



UNIVERSITI PUTRA MALAYSIA

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

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CYCLODEXTRIN GLYCOSYLTRANSFERASE
FROM A LOCALLY ISOLATED *BACILLUS* SP.**

By

SAUVAPHAP A/P AI NOI

**Thesis Submitted to the School of Graduate Studies, Universiti
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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

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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 *Bacillus* sp with 85% similarities. CGTase isolated from alkalophilic *Bacillus* sp. was further characterized. Optimum activity obtained at temperature of 70°C and the enzyme shows a wide range of pH stability ranging from 4 -10 when stored at 4°C for 24 hours and temperature stability ranging from 30°C -



80°C at 1 h incubation period. The CGTase activity was even maintained at 0.4 U/ml at 90°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°C dan kestabilan suhu pada julat 30°C-80°C. Enzim yang dipencilkan ini masih mengekalkan aktivitinya pada 0.4 U/ml untuk 40 minit bagi suhu pengeraman 90°C. Untuk proses pengoptimuman, saringan bagi penentuan sumber karbon untuk penghasilan enzim CGTase dilakukan menggunakan kaedah terubahsuai fenoltalein 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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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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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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TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	iv
ACKNOWLEDGEMENTS	vi
APPROVAL SHEETS	vii
DECLARATION FORM	ix
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvi
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	5
2.1 Starch	5
2.1.1 Raw Starch Degrading Enzymes	6
2.1.2 The Use of Sago Starch	10
2.2 Cyclodextrin Glycosyltransferase (CGTase)	12
2.2.1 CGTase Catalysed Reactions	12
2.2.2 Raw Starch Binding Domain of CGTase	14
2.2.3 Substrate Binding of CGTase	15
2.2.4 Production of Bacterial CGTase	16
2.2.5 Properties of Bacterial CGTase	18
2.3 Isolation and Distribution of Alkalophilic Microorganisms	21
2.4 Cyclodextrin	22
2.4.1 History	22
2.4.2 Production of Cyclodextrin	25
2.4.3 Application of Cyclodextrins	27
2.5 The use of CDase to Understanding CD?	31
3 GENERAL MATERIALS AND METHODS	33
3.1 Chemicals	33
3.2 Screening and Isolation of Microorganism	33
3.3 Assay for CGTase Activity on Phenolphthalein-Methyl Orange-containing (PHP) Solid Medium	34
3.4 Modified Horikoshi-Phenolphthalein (PHP) Method	34
3.5 Culture Conditions	35
3.6 Preparation of Bacterial Inoculums	35
3.7 Total Cells Count	36
3.8 Colony Morphology	37
3.9 Cellular Morphology	37
3.9.1 Gram Staining	37



3.9.2	Endospore Staining	38
3.9.3	Motility Testing	38
3.10	Biochemical Identification of Microorganism	38
3.10.1	Catalase Test	38
3.11	Production of Crude Cyclodextrin Glycosyltransferase (CGTase)	39
3.12	Optimisation using Response Surface Methodology (RSM) Approach	39
3.13	Analytical Methods	42
3.11.1	Assay of CGTase	42
3.11.2	Protein Determination	42
3.11.3	Determination of Cyclodextrin and Sugars	43
4	ISOLATION, SCREENING AND IDENTIFICATION OF CGTase PRODUCING MICROORGANISMS	44
4.1	Introduction	44
4.2	Materials and Methods	45
4.2.1	Chemicals	45
4.2.2	Isolation of Microorganism	45
4.3	Morphological Characterisation	45
4.3.1	Colony Morphology	45
4.3.2	Cellular Morphology	46
4.3.3	Biochemical Identification of Microorganism	46
4.3.4	Catalase Test	46
4.4	Physiological Study	46
4.5	Results and Discussion	47
4.5.1	Isolation of Microorganisms	47
4.5.2	Morphological Characteristics	49
4.5.3	Biochemical Identification of Strain MK 6 using API CHB/E Medium	51
4.5.4	Physiology Characterisation	52
4.6	Conclusions	57
5	OPTIMISATION AND CHARACTERISATION OF CGTase PRODUCTION	58
5.1	Introduction	58
5.2	Materials and Method	59
5.2.1	Chemicals	59
5.2.2	Characterisation of CGTase Enzyme Produced	59
5.3	Optimisation on CGTase Production	60
5.3.1	Preparation of Bacterial Inoculums	60
5.3.2	Cell Growth Estimation	60
5.3.3	Assay of CGTase	59
5.4	Results and Discussion	61
5.4.1	Effect of pH on CGTase Activity and Stability	61

5.4.2	Effect of Temperature on CGTase Activity and Stability	64
5.4.3	Growth Profile for CGTase Production	67
5.4.4	Different Types of Starches on the Effect of CGTase Production	69
5.4.5	Optimisation of CGTase Production by RSM Approach	72
5.5	Conclusions	80
6	PRODUCTION OF CYCLODEXTRIN (CDs)	82
6.1	Introduction	83
6.2	Materials and Method	83
6.2.1	Materials	83
6.2.2	Optimisation of the Solvent System and Separation of CDs	83
6.2.3	Production of Cyclodextrins	83
6.2.4	Sample Preparation	84
6.3	Results and Discussion	84
6.3.1	Optimisation of Solvent System for Separation of CDs	84
6.3.2	Determination of Cyclodextrin	86
6.4	Conclusions	92
7	CONCLUSIONS	94
	REFERENCES	97
	APPENDICES	107
	BIODATA OF AUTHOR	123
	LIST OF PUBLICATIONS	125



