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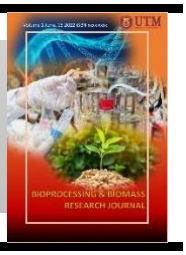

Research Article

Screening Effect of Amino Acid on Xylitol Production By Recombinant *Escherichia coli* System

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ABSTRACT

Numerous studies have been conducted to source for safer biological methods to produce xylitol. In view of this concern and the benefits of xylitol, a fermentation process is formulated to yield the highest xylitol that is both favourable and profitable. In this study, xylitol production from xylose by recombinant *Escherichia coli* system was conducted by modulating both carbon source and amino acid composition of the media for the relative growth delay of the strain. The key enzyme for xylitol production in this recombinant system is xylose reductase, XR which utilizes NADPH to reduce D-xylose to xylitol. By adding 20 types of amino acids individually and substituting glycerol as the carbon source each time, it showed an increase of xylitol to 5.24 g/L and yield biomass production of 1.536. It is hypothesized that the supply of single amino acid act as a tool to enhance (NAD(P)H)/(NADP+) ratio. Reduced NAD(P)H competition from other bioprocesses help the cell replenished the reduced cofactor pool. Xylitol has a remarkable benefit as a healthy replacement of table sugar. Therefore, the success of this study will definitely bring advancement in the production technology and acts as a reference for future research.

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INTRODUCTION

Xylitol is the first sugar of its kind to have global market. It is a favorable substitute for sucrose as a sweetener, and its independence from insulin in metabolic regulation provides diabetic patients with an alternative sweetener (Damião *et al.*, 2018).

Xylitol is derived from xylose via chemical synthesis or biological conversion by microorganisms. The biotechnological method of producing xylitol has surpassed the chemical method because the latter is not eco-friendly and requires a large amount of energy for xylose hydrogenation, resulting in a high production cost (Mathew *et al.*, 2018). Currently, microbial processes, such as fermentative and biocatalytic processes in bacteria, fungi, and yeast cells, are the predominant technology for xylitol production. Common yeast cells used in fermentation to produce xylitol are *Candida tropicalis, Neurospora crassa and Candida guilliermondii.* Yuvadetkun & Boonmee (2016) demonstrated that xylose could be transported into yeast cells and be converted to xylitol by xylose reductase and then to xylulose by xylitol dehydrogenase.

Although microbial application able to eliminate problems occur during chemical synthesis of xylitol, higher xylitol production is in demand for sectors including food, pharmaceutical, and cosmetic. Besides, low concentration of xylitol along with others considerations had become a primary bottleneck in pentose fermenting yeast. Hence, there were efforts to design more cost-effective production

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methods including using a co-substrate, controlling the dissolved oxygen or redox potential, amplifying xylose reductase (hereafter XR) activity (Ko *et al.*, 2006), as well as production using NAD(P)+-dependent polyol dehydrogenases (Kim *et al.*, 2019).

Concurrently, recombinant DNA technology is a fast growing field and new approaches, devices, and engineered products have been developed by scientist around the world for extensive application covering agriculture, health, and environment sectors (Khan *et al.*, 2016). The cloning of foreign DNA in *Escherichia coli* is cornerstone for industrial production. The main reason is because of its ability to accustom both hexose and pentose sugars, rapid growth rates, ease of manipulation and an economical growth medium requirements, as evidence by its ongoing industrial implementation for production of 1,3-propanediol and insulin (Xu & Ma, 2019; Zieliński *et al.*, 2019).

There are several challenges in downstream phase of recombinant *E. coli* as stated by Rosano & Ceccarelli (2014) including low protein expression levels, difficulty in obtaining correctly folded recombinant proteins and inactive insoluble aggregates formation (Rosano & Ceccarelli, 2014) which need to be addressed.

Composition of outgrowth media portrays an important role in the wake up kinetics and ampicillin sensitivity of *E. coli,* as mentioned by Varik et al. (2016). Furthermore, *E. coli* needs to adapt to rapid changes in nutrient availability, for example in periods of famine, the bacteria need to slow down their metabolism and growth, and vice versa. Another aspect in the production of recombinant products that needs to be embarked upon is the significance availability of cofactors, such as NAD(P)H and its important role in amino acid-producing strains for biochemical reactions and physiological function.

Essentially, NAD(P)H availability and form can be manipulated by redirecting the carbon flux in industrial strains for amino acid biosynthesis and it is an efficient and easier conduct. This would in turn increase the efficacy of *E. coli* recombinant products production. However, the question, now, is which control strategy that fits and can be chosen to accelerate the carbon metabolic fluxes and rapidly guide them to a target amino acid via the regulation of intracellular NAD(P)H levels.

