Journal of Biotechnology **18**(2): 363-372, 2020

CLONING AND EXPRESSION OF MALTOOLIGOSYLTREHALOSE TREHALOHYDROLASE FROM *SULFOLOBUS SOLFATARICUS* DSM 1616 IN *BACILLUS SUBTILIS* WB800

Nguyen Tien Cuong[1], Nguyen Thi Hien Trang[1], Nguyen Thi Thao[1], Le Thanh Hoang[1], Nguyen Sy Le Thanh[1], Nguyen Thi Anh Tuyet[1], Nguyen Manh Dat[2], Le Duc Manh[2], Do Thi Thanh Huyen[2], Nguyen Thi Thu[2], Nguyen Thi Trung[3], Hoang Thi Yen[1], Do Thi Tuyen[1,3,✉]

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology*
[2]*Food Industries Research Institute*
[3]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology*

✉To whom correspondence should be addressed. E-mail: dttuyen@ibt.ac.vn

Received: 19.3.2020
Accepted: 02.4.2020

SUMMARY

Maltooligosyltrehalose trehalohydrolase (MTHase) is an industrial enzyme for the production of trehalose. A DNA fragment of 1680 bp encoding for MTHase was cloned from *Sulfobolus solfataricus* DSM 1616 then fused with promoter acoA-amyE already amplified from pMSE3 vector by PCR to generate an expression cassette acoMTH. Afterward the cassette was inserted into pAC7 vector for expression of the gene in *Bacillus subtilis* WB800 – a conventional expression system. Gene MTH was inserted into the genome of *B. subtilis* WB800 by cross-exchange event of pAC7 vector with the host genome for expression of high quality and high quantity of extracellular recombinant protein. By crossing-exchange event at 3'amyE-5'amyE, the expressional cassette was integrated into *B. subtilis* WB800 genome. The expressional cassette was integrated into *B. subtilis* WB800 genome replacing 3'amyE-5'amyE, hindering the native amylase activity of the host. Expression of expected protein was confirmed by electrophoresis SDS-PAGE. From our results, it indicates that gene MTH was expressed successfully in *B. subtilis* WB800. After 0.5% acetoin induction for 48 h, the data showed that the protein with a molecular mass of ~64 kDa on SDS-PAGE was expressed. The level of recombinant protein in WBpAacoMTH was increased and reached 2.5%, 15.2% and 21.95%, respectively comparing with native *B. subtilis* WB800.

Keywords: cloning, expression, maltooligosyltrehalose trehalohydrolase, *Sulfobolus solfataricus* DSM 1616, *Bacillus subtilis* WB800

INTRODUCTION

Maltooligosyltrehalose trehalohydrolase (MTHase) or glycosyltrehalose trehalosehydrolase (EC 3.2.1.141) breaks down the linkage of α-1, 4 -glucosidic contiguous to α-1, 1-glucosidic on the reducing end of maltooligosaccharides to produce trehalose and remained small molecules of maltooligosaccharides. In the combination with maltooligosyl trehalose synthase (MTSase), MTHase and MTSase form an economical enzymatic system to catalyze maltooligosaccharides into trehalose (Maruta *et al.*, 1995; Nakada *et al.*, 1995) by two steps. Firstly, MTSase breaks the linkage of the reducing end of maltooligoscaccharides from α-1, 4 bond to the α-1, 1 bond producing maltooligosaccharides. Then, trehalose residue at the reducing end of saccharide

marcromolecule is cleaved by MTHase. This method has been playing a significant role in producing trehalose for industrial use such as pharmaceutical, food, and cosmetic (Roser, Colaco 1993; Schiraldi *et al.*, 2002; Sola-Penna, Meyer-Fernandes 1998).

The two enzymes were first isolated from non-pathogenic bacteria strains from soil *Arthrobacter* sp.Q36 (Nakada *et al.*, 1995) and *Sulfolobus acidocaldarius* (Nakada *et al.*, 1996). Later, more bacteria strains have been reported for the capability of synthesizing MTSase and MTHase including *Arthrobacter ramosus* S34 (Yamamoto *et al.*, 2001), *Corynebacterium glutamicum* (Carpinelli *et al.*, 2006), *Deinococcus radiodurans* (Timmins *et al.*, 2005), *Rhizobium sp*. M-11. (Maruta *et al.*, 1996). Therefore, many attempts for mass-producing of these two enzymes for industrial uses have been recorded. Previous studies have successfully cloning of 2.2 kb DNA encoding for MTSase from *Sulfolobus shibatae* by expressing the recombinant plasmid of the targeted gene and plasmid pSBTG1 in *E. coli* (Chen *et al.*, 2000). The gene encoding MTHase from *Sulfolobus solfataricus* ATCC 35092 is also investigated, cloned and expressed in *E. coli*. MTHase activity reached 13.300 U/g (dry cell) and with the optimum temperature at 84°C and pH 5 (Fang *et al.*, 2006).

