PRODUCTION OF POLYHYDROXYALKANOATES FROM XYLOSE BY *ESCHERICHIA COLI* CARRYING WILD TYPE AND MUTANT XylE

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by

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LIST OF SYMBOLS

%	Percentage
β	Beta
γ	Gamma
~	Approximately
bp	Base pair
ΔHm	Heat of fusion
°C	Degree Celsius
C	Carbon atom
^{13}C	Carbon-13
Da	Dalton
^{1}H	Proton
h	Hour
g	Gravity
g	Gram
g/L	Gram per liter
J/g	Joule per gram
kDa	KiloDalton
kg	Kilogram
kb	Kilo-base
L	Liter
M	Molar
Mn	Number-average molecular weight

*M*w	Weight-average molecular weight
*M*w/*M*n	Polydispersity index
MHz	Megahertz
min	minute
mg	Milligram
mg/mL	Milligram per milliliter
mL	Milliliter
mM	Millimolar
Mol%	Mole percentage
MPa	Mega Pascal
N	Normality
ng	Nanogram
nm	Nanometer
ppm	Parts per million
psi	Pounds per square inch
rpm	Rotation per minute
*T*g	Glass transition temperature
*T*m	Melting temperature
*T*c	Crystallization temperature
µ	Micrometers
µg	Microgram
µg/mL	Microgram per milliliter
µM	Micromolar
µL	Microliter
µm	Micrometer
v/v	Volume per volume

LIST OF ABBREVIATIONS

wt%	Weight percent
w/v	Weight per volume
w/w	Weight per weight
DCW	Dry Cell weight
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl-CoA
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxybutyryl-CoA
ACP	Acyl carrier protein
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BLAST	Basic local alignment search tool
C/N	Carbon-to-nitrogen ratio
$CDCl_3$	Deuterated chloroform
CME	Caprylic methyl ester
CoA	CoenzymeA
CoASH	Coenzyme-A with sulfhydryl functional group
CPKO	Crude palm kernel oil
CPO	Crude palm oil
DCW	Dry cell weight
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimeter

EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidiun bromide
FabG	3-ketoacyl-CoA reductase
$FeCl_3$	Iron (III) chloride
FID	Flame ionization detector
GC	Gas chromatography
GP	Glycerine pitch
GPC	Gel permeation chromatography
HA	Hydroxyalkanoate
HA-CoA	Hydroxyacyl-CoA
MCL	Medium-chain-length
MM	Mineral salts medium
Mw	Molecular weight (g/mol)
NA	Nutrient agar
NR	Nutrient rich
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
OD	Optical density
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PDI	Polydispersity index

PHAs	Polyhydroxyalkanoates
PhaA	*β*-ketothiolase
PhaB	NADPH-dependentacetoacetyl-CoAdehydrogenase
PhaC	PHA synthase
PhaD	Polyhydroxyalkanoate synthesis repressor
PhaE	Polyhydroxyalkanoate granule associated protein
PCR	Polymerase chain reaction
pH	Potential hydrogen
PO	Palm olein
rRNA	Ribosomal ribonucleic acid
SCL	Short-chain-length
sp.	Species
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TMS	Tetramethylsilane
UV-Vis	Ultraviolet-Visible
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

POLYHYDROXIALKANOAT PRODUKSI DARIPADA XILOSA OLEH *ESCHERICHIA COLI* REKOMBINAN MEMBAWA GEN XylE JENIS LIAR DAN MUTASI

ABSTRAK

Xilosa merupakan gula karbon yang banyak didapati dalam sisa sektor pertanian. Maka ini membuka ruang untuk mentransformasikan sisa pertanian xilosa kepada tenaga dan bahan stok suapan kimia. Oleh yang demikian kajian ini mengutamakan penukaran xilosa kepada bioplastik melalui proses fermentasi oleh bakteria rekombinan. Proses fermentasi ini dijalankan dengan mengunakan *E.coli* yang mempunyai dua jenis plasmid iaitu pACYCxylE untuk mengangkut xilosa dan pGEMCABcn untuk proses fermentasi xilosa kepada PHA. Berdasarkan kriteria tumbesaran yang stabil dan pesat, kestabilan dwiplasmid dan kebolehannya untuk bertumbuh dalam medium minimal, *E. coli* JM109(DE3) digunakan sebagai bakteria pilihan untuk kajian ini sepenuhnya. Ini kerana, bakteria rekombinan JM109(DE3) yang mempunyai dwiplasmid amat stabil dalam medium nutrisi selepas 10 hari tanpa sebarang antibiotik. Berikutan itu, JM109(DE3) dapat mengumpul PHA 62% CDW, selepas 120 jam inkubasi dalam medium yang mengandungi kandungan kepekatan1% xilosa dan tiada pertambahan dalam tahap pengumpulan PHA sehinggga 5% kepekatan xilosa. Selain itu data juga menunjukkan 1% xilosa menjadi faktor penghad proses fermentatsi xilosa kepada PHA. Apabila JM109 (DE3) rekombinan ditumbuhkan dalam medium yang mengadungi 1% glukosa hanya 12% PHA dapat dikumpul. Angka ini semakin menurun sehingga 6% apabila kepakatan glukosa

semakin ditambah sehingga 5%. Ini membuktikan glukosa merencatkan proses fermentatsi oleh rekombinan JM109(DE3). Ini diikuti experimen seterusnya yang menggunakan medium tumbesaran yang mengandungi nisbah kombinasi xilosa kepada glukosa daripada 1:0.1 sehingga 1:1. Data menunjukkan pengumpulan PHA menurun daripada 58% ke 32% CDW apabila nisbah kepekatan sumber karbon campuran meningkat daripada 1% kepada 5%. Oleh sebab perencatan proses fermentatsi berlaku apabila medium tumbesaran mengandungi glukosa selain daripada xilosa, maka enam jenis gen mutan digunakan dalam kajian ini untuk mengatasi masalah ini. Enam jenis gen mutan XylE iaitu, N326F, N326V, F25S, 5V(F25S and N326V) and 5M (F25S and N326M) telah dikaji. Hasil kajian mendapati tiada perencatan tumbesaran berlaku dalam medium yang mengandungi campuran xilosa dan glukosa. Gen mutan N325M, N326F, N326V menghasilkan PHA sebanyak 40% CDW apabila bertumbuh dalam medium yang mengandungi kepekatan 1% xilosa dan 1% CDW dalam medium yang mengandungi kepekatan 1% glukosa. Manakala mutan yang melibatkan residu 25 XylE menghasilkan PHA yang kurang berbanding dengan mutan lain dalam medium yang mengandungi 1% xilosa. Mutan F25S menghasilkan PHA sebanyak 25% CDW dan dalam mutan berganda (F25S+N326V, F25S+N326M) sebanyak 17% CDW. Dalam 1% glukosa kesemua mutan yang dikaji dapat menghasilkan PHA tetapi pada tahap yang kurang berbanding xilosa. Akan tetapi tiada perencatan penghasilan PHA berlaku dalam medium yang mengadungi glukosa sebagai sumber karbon. Maka ini membuktikan mutan-mutan tiada lagi sensitif terhadap glukosa kerana mencatatkan PHA sebanyak 40% CDW apabila nisbah xilosa:glukosa ditingkatkan daripada 1:0.1 sehingga 1:1.

