The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Detection and Biosynthesis of Puffer Fish Toxin from Bacterial Culture for Novel Medical Application

Yu Chung Him

A Thesis submitted in partial fulfillment of the requirements for the Degree of Master of Philosophy

September 2007

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____

Yu Chung Him

(Name of student)

Abstract of thesis entitled

Detection and Biosynthesis of Puffer Fish Toxin from Bacterial culture for Novel Medical application

Submitted by

Yu Chung Him

For the degree of Master of Philosophy

The Hong Kong Polytechnic University

Abstract

Tetrodotoxin (TTX), commonly known as puffer fish toxin, is one of the most lethal neurotoxins. Recent studies demonstrated that, besides puffer fish, TTX is widely distributed amongst a diversity of animals, which indicates TTX has an exogenous microbial origin, rather than being produced by puffer fishes. Due to its specific blocking action towards voltage gated sodium channel that can cease the transmission of action potential, TTX has the potential to develop as a drug lead candidate for local anesthetics or analgesics.

In the present study, it aims to investigate a novel direction for the acquisition of TTX through fermentation technology using the TTX producing microbes isolated from the puffer fish. Also, the development of detection methods of TTX for the quantification of toxin in the culture medium and for the clinical diagnosis of TTX in poisoned patients.

In order to study the production of TTX in the culture medium by TTX producing bacteria, several detection methods of TTX have been studied. Our research group has developed the use of traditional mouse bioassay for the measurement of TTX culture medium without much purification. Another biological based method for the detection of

TTX is a tissue culture assay using neuroblastoma cell line (ATCC, CCL 131). HPLC is a chemical method exploited for TTX detection. HPLC with post column modification using fluorescent detector was developed and it was commonly used for the detection of TTX in research area. However, the detection would denature TTX into C-9 compound which preparative HPLC was not feasible. Therefore, our research group developed the use of HPLC with UV detector for TTX measurement. By using the novel developed heptansulfonic acid (HSA)-based buffer as mobile phase with UV detector at 197nm, TTX could be measured with a detection limit of 10ng/ml. Also, under this HPLC UV detection system, we have developed a solid phase extraction system of TTX for the measurement of TTX in the urine of TTX poisoned patient. There was a TTX poisoning outbreak in Hong Kong last winter and the urine samples were collected from the Queen Mary hospital. TTX was detected in the urine sample and it was found to correlate to the severity of patient's clinical symptoms with urinary creatinine correction.

For the production of TTX using TTX producing bacteria, puffer fish organs were used as screening targets for TTX producing bacteria and the screened bacteria were identified using MIDI system. Several species of bacteria including *Bacillus-cereus, Raoultella-terrigena, Pseudomonas-putida, Microbacterium-arabinogalactanolyticum and Serratia-marcescens* were studied for the production of TTX using fermentation. Our result suggested that aerobic condition is more favorable to the growth of TTX producing bacteria and TTX was produced in higher concentration in this condition. Shake flask and fermenter with different carbon sources, salt concentrations and the addition of puffer fish organ extracts into addition to the Ocean Research Institute (ORI) medium were used to study the growth and the TTX accumulation profile of different TTX producing bacterium. The result suggested that a suitable carbon source specific to the bacteria

species could significantly enhance the growth of the bacteria. Fermenter study was done to provide a monitored pH and dissolved oxygen condition to study the growing pattern, the consumption of carbon source and the TTX accumulation profile of the TTX producing bacteria. It was found that the addition of puffer fish ovary extract would enhance the TTX production. Therefore, we proposed that some proteins inside the extract were involved in the production of TTX. Total proteins of the puffer fish ovary were extracted and added to the ORI culture medium in a fermenter study. The result of the fermenter study with the addition of total protein suggested that protein extract of puffer fish organ could affect the production of TTX from mouse bioassay analysis.

In conclusion, a novel detection and purification method of TTX in urine and bacterial culture medium were developed. Potential TTX producing bacteria were screened from puffer fish organ. The nutrient requirement, growing condition, growing pattern and the TTX accumulation profiles were studied in shake flasks and fermenter. Puffer fish ovary extract and its protein were found to up regulate the production of TTX in the screened TTX producing bacteria.

PUBLICATIONS ARISING FROM THE THESIS

A. Conference Presentation

Yu, C.H., Yu, P.H.F. Tetrodotoxin: Neurotoxin as potential painkiller of chronic pain. World Institute of Pain 4th World Congress, Budapest, Hungary, September 25-28, 2007.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my chief supervisor Dr. Peter Yu, Associate Professor of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, who gave me the chance to have the MPhil study, as well as his continuous support during the two years study which allows the smooth progress of this project.

