DEVELOPMENT OF PROTOCOLS FOR THE GENOMIC CHARACTERIZATION AND EST LIBRARY CONSTRUCTION OF *Cochlodinium polykrikoides*

KALNISHA DEVI

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2011

DEVELOPMENT OF PROTOCOLS FOR THE GENOMIC CHARACTERIZATION AND EST LIBRARY CONSTRUCTION OF *Cochlodinium polykrikoides*

KALNISHA DEVI

THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2011

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS@

JUDUL: DEVELOPMENT OF PROTOCOLS FOR THE GENOMIC CHARACTERIZATION AND EST LIBRARY CONSTRUCTION OF *Cochlodinium polykrikoides*

IJAZAH: SARJANA SAINS

SAYA KALNISHA DEVI A/P PALANI VELU SESI PENGAJIAN: 2009/2011

mengaku membenarkan tesis Sarjana Sains ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan berikut:

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

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau Kepentingan Malaysia seperti termaktub di dalam AKTA RAHSIA RASMI 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
☑	TIDAK TERHAD	

(TANDATANGAN PENULIS)

Disahkan Oleh

(TANDATANGAN PUSTAKAWAN)

Nama Penyelia

Tarikh: 31 OGOS 2011

Tarikh:

CATATAN:- *Potong yang tidak berkenaan.

**Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai sulit atau terhad.

@Tesis dimaksudkan sebagai tesis Ijazah Doktor Falsafah dan Sarjana secara penyelidikan atau disertai sebagai pengajian secara kerja jursus dan Laporan Projek Sarjana Muda (LPSM)

DECLARATION

I hereby declare that the materials used in this thesis are my own except for excerpts, citations, summaries and references, which have been duly acknowledged.

31 August 2011

Kalnisha Devi

PB20098014

CERTIFICATION

NAME : **KALNISHA DEVI**

MATRICS NO. : **PB20098014**

TITLE : **DEVELOPMENT OF PROTOCOLS FOR THE GENOMIC CHARACTERIZATION AND EST LIBRARY CONSTRUCTION OF *Cochlodinium polykrikoides***

DEGREE : **MASTER OF SCIENCE (MOLECULAR BIOLOGY)**

VIVA DATE : **4 AUGUST 2011**

DECLARED BY

1. **SUPERVISOR**
   Mdm. Grace Joy Chin Wei Lie **Signature**

2. **CO-SUPERVISOR**
   Dr. Kenneth Francis Rodrigues **Signature**

3. **CO-SUPERVISOR**
   Prof. Datin Seri Panglima Dr. Ann Anton **Signature**

ACKNOWLEDGEMENT

It is my pleasure to thank the many people who made this thesis possible. My heartfelt thanks goes to my supervisor, Mdm. Grace Joy Chin Wei Lie and also my co-supervisors Dr. Kenneth Francis Rodrigues and Prof. Datin Seri Panglima Dr. Ann Anton who were generous in providing me with continuous encouragement, sound advice, good teaching and lots of good ideas.

I would like to express my deepest gratitude to my fellow coursemates and labmates, Ms. Cassandra Chew Sze Yii, Ms. Joveen Neoh Wan Fen, Ms. Seng Sook Cheng, Mr. Foong Choong Ping and Ms. Hydayaty, who gave me extra guidance on how to go about completing this project, including useful suggestions and tips which I had tend to have overlooked or neglected on my side. I have furthermore to thank the excellent team of laboratory staffs of the Biotechnology Research Institute as well as the Borneo Marine Research Institute, in particular Mdm. Vidarita Maikin, Mr. Mony Mian and Mr. Yusdi Ismail for their tremendous efforts in providing me with the necessary tools, equipments and useful techniques required to carry out my research.

A special and huge thank you goes out to my friends Ms. Jananie Audimulam, Ms. Subashini Ranganathan, Ms. Sathiya Maran, Ms. Sarita Paskaran, Ms. Roopin Kaur, Ms. Lee Hui Yee, Ms. Cindy Chew and Mr. Yeow Chun Liew for their endless support and motivation which helped keep me focused on my project. Thank you for extra helping hand as well as the friendship bond shared together. I love you all. God bless.

