UNIVERSITI PUTRA MALAYSIA

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2013

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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Chair : Mohd Noriznan Mokhtar, PhD

Faculty : Faculty of Engineering

Cyclodextrin glycosyltransferase (CGTase, 2.4.1.19) is an extracellular hydrolytic enzyme which capable to convert starch into cyclodextrins (CDs) via cyclization activity. CDs are cyclic oligosaccharides consisted of six or more glucose units which are widely applied in various industries owing to their unique structure of hydrophobic inner cavity and hydrophilic exterior, which enable the CDs to form inclusion complexes with a variety of guest molecules. The purpose of this study was to introduce an effective approach on the recovery of CGTase from newly isolated *Bacillus cereus* fermentation broth and CDs from production media by reducing time and steps involved in the purification and recovery processes.

Aqueous Two Phase Systems (ATPSs) were applied for the recovery of CGTase and CDs to simplify the downstream processing of CGTase. Basic polyethylene-glycol (PEG)/citrate ATPS was performed to capture the enzyme CGTase from fermentation broth. Several ATPS parameters such as tie-line length (TLL), molecular weight of PEG, volume ratio (V_R), crude load and addition of neutral salt

were investigated and optimized in order to obtain the most effective ATPS for the CGTase recovery. Partial purification of *B. cereus* CGTase with yield (Y_T) of 70% was achieved on the 19.0% PEG and 11.5% citrate ATPS with TLL of 38.89% (w/w), V_R of 2.0, 20% (w/w) crude load and additional 4% (w/w) NaCl at pH 7.0.

The recovery of CGTase by ATPS was then improved by developing a recyclable ATPS in which the polymer PEGs were substituted by using copolymer, ethylene oxide-propylene oxide (EOPO). The capability of EOPOs to separate into two phase after heating above certain temperature enables the polymers to be recovered and re-utilized in subsequent ATPS. This novel study on the CGTase recovery is not only to simplify the CGTase purification steps, but also to reduce the cost and environmental impact. The purified *B. cereus* CGTase with a Y_T of 87% and purification fold (P_{FT}) of 13.1 was obtained from the EOPO/phosphate ATPS comprising TLL of 41.2% (w/w), V_R of 1.25 and crude load of 20% (w/w) at pH of 7.0.

Ionic liquids-based ATPS (ILATPS) was introduced as another ATPS approach for the purification of CGTase. Ionic liquids (ILs) were well known for their green properties in which they can be easily recycled, causing negligible impact to the environment. The rapid phase separation time and ability to enhance the biological activity of biomolecule has made the ILATPS an attractive purification method for CGTase. ILATPS was proved to be a better system for purification of CGTase which was able to purify *B. cereus* CGTase up to 13.9-fold with a Y_T of 96.2%.

Another aim of the study was focus on the extractive bioconversion of CGTase using ATPS. PEG/dextran ATPS has been constructed and sago starch was used as the substrate in the starch bioconversion of CDs. Optimum recovery (13.7mg/mL) of CDs was achieved in PEG 20000/dextran T500 ATPS at TLL of 26.2% (w/w) with

V_R of 4.0, addition of 20% (w/w) crude CGTase and 6% (w/w) of sago starch. ATPS enabled the production and recovery of CDs in a single step by replenishment of substrate and phase components at a regular time interval. A dynamic model of this ATPS was implemented to understand and simplify the reaction kinetics of starch bioconversion by CGTase.

The principal conclusion of this study was that ATPS has the potential to be practiced industrially for the recovery of CGTase and CDs for large scale productions and recoveries. In addition, the aqueous environment of ATPS provided a stable condition for the biological materials, which is well-suited as an extraction method for enzyme CGTase and CDs.

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

APLIKASI SISTEM DUA FASA AKUEUS DALAM PEMULIHAN CYCLODEXTRIN GLYCOSYLTRANSFERASE DAN SIKLODEKSTRINS

Oleh

NG HUI SUAN

Januari 2013

Pengerusi : Mohd Noriznan Mokhtar, PhD

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Cyclodextrin glycosyltransferase (CGTase, 2.4.1.19) merupakan enzim ekstrasellular dan hidrolitik yang berupaya untuk menukar kanji kepada siklodekstrins (CDs) melalui aktiviti pensiklikan. CDs adalah oligosakarida bulat yang terdiri daripada enam atau lebih unik glukosa dan telah digunakan secara meluas dalam pelbagai industri disebabkan oleh strukturnya yang unik di mana terdapat rongga dalaman yang hidrofobik dan luaran yang hidrofilik membolehkan CDs menbentuk kompleks dengan pelbagai molekul. Tujuan kajian ini adalah untuk memperkenalkan strategi yang ekonomik dan berkesan bagi penulenan enzim CGTase dari *Bacillus cereus* dan CDs dengan mengurangkan masa dan langkah-langkah yang terlibat dalam proses penulenan dan penangkapan CGTase dan juga CDs.

Sistem Dua Fasa Akueus (ATPS) telah digunakan dalam penulenan dan penangkapan CGTase dan CDs bagi memudahkan pemprosesan hiliran CGTase. ATPS asas yang terdiri daripada polietilena glikol (PEG)/sitrat telah digunakan untuk memperoleh enzim CGTase dari bahan fermentasi. Beberapa parameter ATPS

seperti panjang *tie-line* (TLL), berat molekul PEG, nisbah isipadu (V_R), berat enzim dan penambahan garam neutral telah dikaji dan dioptimumkan untuk memperoleh ATPS yang paling berkesan bagi pemulihan CGTase. Penulenan separa bagi enzim CGTase dari *B. cereus* dengan 70% hasil (Y_T) telah dicapai dengan ATPS yang terdiri daripada 19.0% (w/w) PEG dan 11.5% (w/w) sitrat dengan 38.89% (w/w) TLL, 4.0 V_R, 20% (w/w) berat enzim dan penambahan 4% (w/w) NaCl pada pH 7.0.

