Madras Agric. J., 2019; doi:10.29321/MAJ 2019.000247

RESEARCH ARTICLE

Xylitol Production by Xylose Reductase over producing Recombinant *Escherichia coli* M15

Manikandan Ariyan[1] and Sivakumar Uthandi[1*]
[1]Biocatalysts Lab, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore - 641003

Received : 29th May, 2019
Revised : 3rd June, 2019
Accepted : 4th June, 2019

ABSTRACT

Xylitol is a functional sugar alcohol widely used as a sugar substitute. Chemical route of xylitol production is highly expensive, whereas microbial sources offer lower yield and conversion efficiency. In the present investigation, xylose reductase (XR) gene from Candida tropicalis GRA1 was cloned in pQE30Xa vector and transformed into *E. coli M15*. The resultant recombinant strain produced xylitol from xylose that harboured Xr gene transcribed under the control of lac operon. Such transcribed Xr protein converts xylose into xylitol using NADPH as a co-factor. Xylitol production was achieved by the recombinant *E. coli M15* pQE30XrCt under shake-flask fermentation (2.1 $g.l^{-1}$ in 24 h) with xylose as the sole substrate in the medium. Addition of co-substrate, glucose in the growth medium enhanced the xylitol production to the tune of 3.4$g.l^{-1}$. Furthermore, the addition of co-substrate glycerol along with xylose enhanced a xylitol yield of 6.4$g.l^{-1}$

Keywords: *Candida tropicalis, Xylose Reductase, E. coli M15, Xylose, Xylitol.*

INTRODUCTION

Xylitol, a crystalline pentose sugar alcohol (5 Carbon), renders sweetness same as glucose and is widely substituted for 6C sugar, especially sucrose and glucose. Xylitol is used in food products to improve shelf life, taste and color (Oliger, 2001). The most attractive characteristic feature of xylitol is in the field of medicine for its nature of insulin-independent digestion with low calorific value (Ur-Rehman *et al*., 2013). Xylitol has the potential to prevent many bone-related diseases like osteoporosis. It helps in oral health maintenance by a daily intake of 6.5 g xylitol for six months and makes plaque bacteria undetectable in saliva, thus eliminating the oral cavity (Mäkinen, 2000). Xylitol has the capacity to reduce the initial infectious stages of cystic fibrosis and it is able to decrease the pathogenicity of *Streptococcus pneumonia* and the mutant *Streptococci* (Tapiainen *et al*., 2004)

Xylitol occurs naturally in some fruits (yellow plum, strawberry, raspberry, and banana) and vegetables (cauliflower and lettuce) in very low quantity (Da Silva and Chandel, 2012; Emodi, 1978). Xylitol is produced commercially by the chemical method by hydrogenating xylose using metal catalysts at high temperature ranging from 353-413 K at 5MPa. In addition to the difficult separation and purification steps, xylitol production by chemical reduction has other drawbacks such as requirements of high pressure (up to 50 atm) and temperature (80–140^{0}C), use of an expensive catalyst, etc. Commercial extraction of xylitol faces hindrances in its production due to its uneconomical quantity. Therefore, it has become necessary and worthwhile to explore methods for effective production of xylitol using microorganisms (Saha *et al*., 2003).

Among the beneficial microbes, yeast is capable of fermenting xylose and glucose to produce ethanol. Yeasts typically convert xylose to xylitol in the presence of Xylose Reductase (*XR*) using NADPH as a co-factor and this xylitol is dehydrogenated into xylulose using the enzyme Xylose Dehydrogenase (*XDH*), which also uses NADPH as co-factor. Later, xylulose is converted into Xylose-5-phosphate which enters into the pentose phosphate pathway. Nowadays, improvement of xylitol production using *XR* gene with recombinant microorganisms has been paid much attention to achieve more production and conversion potential (Kötter *et al*., 1993, Harcus *et al*., 2013). In this context, *E. coli* is considered as an ideal host strain for efficient biotechnological production of various high-value chemical building blocks. Such *E. coli* based expression system is a popular host for heterologous protein expression as it is easy to handle, inexpensive and grows rapidly (Yu *et a*l., 2011). Recombinant *E. coli* strains have been developed to produce xylitol from D-xylose sugars (Zhao *et al*., 2011). In this study, the gene encoding Xylose reductase from *C. tropicalis* GRA1 (GenBank: KY873300.1) was expressed in *E. coli* M15 for the direct synthesis of xylitol from xylose.

