CHAPTER 2

LETIRATURE REVIEW

2.1 History

Sugar production was established in the seventh century AD. Since then, the sugar industry has grown immensely. In the year 2002, global sugar production exceeded 133 million tons per year (Knapp, 2002). The earliest examples of starch hydrolysis are as old as ancient cooking, brewing of wine making. The history of enzymes dates back to 1833 when the isolation of an amylase complex from germinating barley was reported by Anselme Payen and Jean-Francois Persoz. However, it was not until the early 20th century that scientists were able to explain what was happened to starch and what was caused it. The first patent on the production of D-fructose from D-glucose by D-xylose isomerase was issued in 1960 (Jensen and Rugh, 1987).

Increasing amounts of sugars in food, sweets soft drinks, and so on has raised some concerns about their health effects. The number of people suffering from diabetes, obesity, cancer, and cardiovascular diseases is increasing every year in the developing world according to the WHO (press release, March 3, 2003). However, the success of xylitol has shown that alternatives existing dominant sweeteners are possible. Xylitol is a five-carbon polyol (pentitol) that has beneficial health properties. For example, it can prevent tooth decay and acute otitis media in children, when used regularly (Mäkinen, 1992; Uhari *et al*., 1996). It is also a polyol, and is produce industrially and represents an alternative to current conventional sweeteners.

The International Society of Rare Sugars (ISRS) has defined rare sugars as monosaccharides and their derivatives that rarely exist in nature (Granström *et al*., 2004). In our laboratory, we have been studying the production of rare sugars using microbial and enzymatic reactions. We have devised a scheme to display all the rare sugars in a ring-form called an "Izumoring". Using this display, we can illustrate their relation to each other. In addition, we can describe the biochemical reactions needed to produce them and the connection between the D- and L-configurations of these sugars. There are three different Izumoring, one for tetroses, one for pentoses, and one for hexoses.

2.2 Rare sugars definition

According to the quantity of sugars abundance in nature, they can be divided into two groups, natural sugars and unnatural sugars (or rare sugars) (Table 2.1). Natural carbohydrates are inexpensive because of their abundance in the nature. However, the carbohydrates that fall into the rare category are very expensive. In last 10-15 years, some laboratories have reported several strategies for that mass production of rare carbohydrates. This fact prompted researchers to find the usefulness of rare carbohydrates in basic and applied research.

Rare sugars are rarely distributed monosaccharides in nature, because of its very limited amount and expensiveness, the biological effect has almost not been known so far. Recently, an effective strategy for mass production of rare sugars has been developed (Takeshita *et al*., 2000). Rare sugars are usually sweet like the natural sugars, but unlike natural sugars, rare sugars are either not metabolized by the body or are metabolized, but to a lesser extent than natural sugars. Due to these features, rare sugars are desirable as sweeteners for individuals wishing to reduce caloric intake or

for those unable to metabolize common sweetening agents without detrimental effects, such as diabetics. Other advantages of rare sugars are the absence of an objectionable aftertaste, commonly experienced with artificial sweeteners such as saccharin or cyclamates, and their use as potential inhibitors of various glycosidases. However, in spite of the demand for these rare sugars, their commercial availability is negligible as they are expensive to prepare and unavailable in nature (Ahmed *et al*., 1999).

Rare sugar has turned on the interests of researchers since it showed some extraordinary properties, which would be apply in many field of sciences, i.e. non-calorie sweeteners, bulking agents, precursor substances of other unnatural compounds, starting material for antiviral or antitumor agents, food additives, inhibitor for unnecessary proteins, and etc. Reports on various applications of rare sugars were shown in Table 2.2. Because of those valuable benefits, chemists and biologists deliberately focus the development of production of rare sugars.

Rare sugars have been previously originated by chemical methods, however, this reaction was costly, required multiple steps of catalyst activation and faced the occurrence of unnecessary by-products which being not feasible for the mass production (Giffhorn *et al*., 2000). Therefore, bioconversion, the more effective way of natural carbohydrates to rare sugar, is closely focused from many areas of researches.

Table 2.1 Sugar classification by their existent in the nature

	Natural sugar			Unnatural/Rare sugar		
Tetrose (C4)	**Tetritol**			**Aldotetroses**	**Ketotetroses**	**Tetritols**
	Erythritol			D-Erythrose	D-Erythrulose	D-Threitol
				D-Threose	L-Erythrulose	L-Threitol
				L-Erythrose		
				L-Threose		
Pentoses (C5)	**Aldopentoses**	**Ketopentoses**	**Pentitols**	**Aldopentoses**	**Ketopentoses**	**Pentitols**
	L-Arabinose	D-Ribulose	D-Arabitol	D-Arabinose	D-Xylulose	L-Arabitol
	D-Ribose		Ribitol	D-Lyxose	L-Ribulose	
	D-Xylose		Xylitol	L-Lyxose	L-Xylulose	
				L-Ribose		
				L-Xylose		
Hexoses (C6)	**Aldohexoses**	**Ketohexoses**	**Hexitols**	**Aldohexoses**	**Ketohexoses**	**Hexitols**
	D-Galactose	D-Fructose	Galactitol	D-Allose	D-Psicose	Allitol
	D-Glucose	L-Sorbose	L-Gulitol	D-Altrose	D-Sorbose	D-Iditol
	D-Mannose		D-Mannitol	D-Gulose	D-Tagatose	D-Talitol
			D-Sorbitol	D-Idose	L-Psicose	L-Iditol
				D-Talose	L-Tagatose	L-Mannitol
				L-Allose		L-Sorbitol
				L-Altrose		L-Talitol
				L-Galactose		
				L-Glucose		
				L-Idose		
				L-Gulose		
				L-Mannose		
				L-Talose		

Ref: Poonperm (2007)

