UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND CHARACTERISATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM A LOCALLY ISOLATED BACILLUS SP.

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By

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PRODUCTION AND CHARACTERISATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM A LOCALLY ISOLATED *BACILLUS* SP.

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Chairman: **Associate Professor Suraini Abd. Aziz, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Cyclodextrin glycosyltransferase (E.C.2.4.1.19) synthesise cyclic oligosaccharide which is also known as cyclodextrin, from starch. Most of the known CGTases produce a mixture of α-, β- and γ-CD at different ratios. CGTase producing microorganism was isolated from local soils on selective agar medium containing soluble starch which produced clear zones as qualitative measurement of the enzyme present. A total of 250 isolates were collected but only one isolate (Strain MK 6) was selected for further studies based on its highest activity. Strain MK 6 was identified as gram positive rod, motile and produced spore. Biochemical identification using API CHB/E medium confirmed the strain MK 6 was the *Bacilllus* sp with 85% similarities. CGTase isolated from alkalophilic *Bacillus* sp. was further characterized. Optimum activity obtained at temperature of 70^{o}C and the enzyme shows a wide range of pH stability ranging from 4 -10 when stored at 4^{o}C for 24 hours and temperature stability ranging from 30^{o}C -

80^{o}C at 1 h incubation period. The CGTase activity was even maintained at 0.4 U/ml at 90^{o}C for 40 min incubation. Prior to optimisation of CGTase production, selection for the best carbon source through detection on modified phenolphthalein method containing different types of starch were performed. Sago starch gave significant result and was used for further optimisation using statistical analysis namely Response Surface Methodology (RSM). The optimal calculated values were 3.34% sago starch, initial pH of 10.15 and agitation speed of 187 rpm; with predicted activity of 2.07 U/ml of CGTase. These predicted optimal parameters were confirmed in the laboratory and the final CGTase activity obtained was very close to the predicted value at 2.56 U/ml. The optimised crude enzyme produced mainly β-CD (61.6% of the total cyclodextrin amount) with only α-CD as minimal product without detection of γ-CD.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN DAN PENCIRIAN ENZIM SIKLODEKSTRIN GLIKOSILTRANSFERASE DARIPADA *BACILLUS* SP. TEMPATAN

Oleh

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Enzim siklodekstrin glikosiltransferase, CGTase (E.C. 2.4.1.19) merupakan enzim yang bertanggungjawab dalam penghasilan gelung oligosakarida atau lebih dikenali sebagai siklodekstrin (CD) daripada kanji. Kebanyakan enzim CGTase menghasilkan campuran α-, β- dan γ-CD pada nisbah yang berbeza. Pemencilan mikroorganisma penghasil enzim CGTase, dari sumber tanah tempatan menggunakan agar khusus yang mengandungi kanji terlarut, akan membentuk zon cerah dan lutsinar di sekeliling koloni mikroorganisma sebagai ukuran kualitatif kehadiran enzim. Sejumlah 250 koloni bacteria berbeza yang menghasilkan enzim CGTase telah berjaya dipencilkan, tetapi hanya satu koloni strain (MK 6) telah dipilih berdasarkan aktiviti enzimnya yang tertinggi, untuk kajian selanjutnya. Strain MK 6 dikenalpasti sebagai bakteria gram positif, berbentuk rod, bersifat motil dan menghasilkan spora. Pengenalpastian menggunakan medium API CHB/E ini mengesahkan bahawa strain MK 6 adalah dari genus *Bacillus* dengan 85% persamaan. Pencirian enzim CGTase