Previously, *E. coli* BL21 (DE3) is genetically modified to produce xylitol with the addition of Δxy1AB +P21XR. The Δxy1AB represents the removal of *E. coli* wild strain ability to transform xylose to xylulose (deleted chromosomal xylose isomerase gene (xylA) and xylulokinase gene (xylB)); and P21XR indicates the added plasmid into the recombinant *E. coli* that enable the production of XR (Abd Rahman *et al.*, 2020). These were subsequently amplified with Polymerase Chain Reaction (PCR) strategy and ultimately cloned into vectors with inducible promoter of both xylose and Isopropyl β-D-1-thiogalactopyranoside (IPTG). The mechanism of xylitol production by *E. coli* strain BL21 (DE3) Δxy1AB +P21XR is shown in **Figure 1.**

The purpose of this study is to identify amino acids that would significantly contribute to xylitol production from xylose by recombinant *E. coli* system. Navaneethan et al. (2021) claimed that group A amino acids consisting of valine, leucine, and threonine are essential for bacterial growth. The addition of 20 different amino acids supplemented individually in cultures was observed through the effect of amino acids and codon usage of XR with aim to further enhance xylitol output. Next, the expression problem in *E. coli* that would cause metabolic burden was tackled by analysing rare codon (RC) usage of both recombinant *E. coli* and the original XR gene sequence from *N. crassa*. Pellizza et al. (2018) stated that to save time, labor and cost, sequence-based methods can be performed prior real laboratory experiment is conducted as it can be considered as a valuable tools for prediction of recombinant protein overexpression results. In this case, RC usage analysis is a value added information that would help in better understanding the factors governing protein solubility, as well as to provide insight into protein aggregation or protein misfolding.

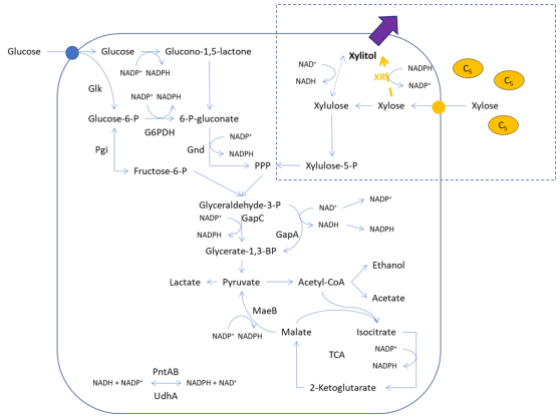

Figure 1 Xylose and glucose pathways expressed in recombinant *E. coli*. Xylose reductase-based pathway for production of xylitol is depicted using dashed lines in the box.

MATERIALS AND METHOD

A recombinant *E. coli* strain that can produce xylitol was obtained from the Genetic Laboratory, Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, as previously stated by Abd Rahman *et al.* (2020). *E. coli* BL21 (DE3) was used as a host organism. An engineered *E. coli* strain carrying *xylose reductase (XR)* gene from *N. crassa* was constructed. Plasmid pET21 under the promoter system was used as a template vector for cloning. The one-step gene inactivation strategy, previously reported by Detsenko and Wanner (2000), was employed to disrupt the *xylA* (xylose isomerase) and *xylB* (xylulokinase) genes in the engineered *E. coli* BL21. Polymerase chain reaction (PCR) amplification of the *xylA* and *xylB* genes region was carried out to verify gene deletion. *E. coli* strains harbouring recombinant plasmids were cultured in Luria-Bertani (LB) agar plate, added with 100 mg/ ml Ampicilin. For induction, one single colony was inoculated into fresh Luria-Bertani (LB) medium (10 g/L yeast extract, 10 g/L sodium chloride, 20 g/L meat peptone), added with 100 mg/ ml Ampicilin at 37 °C, 200 rpm, grown overnight.

The concentrations of xylose and xylitol were quantified using a Waters 1525 binary pump high-performance liquid chromatograph (HPLC) equipped with a Waters 2414 refractive index detector and a Phenomenex Rezex column (300×7.80 mm) at a 75 °C external temperature. Deionized water was used as the mobile phase at a flow rate of 0.6 ml min^{-1}. The standard curves of xylose and xylitol were determined (Abd Rahman *et al.*, 2020).

