Journal of Industrial Microbiology, 4 (1989) 1–5
Elsevier

SIM 00150

Thermal inducible expression of xylose isomerase and its performance in a hollow fiber bioreactor

Dominic W.S. Wong, Linda N.H. Yee and Carl A. Batt

Department of Food Science, Cornell University, Ithaca, NY, U.S.A.

Received 24 November 1987
Revised 3 June 1988
Accepted 9 June 1988

Key words: Xylose isomerase; Enzyme expression, thermally inducible; Hollow fiber bioreactor; *Escherichia coli*

SUMMARY

The *Escherichia coli* xylose isomerase (EC 5.3.1.5) has been expressed under the control of a thermal inverting promoter system (att-nutL-p-att-N block) and its performance in a hollow fiber bioreactor measured. The conversion of xylose to xylulose was inversely proportional to the flow rate and the system operated up to 60°C. The maximum conversion efficiency observed was 19.05% at 55°C.

INTRODUCTION

Xylose isomerase catalyzes the isomerization of xylose to xylulose as well as glucose to fructose. The latter reaction is important for the production of high-fructose corn syrup, making 'glucose isomerase' one of the most widely used industrial enzymes [7]. The conversion of xylose, a pentose sugar abundant in hemicellulosic biomass, is also important since most yeast (e.g. *Saccharomyces cerevisiae*) used for the production of ethanol cannot ferment xylose. *S. cerevisiae* cannot utilize xylose due to its inability to recycle $NADH^+$, accumulating from xylitol oxidation [2,6]. It has been demonstrated that xylose isomerized to xylulose can then be readily fermented by *S. cerevisiae* to ethanol [8,9,12,15]. Numerous attempts to express an active xylose isomerase in *S. cerevisiae* have not been successful (Picataggio, Batt and Sinskey, unpublished results). As an alternative to the direct fermentation of xylose by *S. cerevisiae,* a two-stage system has been developed consisting of an *Escherichia coli* strain which overproduces xylose isomerase to produce a xylose-xylulose isomerate [4] and feeding the isomerate to *S. cerevisiae* (Yee et al., manuscript in preparation). Other laboratories have developed similar strategies to overexpress xylose isomerase in *E. coli* [13,19,20]. This report details the construction of a thermal inducible system for the production of xylose isomerase and its performance in a hollow fiber bioreactor. The thermal inducible system consists

Correspondence: C.A. Batt, Department of Food Science, Cornell University, Ithaca, NY 14853, U.S.A.

of a *lac* promoter carried on an att-nutL-p-att-N gene block which inverts upon heat pulse at 42°C [16]. The *lac* promoter is initially oriented away from the targeted gene. Upon heat induction, the att-nutL-p-att-N block inverts, orienting the promoter toward the targeted gene, resulting in its expression.

A variety of strategies have been developed for the immobilization of whole cells or purified enzymes which carry out xylose or glucose isomerizations [1,7,10]. These include, for purified enzyme, adsorption onto DEAE-cellulose, entrapment in polyacrylamide and porous glass particles. For whole cells, flocculation with magnesium chloride or diatomaceous earth, and glutaraldehyde crosslinking, among other methods, have been reported [7]. We chose to use a hollow fiber bioreactor for carrying out the isomerization, since it provides a simple method for entrapping whole cells. A major advantage of hollow fiber bioreactors is that the cells are not exposed to any harsh chemical treatment and/or shear during immobilization. Recently, Chung et al. [10] have reported the use of a dual hollow fiber reactor for immobilization of *Streptomyces griseus* for glucose isomerization.

MATERIAL AND METHODS

Bacterial strains and plasmids

E. coli JM105 carrying pKKX.7 has been described previously [4]. It carries the *xylA* gene under the expression of the *tac* promoter. *E. coli* D1210 (*λxis*$^-$, *cI857*) carrying pNH7a was kindly provided by W. Szybalski (University of Wisconsin, Madison, WI) and has been described previously [16].

