ISOLATION OF STARCH DEGRADING MICROORGANISM FROM LOCAL HOT SPRING

AFNIL DANIAL BIN AHMAD MERI

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DECLARATION

I declare that this thesis entitled “Isolation of starch degrading microorganism from local hot spring” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature :…….

Name of Candidate : Afnil Danial Bin Ahmad Meri

Date : May, 2008

Special dedication to my beloved mother and father, family members that always love and take good care of me

My friends, my fellow colleague and all faculty members

For all your care, support and believe in me.

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ABSTRACT

This study was performed to isolate starch-degrading microorganism from hot spring which located in Sungai Klah, Perak The objective of this study was to isolate a microorganism that can degrade starch from local hot spring. This research begin with the sample collection from local hot spring and screened for the amylase producer by observing the halo zone appeared around the colonies after pouring with iodine. After that, the microorganism was characterized by using four staining methods which were simple staining, Gram staining, spore staining, and acid-fast staining. In conclusion, the microorganism that had been isolated was rod shaped, Gram positive, spore former, and non acid-fast cells. It can be concluded that microorganism was *Bacillus* type.

ABSTRAK

Kajian ini dijalankan untuk mencari mikroorganisma daripada sumber kolam air panas Sungai Klah, Perak yang memecahkan struktur kanji. Objektif bagi kajian ini adalah untuk mencari mikroorganisma yang memecahkan struktur kanji daripada kolam air panas tempatan. Kajian ini dimulakan dengan mengumpulkan sample dari kolam air panas tempatan dan memerhatikan perembes enzim amylase terbaik daripada sample dengan melihat zon yg menukarkan warna iodin melalui kaedah lumuran iodin ke atas plat agar nutrien. Selepas itu, mikrob tersebut akan di karekterkan mengikut morfologinya melalui empat kaedah lumuran iaitu lumuran ringkas, lumuran gram, lumuran spora, dan lumuran keasidan. Mikroorganisma tersebut berbentuk rod, Gram positif, penghasil spora, dan bukan sel asid. Kesimpulannya, mikroorganisma tersebut ialah tergolong daripada genus *Bacillus*.

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%	-	percent
°C	-	degree Celcius
µg/ml	-	microgram per mililiter
g	-	gram
g/l	-	gram per liter
h	-	hour
kg	-	kilogram
L	-	liter
L/h	-	liter per hour
min	-	minute
ml	-	mililiter
mm	-	milimeter
rpm	-	rotation per minute
v/v	-	volume per volume
wt %	-	weight percent

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish & Mhatre, 1965). These enzymes have a great significance with extensive biotechnological applications in bread and baking, food, textile, and paper industries (Pandey *et al*., 2000).

The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this useful raw material. The starch polymer, like other such polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases. The enzymes are classified into endo-acting and exo-acting enzymes. α-amylase is an endo-acting enzyme and hydrolyses linkages in a random fashion and leads to the formation of linear and branched oligosaccharides, while the rest are exo-acting enzymes and attack the substrate from the non-reducing end, producing oligo and monosaccharides. The starch hydrolytic enzymes comprise 30% of the world's enzyme consumption (van der Maarel *et al*., 2002).

Developments in the starch-processing industry require continued discovery and development of new enzymes. Until now all commercial enzymes have been derived from cultivated bacteria or fungi. Notably, intensive research has been performed aiming at the isolation of unique thermostable and thermoactive amylases from thermophilic and hyperthermophilic microorganisms, therefore allowing more

industrial processes to run at higher temperatures (Niehaus *et al.,* 1999). Thermostable α-amylases have had extensive commercial applications in starch processing, brewing and sugar production (Leveque *et al.,* 2000), desizing in textile industries (Hendriksen *et al.,* 1999) and in detergent manufacturing processes (Hewitt & Solomons, 1996)

Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable α-amylases are available from the mesophile *Bacillus licheniformis* (Morgan & Priest, 1981), *Bacillus* sp. ANT-6 (Burhan *et al.,* 2003) and *Bacillus* sp. ASMIA-2 (Teodoro & Martin, 2000).

1.2 Problem statement

Thermostable α-amylases were isolated a long time ago from *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* (Underkofler, 1976). Thermophilic microorganisms produce thermostable enzymes and it is suitable with the starch-processing industry that using a lot of thermostable enzymes. In order to find other local sources that has starch degrading microorganism, local hot spring is the target place to make a research about isolation of thermostable microorganism that can produce amylase enzyme.

