STARCH DEGRADERS: SCREENING AND PARTIAL CHARACTERIZATION

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I declare that this thesis entitled “Starch Degraders Degraders: Screening and Partial Characterization” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....
Name : PRAVENTHRAN JANARDANAN
Date :18TH OCTOER 2006

To my beloved parents, brothers and sister

LIST OF TABLES AND DIAGRAMS

LIST OF SYMBOLS

°C	-	Degree Celcius
w/vol	-	Weight per volume
mmol	-	Mili mole
h	-	Hour
μm	-	Micrometer
%	-	Percentage
g	-	gram

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Abstract

Starch as we know is the main source of carbohydrate. Since carbohydrate is always somehow linked to industry, we need to produce enzyme that can degrade carbohydrate. Presently the enzyme used is amylase but lately many are venturing into other enzymes especially the ones extracted from bacteria. My research here is concentrating on a specific enzyme named cyclomaltodextrin glycosyltransferase or CGTase. Firstly the bacteria are carefully screened from the sample soil collected from a local corn field. Then after the bacteria have been identified it will be analyzed for its characteristics and the crude enzyme will be tested on its effect by changes of temperature, pH, and carbon and nitrogen source. From this research I have managed to screen a typical strain of CGTase producing bacteria where after running tests such as gram staining, spore staining, and acid fast the type and characteristics of the bacteria was known.

Abstrak

Memang menjadi pengetahuan umum bahawa kanji merupakan sumber karbohidrat utama .Memandangkan potensi kanji dalam industri, banyak kajian telah diambil dalam memperoleh enzim yang boleh mengurai kanji daripada tumbuhan untuk dijadikan bahan yang lebih ringkas. Buat masa ini enzim yang digunakan secara meluas ialah amilase. Namun sejak kebelakangan ini pelbagai kajian telah dijalankan untuk mencari alternatif bagi enzim yang boleh menguraikan kangi. Dalam kajian saya ini saya telah mengkhusukan skop kajian kepada enzim cyclomaltodextrin glycosyltransferase atau CGTase. Kajian saya ini telah dibahagikan kepada dua bahagian iaitu penskrinan bakteria dari sample tanah dan keduanya pencirian enzim serta bakteria yang diperoleh. Daripada kajian ini saya telah dapat mengenalpasti dan mengasingkan sejenis bakteria dari sampel tanah yang diperoleh dari kebun jagung. Daripada ujian ujian yang telah dijalankan saya telah dapat pelajari ciri-ciri bacteria tersebut dan juga kesan faktor faktor seperti suhu, pH,sumber karbon dan nitrogen.

Chapter 1

Introduction

1.1 Introduction

This study is mainly on the research of isolation in the search of starch degrading bacterial enzyme. Starch can be degraded by several enzymes but the enzyme in interest of this study is CGTase. Commercially at the time being enzyme is collected from fungi and other microorganism. However recently have been enzyme produced by fungi or other microorganism are not being the first choice of enzyme to be used in industry. Therefore bacterial enzyme is the prospect for the future. This is due to the fact that fungi are hazardous while microorganisms are hard to be cultivated to extract the enzyme from them. These problems are solved with the cultivation of bacteria for the purpose of enzyme production.

1.2 Problem Statement

Carbohydrate or more specifically starch can be found commonly in nature. Since starch is a large molecule of polymer it is usually considered a nuisance especially in detergent manufacturing industry. Starch from food stains are considered as tough stains to be removed when washing. Addition of starch degrading enzyme in detergent

will overcome this problem. Before being produced as paper, the timber will undergo certain treatment to change its hard and woody characteristics. At present the most commonly used enzyme is amylase from microorganism and fungi. The problem with this method is first the production of enzyme from microorganism is not efficient meanwhile amylase from fungi can be dangerous as it spores can be harmful. That is why now the industry is looking at the prospect of cultivating enzymes from bacteria. Not only that bacteria can be cultivated easily in laboratory but also it produces enzyme much efficiently and with less risk. Although amylase is the most common enzyme used in starch degrading process, cyclomaltodextrin glycosyltransferase (CGTase) has also great potential in industrial sector for the purpose of degrading starch.

