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MOLECULAR CLONING AND EXPRESSION OF A THERMOSTABLE α-AMYLASE FROM *GEOBACILLUS* SP.

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By

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MOLECULAR CLONING AND EXPRESSION OF A THERMOSTABLE α-AMYLASE FROM *GEOBACILLUS* SP.

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Elias Kebede Kassaye

September 2009

Chairman: Abu Bakar Salleh, PhD

Faculty: Biotechnology and Biomolecular Sciences

Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. Selection of a suitable strain is the most significant factor in the amylase production process. On the other hand the screening for a single amylase is difficult because one strain can produce different amylases with different specificities or the amount of amylase produced may be very low. Thus the cloning of one gene directing the synthesis of the desired amylase in a well characterized host like *E. coli* should help greatly in the characterization of new amylases and also allow a significant yield increase. Studies on the screening of amylase producing bacterial strains were carried out on soil and water samples collected from a hot spring located in Slim River, Perak, Malaysia. The bacteria were cultivated in a mineral medium containing soluble starch as the sole carbon source. Three of the isolates namely SR37, SR41, SR74 have demonstrated good activity based on the assay performed using DNS method at 60^{o}C and pH 7.0. The

isolates showed activity of 2.44 U/ml, 4.5 U/ml, 2.05 U/ml for amylopectin and 1.78 U/ml, 3.54 U/ml, 1.65 U/ml for soluble starch, respectively. None of the isolates except SR74 (1.65 U/ml) showed activity at 70^{o}C. Since it showed activity at 70^{o}C, further study was conducted on the isolate SR74 for identification, gene cloning, sequencing and expression for the α-amylase enzyme. Gram staining and morphological studies revealed the isolate was a Gram positive *Bacillus.* Molecular characterization using the 16S rDNA for the isolate SR74 revealed the organism closely related to the members of the genus *Geobacillus.* The fatty acid methyl ester analysis using the Sherlock system also resulted in a typical fatty acid profile of a thermophilic *Geobacillus* and other bacilli. Among them the iso-branched pentadecanoic acid (iso-15:0), haxadecanoic acid (iso-16:0) and heptadecanoic acids (iso-17:0) accounted for 82.26% of the total fatty acids.Iso-15:0 and iso-17:0 were especially abundant. This isolate exhibited anteiso-15:0 (1.05%) and anteiso-17:0 (6.5%) as minor components (7.55% of the total). The isolate was identified as *Geobacillus* sp. SR74. The gene coding for a thermostable α-amylase from *Geobacillus* sp. SR74 was isolated, sequenced and expressed in *Escherichia coli* BL21 (DE3) pLysS. Gene sequencing showed that the enzyme secreted by this isolate shared 98% similarity with *Geobacillus stearothermophilus* α-amylase gene. The ORF of the gene codes for 549 amino acids. The signal peptide comprised 34 amino acids and the remaining 515 amino acids belong to the mature polypeptide. The region encoding the mature α-amylase was heterogeneously expressed in *E. coli* BL21 (DE3) pLysS cells using the pET-32b expression system under the control of the T7 promoter. The mature enzyme had a theoretical molecular weight of 58,547 Daltons and a theoretical *pI* of 5.61. Optimization studies revealed that the highest enzyme activity was

obtained at 16h post induction (32.414 U/ml). The optimum inducer concentration was found to be 0.15mMol L^{-1} IPTG (39.73 U/ml). With regard to production media, LB (49.53 U/ml) and 0.75YT (51.06 U/ml) were found to be best for optimum production of the recombinant enzyme, while the A_{600nm} of 0.75 (58.3 U/ml) being the best microbial density for inducing the production of the enzyme.

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

PENGKLONAN MOLEKUL DAN PENGEKSPRESAN α-AMILASE TERMOSTABIL DARIPADA *GEOBACILLUS* SP.

Oleh

Elias Kebede Kassaye

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Enzim pendegradasi kanji seperti amylase adalah, merupakan antara enzimyang mendapat perhatian ramai penyelidik memandangkan kepentingannya dalam bidang sains, teknologi dan ekonomi. Oleh itu, pemencilan dan penyaringan strain yang bersesuaian merupakan faktor yang amat penting dalam proses penghasilan amilase. Pada masa yang sama, pemencilan amilase tunggal merupakan satu proses yang sukar berdasarkan kepada kemampuan satu strain untuk menghasilkan pelbagai jenis amilase yang mempunyai spesifikasi berlainan dan ditambah dengan penghasilan enzim yang amat rendah. Oleh yang demikian, pengklonan satu gen yang mengkodkan amilase yang dikehendaki di dalam *E. coli* akan membantu dalam pencirian enzim tersebut di samping meningkatkan penghasilannya. Kajian mengenai pencarian strain bakteria yang menghasilkan amilase telah dijalankan ke atas sampel tanah dan air yang telah diambil daripada