PRODUCTION OF POLYHYDROXYALKANOATES FROM XYLOSE BY *ESCHERICHIA COLI* CARRYING WILD TYPE AND MUTANT XylE

ABSTRACT

The abundance of xylose in agricultural waste represents an opportunity to turn it into energy or chemical feedstock. This study explores using *E. coli* JM109(DE3) harbouring pACYCxylE coding for a xylose transporter and pGEMCABcn carrying the PHA operon from *C. necator* to convert xylose into polyhydroxyalkanoate. Based on the criteria of fast growing, plasmid stability and ability to grow on minimal medium, strain JM109(DE3) was found to be the most suitable host for PHA production from xylose. The two plasmids were found to be stable after 10 serial overnight subcultures even without antibiotics selection. The strain was able to accumulate PHA up 62% CDW after 120 hours of incubation in mineral medium containing 1% xylose. Increasing xylose concentration up to 5% did not cause any further increase in PHA content, nor toxicity. Even at 1% xylose or glucose, the data suggests that the mineral medium was already limiting. When grown in 1% glucose, JM109(DE3) yield only 12% CDW PHA. Increasing glucose concentration to 5% leads to lower PHA content of only 6% CDW, implying glucose inhibition. In an experiment using a range of xylose:glucose ratio of 1:0.1 to 1:1, the PHA content fell from 58% to 32% CDW. In order to overcome glucose toxicity, six XylE mutants were tested for PHA production. The mutants were N326M, N326F, N326V, F25S, 5V(F25S and N326V) and 5M (F25S and N326M). Mutants N325M, N326F, N326V

produced PHA around 40% CDW when grown in medium containing 1% xylose. In 1% glucose the PHA content was around 31%. Mutations involving residue 25 of XylE produced lower PHA content of 25% (F25S), and 17% CDW in double mutation mutants (F25S+N326V, F25S+N326M) when grown in 1% xylose. Mutants N326M, N326F, N326V were found to be insensitive to glucose inhibition as it maintained PHA content of 40% when the xylose:glucose ratio in the medium was raised from 1:0.1 to 1:1.

Chapter 1 Introduction

Agricultural waste is the residual material obtained from crop production. Usually the agricultural waste is burnt or converted into natural fertilizer in the past. These residuals could be harmful for the environment and cause pollution when not disposed properly. Currently, agricultural waste is used to generate energy and to produce bioproducts like biofuel, xylitol and PHA.

The main constituent of agricultural waste is cellulose, which is widely distributed in higher plants, some algae, fungi and bacteria (Habibi et al., 2010). Dry substance in most straw and grass species hold approximately 35–45% cellulose, where it is in the secondary cell wall (Sun & Thomkinson, 2000). Cellulosic residues contain 10-20% of hemicellulose which is often repeated polymers of pentoses and hexoses sugars like D-xylose, D-arabinose, D-glucose D-galactose, and D-mannose (Sun & Thomkinson, 2000; Marques et al., 2010).

Hemicellulose is a smaller branched carbohydrate which can be made of different monosaccharides. Hemicelluloses is a heterogeneous biopolymer with xylan being the most abundant component (Saha[rs1], 2003). It is the substrate of xylanase and is made up of a complex of polymeric carbohydrates, including xyloglucan (heteropolymer of D-xylose and D-glucose) etc. Xylan is primarily present in the plant secondary cell wall together with cellulose and lignin, where it makes up the major polymeric constituent (Scheller and Ulvskov, 2010). Structurally, xylan is a polysaccharide with a backbone composed of β-1,4 linkages between d-xylose residues (Ebringerová and Heinze, 2000; Heinze et al., 2004; Mussatto et al., 2008). Xylose, glucose and their positional isomers are the fragments of xylanase biosynthesis regulation where the most predominant sugar in xylan is xylose.

D-xylose is a five-carbon aldose which can be catabolized into useful products mainly by fungus and bacteria. Due to carbon catabolic repression, xylose is always used as a secondary carbon source in a metabolic process where glucose is the most. The microbial production of xylitol is closely connected with ethanol production where it was considered only as a by-product in ethanol fermentation processes from D-xylose.

One of the most interesting product from xylose is polyhydroxyalkanoates (PHA). PHA is a class of organic compounds that occur naturally in bacteria and serve primarily as a carbon reserve under environmentally and metabolically unfavourable situations. PHA is produced by bacteria from renewable resources and was first described by Lemoigne in 1926 by *Bacillus megaterium*. There have been 250 different types of bacteria reported as natural PHA producers (Steinbüchel, 1991; Yang et al., 2010; Fatehi and Ataei, 2013). In addition to natural producers, genetically-modified organisms are also used for the industrial production of PHA (Slater et al., 1988; Braunegg et al., 1998) as well.

The focus of this study is the use xylose, especially from cellulosic hydrolysates as a carbon source to produce PHA by recombinant *E.coli*. One of the questions to be investigated is, whether increasing XylE can enhance xylose transport. To answer this question, the xylose transporter gene *xylE* is transformed into selected *E.coli* strain, JM109(DE3) and determine whether additional XylE enhance xylose uptake in bacterial cells leading to high PHA content. The conversion of the xylose to PHA is enhanced by adding *C.necator* PHA operon into $PGEMCAB_{Cn}$.

Initially hexose sugars are widely used as major carbon sources for PHA production by bacteria. However, a group of researchers proposed~~t~~ PHA production from pentoses or hemicellulose due to its availability and cost. Hence, they carry out their research using *Pseudomonas pseudoflava* strain after knowing its nutritional

versatility and ability to accumulate PHA from pentose sugars (Bertrand et al., 1990). This showed pentose and hexose- sugars are possible carbon sources for PHA production. However, pentose sugars are only get to utilize in the absence of glucose (Zha et al., 2014). This phenomenon is called carbon catabolite repression (CCR). Carbon catabolite repression is an important regulatory mechanism allowing bacteria, yeast and fungi to preferentially use easily metabolizable carbon sources (like glucose) over relatively less favorable carbon sources (for example, organic acids and alcohols). This phenomenon is illustrated by diauxic growth during which bacteria assimilate firstly energy-efficient and rapidly sugars then less-favored carbohydrates. A variety of molecular mechanisms are involved in carbon catabolite repression in order to control not only the expression of genes involved in the utilization of alternative carbon sources but also the expression of genes involved in several processes like virulence, competence etc.

Recent studies (Ferreira et al., 2015; Leu Meur et al., 2012) proved that xylose could be a promising substrate for conversion to PHA. For example, the maximum PHA accumulation could be up to 89% of total biomass by using *Bacillus sp.* with xylose as the substrate (Sindhu et al., 2013). Other strains such as recombinant *E. coli* (Lee, 1998), *Pseudomonas cepacia* (Young et al., 1994), *Burkholderia cepacia* (Keenan et al., 2004), and *Alcaligenes eutrophus* (Linko et al., 1993), were also reported to have the ability to produce PHA from xylose. Thus, these findings only focus on xylose used as sole carbon source for PHA production without ~~any~~addition of recombinant xylose transporter gene in the bacteria.