DNA manipulations

All restriction enzymes and other DNA-modifying enzymes were used according to the directions supplied by the manufacturers. *E. coli* D1210 was transformed as described by Maniatis et al. [14] except that the transformants were plated out and grown at 30°C. Plasmid DNA was isolated by the alkaline lysis method described by Silhavy et al. [18].

Hollow fiber reactor

A MINIKROS KF-200-010-2 hollow fiber reactor was graciously provided by Microgon Inc. (Laguna Hills, CA). Its fibers have a pore size of 0.2 μ, with a shell volume of 27 ml and a total volume of 47 ml.

Enzyme and metabolite assays

Xylose isomerase was assayed using toluenized cells as described previously [4]. Xylose and xylulose concentrations were determined by HPLC using a Bio-Rad HP87C column with water as the mobile phase and the sugars were detected using a refractive index detector.

RESULTS AND DISCUSSION

Construction of the thermal inducible xylose isomerase gene

The plasmid pKKX.7 is a pBR322 derivative which carries the *xylA* gene under the control of the *tac* promoter [4]. It produces up to 28% of the cell protein as xylose isomerase. It does, however, require the addition of isopropyl-β-D-galactopyranoside (IPTG) for induction. (Note: The *lac* promoter from pNH7a has a functional *lac*O which can be used to regulate the *lac* promoter. In *lac*I$^-$ strains, the *lac* promoter is constitutive and therefore the expression of the target gene can be regulated by the inversion.) To obviate the need for this inducer a promoter system consisting of the att-nutL-p-att-N gene block was removed on a 1.5 kb *Eco*RI fragment (a partial digest was performed on pNH7a and a fragment whose internal *Eco*RI site was not cut was extracted from an agarose gel). This 1.5 kb *Eco*RI fragment was inserted into the unique *Eco*RI site in the polylinker between the *xylA* gene and the *tac* promoter of pKKX.7. The resulting construct (pKKXNH.6a) thus contained two promoters, the *lac* promoter from the att-nutL-p-att-N gene block (selected in the opposite orientation) and the *tac* promoter in the correct orientation with respect to the *xylA* gene. Unfortunately, this construct displayed a high level of xylose isomerase activity before induction, presumably due to readthrough

from the *tac* promoter (data not shown). Restriction analysis of the plasmid DNA after heat shock revealed that only 20% of the plasmid population had the att-nutL-p-att-N gene block inverted in the correct orientation with respect to the *xylA* gene. Extending the length of heat shock from 15 min to 30 min did not result in an increased inversion frequency. Podhajska et al. [16] reported greater than 80% inversion of the att-nutL-p-att-N gene block in pNH7a. The lower inversion frequency observed for our construct may be due to the high level of transcription from the *tac* promoter which interferes directly with the expression of one or more genes on the att-nutL-p-att-N block required for inversion.

The upstream *tac* promoter was removed by partial *Sal*I digestion and religation. The resulting construct containing only the att-nutL-p-att-N gene block and the *xylA* gene is designated pKKXNH7.a (Fig. 1). Deletion of the upstream *tac* promoter increased the inversion frequency upon heat induction from approximately 20% to 70%. The xylose isomerase activity detected in uninduced *E. coli* D1210 [pKKXNH7.a] was 1066 units and probably results from a small fraction of the plasmid DNA which has inverted at the lower temperature. After

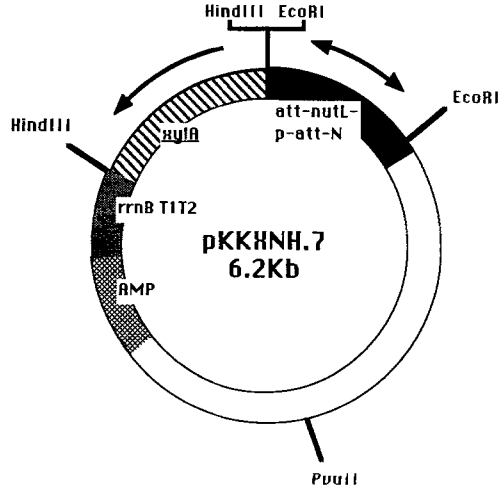

Fig. 1. Diagram of pKKXNH.7. The att-nutL-p-att-N block was removed as a 1.5 kb *Eco*RI fragment and inserted into the unique *Eco*RI site upstream of the *xylA* gene in pKKX.7 [4]. The *tac* promoter upstream was removed on a 1 kb *Sal*I fragment resulting in the final construct shown.

heat shock at 42°C, the xylose isomerase activity was 2000 units. The 70% inversion frequency observed for pKKXNH7.a compares favorably with the results reported for pNH7a [16].

