CLONING, EXPRESSION AND CHARACTERIZATION OF COLD-ADAPTED CHITINASE FROM *Arthrobacter* sp. 6A1

MCMARSHALL M. ONGKUDON

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2018

CLONING, EXPRESSION AND CHARACTERIZATION OF COLD-ADAPTED CHITINASE FROM *Arthrobacter* sp. 6A1

MCMARSHALL M. ONGKUDON

THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2018

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : _____
_____
_____

IJAZAH : _____
_____

SAYA : _____ **SESI PENGAJIAN** : _____
(HURUF BESAR)

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☐ TIDAK TERHAD

Disahkan oleh:

_____ _____
(TANDATANGAN PENULIS) (TANDATANGAN PUSTAKAWAN)

Alamat Tetap: _____
_____
_____
_____

_____
(NAMA PENYELIA)

TARIKH: _____ TARIKH: _____

Catatan:
*Potong yang tidak berkenaan.
*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

4 May 2018

.....

Mcmarshall M. Ongkudon

MZ1612007T

ACKNOWLEDGEMENT

First and foremost, I want to give thanks to God for His blessings throughout the accomplishment of this research project.

Secondly, I would like to express my appreciation to my supervisor, Dr. Cahyo Budiman and co-supervisor, Prof. Dr. Clemente Michael Wong Vui Ling for their supports, advises, and guidance throughout this project.

I would also like to thank Universiti Malaysia Sabah for the research grant (Code: GUG0105-01/2017) for funding this research.

For my family and friends, I appreciate their supports and prayers very much, which help me a lot throughout this project.

To my entire colleagues, especially my lab mates, thank you for assisting me in my lab works which gives me many new and useful knowledge and skills throughout this project.

Last but not least, to all the lecturers and staffs of Biotechnology Research Institute UMS (BRI UMS), I appreciate all the advices and support throughout my study in BRI UMS.

ABSTRACT

Chitin is the second most abundant natural polymer after cellulose and received special interest as a reusable substance. It consists of a chain of N-acetyl glucosamine (GlcNAc) linked via β-1,4 glycosidic bond. Chitinase enzymes catalyse the hydrolysis of the β-1,4 linkages between the GlcNAc. Microbial chitinase is gaining interest for application such as chitinous waste disposal, pest bio-control, and production of GlcNAc for disease treatments and single cell protein production. In a recent discovery, whole genome sequencing of a novel cold-adapted *Arthrobacter* sp. 6A1 isolated from Schirmacher Oasis, Antartica revealed the presence of a chitinase encoding gene, designated as *6A1Chi*. The gene encodes a chitinase (6A1Chi) with the size of 60.88 kDa and contains a catalytic domain (6A1ChiCAT) with the size of 39.89 kDa. This chitinase is hypothesized to be active at low temperature and considered as a cold-adapted enzyme. In the present study, the functionality of the gene encoding 6A1Chi enzyme (6A1Chi) was confirmed under plate agar system. The result showed that 6A1 strain exhibited chitinolytic activity at 15°C, but not at 37°C which suggested that 6A1Chi gene is indeed functional. Further, the gene encoding full-length of this enzyme (6A1Chi) and its catalytic domain (6A1ChiCAT) were ligated onto pET22b+ expression vector and heterologously expressed in *E. coli* BL21 (DE3) CodonPlus. The 6A1Chi enzyme was produced as an insoluble protein and was successfully solubilized using 8M urea solution. However, further purification of this enzyme was unable to produce sufficient amount of protein for further analysis. Meanwhile the 6A1ChiCAT enzyme was produced as a soluble protein. These enzymes were then purified using IMAC. Characterization was conducted only on 6A1ChiCAT by using *p*-NP-$(GlcNAc)_2$ as the substrate at pH 8. Optimal temperature for 6A1ChiCAT activity is 30 °C and shows activity at 5 °C - 15 °C. The catalytic efficiency of 6A1ChiCAT at 15 °C and 30 °C is 0.032 sec^{-1} mM^{-1} and 0.061 sec^{-1} mM^{-1}, respectively. Similar results were also found for chitinases from psychrophilic *Moritella marina*, which display optimum temperature for their catalytic activity at around 30°C. Besides, the nature of *Arthobacter* sp. 6A1 as a facultative psychrophile may also account for this issue. In conclusion, the 6A1Chi enzyme was expressed as an active protein under the native expression system and shows activity and inactivity at 15 °C and 37 °C respectively. The 6A1Chi and 6A1ChiCAT proteins were also successfully expressed in a recombinant system. The hypothesis is accepted in which the 6A1ChiCAT shows cold-adaptive properties whereby, its optimal temperature is lower than most of its mesophilic homologs, and shows activity at low temperature (5 °C - 15 °C). Altogether, we proposed that 6A1Chi, the catalytic domain of this enzyme (6A1ChiCAT) is more promising to be used for further applications as the domain was able to be expressed in soluble form and catalytically active.