*Corresponding author's e-mail: usivakumartnau@gmail.com

MATERIAL AND METHODS

Strains and Vector

Xr gene from *Candida tropicalis* GRA1 was cloned into pQE30Xa expression vector (Qiagen, USA) and transformed into *E. coli* M15 strain (gifted by Dr. Amirul Mallick from Indian Institute of Science Education and Research, Kolkata) for expression and purification of protein (Unpublished work). *E. coli* M15 strain harbouring *XR* gene was used for fermentation studies.

Inoculum preparation

Luria Bertani (LB) broth was used for the inoculum preparation. A single colony of *E. coli M15 pQE30XrCt* from fresh LB plates was inoculated into 5 ml LB broth, and grown overnight at 37^{0}C. The overnight grown fresh culture was used for further fermentation.

Fermentation

Minimal medium (pH7) that Contained: 3.5 g KH_2PO_4; 5.0 g K_2HPO_4; 3.5 g (NH_4) H_2PO_4, 0.25 g $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, and 1 ml.l^{-1} of trace metal stock per litre was prepared and sterilized at 121^{o}C and 15psi.The trace metal stock was prepared as described by Causey *et al*. (2003). For fermentation, xylose (100mM), glucose (50mM) and glycerol were supplemented with 100 µg.ml^{-1} ampicillin and 25 µg.ml^{-1} kanamycin. The culture was maintained at 30^{0}C with the shaking condition (200 rpm). Isopropyl-β-D-thiogalactoside (IPTG) was used for protein induction.

Xylitol Production

Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml minimal medium with 25 µg.ml^{-1} kanamycin and 100 µg.ml^{-1} ampicillin. The overnight grown culture was inoculated at 2% in minimal medium and incubated at 37^{0}C. The optical density of culture was measured periodically at OD_{600} in Micro plate reader (SpectraMax i3X, USA). After attaining the OD_{600} at 0.6, IPTG was added at 1mM final concentration, xylose, glucose and glycerol were amended in three different combinations *viz.,* xylose alone (100 mM), xylose (100 mM) with glucose (50 mM) and xylose (100mM) with glycerol 15ml.l^{-1} individually. The inoculated culture broth was incubated at 30^{0}C and samples withdrawn periodically were centrifuged. The filtered supernatant served as samples for HPLC analysis. Further, cell density and xylitol production were also measured periodically.

Quantification of xylitol

Quantification of xylitol was carried out in HPLC using Agilent system equipped with Agilent technologies 1260 infinity ELSD (Palo Alto, USA). All samples and standards were filtered through 0.25 µm (Millipore) filters. The separation was achieved on Shimadzu-NH_2 column (250mm×4.6mm *i.e.,* 5µm particle size). The mobile phase consists of acetonitrile: water (80:20, v/v) in isocratic elution with a flow rate of 1.5 ml.min^{-1}. The column temperature was maintained at 20^{0}C. The detection was carried out by using evaporative light scattering detection (ELSD) technique. The evaporative temperature and nebulizing temperature were set at 95^{0}C and 80^{0}C, N_2 gas flow rate was 1.60 Standard Litre per Minute (SLM).

RESULTS AND DISCUSSION

Xylose uptake metabolism

Plant biomass and agro-residues rich in sugars including xylose could be selectively reduced to xylitol using an oxidoreductase system, xylose reductase at an optimal level. Engineering xylose reductase and using an over expressed *Xr* could be an effective way to convert the xylose component of biomass into xylitol, sugar alcohol, which are useful in food and pharma industries. Of the available sugars (5 carbon and 6 carbon) in plant biomass, the uptake and transport of sugars vary. Mechanism of xylose (5C) and glucose (6C) uptake (Fig.1) shows xylose uptake occurred primarily through a high-affinity ABC transporter (*XylFGH*) or a low-affinity proton symporter (*XylE*) (Fraenkel and Neidhardt, 1996), although *XylE* has little activity even under high-xylose concentrations (50 mM) (Hasona *et al*., 2004).