Table 2.2 Various applications of rare sugars and their derivatives

Rare sugar	Application	References
Tetrose		
L-Erythrose	- Its derivative, Dideoxyerythrose, has anti-HIV activity.	Tschamber *et al*., 1996
L-Erythrulose	- Starting material for the production non-proteinogenic amino acids	Murga *et al*., 2005
D-Erythrulose	- D-erythrulose 1-phosphate is as slow reversible inhibitor for rabbit muscle aldolase.	Ferroni *et al*., 1991
L-Threose	- Anti-HIV agent	Wu *et al*., 2005
Threitol	- Its derivative, Treosulfan (L-threitol-1, 4-bis-methanosulfonate, Ovastat), can be used in the clinical chemotherapy of human ovarian carcinomas and was effective against xenografted human breast and lung carcinomas.	Fennely, 1997; Maier and Sass, 1996
Pentose		
D-Arabinose	- Used for the synthesis of azinomycin, antitumor and antibiotics	Moran *et al*., 1993
	- Useful in D-erythroascobic acid and oxalic acid production	Loewus *et al*., 1995
	- Its derivative, 5-O-(trans-feruloyl)-D-arabinofuranose, can be used as anti-microbacterial agent	Vafiadi *et al*., 2007
D-Lyxose	- Useful for cancer therapy	Takagi *et al*., 1996
	- Precursor for phytosphingosine**,**	Chang *et al*., 2007
	- Precursor for the production of monocyclic 4'-Aza-L-Nucleosides (glycosidase inhibitor)	Varaprasad *et al*., 1999
L-Ribose	- L-FMAU, a derivative of L-ribose, is active against hepatitis B virus and Epstein-Barr virus.	Chu *et al*., 1998
	- Starting material for the production of L-allose and L-altrose	Austin and Humoller, 1934
	- Potential anti-hepatitis B virus (HBV) and anti-Epstein-Barr virus (EBV) agents	Tiawanwei *et al*., 1996
L-Xylose	- Its derivative, 9-(-2-deoxy-2-fluoro-β- L-arabinofuranosyl) or purine nucleosides, is active against hepatitis B virus.	Ma *et al*., 1997
L-Xylulose	- Potential inhibitors of various glycosidases	Levin *et al*., 1995

Rare sugar	Application	References
Hexose		
D-Allose	- Protective effect against ischemia reperfusion	Hossain *et al*., 2003
	- Cryoprotective effects on mammalian cells.	Sui *et al*., 2007
	- Growth inhibitory effect of D-allose on human ovarian carcinoma cells *in vitro*.	Sui *et al*., 2005
L-Fructose	- Inhibitors of various glycosidases	Levin *et al*., 1995
	- L- and D-fructose mixture kills ants and house-flies.	Gillbert and Zehner 1990
	- Potential inhibitors of various glycosidases and N-link of oligosaccharide processing	Muniruzzaman *et al*., 1996
	- Starting material for preparation of L-glucose	Leang *et al*., 2004
L-Glucose	- Cytostatic and cytotoxic properties, regards to neoplastic cells, can be used for cancer therapy.	Bicher, 1997
	- Prevention of bacterial growth	Bautista *et al*., 2000
	- Intraperitoneal administration of L-glucose (300 mg/kg body weight) enhanced memory in mice by acting peripherally.	Lawson *et al*., 2002
	- An excellent starting material for the development of glycoconjugate vaccines against disease caused by *Shigella sonnei*	Medgyes *et al*., 1997
D-Gulose	- Drug formulation agents and food additives	James *et al*., 1993
D-Sorbose	- Building blocks for the synthesis of interesting natural and biological active products	Huwig *et al*., 1996 and 1998
	- Its derivative, L-threo-2, 5-hexodiulose, is active against insect control.	
D-Tagatose	- A low-calorie sweetener using as an additive in detergent, cosmetic and pharmaceutical formulation	Oh, 2007
L-Tagatose	- Starting material for the synthesis of L-deoxy-galactonojirimycin, a potent glycosidase inhibitor.	Huwig *et al*., 1998
L-Talose	- L-Talofuranosyladenine, aderivative of L-talose, is and inhibitor for the growth of leukemia L 1210 cells *in vitro*.	Lerner and Mennitt, 1994
D-Psicose	- Enhancing chitosan productivity in *Rhizopus oryzae* YPF-61A	Yoshihara *et al*., 2003
	- Suppressing hepatic lipogenic enzyme activities in rats	Matsuo *et al*., 2001
	- Using as a reduced-calorie sweetener.	Matsuo *et al*., 2002

Successfully, the bioconversion strategies for all rare sugars were recently determined and schematized as “Izumoring” (Figure 2.1) (Granström *et al*., 2004; Izumori, 2006). On the basis of Izumoring, enzymes involving in each conversion reaction can be supposed in order to find novel sources of the enzyme or to develop the existing enzymes for achieving the more effective reaction.

2.3 Biotransformation

Biotransformation, also known as microbial transformation or bioconversion, is defined as the chemical reaction mediated by microorganisms or their enzyme preparations. The essential and typical characteristics for enzyme correlating with biotransformation are its specificity and selectivity for stereo- and region-selection which usually take place under mild condition. Obviously, a number of distinct advantages over conventional chemical catalysts made the biotransformation are being employed increasingly as a major part of biotechnological expansion. Various kinds of substrate, such as steroids, amino acids and antibiotics, were investigated by this method in order to achieve the useful intermediate or more valuable product. Recently, many attempts have been developed to accomplish the conversion of natural carbohydrate into a new configuration, which seldom appeared in the nature. The production of rare sugars was also focused using microbial and enzymatic reactions (Kieslich, 1984). Major enzymes using for rare sugar production were epimerase, isomerase and dehydrogenase. Researches on these enzymatic reactions have long been attempted as summarized in Table 2.3.