KALNISHA DEVI
31 August 2011

ABSTRACT

DEVELOPMENT OF PROTOCOLS FOR THE GENOMIC CHARACTERIZATION AND EST LIBRARY CONSTRUCTION FOR *Cochlodinium polykrikoides*

Cochlodinium polykrikoides is one of the causative agents for harmful algal blooms occurring in the west cost of Sabah, which result in massive economic damage to the aquaculture and mariculture industries. Currently, limited information is available on the genomics of *C. polykrikoides*. The objectives of this study were to develop a protocol for the construction of an expressed sequence tag (EST) library for *C. polykrikoides* cells cultured under normalized conditions, to design gene specific primers using sequence information of related dinoflagellates and to test the gene specific primers against the DNA and RNA of *C. polykrikoides*. *Cochlodinium polykrikoides* stock cultures were obtained from the Unit for Harmful Algal Blooms Studies (UHABs) of the Borneo Marine Research Institute, Universiti Malaysia Sabah and cultured in f/2 medium. Optimization of total RNA isolation from *C. polykrikoides* was performed using five different methods. Among the five methods tried, the RNA isolation protocol using the TRIzol reagent produced the best results. Synthesis of cDNA was performed on successfully isolated RNA samples followed by cloning and direct sequencing of the purified clones. In addition, fourteen gene specific primer pairs were designed and synthesized using sequence information from other harmful algae species. Genomic DNA samples isolated from *C. polykrikoides* cells were used as templates for the PCR amplification carried out using the synthesized gene specific primers. Amplification products from three gene specific primers, psaA1 F/R, COX-1 F/R and ATPsynC F/R were purified and subsequently cloned and directly sequenced. Based on the sequencing results, the cloned products were found to produce identity matches to genes encoding products from similar and related protein families, such as the oxidoreductases and cytochrome oxidases.

ABSTRAK

Cochlodinium polykrikoides merupakan salah satu agen penyebab untuk ledakan mikroalga berbahaya yang berlaku di perairan barat Sabah, yang menyebabkan kerugian besar kepada industri akuakultur dan marikultur. Setakat ini, maklumat terhad berkenaan genomik C. polykrikoides boleh didapati. Tujuan kajian ini adalah untuk mengusahakan satu protokol untuk membina sebuah expressed sequence tag (EST) library untuk sel C. polykrikoides yang dikultur dalam keadaan normal, untuk mereka primer gen khusus dengan menggunakan maklumat penjujukan dinoflagellata berkaitan dan untuk menguji primer gen khusus terhadap DNA dan RNA C. polykrikoides. Kultur induk C. polykrikoides diperolehi daripada Unit Kajian Alga Bahaya (UHABs) dari Institut Penyelidikan Marin Borneo, Universiti Malaysia Sabah dan dikultur di f / 2 media. Optimasi untuk protokol isolasi RNA dari C. polykrikoides dilakukan menggunakan lima methodologi berlainan. Protokol untuk isolasi RNA menggunakan reagen TRIzol didapati memberikan isolasi RNA yang berkualiti tinggi. Sintesis cDNA dilakukan untuk sampel RNA yang diisolasikan dengan lengkap diikuti dengan proses pengklonan dan penjujukan. Di samping itu, empat belas pasang primer gen khusus direka dan disintesis menggunakan maklumat penjujukan dari spesies mikroalga berbahaya yang lain. Sampel genomik DNA yang diisolasi dari sel Cochlodinium digunakan sebagai templat untuk amplifikasi PCR yang dilakukan dengan menggunakan primer khusus gen disintesis. Produk amplifikasi daripada tiga primer gen khusus, psaA1 F/R, COX-1 F/R dan ATPsynC F/R telah ditulenkan dan kemudian diklonkan diikuti dengan proses penjujukan. Berdasarkan keputusan penjujukan, produk pengklonan didapati menghasilkan identiti sesuai untuk produk yang dikodkan oleh gen dari keluarga protein yang sama dan berkaitan, seperti oksidoreductase dan Cytochrome oksidase.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	alpha
β	beta
%	percentage
°C	degree celcius
µm	micrometer
µm	micrometer
µl	microlitre
µM	micromolar
mM	miliMolar
µg/ml	microgram per mililitre
BLASTn	Basic Local Alignment and Search Tool for Nucleotide
bp	base pair
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
et al.	*et alia* (and others)
HAB	Harmful algal bloom
kb	Kilo base pair
MgADP	magnesium-adenosine diphosphate
MgATP	magnesium-adenosine triphosphate
ml	mililitre
LSU	Large Subunit
PCR	Polymerase Chain Reaction
pmol	pico molar
RNA	Ribonucleic Acid
SSU	Small Subunit