~~I~~n 1973, a group of researchers, cloned the gene *xylE*, coding for xylose-proton symport in *E. coli* and determined its DNA sequence (Dawes, 1973). This sugar transporter plays a significant role to transport xylose into bacterial cells to be consumed

as ~~the~~ a sole carbon source for maximum consumption. Apparently, xylose metabolism also involved other genes such as, xylose transport genes ABC (*xylFGH*) and D-xylose: H+ symporter (*xylE*), xylose isomerase (*xylA*), and xylulokinase (*xylB*). ~~In *E. co*~~In *E. coli*, xylose isomerase pathway enzymes are encoded by two catabolic genes *xylA* and *xylB*. The ABC transporter encoded by *xylFGH* genes, the proton/xylose symporter encoded by *xylE*, and -finally the transcriptional regulator encoded by *xylR* (Sumiya et al., 1995; Gonzalez et al., 2002). On a series of test conversion pentose and hexose sugars into PHA using -*E.coli* KJ122 strain, confirmed that XylE system was partially required for xylose transport in KJ122 strain to relieve the energy constraint caused by the XylFGH transporter. In addition, both deletions of *xylFGH* and *xylE* genes almost abolished the ability in xylose transport in KJ12203 strain (Khunnonkwao et al., 2018). This result suggested that *E. coli* KJ122 relied on both xylose-specific transporters for xylose consumption.

Apparently, introduction of *xylAB* from *E. coli* W3110 into *Pseudomonas putida* KT2440 was enough to allow the recombinant to efficiently utilize xylose as the sole carbon source for mcl-PHA production (Leu Meur et al., 2014). Then a group of researchers attempted to enhance xylose uptake– in *Cupravidus necator* strains by adding- xylose transporters *xylE*, *xylA* and *xylB* from *E. coli* to improve PHA production ~~(~~Liu et al., 2014). The recombinant strain produced limited PHB amounts from arabinose or xylose but these studies provide the basis for the development of recombinant bacteria strain to test PHA-producing ability from pentose sugars (Liu et al., 201~~3)~~4). This is followed by another study where *xylA* and *xylB* overexpression as a successful strategy for improving xylose utilization and poly-3-hydroxybutyrate (P3HB) production in *Burkholderia sacchari* (Linda et al., 2018).

Although few studies have been carried ~~y~~out on xylose transporters (*xylE, xylA, xylB, xylAB and xylFGH*) as xylose uptake enhancer and to test effect on PHA production in aforementioned bacteria but there is no precise report made on *xylE* alone in~~s~~ enhancing xylose uptake for PHA production in recombinant *E.coli* JM109 (DE3). Thus, this study mainly focus on the effect of additional *xylE* genes ~~activities for~~on xylose uptake for PHA conversion in *E.coli* JM109(DE3). Besides the wild-type *xylE*, mutants of the enzyme created for resistance to glucose inhibition were also tested. This study is important because cellulosic hydrolysates contain both xylose and glucose. The presence of glucose not only competitively inhibits xylose transport; it also suppresses the metabolism of other sugars.

In the light of the above, the objectives of this thesis were:

i. To analyze the effect of additional D-xylose transporter gene (*xylE*) in the conversion of xylose to PHA

ii. ~~i)~~ To determine ~~-~~the effect of *xylE* mutants in the conversion of xylose to PHA.

Chapter 2

Literature Review

2.1 Introduction to agricultural waste

Agricultural wastes (agro-waste) are defined as residues from the growing and processing of raw agricultural. Agro-waste contains soluble sugars, amino acids and insoluble chemical contents (cellulose and lignin) (Lim et al., 2014; Subba Rao, 1993). The agro-waste such as decaying part of plants are the primary source of organic matter in soil (Lim et al., 2014). Agro-waste contributes a significant proportion of the total waste matter in a developed country. Expanding agricultural production has naturally resulted in increased quantities of livestock waste, agricultural crop residues and agro-industrial by-products. Any significant increase in agricultural waste most likely occurred globally with continuation of farming system of third world countries.

The abundance of agro-waste makes it a cheap material to be converted into bio-based products. Agro-waste contains lignocellulosic materials from plant cell wall which consist of lignin, cellulose and hemicellulose, has the potential to be transformed into bioproduct by bacteria. The composition of lignocellulosic materials are mainly pentose and hexose sugars which can be converted into various industrial products.

2.2 Composition of agrowaste.

2.2.1 Cellulose

The term “cellulose” was first used in 1839 in a report of the French academy on the work of Payen. Apart from plants, cellulose are also produced by certain bacteria, algae, and fungi. In research, cellulose is often used as the model substances for

cellulose structure, crystallinity, reactivity, new materials development and biomaterial studies. Cellulose is a biodegradable, biocompatible, and renewable natural polymer and hence it is considered as an alternate to nondegradable fossil fuel-based polymers.

Cellulose is an extensive, linear-chain polymer with many hydroxy groups present in a thermodynamically preferred conformation. Cellulose represents about 1.5×10^{12} tons of the total annual biomass production and sustainable source of raw material for environmentally friendly and biocompatible products (Klemm et al., 2002). The cellulose chain consists D-glucose unit with an original C_4-OH group at one end; the other end is terminated with an original C_1-OH group, which is in equilibrium with the aldehyde structure (Rohrling et al., 2002). As a result of isolation and purification process, cellulose contains additional carboxy and carbonyl group (Perrin et al., 2014; Jullander and Brune, 1957)

Plants materials are the main raw sources of cellulose because they are abundant and relatively cheap. The main source of cellulose is wood pulp and cotton fibres. There are so many well-known plant material sources for cellulose such as jute, ramie, sisal, flax, hemp, water plants, grasses, and some parts of plants such as leaves, stem, fruit, etc. Agricultural wastes such as wheat and rice straw, sugarcane bagasse, sawdust, cotton and stables are also used to produce cellulose as well (Staiger & Tucker, 2008). Indeed, cellulose has various function to be used in sustainable industries for plant based resources.

2[r2].2.2 Lignin

Lignin is a complex organic polymer which can be found in all terrestrial plants and whose function is to bind cells, fibres and vessels. This organic polymer is the most abundant carbon source apart from cellulose. Lignin components empowers plants to

build rigid chemical structures and provides a barrier against hydrolysis of cellulose and hemicellulose in lignocellulosic biomass (Saake and Lehnen, 2012, Zakzeski et al., 2010). The use of lignin in plants, is to add strength and structure to their cellular, to control fluid flow, protection from microorganism, energy storage etc (Falkehag, 1975). Lignin building blocks such as coniferyl, cumaryl and sinapyl alcohol are linked together in different ratios to form a dense layer (Sarkenen et al., 1971). This causes high temperatures and high pressure required to break the lignin three dimensional network. Due to its properties lignin is used widely in large scale industries to produce high quality bio products.