Performance of E. coli D1210 carrying pKKXNH7.a in a hollow fiber reactor unit

A 3 liter culture of *E. coli* D1210 [pKKXNH7.a] was grown at 30°C to an OD_{600} of 0.35, heat-shocked at 42°C for 15 min and grown for an additional 2 h at 30°C. The cells were harvested by centrifugation, resuspended in LB and packed into a MINIKROS hollow fiber reactor. The isomerization mixture consisting of 2% (w/v) xylose, 50 mM TRIS (pH 7.5) and 20 mM $MnCl_2$ was pumped through the hollow fiber reactor at a flow rate of 0.3 ml/min and the system was tested at various operating temperatures. Samples of the effluent were analyzed for xylose and xylulose concentrations by HPLC. The effect of temperature on the isomerization efficiency is presented in Table 1. Previously, we obtained a maximum conversion efficiency at 37°C of 4% with an initial starting concentration of 10% xylose using alginate encapsulated cells [4]. In this previous study, the *E. coli* strain carrying the *xylA* gene expressed by the *tac* promoter was used.

The highest level of isomerization (19%) was obtained at 55°C and the activity decreased dramatically as the temperature was increased above 60°C. These results agree with our previous determination

Table 1

Effect of temperature on the efficiency of xylose isomerization at a flow rate of 0.3 ml/min

Temperature	Isomerization[a,b]
35	6.39
40	9.94
45	14.41
50	18.69
55	19.05
60	18.40
70	6.53

[a] Xylulose formed as a percentage from xylose (initially 2%).

[b] $\frac{\text{xylulose}}{\text{xylulose + xylose}} \times 100$

of the temperature optimum for the *E. coli* xylose isomerase in permeabilized whole cells [4]. The level of conversion is probably limited by the equilibrium between xylose and xylulose which favors the xylose [9,12]. The effect of flow rate on the efficiency of isomerization was also measured for the hollow fiber reactor unit containing *E. coli* D1210 [pKKXNH7.a] (Table 2). As expected, the efficiency of isomerization decreased as the flow rate was increased. In the configuration used, the substrate is fed through the fibers. The substrate must therefore diffuse into the shell side to be isomerized by the entrapped cells and then the product diffuses back into the fibers to exit the reactor. As the flow rate is increased, the residence time for diffusion in and out of the shell side is reduced, therefore limiting the conversion rate.

The hollow fiber reactor unit containing *E. coli* D1210[pKKXNH7.a] efficiently isomerizes xylose to xylulose. We have used a similar system employing *E. coli* JM105[pKKX.7] (the *tac-xylA* initially constructed) and have demonstrated that the isomerate can be fed directly in a continuous culture of *S. cerevisiae* (Yee and Batt, unpublished data). It would appear that this two-stage system is useful for the conversion of xylose into yeast biomass and/or ethanol.

The thermal inducible system described here has certain advantages over the λ P_L promotor [13]. Most notable is that the att-nutL-p-att-N gene block only requires a brief heat shock for inversion. This could be accomplished by passage through a heat exchanger followed by a return to 30°C for enzyme production.

Table 2

Effect of flow rate through the hollow fiber reactor on the isomerization of xylose to xylulose at 35°C

Flow rate (ml/min)	Xylulose formed (%)[a]
0.2	8.0
0.3	6.4
0.5	5.0

[a] $\frac{\text{xylulose}}{\text{xylose + xylulose}} \times 100$

ACKNOWLEDGEMENTS

This work was supported by the Strauss-Hanauer Foundation. The authors would like to thank Prof. Jean Hunter for her contribution to the development of the hollow fiber reactor.