B. subtilis is a well-known expression system for its capability of producing a high level of recombinant protein both in quantity and quality. Additionally, *B. subtilis* strain has been engineered for overexpression of recombinant protein with extracellular protease deficiency. Several recombinant proteins have been expressed in various modified strains as WB600 (Peng *et al.*, 2004); WB700 (Ye et al. 2000); WB800 (Phuong *et al.*, 2012). Up to date, there haven't been many studies about expression of MTHase in *B. subtilis* WB800. For the first time, in this study, we have cloned gene encoding for MTHase from *Sulfolobus solfataricus*, these fragments have been amplified from vector pJET 1.2 in *E. coli*, and expressed in *B. subtilis* WB800 which is deficient in eight extracellular proteases (Wu *et al.*, 2002) for industrial mass. The recombinant MTHase was purified and bioactivities were investigated.

MATERIALS AND METHODS

Chemical and reagents

Restriction enzyme, *Taq* DNA polymerase, *T4* ligase were from Fermentas (Thermo Fisher Scientific Inc., Waltham, USA). DNS, maltopentaose, maltooligosaccharide were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). Agarose, peptone and yeast extract were supplied by BioBasic Inc. (Ontario, Canada). Kit Probond™ Nikel-Chelating resin was from Invitrogen Corp. (Carlsbad, CA, USA). D-Glucose, methanol, sodium chloride, sodium hydroxide, glycerol was from Merck (Darmstadt, Germany). DNA Gel-extraction kit was from Promega (San Louis Obispo, CA, USA). Sorbitol was from Scharlau (Spain). All other chemicals were of analytical grade otherwise stated.

Bacteria and plasmids

S. solfataricus DSM 1616 was purchased from Leibniz Institute DSMZ (Germany). *B. subtilis* strain WB800 possessing 8 extracellular proteases (nprE, aprE, epr, bpr, mpr :: ble, nprB :: bsr, vpr, wprk :: hyg) was used as a host for expression of MTHase (Greiswald University, Germany). Vector pAC7 with 10600 bp in size harbors the ampicilin (Ap^r) and (Kn^r) kanamycin resistance genes active in *E. coli* and *B. subtilis*, respectively, promoter *lacZ* and 5'amyE-3'amyE. The multiple cloning size containing *Eco*RI, *Xho*I, 6His, and T7 terminator was located between the kanamycin resistance gene and *lacZ*. pJET1.2/blunt vector 2974 bp (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA).

DNA manipulations

Genomic and plasmid DNA isolations were carried out by the method as previously described (Quyen *et al.*, 2006). The DNA fragments and PCR products were separated in 0.8% agarose gel, excised and purified by a gel extraction kit (Qiagen,

Venlo, The Netherlands) according to the manufacturer's instruction. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). *E. coli* DH5α cells were transformed using the heat shock method as previously described (Quyen *et al.*, 2006).