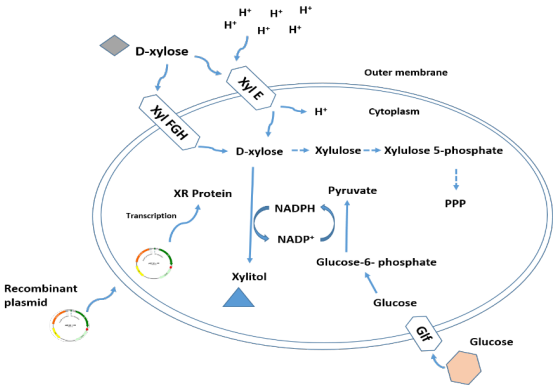

Figure 1. Mechanism of xylose and glucose uptake by recombinant *E. coli* M15 and xylose metabolisms of native yeast and xylitol production

In general, *E. coli* exhibits diauxic growth such that glucose is preferentially assimilated before xylose (Hasona *et al*., 2004; Hernandez-Montalvo *et al*., 2001). Most of the *E. coli* strains (including M15) do not naturally synthesize or metabolize xylitol, although mutants have been isolated which are capable of converting xylitol to D-xylose using an enzyme oxidoreductases (Wu, 1976). Therefore, xylitol production is possible by expression of *Xr* by direct reduction (Fig. 1).

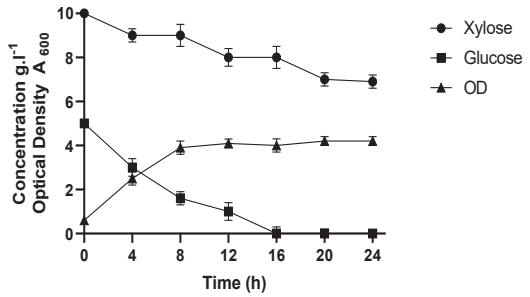

Figure 2. *E. coli* M15 (wild type) grown in minimal medium supplemented with xylose, glucose respectively and culture samples were withdrawn periodically (up to 24 h) for measurement of growth at A600nm, sugar consumption and xylitol production.

Xylitol production under shake flask condition

In the present investigation, xylitol production by the wild *E. coli* M15 in minimal medium (with glucose and xylose) showed an initial uptake of glucose alone on high xylose concentration, thereafter efficient consumption of xylose was observed in the diauxic growth pattern of *E. coli* (fig 2). In addition, the strains tested for xylitol production by neither wild

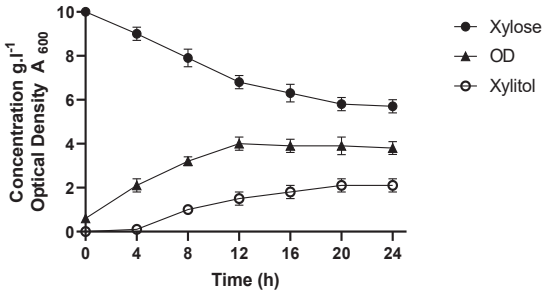

Figure 3. *E. coli M15 pQE30CtXr* grown in minimal medium supplemented with xylose and culture samples were withdrawn periodically (up to 24 h) for measurement of growth at A600nm, sugar consumption and xylitol production.

strain nor IPTG un-induced recombinant *E. coli* M15 pQE30*CtXr* showed the production of xylitol suggesting that xylitol production occurred only in the presence of XR protein.

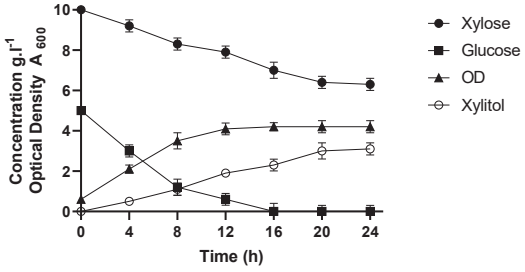

Figure 4. *E. coli M15 pQE30CtXr* grown in minimal medium supplemented with xylose, glucose respectively and culture samples were withdrawn periodically (up to 24 h) for measurement of growth at A600nm, sugar consumption and xylitol production.

Heterologously over expressed *CtXr* gene synthesis xylose reductase, which in turn converts xylose to xylitol in an NADPH dependent reaction (Verho *et al.,* 2005). The NADPH/ NADP+ cofactor plays a major role in the reductive pathway of xylitol production by recombinant organisms, which oxidize NADPH to NAPD+. Similarly, Suzuki *et al.* (1999) were successful in the production of xylitol from xylose using whole cell biocatalyst, recombinant *E. coli* JM109.

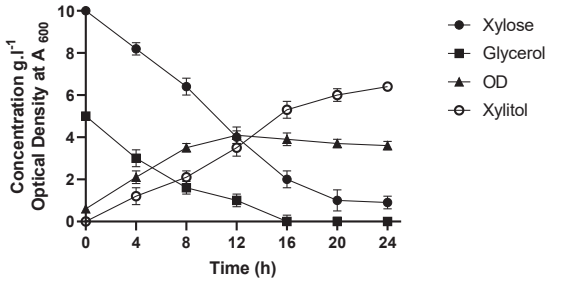

Fig 5. *E. coli M15 pQE30CtXr* grown in minimal medium supplemented with xylose, glycerol respectively and culture samples were withdrawn periodically (up to 24 h) for measurement of growth at A600nm, sugar consumption and xylitol production.

