CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT *ESCHERICHIA COLI* FOR PRODUCTION OF XYLITOL FROM MIXED SUGARS

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ABSTRACT

Development of a microbial system for production of value-added chemicals has garnered interest for its benefits of low operational cost and greater substrate specificity. Microbial production of xylitol is highly desired for its ability to produce xylitol from unpure carbon sources mainly from lignocellulosic waste. While recent studies on xylitol production focused on utilizing mixed sugars for xylitol production, this required the expression of multiple genes to enable simultaneous xylitol conversion from glucose, xylose and arabinose which leads to cell's metabolic burden. Moreover, manipulation on the cell is also needed to remove catabolite repression present in the cell. This is the first study that describes xylitol production from multiple sugars (glucose, xylose and arabinose) in *Escherichia coli* BL21 expressing only a single gene, xylitol 5-phosphate dehydrogenase (XPDH). XPDH converts $_{D}$-xylulose-5-phosphate, an intermediate in *E. coli* pentose phosphate pathway to $_{D}$-xylitol-5-phosphate which is then hydrolyzed to $_{D}$-xylitol by phosphatase. XPDH from *Clostridium difficile* was cloned into *E. coli* and screened for xylitol production using high pressure liquid chromatography analysis. Then, xylitol production was improved through metabolic engineering by deleting competing pathways and process optimized using one factor-at-a-time (OFAT) method. Initial screening of xylitol production revealed that *E. coli* BL21 expressing XPDH (NA116) was able to produce xylitol from each sugar, glucose, xylose and arabinose (supplied at 10 g/L) with final xylitol of 0.283 g/L, 0.518 g/L and 2.09 g/L respectively. Metabolic manipulation of the *E. coli* was made by deleting competing pathways in glycolysis and pentose phosphate pathway, namely phospoglucose isomerase (*pgi*), ribose isomerase A (*rpiA*), and ribose isomerase B (*rpiB*) genes. Screening of the mutants revealed highest xylitol production from arabinose by NA207 (Δ*rpiA*) mutant, with final xylitol produced of 3.91 g/L. Further manipulation of NA207 strain was made by introducing *ptsG* deletion to allow simultaneous carbon uptake in the presence of glucose for mixed sugars fermentation, yielding NA223 (Δ*rpiA*Δ*ptsG*) strain. The result revealed that NA223 showed 4 times more arabinose uptake in the mixed sugar culture compared to NA116, with final xylitol production of 1.18 g/L and 0.815 g/L respectively. Optimization of NA223 mutant was done by using OFAT method manipulating several parameters; inducer concentrations, temperature, media type and initial pH. The final parameters manipulation showed to have improved xylitol production to 5 times compared to initial conditions with 1.674 g/L in 0.05mM IPTG, 3.687 g/L in 25 °C, 3.95 g/L in buffered YT broth (BYT) medium, and 5.216 g/L in initial pH 8.5 of BYT. This study shows that xylitol conversion from mixed sugars is possible by expressing only a single heterologous gene, XPDH in *E. coli* while *ptsG* deletion alleviates carbon catabolite repression by allowing simultaneous arabinose uptake in the presence of glucose.

