Faculty of Resource Science and Technology

SCREENING AND ISOLATION OF CYCLODEXTRIN PRODUCING FUNGI AND BACTERIA FROM SELECTED UNIMAS AAS COLLECTION

Kathleen Michelle Mikal (18680)

Bachelor of Science with Honours
(Resource Biotechnology)
2010

ACKNOWLEDGEMENT

Above all, I would like praise God the highest for His blessing and for granting me the strength and patience to accomplish my final year project. A special thanks and sincere appreciation I dedicate to my supervisor, Dr. Mohd. Hasnain bin Md. Hussain and my co-supervisor, Dr. Awang Ahmad Sallehin Awang Husaini for their advices, guidance and encouragement in this project. I am deeply indebted to the members of Proteomic Laboratory and Molecular Genetic Laboratory, especially to postgraduate students, Kak NurHidayah and Fraser for all their help and valuable guidance I am especially grateful and thankful to my beloved family and my dearest best friends for their great help and support in times of difficulties. And finally, thank you for those who helped me throughout this project, fellow course mates, lecturers, lab assistants and all those who involve directly and indirectly in this project.

TABLE OF CONTENTS

LIST OF ABBREVIATION

AAS : Awang Ahmad Sallehin

ATCC : American Type Culture Collection

CD : Cyclodextrin

CGTase: Cyclodextrin glycosyltransferase

dH_2O : Distilled water

EtOH : Ethanol

HII : Horikoshi II

HPLC : High Performance Liquid Chromatography

Km : Kinetic value

LB : Luria Bertani

mM : miliMolar

OD : Optical Density

PDA : Potato Dextrose Agar

LIST OF TABLES

TABLES **PAGE**

LIST OF FIGURES

Screening and Isolation of Cyclodextrin Producing Fungi and Bacteria from Selected UNIMAS AAS Collection

Kathleen Michelle Mikal

Resource Biotechnology Programme
Faculty of Science and technology
Universiti Malaysia Sarawak

ABSTRACT

Cyclodextrin (CD) is cyclic oligosaccharides consisting of α-1,4-glucosidic linkage produced by the reaction of cyclodextrin glycosyltransferase (CGTase EC 2.4.1.19) and starch. CGTase are usually produced by bacteria mostly from the *Bacillus* genera. There is no research done on fungi for production of CD. In this study, screening was done on fungi and bacteria to identify which fungi and bacteria could produce CGTase that will later form CD. The screening was done on the Horikoshi II media containing phenolphthalein and methyl orange as CGTase indicator. Five fungi and three bacteria from the AAS UNIMAS collection were screened. Screening and enzyme assay showed that two fungi, *Aspergillus nomius* and *Aspergillus flavus* produce CGTase. Both fungi have high enzyme activity with 12.97U/ml for *A.nomius* and 10.66U/ml for *A.flavus* based on CGTase assay.

Key words: cyclodextrin (CD), cyclodextrin glucosyltransferase (CGTase), screening, fungi

ABSTRAK

Siklodekstrin (CD) merupakan oligosakarida siklik yang terdiri daripada rantaian α-1,4-glucosidik, hasil tindak balas siklodekstrin glucosiltransferase (CGTase, EC 2.4.1.19) dengan kanji. Enzim ini kebanyakannya dihasilkan oleh bakteria dari genus Bacillus. Belum ada penyelidikan dijalankan ke atas fungi untuk menghasilan CD. Dalam kajian ini, penyaringan dilakukan ke atas fungi dan bakteria untuk mengenal pasti fungi dan bakteria yang boleh mehasilkan CGTase yang akan membentuk CD. Penyaringan dilakukan pada media Horikoshi II yang mempunyai fenolftalein dan metil oren sebagai penunjuk (indikator) penghasilan CGTase. Lima fungi dan tiga bakteria yang diambil dari pada koleksi AAS UNIMAS telah diuji. Penyaringan dan ujian enzim menunjukkan dua fungi, Aspergillus nomius dan Aspergillus flavus menghasilkan CGTase. Kedua-dua fungi tersebut juga mempunyai aktiviti enzim yang tinggi dengan 12.97U/ml bagi A.nomius dan 10.66U/ml bagi A.flavus berdasarkan ujian enzim.