Construction of recombinant vector pAC7 integrated gene MTH

To design the expressional vector which contains a promoter and a terminator, we inserted a cassette contained a promoter acoA-aceton induction, amyE signal, target gene MTHase. First, the DNA fragment composed of a promoter acoA and amyE (~360 bp) was cloned from a recombinant plasmid pMSE3 (Greifswald University, Germany) using primers acoAF and acoAR. Next, the target gene encoding MTHase (MTH) was amplified by amyE-MTHF and MTHR primers. Then, these two sequences were fused together by PCR reaction which composed of: 2 µL of PCR product of each DNA, 1 U *Pfu* polymerase, 2 mM $MgSO_4$, 2 µL 10x *Pfu* buffer, 2 mM dNTP, suspended in deionized water for the total volume of the mixture at 20 µL. The PCR was performed using the following cycling conditions 95°C for 4 min, 15 cycles (95°C for 45s, 56°C for 1 min, 72°C for 1 min 45 s), 72°C for 10 min. The PCR product continuously applied for next reaction using two primers acoAF and MTHR. The reaction mixture included: 2 µL of previous PCR product, 1 U *Pfu* polymerase, 2 mM $MgSO_4$, 2 µL 10x *Pfu* buffer, 2 mM dNTP, 10 pmol primer acoAF, 10 pmol primer MTHR; the mixture was suspended to a total volume of 20 µL. The reaction was performed using the following condition of 95°C for 4 min, 35 cycles (95°C for 45 s, 56°C for 1 min, 72°C for 1 min 45 s), 72°C for 1 min. The final product was the cassette promoter acoA-amyE-gene encoding MTHase (acoMTH). Cassette was cloned in plasmid pJET1.2 to form plasmid pJacoMTH.

An engineered pAC7 vector with a 6 histidine tag and a terminator (Nguyen *et al.*, 2013), and pJacoMTH plasmid were cut by *EcoR*I and *Xho*I. The mixture was run on DNA electrophoresis to acquire a single band of linear plasmid-pAC7 and cassette acoMTH. These two DNA fragments were then purified by AccuPrep® Gel Purification Kit (Bioneer, South Korea) and ligated. Each 10 µL of a reaction composed of 2 µL pAC7, 2 µL DNA cassette acoMTH, 1 µL 10X buffer, 0.5 µL 10U/µL *T4* DNA ligase, 5.5 µL H_2O. Then, the resulting recombinant plasmid pAacoMTH was amplified by *E. coli* DH5α.

Table 1. Primers for cloning and fusion of expressional cassette acoMTH.

Primers	Nucleotides sequence 5'→3'	Product
acoAF	gaattcgcgaattctcagtcaaacgatgcag *EcoR*I	Promoter acoA and signal peptide amyE
acoAR	agcgctcgcagccaccggtcctgc	
AmyE-MTHF	ggctgcgagtgct ACGTTTGGTTATAAATTA	Encoding gene MTH
MTHR	CTCGAGAAGTTTATATAAAGCAA *Xho*I	

Transformation of recombinant plasmid into *B. subtilis* WB800

The pAacoMTH was transformed into *B. subtilis* WB800 by replacement of homologous sequences of 5'amyE and 3'amyE in the host by those of pAC7 vector. First, the recombinant plasmid was digested by *Sca*I in the following mixture composed of 200 µL (5 µg) plasmid, 50 µL 10X *Sca*I buffer, 5 µL *Sca*I (50 U), 245 µL (H_2O). A total volume of 500 µL was incubated for 10 min at 37°C and precipitated in isopropanol. The sediment was dissolved in deionized water.

One colony of *B. subtilis* WB800 was inoculated in 2 mL SPII media, shaking at 200 rpm and 37°C overnight until OD_{600} reached 0.6-0.8. 1 mL of cell culture was centrifuged at 5000 rpm in 4 min. Next, 900 µL of top supernatant was removed. 100 µL including sediment was added 30 µg of digested pAacoMTH vector. The mixture was incubated at 37°C, shaking 200 rpm in 30 min. Following this, 0.5 mL LB media was added into the sample and shaking continually for 1 hr. Recombinant *B. subtilis* WB800 colony was selected on kanamycin (10 µL/mL) embedded LB.

Gene expression

For expression of MTHase in recombinant *B. subtilis* WB800 (WBpAacoMTH) 2.5 mL of an overnight culture were inoculated into 250 mL LB medium in a 1-liter Erlenmeyer flask and grown at 37°C with agitation at 200 rpm. The culture was cultivated until an optical density (OD) at 600 nm reached 1.5 (approximately at the end of the exponential growth) and then induced by the addition of acetoin to a final concentration of 0.5% (w/v). After the acetoin induction for 48 h, the culture supernatant containing the extracellular recombinant MTHase was collected.

SDS-PAGE analysis and lugol test for expression of MTHase in *B. subtilis* WB800

The molecular mass of MTHase was determined by 12.5% SDS polyacrylamide gel electrophoresis (Laemmil 1970). Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250. Integration of gene MTH into the genome of *B. subtilis* WB800 was identified using the amylase test with lugol.