I would like to thank Dr. C.F. Yu, Research Associate of Dr. P. Yu, who continuously gave me technical support throughout my project and his kindness during the study visit in Nagasaki University.

I am also grateful to Prof. Sidney Tam and Dr. Tsui Sik-Hon of Queen Mary Hospital, who kindly given the patient's urine samples and useful clinical information for the case study of puffer fish poisoning in Hong Kong.

Thanks are given to Miss Connie Chan, Miss Tammy Cheng and Miss Irene Chan, Dr Chen Cheng for their continuous support, kindly help and their encouragement during the study. Also, they provided a good, harmonic environment for the two years research study.

Table of CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ACT	Activated carbon treatment
B.Cereus	*Bacillus-cereus*
CFP	Ciguatera fish poisoning
C. patoca	*Chelonodon patoca*
CMU	Corrected mouse unit
FBS	Fetal Bovine Serum
HAS	1-heptanesulfonic acid sodium salt monohydrate
HPLC-FLD	High Performance Liquid Chromatography with Fluorescent detector system
HPLC-UVD	High Performance Liquid Chromatography with Ultra-violet detector system
Hr	Hour
ICR	Institute of Cancer Research
ICU	Intensive Care Unit
LB	Luria-Bertani
MEM	Minimum Essential Medium
M. arabinogalactanolyticum	*Microbacterium arabinogalactanolyticum*
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MU	Mouse unit
OD	Optical density

ORI	Ocean Research Institue
PBS	Phosphate buffered saline
PFP	Puffer fish poisoning
PMS	Phenazine methosulfate
PO_2	Partial pressure of oxygen
P.putida	*Pseudomonas putida*
PSP	Paralytic shellfish poisoning
R.terrigea	*Raoultella-terrigea*
S. marcescens	*Serratia marcescens*
STX	Saxitoxin
T. alboplumbeus	*Takifugu alboplumbeus*
T. niphobles	*Takifugu niphobles*
UCC	Urinary creatinine correction
VGSCs	Voltage gated sodium channels
TTX	Tetrodotoxin

1. Background and Literature Review

Puffer fish toxin is one of the most common lethal marine neurotoxin that causes poisoning all over the world. Tetrodotoxin (TTX) is the major toxin present in the puffer fish toxin, which was discovered by the Japanese in 1909. TTX, which is extremely potent, is a non-protein small molecule with a low molecular weight. It is a kind of marine neurotoxin which blocks the voltage-gated sodium channels (VGSCs) in nerve cell membrane. TTX is named after the fish, the Tetraodontiformes (Tetraodon puffer fish) which is a family of puffer fish in which TTX is usually present.

TTX is water soluble, colorless, odorless, heat stable and would not be degraded by cooking. In Japan, puffer fish (fugu) is considered a great delicacy and 30-100 people are poisoned by TTX intoxication as a result of the consumption of puffer fish annually. In Hong Kong, puffer fish poisoning happen occasionally and usually involves the consumption of puffer fish by fishermen since puffer fish is not allowed to be sold in the market and only restaurants with a licensed chef can prepare non-toxic puffer fish directly imported from Japan. However, apart from TTX poisoning, there are some other marine neurotoxins that cause substantial food poisoning. The most common marine neurotoxin includes Ciguatera poisoning caused by the consumption of coral fish. Saxitoxin from bivalve, brevetoxin and domic acid from shellfish are also some other common marine neurotoxins which cause poisoning.

Neurotoxin is a kind of toxins that specifically acts on the nerve cells by interacting with the membrane proteins in the neuron and ion channels. The most common effect of neurotoxins is a rapid onset of paralysis. Neurotoxins and venoms are usually found in organisms to protect themselves against vertebrate predators. Though neurotoxins are

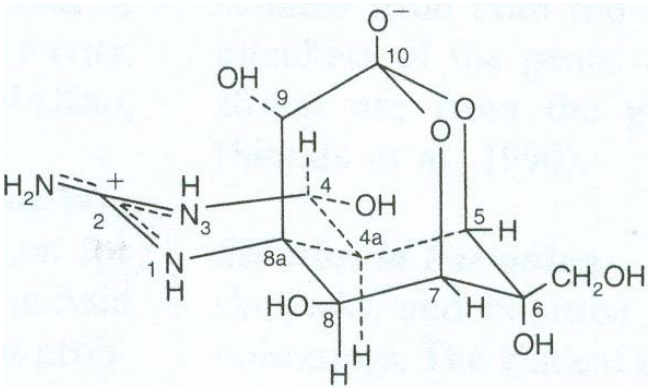
hazardous to human, venoms from snakes and lizards have been identified as a potential drug with an anti-cancer effect and a drug of diabetes (Poulsen *et al*., 2005).