Kata Kunci: Siklodekstrin, siklodekstrin glucosiltransferase (CGTase), penyaringan, fungi

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cyclodextrin has been an important molecule due to its ability to form inclusion complexes which allow changes of the physical and chemical properties of guest molecules which offers various usages in food industry, cosmetic, pharmaceutical industries, perfumes and agricultural chemistry (Bonilha *et al*., 2006).

Since the scientific discovery of cyclodextrin in 1891, most research in the latter years is performed on bacteria such as *Bacillus*, *Klebsiella*, *Pseudomonas*, *Brevibacterium*, *Thermoanaerobacterium*, *Micrococcus* and *Clostridium*. The main bacteria genera used for the production of cyclodextrin are *Bacillus* (Volkova *et al*., 2000; Bonilha *et al*., 2006). Manipulation of fungi in production of cyclodextrin is very rare. Therefore, there is no reported study on the production of cyclodextrin using fungi. Bacteria have an advantage over fungi because the time needed for production of the CGTase for bacteria are shorter.

This main aim of this study is to screen, isolate and identify new cyclodextrin-producing fungi and bacteria. Screening of the fungi and bacteria was done on the Horikoshi II medium (which includes phenolphthalein and methyl orange dye). Although this study aims to isolate new strain of bacteria, the study focuses more on the cyclodextrin-producing fungi. The fungi were identified macroscopically and microscopically. The characteristic observed were the shape of the spore, the color of the colony, the shape of the spore and the fruiting body. Quantitative

analysis was done on the CGTase produced by the fungi to measure the activity of the enzyme. However, since the bacteria failed to produce CD, the screening of bacteria was stopped and further study was done on the CD-producing fungi. Through this research, it is hope that it can fulfill the needs of increasing demand of cyclodextrin and benefit various areas that utilizes cyclodextrin especially in a large scale industry.

1.2 Objectives

The objectives for this study are:

- To screen cyclodextrin-producing fungi and bacteria that produces the CGTase from nature.
- To isolate and identify the fungi and bacteria that produces CGTase activity.
- To analyze quantitatively the CGTase produced by the isolated and identified cyclodextrin-producing fungi and bacteria.
- To compare the CGTase production between the newly isolated cyclodextrin-producing fungi and bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Cyclodextrin

2.1.1 History of Cyclodextrin

The first paper by Villiers, published in 1891 reported the observation on the formation of unidentified crystalline substance during fermentation of starch. The French author assumed that the substance is a type of cellulose and named it cellulosine. 15 years later, Franz Schardinger, an Austrian microbiologist had isolated a microorganism, *Bacillus macerans* which produced two distinct crystalline substances when cultivated on medium that contains starch. The properties of these crystalline substances were similar to the properties of the known partial degradation products of starch, the dextrins. He, therefore, named them α- and β-dextrin (Szejtli, 2004). In the 1930s, Freudenberg discovers γ-CD and suggested that there is a possibility that larger cyclodextrin exists. At the same time, he and his co-workers came to conclusion that the crystalline Schardinger-dextrins are built from maltose units and contains only α-1,4-glycosidic linkages (Loftsson and Duchêne, 2006). They first postulated the cyclic structure of these crystalline dextrins in 1936 (Szejtli, 2004).

2.1.2 Structure and Properties of Cyclodextrin

Cyclodextrin are cyclic oligosaccharides consisting of α-1,4-glucosidic linkage which joins 6, 7 and 8 D-glucose units. These joined glucose units are known as alpha-cyclodextrin, beta-cyclodextrin and gamma-cyclodextrin respectively (Allenza *et al*., 1991; Yim *et al*., 1997). In

a report by Volkova *et al*. (2000), it is noted that these doughnut-shaped molecule possesses hydrophilic surface and hydrophobic central cavity. Hydrophilic characteristic is due to the presence of hydroxyl groups on the outer surface (Hartong *et al.,* 2007). This enables the cyclodextrin to form inclusion complexes with different organic and inorganic guest molecules. Cyclodextrin encapsulate other molecules within their cyclic structure, in their hydrophobic cavity. Their ability of changing the physical and chemical properties of a wide variety of molecules offers a wide uses in food industry, cosmetic, paper industry and pharmaceutical industries (Doukyu *et al*., 2003; Bonilha *et al*., 2006).

