UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF CYCLODEXTRIN GLUCANOTRANSFERASE BY BACILLUS CIRCULANS P28 USING SAGO STARCH AS SUBSTRATE

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By

AZLINA MANSOR

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

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

PRODUCTION OF CYCLODEXTRIN GLUCANOTRANSFERASE BY *BACILLUS CIRCULANS* P28 USING SAGO STARCH AS SUBSTRATE

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Chairman: **Professor Arbakariya Ariff, PhD**

Faculty: **Institute of Bioscience**

The present study was undertaken to address the needs to search for novel cyclodextrin glucanotransferase (CGTase) with improved properties due to the vast diversity of cyclodextrin (CDs) applications. Lack of information pertaining to the limitation in CGTase production using sago starch, an abundant raw material available in Malaysia as substrate, has also led to this research direction. In this study, fermentation of CGTase by a locally isolated bacterium, *Bacillus circulans* P28, was first performed in shake flasks to optimize medium formulation and culture conditions. The fermentation process was then transferred to 2 L stirred tank bioreactor, where the requirement for aeration and agitation were studied in batch fermentation mode. Subsequently, fed-batch cultures were conducted to overcome the problem related to the effect of catabolite repression for enhancement of CGTase production.

CGTase from *B. circulans* P28 was purified to homogeneity up to 20 fold by 40-60% ammonium sulphate precipitation and anion exchange DEAE-Cellulose with 23% recovery. The enzyme was a monomer with an estimated molecular weight of 33 kDa

on the SDS-PAGE using 12% acrylamide gel electrophoresis. The enzyme was stable and active at a broad pH range (6 to 10) and was optimally active at 65°C, indicating that this enzyme may have potential for industrial application in CD production. Thermal stability was improved in the present of 2 mM $CaCl_2$ and was stable up to 60°C for 1 h. Sago starch was the most preferred substrate for CD production by CGTase from *B. circulans* P28 as compared to other starches tested in this study (potato, soluble and tapioca starch). In the enzymatic reaction, mainly β-CD (78%) and γ-CD (22%) were produced, suggesting a simpler and easier CDs separation processes required in the downstream processing.

The optimal conditions for CGTase fermentation by *B. circulans* P28 in shake flask were at 30°C and in the presence of 1% (w/v) Na_2CO_3 (which associated with an initial culture pH of 10.2). Optimization of the culture medium using full factorial design (FFD) approach has revealed that sago starch, yeast extract and interaction of sago starch and yeast extract significantly affected CGTase production by *B. circulans* P28. In fermentation using the optimized medium composition (3 g/L sago starch and 17.83 g/L yeast extract) which corresponded to C/N ratio of 0.8, the maximum CGTase activity obtained was 6.96 U/mL, which was about 45% higher than that obtained in non-optimized medium (4.6 U/mL).

Production of CGTase by *B. circulans* P28 in 2 L stirred tank bioreactor increased propotionally with the increase in agitation speed, ranging from 400 to 900 rpm though growth was slightly inhibited at agitation speed of above 600 rpm. The shear forces created by the impeller at high agitation speed had caused cell distruption and affected the cell viability, thus, resulted in an increase in CGTase activity. The highest CGTase activity (9.6 U/ml) in batch fermentation using stirred tank bioreactor was obtained at; agitation speed of 600 rpm; air-flow rate of 0.5 vvm; initial culture pH of 10.8 without

pH control during the fermentation and temperature of 30°C. This gave an improvement of 44% as compared to that obtained in optimum fermentation using shake flask.

Further improvement in CGTase production by *B. circulans* P28 was achieved through the implementation of fed-batch fermentation. The highest overall enzyme yield (7400 U/g) and productivity (1500 U/L/h) was obtained in fed-batch fermentation fed with low concentration of starch (3 g/L) in the feed at a constant feeding rate of 0.36 g/L/h. This gave an improvement in term of maximum CGTase activity and productivity, by 104% and 49%, respectively, as compared to conventional batch fermentation. This study indicated that fed-batch fermentation is a good alternative for CGTase production by overcoming the catabolic repression effect. Many different approaches used in this study to overcome the limitations of CGTase production may find vast applications in obtaining high enzyme productivities using other CGTase-producing microorganisms and perhaps also for other starch-converting enzymes.