Table 2.3 Enzyme involved in some rare sugar producing reactions

Enzyme group	Enzyme name	Organisms	Reactions	References
epimerase	D-Tagatose-3-epimerase [a]	*Pseudomonas chichorii* ST-24	D-Fructose= D-Psicose	Itoh *et al*., 1994
	D-Psicose-3-epimerase [a]	*Agrobacterium tumefaciens*	D-Fructose= D-Psicose	Kim *et al*., 2006
isomerase	L-Rhamnose isomerase [a]	*P. stutzeri*	D-Allose= D-Psicose	Leang *et al*., 2004
	L-Arabinose isomerase [a]	*T. neapolitana*	D-Tagatose= D-Galactose	Hong *et al*., 2007
	L-Ribose isomerase [a]	*Acinetobacter* sp.	L-Ribose= L-Ribulose	Mizanur *et al*., 2001
	D-Lyxose isomerase	*Cohnella laevoribosii* RI-39 sp. nov.	L-Ribose= L-Ribulose	Cho *et al*., 2006
Oxido-reductase	Mannitol dehydrogenase [a]	*Thermotoga maritime*	D-Mannitol= D-Fructose	Puttick *et al*., 2007
	Galactitol dehydrogenase	*Rhodobacter sphaeroides*	L-Tagatose= Galactitol	Huwig *et al*., 1997
	Sorbitol dehydrogenase	*Gluconobacter oxydans* G624	D-Sorbitol= L-Sorbose	Shibata *et al*., 2000
	D-Arabitol dehydrogenase [a]	*G. oxydans*	D-Arabitol= D-Xylulose	Cheng *et al*., 2005
	Ribitol dehydrogenase	*Klebsiella pneumonia* IFO 3321	Allitol= D-Psicose	Takeshita *et al*., 2000

[a] recombinant protein

2.4 Biocatalysts for production of rare sugars

It is advantageous to use cheap natural resources, such as starch, wood or whey, as starting materials for the production of rare sugars. D-Glucose is obtained from starch whereas D-xylose can be derived from the hemi-cellulosic fraction of wood. Whey contains lactose, which can be hydrolyzed into D-glucose and D-galactose. The production of the various rare sugars using whole cells and enzymes

were studies Rare Sugar Research Center at Faculty of Agriculture Kagawa University by Prof. Ken Izumori and his colleagues. The main biocatalysts are enzymes such as D-tagatose 3- epimerase, various oxidoreductases, polyol dehydrogenases, and aldose isomerases. D-Tagatose 3- epimerase (DTE) catalyses the epimerization of all ketohexoses and ketopentoses at the C-3 position, producing the corresponding ketoses (Itoh *et al*., 1994). The amino acid sequence of this enzyme was determined and no homology to other DTE enzymes was reported (Ishida *et al*., 1997). This enzyme can used for the mass production of various rare ketohexoses such as D-psicose form D-fructose (Itoh *et al*., 1995a; Takeshita *et al*., 2000), D-sorbose from D-tagatose (Itoh *et al*., 1995b), L-fructose from L-psicose, and L-tagatose from L-sorbose (Itoh and Izumori, 1996).

Oxidoreductases catalyze oxidation-reduction between ketoses and polyols. Oxidoreductase reactions using microbial cells as catalysts instead of enzymes has been reported. D-Tagatose was produced from galactitol (Izumori *et al.*, 1984) and L-tagatose from galactitol (Shimonishi *et al*., 1995). Using the reverse reaction of polyol dehydrogenase, various rare hexitols were prepared from ketohexoses by microbial reactions. These include D-talitol production from D-tagatose (Muniruzzaman *et al*., 1994), allitol from D-psicose (Muniruzzaman *et al*., 1995), D-talitol from D-psicose (Sasahara *et al*., 1998), and D-iditol from D-sorbose (Sasahara et al., 1999).

Various aldose isomerases transform ketohexoses to the corresponding aldohexoses. Bhuiyan *et al.* (1997 and 1998) reported L-mannose and D-allose production from L-fructose and D-psicose, respectively. D-Xylose isomerase is used in the conversion of D-glucose into D-fructose in the industrial production of high

fructose corn syrup (HFCS). However, Pastinen *et al.* (1999) found that D-xylose isomerase can be used in the production of various rare aldohexoses from ketohexoses, such as D-altrose, and D-allose from D-psicose, as well as D-gulose and D-idose from D-sorbose. They have also shown that active cross-linked D-xylose isomerase crystals can be used for the separation of enzyme inhibitors such as xylitol and D-sorbitol from impure mixtures of D-arabinitol, D-mannitol, ribitol, and monosacchrides (Pastinen *et al*., 1998).

2.5 Izumoring for hexoses (Granström *et al*., 2004)

As shown in Figure 2.1, a circular map was drawn in the form of a ring based on a cycle composed of eight ketohexoses and four hexitols connected by the two enzymes. The ring is divided into three different vertical zones illustrated with different colors, *i.e.*, L-zone (pink), DL-zone (yellow), and D-zone (blue). The point of symmetry of D- and L-sugars is located at the center of the ring. The D-series compounds are placed in the D- and L-zone, respectively. Those compounds that have both D- and L-symmetry belong to the DL-zone in the center of the ring. Galactitol (D-, L-galactitol) and allitol (D-, L-allitol) are placed at the center of the ring because both of these hexitols are optically inactive among the 18 compounds (8 ketohexoses and 10 hexitols). The remaining four hexitols, D-mannitol, D-iditol, L-mannitol, and L-iditol, are placed at the outer ring.

