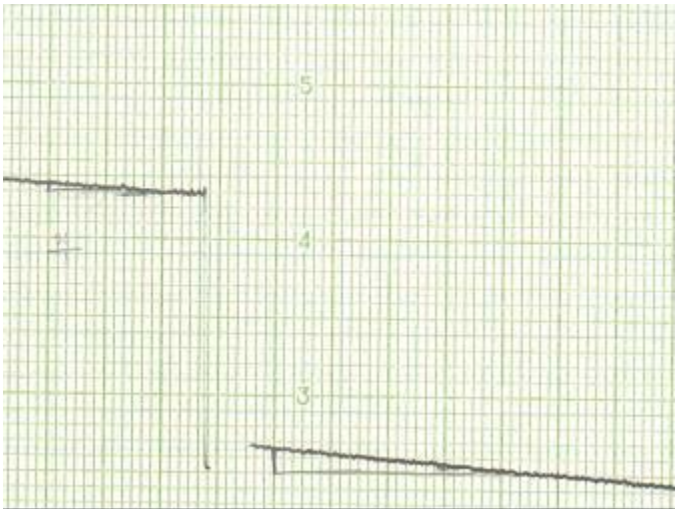
Where

ΔA - difference in absorbance at 340 nm
Δt - difference in time in min
V - assay volume in ml
ξ - linear millimolar extinction co-efficient of NADPH or NAD in l/mmol.mm
(0.632 l/mmol.mm at 340 nm)
d – light path in mm = 10 mm
v – volume of the enzyme solution in the assay in litres
m – mass of the protein in mg/ml

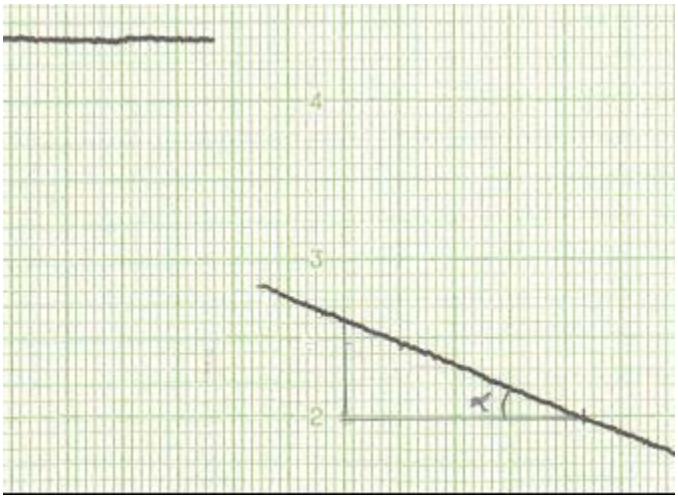
*Methods in Enzymatic analysis (1983) Vol VI p.689