ABSTRAK

Chitin adalah polimer semulajadi yang kedua terbanyak selepas selulosa dan menjadi tarikan sebagai bahan yang boleh diguna semula. Chitin terhasil daripada rantaian N-acetyl glucosamine (GlcNac) dihubungkan oleh ikatan glikosidik β-1,4. Enzim chitinase berfungsi sebagai pemangkin hidrolisis ikatan glikosidik β-1,4 pada rantaian GlcNAc. Chitinase daripada mikroorganisma mendapat perhatian sebagai aplikasi untuk penghapusan sisa-sisa chitin, kawalan biologi mahkluk perosak dan penghasilan GlcNAc untuk tujuan rawatan penyakit dan penghasilan *single cell protein.* Dalam penemuan terbaru, keputusan penjujukan genom *Arthrobacter* sp. 6A1 daripada Schrimacher Oasis, Antartica menunjukkan terdapatnya gen yang menghasilkan chitinase, *6A1Chi* dalam genom tersebut. Gen ini mengkodkan enzim chitinase (6A1Chi) dengan berat molekul 60.88 kDa dan mempunyai domain katalitik (6A1ChiCAT) dengan berat molekul 39.89 kDa. Maka, hipotesis dalam kajian ini ialah, 6A1Chi adalah aktif pada suhu yang rendah dan dikira sebagai enzim tahan sejuk. Dalam kajian ini, kefungsian gen *6A1Chi* dan domain katalitik, *6A1ChiCAT* telah ditentukan melalui sistem plat agar. Keputusan menunjukkan bahawa strain 6A1 ini berupaya untuk meleraikan molekul chitin pada suhu 15 °C tetapi tidak pada 37 °C yang mana menunjukkan kefungsian gen 6A1Chi. Gen 6A1Chi telah diklon pada plasmid pET22b+ dan diekspres menggunakan sel *E. coli* BL21 (DE3) CodonPlus. Enzim 6A1Chi dihasilkan sebagai protein tidak larut dan berjaya dilarutkan menggunakan larutan 8M urea. Walaubagaimanapun, penulenan enzim ini gagal memberi amaun yang cukup untuk pencirian. Manakala 6A1ChiCAT dihasilkan sebagai protein yang larut air. Enzim-enzim tersebut kemudian ditulenkan menggunakan IMAC. Pencirian hanya melibatkan 6A1ChiCAT, dengan menggunakan *p*-NP-$(GlcNAc)_2$ sebagai substrat, pada pH 8. Suhu optima untuk 6A1ChiCAT ialah 30 °C dan aktif pada suhu 5 °C - 15 °C. Efisiensi katalisis 6A1ChiCAT pada 15 °C dan 30 °C ialah 0.032 s^{-1} mM^{-1} and 0.061 s^{-1} mM^{-1}. Penemuan yang sama dilaporkan pada chitinase psikrofilik daripada *Moritella marina* yang mempunyai suhu optima sekitar 30 °C. Selain itu, sifat anaerob fakultatif pada *Arthrobacter* sp. 6A1 juga dapat dikaitkan dengan ciri-ciri 6A1ChiCAT. Kesimpulannya, enzim 6A1Chi diekspres sebagai protein yang aktif melalui sistem ekspresi yang asli. 6A1Chi aktif pada suhu 15 °C dan tidak aktif pada suhu 37 °C. Protein 6A1Chi dan 6A1ChiCAT juga berjaya dihasilkan melalui sistem rekombinan. Hipotesis diterima, di mana 6A1ChiCAT menunjukkan ciri-ciri tahan sejuk, di mana suhu optimanya adalah lebih rendah daripada kebanyakkan homolog mesofiliknya, dan aktif pada suhu rendah (5 °C- 15 °C). Keseluruhannya, domain katalitik bagi 6A1Chi (6A1ChiCAT) menunjukkan potensi yang baik untuk aplikasi kerana domain ini mampu diekspres sebagai protein yang larut dan aktif.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Csps	-	Cold shock protein
CBD	-	Chitin binding domain
CSD	-	Cold shock domain
FnIII	-	Fibronectin type III domain
FPLC	-	Fast protein liquid chromatography
GlcNAc	-	N-acetyl glucosamine
SCP	-	Single cell protein
SDS-PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
***p*-NP**	-	para-Nitrophenyl