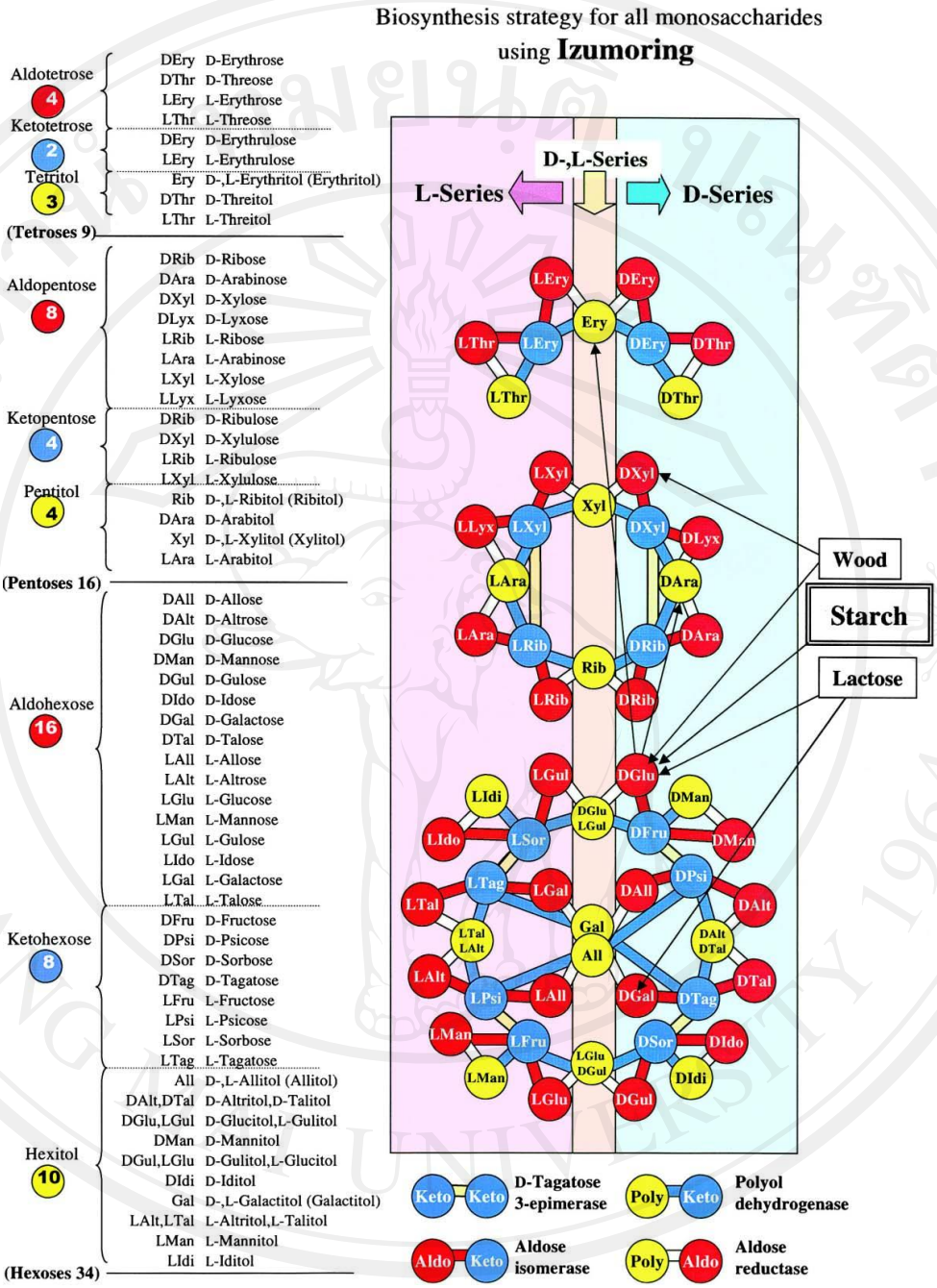


Figure 2.1 Izumorings for tetroses, pentoses and hexoses are presented in tree form. The D-, DL-, and L-sugars are divided in the three different vertical zones. The Izumoring tree illustrates the strategy to design the production processes of all monosaccharides from readily available raw materials; starch, wood and lactose (Granström *et al.*, 2004).

Furthermore, aldohexoses can be converted to the corresponding ketohexoses by aldose isomerase reaction, and also converted to the corresponding hexitols by a hydrogenation reaction. A symmetric Izumoring comprising of 16 aldohexoses, 8 ketohexoses, and 10 hexitols (Figure 2.1, the biggest ring). Enzyme reaction or hydrogenation can convert all compounds to each other. So Izumoring named this symmetric ring which is complete design for the bioproduction of ketohexoses and hexitols as well as aldohexoses.

A ketohexose synthesis strategy using the Izumoring clearly suggests a way for designing a production process for any ketohexose from D-fructose that can be produced from D-glucose. For example, L-fructose has some interesting properties as a source of energy (Klein *et al*., 1993; Livesey and Brown, 1995) and as an inhibitor for glycoproteins (Muniruzzaman *et al*., 1996). Following the route of the Izumoring from D-fructose to L-fructose the production method for L-fructose via four reactions can be predicted. The first step is epimerization of D-fructose to D-psicose by D-tagatose 3-epimerase (Itoh *et al*., 1995a; Takeshita *et al*., 2000). The second step is reduction of D-psicose to allitol (D-, L-allitol) by oxidoreductase (Muniruzzaman *et al*., 1995; Takeshita *et al*., 2000). The third reaction is transformation of allitol (D-, L-allitol) to L-psicose by oxidoreductase (Takeshita *et al*., 1996). The final step is epimerization of L-psicose to L-fructose by D-tagatose 3-epimerase (Itoh and Izumori, 1996).

2.6 Izumorings for pentoses and tetroses

The construction of Izumorings for pentoses and tetroses follows the same pattern as that for hexoses (Figure 2.1, the top ring and middle one). In the case of

pentoses there are eight aldopentoses, four ketopentoses, and four pentitols. The entrance from a D-pentose configuration to an L-pentose configuration occurs through xylitol (D-, L-xylitol) and ribitol (D-, L-ribitol). D-Xylose can be used as a starting material for the synthesis of all pentoses. Granström *et al*. (2002) have purified D-xylose reductase that reduces D-xylose into xyliyol from *Candida guilliermondii*. It was shown to reduce D- and L-forms of arabinose, ribose and lyxose, in addition to D-, L-xylose. Bhuiyan *et al*. (1998) reported the preparation of L-lyxose from ribitol using microbial and enzymatic reactions. First ribitol was oxidized to L-ribulose by *Acetobacter acetii* IFO 3281. L-Ribulose was epimerized to L-xylulose by D-tagatose 3-epimerase and finally isomerized to L-lyxose by L-rhamnose isomerase. Kylmä *et al*. (2004) was able to determine a specific production rate of 1.2 g/g/h in the bioreactor for L-ribulose conversion from ribitol using resting cells of *A. acetii* IFO 3281.