2.2.3 Hemicellulose

Hemicellulose is a heteropolymer and is the next significant wood constituent along with cellulose. It's a branched polymer of pentose and hexose sugars, found in the plant cell wall and is synthesised by glycosyltransferases in the Golgi membranes (Holtzapple, 2003; Gírio et al., 2010). Apart from glucose, hemicellulose consists of several other sugar polymers as well. Those polymers made 15-35% of plant biomass and contain pentoses (b-D-xylose, a-L-arabinose), hexoses (b-D- b-D-glucose) etc. (Gírio et al., 2010; Ebringerová et al., 2005; Theander, 1985). The most relevant hemicelluloses are xylans and glucomannans, with xylans being the most abundant. Xylans are the main hemicellulose components of secondary cell walls constituting about 20–30% of the biomass of hardwoods and herbaceous plants. Grasses and cereals tissues consists approximately 50% of xylans (Ebringerová et al., 2005). Xylans are usually available in huge amounts as by-products of forest, agriculture, agro-industries, paper industries. There are different methods used to obtain hemicelluloses from plant sources and the extracted composition can be highly dependent on the isolation process. Some process used in hemicellulose extraction were alkali extraction, dimethyl

sulfoxide, methanol/water steam and microwave treatment (Celebioglu et al., 2012). Due to its availability from natural resources and the presence of other sugar compounds apart from glucose during photosynthesis, hemicellulose is widely used for xylose production.

2.3 Xylose

Xylose is classified under pentose and is commonly found in plant fibers and vegetable gum. It was first isolated and discovered from wood by Koch in 1981(Koch, 1887; Lewis, 1932). Xylose (Figure 2.1) is generally known as wood sugar, is a monosaccharide consist of five carbon atom and an aldehyde functional group. It has a chemical formula of $HOCH_2(CH(OH))_3CHO$ and can adopt several structures. These structures include D-xylose which occurs in living things and L-xylose which is synthesized. D-Xylose is dissimilated in *E.coli* K-12 through the pentose phosphate pathway (Lin, 1987). This sugar is first isomerized into D-xylulose by xylose isomerase (XylA) and then phosphorylated by xylulokinase (XylB) to produce D-xylulose 5-phosphate (David, 1970; Shamanna, 1979). The transport of D-xylose (Figure 2.2) in its aldo-pentose form is mediated by either the binding protein dependent or the low-affinity transporter which is *xylE* gene. The characteristics of D-xylose transport systems in different microorganisms are reviewed by Jeffries where bacteria commonly employ active (energy-requiring) mechanisms for sugar uptake. As for example *E. coli* uses a chemo static transport mechanism for xylose uptake. Most of the xylose uptake system are chemosmotic proton symport and highly pH dependent. It also subjected to competitive inhabitation by different sugar due to carrier protein specificity.

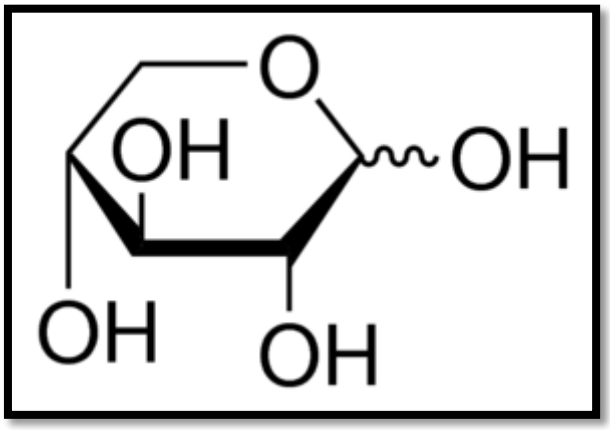

Figure 2.1 D-Xylose, a carbohydrate that consist of 5 carbons.

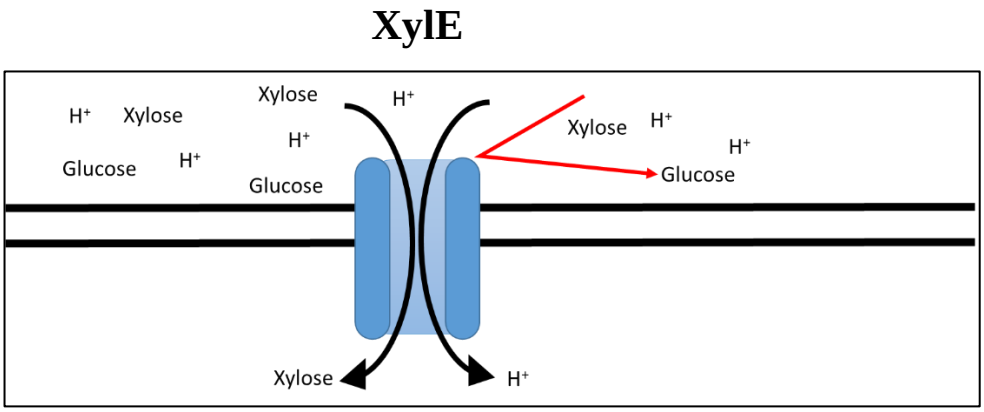

Figure 2.2 Xylose transport systems in *E. coli*. XylE is a D-xylose proton symporter that uses the proton gradient as a source of energy. This figure shows how xylose transported from extracellular cell into intracellular by XylE.

2.3.1 Amino acid that form hydrogen with xylose

Monosaccharides are the major building blocks of many important carbohydrate systems. Although the chemical composition within a class of monosaccharides, such as aldopentose sugars, is identical (C5H10O5), the orientation of hydroxyl groups can differ across stereoisomers. The subtle variation in hydroxyl arrangement is thought to account for differences in chemical and physical properties of the sugars. As for biologically significant monosaccharides, D-xylose is a model system for studying the effects of both primary stereo electronics and hydrogen bonding. (Zhao et al., 2020)

D-Xylose is specifically recognized by residues in XylE through hydrogen bonds. D-Xylose is coordinated by both polar and aromatic residues. Except for Gln 415, all other residues involved in D-xylose recognition are completely invariant in GLUT1–4 The hydroxyl groups of D-xylose are specifically recognized, through a total of eight hydrogen bonds, by polar residues including Gln 168 on TM5, Gln 288/Gln 289/ Asn 294 on TM7, Trp 392 on TM10, and Gln 415 on TM11 (Fig. 2b and Supplementary Fig. 7b). Tyr 298 and Gln 415 also contribute to substrate binding through water-mediated hydrogen bonds (Supplementary Fig. 7c). Several aromatic residues, including Phe 24 on TM1, Tyr 298 on TM7, Trp 392 on TM10, and Trp 416 on TM11, are located in the vicinity of D-xylose (Sun at al., 2012)

The transfer of hydrogen or hydride ions in the active site is commonly found in many enzyme reaction mechanisms. These hydrogen atoms or ions are, however, difficult to locate. This problem of locating hydrogen atoms in proteins, however, has been successfully addressed by neutron studies (Katz et al., 2006) because the neutron scattering power of an atom, unlike that for x-ray scattering, does not depend directly on its atomic number (Bacon, 1975; Sears, 1992)