kawasan air panas di Slim River, Perak. Bakteria telah ditumbuhkan di dalam medium mineral yang mengandungi kanji terlarut sebagai sumber utama karbon. Tiga pencilan iaitu SR37, SR41, SR74 telah menunjukkan aktiviti yang baik berdasarkan asai yang dijalankan menggunakan kaedah DNSA pada suhu 60°C dan pH 7.0. Pencilan tersebut masing – masing menunjukkan aktiviti sebanyak 2.44 U/ml, 4.5 U/ml, 2.05 U/ml untuk amilopektin dan 1.78 U/ml, 3.54 U/ml, 1.65 U/ml untuk kanji terlarut. Memandangkan ia telah menunjukkan aktiviti pada suhu 70°C, kajian lanjut telah dijalankan pada pencilan SR74 untuk pengenalpastian (identifikasi), pengklonan gen, penjujukan dan penghasilan untuk enzim α-amilase. Pewarnaan Gram dan ciri morfologi menunjukkan yang pencilan ini adalah *Bacillus* yang mempunyai dinding sel gram positif. Pencirian molekular yang menggunakan 16S rDNA untuk pencilan SR74 menunjukan organisma ini mempunyai hubungan/pertalian yang rapat dengan genus dari kumpulan *Geobacillus*. Analisis ester metil asid lemak menggunakan sistem Sherlock juga menunjukkan profil asid lemak yang sama dengan *Geobacillus* yang termofilik dan lain – lain *bacillus*. Dalam profil tersebut, cabang asid iso-pentadekanoik (iso-15:0), asid heksadekanoik (iso-16:0) dan asid heptadekanoik (iso-17:0) menyumbang sebanyak 82.26% daripada keseluruhan asid lemak. Iso-15:0 dan iso-17:0 paling banyak didapati. Pencilan ini mempamerkan anteiso-15:0 (1.05%) dan anteiso-17:0 (6.5%) sebagai komponen minor (7.55% daripada keseluruhan). Pencilan ini dinamakan sebagai *Geobacillus* sp. SR74. Gen yang mengkodkan α-amilase termostabil daripada *Geobacillus* sp. SR74 ini telah dipencilkan, dijujukkan dan diekspreskan di dalam *Escherichia coli* BL21 (DE3) pLysS. Penjujukkan gen menunjukkan enzim yang dirembeskan oleh pencilan ini berkongsi 98% persamaan dengan gen α-amilase daripada *Geobacillus*

stearothermophilus. Gen α-amilase daripada ORF gen ini mengkodkan 549 asid amino. Peptida isyarat terdiri daripada 34 asid amino dan selebihnya iaitu 515 asid amino adalah polipeptida matang. Gen yang mengkodkan α-amilase matang telah diekspreskan secara heterogenus di dalam sel *E. coli* BL21 (DE3) pLysS menggunakan vektor pengekpresan, pET 32-b yang dikawal oleh promoter T7. Secara teori, enzim matang ini mempunyai berat molekul sebanyak 58,547 Daltons dan *pI* 5.61. Tempoh aruhan yang optimum dapat diperhatikan pada 16 jam selepas aruhan (39.734 U/ml). Aruhan optimum menggunakan IPTG adalah pada kepekatan 0.15 mmol/L (39.73 U/ml). Medium terbaik adalah dengan menggunakan 0.75YT (51.06 U/ml) dan LB (49.53 U/ml), sementara ketumpatan mikrob yang terbaik untuk aruhan penghasilan enzim ialah pada A_{600nm} 0.75 (58.3 U/ml).

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I certify that a Thesis Examination Committee has met on September 16 2009 to conduct the final examination of Elias Kebede Kassaye on his thesis entitled "Molecular Cloning and Expression of a Thermostable α-Amylase from *Geobacillus* sp." in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 march 1998 The Committee recommends that the candidate be awarded the relevant degree.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that this thesis is the result of my own 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 any other institution.

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ELIAS KEBEDE KASSAYE

Date: 10 February 2010

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LIST OF ABBREVIATIONS

A: Adenine

α: Alpha

β: Beta

C: Cytosine

CAZy: Carbohydrate active enzymes

Da: Daltons

dH_2O: distilled water

DNA: Deoxyribonucleic acid

DNSA: Dinitrosalicylic acid

DSMZ: Deutche Slammlung Von Microorganismen

EDTA: Ethylene di-amine tetrachloro acetate

g/L: Gram per Litre

g :Gram

G: Guanine

GH: Glycosyl hydrolases

GOC: Groundnut oil cake

GTE: Glucose/Tris-Hcl/EDTA

h :Hour

IPTG: Isopropyl-β-D-galactoside

IUB-MB: International union for biochemistry and molecular biology

KDa: Kilo Daltons

L: Litre

LB: Luria Bertani

M: Molar

µg: Microgram

µl: Microliter

µm: Micrometer

mg: milligram

min: Minute

ml: milliliter

mM: millimolar

mRNA: messenger RNA

NB: Nutrient broth

nm: nanometer

OD: Optical density

Ori: Origin of replication

ORF: Open Reading Frame

PCI: Phenol/Chloroform/Isoamyl alcohol

PCR: Polymerase Chain Reaction

RBS: Ribosomal Binding Site

RNA: Ribonucleic acid

rRNA: ribosomal RNA

SD : Shine Dalgarno

SDS–PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Smf : submerged fermentation