The Izumoring for tetroses consists of four aldotetroses, two ketotetroses, and three tetritols. The entry point from a D-configuration to an L-configuration goes through erythritol. Erythritol is currently used as a bulk sweetener in low calorie foods such as cookies, candies, and yoghurts. Mizanur *et al*. (2001) used erythritol as a starting material for preparing the rare and expensive aldotetrose L-erythrose. Erythritol was first converted to the ketotetrose L-erythrulose by *Gluconobacter frateurii* IFO 3254 and then isomerized to L-erythrose with L-ribose isomerase enzyme. Vuolanto *et al*. (2002) showed that D-xylose isomerase is able to isomerase and epimerize all the different D- and L-tetroses.

2.7 Application of rare sugars

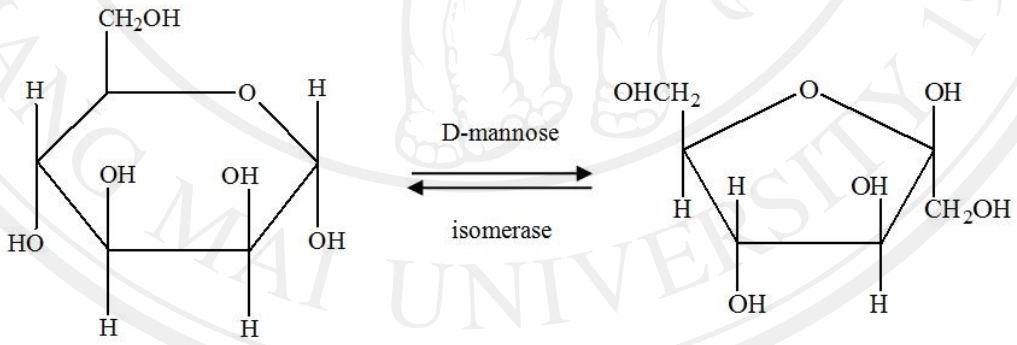
The production methods for various rare sugars require multidiscipline approaches that include fermentation technology, molecular biology, enzyme technology, and organic chemistry using Izumoring should be a base enzyme chart. The first task was the construction of the three Izumorings, following which the key biocatalysts have been isolated and purified (Izumori, 2002). Developing methods for the mass production of rare sugars from readily available raw materials such as starch, hemi-cellulosic waste, and whey should be produced low cost production. Unidentified novel characteristics from these rare sugars which will have a profound effect on the everyday life and health of people could be discovered. Rare sugars, such as D-psicose was successful for large scale production at Kagawa University. It produce from D-fructose using D-tagatose 3-epimerase. In addition, various novel physiological functions of rare sugars have been discovered. For example, D-allose has a potent inhibitory effect on the production of reactive oxygen species (Murata *et al*., 2003), and thus, may be used for medical purposes. The potential for a wide range of applications is a characteristic of rare sugars.

2.8 Mannose isomerase

Entry	EC 5.3.1.7
Name	D-Mannose isomerase
	Mannose isomerase
Class	Isomerase
	Intramolecular oxidoreductase
	Interconverting aldoses and ketoses

Sysname	D-Mannose ketol-isomerase
Reaction	D-Mannose = D-Fructose
Product	D-Fructose
Comment	Also acts on D-lyxose and D-rhamnose

Mannose isomerase (D-mannose isomerase (D-MI); EC 5.3.1.7), which is an intracellular enzyme, is known to catalyze the reversible isomerization of D-mannose to D-fructose (Figure 2.2) and has been found in D-mannose-grown from some bacterial species. Several reported describes culture conditions of the microorganisms, recovery and purification methods of the produced mannose isomerase (Takasaki *et al*., 1993; Stevens *et al*., 1981; Palleroni and Doudoroff, 1956).



D-mannose D-fructose

Figure 2.2 Reaction catalyzed by D-mannose isomerase

All D-MI reported to date are inducible enzymes. In order to develop new techniques and new sources productions of D-MI, there are several researches with reported. Palleroni and Doudoroff (1956) isolated D-MI from *P. saccharophila* grown

on fructose as the sole carbon source, it was the starting point of the exploitation of this enzyme, because of, it is not only the first isomerase with a high affinity for free hexose, but also because it appears almost invariably in a mutant strains of *Pseudomonas saccharophila* which are capable of growing with fructose as substrate. The enzyme was found to be active towards D-lyxose and D-rhamnose as well as D-mannose. The product of the reaction with D-lyxose was shown to be D-xylulose. The product of the reaction with D-rhamnose was presumed to be D-rhamnulose. The enzyme was capable of catalyzing the isomerization of D-mannose and D-fructose but was not induced by growing the cells on mannose. The mannose isomerase isolated from *Mycobacterium smegmatis* and *Agrobacterium radiobacter* M-1 are capable of catalyzing the conversion of D-lyxose to a ketose, presumably D-xylulose (Hirose *et al*., 2001; Hey-Ferguson and Elbein, 1970).

Indeed, some rare sugars production involves in the reaction of rare sugars enzyme were known as the popular enzyme, e.g., D-xylose isomerase, L-arabinose isomerase, D-MI, and so on. Takeshita *et al*. (2000) reported D-allose, one of rare sugars, is produced from D-psicose using L-rhamnose isomerase, while D-psicose is produced from D-fructose, and D-fructose produced from D-mannose using D-MI (Takada and Izumori, 2002).

2.8.1 Source of microorganisms

D-mannose isomerase is widely distributed in prokaryotes (Table 2.4) after its discovery in *Pseudomonas* sp., some number of bacteria was found to produce mannose isomerase that is active in the enzymatic conversion of D-mannose to D-fructose.