The recombinant colonies hindering amylase activity in which 5'amyE-3'amyE sequence was replaced by cross-exchange event of acoAMTH was screened on LB agar plate dying lugol. The putative strains were streaked on the plates embedding with 0.5% starch (w/v), 10 mg/mL kanamycine (w/v) and incubated at 37°C overnight. Next, they were screened with lugol 0.1% (w/v) in 1 min. After washing with 0.9% NaCl (w/v), the circle-starch hydrolysis can be observed. If the integration is successful, the host cell can not secret amylase, thus colorized lugol test will show blue color of lugol. Otherwise, the gene was not inserted.

RESULTS AND DISCUSSION

Gene cloning and analysis

Trehalose-a non reducing disaccharides sugar contained two glucose molecules which are linked by α-glucosidic linkage. Since the initial study reported in the plant in 1862 (Wiggers 1963), trehalose was found in many sources of organisms up to date. Recently, pharmaceutical, food, and cosmetic industry have been utilized trehalose as a supplemental agent, the compound showed high efficacy in stabilizing and modulating enzyme function (Sola-Penna and Meyer-Fernandes 1998). Therefore, many studies were carried out to mass-produce the non reducing sugar. MTSase and MTHase are the two main enzymes involved in the production of trehalose by 2 steps hydrolysis. In the final step, MTHase reduces MTSase-catalyzed products into maltooligosaccharide and trehalose. Consequently, MTHase and MTSase are being produced on a large scale.

The gene encoding for MTHase was amplified PCR with specific primers and sequenced. It showed that the putative gene MTH has the size of 1680 bp encoding for a protein molecule of 560 amino acids with molecular weight of ~ 64 kDa. The gene sequence from *S. solfataricus* DSM 1616 showed 99.82% identity to the corresponding sequence of *S. solfataricus* strain (WP-009989788.1), and 90% with one from *S. islandicus* strain. The sequence was deposited in GenBank with an accession number of MN163002.

Construction of expression plasmid

The construction of expression plasmid was illustrated as the following steps (Figure 1). A promoter of 370 bp was cloned from pMSE3 by two

primers acoAF and acoAR (Figure 2A). Gene MTH was amplified from the total DNA of *S. solfatarius* DSM 1616 whose PCR product showed the two bands of approximately 1700 bp (Figure 2B). The ligation of acoA and gene MTH forms a cassette acoMTH (Figure 1A and 2C). The gene MTH encodes for 560 amino acids with an estimated size of 64 kDa composing of 68 strong alkaline amino acids, 85 strong acidic amino acids, 180 hydrophobic amino acids and 139 polar amino acids (Figure 3).