St.	Saint
Taq	*Thermus aquaticus*
U	Unit
US$	US Dollars
rcf	relative centrifugal force
rpm	revolutions per minute

CHAPTER 1

INTRODUCTION

1.1 Introduction

The dinoflagellates (division Pyrrhophyta, class Dinophyceae) are an important group of phytoplankton in marine and fresh waters. Their adaptation to a wide variety of environments is reflected by a tremendous diversity in form and nutrition and an extensive fossil record dating back several hundred million years (Graham and Wilcox, 2000).

A number of dinoflagellate species are known to produce potent neurotoxins, which are often associated with the phenomena commonly called "red tides." This term can be quite misleading, since many toxic blooms occur when waters are not discoloured, but other blooms which turn the water red due the high biomass and pigments of the dinoflagellates are not toxic (Smayda, 1997). These outbreaks are now called harmful algal blooms or HABs.

Documentation of HABs has expanded greatly over the last few decades, and presently, nearly every country with marine waters is known to be affected by these blooms (Hallegraeff, 1993). HAB toxins can affect humans, other mammals, seabirds, fish, and many other animals and organisms. HABs caused by the marine ichthyotoxic dinoflagellate *Cochlodinium polykrikoides* Margalef are responsible for mass mortalities of wild and farmed fish worldwide, with catastrophic impacts to aquaculture and local economies. This species was first described from Puerto Rico in the Caribbean by Margalef (1961). The geographic distribution of *C. polykrikoides* is extensive, and populations have been documented in tropical and warm-temperate waters around the world, including the Caribbean Sea, eastern and western Pacific Ocean, the eastern Atlantic Ocean, Indian Ocean, and Mediterranean Sea (Kudela *et al.*, 2008; Matsuoka *et al.*, 2008).

Notorious for causing mass mortalities of wild and farmed fish, *C. polykrikoides* blooms is an issue of national concern in Japan and Korea due to their catastrophic impacts on aquaculture industries and coastal economies. A single bloom season can have devastating impacts to fisheries such as the incident in 1995, whereby a particularly severe and widespread *C. polykrikoides* bloom persisted for nearly eight weeks along the entire south coast of Korea, ultimately costing US$ 95 million in economic losses (Kim, 1998). In Sabah, a large bloom of *C. polykrikoides* cost losses amounting to millions of dollars of high value farmed fish in 2003 (*Daily Express*, 2007). Fish kills associated with *C. polykrikoides* blooms have also been reported in other countries such as the Philippines, China and Japan (Kim *et al.*, 2004; Imai *et al.*, 2006; Azanza *et al.*, 2008; Gobler *et al.*, 2008).

In recent decades, the rapid emergence of *C. polykrikoides* as a major HAB problem has garnered considerable attention from governmental and commercial organizations, and spurred research by the scientific community into the causes and management of these events. To date, limited information pertaining to the genomics of *C. polykrikoides* has been gathered via research. Many aspects of biology, such as its taxonomy, optimum environmental conditions for reproduction, life history including cyst formation and ichthyotoxicity, are still unclear at this time (Matsuoka *et al.*, 2008).

1.2 Objectives of Study

The objectives of this project were:

(a) to develop a protocol for the construction of an expressed sequence tag (EST) library for *C. polykrikoides* cultured under normalized conditions.

(b) to design gene specific primers using sequence information of related dinoflagellates.

(d) to test the gene specific primers against the DNA and RNA of *C. polykrikoides*

1.3 Significance of Study

In the west coast of Sabah, large blooms of *Cochlodinium polykrikoides* pose a serious threat to aquacultulture and fisheries as it causes local aquaculture companies to suffer from revenue loss accounting from the massive kills of high value farmed fish. Such massive economic damage impacts the country as whole, but is most fatal on the local people of Sabah who depend on marine resources as their source of daily income.