SOC: Super optimal broth with catabolite repression

SP: Signal peptide

SR: Slim River

SSF: Solid state fermentation

T: Thiamine

TIM: Triose phosphate isomerase type barrels

TIR: Translation initiation region

U/mg: Unit per milligram

U/ml: Unit per milliliter

UV: Ultra violet

v/v :Volume per volume

WB: Wheat bran

w/v : Weight per volume

YT: Yeast Trypt

CHAPTER 1

Introduction

Enzymes or biocatalysts are the key components of industrial biotechnology. Their abilities to convert the substrates to desired products at mild conditions and in greatly specific manners are essential for sustainable development of various industries including food, textile and pharmaceuticals. In recent years the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extra-cellular enzymatic activity in several microorganisms (Gupta *et al*., 2003; Omemu *et al*., 2005; Yamabhai *et al.*, 2008).

Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. In terms of energy utilization and process simplicity, amylase conversion of raw starches is believed to be superior to the conventional method that makes use of pre-gelatinized starch as substrate. Amylases are among the most important enzymes having a great significance with extensive biotechnological application in a number of industrial processes such as in the food, fermentation, textiles, paper industries and in detergent producing processes comprising about 30% of the world's enzyme production. They would be potentially useful in the pharmaceutical and fine chemicals industries if enzymes with suitable properties can be prepared (Omemu *et al.*, 2005; Hmidet *et al*., 2008).

Due to the increasing demand for amylase enzymes in various industries, there is enormous interest in developing enzymes with novel properties such as raw starch degrading amylases suitable for industrial applications and their cost effective

production techniques. Amylases can be derived from several sources such as plants, animals and micro-organisms; however the enzymes from microbial sources generally meet industrial demands. Microbial amylases have successfully replaced the chemical hydrolysis of starch in starch processing industries. The major advantages of using microorganisms for the production of amylases is the cost effective bulk production capacity, consistency, less time and space required for production, ease of process modification and optimization and microbes are relatively easy to manipulate to obtain enzymes of desired characteristics (Pandey *et al.*, 2000; Gupta *et al*., 2003; Asgher *et al.*, 2007).

Genetic engineering has been used extensively for cloning of amylase producing strains, mainly on α-amylases and glucoamylases in order to achieve desirable characteristics in the cloned host. The purpose of gene cloning can be amongst others, the expression of thermo stable enzymes, higher enzyme productivity and co-expression of two enzymes by the same organism (Pandey *et al*., 2000).

Escherichia coli is one of the most useful bacteria as a host cell in the field of gene engineering because of high-level expression and synthesis of gene products by utilizing *E. coli*. This organism has been used extensively for cloning of exogenous gene and expression of exogenous proteins. *E. coli* has been the "factory" of choice for the expression of many proteins because its genome has been fully mapped and the organism is easy to handle; grows rapidly; requires an inexpensive, easy-to-prepare medium for growth; and secretes protein into the medium which facilitates recovery of the protein (Coronado *et al*., 2000; Kang *et al*., 2004; Shiina *et al*., 2004).

Demand for novel amylases worldwide is increasing day by day, as these enzyme application spectra are spreading in various industrial sectors. The classical approach is the isolation of microbial species, which produce novel enzyme from exotic environments and would offer a competitive advantage over the existing products. Subsequent characterization of these microbes under fermentation conditions to optimize the enzyme production properties plays vital role in evaluation of economic significance (Prakasham *et al.*, 2007).

Although amylases can be produced by several micro-organisms, it remains a challenging task to obtain a strain capable of producing commercially acceptable yields. Selection of a suitable strain is the most significant factor in the amylase production process. Each application of amylases requires unique properties with respect to specificity, stability, temperature and pH dependence. Screening of microorganisms with higher amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Pandey *et al*., 2000; Asgher *et al.,* 2007). On the other hand the screening for a single amylase is difficult because one strain can produce different amylases with different specificities or the amount of amylase produced may be very low. Thus the cloning of one gene directing the synthesis of the desired amylase in a well characterized host like *E. coli* should help greatly in the characterization of new amylases and also allow a significant yield increase (Ozcan *et al*., 2001).

The general objective of the current study was therefore to isolate thermostable α-amylase producing bacteria, to clone and express the thermostable α-amylase gene.