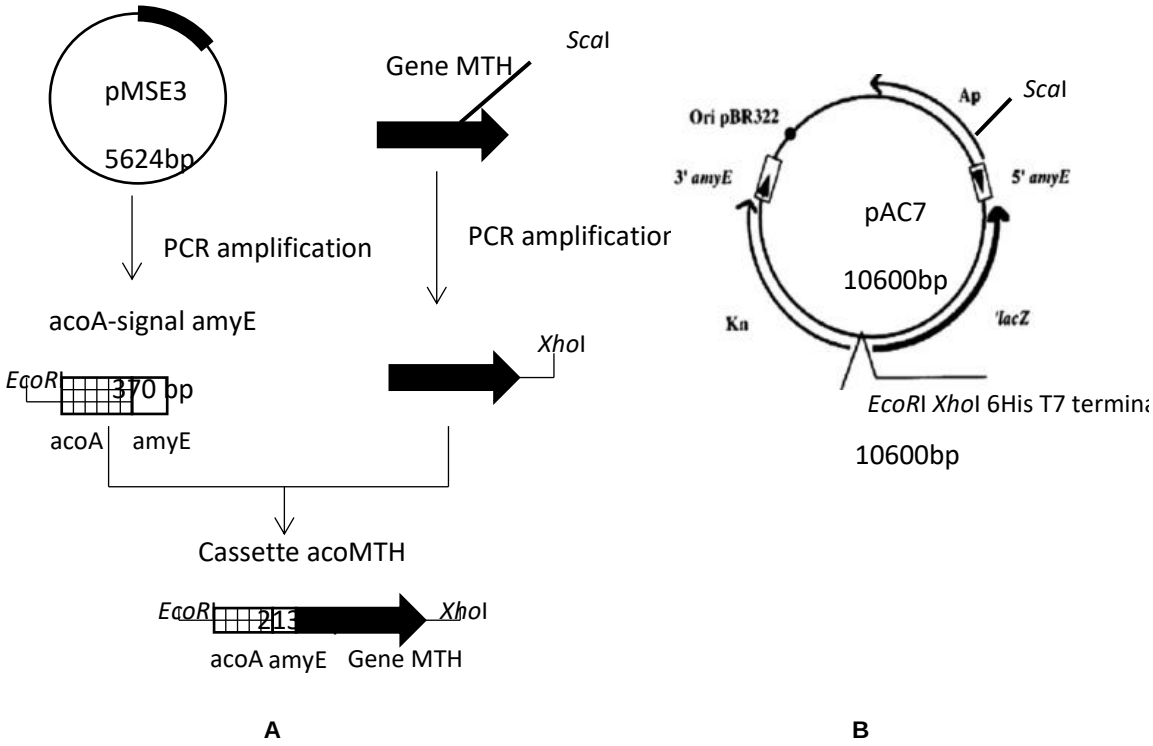

Figure 1. Diagram representation of construction plasmid. A: Expressional construction of cassette acoMTH. B: Vector pAC7 + T7 terminator

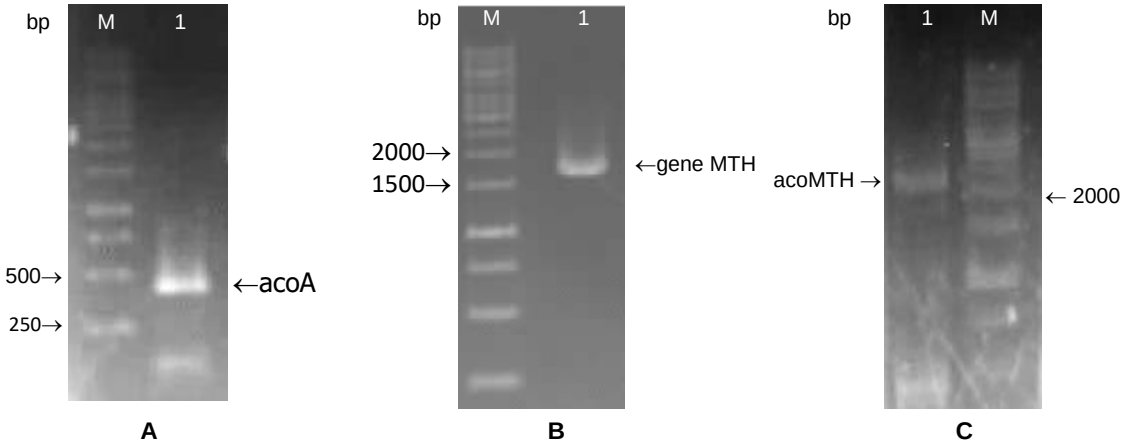

Figure 2. DNA electrophorensis of PCR product: A: acoA. B: gene MTH. C: Intergration of cassette acoMTH.

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TFGYKLDEDG VTFNLWAPYQ RKVKLKILNR GIYEMERDDK GYFTITLDNV RVGDRYKYIL  60

DDNSEVPDPA SRYQPEGVHG YSEIISPDFE WDDENSVKVK REDLVIYELH IGTFTSEGTF  120

EGVIKKLNYL KEPGVTAIEI MPIAQFPGKK DWGYDGVYLY AVQNSYGGPS GFRKLVNEAH  180

KLGLAVILDV VYNHVGPEGN YMVKLGPYFS EKYKTPWGLT FNFDDAGSDE VRKFILENVE  240

YWINEFHVDG FRLDAVHAII DNSPKHILED IADVVHKYDK IVIAESDLND PRVVNPKEKC  300

GYNIDAQWVD DFHHAIHAFL TGERQGYYSD FGSIGDIVKS YKDVFIYDGK YSNFRRKTHG  360

KSVGDLDGCK FVVYIQNHDQ VGNRGGGERL IKLVDKESYK IAAALYILSP YIPMIFMGEE  420

YGEENPFYYF SDFSDPKLIQ GVREGRRREN GQETDPQSDC TFNDSKLSWK INDDILSFYK  480

SLIKIRKEYG LACNRKLSVE NGNYWLTVKG NGCLAVYVFS KSVIEMKYSG TLVLSSNSSF  540

PSQITESKYE LDKGFALYKL  560
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Figure 3. Amino acid sequence analysis of MTHase from *S. solfataricus* DSM 1616.