1.3 Objectives of Study

The objective of this study is to screen and characterize microbe or bacteria that can produce cyclomaltodextrin glycosyltransferase (CGTase) enzyme. Besides that the result and effect of nitrogen and carbon sources is also examined

1.4 Scope of Study

The scope of the experiment consists of;

i) Screening of CGTase producing microbes from sample soil.

ii) Characterization of the microbes by using Morphology and Physiological tests .

iii) Study the effect of various carbon and nitrogen source on production of CGTase by microbes.

Chapter 2

Literature Review

2.1 Starch

Starch is a type of carbohydrate found vastly in plant tubers and seed granules. It is also is the main energy source in animals and also human beings. Among starch sources are maize, potato, rice, wheat and tapioca with maize being the largest source among all. Starch is constructed by linkages of smaller components called amylose and amylopectin.

2.1.1 Amylose

Amylose consists of polymers of α-D-glucose units in the $^4C_1$ conformation. Amylose molecules consist of single mostly unbranched chains with 500 to 20,000 α-(1,4) - D-glucose units dependent on source. Amylose can form an extended shape but

generally tends to wind up into rather stiff left handed single helix or form even stiffer parallel left handed double helical junction zones. The molecular structure of amylose is as shown in figure 1.0

Recently, a method for the determination of the amylose content in starch has been developed which is based on the colorimetric measurement of the iodine complexes formed with amylose and amylopectin. The method requires measurement at only one wavelength and avoids the use of harsh dispersants for the starch. Dimethyl sulphoxide is used as the dispersant and a wavelength of 600 nm can be used for measurement of the amylose content of starches from different botanical sources. A linear relationship was obtained between absorbance and amylose concentration for mixtures of amylose and amylopectin standards, and this forms the basis of the determination. The method is rapid, simple, and accurate and does not require the use of multi-component analysis of spectra, since a wavelength is chosen that suits the particular starch being analysed. [McGrance *et al*.]

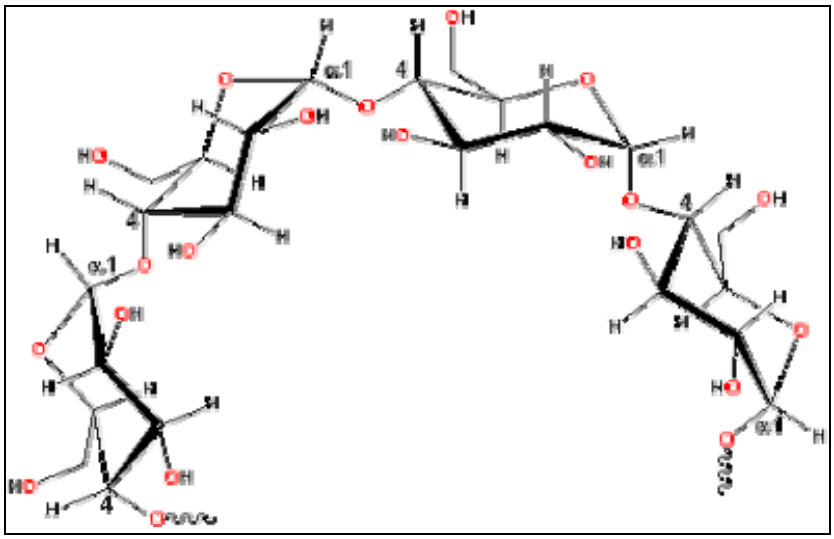

Figure 1.0: Structure of Amylose molecule(Source: *J. McGrance et al 1990*)

2.1.2 Amylopectin

Amylopectin in the other hand consist of non-random α-(1,6) branching of the amylose-type α-(1,4)-D-glucose structure. Amylopectin is a highly branched polymer of glucose found in plants. It is one of the two components of starch, the other being amylose. Glucose units are linked in a linear way with α (1,4) bonds. Branching takes place with α (1,6) bonds occurring each 24 to 30 glucose units. Its counterpart in animals is glycogen which has the same composition and structure except for branching that occurs each 8 to 12 glucose units.The branching of amylose is determined by branching enzymes that leave each chain with up to 30 glucose residues. Each amylopectin molecule contains a million or so residues, about 5% of which form the branch points. There are usually slightly more 'outer' unbranched chains (called A-chains) than 'inner' branched chains (called B-chains). There is only one chain (called the C-chain) containing the single reducing group.