Cultures with overnight grown *E. coli* strain were prepared in 150 mL shake-flasks containing 30 mL of M9 modified auto induction medium [M9 salts (per 1 L: 40.0 g $Na_2HPO_4.2H_20$, 15.0 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl, 0.5g

glucose, 5mL glycerol; 2g lactose) and other salts per 1 L: 2 mL 1M $MgSO_4$ & 100 µL 1M $CaCl_2$ in a shaking incubator, 37 °C, 150 rpm. A 5% inoculum size is applied with 20 g/L of xylose at 26 h of fermentation. Optical density (OD) is analysed with Thermo Spectrophotometer every two hours.

Amino acids (each at 100 µg/mL) were purchased from Himedia, India. Glycerol was added because complex components including yeast extract, tryptone or amino acids added into medium able to functionalize glycerol (Trinh & Srienc, 2009).

Calculation of rare codon was performed using codon usage database, as previously mentioned by Rekha *et al.* (2019). Codon-optimized gene of XR was obtained from National Center for Biotechnology Information (NCBI) for enquery *Neurospora crassa* xylose reductase mRNA, complete cds.

RESULTS AND DISCUSSION

Biomass and Xylitol Production

In this study, the optimization of xylitol production was improved by combining the effect of using single step XR pathway, RC analysis, supplementation of 20 types of amino acids individually and substitution of glycerol as the main carbon source in the media. Overall, xylitol productivity and biomass yield were enhanced from 3.58 to 5.24 g/L and from 0.0637 to 0.0823 g/L/h in glycerol fermentation respectively, as shown in **Figure 2.**

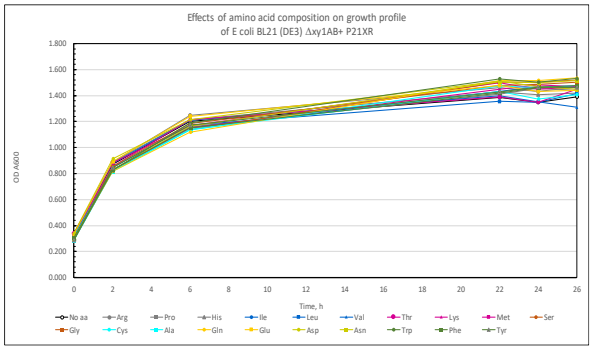

Figure 2 Xylitol production rate varies according to amino acid supplementation.

In **Table 1,** the biomass production rate and xylitol production resulted by different amino acid are tabulated. With supplementation of serine, the highest xylitol yield (0.262 g xylitol/g xylose) were obtained while the lowest yield (0.063 g xylitol/g xylose) was obtained with supplementation of tryptophan lower than the yield without amino acid (0.179 g xylitol/g xylose). In general, there was an increase in xylitol production with additional supplementation of 20 types of amino acid individually, with serine, aspartic acid and glutamic acid supported xylitol production while tryptophan suppressed the production.

Accordingly, serine, aspartic acid, and glutamic acid act as initial precursors to produce xylitol while tryptophan will provide metabolic competition in the recombinant *E. coli* system. For xylitol production, xylose must first be converted to xylulose, which is then reduced to xylitol. Serine and glutamic acid can be metabolized to form intermediates which are subsequently changed into xylulose by a sequence of enzyme processes in the xylitol synthesis pathway, while aspartic acid aid in the production of the necessary xylitol biosynthesis intermediates by acting as an additional carbon source (Hedblom & Adler, 1983). On the other hand, tryptophan competes with other carbon sources like glucose or xylose for uptake and utilization. It is possible for those substrates to be used and the yield of xylitol to decrease if tryptophan is preferentially metabolized or used as a carbon source over those needed for xylitol synthesis (Mesibov & Adler, 1972). Therefore, by adding another precursor to the xylitol synthesis pathway such as increasing serine, aspartic acid and glutamic acid availability in this situation may possibly increase xylitol yield by supplying more building blocks for the xylitol synthesis pathway.

Table 1 Xylitol yield obtained when different amino acid supplemented by *E. coli* strain BL21(DE3) Δxy1AB +P21XR at 150 rpm, 37°C with 20 g/L of xylose at 26 h of fermentation.