menunjukkan aktiviti optimum pada suhu 70^{o}C dan kestabilan suhu pada julat 30^{o}C-80^{o}C. Enzim yang dipencilkan ini masih mengekalkan aktivitinya pada 0.4 U/ml untuk 40 minit bagi suhu pengeraman 90^{o}C. Untuk proses pengoptimuman , saringan bagi penentuan sumber karbon untuk penghasilan enzim CGTase dilakukan menggunakan kaedah terubahsuai fenolftalein menggunakan pelbagai jenis kanji terlarut yang lain. Kanji sagu didapati merupakan kanji yang paling sesuai bagi penghasilan enzim CGTase yang tinggi. Penghasilan enzim CGTase seterusnya dioptimumkan menggunakan analisa statistic yang dikenali sebagai kaedah *tidakbalas permukaan* (RSM). Nilai optima yang diperolehi adalah seperti berikut: 3.34% kepekatan kanji sagu, pH awalan 10.15 dan kadar goncangan pada 187 rpm untuk memperolehi nilai jangkaan enzim sebanyak 2.07 U/ml. Eksperimen sebenar menggunakan nilai optima parameter yang diberikan, memberikan kepekatan enzim sebanyak 2.56 U/ml, dimana ia adalah hampir dengan nilai jangkaan. Enzim CGTase yang dioptimumkan ini didapati menghasilkan β-CD sebagai hasil utama (61.6% daripada jumlah CD yang dihasilkan), manakala α-CD sebagai hasil sampingan tanpa penghasilan γ-CD.

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My deepest appreciation to my parents and siblings for their unconditional love, sacrifice and support throughout these years. To Kok Ming, thank you for putting up with me, standing by me through high and low. All of you have made my life more colourful and no words could express my gratitude and my love for all of you.

I certify that an Examination Committee met on 14th November 2006 to conduct the final examination of Sauvaphap a/p Ai Noi on her Master of Science thesis entitled "Isolation and Characterisation of Cyclodextrin Glycosyltransferase (CGTase) Producing ***Bacillus***" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the examination Committee are as follows:

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Date: 8 FEBRUARY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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Name: SAUVAPHAP A/P AI NOI

Date : 28 OCTOBER 2006

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CD	Cyclodextrin
CGTase	Cyclodextrin Glycosyltransferase
DF	Degree of freedom
g	gram
g/L	gram per liter
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
M	Molar
mg	milligram
mg/ml	milligram per milliliter
$MgSO_4.7H_20$	Magnesium sulphate heptahydrates
NA	Nutrient Agar
NaCl	Sodium Chloride
MBS	Maltose Biding Site
PHP	Phenolphthalein
Rpm	Revolutions per minute
U/ml	Unit per milliliter
% w/v	Percentage weight per volume
μm	micrometer

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CHAPTER 1

INTRODUCTION

Researches around the world had, are and still isolating powerful microorganisms, which are able to secrete powerful enzyme. Isolation of microorganisms was done in all types of environment, ranging from acidic to alkaline environment. However it is of importance to note that "moderate" environment is essential to support life. Moderate environment usually means growth if living beings at near neutral to neutral pH, temperature between 20^{o}C and 40^{o}C, air pressure of 1 atm and adequate concentration of nutrients and salt (Horikoshi, 1990). In nature, the existence of extreme environment for instance acidic or hot springs, saline lakes, desserts and alkaline lakes would seem too harsh for life. Surprisingly, many organisms of industrial importance have been found in such extreme environment. For instance CGTase enzyme has been isolated mostly from alkaline lakes. Some researchers even have isolated the enzyme from hot springs. Malaysia (a humid country with moderate temperature) however, does not possess any alkaline lakes or soils. Isolation of CGTase enzyme was done mainly from hot springs.

In this research however, attempt to isolate CGTase enzyme from local soils organisms were done. Local soils, although mainly of neutral or a little acidic, may contain some alkalophillic microorganisms. However, the chances of occurrence of alkaline organisms in non-alkaline environment are only about 1/10 (Horikoshi, 1990). Only with the establishment of a rapid and sensitive method in detection of alkaline microorganism that produces CGTase enzyme

was done, further enzyme studies such as optimisation and characterisation can be carried out.

Cyclodextrin glycosyltransferase (CGTase) or [1, 4 - α-D-glucopyranosyl]-transferase is an extracellular enzyme, which degrades starches into cyclodextrin (CDs) molecules via cyclisation reaction. Cyclisation happens when a linear oligosaccharide (starch) chain is cleaved and the new reducing end sugar is transferred to the non-reducing end sugar of the same chain. Therefore cyclodextrins are cyclic oligosaccharides consisting of 6-12 units of glucose joined by the α-1, 4-linkages. CGTases also catalyses two intermolecular transglycosylation reactions: coupling, in which a cyclodextrin ring is cleaved and transferred to an acceptor maltooligosaccharide substrate and disproportionation, in which a linear maltooligosaccharide is cleaved and the new reducing end sugar is transferred to an acceptor maltooligosaccharide substrate. Besides these reactions, the enzyme has a weak hydrolysing activity (Penninga *et al*., 1995; Bart *et al*., 2000) (see Figure 1.1). Cyclodextrins with 6, 7 and 8 glucose units are most common and also known as α-, β- and γ- cyclodextrin, respectively.