ABSTRAK

Pembangunan sistem mikrob untuk menghasilkan bahan kimia nilai tambah menjadi satu tarikan kerana kos operasinya yang rendah dan spesifikasi substrat yang lebih luas. Penghasilan xilitol menggunakan mikrob sangat dikehendaki kerana ia boleh menghasilkan xilitol daripada sumber karbon tidak bersih daripada sisa lignoselulosa. Kebanyakan kajian terkini mengenai pengeluaran xilitol memberi fokus kepada penggunaan gula campuran untuk penghasilan xilitol, ia memerlukan ekspresi beberapa gen untuk membolehkan penukaran xilitol secara serentak daripada glukosa, xilosa dan arabinosa yang boleh mengakibatkan terjadinya beban metabolik kepada sel. Selain itu, manipulasi pada sel juga perlu dilakukan bagi membolehkan penindasan katabolit dalam sel dihapuskan. Kajian ini adalah kajian pertama yang melaporkan penghasilan xilitol daripada beberapa gula secara serentak (glukosa, xilosa dan arabinosa) dalam *Escherichia coli* BL21 dengan ekspresi satu gen sahaja, iaitu xilitol 5-fosfat dehidrogenase (XPDH). XPDH menukarkan $_{D}$-xilulosa-5-fosfat iaitu satu metabolit perantara dalam laluan pentosa fosfat *E. coli* kepada $_{D}$-xilitol-5-fosfat, yang kemudiannya dihidrolisiskan kepada xilitol oleh fosfatase. XPDH daripada *Clostridium difficile* telah diklonkan ke dalam *E. coli* dan disaring untuk penghasilan xilitol menggunakan analisis kromatografi cecair tekanan tinggi. Kemudian, pengeluaran xilitol juga dipertingkatkan melalui kejuruteraan metabolik dengan membuang laluan saingan dan melakukan proses fermentasi yang dioptimakan melalui kaedah satu faktor pada satu masa (OFAT). Saringan awal pengeluaran xilitol menunjukkan bahawa *E. coli*-XPDH (NA116) mampu menghasilkan xilitol daripada setiap gula yang diuji iaitu glukosa, xilosa dan arabinosa (dibekalkan pada 10 g/L) dengan xilitol sebanyak 0.283 g/L, 0.518 g/ L dan 2.09 g/L mengikut turutan. Manipulasi laluan metabolik *E. coli* dibuat dengan memadamkan laluan bersaing dalam laluan glikolisis dan pentosa fosfat, iaitu fospoglukosa isomerase (*pgi*), ribosa isomerase A (*rpiA*), dan ribosa isomerase B (*rpiB*). Saringan terhadap setiap mutan mendapati mutan NA207 (Δ*rpiA*) menghasilkan xilitol tertinggi sebanyak 3.91 g/L menggunakan arabinosa. Justeru, manipulasi pada mutan NA207 dilakukan dengan memadamkan gen *ptsG* untuk membolehkan pengambilkan karbon serentak bersama glukosa bagi fermentasi menggunakan campuran gula, yang menghasilkan mutan NA223 (Δ*rpiA*Δ*ptsG*). Keputusan kajian menunjukkan bahawa NA223 dapat menggunakan campuran gula lebih banyak semasa glukosa masih ada dalam media fermentasi berbanding NA116, dan menghasilkan xilitol yang lebih tinggi sebanyak 1.18 g/L berbanding 0.815 g/L pada asalnya. Pengoptimuman mutan NA223 seterusnya dilakukan dengan menggunakan kaedah OFAT, dengan manipulasi beberapa parameter iaitu kepekatan induksi, suhu, jenis media dan pH awal. Manipulasi parameter terakhir menunjukkan pengeluaran xilitol bertambah sebanyak 5 kali ganda berbanding dengan kondisi awalnya, dengan peningkatan pada setiap parameter iaitu 1.674 g/L dalam 0.05mM IPTG, 3.687 g/L dalam 25 °C, 3.95 g/L dalam medium buffer YT (BYT), dan 5.216 g/L dalam pH awal 8.5 BYT. Kajian ini menunjukkan bahawa xilitol berjaya dihasilkan daripada campuran gula dengan ekspresi hanya satu gen heterolog, XPDH dalam *E. coli* manakala pemadaman *ptsG* berjaya mengurangkan tindakan penindasan katabolit dengan membenarkan pengambilan gula arabinosa dan xilosa secara serentak dalam kehadiran glukosa dalam sel.

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LIST OF ABBREVIATIONS

rpiA	-	Ribose isomerise A
rpiB	-	Ribose isomerise B
pgi	-	Phosphoglucose isomerise
ptsG	-	Phosphotransferase G
PTS	-	Phosphotransferase system
CCR	-	Carbon catabolite represssion
PEP	-	phosphoenolpyruvate
XPDH	-	Xylitol-5-phosphate dehydrogenase

LIST OF SYMBOLS

Δ	-	Delta, deletion
°C		Degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

From our food additives to our medicines, the use of fine chemicals encompasses a broad range of applications in our lives. Fine chemicals are defined as chemicals with specific molecular characteristics which are produced at low quantity but expensive (Mullin, 2012). Being essential building blocks for various manufacturing products, the demands for fine chemicals has driven the chemical industry forward for its production and development. Generally, chemical industry heavily relies on fossil feedstocks as raw material for chemical production. This pose as a major problem due to its environmental issue and being a non-renewable carbon source (Straathof et al., 2019). Meanwhile, lignocellulosic biomass is sustainable, abundant and readily available source for chemical feedstock with the advantage of releasing zero net carbon emission to the atmosphere (Nanda et al., 2016). Therefore, the shift to using renewable lignocellulosic biomass as a feed source has become a huge topic explored for fine chemicals production.

Lignocellulosic biomass is an organic material derived from municipal or industrial wastes, agricultural residues, wood residues and dedicated crops such as switch grass (Fuente-Hernandez et al., 2013; Mohamad et al., 2015). The biomass is composed of three main components which are cellulose, hemicellulose and lignin. The two most abundant components, cellulose and hemicellulose are the main source for sugar monomers for bioconversion. Cellulose is made up from glucose monomers linked by β-(1→4)-glycosidic bonds while hemicellulose is made up from a variety of pentose (i.e D-xylose and L-arabinose) and hexose sugars (i.e D-glucose, D-mannose, and D-galactose) (Hayes, 2015; Kim et al., 2010). Hydrolysis of lignocellulosic biomass release the sugar monomers and later used for bioconversion

to produce value-added chemicals and biofuels, either by biological or chemical means. Bioconversion of lignocellulosic biomass generated a wide variety chemicals such as succinic acid, acetic acid, lactic acid, acetone, furfural, butyric acid and xylitol (Peng et al., 2011).