1.1 Tetrodotoxin and its derivative

Tetrodotoxin (TTX) is a non-protein small molecule. It has a low molecular weight of 319.27, and a chemical formula of $C_{11}H_{17}O_8N_3$. It has a unique complex chemical structure as shown in Figure 1. The IUPAC name of TTX is Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-7,10,11,12-pentol with a CAS number: 4368-28-9. TTX is soluble in acidic environment with pKa 8.76, but not in any neutral organic solvent, such as DMSO or acetone, which limits the extraction of TTX using aqueous solutions. TTX is odorless, colorless and thermal stable with a color change from colorless to dark at above 220^{o}C without decomposition.

TTX contains one carbocyclic ring, six hydroxyl groups, a hemilactal group, a series of chiral carbons and a guanidine group which poses the specific toxicity towards the sodium channel of the neuron (Yotsu-Yamashita et al., 1999). TTX has a positively charged guanidinium group that produces stabilizing resonance effect, and a pyrimidine ring fused with additional ring systems containing hydroxyl groups (Goto *et al*., 1965). TTX can exist as an equilibrium mixture in the form of lactone and hemilactal due to the tautomerism of TTX (Yasumoto *et al*., 1989). TTX is not stable in extreme acidic or alkaline environment and its toxicity would diminish with long term storage in an aqueous medium. The dry crystalline form of TTX is regarded as a stable storage of TTX without affecting its toxicity.

Figure 1 Structure of Tetrodotoxin



Many analogs of TTX have been synthesized and are currently being studied, as these structural modifications are a useful tool for studying the mechanism involved in sodium channel in neuroscience research.

Around 10 TTX derivatives have been identified in different TTX bearing organisms. Among those derivatives, 4-epi TTX, anhydro TTX and tetrodonic acid are the most commonly found derivatives (Yasumoto *et al*., 1996). They were formed from TTX by acid catalyzed epimerization and dehydration in which they usually present in the form of mixture with TTX. TTX derivatives have different molecular formula, physical properties, chemical properties and have lower toxicity compared to TTX (Nakamura *et al*., 1984). The toxicity of 4-epitetrodotoxin and anhydrotetrodotoxin are 710 and 92 mouse units (MU) per mg, respectively, which were less potent compared to the TTX with 5000 MU per mg. Due to the non-specificity and low toxicity of TTX derivatives towards traditional mouse assay, the use of mouse assay for the quantification of TTX derivatives is not possible. HPLC with fluorescent detector, LCMS and H-NMR are usually used for the detection and quantification of TTX derivatives.

1.2 Tetrodotoxin in Puffer fish

Puffer fish, also known as “Fugu” in Japanese, has a long history with TTX as early as 2500 BC in Egypt, and at about the same time in China due to the value of puffer fish, Tetradontiformes, were consumed as food (Fuhrman, 1986). Puffer fish is the most recognizable living organism that contains TTX. It causes poisoning to human beings all over the world, especially in Japan. The amount of TTX in the puffer fish is not evenly distributed, but concentrated in several organs. The level of toxicity, the distribution of toxin in different organs and the toxicity of organs throughout a year are species specific. Also, the toxicity of the puffer fish is believed to be related to its living environment and food source.

The ovary generally has the highest concentration of TTX compared with the among other organs of the puffer fish and it is used as a TTX source to purify TTX. There are several patented methods involving the use of acetic acid to extract TTX from the puffer fish ovary followed by a series of purification steps involving activated carbon, weak ion-exchange column, etc. Also, TTX is usually present in high concentrations in the liver of the puffer fish. The “Fugu Liver” is considered as a delicacy in Japan and the Japanese would consider the numbness of the tongue after consuming the Fugu liver a wonderful experience and a sign of courage. The tiny amount of TTX present in the Fugu liver is classified as good quality, though there are strict regulations in Japan to ensure the puffer fish in the market are free from TTX.