CHAPTER 1

INTRODUCTION

1.1 Background

Chitin is the second most abundant natural polymer after cellulose and received special interest as a reusable substance (Kubicek et al., 2001; Lonhienne et al., 2001). Chitin consist of a chain of N-acetyl glucosamine (GlcNAc) linked via β-1,4 bond. It is an important component in fungal cell walls and certain green algae. Chitin exists abundantly in the shells, cuticles and exoskeletons of worms, molluscs, and arthropods (Stefanidi and Vorgias, 2008). Chitin made up 20 to 58% of the marine invertebrates dry weight (Dahiya et al., 2005). The vast quantities of chitin continuously produced in nature require large scale of removal and recycling (Ramli et al., 2011). Previous studies reported the various applications of chitin monomer in the industries such as single cell protein production and used as an anti-inflammatory drug (Patil et al., 2000).

In the current centuries, chitin monomer, GlcNac, has been massively produced via acid hydrolysis. However, this method is causing several problems including high production cost, low yield (<65%), and acidic pollution (Chen et al., 2010). Hence, enzymatic method is gaining attention due to the environment friendly features, and specific degradation activity. For this purpose, chitinase enzyme is preferred.

Chitinase enzymes catalyse the hydrolysis of the β-1,4 glycosidic linkages between monomers of the chitin. Due to the abundance, low cost, high stability and rapid production, microbial chitinase is gaining interest for application such as chitin waste disposal, pest bio-control, and disease treatments (Das et al., 2017; Rathore and Gupta, 2015).

A novel *Arthrobacter* sp. 6A1 was isolated by Wong et al., (2005) from the soil at Schirmacher Oasis, East Antarctica. This bacteria display optimum growth temperature at 15oC which was then considered as a cold-adapted bacterium. Whole genome sequencing revealed the presence of a putative chitinase gene in its genomic DNA, designated as 6A1Chi. Nevertheless, whether the gene is functional or not remain to be further studied. The 6A1Chi enzyme produced by this strain was hypothesized to be a cold-adapted chitinase as it comes from a psychrophilic bacterium.

Cold-adapted enzymes are characterized by their optimum activity at temperatures ranging from 4 °C - 20 °C which usually lower than their mesophilic and thermophilic homologs (Singh et al., 2016). Cold-adapted enzymes provide several advantages in the industries. These include energy saving and minimized chemical side-reactions which may occur at higher temperatures (Santiago et al., 2016).

To compare, the optimal temperature for mesophilic and thermophilic chitinase ranges from 40 °C - 60 °C and 70 °C - 85 °C respectively, which generally higher than the psychrophilic chitinase (Elleuche et al., 2015; Nagpure et al., 2014). The differences in their adaptability towards low temperatures are known to be related to their unique structural features. These features include increased glycine residues and reduced proline in loop regions residues to enhance conformational flexibility, and reduced arginine residues which results in reduced salt bridge and hydrogen bond formation (Feller, 2010). Additionally, the size of non-polar residues in the protein core is also reduced which causes weaker hydrophobic interactions (Feller, 2010).

Nevertheless, cold-adapted enzymes provide several advantages in the industries. These include energy saving and minimized chemical side-reactions which may occur at higher temperatures (Santiago et al., 2016). Unfortunately, exploration and studies on cold-adapted chitinases are not as many as their mesophilic and thermophilic counterparts. This might be due to limited sources of cold-adapted chitinases.

Currently, several bacteria that produce cold-adapted chitinase have been characterized, which include *Arthrobacter* sp. TAD20, *Alteromonas* sp. O-7, *Moritella marina*, *Sanguibacter antarticus* KOPRI 21702 and *Vibrio* sp. strain Fi:7 (Bendt et al., 2001; Lonhienne et al., 2001; Orikoshi et al., 2005; Park et al., 2009; Stefanidi and Vorgias, 2008). Optimum pH for their catalytic activity was found to be in the range of 4.0 – 7.6. Interestingly, the optimum temperature for the catalytic activity was reported to be in the range of 15 °C– 37 °C which is beyond the optimum growth temperature of the host cells. Sato et al. (2011) previously suggested that some proteins or enzymes from psychrophilic or cold-adapted bacteria do not follow the optimum growth temperature of the host cells. This unusual property might be related to the specific requirement for biological function. It is interesting to confirm if chitinases also follow this assumption. Studies on chitinases from wider cold-adapted or psychrophilic bacteria are then unavoidable to confirm if this assumption applies to chitinases.