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

PENGHASILAN SIKLODEKSTRIN GLUKANOTRANSFERASE OLEH *BACILLUS CIRCULANS* P28 MENGGUNAKAN KANJI SAGU SEBAGAI SUBSTRAT

Oleh

AZLINA BINTI MANSOR

Januari 2013

Pengerusi: **Prof. Arbakariya Ariff, PhD**

Fakulti: **Institut Biosains**

Penggunaan siklodekstrin (CDs) yang sangat meluas telah mendorong pencarian enzim siklodekstrin glukanotransferase (CGTase) baru dengan ciri-ciri penambahbaikan. Maklumat mengenai penghasilan enzim CGTase menggunakan kanji sagu sebagai substrat yang amat terhad juga telah mendorong supaya penyelidikan ini dijalankan. Dalam kajian ini, fermentasi enzim CGTase oleh bakteria pencilan tempatan, *B. circulans* P28 telah dijalankan menggunakan kelalang goncangan bagi tujuan pengoptimuman formulasi medium dan keadaan pengkulturan. Seterusnya, proses fermentasi dijalankan menggunakan bioreaktor 2 L bagi mengkaji keperluan pengudaraaan dan pengadukan melalui kaedah sistem fermentasi sesekelompok. Masalah penindasan katabolit telah dapat diatasi melaui pendekatan fermentasi suapan sesekelompok telah dapat meningkatkan penghasilan enzim CGTase.

Tahap penulenan enzim CGTase daripada *B. circulans* P28 telah dijalankan sehingga 20 kali ganda melalui kaedah pemendakan menggunakan ammonium sulfat (40-60%) dan

pertukaran anion DEAE-selulose dengan peratus perolehan semula sebanyak 23%. Enzim ini merupakan sejenis monomer dengan berat anggaran 33 kDa yang ditentukan melalui kaedah SDS-PAGE menggunakan 12% gel akrilamida. Enzim ini berpotensi untuk diaplikasikan dalam industri penghasilan siklodektrin (CD) kerana cirinya yang aktif dan stabil pada julat pH yang luas (6-10) serta optimum pada suhu 65°C. Kestabilan terma dapat ditingkatkan melalui penambahan 2 mM $CaCl_2$, yang membolehkan ia stabil sehingga suhu 60°C untuk tempoh 1 jam. Kanji sagu merupakan substrat terbaik bagi menghasilan siklodekstrin daripada enzim CGTase daripada *B. circulans* P28 berbanding kanji jenis lain (kanji ubi kentang, kanji larut dan kanji ubi kayu). Tindakbalas enzimatik yang spesifik telah menghasilkan β-CD (78%) dan γ-CD (22%) akan menyumbangkan kepada pengasingan siklodekstrin yang lebih mudah dan ringkas dalam proses hiliran.

Hasil kajian mendapati keadaan pengkulturan yang optimum bagi penghasilan enzim CGTase oleh *B. circulans* P28 di dalam kelalang goncangan adalah pada suhu 30°C dengan kehadiran 1% Na_2CO_3 (menghasilkan pH awal medium 10.2). Pengoptimuman kultur medium menggunakan rekabentuk penuh faktorial (FFD) telah membuktikan bahawa kanji sagu, ekstrak yis dan interaksi di antara kanji sagu dan ekstrak yis mempunyai kesan signifikan terhadap penghasilan enzim CGTase oleh *B. circulans* P28. Fermentasi menggunakan komposisi medium yang telah dioptimumkan (3 g/L kanji sagu dan 17.83 g/L ekstrak yis) dengan nisbah C: N bersamaan 0.8, telah menghasilkan aktiviti maksimum CGTase sebanyak 6.96 U/ml, iaitu 45% lebih tinggi berbanding penghasilan enzim menggunakan medium tidak optimum (4.6 U/mL).

Penghasilan enzim CGTase oleh *B. circulans* P28 dalam bioreaktor 2 L telah meningkat secara perkadaran terus dengan peningkatan halaju adukan pada julat 400 ke 900 rpm, walaupun pertumbuhan sel adalah sedikit direncatkan pada halaju adukan melebihi 600

rpm. Daya ricihan yang dihasilkan oleh pengaduk pada halaju adukan yang tinggi telah memberi kesan terhadap viabiliti sel dan menyebabkan pemecahan sel, seterusnya meningkatkan aktiviti enzim CGTase di dalam kultur. Melalui kaedah fermentasi sesekelompok, aktiviti enzim CGTase tertinggi (9.6 U/mL) dapat dihasilkan pada keadaan pengkulturan berikut: halaju adukan pada 600 rpm; pengudaraan pada 0.5 vvm; pH awal medium 10.8 pada suhu 30°C. Penghasilan enzim menggunakan bioreaktor adalah 44% lebih tinggi berbanding fermentasi menggunakan kelalang goncangan.

