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Full Length Article

Lactose Induced Expression of *Thermotoga petrophila* α-amylase Gene Regulated by T7 Promoter in *E. coli* Codon Plus (DE3)

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Abstract

Current work is aimed to evaluate the lactose induced expression of *Thermotoga petrophila* α-amylase gene under T7 promoter in *E. coli*. Recombinant protein was expressed in nine different media (LB, ZB, 4xZB, ZBM, ZYB, 3xZYB, M9, ZYBM9 and 3xZYBM9) by using Isopropyl β-D-1-thiogalactopyranoside (IPTG) and lactose as inducer. Lactose was found to be the better inducer in all the media with maximum expression in tryptone rich ZBM medium. The enzyme expression increased when host cells were given heat shock (42ºC) for 1 h immediately after induction with lactose. The parameters such as lactose concentration (200 mM), cell density at the time of induction (OD_{600} 0.8), and incubation temperature (37ºC) were also optimized, that resulted in 1.5 fold increase in intracellular α-amylase activity. Thus, lactose as inducer is recommended to be used for the enhanced expression of recombinant α-amylase in tryptone rich medium. © 2014 Friends Science Publishers

Keywords: Lactose; pET expression system; Protein expression; Tryptone rich medium; Thermostable α-amylase; *Thermotoga petrophila*; *E. coli*

Introduction

Starch is heterogeneous polysaccharide and pervasive reserve food compound in plants (Elleuche and Antranikian, 2013) and is major food source for human. Starch hydrolyzing enzymes i.e. amylases constitute about 30% of the industrial enzyme market. These are being employed extensively in medicine, analytical chemistry, pharmaceutical, food, paper, textile and distillery industries (Asgher *et al*., 2007; Sarethy *et al.*, 2012; Khemakhem *et al.,* 2013). α-amylase (EC 3.2.1.1) is endoacting enzyme that cleaves α-1,4-glycosidic bonds within starch and related polysaccharides to form maltooligosaccharides and maltodextrin (Li *et al*., 2011; Derde *et al*., 2012; Lakshmi *et al*., 2013; Presecki *et al.* 2013; Puspasari *et al*., 2013; Roy and Mukherjee, 2013).

The quest for thermostable α-amylases with novel properties to accomplish the demand of industrial sector is continuous (Karakas *et al*., 2010; Sidkey *et al*., 2011). Members of genus *Thermotoga* are hyperthermophile and proficient to metabolize variety of simple to complex carbohydrates including starch (Van Ooteghem *et al*., 2002; Blumer-Schuette *et al*., 2008). Hence, this species is excellent source of thermostable enzymes. However, due to stringent growth requirements, these hyperthermophiles are difficult to grow for industrial scale production (Leveque *et al*., 2000). Recombinant DNA technology has excelled by providing methods for cloning of desired genes in to a mesophilic host (Oganesyan *et al*., 2007). However, after gene cloning, the most important requirement is process development for the achievement of high level of recombinant protein expression (Gombert and Kilikian, 1998).

For the proteins expression in *E. coli*, a very strong and effective T7 promoter system has been developed. The isopropyl-β-D-thiogalactopyranoside (IPTG) is commonly used inducer to regulate the expression of target proteins under the influence this promoter in BL21 (DE3) strain. However, IPTG is not only expensive but toxic to host cell leading to urge for alternative inducers (Lin and Hsu, 1997; Killikian, 2000). Lactose, a natural inducer of lac operon, is inexpensive and nontoxic to *E. coli* cells (Yan *et al*., 2004; Studier, 2005; Zhang *et al*., 2009). In addition, within the cell it can also serve as energy as well as carbon source (Killikian, 2000; Howhan and Pornbanlualap, 2003). Hence, it is mandatory to develop the cultivation strategies to attain high cell concentrations and establishment of conditions for adequate induction to maximize the expression (Gombert and Kilikian, 1998).

Very little literature describes the lactose induced expression of the foreign gene regulated by T7 promoter in pET expression system perhaps due to difficulties encountered in establishing the ideal conditions for the induction (Gombert and Kilikian, 1998). We predict that the lactose can be used to replace IPTG for the enhanced expression of heterologous protein in pET expression system if suitable medium and cultural conditions are provided. Therefore, the goal of present work is to evaluate

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lactose as alternative inducer and optimization of cultural conditions for the expression of α-amylase cloned from *Thermotoga petrophila*. While, the expression of the enzyme was analyzed in term of intracellular enzyme activity.