2.1.3 Types of Cyclodextrin

There are three major cyclodextrins; α-, β- and γ-cyclodextrin are crystalline, homogeneous, nonhygroscopic substances, torus-like macro-rings which is built from glucopyranose units. The α-cyclodextrin (Schardinger's α-dextrin, cyclomaltohexaose, cyclohexaglucan, cyclohexaamylose, αCD, ACD, C6A) comprises six glucopyranose units, β-CD (Schardinger's β-dextrin, cyclomaltoheptaose, cycloheptaglucan, cycloheptaamylose, βCD, BCD, C7A) comprises seven such units and γ-CD (Schardinger's γ-dextrin, cyclomaltooctaose, cyclooctaglucan, cyclooctaamylose, γCD, GCD, C8A) comprises eight such units (Szejtli, 2004).

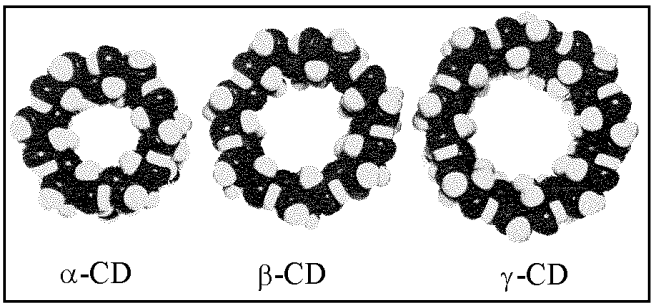

Figure 2.1 Molecular models of Alpha-,Beta- and Gamma-cyclodextrin (Volkova *et al.*, 2000)

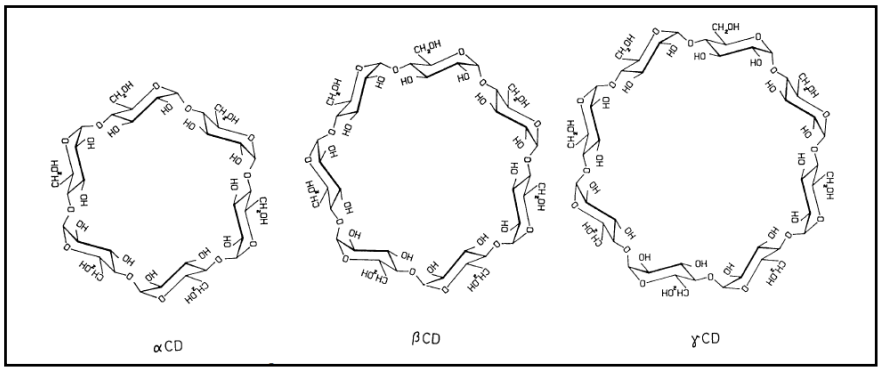

Figure 2.2 Chemical structure of Cyclodextrin (Szejtli, 2004)

Table 2. 1 Comparison between Alpha-, Beta- and Gamma-cyclodextrin (Horikoshi *et al*., 1979; Szejtli, 2004).

Properties	**α-Cyclodextrin**	**β-Cyclodextrin**	**γ-Cyclodextrin**
Number of Glucose Units	6	7	8
Solubility (at 25˚C in water)	140 mg/cm^3	18 mg/cm^3	220 mg/cm^3
Color of Reaction (With Iodine)	Blue colour	Yellowish brown/ Reddish brown (Hardly react with iodine)	Yellowish brown/ Reddish brown (Hardly react with iodine)

Crystallography	Hexagon/ Blade	Pararellogram	Quadrilateral
Molecular Weight (g mol^{-1})	970	1,140	1,300
Melting Point (˚C)	200	200	200
Optical Rotation [α] D	+150	+160	+170

2.1.4 Production of Cyclodextrin

Typical enzymatic degradation of starch produces dextrins, a linear or branched chain of malto-oligomers. This is a true hydrolytic process as the product is produced from the splitting of glycosidic linkage reacts with a molecule of water. However, the degradation of starch by CGTase undergoes an intramolecular reaction and chain splitting without involving water molecules and α-1,4-linked cyclic products are produced. These cyclic products are called cyclodextrins (Augusto, 1996). Based on previous study by Abd-Aziz and her collegues (2007), sources of starch that could be used in the production of cyclodextrin are sago, tapioca, corn, rice, green peas and glutinous starch. Sago has the highest rate of CGTase production. The three major types of cyclodextrin, α-CD, β-CD and γ-CD, although produced by the same CGTase, have different optimum condition for their production. α-CD production requires the action of CGTase at neutral condition with the pH of 6.0-7.0 at the temperature of 35-40˚C (Prakash and Abbot, n.d). Meanwhile, the optimum pH and temperature for the production of β-CD is pH 7.5-8.5 and 65˚C respectively (Yim *et al*., 1997).