1.3 Objectives

To isolate a microorganism that can degrade starch from local hot spring.

1.4 Scopes

The scopes of this study are as follows:

a) To screen the microorganism

b) To isolate the microorganism that can degrade starch.

c) To characterize the microorganism that involved in starch degrading

CHAPTER 2

LITERATURE REVIEW

2.1 Thermophile

A thermophile is an organism in type of extremophile which thrives at relatively high temperatures, above 45°C. Thermophiles are found in assholes various geothermally heated regions of the earth such as hot springs like those in Yellowstone National Park and deep sea hydrothermal vents as well as decaying plant matter such as peat bogs and compost. As a prerequisite for their survival, thermophiles contain enzymes that can function at high temperature. Some of these enzymes are used in molecular biology. For example heat-stable DNA polymerases for polymerase chain reaction and also in washing agents. Thermophiles are classified into obligate and facultative thermophiles. Obligate thermophiles (extreme thermophiles) require such high temperatures for growth, while facultative thermophiles (moderate thermophiles) can thrive at high temperatures but also at lower temperatures (below 50°C). Hyperthermophiles are particularly extreme thermophiles for which the optimal temperatures are above 80°C (Brock, 1978).

2.1.1 Importance of enzymes from thermophiles

The enzymes isolated from some extremophiles have proven to be of great use in the biotechnology industry, able to function under conditions that would denature enzymes taken from most normal organisms. The most commonly used DNA polymerase for the polymerase chain reaction technique is Taq DNA

polymerase, originally isolated from *Thermus aquaticus*, a bacterial species found in surface aquatic locations such as Yellowstone National Park hot springs. For a few PCR applications, the lack of proofreading by Taq DNA polymerase is a problem. The DNA polymerase from *Thermococcus litoralis* was shown to have a proofreading exonuclease activity (Mattila *et al*., 1991). Another heat stable polymerase comes from the organism *Pyrococcus furiosus* (Pfu). This organism grows optimally at 100°C, making it a hyperthermophile. Taq DNA polymerase is adequate for most PCR, but one study (Hamilton *et al.,* 2001) reported that higher fidelity thermostable DNA polymerases such as Vent account for as much as 30% of DNA polymerase sales.

2.2 Amylase enzyme classification

2.2 1 α-Amylase

The α-amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) are calciumetallo enzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. Because it can act anywhere on the substrate, α-amylase tends to be faster acting than β-amylase. In animals, it is a major digestive enzyme. In human physiology, both the salivary and pancreatic amylases are α-Amylases. α-Amylases are produced by microorganism isolated from various sources such as hot spring, deep sea hydrothermal vent and many more (Maton *et al*., 1993). Table 2.1 summarizes the sources of microorganism which can provide thermo tolerant enzymes.

Table 2.1: Examples of sites serving as sources of microorganisms which can provide thermo tolerant enzymes (Haki and Rakshit, 2003)

Source	Microorganism	Enzyme
Hot spring	*Thermus* sp.	α-Amylase
Hot spring	*Basillus* sp. WN.11	α-Amylase
Deep sea hydrothermal vent	*Staphilothermus marinus*	α-Amylase
Marine solfatare	*Thermococcus litoralis*	Pullulanase
Decomposed plant sample from a lake	*Clostridium absonum* CFR-702	Cellulase free xylanase
Hot spring	*Basillus thermoleovocans* ID-1	Lipase
Compost of fermenting sitrus peels, coffee, and tea extract residues	*Basillus stains* MH-1	Endochitinase
Compost	*Basillus stearothermophilus* CH-4	β-N-acetylhexosaminidase
Korean salt fermented anchovy	*Basillus* sp. KYJ963	β-Amylase
Deep sea hydrothermal vent	*Pyrococcus abyssi*	Alkaline phosphatase
Sediment of hot spring	*Basillus* sp. 3183	α-Amylase-like pullulanase
Garbage dump	*Basillus circulans*	Xylanase
Compost treated with artichoke juice	*Basillus* sp.	Inulinase

2.2.2 β-Amylase

Another form of amylase, β-amylase (EC 3.2.1.2, 1,4-α-D-glucan maltohydrolase) is also synthesized by bacteria, fungi and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into sugar, resulting in the sweet flavor of ripe fruit. Both are present in seeds, β-amylase is present prior to germination whereas α-amylase and proteases appear once germination has begun. Cereal grain amylase is key to the production of malt. Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not contain β-amylase, although it may be present in microrganisms contained within the digestive tract (Maton *et al*., 1993).