Table 1.1 Puffer fish species and TTX distribution toxicity level in different organs (Noguchi, 2006)

Family		Species	Maximal toxicity*						
			Ovary	Testes	Liver	Skin	Intestine	Muscle	Blood
Tetradontidae	Marine	Japanese pufferfish							
		Takifugu niphobles (grass puffer)	●	○	●	◎	●	○	-
		T. poecilonotus (finepaterned puffer)	●	◎	●	◎	◎	○	-
		T. pardalis (panther puffer)	●	○	●	◎	◎	X	X
		T. snyder (vermiculated puffer)	●	X	●	◎	◎	○	-
		T. porphyreus (genuine puffer)	●	X	●	◎	◎	X	-
		T. chinensis (eye spot puffer)	●	-	●	-	-	-	-
		T. obscurus (“mefugu”)	●	X	◎	◎	◎	X	-
		T. exascurus (“mushifugu”)	●	X	◎	◎	-	X	-
		T. pseudommus (“numeradamashi”)	●	X	○	○	○	X	-
		T. chrysops (red-eyed puffer)	◎	X	◎	◎	○	X	X
		T. vermicularis (pear puffer)	◎	X	◎	◎	○	X	-
		T. rubripes (tiger puffer)	◎	X	◎	X	○	X	X
		T. xanthopterus (striped puffer)	◎	X	◎	X	○	X	-
		T. stictonotus (spotty back puffer)	◎	X	◎	○	X	X	-
		Tetraodon alboreticulatus (“shiroamifugu”)	●	-	○	○	◎	○	-
		Pleuranacanthus sceleratus (slack-skinned puffer)	●	-	○	○	◎	○	-
		Chelonodon patoca (“okinawafugu”)	◎	◎	◎	●	-	◎	-
		Arothron firmamentum (starry toad)	◎	X	X	○	X	X	-
		Canthigaster rivulata (scribbled toby)	X	-	○	◎	○	X	-
		Lagocephalus lunaris (“dokusabafugu”)	X	X	X	◎	X	●	-
		L. inermis (smooth-backed blowfish)	X	X	◎	X	X	X	-
		L. wheeleri (blowfish)	X	X	X	X	X	X	-
		L. gloveri (brown-backed toadfish)	X	X	X	X	X	X	-
		Sphoeroides pachygaster (slackskinned puffer)	X	X	X	X	X	X	-
		Chinese pufferfish							
		Takifugu flavidus (“sansaifugu”)	●	◎	●	◎	◎	○	-
	Brackish	Thai pufferfish							
		Tetraodon nigroviridis	-	-	X	◎	○	○	-
		T. steindachneri	-	-	X	◎	X	X	-
Diodontidae	Marine	*Diodon holocanthus*	X	-	X	X	X	X	-
		Chilomycterus affinis	X	-	X	X	X	X	-
Ostraciidae	Marine	*Ostracion immaculatum*	X	X	X	X	X	X	-
		Lactoria diaphana	X	X	X	X	X	X	-
		Aracana aculeate	X	X	X	X	X	X	-

*○: 10-100 MU/g tissue (weakly toxic). ◎ : 100-1000 MU/g tissue (moderately toxic); ● : > 1000 MU/g tissue (strongly toxic), where 1 MU (mouse unit) is defined as the amount of toxin that skills a male mouse of ddY strain (20g body weight) in 30 min after intraperitoneal administration. The amount is equivalent to about 0.2 μg of TTX. △ : derivatives of TTX were detected (toxicity data are unavailable).

1.3 Biogenesis and Distribution of TTX in nature.

Puffer fish is the most recognizable living organism that contains TTX and it was the only known source of TTX until the discovery of TTX in *Taricha* genus newts in 1964. Afterward, TTX bearing organisms were reported in a wide range of animals other than puffer fish as shown in Table 1.2, which led to controversies between the endogenous and exogenous origins of TTX. TTX is widely distributed in nature, in both marine and terrestrial organisms from four different phyla. TTX has now been identified in at least 14 families and there is little or even no correlation among those TTX bearing species. Most of them are discovered as a result of human's fatal interactions with them. The blue ring octopus was identified as TTX bearing through fatal bites to humans, the ivory shell through fatal human poisoning.