2.2 Cyclodextrin glycosyltransferase (CGTase)

Cyclodextrin glycosyltransferase (CGTase EC 2.4.1.19) is an enzyme that converts the starch and other α-1,4-linked glucans to produce a non reducing and cyclic malto-oligosaccharides cyclodextrin (Higuti *et al*., 2003; Bonilha *et al*., 2006). CGTase is a member of the the α-amylase or the glycosyl hydrolase family 13 (Wind *et al.*, 1998). *Bacillus* is the main genus of bacteria that produces CGTases but various other genera of bacteria such as *Klebsiella*, *Pseudomonas*, *Brevibacterium*, *Thermoanaerobacterium*, *Micrococcus* and *Clostridium* also produces CGTase. However, there are no published papers on CGTase produced by fungi as reported by Kuriki and Okada (1995) in their book. CGTase are an extracellular enzyme which is known to catalyze four different transferase reactions: cyclization (convertion of starch and related to α-1,4-glucans into cyclodextrins), coupling (opening of the cyclodextrin ring and transfer of linear malto-oligosaccharides to acceptors), disproportionation (transfer of linear malto-oligosaccharides to acceptors) and a weak hydrolysis activity as shown in the schematic diagram in Figure 3. CGTases produces α-, β- and γ- CDs from starch in different reactions and in the different amount (Volkova *et al*, 2000; Bonilha *et al*, 2006).

2.3 Uses of Cyclodextrin

The ability of these unusual molecules to form inclusion complexes offers a variety of potential uses for food, cosmetic, agricultural chemistry, perfumes and pharmaceutical industries (Bonilha *et al*, 2006; Doukyu, 2003). It is undoubted that cyclodextrin is a very valuable chemical. Another characteristic that made cyclodextrin a famous chemical is non-toxic (Hartong *et al.*, 2007). Most chemical are toxic and is hazardous to human's health.

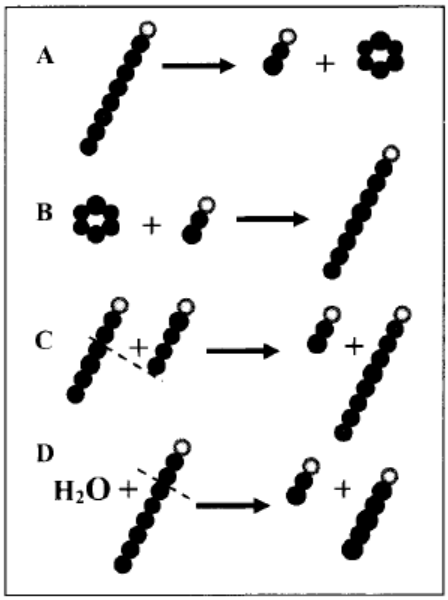

Figure 2.3 Schematic diagram of the four different transferase catalyzed by CGTase (Kaulpiboon and Hansakul, 2007).

The (●) represent glucose residues; the open circles(o) indicate the reducing end sugars. (A) Cyclization, (B) coupling, (C) disproportionation, and (D) hydrolysis.

2.3.1 Cyclodextrin in Perfuming Industry

In perfuming cosmetic industry, it is essential to produce fragrance materials with pleasant odor. However, perfumes are poorly water-soluble and commonly exist in a liquid state which hardens the perfuming process. For many years, surfactant is used in the solubilization process in preparation of perfumes but this rapidly decreases the fragrance material in the product during storage. This is solved by adding cyclodextrin into the product. Cyclodextrin complexation with fragrance materials increases the product solubility and reduces evaporation as it became a high energy barrier to prevent volatilization. Through CD

complexation, release of fragrances can be controlled and long-lasting fragrance is produced (Numanoğlu *et al*, 2006).

2.3.2 Cyclodextrin in Food Industry

Cyclodextrin has many applications in food industry. The ring structure of cyclodextrin has found to be useful in the production of cholesterol free products. The hydrophobic cholesterol will be easily trapped inside the ring. When the cyclodextrin is being removed from the body, the cholesterol will be removed as well (Anon, 2009a). In foods such as baked goods and instant coffee and tea, α-CD function as carriers for colours, sweeteners and flavours. Meanwhile, in dry mixes and dietary supplements, α-CD acts as stabilizer that stabilizes the colour, flavours, vitamins and polyunsaturated fatty acids (Prakash and Abbot, n.d).