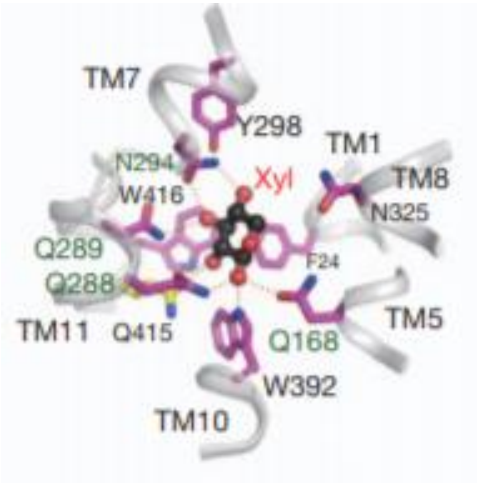

Figure 2.3 D-Xylose is coordinated mainly by residues from the C domain. The surrounding polar and aromatic residues are shown in magenta and orange sticks, respectively. D-Xylose is shown in black ball-and-sticks (Sun et al., 2012)

2.3.2 Xylose fermentation by bacteria

D-xylose is abundant in nature and is utilized by certain bacteria as a carbon source. D-xylose isomerase (*XylA*) and D-xylulose kinase (*XylB*) are two intracellular enzymes used to metabolize D-xylose in a bacteria cell. There are only two mechanisms of D-xylose transport have been characterized in bacteria till date. First mechanism was identified in *E. coli* involves a D-xylose–H1 or Na1 symporter (Paulsen et al., 1998; Kremling et al., 2015). The second mechanism consists of a high-affinity D-xylose transport system involving a periplasmic binding protein which driven by ATP in *E. coli* and thermophilic *Bacillus* species (Sumiya et al., 1995; Chaillou et al., 1999). These mechanisms occur during bacterial fermentation where a xylose isomerase (XI) converts

xylose to xylulose, which after phosphorylation, is metabolized through the pentose phosphate pathway (PPP).

Currently, there are three main bioproducts that can be obtained from this bacterial fermentation process that uses xylose as the major carbon source namely, biofuel (ethanol), xylitol (natural sweetener) and PHA (biopolymer). Production of biofuel uses xylose obtained mainly from natural substrates such as sugar cane, corn starch, cassava, etc. The cost of raw material to produce bioethanol is as high as 40% of the overall production cost (Sivers et al., 1994; Wyman, 1999).

There is a high amount of pentose present in agricultural residues (Saini et al., 2015) which consist of 95% D-xylose and L-arabinose. Xylan (found in hemicellulose) is easily hydrolysed to xylose, which can be converted into xylitol (Hao and Mohnen, 2014; Jiang et al., 2016).

Another product of xylose fermentation by bacteria is polyhydroxyalkanoate (PHA). PHA is a biodegradable polyester that is a strong candidate to replaced fossil-based plastic. As a conclusion, xylose can be a carbon source in bacteria fermentation transforming into three major bioproducts which are widely used in industry.

2.3.3 Selection of Host and Plasmid

To successfully perform molecular genetic techniques it is essential to have a full understanding of the properties of the various *E.coli* host strains commonly used for the propagation and manipulation of recombinant DNA. *E. coli* is an enteric rod-shaped Gram-negative bacterium with a circular genome of 4.6 Mb (Blattner et al., 1997). It was originally chosen as a model system because of its ability to grow on chemically defined media and its rapid growth rate. The ease of its trans-formability and genetic manipulation has subsequently solidified the role of *E. coli* as the host of choice for the

propagation, manipulation, and characterization of recombinant DNA. Comparatively plasmid transformation of *E. coli* can be performed quickly (Pope and Kent, 1996). *E. coli* has also been engineered to directly synthesize polyhydroxyalkanoates (PHA) with different monomer composition (Park et al., 2002). In this research several *E.coli* strains have been tested for continuous experiment. In particular, JM109 and JM109(DE3) a K-12 strains bacterium were used as host due to its ability that provides minimized recombination and aids in plasmid stability which results in high quality plasmid DNA preparation.

The plasmids most commonly used in recombinant DNA technology highly replicate in *E. coli.* An expression vector that contains the appropriate pieces for a host cell to produce the protein must be used for a gene to give rise to its protein product. In this research two plasmid have been used on host strain first was pACYCDuet-1 is designed for the co-expression of two target genes. The vector encodes two multiple cloning sites (MCS) each of which is preceded by a T7 promoter, lac operator and ribosome binding site. The next plasmid was, pGEMCABcn is convenient for "capturing" our PCR product for subsequent cloning and its cloning is quite inexpensive and reliable, and a good way to ensure that PCR product stored in a simple and well characterized cloning vector.

2.4 Metabolic pathway

2.4.1 XylE for uptake xylose

Figure 2.3 shows the transport of D-xylose into *E.coli* cells by xylose transporters *xylE* (in this study)/ *xylFGH*. D-xylose is then converted into xylulose by native xylose isomerase (*xylA*), which is subsequently phosphorylated to xylulose-5-

phosphate by xylulose kinase *(xylB*). Xylulose-5-phosphate is an intermediate of the pentose phosphate pathway (Weinberg, 1962; Cirino et al., 2006; Kim et al.,2015)

2.4.2 CAB$_{Cn}$ for PHA synthesis

Figure 2.4 shows the pathway used in PHB-producing organisms, such as *C.necator*. In *C. necator*, multiple carbon sources can be converted into acetyl-CoA. The acetyl-CoA can then be directed toward tricarboxylic acid (TCA) cycle (biomass production) or polyhydroxybutyrate (PHB) (under nutrient limitation). An *E. coli* cell factory can be constructed by expressing the *phaCAB* gene cluster from *C. necator*. Three enzymes are necessary for new synthesis of PHB in *C. necator* are: 3-ketoacyl