Table 1.2 Distribution of TTX in animals apart from the puffer fish (Noguchi, 2006)

Animals		Toxic parts	Maximal toxicity*	References
Platyhelminthes				
Turbellaria				
Flatworms	*Planocera spp.*	Whole body	●	Miyazawa et al. (1986)
Nemetinea				
Ribbonworms	*Lineus fuscoviridis*	Whole body	●	Miyazawa et al. (1988)
	Tubulanus punctatus	Whole body	◎	Miyazawa et al. (1988)
	Cepholothrix linearis	Whole body	●	Ali et al. (1990)
Mollusca				
Gastropoda	*Charonia sauliae*	Digestive gland	●	Narita et al. (1981)
	Babylonia japonica	Digestive gland	○	Noguchi et al. (1981)
	Tutufa lissostoma	Digestive gland	◎	Noguchi et al. (1984)
	Zeuxis siquijorensis	Whole body	●	Narita et al. (1984)
	Niotha clathrata	Whole body	●	Jeon et al. (1984)
	Natica lineate	Whole body	◎	Hwang et al. (1990)
	Cymatium echo	Digestive gland	○	Narita (1991)
	Pugilina ternotoma	Digestive gland	○	Narita (1991)
Cephalopoda	*Hapalochlaena maculosa*	Posterior salivary gland	●	Sheumack et al. (1984)
Annelida				
Polychaeta	*Pseudopolamilla occelata*	Whole body	○	Yasumoto et al. (1989)
Arthropoda				
Xanthidae crabs	*Atergatis floridus*	Whole body	○	Noguchi et al. (1983)
	Zosimus aeneus	Whole body	○	Yasumura et al. (1986)
Horseshoe crab	*Carcinoscorpius rotundicauda*	Egg	○	Kungsuwan et al. (1987)
Chaetognatha				
Arrowworms	*Parasagitta spp.*	Head	△	Thuesen et al. (1988)
	Flaccisagitta spp.	Head	△	Thuesen et al. (1988)
Echinodermata				
Starfish	*Astropecten spp.*	Whole body	◎	Maruyama et al. (1984, 1985)
Vertebrata				
Pisces				
Goby	*Yongeichthys criniger*	Skin, viscera, gonad	◎	Noguchi and Hashimoto (1973)
Amphibia				
Bewts	*Taricha spp.*	Skin, egg, ovary, muscle, blood	◎	
	Notophthalmus spp.	Skin, egg, ovary	◎	Mosher et al. (1965)
	Cynopsis spp.	Skin, egg, ovary, muscle, blood	○	Yotsu et al. (1990)
	Triturus spp.	Skin, egg, ovary, muscle, blood	△	Yasumoto et al. (1988)
Frogs	*Atelopus spp.*	Skin	●	Yotsu et al. (1990)
	Colostethus sp.	Skin	◎	Kim et al. (1975)
	Polypedates sp.	Skin	◎	Daly et al. (1994) Tanu et al. (2001)

*○: 10-100 MU/g tissue (weakly toxic). ◎ : 100-1000 MU/g tissue (moderately toxic); ● : > 1000 MU/g tissue (strongly toxic), where 1 MU (mouse unit) is defined as the amount of toxin that skills a male mouse of ddY strain (20g body weight) in 30 min after intraperitoneal administration. The amount is equivalent to about 0.2 μg of TTX. △ : derivatives of TTX were detected (toxicity data are unavailable).

Furthermore, a breakthrough in TTX research came about when it was found that the TTX producing bacteria include the *Vibrio* family, *Vibrio alginolyticus* (Narita et al., 1987) which showed TTX toxicity in fermentation. Other species including *Microbacterium arabinogalactanolyticum, Serratia marcescens* (Yu et al., 2004) and *Pseudoalteromonas tetradonis* were also identified as TTX producing and they were usually isolated from the organs of the TTX bearing organisms. Apart from isolation from living organism, TTX producing bacteria were also identified in both marine and freshwater sediments (Do *et al*., 1991; Do *et al*., 1993).

With the discovery of TTX in four different phyla (Chordata, Mollusca, Echinodermata, Arthropoda), scientists started to question the origin of TTX, whether it was exogenous acquired or endogenous produced by TTX bearing organisms. The origin of TTX in the TTX bearing organisms was extensively studied in the case of puffer fish. It was reported that cultivated puffer fish above the sea bed or in an enclosed water system was found to be non-toxic, but the puffer fish can become toxic when it was grown in open water again or was fed with toxic puffer livers (Shimizu, 1986). These experiments suggested that TTX can be obtained and accumulated from the food chain in the higher level TTX bearing organism, like puffer fish. Furthermore, the wide distribution of TTX in organisms from different phyla of unrelated TTX bearing organisms suggested that the origin of TTX in puffer fish should not be endogenous, but obtained from an exogenous origin instead. It was further suggested that TTX producing bacteria is the primary producer of TTX and TTX was passed in the food chain and resulted in a wide distribution of TTX bearing organisms from different phyla and habitat.