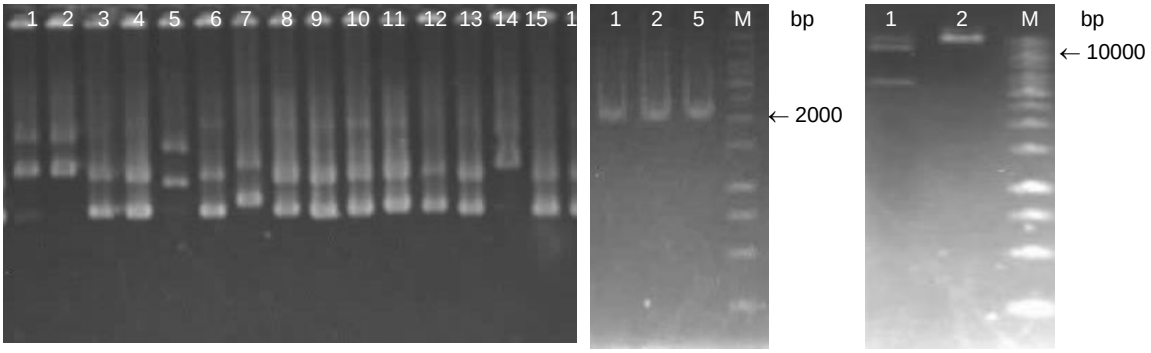
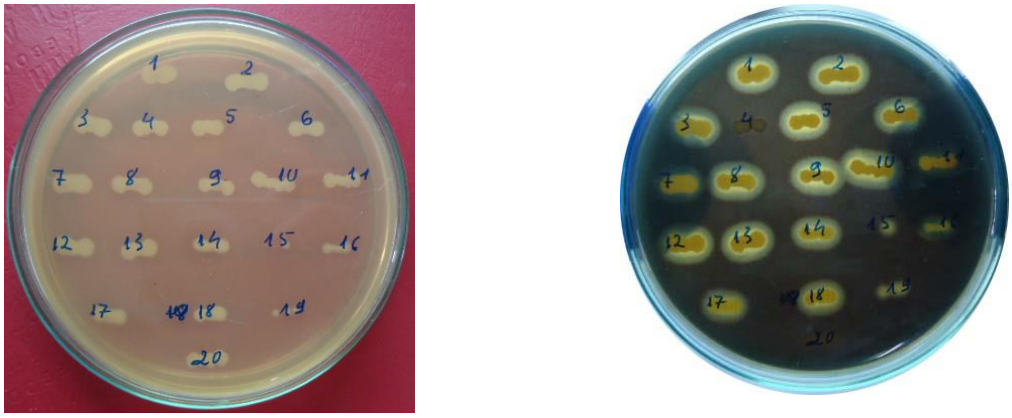


Figure 4. A: 1-16 Total DNA of putative recombinant colonies in which the integrated pAacoMTH posing heavier molecular weight in comparison with control constraining a higher position on DNA electrophoresis B.Lane 1; 2; 5 showed the molecular weight ~2100 bp corresponds to the size of cassette acoMTH, this confirms the integration of acoMTH in pAC7 by PCR. C: Recombinant plasmid pAacoMTH was digested by *Sca*I; Lane 1: undigested pAacoMTH, Lane 2: digested pAacoMTH.

Cassette acoMTH of ~ 2100 bp was synthesized by a fusion PCR reaction using acoAF and MTHR primers. A plasmid pAC7 with inserted T7 terminator (Nguyen *et al.*, 2013) was cut by *Eco*RI and *Xho*I, and ligated to acoMTH to form a recombinant plasmid pAacoMTH. (Figure 4A). The bands of lane 1; 2; 5 and 14 constrained at the higher position in comparison with control (lane 9). The recombinant plasmid was then checked by PCR with primers acoAF and MTHR. The results indicated that PCR products from the

plasmids 1; 2 and 5 was about 2100 bp as required (Figure 4B). Hence we got the recombinant plasmid pAacoMTH.