Appendix B

***Corynebacterium* sp. B 4247 xylose reductase activity with substrates: D-xylose (A), Glyceraldehyde (B)**



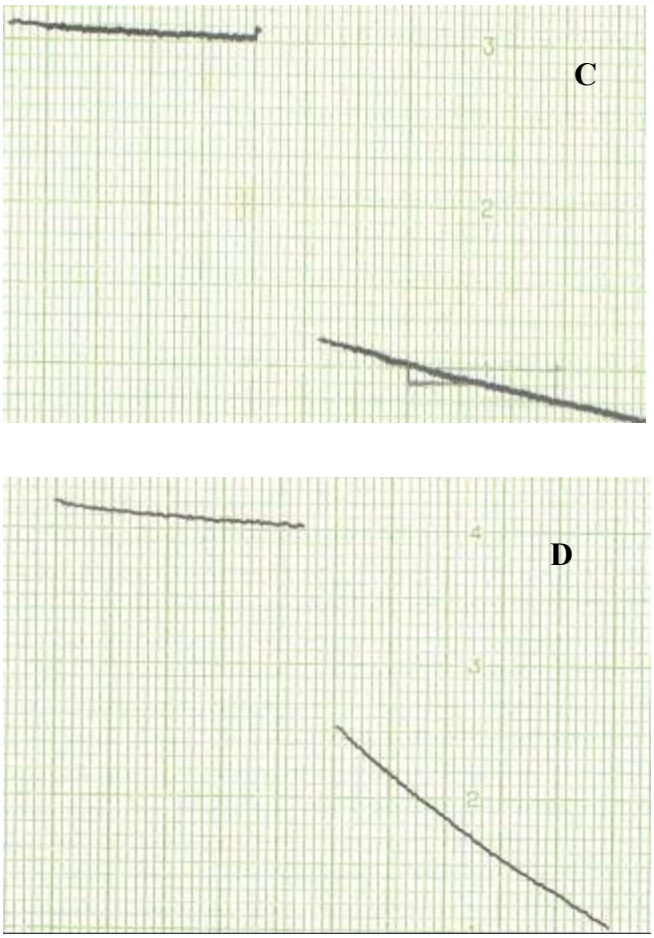
A



B

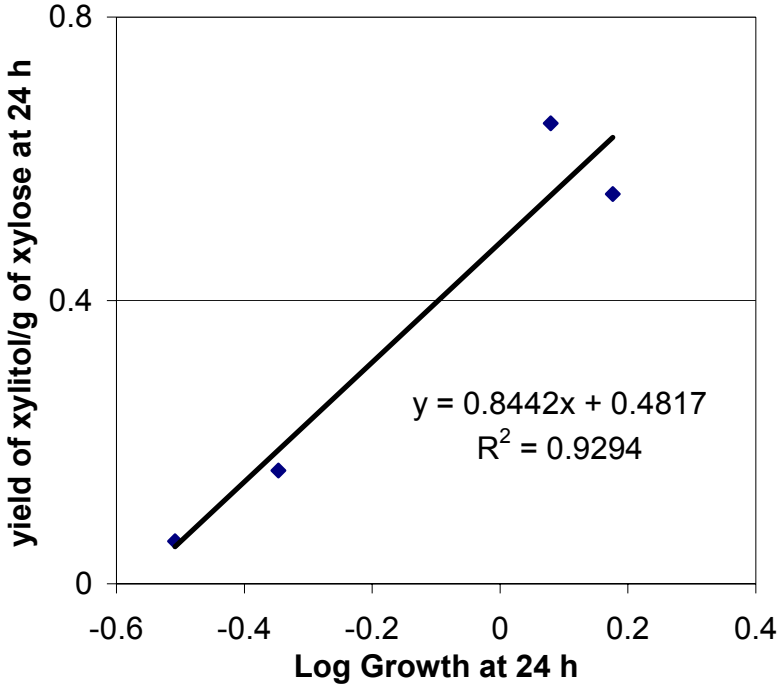
Appendix C

***Candida tropicalis* ATCC 96745 xylose reductase activity with substrates: D-xylose (C), Glyceraldehyde (D)**



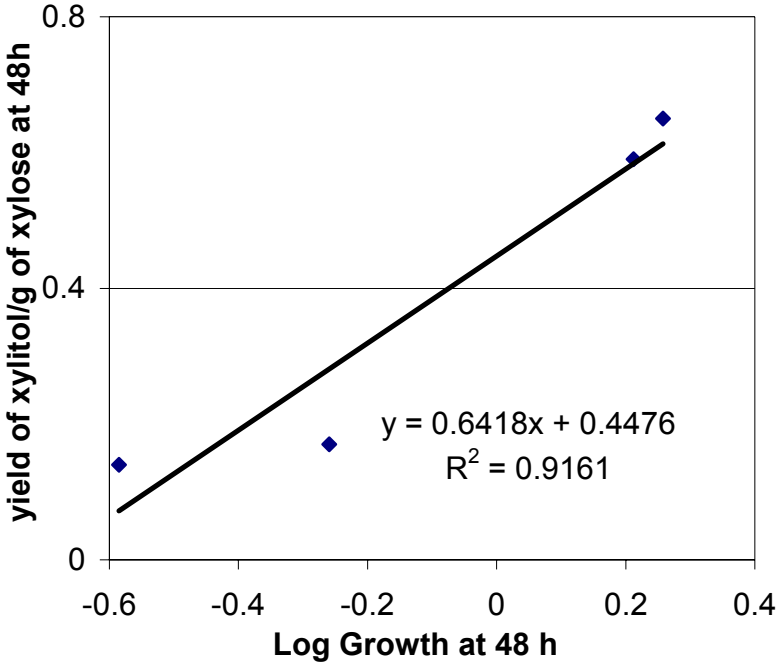
Appendix D

***Corynebacterium* sp. B 4247 growth *vs* yield in 100 ml, 200 ml, 350 ml and 450 ml volume cultures in 500 ml flasks at 24 h**