Apart from the food chain hypothesis of TTX being accumulated in TTX bearing organisms, scientists also suggested a symbiotic relationship between the TTX-producing bacteria and the TTX bearing organisms. In this symbiotic relationship, the TTX-producing bacteria can obtain nutrients from the TTX bearing animals while the TTX produced by the bacteria can provide protection or predation advantage to the animals. For example, the slow swimming puffer fish accumulates the TTX produced by the symbiotic bacteria as a biological defense mechanism to protect itself from predators (Yu and Yu, 2002) and the blue-ringed octopus can inject TTX into the prey for predation.

1.4 Pharmacology of TTX

TTX is a potent neurotoxin which binds selectively to the site 1 of the voltage-gated sodium channels (VGSC) situated at the extracellular pore openings. The binding to the sodium channels is extremely tight and works as an irreversible, competitive inhibitor which mimics the hydrated sodium cation and results in an inhibition of sodium ions conductance. The tight binding is the result of the positively charged guanidiunium group of TTX binding to the side of the VGSCs which is negatively charged while the rest of the TTX molecule blocks the sodium channel. The binding is further strengthened by the hydroxyl groups of ringed systems in TTX which produce an electrostatic force between TTX and the VGSCs at aqueous interfaces in the cell. The binding results in a conformational change in the side group of VGSCs which make the binding difficult to reverse and extremely tight.

This sodium channel binding site is also active to another neurotoxin (Saxitoxin) presented in dinoflagellates (Denac *et al.*, 2000). So, it is difficult to use the traditional

mouse assay or some in vitro bioassays like tissue culture assay to distinguish between TTX and saxitoxin intoxication. The binding of TTX to the VGSC obstructs the depolarization and propagation of the action potential along the neurons by affecting the permeability to potassium ions. As a result, TTX intoxication victims usually die of asphyxiation caused by the paralysis of diaphragm and intercostals muscles.

1.5 Therapeutic Applications of Tetrodotoxin

Though TTX is known as a potent neurotoxin which can be fatal in even a tiny amount, it can serve as a potential therapeutic drug with its specific action over the sodium channel with a careful dosage control. TTX has the potential to serve as an anti-cancer drug by showing an inhibitory effect on the invasiveness of metastatic prostate cancer (Prasad *et al*, 2004). Also, TTX was found to have the potential to be developed as an anaesthesia (Schwartz *et al*., 1998), pain killer for chronic cancer pain and neuroprotective drug (Narahashi, 2001). With its selective blockage action towards VGSCs in the nerve, TTX can cease the transmission of action potential by blocking the conduction of nerve impulse along the nerve axons. TTX has been developed as a local anesthetic or analgesic which produces a loss of sensation in a circumscribed area by affecting the transmission of nerve signal to the brain.

Moreover, a Canadian based company (International Wex Technologies Inc.) has developed several drugs from TTX for the relief of chronic pain (cancer pain), to function as anaesthesia and to relieve the withdrawal effect of heroin. The company claims that drug from TTX has a function similar to morphine and they have many advantages than

morphine. TTX is non-opioid, would not cause addictive problems, fast in action, long-lasting and 3200 times more powerful than morphine with the same amount of drug.

1.6 Acquisition of Tetrodotoxin

With the potential therapeutic application of TTX to be developed as drug, the acquisition of TTX should be obtained in large scale with a cost-effective method. Several methods have been developed for the preparation and collection of TTX including the extraction from puffer fish, chemical synthesis and microbial biosynthesis.

1.6.1 Extraction of TTX from puffer fish

TTX extraction from puffer fish is a traditional and the most common method. It is usually done by collecting the ovaries of puffer fish, followed by extraction in an acidic environment and a series of purification steps, which include centrifugation, filtration and purification with activated charcoal and weak ion exchange column. Crystalline TTX could be obtained after a volume reduction of eluate followed by freeze drying. However, a large scale extraction of TTX from puffer fish ovaries poses several difficulty and problem. The availability of puffer fish, the seasonal variation of TTX content in the puffer fish and the complex purification steps are the limitations for a large scale production. Furthermore, the large scale consumption of puffer fish for TTX extraction involves an extensive fishing of puffer fish which would damage the ecosystem.