In the present study, xylitol production was evaluated under three different combinations. Of which, the first combination included xylose in the minimal medium revealed lower xylitol yield compared to xylose supplemented with co-substrates.. This might be due to the high enzyme concentration and insufficient cofactor regeneration in the cells. Therefore, external and genetic means of increasing the availability of cofactors has been developed in many whole-cell biocatalysts (Zhao *et al*., 2003).

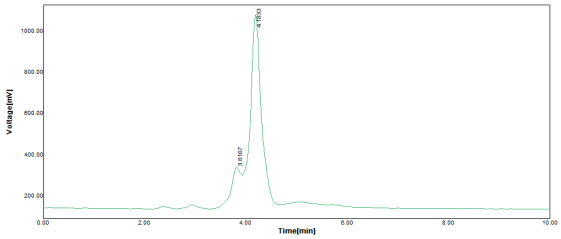

Figure 6. HPLC chromatogram of 24 h old culture grown in xylose and glycerol mixture. Peaks corresponding to xylose and xylitol were observed in 3.8 and 4.1 min. retention time respectively

Minimal medium supplemented with xylose induced Lac promoter to synthesize XR protein in the *E. coli* cytoplasm upon IPTG induction. Furthermore, xylitol production measured periodically by HPLC resulted in a minimum quantity of 1.5g.l^{-1} at 12 h of fermentation by utilizing very low xylose. Further,

extending the fermentation up to 20 h, slightly improved the rate of xylose utilization. On the contrary, the conversion rate of xylitol was relatively low (2.1 $g.l^{-1}$) Fig.3.

In the second combination (xylose and glucose), initial uptake of xylose was lower compared to glucose, subsequently, after 24 h of fermentation, glucose was completely utilized, leaving xylose unutilized in the medium by the *E. coli* M15 pQE30*CtXr* (Fig.4) registering a xylitol yield of 3.4 $g.l^{-1}$. This can be explained by the presence of glucose *ptsG* gene, which regulates glucose uptake and phosphorylation, thereby results in a reduction of xylose consumption (Gosset, 2005).

On the other hand, the third combination of xylose amended with co-substrate glycerol, significantly increased the consumption of xylose, resulting in higher levels of xylitol. After 24h of fermentation, production of xylitol was 6.4 $g.l^{-1}$. Glycerol incorporation as co-substrate in the growth medium is hypothesized to enhance the conversion of xylose to xylitol by maintaining redox balance by regulation of glycerol metabolisms; thereby the entire reaction of the XR pathway is shifted towards xylitol synthesis. The total xylose and glycerol present in the spent fermentation broth were completely utilized by the metabolically recombinant *E. coli* M15 pQE30CtXr (Fig.5). This might be due to internal redox balance in which the cells must recycle NADPH generated through glycerol catabolism suggesting efficient utilization of xylose in the presence of glycerol.

CONCLUSION

Cost-effective, continuous delivery of reduced cofactors remains an important challenge in biocatalysts. While the use of whole-cells can have many advantages, the efficiency with glucose or other sugars are utilized as ''co-substrates'' can be improved considerably. Maximum xylitol production of 6.1 $g.l^{-1}$ was achieved with an addition of glycerol as a co-substrate. Metabolically engineering xylose pathway for enhanced xylose uptake would pave the way for utilization cheaply available substrates such as corncob, crop residues and wood Hydrolysate for efficient conversion of xylose into xylitol.

ACKNOWLEDGEMENT

This work was carried out with financial assistance from University Core Project (B27NV-CP016) and previous financial assistance to SU through Indo-Russia joint collaboration supported by DBT, GoI, New Delhi (No.DBT/IC2/Indo-Russia/2014-16/04). The authors are grateful to the instrumentation facility extended by the metabolomics and proteomics analytical facility, Dept. of AgrI. Microbiology, TNAU, established under GoI-MHRD (FAST)-Microbes to feed the world (Grant F.No 5-5/2014-75-VII dt.04.09.2014). I also gratefully thank Dr. Amirul Mallick (Indian Institute of Science Education and Research, Kolkata) who spared *E. coli* M15.

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