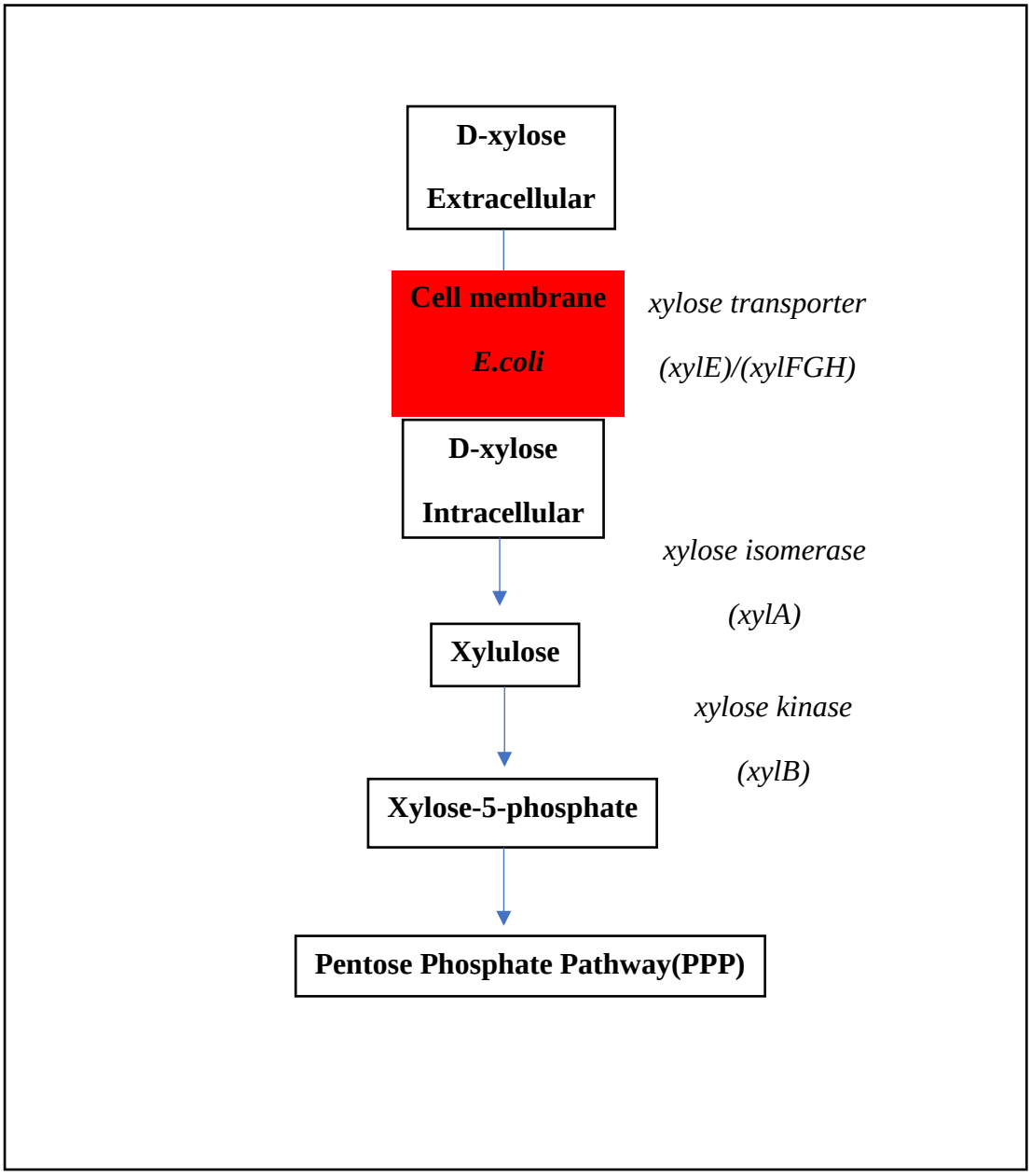

Figure 2.4 *xylE* in xylose metabolic pathway (Sandström et al., 2015). A condensed view of the glycolytic pathway steps, as well as the XR and PHB pathway steps that are relevant for the PHB accumulation in the constructed strain.

- Enzyme shown in *italic*.
- *xylE, xylFGH, xylA* and *xylB* worked as enzyme in this metabolic pathway.

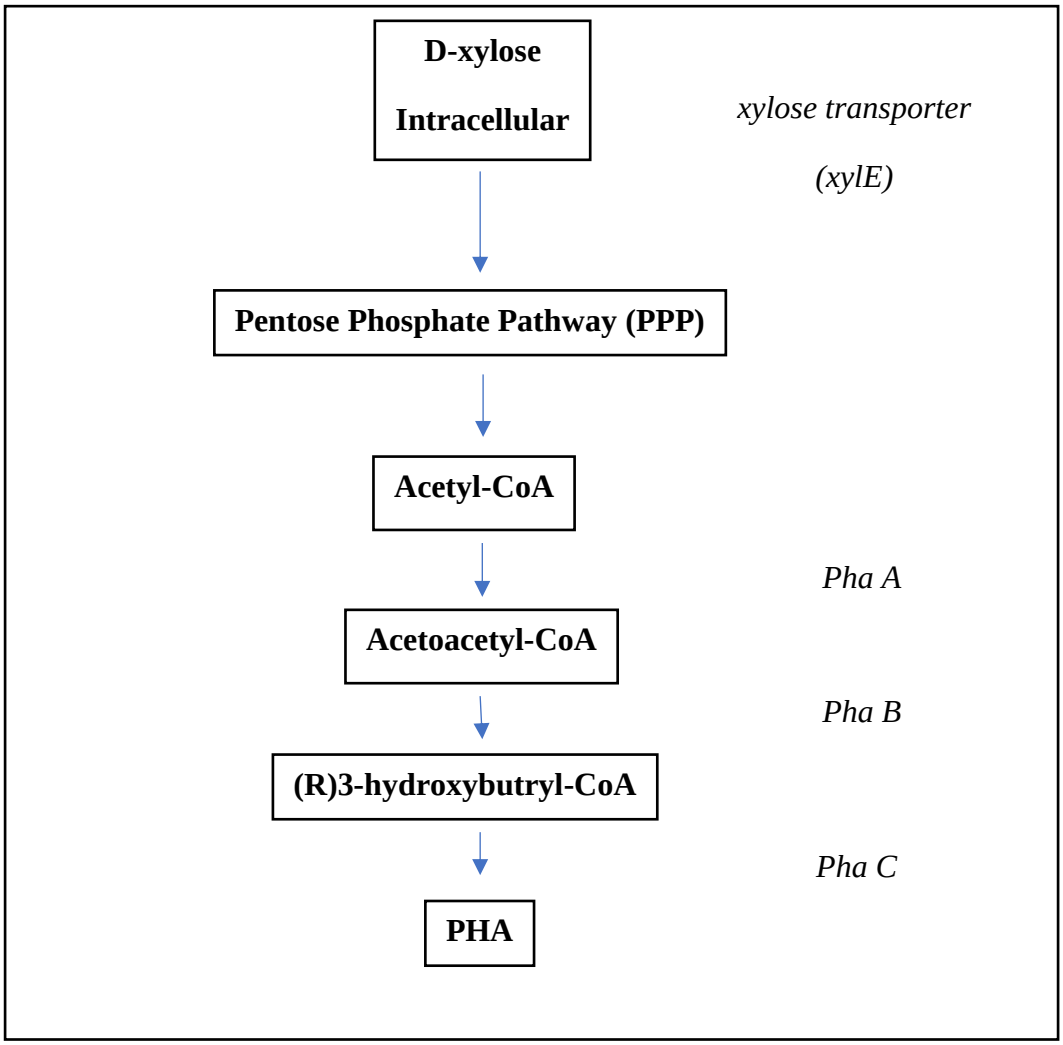

Figure 2.5 Pathway of conversion of xylose into PHA. (Sandström et al., 2015).

A condensed view of the glycolytic pathway steps, as well as the XR and PHB pathway steps that are relevant for the PHB accumulation in the constructed strain.

- Enzyme shown in *italic*. The rate limiting step occurred after xylose entered into PPP.

PhaA - 3-ketothiolase, *PhaB* – NADPH-dependent acetoacetyl-CoA reductase, *PhaC* – PHA synthase worked as enzyme in this metabolic pathway,

-coenzyme A (CoA) thiolase (PhaA), NADPH-dependent 3-acetoacetyl-CoA reductase (PhaB), and PHB synthase (PhaC). PhaA and PhaB catalyze the condensation of two molecules of acetyl-CoA to 3-acetoacetyl-CoA and the reduction of acetoacetyl-CoA to R-(–)-3-hydroxybutyryl-CoA, respectively. PhaC polymerizes these monomers to PHB releasing one CoA-SH molecule per monomer per reaction. The resulting PHB polymer is stored as water-insoluble granules in the cytoplasm of the cells (Anderson et al., 1990, Nikel et al., 2006, Matsumoto et al., 2010).