1.6.2 Chemical synthesis of TTX

The chemical synthesis of TTX was reported in 1972 with a complex 29-steps racemic synthetic pathway. The multiple steps of the chemical synthesis result in a low and inefficient yield which hinders the use of chemical synthesis in the production of TTX (Kishi *et al*., 1972)

1.6.3 Microbial biosynthesis of TTX

The microbial biosynthesis of TTX is a novel direction for the production of TTX which is not well documented in research journals. The most common isolated TTX producing bacteria belongs to the *Vibrio* and *Pseudomonas* species and some other species have also been identified, such as *Aeromonas, Bacillus, Microbacterium* species, etc. Though some of TTX producing bacteria have been identified, the amount of TTX production by these species is limited. So, the mass production of TTX using microbial synthesis is not possible at present as the optimal condition, nutrient requirement for the maximal production of TTX by the TTX producing bacteria are still unknown.

Also, with the development and identification of the biosynthetic pathway of TTX of the TTX producing bacteria, it is expected that the method of microbial synthesis of TTX could provide a stable supply of TTX without seasonal variation and thus the killing of wild puffer fish for a tiny amount of TTX could be avoided. This method could increase the supply and lower the unit price of TTX, which the unit price is US$ 260 for 1mg of TTX (Sigma, USA)

Among these three methods, the microbial biosynthesis of TTX showed the highest potential for the large scale production of TTX. Though the yield of TTX synthesized is limited now, it is expected that the yield could significantly enhanced with extensive research and development. Since bacteria should be the primary producer of TTX, the production would be enhanced with the identification of TTX synthetic pathway in the bacteria that allows the provision of suitable substrate with optimal fermentation condition for the maximal production of TTX.

1.7 Detection of TTX

1.7.1 Mouse Bioassay

Mouse bioassay is a widely accepted traditional detection method and is currently used by the Japanese government for the detection of puffer fish poisoning content in the seafood available in the market (Ministry of Health and Welfare, 1991). It is also the official method for the detection of some other marine neurotoxins including paralytic shellfish poisoning (PSP) and Ciguatera fish poisoning (CFP) which may be present in seafood.

The mouse assay is done by injecting sample extract into the mouse intraperitoneally and the toxicity of the sample extract is determined by the time of death of the mice. The time of death is related to the toxicity of the sample extract and the toxicity could be translated into a mouse unit (MU) from the conversion table of the time of death and mouse unit (Appendix 1). One MU is defined as the amount of toxin required to kill a 20g ICR (Institute of Cancer Research) strain mouse in 30 minutes after intraperitoneal injection.

There are some advantages in using the mouse bioassay. The preparation for the detection is simple and requires no sophisticated skill. Also, it can be done within an hour since the death time for a reliable result should be within 25mins. However, there is an increasing ethical concern in sacrificing animals for the mouse bioassay. Also, the detection is not specific to TTX only, but also other toxins acting in the nervous system. For example, Saxitoxin (STX) also acts on the sodium channel of the nerve which produce similar intoxication symptoms in the mouse that could not be distinguished by the mouse bioassay.

With the increasing ethical concern and the limitations of the mouse bioassay in the detection of TTX, different assays for the detection of TTX have been developed and it is expected that the mouse bioassay will be replaced in the future.

1.7.2 High Performance Liquid Chromatography (HPLC) Detection

The HPLC detection has been developed using post column derivation followed by fluorescent detector for both qualitative and quantitative analysis of TTX and its analogues. TTX and its derivatives are separated by reverse phase C-18 column or ion-exchange column. The separated toxin eluate would enter the post column system where it is mixed with concentrated sodium hydroxide and fit in the heating coil at around 100°C. TTX and its related toxins are converted into fluorescent C9 compound which could be measured by a fluorescent detector. The HPLC followed by post column derivation using fluorescent detector (HPLC-FLD) is capable for separating and detecting TTX analogues, such as 6-epi-TTX, 4,9-anhydro-6-epi-TTX, 4-epi-11-deoxy-TTX, 4-epi-TTX, 4,9-anhydro-TTX, 11-deoxyTTX and 4,9-anhydro-11-deoxyTTX which could

be isolated from puffer fish (Yotsu *et al.*, 1989). However, the purification step for the samples is complicated in which impurities would affect the chromatographic result significantly. Also, the HPLC with post column is an indirect measurement of C9 compound, in which there may have C9 compound may have presented in the samples which are not from TTX and alter the result.