Materials and Methods

Strains and Plasmid

In present work, previously cloned α-amylase gene (1.6 kb) in to pET21a (+) from *T. petrophila* was used. *E. coli* BL21 Codon Plus was used for the expression of protein.

Plasmid Isolation and Transformation

Recombinant pET 21a (+) was isolated from 16 h old culture of *E. coli* DH5α by alkaline lysis method. The isolated plasmid was transformed to competent cells of *E. coli* BL21 Codon Plus by heat shock (Sambrook and Russel, 2001).

Expression of Recombinant α-amylase

Bacterial cells were grown in flasks 50 mL containing sterilized medium and 100 µg/mL ampicillin using 0.5 mL (1%, v/v) of overnight grown starter culture at 37ºC (200 rpm). When cell growth reached at 0.6. $O.D_{600}$, heat shock was given by keeping these flasks at 42ºC for 1 h in a shaking incubator at 200 rpm. At this point, induction was made with inducer (IPTG or lactose) and transferred the cells at 37ºC for the expression of recombinant protein. Basic media used in present work were M9, LB, ZB, ZYB (Studier, 2005) with modification in their composition and in different combination.

LB:	1 g NaCl, 0.5 g Yeast extract, 1 g Tryptone/L
ZB:	5 g NaCl, 10 g Tryptone /L
ZBM:	5 g NaCl, 40 g Tryptone/L
4xZB:	20 g NaCl, 40 g Tryptone/L
ZYB:	5 g NaCl, 5 g yeast extract and 10 g Tryptone /L
3xZYB:	15 g NaCl , 15 g Yeast extract and 30g Tryptone/L
M9:	4 g glucose, 1 g NH4Cl, 6g Na2HPO4, 3 g KH2PO4 and 1 mL of 1 M $MgSO_4$/L
ZYBM9:	5 g NaCl , 5 g Yeast extract, 10 g Tryptone and M9 /L
3xZYBM9:	15 g NaCl , 15 g Yeast extract, 30 g Tryptone and M9/L

After the completion of incubation, centrifuged the culture at 6000 rpm. The cell pellet (in 500 µL Tris HCl, pH 8.0) was sonicated followed by centrifugation at 13000 rpm at 4°C and used the supernatant to determine intracellular α-amylase.

Enzyme Assay

α-amylase assay was determined after Liebl *et al.* (1997). Reaction was carried out in heat resistant 2 mL eppendrof tubes. Reaction mixture containing 100 µL starch prepared in 50 mM Tris HCl (pH 8.2) and 100 µL of diluted enzyme was incubated at 98°C for 5 min. Reaction was stop by adding 200 µL of DNS and boiled it for 5 min and recorded the OD at 540 nm after addition of 1 mL distilled water (Miller, 1959). One unit of enzyme is defined as "the amount of enzyme that liberates 1.0 µM of reducing sugar as maltose per mL per min".

Statistical Analysis

Each experiment was performed in completely randomized design with three replications and data collected was statistically analyzed using one-way analysis of variance and comparison of means was calculated by Duncan's multiple range test at 5% level of significance using COSTAT computer software (COHROT, Monterey, California, USA).

Results

Comparison of Lactose and IPTG as Inducer in Different Media

Screening of nine different media given above was carried out individually with lactose and IPTG (Fig. 1 and 2). In all the media intracellular activities were higher when induction was carried out with lactose. However, maximum expression was obtained with modified ZB medium (ZBM) 48 h after the induction in present work. Whereas, M9 gave the minimum enzyme activities with both the inducers.

Effect of Heat Shock

Effect of heat shock on the lactose induced intracellular expression of α-amylase of *T. petrophila* is shown in Fig. 3. Direct incubation of host cells at 37°C immediately after induction resulted in comparatively lower expression. However, the enzyme activity was maximum when cell culture was kept at 42°C (200 rpms) for 1 h after lactose induction prior to incubation at 37°C for 48 h.

