UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A LOCAL BACTERIAL ISOLATE EXPRESSING CYCLODEXTRIN GLYCOSYLTRANSFERASE THROUGH MOLECULAR CLONING APPROACHES

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By

NORHAYATI BINTI RAMLI

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfillment of the requirements for the Degree of Doctor of Philosophy

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DEDICATED TO MY LATE MOTHER, FATHER AND FAMILY

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy

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Chairman : Professor Suraini Abd. Aziz, PhD

Faculty : Biotechnology and Biomolecular Sciences

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) exhibited as an important member of the amylolytic glucosylase family that catalyzed the formation of cyclodextrins (CDs) through cyclization reaction. The ability of CGTase to convert starch into CD brings a great interest to the researchers. The formation of CD-inclusion complex with variety of guest molecules was advantageous as the enhancement of the physical and chemical properties of the inclusion complex formed were beneficial in biotechnology, pharmaceutical, food, cosmetic, chemical and agricultural field. However, the obstacle in producing the CD in industrial scale was due to the production of different ratios of α-, β- and γ-CD catalyzed by CGTases. The above phenomenon contributed to the complicated and tedious purification strategy of the final targeted product. Due to the high viscosity of starches, pre-treatment stage was needed in order to prevent the blockage in the membrane system in filtration stage. Furthermore, the low concentration of CGTase produced by wild type strain at longer incubation time caused the major problem for

CGTase production in large scale. Hence, the objectives of this study were to isolate and screen the potential CGTase producer. The isolated strain was then subjected to the isolation of CGTase gene using primer screening technique and used for construction of CGTase expression system in *Escherichia coli*. The enzyme produced from recombinant strains was evaluated.

In this study, the CGTase-producing bacteria were successfully isolated from the soil in Malaysia. Out of 65 strains, eleven CGTase producers has been further screened using modified Horikoshi agar type II with specific indicator. The size of halo zones formation on the plate indicates a good qualitative measurement of CGTase producer as the biggest diameter formed is indicating the highest CGTase activity obtained. All eleven isolates showed the characteristics of Gram positive and identified as *Bacillus* sp. Furthermore, the selection for the best CGTase producer was carried put with the highest CGTase activity (11.709 U/mL) and CD concentration (0.011, 2.504 and 0.188 mg/mL for α-, β- and γ-CD, respectively) were produced by *Bacillus* sp. NR5 UPM using raw soluble starch as a substrate at 48 hours of fermentation. This isolate also showed the highest CGTase activity (15.514 U/mL) at 32 hours of fermentation with the used of sago starch as a substrate. The isolate *Bacillus* sp. NR5 UPM has the capability in predominantly producing β-CD as a main product and has a potential in degrading the raw starch, thus known as a raw-starch degrading enzyme producer.

Further study on the isolation of CGTase gene from *Bacillus* sp. NR5 UPM DNA was successfully carried out using DNA walking strategy. Analysis of the nucleotide sequences revealed the presence of an open reading frame of 2112 bp which encoded

a protein containing 704 amino acids with a putative molecular weight of 78.6 kDa. The deduced amino acids sequence showed about 98% homology with the CGTase from *Bacillus* sp. KC201. The recognition of TTG as a start codon was assisted by the presence of Shine-Dalgarno sequence, which located at 6 bp upstream from the initiation codon. Meanwhile, the presence of 29 amino acids residues of the isolated CGTase was functioned as signal peptide which facilitated in the secretion of protein.

The pUC19CGT-SS expression system has been constructed by cloning the full length of CGTase gene under the transcriptional control of *lac* promoter of pUC19 into an *E. coli*. Compared to wild type, the CGTase that was produced in recombinant strain only required one-fourth of culture time and neutral pH to produce CGTase. After 12 hours of cultivation, the CGTase activity in the culture medium reached 29.6 U/mL, which was approximately 2.5-fold higher than the CGTase produced by the parental strain. Interestingly, 94% of the CGTase activity was detected in an extracellular space, indicating the signal peptide was functional in *E. coli*. In addition, the isolation of the promoter and transcriptional terminator of CGTase from *Bacillus* sp. NR5 UPM was carried out. The functionality of an insertion of putative promoter regions upstream of CGTase gene was verified by the construction of *E. coli* strain harbouring pTZCGT-BS, which showed approximately 3.2-fold increment of CGTase activity compared to the parent strain. These findings support the important function of the strong promoter in regulating the expression level of enzyme. In conclusion, the study on the strain development from *Bacillus* sp. NR5 UPM by expressing CGTase gene into *E. coli* expression system was successfully carried out with the improved enzyme activity from the recombinant strain.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN BAGI EKSPRESI SIKLODEKSTRIN GLIKOSILTRANSFERASE OLEH BAKTERIA PENCILAN TEMPATAN MELALUI KAEDAH PENGKLONAN MOLEKUL