The commercial potentials of cold-adapted chitinases include their application as bio-fungicide and for the production of the pharmaceutically beneficial glucosamine (Cavicchioli et al., 2011). Unfortunately, only limited reports on cold-adapted chitinase are available as compared to their mesophilic and thermophilic counterparts. This leads to the need for further exploration and studies on cold-adapted chitinases. Accordingly, studies on 6A1Chi enzyme should contribute to addressing this issue. Besides, microbial enzymes offer more advantages for applications as compared to enzymes from animal or plant. Production of microbial enzymes involves lower cost and the raw materials for microbial cultivation are widely available. Additionally, microbes contains less injurious constituents and they are more predictable and controllable in terms of enzyme contents (Kuddus, 2018).

Besides, further studies and upstream applications of cold-adapted chitinases are often limited by the production system for having sufficient amount of the enzymes. As an example, a high concentration of protein is needed for structural studies using X-ray crystallography or NMR spectroscopy. In addition, mass production of enzymes is certainly needed in industrial applications. To this respect, heterologous expression system is known to be an efficient system for mass-production of the enzyme.

Heterologous gene expression system allows a desired protein encoding genes from a particular species to be expressed in the cell of a different species (Gagnon, 2009). Heterologous gene expression system is constructed by isolating the gene of interest (GOI) from the native host genome followed by the ligation of the GOI onto the cloning site of an expression vector to yield a recombinant plasmid. The expression construct is then used to transform the heterologous expression host cells, followed by expression induction of the GOI in the expression host (El-Gayar, 2015).

The present study attempts to address two main hypotheses as follows. First, the gene encoding 6A1Chi enzyme is hypothesized to be functional, which enable this strain to produce chitinase enzyme that capable of degrading chitin-containing substrates for their energy purposes. Second, the 6A1Chi enzyme is hypothesized to exhibit cold-adapted properties, particularly to be active at low temperature (5 °C- 15 °C). Accordingly, this study involves confirmation of functionality of the gene encoding 6A1Chi of *Arthrobacter* sp. 6A1. Further, the study also describes the cloning of the full length and a catalytic domain of *6A1Chi* gene followed by heterologous expression of the gene in *E. coli BL21* (DE3) CodonPlus and purification of the expressed chitinase using Immobilized Metal Affinity Chromatography (IMAC). Subsequently, optimum temperature and the kinetic of catalytic activity of this enzyme were determined. This study is important as the outcome provides a platform for further studies and industrial applications of the 6A1Chi enzyme.

1.2 Research Objectives

The objectives of the study are:

1. To determine the functionality of the gene encoding chitinase enzyme in genomic DNA of *Arthobacter* sp. 6A1 (*6A1Chi*).
2. To determine the expressibility and solubility of 6A1Chi enzyme under a heterologous expression system.
3. To characterize optimum temperature for the catalytic activity and the kinetic activity of 6A1Chi enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Chitin

Chitin is a natural polymer which made up of GlcNAc monomer linked by β-1, 4 glycosidic bonds (Figure 1). Chitin is the second most abundant natural polymers following cellulose and a continuous source of renewable raw materials (Tharanathan and Kittur, 2003). Currently, commercial production of chitin and chitosan are available in Norway, India, Japan, Poland, the US, and Australia (Zargar et al., 2015).

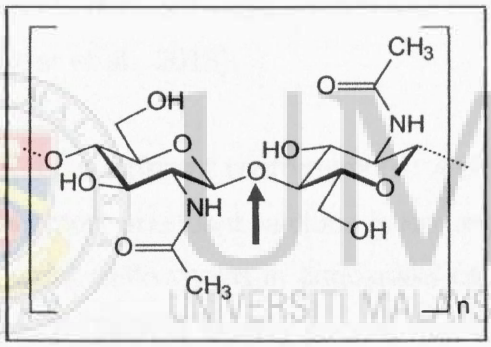

Figure 1: **Chitin structure. The red arrow indicates the glycosidic oxygen**
Source: Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*, *13*(3), 1133–1174

2.1.1 Chitin Occurrence

Chitin exists naturally as a constituent of crustacean and insect exoskeleton, diatoms, fungal cell walls, and squid pens. Crabs and shrimps contain the highest percentage of chitin which produces 90% of chitinous waste (Rathore and Gupta, 2015). Chitin in the exoskeleton of arthropods or the fungal cell walls is naturally exists as ordered crystalline microfibrils (Zargar et al., 2015). More than 80,000 tons of chitin are produced from industrial wastes every year (Kim et al., 2007), mostly from the seafood processing industry.

2.1.2 Physicochemical Properties of Chitin

Chitin is a hydrophobic substance and is insoluble in most organic solvents. It has a comparable function to that of collagen in animals and cellulose in plants. While plants produce cellulose in their cell walls, insects and crustaceans produce chitin in their exoskeleton (Zargar et al., 2015).