As to date, genomic data on *C. polykrikoides* is limited. Although many possible reasons have been suggested as the cause, the exact factors that induce the occurrence of *C. polykrikodes* blooms in Sabah are yet unknown. Expressed sequence tags can be used to identify the set of genes that are actively transcribed, as well as those whose expression have been repressed in the genome of the *C. polykrikoides* cells cultured under normalized conditions.

This study can help to elucidate the gene expression patterns of *C. polykrikoides* and also the factors associated with the induction of blooms. Such information may allow for the development of a better understanding of this harmful algae as well as the formation of strategies that can control the further spread of harmful algal blooms in the future. These efforts may help the relevant state agencies to regulate and mitigate blooms, preventing massive economic losses and public health problems.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Harmful Algal Blooms

2.1.1 Harmful Algal Blooms

Algae are photosynthetic organisms in marine ecosystems which serve as vital components of marine food webs, supporting zooplanktons and other higher life forms. Organisms known to feed and survive on microscopic algae are mainly composed of filter feeding bivalve shellfish such as mussels, oysters, clams and larvae of commercially important crustaceans and finfish feed (Camacho *et al.*, 2007). As a result of massive proliferations of phytoplankton, algal blooms with high concentration of algal biomass occur. Regions that are temporarily rich in inorganic nutrients such as nitrogen, phosphorus and iron encourage the formation of blooms (Camacho *et al.*, 2007). Rensel and Whyte (2003) noted that such blooms are known to occur more frequently during summer time, when sunlight levels are high; and are mostly harmless as they are beneficial to aquaculture and wild fisheries. On the contrary, such high density blooms may have negative effects (under variable circumstances), resulting in massive economic losses to aquaculture, fisheries and tourism operations with major impacts on the environment and human health.

Harmful algal bloom (or HAB) species represent a small subset of algal species that negatively impact humans or the environment by producing toxic blooms. In broader terms, they include potentially toxic (both autotrophic and heterotrophic) species and high-biomass producers that can cause hypoxia and anoxia and also indiscriminant mortalities of marine life after reaching dense concentrations, whether or not toxins are produced (GEOHAB, 2001). Over the past 25 years, HABs have been escalating globally in terms of their frequency, distribution, and in the ramifications of their effects on public health, living resources, and local economies (Burkholder, 1998; Hallegraeff, 1995; Hoagland *et al.*, 2002; Landsberg, 2002). Humans and animals face health risks when exposed

to the toxins or bioactive compounds released by HABs. In addition, the build-ups of high biomass result in deterioration of water quality which degrades aesthetic, ecological and recreational values.

At least 90 species of marine microalgae are known to produce toxins with 70 of these species being dinoflagellates (Camacho *et al.*, 2007). While not strictly pathogenic, approximately 3.5% of the known species of marine microalgae are considered to be harmful or toxic with uprising numbers being reported annually (Landsberg, 2002). Marine algal toxins have been observed to cause more than 60,000 poisoning events yearly, with an associated mortality rate of 1.5% globally (Gill *et al.*, 2003). Based on their modes of action, HAB species can be categorized into three distinct groups (Table 2.1).

Table 2.1: Harmful Algae species and their modes of action

Mode of Action	HAB species involved
Indiscriminate kills of fish and invertebrates via oxygen deprivation	*Heterocapsa triquetra*, *Noctiluca scintillans*
Potent toxin producers with capacity to harm marine organisms and humans	*Prorocentrum spp.*, *Pyrodinium bahamense* var. *compressum*
Harm fish and invertebrates via clogging or damaging of gills	*Cochlodinium polykrikoides*, *Pfiesteria piscicida*, *Heterosigma akshiwo*

Source: Haellegraeff (2003)

2.1.2 Causes of Harmful Algal Blooms Phenomena

Although known as a natural phenomenon, HABs have increased in their frequency, geographic range and intensity since the 1970s, with their economic impact becoming greater now than in the past (Richlen *et al.*, 2010). Several possible reasons have been speculated regarding the reported global expansion, in addition to nutrient pollution. The exact factors contributing to the occurrence of such phenomena are however, yet unknown (Heisler *et al.*, 2008). Species distribution or introduction can occur via natural currents and storms, in the course of transport of cells or cysts. Algal cells or cysts may also be transported by human activities such as dispersal by ballast water exchange or shellfish seeding operations (Schwinghamer *et al.*, 1994). Human activities such as overfishing and increased