For insertion of pAacoMTH into the genome of *B. subtilis*, recombinant plasmid pAacoMTH was digested with restriction enzyme *Sca*I. However, we found out that *Sca*I located in both pAC7 plasmid and gene MTH, in which, the postion of *Sca*I on pAC7 is located in the sequence encoding for ampicilin-resistance (Figure 1). Thus, we carried out a partially cutting reaction by shortening the digested time of pAacoMTH to eliminate the negative effect on the MTH gene by exploiting the incompletion of cleaving reaction, and chose kanamycin for selection of recombinant colonies. As expectation, we got the intact product of pAC7 and gene MTH with the corresponding molecular weight separated in agarose gel 0.8% (Figure 4C). After recombinant pAacoMTH was transformed into WBpAacoMTH, cassette acoAMTH would be integrated by the crossover of 2 homologous sequences 3'amyE and 5'amyE on plasmid vector to the homologous sequence of the gene encoding the synthesis of amylase in the genome of *B. subtilis* WB800. The recombinant *B. subtilis* colonies was streaked on LB agar plate supplementing with 0.5% starch. There was no apparent circle-starch hydrolysis. Hence, cassette acoMTH must be integrated into the genome of *B. subtilis* WB800 (number 4 and 20) (Figure 5).



A **B**

Figure 5. Activity of starch hydrolytic of recombinant colony WBpAacoMTH. Recombinant strains WBpAacoMTH (2-20) and WB800 (1) on Agar plate embedding 1% starch before (A) and after (B) dying lugol.

To confirm the integration of the expression cassette into genomic DNA of *B. subtilis* WB800, total DNA of colonies WBpAacoMTH number 4 and 20 was extracted and used as template for PCR reaction amplifying acoMTH cassette using 2 primers acoAF and MTHR. The result (Figure 5) showed the expression cassette about 2100 bp was amplified from both templates of colonies 4 and 20 only. At the same time, the amylase test indicated numbers 4 and 20 retained the blue color of lugol which corresponding to the inactivation of amylase activity of native *B. subtilis* WB800. Accordingly, the 3'amyE-5'amyE sequence is responsible for biosynthesis of amylase has been replaced. This result revealed that the expression cassette was integrated into genome of *B. subtilis* WB800.

Expression of MTHase

WBpAacoMTH were cultivated in LB medium for the MTHase production. After 0.5% acetoin induction for 48 h, the culture supernatant was collected and analyzed on SDS-

PAGE to evaluate level of protein expression by Dolphin 1D software. The data showed that the protein with a molecular mass of ~64 kDa on SDS-PAGE was expressed. The level of recombinant protein in WBpAacoMTH was increased and reached 21.95% (lane 2- Figure 6), 15.2% (lane 3- Figure 6) and 2.5% (lane 4- Figure 6), respectively comparing with native *B. subtilis* WB800 (lane 1- Figure 6). The expression of MTHase in *B. subtilis* WB800 haboring a dominant protein with the molecular weight is almost the same as *S. solfataricus* ATCC 35092 of 64 kDa expressing in *E. coli* (Fang *et al.*, 2006).

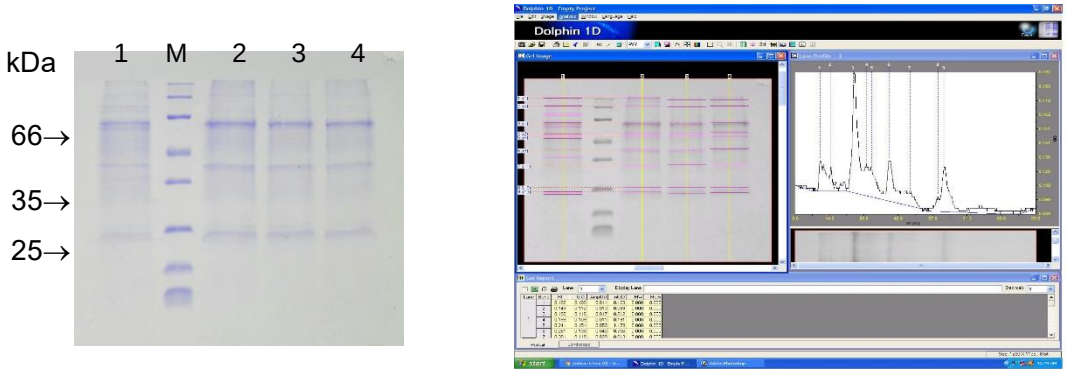


Figure 6. SDS-PAGE of expression MTHase (A) Lane: 1 represents native *B. subtilis* WB800; 2-4: the supernatants of recombinant *B. subtilis* WB800 haboring pAC7 after induction with 0.5% acetoin for 48 hrs; M: pre-stained molecular weight of protein marker (Fermentas). (B) Evaluated level of protein expression by dolphin 1D software.