Amylopectin interferes with the interaction between amylose chains (and retrogradation) and its solution can lead to an initial loss in viscosity and followed by a more slimy consistency.

Amylopectin is also a glucan with α (1,4) residues on the main chain. It contains branches linked α (1-6), and occurs about once every 12-25 residues along the chain. The branch chains are typically 20-25 residues long. Hydrolysis of the chains into fragments yields maltose, usually done by enzymes such as alpha-amylase.

Amylopectin together with amylose compose starch, the primary storage polysaccharides of plants. Amylopectin is a polymer of glucose. It differs from amylose and resembles the animal storage polysaccharide, glycogen, in containing (1,6) branches in addition to (1,4) links between glucose units. Amylopectin is less branched, however, than glycogen, having branches approximately every 10-20 residues, versus every 8 residues in glycogen. Amylose, amylopectin, and glycogen all differ from the polysaccharide, cellulose, in containing exclusively 1,4 bonds in contrast to the 1,4 bonds of cellulose

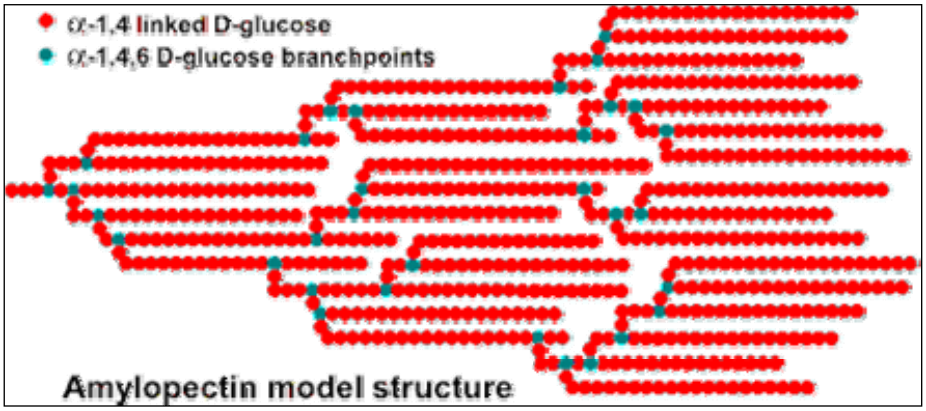

Figure 1.1: Structure of Amylopectin molecule

2.2 Cyclodextrin

Cyclodextrins or also called cycloamyloses make up a family of cyclic oligosaccharides, composed of 5 or more α-D-glucopyranoside units linked 1,4, as in amylose (a fragment of starch). Typical cyclodextrins contain a number of glucose monomers ranging from six to eight units in a ring, thus denoting:

- α-cyclodextrin: six sugar ring molecule
- β-cyclodextrin: seven sugar ring molecule
- γ-cyclodextrin: eight sugar ring molecule

Cyclodextrins are produced from starch by means of enzymatic conversion. Over the last few years they have found a wide range of applications in food, pharmaceutical and chemical industries as well as agriculture and environmental

2.3 CGTase

Cyclomaltodextrin glucanotransferase (CGTase) plays a role in the starch utilization pathway of some bacteria [Fiedler *et al*.,1981] and catalyzes various glucan transfer reactions with starch. The action of CGTase starts with the cleavage of one α-1,4-linkage within the glucan molecule. The newly produced reducing end is then transferred either to the nonreducing end of another molecule (disproportionation reaction) or to its own nonreducing end (cyclization reaction). CGTase also catalyzes the reverse reaction of cyclization, in which cycloamylose is opened by the enzyme and a linearized fragment is transferred to an acceptor (coupling reaction). At a certain frequency, the newly produced reducing end is transferred not to a carbohydrate acceptor but rather to a water molecule, which results in either the hydrolysis of amylose or the linearization of cycloamyloses (hydrolytic reaction).