2.2.3 γ-Amylase

In addition to cleaving the last α(1-4)glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose, γ-amylase (EC 3.2.1.3, Glucan 1,4-α-glucosidase) will cleave α(1-6) glycosidic linkages (Maton *et al*., 1993).

2.2.4 Amylase enzyme uses

Amylase enzymes are used extensively in bread making to break down complex sugars such as starch (found in flour) into simple sugars. Yeast then feeds on these simple sugars and converts it into the waste products of alcohol and CO_2. This imparts flavour and causes the bread to rise. While amylase enzymes are found naturally in yeast cells, it takes time for the yeast to produce enough of these enzymes to break down significant quantities of starch in the bread. This is the reason for long fermented doughs such as sour dough. Modern bread making techniques have included amyalse enzymes into bread improver thereby making the bread making process faster and more practical for commercial use. Bacilliary amylase is also used in detergents to dissolve starches from fabrics (Udani *et al*., 2004). Bioconversion reactions and applications of thermostable enzymes is showed in Table 2.2.

Table 2.2: Bioconversion reactions and applications of thermostable enzymes (Haki and Rakshit, 2003)

Enzyme	Temperature range(^{0}C)	Bioconversions
α-Amylase(bacterial)	90-100	Starch-dextrose syrups
α-Amylase(fungal)	50-60	Starch-dextrose syrups
Pullulanase	50-60	Starch-dextrose syrups
Xylanase	45-65	Craft pulp-xylan + lignin
Chitisinase	65-75	Chitin-chitobiose Chitin-chitosan
Cellulase	45-55	Cellulose-glucose
Protease	65-85	Protein-amino acid and peptides
Lipase	30-70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis
DNA polymerase	90-95	DNA amplification

2.3 Starch

Starch is a complex carbohydrate which is soluble in water. It is used by plants as a way to store excess glucose and can be used as a thickening agent when dissolved and heated. The word is derived from Middle English sterchen, meaning to stiffen. The formula for starch is $C_6H_{10}O_5$. In terms of human nutrition, starch is by far the most important of the polysaccharides. It constitutes more than half the carbohydrates even in affluent diets, and much more in poorer diets. It is supplied by traditional staple foods such as cereals, roots and tubers. Starch contains a mixture of two molecules: amylose and amylopectin. Usually these are found in a ratio of 30:70 or 20:80, with amylopectin found in larger amounts than amylose. Starch is often found in the fruit, seeds, rhizomes or tubers of plants. The major resources for starch production and consumption worldwide are rice, wheat, corn, and potatoes (Raven *et al*., 1999).

2.3.1 Composition and structure of starch

Starch is produced as granules in most plants cells and is referred to native when in this particular granular state. Native starches from different botanical sources vary widely in structure and composition, but all granules consist of two major molecular components, amylose and amylopectin, both of which are polymers of α-D-glucose units in the $^4C_1$ conformation (Parker, 2001). In amylose (Figure 2.1), these are linked α -(1 → 4)-, with the ring oxygen atoms all on the same side, whereas in amylopectin about one residue in every twenty is also linked α -(1 → 6)-forming branch-points as shown in Figure 2.2.

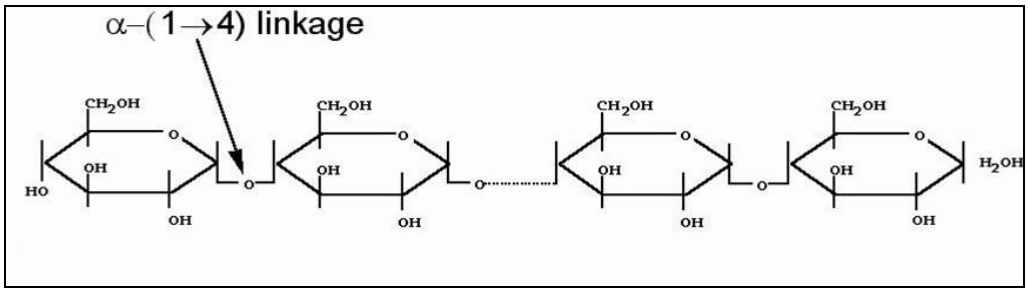

Figure 2.1: Amylose molecule structure (Parker, 2001)