CONCLUSION

In this study, we have successfully cloned and expressed of a MTHase from *S. solfataricus* DSM 1616 in *B. subtilis* WB800 which is eight protease gene deficient.

Acknowledgements: *This work was supported by a grant from the project "Research on the production of trehalose from starch by enzyme technology applied in food technology" of the Ministry of Industry and Trade of the Socialist Republic of Vietnam. We appreciated Prof. Dr. Uwe Bornscheuer Institute of Biochemistry, Greifswald University, Germany for providing the expression vector pMSE3 and B. subtilis WB800. We would like to thank reviewer's valuable comments to improve this paper.*

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NHÂN DÒNG VÀ BIỂU HIỆN ENZYME MALTOOLIGOSYLTREHALOSE TREHALOHYDROLASE TỪ CHỦNG VI KHUẨN CỔ *SULFOLOBUS SOLFATARICUS* DSM 1616 TRONG *BACILLUS SUBTILIS* WB800

Nguyễn Tiến Cường[1], Nguyễn Thị Hiền Trang[1], Nguyễn Thị Thảo[1], Lê Thanh Hoàng[1], Nguyễn Sỹ Lê Thanh[1], Nguyễn Thị Ánh Tuyết[1], Nguyễn Mạnh Đạt[2], Lê Đức Mạnh[2], Đỗ Thị Thanh Huyền[2], Nguyễn Thị Thu[2], Nguyễn Thị Trung[3], Hoàng Thị Yến[1], Đỗ Thị Tuyên[1,3]

[1]Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
[2]Viện Công nghiệp thực phẩm
[3]Học viện Khoa học và Công nghệ, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÓM TẮT

Maltooligosyltrehalose trehalohydrolase (MTHase) là một enzyme công nghiệp dùng cho việc

sản xuất đường trehalose. Nghiên cứu biểu hiện enzyme MTHase trong *B. subtilis* WB800 là một hệ biểu hiện protein ngoại bào có năng suất biểu hiện cao và ổn định. Hiện nay một số chủng *B. subtilis* như *B. subtilis* WB600, WB700, WB800 đã loại bỏ tương ứng 6, 7 và 8 protease ngoại bào, do đó hạn chế việc phân cắt protein ngoại lai, dẫn tới làm tăng năng suất protein biểu hiện. Vì vậy, MTHase sẽ được nghiên cứu biểu hiện trong *B. subtilis* WB800 để nâng cao năng suất biểu hiện sinh tổng hợp enzyme. Gene mã hóa cho MTHase được chèn vào genome của *B. subtilis* WB800 bằng sự kiện trao đổi chéo với đoạn tương đồng trên vector biểu hiện pAC7. Đoạn DNA mã hóa cho MTHase được nhân lên từ *S. solfataricus* DSM 1616, đồng thời đoạn signal biểu hiện acoA-amyE được nhân từ vectơ pMSE3. Hai sản phẩm từ hai quá trình nhân dòng riêng biệt được nối lại với nhau bằng phản ứng PCR dung hợp để tạo cassette acoMTH. Thông qua sự kiện trao đổi chéo ở hai đầu 3'amyE-5'amyE, cassette biểu hiện được chèn vào genome của *B. subtilis* WB800. Từ những số liệu thu được cho thấy cassette biểu hiện được chèn thành công vào genome *B. subtilis* WB800 và thay thế đoạn 3'amyE-5'amyE, làm mất khả năng sản xuất amylase của vật chủ. MTHase được biểu hiện sau khi được cảm ứng 0,5% acetoin sau 48 giờ, có kích thước khoảng 64 kDa, mức độ protein biểu hiện ở các dòng tăng tương ứng đạt 2,5%, 15,2% và 21,95% sau khi được đánh giá bằng phần mềm dolphin 1D so với chủng gốc *B. subtilis* WB800.

Từ khóa: *Bacillus subtilis* WB800, biểu hiện, maltooligosyltrehalose trehalohydrolase, nhân dòng, *Sulfobolus solfataricus* DSM 1616