2.5 Sugar transport system

Sugar transport in bacteria involves highly specific membrane proteins that catalyze translocation and accumulation of various sugars from the environment across the cytoplasmic membrane and metabolized into the cell (Mitchell,1987). [r3][U4]Active transport in bacteria require energy when the translocation of sugar takes place against a concentration gradient (from a low concentration outside of the cell to higher concentrations inside). In bacteria, there are three types of transport systems involved for transporting sugar which require energy (Pieter, 1982; Epstein, 1974).

The Phosphotransferase System (PTS) represents a distinct family found only in certain bacteria. It is a bacterial sugar transport system that is found in *E. coli* which plays an essential role in catabolite repression and inducer exclusion. The PTS is a multicomponent system and catalyses vectoral phosphorylation of various sugars. (Epstein, 1974; Weigels, 1981; Lengeler, 1996). Therefore, translocation across the membrane and the first step in metabolism are same. Many sugars are transported by PTS (glucose, mannitol, fructose, mannose, galactitol, sorbitol and xylitol), and specificity for each sugar is provided by the membrane-embedded component of the system itself. PTS in *E. coli*, is the source of monosaccharides and their derivatives, but in other bacteria, this system also utilized disaccharides (Pieter, 1982; Bettenbrock,

2012). The PTS is also involved in the regulation of uptake and metabolism of various sugars as stated above as well as bacterial chemotaxis toward these sugars. Hence, sugar transport system in bacteria plays vital role to balance and translocate sugar accumulation in cell membrane and cell wall.

2.5.1 XylE

XylE protein is the first member of the Major Facilitator Superfamily (MFS) that has been structurally characterized in multiple transporting conformations that include both outward-and inward-facing (Figure 2.4). The MFS is largest membrane family present in bacteria, archaea, and eukaryotes (Pao, 1998). In bacteria, GLUT homologues such as the *E.coli* xylose permease (XylE) couple the transport of a H+ with the transport of a sugar, D-xylose against its concentration gradient from the periplasm to the cytoplasm. Currently, crystal structures of XylE in the outward partially occluded conformation with substrate bound and the inward partially occluded conformations as well as the inward open conformation have been reported (Abramson, 2003; Wisedchaisri et al., 2014). Based on an overlay of these structures, the transition from outward facing to inward facing in XylE was described as a symmetric rigid body movement. Structural analyses and comparisons suggest that 12 membrane-spanning segments may occur in the XylE protein (Davis, 1987; Pao et al., 1998; Yan, 2013). The gene for this protein is called *xylE* which is responsible for xylose transport in *E.coli* (Davis, 1987). The cloning strategy utilized λplacMu insertions and exploited the proximity of *xylE* to *malB* in a 2.8 kbp fragment.

The *xylE* gene was identified as a 1473 base pair open reading frame, located 373 base pairs downstream of *malG*, encoding a hydrophobic protein with a molecular weight of 53,607 (Davis, 1987).

The XylE structure in figure 2.5 contains residues 8–471 forming 12 TM helices divided into two halves, the N- and the C-domains, each containing six TM helices. These two domains are connected through a helical linker (residues 221–275), which is partially disordered in this structure. In the crystal structure of the outward partially occluded conformation the sugar-binding site in XylE is formed by several highly conserved residues and contribute a total of eight hydrogen bonds with D-xylose. In addition, Phe24 (TM1), Tyr298 (TM7) and Trp416 (TM11) form parts of the sugar binding pocket. Mutation of these residues severely diminished XylE function (Wisedchaisri et al., 2014; Pao et al., 1998).

As seen in figure 2.6, structural analysis of XylE reveals two such residues which are Asp27 (TM1) and Glu206 (TM6). Sequence analyses of sugar porter members indicate that Asp27 is conserved in homologues that are known to couple the transport of sugar with protons. Mutations of Asp27 in XylE abolished transporter function. In the crystal structure of the outward partially occluded conformation of XylE, Asp27 is stabilized by interactions with the conserved Arg133 from TM4 (Sun et al., 2012). The protonation of Asp27 and the binding of sugar to the substrate-binding site together, may have triggered and accelerate the conformational change in the transporter to switch from the outward- to the inward-facing conformation.

In Figure 2.7 the conformational change also assists the sugar movement along the transport pathway for the sugar to exit to the cytoplasm. After both the proton and the sugar have dissociated to the cytoplasm, the interaction between Asp27 and Arg133

could then be re-established and accelerate XylE to transition back to the outward facing conformation to complete the transport cycle. This mechanism helped to explain MFS members' remarkable characteristics of high affinity for substrates and high efficiency of transport in sugar sources (Sun et al., 2012; Pao et al., 1998). Figure 2.9 explains the sequence of sugar transport mechanism in XylE structure.

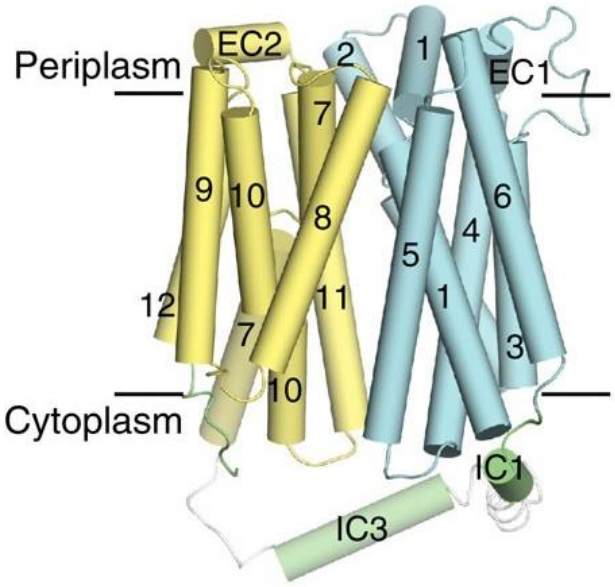

Figure 2.6 Structure of XylE in the inward-facing open conformation with a detached cytoplasmic domain. The structure folds into 12 transmembrane (TM) domains as labelled which separated by TM1-6- N Domain (Light Blue) and TM 7-12-C Domain (Yellow). This figure explained significant accumulation of (3H)-D-xylose in cell-based uptake assay (Wisedchaisri et al., 2014)