Xylitol is a five-carbon sugar alcohol that is obtained from hemicellulosic sugar monomer, xylose. Its structure is composed of five hydroxyl groups which makes it highly soluble in aqueous solution (Grembecka, 2015). Xylitol is included among ten most promising fine chemicals in the production of fuels and pharmaceutical/chemical products (Bozell and Petersen, 2010), due to the presence of multiple functional groups which makes it an excellent building block for transformation into high-value bio based chemicals or materials (Peng et al., 2011). The industrial application of xylitol is diverse, ranging from food additives to pharmaceutical products given to its properties of having higher sweetness level than sucrose, anticariogenic, insulin dependent metabolism and pharmacological properties (O'Donnell and Kearsley, 2012). Demand for xylitol has steadily increased over the years with more than forty times increment in its production over four decades, from 6000 tons in 1978 to 190.9 thousand tons in 2016. It was forecasted to further increase to 266.5 thousand tons by 2022 (Delgado Arcaño et al., 2020; Hyvönen et al., 1982).

Current industrial production of xylitol is done through chemical hydrogenation of xylose using nickel catalyst, which operated at high temperature and high pressure (Albuquerque et al., 2014). The process requires the use of pure xylose which is obtained from xylan-rich hemicellulosic hydrolysate, typically prepared by acid hydrolysis. However, due to the presence of other sugar monomers (mannose, arabinose, galactose, and glucose) and other impurities (proteins, metal ions and color) in the hydrolysate, extensive purification steps to separate xylose are necessary. The xylose-rich hydrolysate is then hydrogenated to produce xylitol at high temperature of 8-140 °C and pressure up to 50 atm (Pal et al., 2016; Parajó et al., 1998). Other than being energy intensive, the process also requires the use of highly sensitive catalyst and multiple steps for xylitol separation and purification (Delgado Arcaño et al., 2020; Rafiqul and Sakinah, 2013). These factors led to high

production cost of xylitol, with more than 10 times of sucrose conversion to sorbitol (Peng et al., 2011). Therefore, a milder alternative for xylitol production using microbial bioconversion is highly desirable as it operates at milder conditions and also environmentally sustainable.

Biotechnological methods have been used to study a variety of microorganisms for the conversion of xylitol, which ranged from fungi, yeast and bacteria. Compared to chemical process, this system is more desirable because biotechnological xylitol synthesis is less expensive, and allows the use of unpure xylose, which reduce much cost on the initial multiple purification steps (Venkateswar Rao et al., 2016). Naturally, certain yeast, bacteria and fungi can synthesize xylitol from xylose such as *Candida sp* (Barbosa et al., 1988; Granström et al., 2007a), *Debaromyces hansenii* (Sampaio et al., 2008), *Pichia stipites* (Hahn-Hägerdal et al., 2007), *Corynebacterium sp* (Yoshitake et al., 1971), *Mycobacterium smegmatis* (Izumori and Tuzaki, 1988), and *Enterobacter liquifaciens* (Yoshitake et al., 1973), *Aspergillus niger* and *Penicillum sp* (Sampaio et al., 2003). Among these, highest xylitol production from natural producers were recorded by yeasts, with 77.2 g/L xylitol yield in *Candida guilliermondii* FTI-20037118 (Barbosa et al., 1988). The main enzyme for xylitol conversion in microbes is xylose reductase, XR which hydrolyzes xylose into xylitol by the aid of NADPH cofactor. Xylitol is then secreted out of the cell or further metabolized by xylitol dehydrogenase, XDH into xylulose for metabolism (Chen et al., 2010). In recent decades, many studies have explored to modify XR and XDH through mutation or genetic engineering to improve xylitol production in microbes.

While xylitol conversion from xylose has developed widely, several studies have also reported xylitol conversion from $_{D}$-glucose and $_{L}$-arabinose which are another two major sugar components in hemicellulose. The earliest report of xylitol conversion from glucose was done through sequential fermentations of three microbes, *Debaryomyces harseniii*, *Acetobacter suboxydans*, and *Candida guilleiermondii* var. *soya* which converted glucose to $_{D}$-arabitol, then $_{D}$-arabitol to $_{D}$-xylulose and $_{D}$-xylulose to xylitol respectively. The xylitol yield was 18 g/L from 155 g/L supplied glucose, with 11% xylitol conversion (Onishi and Suzuki, 1969).