Effect of Lactose Concentration

The effect of different lactose concentrations (100-400 mM) was also investigated on the expression of recombinant protein in ZBM (Fig. 4). The enzyme activity of cloned α-amylase increased with increase in inducer concentration and reached maximum when the medium was supplemented with 200 mM lactose.

Effect of Incubation Temperature

Fig. 5 depicts the effect of incubation temperature on the expression of α-amylase in term of enzyme activity. After induction and heat shock, incubation was carried out at 20, 25, 30, 37 and 40°C at 200 rpm. Samples were taken periodically from each flask for the enzyme activity analysis. The maximum activities were obtained at 37°C.

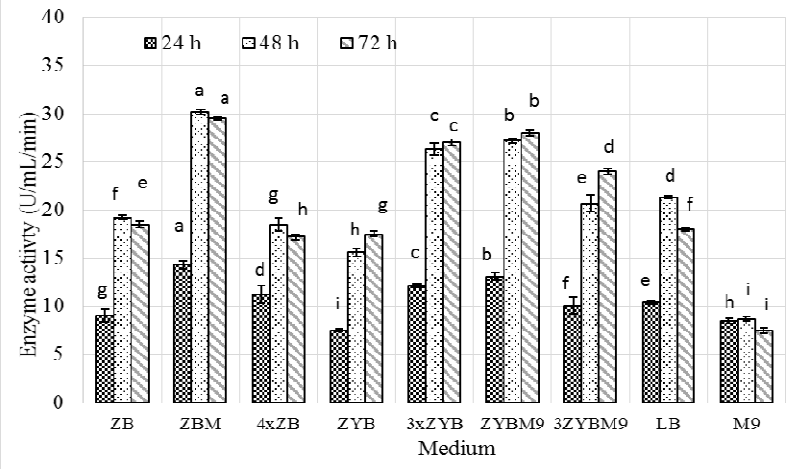

Fig. 1: Lactose induced intracellular expression of α-amylase in different media
The Y-error bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤ 0.05

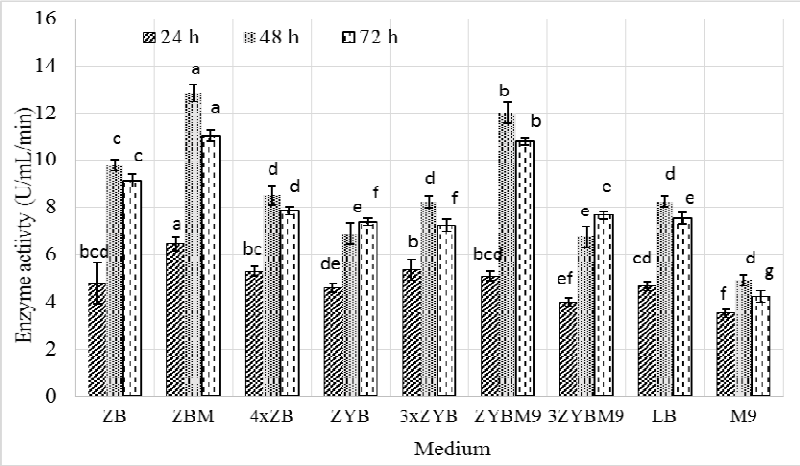

Fig. 2: IPTG induced intracellular expression of α-amylase in different media
The Y-error bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤ 0.05

Effect of Cell Density with Reference to Induction Time

The effect of cell density of *E. coli* BL21 Codon Plus in term of OD at A_{600} (0.40, 0.6, 0.8, 1.0 and 1.2) at the time of induction was also investigated (Fig. 6). Maximum expression was obtained when host cell OD_{600} was at 0.8 at the time of induction. However, further increase in the bacterial cell concentration did not increase the enzyme expression.

Discussion

In present work when comparative analysis of IPTG and lactose as individual inducer for the expression of recombinant α-amylase was carried out in nine different media, latter was found to be the best. Lactose being natural inducer of lac operon causes less metabolic load on host machinery. Moreover, lactose can also act as carbon source