Among known microorganisms for their ability to produce D-MI, the enzyme was active at pH 6.0-10.0 but unstable at high temperature. Intracellular D-MI has been reported to be produced by *Escherichia coli* (Stevens and Wu, 1976). The intracellular D-MI from *Mycobacterium smegmatis* have been purified to homogeneity by conventional purification techniques such as gel filtration, ion-exchanges chromatography and preparative Diethyaminoethyl (DEAE)-cellulose column chromatography (Hey-Ferguson and Elbein, 1970).

Table 2.4 D-Mannose isomerase producing by microorganisms

Microorganisms	References
Agrobacterium radiobacter M-1	Hirose *et al*., 2001
Escherichia coli K12	Stevens and Wu, 1976
Mycobacterium smegmatis	Hey-Ferguson and Elbein, 1970
Pseudomonas cepacia	Allenza *et al*., 1990
Pseudomonas saccharophila	Palleroni and Doudoroff, 1956
Pseudomonas sp.	Takasaki *et al*., 1993
Xanthomonas rubrilineans	Takasaki and Tanabe, 1964
Streptomyces aerocolonigenes	Takasaki, 1967
Streptomyces sp.	Tangjaiatitharn, 2003

2.9 Actinomycetes

Actinomycetes are one of the most investigated groups since they constitute a potential source of biotechnological interesting substances. They are well known as a group of filamentous, Gram positive bacteria, which possess many important and

interesting features. They are of considerable value as producers of antibiotic and enzymes, pigment, and vitamin (B12), they exhibit a range of life cycles which are unique among the prokaryotes, and they are appear to play a major role in the cycling of organic matter in the soil ecosystem (William *et al*., 1993; Hirsch and Christensen, 1983; Sykes and Skinner, 1973).

Actinomycetes are a heterogeneous group of organisms characterized by their ability to form branching filaments at some point during their life cycle. Whereas other bacteria are classified in genetically related taxonomic families and sub grouping, the aerobic actinomycetes are grouped with purely descriptive names. When they growing a solid substratum such as agar or the branching network of hyphae which grows on above the level of medium (aerial mycelium and the part of the mycelium which remains at the level of the substrate, e.g., in contact with the medium is substrate mycelium) (Figure 2.3). Most actinomycetes are not motile. When motility is present, it is confined to flagellated spores (Prescott *et al*., 1999; Hollick, 1995; Singleton and Sainsbury, 1978).

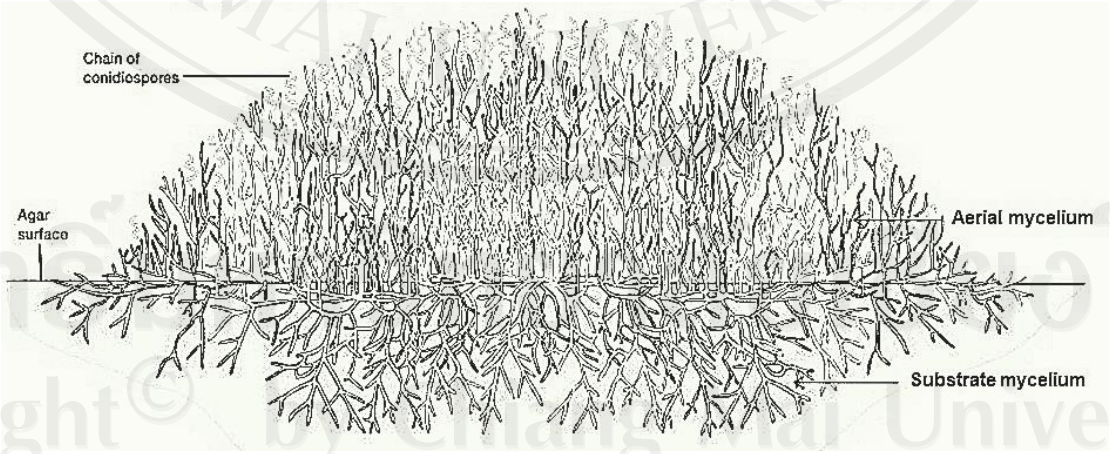


Figure 2.3 The cross section of an actinomycete colony.

2.9.1 Identification of actinomycetes

Identification of actinomycetes to genus level needs the data for analysis based on classical approach, chemotaxonomic characterization, and molecular information (species level). Classical approach including morphological, physiological, and biochemical characters. The classical method described in the identification key by Nonomura (1974), Bergey's Manual of Systematic Bacteriology (Locci, 1989) and using a variety of standard culture media recommended for International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) is useful in the identification of streptomycetes. Morphological observations including germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium, melanoid pigment, reverse side pigment, soluble pigment, spore chain morphology and spore surface have been used to identify actinomycetes (Locci, 1989). Chemotaxonomy is the study of chemical variation in organisms and using their chemical characters to classify and identify. One valuable method has been used to identify the genera of actinomycetes is presence of diaminopimelic acid (DAP). DAP isomers are the most important cell-wall properties of Gram-positive bacteria and actinomycetes. Most bacteria have a characteristic of peptidoglycan as wall envelope. The 2, 6-DAP is widely distributed as a key amino acid and use with two amino bases in characterization. If DAP is present, bacteria generally contain one of the isomers, the LL-form or the *meso*-form, mostly located in the wall envelope. Moreover, the sugar composition often provides valuable information to classify and identify of actinomycetes. Normally, actinomycete cells contain some kinds of sugars, in addition to the glucosamine and muramic acid of peptidoglycan. The sugar pattern of actinomycete presents as a key role in the identification of sporulating

actinomycetes when the isolates have *meso*-DAP in their cell walls. On the other hand, the actinomycetes that have LL-DAP along with glycine (wall chemo type-I) have no characteristic pattern of sugars (Lechevalier and Lechevalier, 1970). Most components of the cell wall and whole-cell of actinomycetes are shown in Table 2.5

Table 2.5 Type of cell wall diaminopimelic acid isomers (DAP) and whole-cell sugars of actinomycetes (Lechevalier and Lechevalier, 1970)

	Cell-wall types			
	I	**II**	**III**	**IV***
DAP isomers	LL	meso	meso	meso
Glycine	+	+	-	-
	Whole-cell sugars pattern (WCSP)			
	A	**B**	**C**	**D**
Arabinose	+	-	-	+
Galactose	+	-	-	-
Xylose	-	-	-	+
Madurose	-	+	-	-

*Type IV was differentiated from type III by the presence of arabinose and galactose in the whole cell hydrolysates.