Amino acid	Biomass production (OD)	Xylitol (g/L)
Threonine	1.464	4.54
Glutamic Acid	1.441	4.97
Lysine	1.471	3.59
Histidine	1.420	3.51
Tyrosine	1.460	2.55
Isoleucine	1.468	3.59
Valine	1.310	4.45
Proline	1.442	2.80
Tryptophan	1.532	1.25
Glutamine	1.536	2.76
Serine	1.518	5.24
Aspartic Acid	1.448	5.22
Asparagine	1.514	3.51
Glycine	1.504	2.40
Alanine	1.477	3.61
Phenylalanine	1.476	3.87
Arginine	1.466	3.99
Methionine	1.448	4.47
Leucine	1.414	4.81
Cysteine	1.408	4.42
No amino acid	1.388	3.58

From the results, it showed that the key enzyme for xylitol production in this recombinant system is XR which utilize NAD(P)H to reduce D-xylose to xylitol. The cultures were improved approximately 50% compared to when no amino acid was supplied. This shows that when certain amino acids were present, these amino acids might compensate the loss of NAD(P)H from de novo biosynthesis. On the other hand, it also indicates that an improper redox cofactor level or balance occurred when amino acid was absent (Rodionova *et al.*, 2014).

This correlates with findings by Nidetzky et al. (2003) that claimed substrate-saturated reaction conditions is dependent on the dissociation of NAD(P)H which is a rate-limiting step. Kim et al. (2019) also mentioned that the NAD(P)H cause enzymes to suffer from product inhibition in a competitive manner, revealing a rate-limiting step after the binding of NAD(P)H. Therefore, NAD(P)H product inhibition must be overcome for rare sugar production.

Another interesting claim is that direct conversion of xylose to xylitol in single step caused overexpressing of endogenous XR, thus improving NAD(P)H recycle in recombinant *E. coli*. Adequate NAD(P)H recycle along with the supplementation of amino acids individually resulted in lowering the rate of biochemical synthesis due to the

presence of external amino acids in the media. Additionally, the metabolic flux and demand for NADPH may be affected. The consumption of NADPH by amino acid metabolism makes amino acid replenishment potentially less necessary for cellular NADPH production. The rate of NADPH-dependent biosynthetic reactions may be impacted by this, which would diminish the rate of biochemical synthesis as a whole. It may be not as essential for cells to synthesize specific amino acids when there is an abundance of those amino acids available. The metabolic pathways in charge of amino acid biosynthesis may consequently be downregulated, which would result in a slower rate of biochemical synthesis (Chin & Cirino, 2011). When external amino acids were present, recombinant *E. coli* might able to reduce the amount of NAD(P)H required for biochemical synthesis and the NAD(P)H can be directed for maximum xylitol production and restore growth rate.

Rare Codon Analysis

Databases obtained from computational analysis of the available *E. coli* genome and protein structure revealed that high-frequency-usage codons are always associated with structural elements such as α–helices, while clusters of lower frequency usage codons are more likely to be associated with β-sheets, coils, and disordered regions, as mentioned by Pellizza *et al*. (2018).

In this context, codon-optimized gene of XR *N. crassa* is taken and integrated into the genome of recombinant *E. coli* BL21. Codon usage data for codon-optimized gene of XR *N. crassa* extraction from kazUSA revealed that out of the 8 rare codons (AGG, AGA, AUA, CUA, CGA, CGG, CCC, UCG) CCC for Proline is highly expressed with sets of codons (**Figure 3).**

Neurospora crassa "xylose reductase" 2 CDS's (819 codons)

fields: [triplet] [frequency: **per thousand**] ([number])

UUU	6.1(	5)	UCU	13.4(	11)	UAU	4.9(	4)	UGU	6.1(	5)
UUC	29.3(	24)	UCC	23.2(	19)	UAC	28.1(	23)	UGC	7.3(	6)
UUA	2.4(	2)	UCA	6.1(	5)	UAA	0.0(	0)	UGA	1.2(	1)
UUG	9.8(	8)	UCG	7.3(	6)	UAG	1.2(	1)	UGG	22.0(	18)
CUU	11.0(	9)	CCU	13.4(	11)	CAU	3.7(	3)	CGU	9.8(	8)
CUC	40.3(	33)	CCC	26.9(	22)	CAC	15.9(	13)	CGC	33.0(	27)
CUA	6.1(	5)	CCA	9.8(	8)	CAA	17.1(	14)	CGA	4.9(	4)
CUG	19.5(	16)	CCG	8.5(	7)	CAG	26.9(	22)	CGG	4.9(	4)
AUU	12.2(	10)	ACU	7.3(	6)	AAU	1.2(	1)	AGU	4.9(	4)
AUC	31.7(	26)	ACC	33.0(	27)	AAC	36.6(	30)	AGC	12.2(	10)
AUA	6.1(	5)	ACA	12.2(	10)	AAA	7.3(	6)	AGA	3.7(	3)
AUG	17.1(	14)	ACG	13.4(	11)	AAG	39.1(	32)	AGG	12.2(	10)
GUU	3.7(	3)	GCU	15.9(	13)	GAU	22.0(	18)	GGU	13.4(	11)
GUC	28.1(	23)	GCC	47.6(	39)	GAC	29.3(	24)	GGC	33.0(	27)
GUA	4.9(	4)	GCA	4.9(	4)	GAA	15.9(	13)	GGA	8.5(	7)
GUG	17.1(	14)	GCG	24.4(	20)	GAG	47.6(	39)	GGG	13.4(	11)