Chitin exists in three different conformations: α-chitin, β-chitin, and γ- chitin with α-Chitin being the most abundant. α-chitin is isomorphic and more condensed due to the organization of chitin chains in antiparallel configuration which enhance the hydrogen bonding. β-Chitin is loosely packed due to parallel configuration of the chain with reduced intermolecular forces. γ-chitin has a combination of both α- and β-chitins configuration (Ramírez-Coutiño et al., 2006). Chitin can be degraded by chitinase to produce disaccharides and longer oligosaccharides (Kurita, 2001).

The degree of acetylation in chitin is usually 0.90, indicating the presence of some amino groups. Amino groups in chitin may vary (5-15%) due to deacetylation during extraction. Chitin solubility and solution properties are highly influenced by the degree of N-acetylation (Zargar et al., 2015).

2.1.3 Application of Chitin and Its Derivatives, Chitosan

Chitin and its derivatives, chitosan are biocompatible and biodegradable substances. Many of its applications for specific purposes have been discovered. These include sponges and bandages to treat wounds and suture threads. Exposure of blood to chitosan activates the formation of clots due to the interaction of the acid groups of the blood cells and the amino group of the chitosan (Zargar et al., 2015).

In food industry, chitin and its derivatives are utilized as antioxidant and antimicrobial agent to enhance food safety, quality, and shelf-life (Hamed et al., 2016). Addition of 1% chitosan results in 70% reduction of 2- thiobarbituric acid reactive substances (TBARS) values of meat within 3 days of storage at 3°C (Tharanathan and Kittur, 2003). Chitosan's inhibition seems to be related to its chelation of the free iron that is released from the heme proteins of meat during heat processing (Tharanathan and Kittur, 2003).

Chitosan has analogous structural characteristics with glycosamino glycans (GAGs) which are found in the extracellular matrix of certain human tissues. Hence, chitosan is highly beneficial in tissue engineering for enhancing cell attachment and the maintenance of tissue differentiation (Zargar et al., 2015).

In the agricultural industry, chitosan has been applied to enhance the germination of *Carom copticum* (ajowan) seed (Mahdavi and Rahimi, 2013). This study reported the highest germination enhancement of the seeds after treatment with 0.2% chitosan solution. In another study, Zeng et al. (2012) reported the potential application of chitosan as bio-pesticides. The study revealed the induction of pest (insects) repellent effect in soybean seeds after coating with chitosan.

2.2 Chitinases

Chitinases belongs to glycosyl hydrolases family 18 (GH18) and family 19 (GH19) which catalyse the hydrolysis of β-1, 4 glycosidic linkage of GlcNAc within chitin chains. Chitinases vary in molecular weight which range from 20 kDa to approximately 90 kDa (Bhattacharya et al, 2007). Chitin degradation by chitinases produces low molecular weight chitooligomers which are industrially important.

Chitinases are classified into 2 main groups: endochitinases (E.C 3.2.1.14) and exo-chitinases. Endochitinases catalyse the hydrolysis of glycosidic bond at the internal region of chitin monomer. This releases the dimer di- cetylchitobiose and multimers of GlcNAc which include chitotriose, and chitotetraose (Sahai and Manocha, 1993). The exo- chitinases are divided into chitobiosidases (E.C. 3.2.1.29) and 1-4-β-glucosaminidase (Harman et al., 1993). Chitobiosidases catalyse the release of di-acetylchitobiose starting at the non-reducing end of the chitin polymer. 1-4-β-glucosaminidases (E.C. 3.2.1.30) catalyses the cleavage of endochitinases and chitobiosidases hydrolysis product which generate monomers of GlcNAc (Sahai and Manocha, 1993).

2.2.1 Microbial Chitinases

Most industrially applied enzymes are acquired from plants, animals and microbes. However, microbial enzymes are more advantageous in the industries. In comparison with plants and animals enzyme, microbial enzymes can be produced at lower cost and the raw materials for microbial cultivation are widely available. Furthermore, microbes comprise less injurious constituents and they are more predictable and controllable in terms of enzyme contents (Kuddus, 2018). Many of the microbial enzymes have been studied well. These include the chitinase enzyme.

Microbes such as *Streptomyces*, *Alteromonas*, *Escherchia*, and *Aeromonas* are capable of producing chitinases (Hamid et al., 2013). Such microbes have been acquired from soil, shellfish waste, garden and park waste compost, and hot springs (Yuli et al., 2004). Most chitinolytic microbes mainly produce GH 18 chitinases (Yan and Fong, 2015).