CGTases with varying properties are produced by bacteria mainly belonging to the *bacillus* species, by submerged culture in a complex medium (Adriana, 2002). Some of the known sources of CGTase producers are *Bacillus macerans*, *Bacillus subtilis*, *Bacillus stereothermophillus*, *Bacillus megaterium*, *Klebsiella pneumonia* and micrococcus species. Alkalophilic microorganism is also known to produce unusual enzyme that can be used in industrial and other processes. All

known CGTases (Bart *et al*., 2000) produce a mixture of cyclodextrins (and linear malto-oligosaccharides) when incubated with starch. The CGTase crude enzyme isolated from local *Bacillus* sp. produces alpha (α) and beta (β) cyclodextrin only. However a CGTase, which only produces a single type of cyclodextrin, is industrially favorable. Figure 1.1 show a schematic representation of the CGTase catalysed reactions.

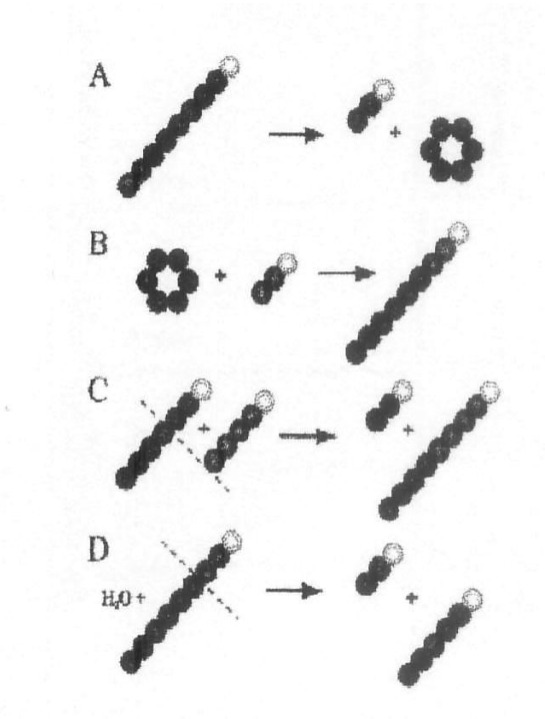

Figure 1.1 Schematic diagram of CGTase catalysed reaction.The circles represent glucose residues whilst the white circles indicate the sugars reducing end. (A) cyclisation, (B) coupling, (C) disproportionation, (D) hydrolysis

Therefore the objectives of this research are divided into three:

1. Screening, isolation and characterization of CGTase producing microorganism from local soils
2. Optimisation using statistical analysis namely response surface methodology (RSM) and characterization of the local isolated microbes in production of CGTase enzyme
3. Production of cyclodextrin (CD)

CHAPTER 2

LITERATURE REVIEW

2.1 Starch

Most green plants produce starch as a means of energy storage. It is deposited (100 μm) in special organelles (chloroplasts and amyloplasts). These tiny white granules exists in various parts of plants, for example in cereal grains (maize, wheat), in roots (potatoes). These granules are insoluble in cold water (Swinkels, 1985). The size and shape of the granules are peculiar to each variety of starch.

Starch is actually a polymer composed of glucose units primarily linked by the α(1-4) glucose linkages that make the amylase and additional α(1-6) linkages that makes amylopectin. Starch usually consists of a mixture of two types of polymers; amylase and amylopectin. Amylose is a much more linear polymer since the frequency (0.3 – 0.7% of total starch content) is much smaller than in amylopectin (4-5%). Amylopectin, a branched polymer consist of linear chains of 20-24 α(1-4)-linked D glucose connected by a α(1-6)-D-glucosidic linkages, thus forming a branched chain (Hizukuri, 1996).

The world production of industrial starch increases steadily and primary demand of starch includes:

i) High fructose syrups (especially in the USA and to a lesser extend in Europe.

ii) Glucose syrups for fermentation purposes