Protokol penulenan CGTase menggunakan ATPS kemudian diperbaiki dengan menggunakan ATPS kitar semula di mana PEG polimer telah digantikan dengan kopolimer, etilena oksida-propilena oksida (EOPO). Kebolehan EOPOs berpisah kepada dua fasa selepas pemanasan atas suhu tertentu membolehkan polimer ini digunakan semula dalam ATPS berikutnya. Kaedah penulenan CGTase ini bukan sahaja mempermudahkan langkah-langkah penulenan CGTase, tetapi juga mengurangkan kesan kepada alam sekitar. Hasil (Y_T) pemulihan sebanyak 87% dan 13.1 penulenan kali ganda (P_{FT}) enzim CGTase telah diperoleh dari *B. cereus* dengan menggunakan ATPS EOPO/fosfat yang terdiri daripada 41.2% (w/w) TLL, V_R sebanyak 1.25, dan 20% (w/w) berat enzim pada pH 7.0.

ATPS berasaskan cecair ionik (ILATPS) telah diperkenalkan sebagai ATPS baru untuk penulenan CGTase di mana cecair ionik (IL_S) yang terkenal dengan ciri-ciri mereka yang mudah dikitar semula dan tidak membahayakan alam sekitar. Masa permisahan fasa yang pendek dan keupayaan ILs untuk meningkatkan aktiviti biologi biomolekul telah menjadikan ILATPS satu kaedah penulenan yang menarik bagi CGTase. ILATPS telah dibuktikan sebagai satu sistem yang lebih baik bagi penulenan enzim CGTase dari *B. cereus* di mana ILATPS berupaya untuk

menulenkan enzim CGTase dari bahan fermentasi sehingga 13.9 kali ganda penulenan dengan Y_T sebanyak 96.2%.

Satu lagi matlamat kajian ini adalah untuk melakukan pemulihan dan penukaran kanji kepada CDs dengan menggunakan ATPS. ATPS PEG/dextran telah dibina dengan menggunakan kanji sagu sebagai substrat dalam proses penukaran kanji ini. Pemulihan optimum CDs (13.7 mg/mL) telah dicapai dalam ATPS PEG 20000/dextran T500 pada 26.2% (w/w) TLL, 4.0 V_R, penambahan enzim CGTase sebanyak 20% (w/w) dan 6% (w/w) kanji sagu. ATPS membolehkan penghasilan dan penulenan CDs dicapai dalam satu langkah dengan penambahan substrat dan komponen fasa pada masa tertentu. Satu ATPS model telah dilaksanakan bagi memahami dan memudahkan penukaran kanji oleh CGTase dengan penghasilan CD ini.

Kesimpulan utama kajian ini menyatakan bahawa ATPS mempunyai potensi sebagai amalan perindustrian dalam penulenan CGTase dan penghasilan CDs yang berskala besar. Tambahan pula, persekitaran akueus ATPS yang berkeadaan stabil telah menggalakkan penggunaannya dalam penulenan enzim CGTase dan CDs.

ACKNOWLEDGEMENTS

All of this work was performed at the Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia (UPM), Malaysia.

I sincerely thank to my supervisor, Dr.-Ing. Mohd Noriznan Mokhtar, Department of Process and Food Engineering, Faculty of Engineering, UPM for his priceless advices, guidance, valuable support and above all the confidence and optimism he showed in my ability and work.

My heartfelt gratitude to Professor Dr. Ling Tau Chuan, Institute of Biological Science, Faculty of Science, University of Malaya for granted me permission to pursue my postgraduate study in University Putra Malaysia before his transfer and for his kind assistance and support throughout my PhD study. Further, I would like to thank my co-supervisor, Professor Dr. Arbakariya B. Ariff for his professional guidance and moral support throughout my research.

Many thanks to my former supervisor, Dr. Syahida Ahmad, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, UPM for her inspirations, advices and support for my courage to pursue my PhD study. Also, I would like to thank Mr. Jonathan Lim Chee Woei, Faculty of Medicine, UPM for his kind assistance, motivation and encouragement all through my PhD program.

I would like to express my sincere appreciation to Dr. Edward Ooi Chien Wei, School of Engineering, Monash University Sunway Campus for his enthusiasm encouragement and guidance throughout my PhD study. I wish to thank to my fellow friends in Laboratory of Immunotherapeutic and Vaccines (LIVES) (Chee Wun, Joo

Shun, Pau Loke, Yin Hui and others) for their help and support. Special thanks to the staffs in LIVES and Faculty of Engineering for their kind assistance all these years.

I sincerely thank to my parents and family for their continued support, care and tolerance. Without them, I may not be able to complete my PhD program.

My sincere appreciation and gratitude to Universiti Putra Malaysia for their financial support throughout my postgraduate study by providing me the graduate research fellowship.

I certify that a Thesis Examination Committee has met on 31.01.2013 to conduct the final examination of Ng Hui Suan on her thesis entitled **"Aqueous two phase system application in the recovery of cyclodextrin glycosyltransferase and cyclodextrins"** 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 fulfillment 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.

NG HUI SUAN

Date: 31 January 2013

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