Range of DNA base compositions of most actinomycetes is 63-78 mol% G+C and the value of mol% G+C content has been used to classify group of actinomycetes (Madigan and Martinko, 2006). Additionally, the type of compositions of phospholipids (Table 2.6), fatty acids and menaquinones in actinomycete cells have been used (Boone and Pine, 1968; Lechevalier and Lechevalier, 1980).

Table 2.6 Type of phospholipids of actinomycetes (Lechevalier, 1977)

Type	PIM	PI	PC	PG	PE	PME	GluNU	APG	DPG
I	+	+	-*	V	-*	-	-*	V	V
II	+	+	-*	V	+*	-	-*	V	+
III	V	+	+*	V	V*	+	-*	V	V
IV	?	+	-*	-*	V*	V	+*	-	+
V	?	+	-*	-*	V*	-	+*	V	+

* = diagnostic phospholipids

PIM = phosphatidylinositolmannosides

PI = phosphatidylinositol

PC = phosphatidylcholin

PG = phosphatidylglycerol

PE = phosphatidylethanolamine

PME = phosphatidylmethylethanolamine

GluNU = unknown glucosamine-containing phospholipids

APG = acyl phosphatidylglycerol

DPG = diphosphatidylglycerol

The most efficacious technique to taxonomy is complete the study of nucleic acids because these are either direct gene products or the genes themselves in comparisons of nucleic acids yield considerable information about true relatedness. Molecular systematic that used to classify and identify of actinomycetes includes nucleic acid hybridization and nucleic acid sequence analysis (O'Donnell *et al.*, 1993). Phylogenetic studies based on 16S rDNA sequences are significantly increasing in the systematics of bacteria and actinomycetes (Yokota, 1997). Moreover, sequences of 16S rDNA are basis for actinomycetologists in using phylogenetic tree to identify and investigate of evolution of actinomycetes.

2.10 Statistical analysis

2.10.1 Analysis of variance (ANOVA)

ANOVA is a set of statistical methods used mainly to compare the means of two or more samples. Estimates of variance are the key intermediate statistics calculated, hence the reference to variance in the title ANOVA. One factor analysis of variance (Snedecor and Cochran, 1989) is a special case of ANOVA, for one factor of interest, and a generalization of the two-sample *t*-test. The two-sample *t*-test is used to decide whether two groups (levels) of a factor have the same mean. One-way ANOVA is a simple special case of the linear model. The one-way ANOVA form of the model is

$$y_{ij} = \alpha_j + \varepsilon_{ij}$$

Where y_{ij} is a matrix of observations in which each column represents a different group. $\alpha_{.j}$ is a matrix whose columns are the group means. (The "dot *j*" notation means that α applies to all rows of column *j*. That is, the value α_{ij} is the same for all *i*.) and ε_{ij} is a matrix of random disturbances.

The model assumes that the columns of *y* are a constant plus a random disturbance. You want to know if the constants are all the same. For example, data collected on, say, five instruments have one factor (instruments) at five levels. The ANOVA tests whether instruments have a significant effect on the results.

2.10.2 Statistical experimental design

2.10.2.1 Optimization experiment

Response surface methodology (RSM) is widely used for process optimization and to study the effect of several parameters (Xiong *et al*., 2007; Heck *et al*., 2006). It is useful in studying the interactions of the various parameters affecting

the process (Mundra *et al*., 2007). We usually represent the response surface graphically as shown in Figure 2.4 that plotted the response value (axis Z) versus the level of parameter x_1 and x_2 (axis X and Y).

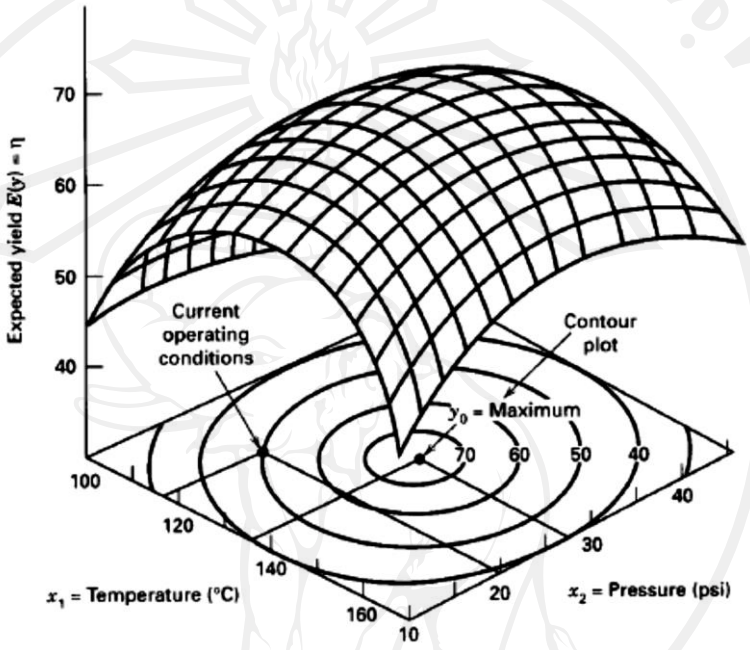


Figure 2.4 Three-dimension responses surface with a contour plot of a response surface (Montgomery, 2001).