CGTase was first observed to be produced by *Bacillus macerans* and has since been found in many bacteria. All CGTases produce mainly cycloamyloses with degrees of polymerization (DP) of 6, 7, and 8 (CD6, CD7, and CD8), which are generally called α-, β-, and γ-cyclomaltodextrin, at equilibrium but with different product specificities (i.e., different amounts of CD6, CD7, and CD8). These cyclomaltodextrins can complex various inorganic or organic compounds in their hydrophobic central cavities. Therefore, these cyclomaltodextrins are widely used in the pharmaceutical, food, and cosmetic industries. Since each cyclomaltodextrin has a distinct spectrum for guest molecules, extensive studies have been carried out to understand the mechanism of the cyclization reaction and to find or engineer CGTases to produce specific types of cyclomaltodextrin.

Although CD6, CD7, and CD8 are its main products at equilibrium, CGTase also produces larger cycloamyloses with DP of from 9 to more than 60. Therefore, to understand the reaction mechanism of CGTase, these larger cycloamyloses must also be considered. Recently, a method we established for quantifying CD6 to CD31 by high-performance anion-exchange chromatography (HPAEC) and showed that this method could be useful for understanding the cyclization reaction of CGTase. In the present

study, this method was used to investigate the actions of three different CGTases: CGTase from *B. macerans* (*Bmac* CGTase), which produces mainly CD6 (4); CGTase from alkalophilic *Bacillus sp*. strain A2-5a (A2-5a CGTase), which produces mainly CD7; and CGTase from *Bacillus stearothermophilus* (*Bste* CGTase), which produces nearly equal amounts of CD6 and CD7.

2.4 Application of CGTase in Industry

Basically CGTase is used in industry for the purpose of producing cyclodextrins in different ratio of α, β and γ depending on the need of the industry.

2.4.1 Pharmaceutical Industry

Cyclodextrin (CD) is used for achieving a better stabilization of drugs, for protecting humidity, oxidization and dissolution from light and heat: extending the expiration time of drugs, for improving the solubility and rate of biological utilization, and for avoiding unpleasant odor or taste and for decreasing toxicity side effects. Therefore cyclodextrin is formed from degradation of starch (amylase) by the action of CGTase enzyme.

2.4.2 Food Industry

CD is used for a better stabilization of food and rough material in its components, for protecting humidity, oxidizing, for keeping original color , fragrance

and taste; for damp-proof an rot-proof; for isolating unpleasant odor and bitter taste, for enhancing emulsification and foamability.

2.4.3 Operational Chemical Industry

Cyclodextrin is used as emulsifier, for keeping fragrance agent and keeping color agent to the cosmetics; for enhancing foamability and dividing out stimulation in the tooth paste; for isolating aroma dextrin and spice. It makes them better and can be kept for a longer time.

2.5 Starch Degrading Bacteria

CGTases are predominantly extracellular enzymes, produced by a variety of bacteria, mainly by *Bacillus*, but also by *Klebsiella, Micrococcus, Thermoanaerobacterium* and others.

Table 2.1: Summary of CGTase production by various bacteria and their production medium

Bacteria	**pH**	**T(**°C)	**Time** (h)	**Medium**	**Reference**
Bacillus circulans	**8**	**37**	**48**	Cassava starch, Ammonium sulphate, Phosphate buffer, $MgSO_4$ and $FeSO_4$	**Adriana *et al.*,(2002)**
Anaerobranca horikoshii	**8.5**	**60**	**55**	**D-Glucose, Yeast extract, Peptone,** $MgSO_4$, KH_2PO_4, Na_2CO_3, $(NH_4)_2SO_4$	Horikoshi **1999**
Bacillus acidopullulyticus	**5**	**37**	**22**	**Soluble starch, yeast extract, peptone,** $MgSO_4$, KH_2PO_4,	**Jensen *et al*, 1984**

				Na_2CO_3		
Thermoanarobacter saccharolyticum	**12**	**75**	**20**	**Soluble starch, yeast extract, peptone,** $MgSO_4$, KH_2PO_4, Na_2CO_3	**Suzuki *et al.* 1986**	

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals

Copper sulfate hydrate, folin-ciocaltieau, sodium sulfite, dinitrosalisylic acid, ammonium oxalate, sodium bicarbonate, carbol-fuschin solution, malachite green, glucose, yeast and peptone are from analytical grade and were obtained from suppliers Sigma-Aldrich. Besides that commercial starch was obtained from local hypermarket.