Coding GC 59.30% 1st letter GC 58.12% 2nd letter GC 45.79% 3rd letter GC 73.99%

Figure 3 *N. crassa* codon usage data extracted from kazUSA

In *E. coli* BL21 (DE3), there are eight common rare codons (AGG, AGA, AUA, CUA, CGA, CGG, CCC and UCG) based on codon usage data extracted from kazUSA and all 8 of these rare codons stated are being expressed only that the expression level is lower.

As previously suggested, since codon bias acts on the translation rate hence it is a pertinent prior analysis for production of target proteins. Proline exhibited high expression as RC and when there is external source of proline being supplied to the amino acid flux, the rate of proline synthesis starts to slow down and NAD(P)H was targeted to xylitol production. However xylitol production from medium supplemented with proline is lower than medium without amino acid. According to Rosano and Ceccarelli (2009), if a host contain foreign mRNA carrying rare codons it will cause depletion of low abundance tRNAs to occur and eventually affects the heterologous protein expression levels and quality by mis incorporation of amino acid and/or polypeptide truncation.

Arginine and serine are rare codons of *E. coli* and supplementing arginine and serine amino acids may aid the growth as both amino acids were expressed lower in cell. Amino acid serine contributes to the highest xylitol production from the 20 amino acid supplied. It is suspected due to deamination by serine which easily converted into pyruvate compared to glucose to enter the Krebs cycle. Traxler *et al.* (2008) stating that primary carbon sources consisted of mainly carbohydrates was utilized strongly at three times points of growth arrest and only amino acid serine is included in this group. Moreover, serine is known as precursor to other amino acids including glycine which makes it an important participant in the biosynthesis of purines and pyrimidines and both glycine and serine are the cheapest amino acids since their biosynthesis is ATP neutral (Traxler *et al.*, 2008).

Just like serine, aspartic acid is a precursor to several amino acids including methionine, threonine, isoleucine and lysine and a hydrogen acceptor in ATP synthase chain. This give idea that these amino acids are used for other purposes such as energy especially in times of carbon starvation as they are employed in larger quantities by cells more than it need for protein synthesis.

While amino acids that shown to have negative effects on the xylitol production rate are tryptophan, glycine, tyrosine and proline which exhibited high expression although proline is categorized as RC in *E. coli* BL21 (DE3). In addition, amino acids synthesis is related to codon translations. The results obtained from effect of individually amino acid supplemented on xylitol production rate is as follows;

Current: serine > aspartic acid > glutamic acid > leucine > methionine > threonine > valine > cysteine > arginine > phenylalanine > alanine > lysine, isoleucine > asparagine, histidine > proline > glutamine > tyrosine > glycine > tryptophan

Another aspect that can affect the accumulation of NAD(P)H is the carbon source used. In the present study, glycerol was used as a main carbon source (originally glucose) with 5052 designed medium (per 1 L: 0.5 g glucose, 5 g glycerol; 2 g lactose). Interestingly, it is a substrate that is reported able to escalate NAD(P)H supply [23]. Glycerol is also able to enter membranes of *E. coli* through direct penetration due to its lipid soluble characteristic and the metabolization occurs in the absence of electron acceptors (Murarka et al. 2008). A 20% amino acids present in biomass were reported by Murarka et al. (2008) originated from glycerol hence confirming that glycerol is able to increase cell mass production.

CONCLUSION

In this study, the supplementation of individual amino acids proven to improve the xylitol production by 50% from xylose through the optimization on medium conditions of recombinant *E. coli* system. Glycerol also contributed to the enhanced yield of biomass when being used as a carbon source. It is possible to increase the production of xylitol by

using the study's findings in practice. As xylitol is recognized to be low in calories and to have more health benefits than table sugar, it may be utilized to replace table sugar as an alternative sweetener in the food manufacturing industry.

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