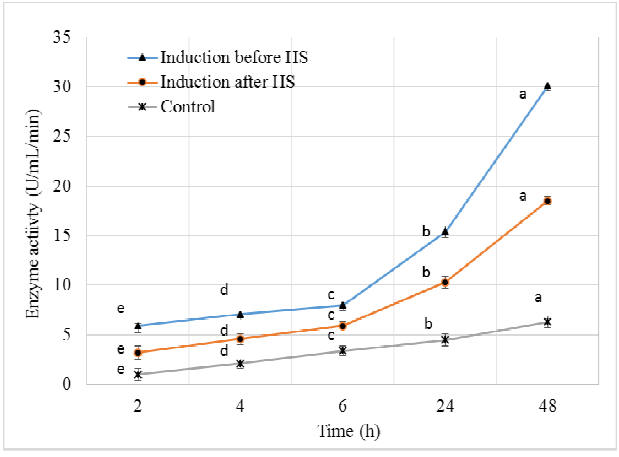

Fig. 3: Effect of heat shock (HS) on the intracellular expression of α-amylase

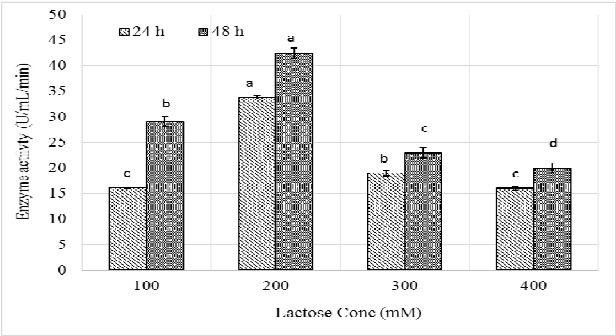

Fig. 4: Effect of lactose on the intracellular expression of α-amylase

The Y-error bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤ 0.05

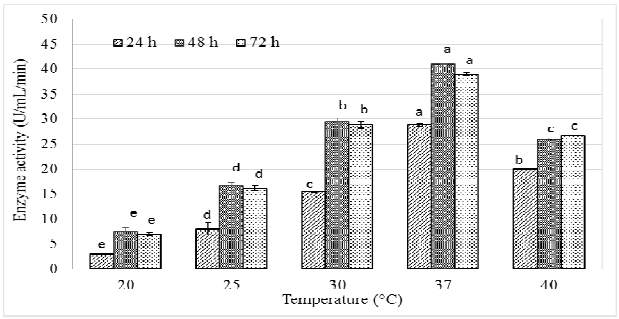

Fig. 5: Effect of growth temperature on intracellular expression of α-amylase

The Y-error bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤ 0.05

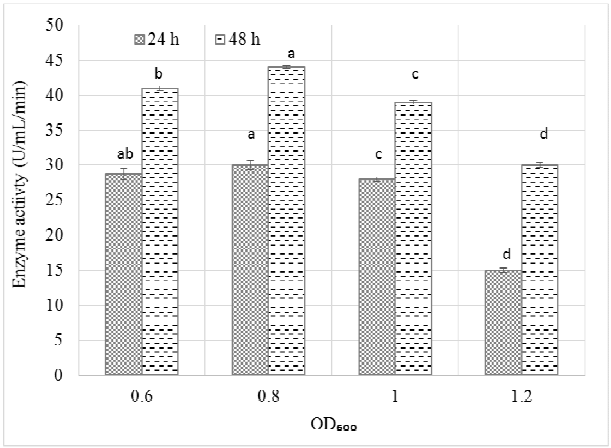

Fig. 6: Effect of cell density with reference to induction on intracellular expression of α-amylase

The Y-error bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤ 0.05

for the continuous growth of cells and is nontoxic than IPTG (Yan *et al*., 2004; Miller, 2006; Zhang *et al*., 2009). Among different media tested, ZBM gave better enzyme activities with both the inducers 48 h after induction when host cell enter the stationary phase. This medium was designed in present work to have appropriate concentration of NaCl and more tryptone compared to others. Trypone is rich source of amino acids and their metabolism repress induction of recombinant protein in host cell even in the presence lactose during log phase. This inhibition is relieved at stationary phase during which full induction occurs (Studier, 2005).

The maximum enzyme activities were obtained when host cell culture was given heat shock immediately after induction followed by incubation at 37°C for 48 h. It is because heat treatment is accountable for the production of heat shock proteins or molecular chaperons that assist the proper expression of proteins, and hence prevent inclusion bodies formation within host (Ananthan, 1986; Oganesyan *et al*., 2007).