3.2 Storage of Microbes

The microbes were stored in 18 %(v/v) glycerol stock in -80°C.

3.3 Medium

3.3.1 Agar Medium

Agar medium is used to cultivate the bacteria collected from sample soil. After sieved the sample is diluted and after the tenth dilution 1 ml of sample is streaked on agar plate in a petri dish. The agar plate contains agar made up of general nutrients that are essential for any bacteria to grow on.

3.3.2 Seed Culture Medium

The microbe was grown on Horikoshi II medium (Park *et* al. 1989) Agar plate medium contained 2%(w/v) of D- Glucose, 1.5% (w/v) agar powder, 0.5% (w/v) yeast extract, 0.5% (w/v)of peptone, 0.1%(w/v) K_2HPO_4 and 0.02% (w/v) $MgSO_4.7H_2O$ and added with 10% (w/v) of Na_2CO_3

3.3.3 Production Medium

After determining the strain of bacteria that can degrade starch, it was isolated from a petri dish into production medium. Production medium is composed of 2%(w/v) corn starch, 1%(w/v) yeast extract, 0.1%(w/v) K_2HPO_4 and 0.02% (w/v) $MgSO_4.7H_2O$ and added with 10% (w/v) of Na_2CO_3

3.4 Characterization of Microbes

3.4.1 Morphology Test

3.4.1.1 Acid Fast

Determination of bacteria was done using Ziehl-Neelsen Method.Before staining, all slides are kept in direct UV light at least 30 minutes for disinfection of smears. Then the slides are flamed to heat fix. The entire slides are flooded with Carbol Fuchsin. Enough stain is added to keep the slides covered throughout the entire staining step.Before any further procedures of thie method can be done,it is necessary to prepare solution of Basic Fuchsin and Methylene Blue. Microbe that was isolated was smeared and covered with carbolfuchsin, and placed put on boiling hot bath for 5 minutes. Then, the slide is cooled and decolorized using acid-alcohol for 15 to 20 seconds. The decolorization was stopped by rinsing with water. The slide was then counterstained with methylene blue for 30 seconds. Excess methylene blue was rinsed with water. Slide was blot dry with bibulous paper, and examined under oil immersion.

3.4.1.2 Gram Staining Method

Individual bacterial cells are hard to see, partly because they are small, but also because they are almost transparent. In addition to magnification under a microscope, optical tricks must also be used to be able to see them such as staining method. This method can make bacterial cells visible under the microscope. Basically bacteria are classified under 2 categories- Gram Positive and Gram Negative. ***Gram-positive bacteria***, when tested with Gram staining method gives the result of purple crystal violet stain trapped by the layer of peptidoglycan which forms the outer layer of the cell meanwhile in ***Gram-negative bacteria***, the outer membrane prevents the stain from reaching the peptidoglycan layer in the periplasm. The outer membrane is then

permeabilized by acetone treatment, and the pink safranin counterstain is trapped by the peptidoglycan layer

Bacteria were smeared on staining rack and slide was stain with crystal violet for 1 to 2 minutes. The slide is then flooded with iodine for another 1 to 2 minutes. The iodine is then poured off. The process was followed by decolorized process using acetone for 2 to 3 seconds, and immediately washed with water. The slide flooded with safranin counterstain for 2 minutes and washed with water. Blot dry with bibulous paper and air dry was examined under the oil immersion

3.4.1.3 Spore Staining Method

The Schaeffer-Fulton method is the most commonly used endospore staining technique, and it uses Malachite green as the primary stain. Microbe was tested of its vegetative spores. Using antiseptic technique, isolated microbe was smeared into a clean slide. Slide with microbe was then air dried. The slide was then covered with paper towel, placed on staining rack and over a boiling water bath. Paper towel with slide was then flooded with malachite green and it was steamed for 5 minutes. After 5 minutes, the slide was removed, and the paper towel was removed. The slide was left to cool and rinsed with deionized water. Safranin was rinsed off and the slide is blot dry with bibulous paper and examined under oil immersion. Once the endospore has absorbed the stain, it is resistant to decolorization, but the vegetative cell is easily decolorized with water (leaving the vegetative cells colorless). When viewed under a microscope, the endospores appear green, while the vegetative cells are red or pink as shown in Figure 3.1