Proses penambahbaikan penghasilan enzim CGTase oleh *B. circulans* P28 selanjutnya telah dicapai melalui kaedah fermentasi suapan sesekelompok. Sebanyak 7400 U/g jumlah hasil enzim dan 1500 U/L/h produktiviti telah diperolehi menerusi fermentasi suapan sesekelompok menggunakan suapan kanji sagu berkepekatan rendah (3 g/L) pada kadar suapan tetap (0.36 g/L/h). Kedua-dua aktiviti dan produktiviti maksimum CGTase masing-masing telah metingkatkan sebanyak 104% dan 49%, berbanding kaedah konvensional fermentasi sesekelompok. Kajian ini telah membuktikan bahawa fermentasi suapan sesekelompok merupakan kaedah alternatif yang lebih baik bagi mengatasi masalah kesan penindasan katabolit dalam penghasilan CGTase. Kepelbagaian pendekatan yang digunakan bagi mengatasi kekangan dalam penghasilan enzim CGTase dalam kajian ini boleh diaplikasikan untuk meningkatkan produktiviti enzim CGTase oleh mikroorganisma lain serta dalam penghasilan enzim pengurai kanji yang lain.

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I certify that a Thesis Examination Committee has met on **29 January 2013** to conduct the final examination of Azlina Mansor on her thesis entitled **"Production Of Cyclodextrin Glucanotransferase By *Bacillus circulans* P28 Using Sago Starch As Substrate"** in accordance with Universities and University College 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.

Members of the Examination Committee were as follows:

Shuhaimi Mustafa, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Siti Mazlina Mustapha Kamal, PhD
Associate Professor
Faculty of Engineering
Universiti Putra Malaysia
(Internal Examiner)

Umi Kalsom Md. Shah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Muhammad Asghar, PhD
Associate Professor
Department of Chemistry and Biochemistry
University of Agriculture,
Faisalabad, Pakistan.
(External Examiner)

_____
NORITAH OMAR, **PhD**
Assoc. Professor and Deputy Dean
School of Graduate studies
Universiti Putra Malaysia

Date: 26 June 2013

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:

Arbakariya Ariff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Rosfarizan Mohamad, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Raha Abd. Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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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AZLINA BINTI MANSOR

Date : 29 January 2013

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LIST OF ABBREVIATIONS

CGTase	Cyclodextrin glucosyltransferase
CD	Cyclodextrin
CCR	Carbon catabolite repression
CCD	Central composite design
FFD	Full factorial design
RSM	Response surface methodology
DP	Degree of polymerization
DOT	Dissolved oxygen tension
DO	Dissolve oxygen
K_m	Michealis Menten constant
V_{max}	Maximum reaction velocity
K_La	Volumetric oxygen transfer rate
QO_2	Specific oxygen uptake rate
OD	Optical density
CFU	Colony forming unit
BSA	Bovine serum albumin
DNS	Dinitrosalicylic acid
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel
ANOVA	Analysis of varians
2 FI	Two factor interaction
DCW	Dry cell weight
P_m	Maximum CGTase production
X_m	Maximum cell concentration

S_{Gmax}	Maximum concentration of accumulated reducing sugar
«$_{max}$	Specific growth rate
$Y_{P/S}$	Yield of enzyme on the basis of starch concentration used
vvm	Volumetric airflow rate/liquid volume
rpm	Rotation per minute
D_i	Diameter of impeller
D_t	Diameter of tank
H_i	Height of impeller
H_L	Height of liquid in reactor
L	Width of impeller
W_i	Height of impeller

CHAPTER 1

INTRODUCTION

Cyclodextrin glycosyltransferase (EC 2.4.1.19) is an enzyme that converts starch and other derivatives into non-reducing, cyclic malto-oligosaccharides called cyclodextrins (CDs) via intramolecular transglycosylation reaction. There are mainly 3 major types of CDs namely; α-, β- and γ-CDs which consist of 6, 7 and 8 glucose molecules, respectively. The CD molecules have a unique structure of a hydrophobic interior cavity and hydrophilic exterior surface that enable them to encapsulate other molecules within their cavities and form inclusion complexes with a wide variety of solid, liquid and gaseous compounds. The molecular encapsulation changes the physical and chemical properties of the included molecules and has therefore open up to a wide application in many industries such as pharmaceutical, cosmetic, agricultural, chemical and food industries (Moriwaki *et al.*, 2007).