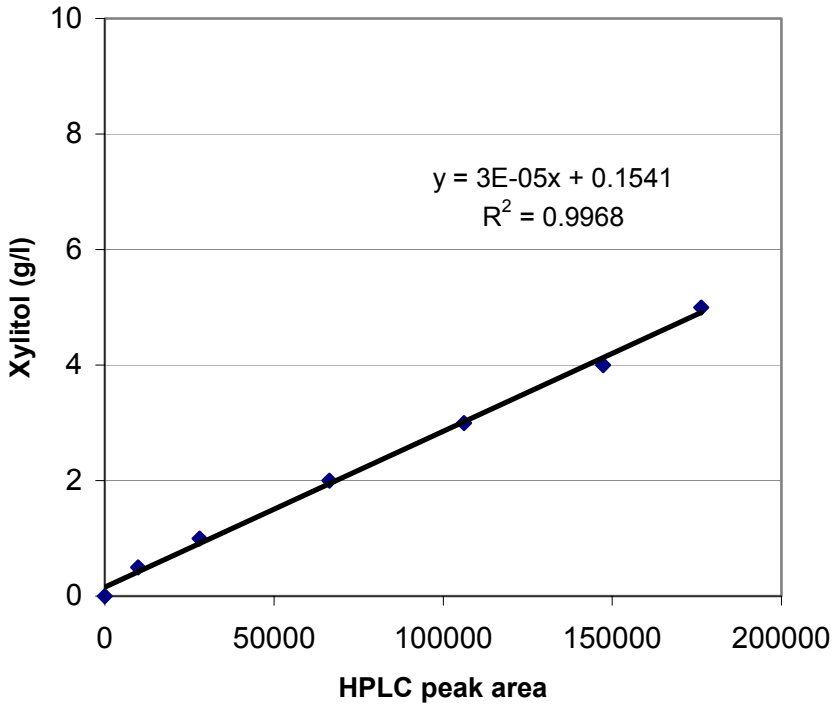
Appendix E

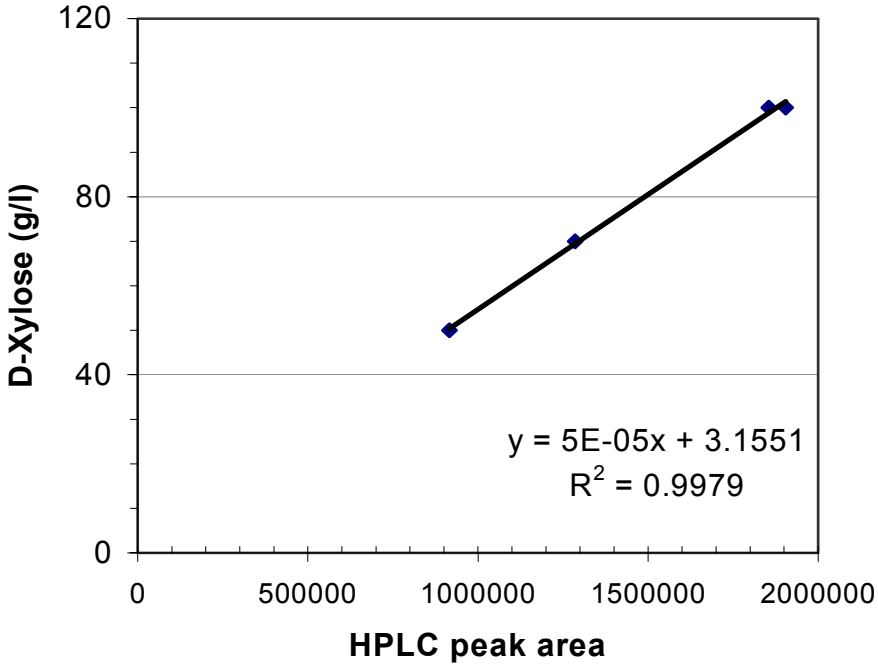
***Corynebacterium* sp. B 4247 growth *vs* yield in 100 ml, 200 ml, 350 ml and 450 ml volume cultures in 500 ml flasks at 48 h**