LIST OF TABLES

Table		Page
2.1	Action of different raw starch degrading enzymes and basis for classification	7
2.2	Cyclodextrins properties	24
3.1	Actual factor levelsl corresponding to the coded factor levels	41
4.1	Summary of morphological characteristics of Strain MK 6	51
4.2	Effect of Na ⁺ ions on Growth of Alkalophilic <i>Bacillus</i> sp.	53
4.3	Effect of Na ⁺ ions on Growth of Strain MK 6	54
5.1	Comparison of the CGTase properties of <i>Bacillus</i> sp. MK 6 with those from other <i>Bacillus</i> sp.	63
5.2	Comparisons of yield of CGTase among different starches used	71
5.3	Analysis of variance for the regression model of response <i>Y</i> obtained from the response surface experiment	74
5.4	Regression coefficients, t-value and p-value of second-order Response surface equation for yield of CGTase enzyme, <i>Y</i>	75
6.1	The retention time of CDs	85
6.2	Production of cyclodextrin at different incubation time	89

LIST OF FIGURES

Figure	Page
1.1 Schematic diagram of the CGTase catalysed reaction	3
2.1 Action of enzymes involved in the degradation of starch.	9
2.2 Scheme of the cyclization reaction of CGTase.	13
2.3 The five domains, A, B, C, D, and E in cyclodextrin glycosyltransferase (CGTase)	14
2.4 Chemical structure of cyclodextrin	24
4.1 Assaying of Cyclodextrin Glycosyltransferase (CGTase) Activity on Modified Phenolphthalein-Methyl Orange Solid Medium of Strain MK 6.	47
4.2 Morphological characteristic of strain MK 6.	49
4.3 Formation of endospore of Strain MK 6 in Gram-stain preparation	49
4.4 Effect of different concentration of NaCl on the growth of strain MK 6.	53
4.5 Growth profile of Strain MK 6 at different temperatures	56
5.1 Effect of pH on the enzyme activity of <i>Bacillus</i> MK 6 after 10 minutes incubation	61
5.2 pH stability of CGTase from <i>Bacillus</i> MK 6 after 24 hours incubation at 4°C, 30°C and 37°C, respectively	64
5.3 Effect of temperature on the activity of CGTase isolated from <i>Bacillus</i> MK 6 after 10 minutes incubation.	65
5.4 Temperature stability of crude enzyme at 60 minutes of incubation time	66
5.5 Production profile of CGTase by <i>Bacillus</i> MK 6	67

5.6 Comparisons between carbon sources in CGTase production by <i>Bacillus</i> MK 6	70
5.7 Influence of pH and % sago starch on production of CGTase	76
5.8 Influence % sago starch and agitation on production of CGTase	78
5.9 Influence of pH and agitation on production of CGTase	78
5.10 Correlation between observed response and predicted value	80
6.1 Liquid chromatogram of the separation of cyclodextrins.	85
6.2 Liquid chromatography chromatogram of sample without any treatment.	88
6.3 Time course of CDs production by <i>Bacillus</i> MK 6's CGTase.	90



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
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	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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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.

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 of living beings at near neutral to neutral pH, temperature between 20°C and 40°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, deserts 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 alkalophilic 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 stercorophilus*, *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.

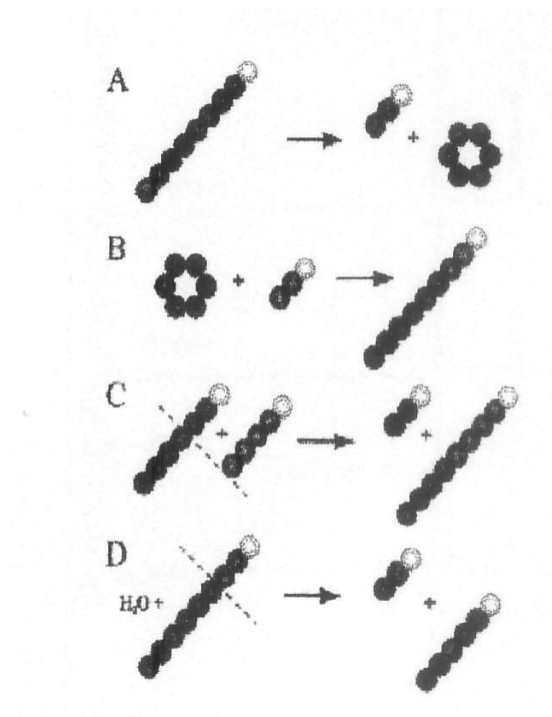


Figure 1.1 Schematic diagram of CGTase catalysed reaction. The circles represent glucose residues whilst the white circles indicate the sugars reducing end. (A) cyclisation, (B) coupling, (C) disproportionation, (D) hydrolysis

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 exist 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 $\alpha(1-4)$ glucose linkages that make the amylose and additional $\alpha(1-6)$ linkages that make amylopectin. Starch usually consists of a mixture of two types of polymers; amylose 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 consists of linear chains of 20-24 $\alpha(1-4)$ -linked D glucose connected by a $\alpha(1-6)$ -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 extent in Europe).
- ii) Glucose syrups for fermentation purposes