Oleh

NORHAYATI BINTI RAMLI

Julai 2012

Pengerusi : Profesor Suraini Abd. Aziz, PhD

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Siklodekstrin glikosiltransferase (CGTase, EC 2.4.1.19) merupakan enzim penting dalam keluarga amilolitik glukosilase yang memangkinkan penghasilan siklodekstrins (CDs) melalui tindak balas penghasilan gelung. Kebolehan enzim CGTase untuk menukarkan kanji kepada CD menarik minat penyelidik. Pembentukan pencantuman kompleks CD dengan pelbagai molekul asing memberi kelebihan di mana peningkatan sifat fizikal dan kimia bagi kompleks yang terbentuk berkepentingan dalam bidang bioteknologi, farmaseutikal, makanan, kosmetik, bahan kimia dan pertanian. Namun, penghasilan CD dalam skala besar adalah sukar kerana penghasilan α-, β- dan γ-CD dalam nisbah yang berbeza yang dimangkinkan oleh CGTase. Fenomena di atas menyumbangkan strategi penulenan yang sulit dan sukar kepada produk yang dikehendaki. Oleh kerana kelikatan kanji yang tinggi, langkah pra-rawatan diperlukan untuk mengelakkan sistem membran tersumbat pada langkah penapisan. Tambahan pula, masalah utama penghasilan CGTase dalam skala besar adalah kerana kepekatan enzim CGTase yang rendah yang dihasilkan oleh strain liar. Oleh itu, objektif kajian ini adalah untuk memencil dan menyaring penghasil

CGTase yang berpotensi. Strain yang dipencilkan akan digunakan untuk pemencilan gen CGTase menggunakan teknik penyaringan pemula bagi pembentukan sistem ekspresi CGTase dengan *Escherichia coli*. Penghasilan enzim dijalankan oleh strain rekombinan yang diperoleh ditentukan.

Dalam kajian ini, bakteria penghasil CGTase telah berjaya dipencilkan daripada sumber tanah di Malaysia. Daripada 65 strain yang dipencilkan, sebelas penghasil CGTase disaring dengan lebih lanjut menggunakan agar Horikoshi jenis II yang dimodifikasi dengan penunjuk spesifik. Saiz zon lingkaran yang terbentuk pada plat menandakan pengukuran kualitatif yang baik bagi penghasil CGTase. Kesemua sebelas bakteria yang dipencilkan menunjukkan ciri Gram positif dan dikenalpasti sebagai spesies *Bacillus*. Tambahan lagi, pemilihan untuk penghasil CGTase terbaik telah dijalankan dengan aktiviti CGTase yang terbaik (11.709 U/mL) and kepekatan CD (0.011, 2.504 and 0.188 mg/mL untuk setiap satu α-, β- dan γ-CD) telah dihasilkan oleh *Bacillus* sp. NR5 UPM menggunakan kanji mentah terlarut pada jam ke-48 proses fermentasi. Bakteria yang dipencil ini juga menunjukkan penghasilan CGTase tertinggi (15.514 U/mL) pada jam ke-32 proses fermentasi dengan menggunakan kanji sagu sebagai substrat. Bakteria pencilan *Bacillus* sp. NR5 UPM mempunyai kebolehan dalam menghasilkan lebih banyak β-CD sebagai produk utama dan berpotensi dalam mengurai kanji mentah, yang juga dikenali sebagai penghasil enzim-pengurai kanji mentah.

Kajian lanjut dalam pemencilan gen CGTase daripada DNA *Bacillus* sp. NR5 UPM telah berjaya dijalankan menggunakan strategi perjalanan DNA. Analisis ke atas jujukan nukleotida menunjukkan kehadiran satu rangka kehadiran terbuka sepanjang

2112 bp, dengan jujukan peptida CGTase sebanyak 704 asid amino, dengan berat molekul putatif 78.6 kDa. Jujukan rantaian asid amino menunjukkan kira-kira 98% homologi dengan CGTase daripada *Bacillus* sp. KC201. Pengenalan TTG sebagai kodon permulaan dibantu oleh kehadiran jujukan Shine-Dalgarno yang terletak 6 bp di sebelah hilir daripada kodon permulaan. Manakala, kehadiran 29 jujukan asid amino daripada CGTase yang dipencilkan mungkin bertindak sebagai peptida isyarat yang mana bertindak membantu dalam perembesan protein.

Sistem ekspresi pUC19CGT-SS telah dibina dengan mengklonkan gen CGTase penuh di bawah kawalan transkripsi pemula *lac* pUC19 ke dalam *E. coli*. Berbanding dengan strain liar, CGTase yang dihasilkan oleh strain rekombinan hanya memerlukan satu perempat daripada masa kultur dan pH neutral untuk menghasilkan CGTase. Selepas 12 jam pengkulturan, aktiviti CGTase dalam medium kultur mencapai 29.6 U/mL, yang merupakan kira-kira 2.5 kali ganda lebih tinggi daripada CGTase yang dihasilkan daripada strain liar. Menariknya, 94% daripada aktiviti CGTase dikesan di luar sel, menunjukkan peptida isyarat berfungsi di dalam *E. coli*. Di samping itu, pemencilan pemula dan penamat transkripsi CGTase daripada *Bacillus* sp. NR5 UPM telah dijalankan. Fungsi pemula yang dipencilkan dengan kehadiran pemula vektor-T7 dibuktikan dengan pembinaan strain *E. coli* yang mengandungi pTZCGT-BS, dengan kenaikan kira-kira 3.2 kali ganda aktiviti CGTase berbanding strain asal. Penemuan ini menyokong fungsi penting pemula yang kukuh dalam mengawal selia ekspresi enzim. Kesimpulannya, kajian ke atas pembangunan strain daripada *Bacillus* sp. NR5 UPM dengan mengekspresi gen CGTase ke dalam sistem ekspresi *E. coli* berjaya dijalankan dengan peningkatan aktiviti enzim daripada strain rekombinan.

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I certify that a Thesis Examination Committee has met on 10 July 2012 to conduct the final examination of Norhayati binti Ramli on her thesis entitled "Development of a Local Bacterial Isolate Expressing Cyclodextrin Glycosyltransferase through Molecular Cloning Approaches" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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NORHAYATI BINTI RAMLI

Date: 10 July 2012

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