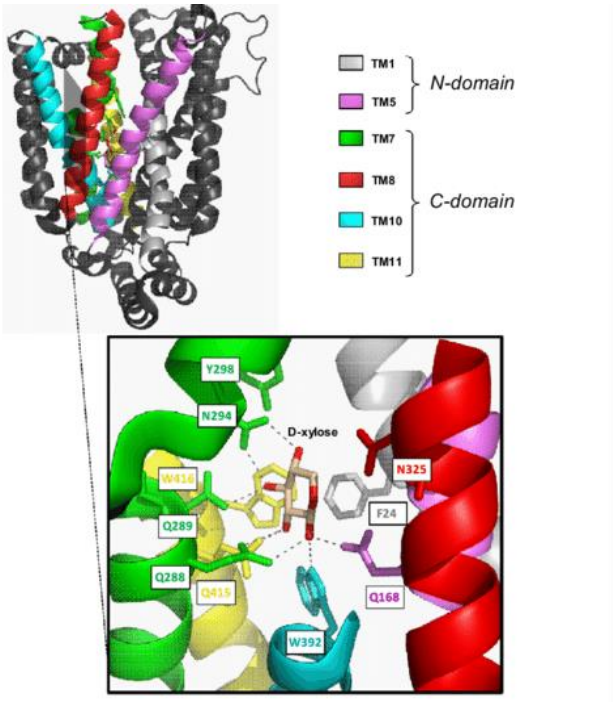

Figure 2.7 Cartoon representation of XylE bound to D-xylose. The Important transmembrane segments (TMs) involved in the binding site are colored. This figure shows, all the binding site formed by amino acids with N and C domain TMs. F24 (TM1), Q168 (TM5), Q288/Q289/N294/Y298 (TM7), N325 (TM8), W392 (TM10) and Q415/W416 (TM11), represented as sticks. The hydrogen bonds are depicted as dotted grey lines (Sun et al., 2012)

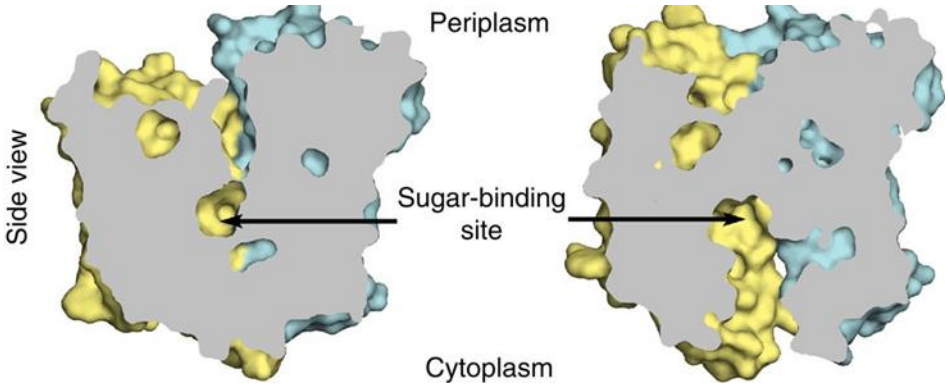

Figure 2.8 shows sugar binding site of Crystal structure of XylE bound to D-xylose. The structure of this bacterial homologue is divided into (N- and C-domain) colored in orange and silver, respectively. Both domains are connected by an intracellular domain represented in grey (Wisedchaisri et al., 2014).

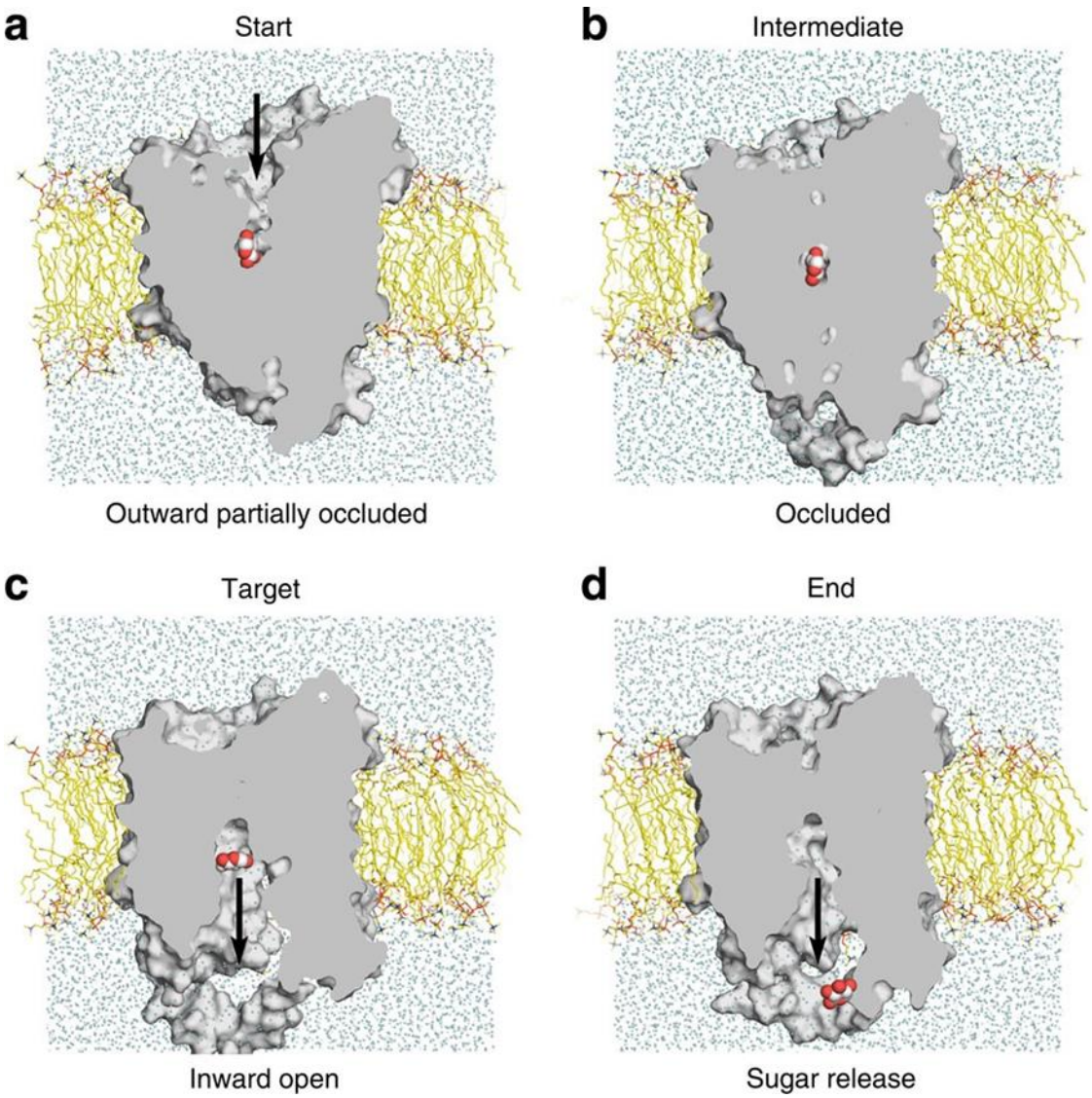

Figure 2.9 This pictures from Wisedchaisri et al., 2014, explained the process of D-xylose binding process in XylE from the beginning till the end. It showing how does xylose (sugar) carried by xylE from extracelluar to intracellular. (a) The starting outward partially occluded conformation with D-xylose bound, (b) the occluded intermediate, (c) the target inward open conformation and (d) the final sugar release conformation of XylE embedded in the membrane system in the MD simulation box. XylE is embedded in the lipid molecules (yellow and orange lines) surrounded by water molecules (light-blue spheres) and Cl− ions (magenta spheres). D-xylose molecule is shown as white and red large spheres.