REFERENCES

1 Antrim, R.L. and A.L. Auterinen. 1986. A new regenerable immobilized glucose isomerase. Starch/Starke 38: 132–137.
2 Batt, C.A., S. Carvallo, D.D. Easson, M. Akedo and A.J. Sinskey. 1985. Evidence for a xylose metabolic pathway in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 28: 549–553.
3 Batt, C.A., M.C. Claps, M.S. Bodis, S. Jamas and A.J. Sinskey. 1985. Analysis of xylose operon regulation by Mud-lac fusion: trans effect of plasmid coded xylose operon. Can. J. Microbiol. 31: 930–933.
4 Batt, C.A., S.R. Novak, E.O. O'Neill, J. Ko and A.J. Sinskey. 1986. Hyperexpression of *Escherichia coli* xylose isomerase. Biotechnol. Prog. 2: 140–144.
5 Blanch, H.W., T.B. VickRoy and C. Wilkes. 1984. Growth of procaryotic cells in hollo-fiber reactors. Ann. N.Y. Acad. Sci. 434: 373–381.
6 Bruinenberg, P., P. deBot, J. vanDijken and W. Scheffers. 1983. NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. Appl. Microbiol. Biotechnol. 19: 256–260.
7 Chen, W.P. 1980. Glucose isomerase. A review. Proc. Biochem. 15: 30–41.
8 Chiang, L., M. Flickinger, L. Chen and G. Tsao. 1982. Ethanol production from pentoses by immobilized microorganisms. Enzyme Microbial Technol. 4: 93–95.
9 Chiang, L., H. Hsiao, P. Ueng and G. Tsao. 1981. Enzymatic and microbial preparation of d-xylulose from d-xylose. Appl. Environ. Microbiol. 42: 66–69.
10 Chung, B.H., H.N. Chang and Y.H. Kho. 1987. Dual hollow fiber membrane bioreactor for whole cell enzyme immobilization of *Streptomyces griseus* with glucose isomerase activity. J. Ferment. Technol. 65: 575–581.
11 Gong, C., L. Chen, M. Flickinger, L. Chiang and G. Tsao. 1981. Production of ethanol from D-xylose by using D-xylose isomerase and yeast. Appl. Environ. Microbiol. 41: 430–436.
12 Hahn-Hagerdal, B., S. Berner and K. Skoog. 1986. Improved ethanol production from xylose with glucose isomerase and *Saccharomyces cerevisiae* using the respiratory inhibitor azide. Appl. Microbiol. Biotechnol. 24: 287–293.
13 Lastick, S., M. Tucker, V. Mackedonski and K. Grohmann. 1986. Overproduction of *Escherichia coli* xylose isomerase. Biotechnol. Lett. 8: 1–6.
14 Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular

Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

15 Olivier, S. and P. Toit. 1986. Sugar cane bagasse as a possible source of fermentable carbohydrates. II. Optimization of xylose isomerase as well as sugar cane bagasse hydrolyzate to xylulose in laboratory scale units. Biotechnol. Bioeng. 28: 684–699.

16 Podhajska, A.J., N. Hasan and W. Szybalski. 1985. Control of cloned gene expression by promoter inversion in vivo: construction of the heat-pulse-activated att-nutL-p-att-N module. Gene 40: 163–168.

17 Sarthy, A., B. McConaughy, Z. Lobo, J. Sundstrom, C. Furlong and B. Hall. 1987. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 53: 1996–2000.

18 Silhavy, T.J., M.L. Berman and L.W. Enquist. 1984. Experiments in Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

19 Stevis, P. and N. Ho. 1985. Overproduction of D-xylose isomerase in *Escherichia coli* by cloning the D-xylose isomerase gene. Enzyme Microb. Technol. 7: 592–596.

20 Wovcha, M., D. Steuerwald and K. Brooks. 1983. Amplification of D-xylose and D-glucose isomerase activities in *Escherichia coli* by gene cloning. Appl. Environ. Microbiol. 45: 1402–1404.