Appendix F

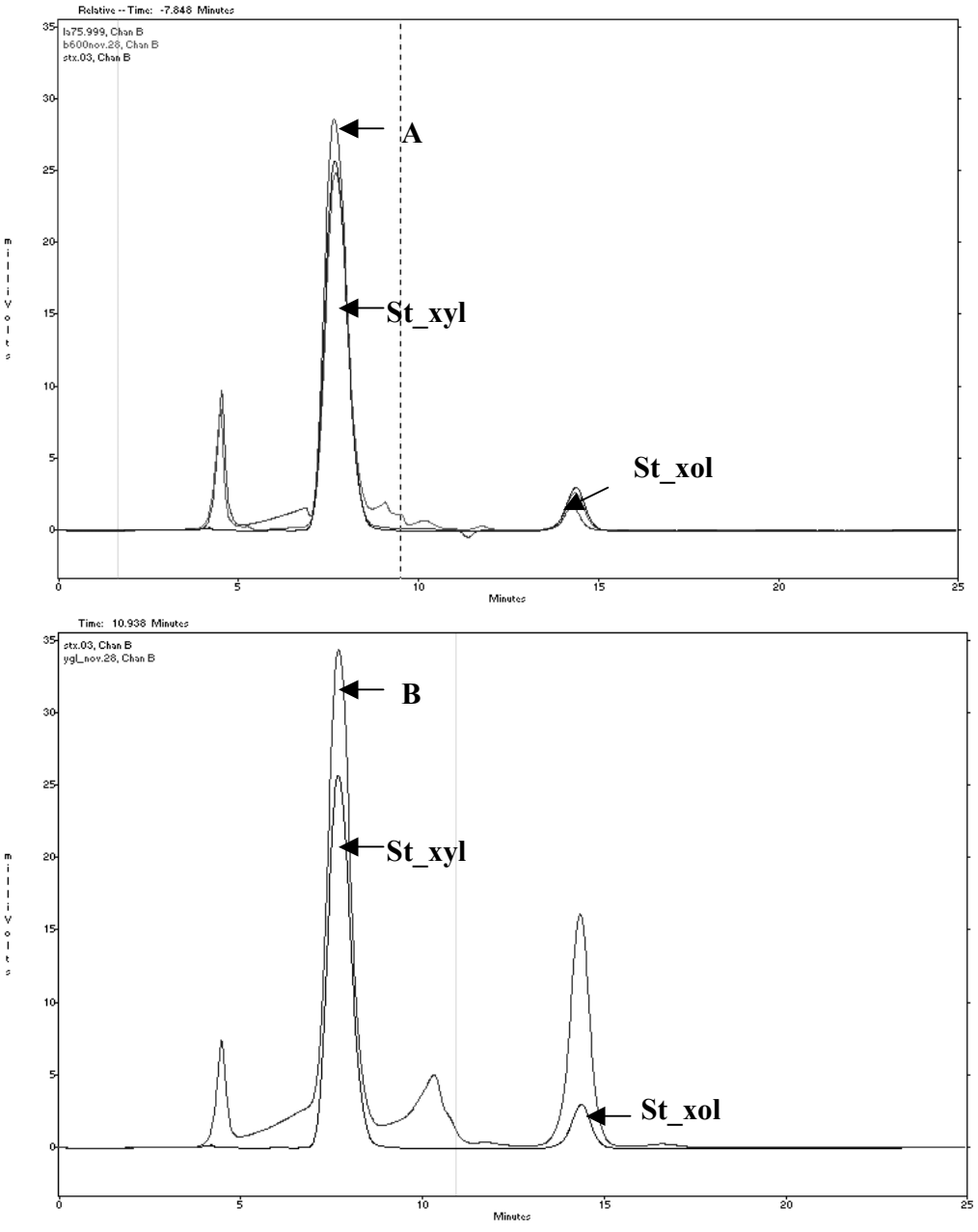
HPLC calibration plots for xylitol and D-xylose





Appendix G

HPLC chromatograms of D-xylose and xylitol standards superimposed with *Corynebacterium* sp. B 4247 culture broths: Corynebacterium medium (A), Corynebacterium medium with potassium gluconate (B), Standard D-xylose (St_xyl), Standard xylitol (St_xol)



VITA

Sendil Rangaswamy
email: srangasw@vt.edu

Education

Doctorate of Philosophy, Biological Systems Engineering,
Virginia Polytechnic Institute and State University, Blacksburg, VA,
Dissertation: Xylitol production from D-xylose by facultative anaerobic bacteria
Advisor: Dr. Foster Agblevor

MS Biotechnology Tamil Nadu Agricultural University, India
MS Thesis work: Plant cell culture in bioreactors for the production of secondary metabolites.

BE. (Ag) Tamil Nadu Agricultural University, India.
Senior Project: Designed and developed single bullock drawn plow as a team project.

Skills:

Molecular Biology

- Enzyme isolation, purification, characterization, assays in yeast and bacteria
- Amino acid sequencing, DNA sequencing -homology
- DNA, RNA-isolation in bacteria and Plant cells
- Cloning in Bacteria, bacterial gene Isolation, sequencing
- Fermentors -Plant and bacterial cell cultures, Screening bacterial cultures
- FPLC, HPLC and Mass Spectrometer.

Work Experience

Research Assistant
Biological Systems Eng., VirginiaTech. 1998-2001

Faculty
Center for Plant Molecular Biology and Biotechnology, TNAU, India.
1995-1998 Jan

Technical officer
Industrial Enzymes for Biocon India Ltd**.** and Nova Enzymes, India. 1992-1995

Publications

Rangaswamy, S. and F. A. Agblevor. 2002**.** Screening of facultative anaerobic bacteria utilizing D-xylose for xylitol production. *Applied Microbiol Biotechnol.* 60:88-93.

Conference Presentations

Xylitol production utilizing D-xylose by facultative anaerobic bacteria. 2001. IBE conference at Sacremento.

Awards

- Award for good Poster Presentation at IBE-ASAE conference
- 2nd Best Project award for Undergraduate project
- Indian Council of Agricultural Research -fellowship award in Undergraduate
- Department of Biotechnology, India – fellowship award in Masters program