2.3.3 Cyclodextrin in Waste Treatment

A study by Hartong and his colleagues in 2007 has shown that, CD, even when applied in a lower amount, its still very beneficial. This has made CD more attractive in commercial application. In waste or sludge treatment, CD is able to increase cake solids in the drainage system. When added along with conventional coagulant and flocculants, CD could increase the rate of dewatering or water removal in biological and fibrous sludge. The rate of sludge capturing during belt pressing also increases. CD also possesses the ability of destabilizing cationic polymers in aqueous solution. All four stated abilities of the CD were the result of full-scale trial that took place at the Stora Enso Wosconsin Rapids water facility and this has made the drainage system better. The ability of CD to destabilize the cationic polymers reduces the turbidity of the suspensions.

2.3.4 Cyclodextrin in Paper Industry

CD is also beneficial in paper industry. One of the examples of CD application is in the deinking process of paper such as old newspaper (ONP), magazine waste (OMG) and mixed office waste (MOW). During ONP deinking, the soluble ink such as non-contact laser inks and xerographic toners will tend to re-deposit back to the pulp fiber which is a problem in deinking. However, this can be solved by adding CD into the process. CD will trap those small ink particles in its hydrophobic cavity. This increases the efficiency of the deinking process. The brightness of the pulp increases and there will be less dirt. The hydrophobic cavity if CD can also capture and caged the hydrophobic lipid materials (fatty acids and esters) thus, enhancing their solubility in water. During deinking too, CD could trap the 'stickies' that causes problem during the reprocessing of the pulp. 'Stickies' are water insoluble and there is high possibility that it can agglomerates on the paper manufacturing machines reducing the paper quality, breaking the paper fibers and it is time consuming when it comes to cleaning the equipment. All three types of deposits that has form inclusions with CD could be removed from the pulp by washing, flotation and press (Xu *et al*., 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection and preparation

3.1.1 Collection and preparation of fungi samples

Samples of fungi were collected from the UNIMAS Awang Ahmad Sallehin (AAS) Collection. There were 4 fungi samples available for this study in the UNIMAS AAS Collection: one unidentified samples from sago humus (NSH6), an identified sample from sago humus (NSH9) and 2 indentified samples from sago waste (SW004 and OSP). All four fungi samples were revived and subcultured in PDA.

3.1.2 Preparation of bacteria samples

There were 3 samples of bacteria taken from many sources: *Bacillus licheniformis* P7, *Bacillus amyloliquefaciens* UMAS 1002 and *Escherichia coli* ATCC 25922. Bacteria was grown overnight at 37˚C in LB broth. The overnight culture was later streaked on a LB agar plate using the inoculating loop. The inoculated LB agar medium was then incubated at 37˚C for 24 hours.

3.2 Screening of CGTase producers

3.2.1 Screening of CGTase - producing fungi

Fungi samples: OSP, SWOO4, NSH6 and NSH9 were cultured on a modified Horikoshi II (HII) agar medium (Park *et al*., 1989; Higuti *et al*., 2003; Kitcha *et al*., 2008) containing 1.0% soluble starch, 0.5% mycological peptone , 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 1.0% Na_2CO_3 (autoclaved separately), 1.5% agar (all autoclaved at 121°C for 15 minutes) and dye: 0.03% phenolphthalein and 0.01% methyl orange that were added after agar was autoclaved (all concentration w/v in distilled water). 1 cm of the fungi samples from PDA was cut and transferred to the HII agar medium. The culture was then incubated at 27°C for 3 to 6 days. Fungi colony that reduced the pink color of phenolphthalein to colorless and did not turn methyl orange color to red was selected for further study and isolation.

3.2.2 Screening of CGTase – producing bacteria

Samples of bacteria were streaked on HII agar media containing (Park *et al*., 1989; Higuti *et al*., 2003; Kitcha *et al*., 2008) containing 1.0% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 1.0% Na_2CO_3 (autoclaved separately), 1.5% agar (all autoclaved at 121°C for 15 minutes) and dye: 0.03% phenolphthalein and 0.01% methyl orange (added after media was autoclaved) (all concentration w/v in distilled water). The bacteria were then incubated at 37°C for 2 days. Colonies surrounded by halo zones were isolated and grown in broth containing the HII components excluding the dyes for further study.