Appropriate concentration of lactose is required within the medium to compensate the growth as well as induction requirement of the growing cells in the absence of additional carbon source (Studier, 2005). In the present work, 200 mM lactose was found to be optimum for the induction in ZBM medium**.** It is due to reason that high level of allolactose is required at the time of induction. Hence to satisfy this need increased inducer concentration in growth medium is mandatory (Babu and Aravind, 2006). Furthermore, presence of lactose in the medium in high concentration can also extend the lag and stationary phase of *E. coli* (Miller, 2006).

Maximum lactose induced expression of α-amylase growth was achieved at 37°C as it is also optimum temperature for the *E. coli* growth and subsequently protein production (Ozturk *et al*., 2013). The enzyme expressions were high when host cell OD_{600} was at 0.8 at the time of induction due to the requirement of enough amount of cells at the time of induction. However, in contrast to present finding Yan *et al*. (2004) reported maximum recombinant proteins expression up to 1.2 OD_{600} at 37°C

In conclusion, an increase of 1.5 folds intracellular enzyme activity was achieved after optimization of process parameter. Therefore, lactose induced expression of *T. petrophila* α-amylase gene in *E. coli* BL21 Codon Plus (DE3) under T7 promoter in tryptone rich medium was found to be quite efficient in present work. Lactose can also be recommended to use in place of IPTG for the enhanced expression of recombinant protein.

References

Ananthan, J., A.L. Goldberg and R. Voellmy, 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science,* 232: 522–524

Asgher, M., M.J. Asad, S.U. Rahman and R.L. Legge, 2007. A thermostable α-amylase from a moderately thermophillic *Bacillus subtilis* strain for starch processing. *J. Food Eng*., 79: 950–955

Babu, M.M. and L. Aravind, 2006. Adaptive evolution by optimizing expression levels in different environments. *Trends Microbiol.*, 14: 11–14

Blumer-Schuette, S.E., I. Kataeva., J. Westpheling., M.W. Adams and R.M. Kelly, 2008. Extremely thermophilic microorganisms for biomass conversion: status and prospects. *Curr. Opin. Biotechnol*., 19: 210–217

Derde, L.J., S.V. Gomand, C.M. Courtin and J.A. Delcour, 2012. Hydrolysis of β-limit dextrins by α-amylases from porcine pancreas, *Bacillus subtilis, Pseudomonas saccharophila* and *Bacillus stearothermophilus*. *Food Hydrocolloids*, 26: 231–239

Elleuche, S. and G. Antranikian, 2013. Starch-hydrolyzing enzymes from thermophiles. *In: Thermophilic microbes in environmental and industrial biotechnology*. Satyanarayana, T., J. Littlechild and Y. Kawarabayasi (eds.). pp: 509–533. Springer, New York, USA

Gombert, A. K., and B. V. Kilikian, 1998. Recombinant gene expression in *Escherichia coli* cultivation using lactose as inducer. *J. Biotechnol.*, 60: 47–54

Howhan, P. and S. Pornbanlualap, 2003. Cloning and effective induction of escherichia coli nucleoside diphosphate kinase by lactose. *Sci. Asia*, 29: 347–353

Karakas, B., M. Inan and M. Certel, 2010. Expression and characterization of *Bacillus subtilis* PY22 α-amylase in *Pichia pastoris*. *J. Mol. Catalysis B: Enzym.*, 64: 129–134

Khemakhem, B., I. Fendri, I. Dahech, K. Belghuitha, R. Kammounc and H. Mejdouba, 2013. Purification and characterization of a maltogenic amylase from Fenugreek (*Trigonella foenum graecum*) seeds using the Box Benkhen Design (BBD). *Ind. Crop Prod*., 43: 334–339

Killikian, B.V., I.D. Suarez., C.W. Liria and A.K. Gombert, 2000. Process strategies to improve heterologous protein production in *Escherchia coli* under lactose and IPTG induction. *Process Biochem.*, 35: 1019–1025

Lakshmi, H. P., U. V. Prasad, S. Yeswanth, O. H. Prasad, M. L. Narasu, P. V. Gurunadha and K. Sarma, 2013. Molecular characterization of α-amylase from Staphylococcus aureus, *Bioinformation*, 9: 281–285