Another approach for xylitol conversion from glucose was made by using xylitol phosphate dehydrogenase enzyme, XPDH from *Lactobacillus rhamnosus* expressed in *Bacillus subtilis.* XPDH catalyzes the conversion of $_{D}$-xyulose-5-phosphate to $_{D}$-xylitol-5-phosphate which is further hydrolyzed by phosphatase into xylitol (Povelainen and Miasnikov, 2007). The study reported xylitol yield of 2.3 g/L from 10 g/L glucose (23% xylitol conversion). Meanwhile, xylitol conversion from arabinose was first reported in engineered *Escherichia coli* by the expression of three heterologous genes for ATX pathway, encoding for $_{L}$-arabinose isomerase (*araA*), $_{D}$-psicose 3-epimerase (*DPE*) and $_{L}$-xylulose reductase (*LXR*) (Sakakibara et al., 2009). In this pathway, $_{L}$-arabinose is converted to $_{L}$-ribulose, then to $_{L}$-xylulose and finally xylitol respectively. The study reported final conversion of 14.5 g/L xylitol from 15.2 g/L arabinose, with 95% conversion after 30 hours. Similar strategies was also applied in *C. glutamicum* for conversion of xylitol from arabinose, which gave a yield of 4.4g/L xylitol from 6.1 g/L arabinose consumed, with 72% conversion (Dhar et al., 2016). These studies showed that manipulation of genes and pathways made conversion of xylitol from other sugars possible with many outlooks for further improvement.

It is interesting to note that many recent microbial xylitol conversion researches have focused on developing a system that could use multiple sugars present in hemicellulose. *E. coli* is among popular hosts as cell biocatalyst for bioproduct conversion has been one of the main focus in this field given to its characteristics of robust growth, prone to genetic manipulation, high cell viability and efficient production. Development of a microbial system for xylitol production involves two approaches, molecular strategies such as metabolic engineering, and modification of process parameters for improved xylitol yield. This study explores the production of xylitol from multiple sugar, xylose, glucose, and arabinose using the expression of a single gene, XPDH in *E. coli*. The host is also subjected to metabolic engineering for improved pathway which aids in xylitol conversion and also enables simultaneous utilization of mixed sugars in *E. coli*. Finally, xylitol yield is also enhanced by manipulation of fermentation parameters to obtain optimal conditions for xylitol production.

1.2 Problem Statement

Biotechnological production of xylitol is advantageous to current chemical production by being environmentally sustainable and cost effective due to its mild operational conditions and reduced multiple purification steps for substrate preparation from hemicellulosic biomass. Since glucose, xylose and arabinose are three major sugars in hemicellulosic biomass, developing a system to utilize these sugars for xylitol production is highly desirable. While significant improvement has been developed in xylitol research, the majority of research has focused on xylitol production only from xylose and only a limited number of reports exist for xylitol production from glucose and arabinose. Moreover, the studies also reported the utilization of multiple gene expressions to allow xylitol production from the other sugars. This could lead to metabolic burden and reduced performance of the cell. Another strategy of using dual or sequential fermentation system has also been applied for the same purpose which requires more preparation steps and time. Thus a simpler system for xylitol production from multiple sugars should be explored to address this problem.

1.3 Objectives of the Study

In order to achieve a microbial system for xylitol conversion from multiple sugars, the objectives of this research are:

(a) To construct and study a recombinant *E. coli* BL21 for xylitol synthesis from glucose, xylose and arabinose.
(b) To modify metabolic pathway for xylitol production in *E. coli* BL21
(c) To optimize fermentation conditions for highest xylitol production from mixed sugars in *E. coli* BL21.

1.4 Scopes of the Study

The scope of study listed below will be used to achieve the objectives stated.

i. The first objective was defined with the following scopes; cloning, construction and evaluation of XPDH enzyme for xylitol conversion in *E. coli* BL21. Investigation of xylitol production from glucose, arabinose and xylose by XPDH in *E. coli* BL21 is done by HPLC analysis.

ii. The scope for second objective is achieved by conducting metabolic pathway optimization through gene deletions for xylitol production from single sugar, and conducting genetic manipulation for mixed sugar utilization in *E. coli* BL21 for xylitol production from mixed sugars.

iii. The final objective has the following scope; optimization of the fermentation conditions by studying the effects of (1) inducer concentration, (2) post induction temperature, (3) media type and (4) initial pH for maximum xylitol production using one-factor-at-a-time (OFAT) method.

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LIST OF PUBLICATIONS

Indexed Journal

1. **Abdullah, N**., Md Illias, R., Low, K.O., Jaafar, N. R., Mohamad Sukri, H.,and Abdul Rahman, R. (2022) Metabolic pathways modification forproduction of xylitol from glucose in *Escherichia coli*. *Jurnal Teknologi*,8(4). Accepted. (**Indexed by SCOPUS)**