The diversity of CDs applications creates the needs to search for CGTase with novel and improved properties. The identification of new microbial sources for CGTase is important in finding enzyme with superior properties and can best suit the industrial processes. Efforts by many investigators have been oriented mainly on the purification and characterization of the bacterial CGTase for the last 30 years (Tonkova, 1998). Although the potential of cyclodextrin is well documented, the market of cyclodextrin is limited by their high cost. Two main constrains that limit the general use of cyclodextrin were due to their high cost and also the limited availability of especially α- and γ-CGTase enzymes. In general, all known CGTases produce a mixture of α-, β- and γ-CDs

in different ratios, depending on the enzyme source (Martins and Hatti-Kaul, 2002; Moriwaki *et al*., 2007). As separation of different CDs from a mixture is time-consuming, tedious and expensive, CGTases that can synthesize predominantly one type of CD can reduce subsequent purification costs and hence, are of interest and commercially desired (Gawande and Patkar, 2001). The low cost processes for CDs production can be achieved only if CGTases that favour kinetically the formation of specifically a particular type of CD can be developed and produced economically.

Medium composition and fermentation conditions greatly influenced the production of CGTases by microorganisms (Rosso *et al*., 2002; Ibrahim *et al*., 2005). The fermentation performance, in term of product yield, final product concentration and overall productivity is varied depending on the medium composition and culture condition, which are highly strain specific. In commercial practice, optimization of medium with regards to development of cost effective fermentation medium is important in order to maintain the balance between various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Furthermore, application of statistical design approach such as response surface methodology (RSM) and full factorial design (FFD) are commonly practiced in bioprocess optimization. Such approach enable the study on the effects of factors influencing the responses simultaneously (Adinarayana and Ellaiah, 2002). This approach is also beneficial to overcome the limitations in the conventional method of optimization which is time consuming and expensive. Optimization of medium composition using Central Composite Design (CCD) has resulted in an increase of 53% in production of β-CGTase from *Bacillus* G1 (Ibrahim *et al*., 2005). Furthermore, application of factorial design for

optimization of CGTase from *Klebsiella pneumoniae pneumoniae* AS-22 have resulted an increase of about 9 fold in enzyme activity compared to that obtained in the basal media (Gawande and Patkar, 1999).

Most CGTases are produced by bacteria of the genus *Bacillus*, mainly by the aerobic alkalophilic strains (Pinto *et al.*, 2007) and fermentation of CGTase production were generally performed in the submerged batch fermentation (Tonkova, 1998). Thus, process variables such as dissolve oxygen tension through the control of fermentation parameters of agitation speed and air flow rates are important and should be taken into account as sufficient oxygen supply may boost enzyme production and reduce production cost (Vassileva *et al.*, 2005).

The bacterial CGTases are generally inducible enzymes and require some form of starch for induction (Gawande *et al.*, 2003). However, employment of starch as carbon source had caused the formation and accumulation of severals types of simple metabolisable sugars or malto-oligosaccharides during the process of starch hydrolysis (Zain *et al.*, 2007) and thus, have led to the carbon catabolite repression (CCR) effect which inhibit the production of CGTases by many microorganisms (Vassilleva *et al.*, 2003; Wang *et al.*, 2006). An alternative way to overcome the effect of CCR is by conducting fed-batch fermentation. Several fed-batch approaches have been conducted using different types of carbon sources or feeding concentration, different fed-batch modes or feeding rates which demonstrated various effects on CGTase production (Wang *et al.*, 2006; Pinto *et al.*, 2009; Gawande *et al*., 2003; Zain *et al.*, 2007).

From the aspects mentioned above, carefully designed experiments need to be conducted with regard to overcome the limitations that commonly occur in the production of bacterial CGTases. The present study was undertaken to establish a high performance CGTase fermentation process by a locally isolated strain, *B. circulans* P28, using sago starch as substrate. The specific objectives of this study were:

1. to screen, isolate and screen for CGTase-producing bacteria from local resources, identify the strains and characterize the enzyme properties produced by the locally isolated strain,
2. to investigate the optimum culture conditions and nutrients requirement that enhance maximum CGTase production (carbon source, nitrogen source, initial culture pH and temperature) via conventional method, followed by factorial design approach and finally determine the effect of C/N ratio on CGTase fermentation performance in shake flasks,
3. to assess the operational of fermentation conditions (agitation speed and air flow rates) effects and their kinetics fermentation performance such as enzyme yield, productivity and specific growth rate in 2 L bioreactor
4. to evaluate the feasibility of using fed-batch for enhanced CGTase production and assess the effect of operation at different starch concentrations in the feed stream and varying the feeding rates on kinetic parameters such as enzyme activity, enzyme and biomass yield and productivity .

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