Leveque, E., S. Janecek., B. Haye and A. Belarbi, 2000. Thermophilic archaeal amylolytic enzymes. *Enzym. Microbiol. Technol*., 26: 3–14

Li, S., Z. Zuo, D.Niu, S. Singh, K. Permaul, B.A. Prior, G. Shi and Z. Wang, 2011. Gene cloning, heterologous expression, and characterization of a high maltose-producing α-amylase of *Rhizopus oryzae*. *Appl. Biochem. Biotechnol*., 164: 581–592

Liebl, W., I. Stemplinger and P. Ruile, 1997. Properties and gene structure of the *Thermotoga maritima* alpha-amylase AmyA, a putative lipoprotein of a hyperthermophilic bacterium. *J. Bacteriol.,* 179: 941–948

Lin, L.L. and W.H. Hsu, 1997. Lactose-induced expression of *Bacillus sp.* TS-23 amylase gene in *Escherichia coli* regulated by a T7 promoter. *Lett. Appl. Microbiol.,* 24: 365–368

Miller, E. 2006. Population growth and lag time assent in lactose induced *Eschericia coli* Stains. *Cantaurus*, 14: 24–26

Miller, G.L, 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.,* 31: 426–428

Oganesyan, N., I. Ankoudinova, S.H. Kim and R. Kim, 2007. Effect of osmotic stress and heat shock in recombinant protein overexpression and crystallization. *Protein Expres. Purif*., 52: 280–285

Ozturk, M.T., N. Akbulut, S.I. Ozturk and F. Gumusel, 2013. Ligase-independent cloning of amylase gene from a local *Bacillus subtilis* isolate and biochemical characterization of the purified enzyme. *Appl. Biochem. Biotechnol.*, 171: 263–278

Presecki, A.V., Z.F. Blazevic and D. Vasic-Racki, 2013. Complete starch hydrolysis by the synergistic action of amylase and glucoamylase: impact of calcium ions. *Bioproc. Biosyst. Eng*., 36: 1555–1562

Puspasari, F., O.K. Radjasa, A.S. Noer, Z. Nurachman, Y.M. Syah, M. van der Maarel, L. Dijkhuizen, S. Janecek, D. Natalia, 2013. Raw starch-degrading α-amylase from *Bacillus aquimaris* MKSC 6.2: isolation and expression of the gene, bioinformatics and biochemical characterization of the recombinant enzyme. *J. App. Microbiol.,* 114: 108–120

Roy, J.K. and A.K. Mukherjee, 2013. Applications of a high maltose forming, thermo-stable α-amylase from an extremely alkalophilic *Bacillus licheniformis* strain AS08E in food and laundry detergent industries. *Biochem. Eng. J.*, 77: 220–230

Sambrook, J. and D.W. Russell, 2001. *Molecular cloning: a laboratory manual*. 3rd ed., Cold Spring Harbor Laboratory Press Cold Spring, New York, USA

Sarethy, I.P., Y. Saxena, A. Kapoor, M. Sharma, R. Seth, H. Sharma, S.K. Sharma and S. Gupta, 2012. Amylase produced by *Bacillus sp* . SI-136 isolated from sodic-alkaline soil for efficient starch desizing. *J. Biochem. Tech.,* 4: 604–609

Sidkey, N.M., M.A. Abo-Shadi, R. Balahmar, R. Sabry and G. Badrany, 2011. Purification and characterization of α-amylase from a newly isolated *Aspergillus flavus* F_2 Mbb. *Int. Res. J. Microbio.*, 2: 96–103

Studier, F.W., 2005. Protein production by auto-induction in high-density shaking cultures. *Protein Expres. Purif.*, 41: 207–234

Van Ooteghem, S.A., S.K. Beer and P.C. Yue, 2002. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. *Appl. Biochem. Biotechnol.,* 98: 177–189

Yan, J., S.F. Zhao, Y.F. Mao and Y.H. Luo, 2004. Effects of lactose as an inducer on expression of *Helicobacter pylori* rUreB and rHpaA, and *Escherichia coli* rLTKA63 and rLTB. *World J. Gastroenterol*., 10: 1755–1758

Zhang, H., X. Mao, Y. Wang and X. Hu. 2009. Optimization of culture conditions for high-level expression of dextransucrase in *Escherichia coli*. *J. Food Agric. Environ.*, 7: 75–78

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