The fundamental methods for quantitative variables involve fitting first-order (linear) or second-order (quadratic) functions of the predictors to one or more response variables, and then examining the characteristics of the fitted surface to decide what action is appropriate. If the response can be defined by a linear function of independent variables, then the approximating function is a first-order model. A first-order model with 2 independent variables can be expressed as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_k x_k + \varepsilon$$

If there is a curvature in the response surface, then a higher degree polynomial should be used. The approximating function with 2 variables is called a second-order model:

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j + \varepsilon$$

Where, Y is the variable response and β is the regression coefficients given by the model and X_i and X_j are the independent factors of the experiment (Akay, 2007).

In general all RSM problems use either one or the mixture of the both of these models. In each model, the levels of each factor are independent of the levels of other factors. In order to get the most efficient result in the approximation of polynomials the proper experimental design must be used to collect data. Once the data are collected, the Method of Least Square is used to estimate the parameters in the polynomials. The response surface analysis is performed by using the fitted surface. The response surface designs are types of designs for fitting response surface. Therefore, the objective of studying RSM can be accomplish by

(1) understanding the topography of the response surface (local maximum, local minimum, ridge lines)

(2) finding the region where the optimal response occurs. The goal is to move rapidly and efficiently along a path to get to a maximum or a minimum response so that the response is optimized

2.10.2.2 Central composite designs (CCD)

A Box-Wilson Central Composite Design, commonly called a central composite design; CCD, contains an imbedded factorial or fractional factorial design with center points that is augmented with a group of star points' that allow estimation of curvature. If the distance from the center of the design space to a factorial point is ±

1 unit for each factor, the distance from the center of the design space to a star point is $\pm\alpha$ with $|\alpha| > 1$. The precise value of α depends on certain properties desired for the design and on the number of factors involved. In addition, the number of center point runs the design is to contain also depends on certain properties required for the design. A CCD always contains twice as many star points as there factors in the design. The star points represent new extreme values (low and high) for each factor in the design. Each studied factor is used at 5 different levels, but not all level combinations occur. Rather, the CCD is composed of three parts (Figure 2.5):

(1) a factorial or "cube" part consisting of 2^{k-p} points from a full 2^k factorial (p=0), each point being replicated r_f times; the level of each factor are coded as -1 and +1; the number of experimental runs in $n_f = 2^{k-p}(r_f)$

(2) an axial or "star" part consisting of $2k$ points on the axis of each factor at α distance a from the center of the design, each point being replicated r_a times; this give rise to $n_a = 2kr_a$ experimental runs

(3) n_0 replication of the center point (0, 0, …, 0)

Therefore, the total number of experimental runs is then $N = n_f + n_a + n_0$.

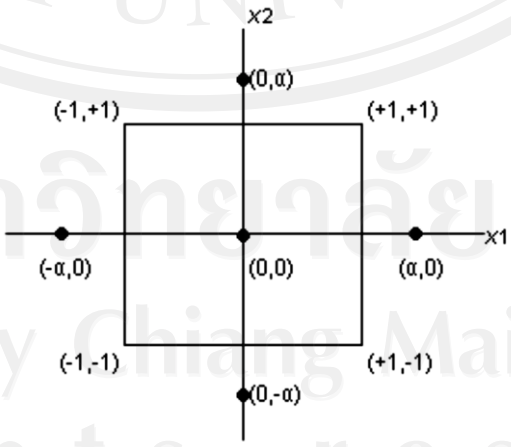


Figure 2.5 Three parts that composed to be CCD, k=2

There are 2 parameters in the design that must be specified: the distance α of the axial runs and the number of replication at the center point (n_0) (Montgomery, 2001). Box and Hunter (1957) suggested that a second-order response surface design should be rotatable. A CCD is made rotatable by the choice of α. The value of α for rotatability depends on the number of point in factorial part of the design. In fact, $\alpha = (n_f)^{1/4}$ yields a rotatable CCD where n_f is the number of point used in the factorial part (2^k) of the design. Rotatability is a spherical property; that is, it makes the most sense as a design criterion when the region of interest is sphere. However, it is not important to have exact rotatability to have a good design. In fact, for a spherical region of interest, the best choice of α from a prediction variance viewpoint for the CCD is to set $\alpha = \sqrt{k}$.

The choice of α in the CCD is dictated primarily by the region of interest. When this region is a sphere, the design must include center runs to provide reasonably stable variance of predicted response. Generally, three to five center runs are recommended.

There are many other response surface designs that are occasionally useful in practice. Particularly, useful designs for $k = 2$ are the pentagon and hexagon design. Some typical values of α as a function of the number of factors are showed in Table 2.7.

Table 2.7 Determining α for rotatability ($\alpha = (n_f)1/4$)

Number of factors	Factorail part	Scaled value for α
2	2^2	$2^{2/4} = 1.414$
3	2^3	$2^{3/4} = 1.682$
4	2^4	$2^{4/4} = 2.000$
5	2^5	$2^{5/4} = 2.378$
6	2^6	$2^{6/4} = 2.828$

2.10.3 The application of statistical experiment in biotechnological processes

The classical method; one-variable-at-a-time, bioprocess design may be effective in only some situations, but fails to consider the combined effects of all involved factors, moreover, time consume and high cost. Factorial design optimization and RSM analysis fulfill this requirement. RSM is a collection of mathematical and statistical techniques widely used to determine the effects of several variables and to optimized different processes; especially, biotechnological processes (Heck *et al*., 2006). In biotechnological processes, experimental designs are widely used in many applications, for example optimization of the medium for the production of enzymes (inuliase: Xiong *et al*., 2007; xylanase: Heck *et al*., 2006; α-amylase: Francis *et al*., 2003; chitinase: Dahiya *et al*., 2005), organic acid (lactic acid: Naveena *et al*., 2005; citric acid: Lotfy *et al*., 2007), bioactive compounds (candicidin: Mao *et al*., 2007).