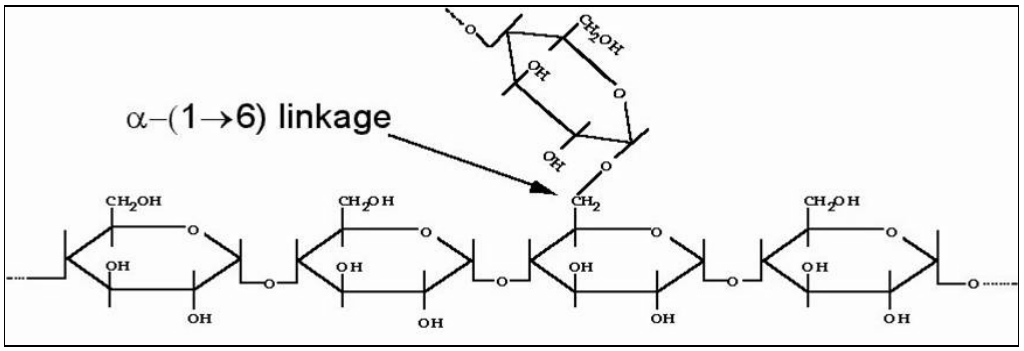

Figure 2.2: Amylopectin molecule structure (Parker, 2001)

2.3.2 Starch derivatives

Starch can be hydrolyzed into simpler carbohydrates by acids, various enzymes, or a combination of the two. The extent of conversion is typically quantified by dextrose equivalent (DE), which is roughly the fraction of the glycoside bonds in starch that have been broken. Food products made in this way include maltodextrin, a lightly hydrolyzed starch product used as a bland-tasting filler and thickener, various corn syrups, viscous solutions used as sweeteners and thickeners in many kinds of processed foods, dextrose, commercial glucose, prepared by the complete hydrolysis of starch, high fructose syrup, made by treating dextrose solutions to the enzyme glucose isomerase, until a substantial fraction of the glucose has been converted to fructose. In the United States, high fructose corn syrup is the principal sweetener used in sweetened beverages because fructose tastes sweeter than glucose and fewer sweeteners may be used (Raven *et al.*, 1999).

2.3.3 Starch applications

2.3.3.1 Papermaking

Papermaking is the largest non-food application for starches globally, consuming millions of metric tons annually. In a typical sheet of copy paper for instance, the starch content may be as high as 8%. Both chemically modified and unmodified starches are used in papermaking. In the wet part of the papermaking process, generally called the “wet-end”, starches that have been chemically modified to contain a cationic or positive charge bound to the starch polymer, and are utilized to associate with the anionic or negatively charged paper fibers and inorganic fillers. These cationic starches impart the necessary strength properties for the paper web to be formed in the papermaking process (wet strength), and to provide strength to the final paper sheet (dry strength). In the dry end of the papermaking process the paper web is rewetted with a solution of starch paste that has been chemically or enzymatically depolymerized. The starch paste solutions are applied to the paper web by means of various mechanical presses (size press). The dry end starches impart

additional strength to the paper web and additionally provide water hold out or “size” for superior printing properties (Raven *et al*., 1999).

2.3.3.2 Corrugating glues

Corrugating glues are the next largest consumer of non-food starches globally. The corrugating glues generally contain a mixture of chemically modified and unmodified starches that have been partially gelatinized to form an opaque paste. This paste is applied to the flute tips of the interior fluted paper to glue the fluted paper to the outside paper in the construction of cardboard boxes. This is then dried under high heat, which provides the box board strength and rigidity (Raven *et al*., 1999).

2.3.3.3 Construction industry

In construction industry starch is used in the sheet rock or wall board manufacturing process. Chemically modified or unmodified starches are added to the rock mud containing primarily gypsum. Top and bottom heavyweight sheets of paper are applied to the mud formulation and the process is allowed to heat and cure to form the eventual rigid wall board. The starches act as glue for the cured gypsum rock with the paper covering and also provide rigidity to the board (Raven *et al*., 1999).

2.3.3.4 Clothing starch

Clothing starch or laundry starch is a liquid that is prepared by mixing a vegetable starch in water (earlier preparations also had to be boiled), and is used in the laundering of clothes. Starch was widely used in Europe in the 16th and 17th centuries to stiffen the wide collars and ruffs of fine linen which surrounded the