For the HPLC detection using an UV detector (HPLC-UVD) without post column modification, our research group has developed such a HPLC system for the measurement of TTX (Yan *et al*., 2005). The detection limit is around 10ug/ml, which is not as sensitive as the HPLC with post column modification using fluorescent detector. However, with the modification of the mobile phase, like optimizing the condition of the HPLC system, and introducing a buffer system into the mobile phase, there is potential to enhance the sensitivity of HPLC detection using an UV detector.

1.7.3 Tissue Culture Assay

Tissue culture assay is a kind of cell cytotoxic bioassay based on the sodium channel blocking activities of TTX. TTX has the ability of sodium channel blocking which antagonizes the combined effect of two sodium channel opening chemicals used in tissue culture assay. Veratridine, a kind of sodium channel opener, and ouabain which is a kind of enhancer of veratrdine would act on the neuroblastoma cell line (ATCC, CCL 131) and cause the cells swell and die. The presence of TTX, which shows sodium channel blocking activities, can compensate the opening effect of the drugs and protect the neuroblastoma cell. By measuring the survival of cells using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H-tetrazolium monosodium salt assay with a

microplate reader (Hamasaki *et al*., 1996), the TTX amount can be determined. The number of cells that survive is proportional to the amount of TTX presented in the sample in the range of 2-70nM. Furthermore, this assay is also reported to be suitable for the measurement of TTX presence in the supernatant of bacterial culture which does not require a series of purification steps.

1.8 Puffer fish Poisoning (PFP)

PFP is the one of the most dangerous food poisoning with a high mortality rate (almost 60%) and it happens all over the world and especially in the East Asia (Isbister and Kiernan, 2005). As puffer fish is considered as a delicacy in Japan and some cities in China, case of TTX intoxication are frequently reported (Ushiyama *et al*., 2001). With the well-established legislation and regulation on the consumption of puffer fish in Japan, TTX poisoning cases have reduced in recent decades. Fishermen are another party who can obtain and consume puffer fish directly from the seas, sometimes resulting in TTX intoxication. Apart from the TTX poisoning by the consumption of puffer fish, marine gastropod TTX-poisoning caused by edible marine gastropods are reported consistently in China and Taiwan (Shui et al., 2002, Hwang et al., 2005) and horseshoe crab TTX-poisoning is documented in Thailand. (Kanchanapongkul *et al*. 1995).

1.8.1 Clinical symptoms TTX poisoning

Tetodotoxin intoxication is usually caused by the improper consumption of puffer fish. The onset of symptoms after the ingestion of TTX contaminated fish are around 30mins

and the severity usually depends on the amount of toxin ingested. There is a grading system to classify the different levels of TTX intoxication as shown in Table 3.

Table 1.3. Grading system for TTX poisoning (Klsbiter *et al*., 2005)

Grade of poisoning	Signs and symptoms	Onset
Grade 1	Perioral numbness and paraesthesia, with our without gastrointestinal symptoms (mainly nausea).	5-45 mins
Grade 2	Lingual numbness, numbness of face, and other areas (distal). Early motor paralysis and incoordination. Slurred speech. Normal reflexes.	10-60 mins
Grade 3	Generalized flaccid paralysis, respiratory failure, aphonia, fixed or dilated pupils. Patient is conscious.	15 mins – several hours
Grade 4	Severe respiratory failure and hypoxia. Hypotension, bradycardia, and cardiac dysrhythmias. Unconsciousness may occur.	15 mins – 24 hours

The symptoms of the early stage of TTX poisoning include numbness of the lips and tongue, sensations of lightness or floating, headache, nausea, diarrhea and vomiting. The symptoms of the second stage are increasing paralysis, difficulty to move or even to sit, weakness, twitching and a loss of coordination of muscles. In severe cases, the poisoning will cause cardiovascular effects, respiratory failure and coma, which may result in death within 4 to 8 hours. Most patients could be recovered after 5 days, but it may take longer for the severe cases.

1.8.2 Clinical diagnosis of TTX poisoning

The causative agent of the food poisoning is usually judged from the symptoms of patients (How *et al*., 2003). Identification of the toxin involved in food poisoning is usually done by analyzing the leftover of the suspected food taken (Noguchi and Ebesu, 2001, Hwang et al. 2003). However, the leftover food is usually not obtainable as it is not brought along with the patient to hospital or it may get lost in most of the time. Hence, the measurement of TTX in urine is essential in the diagnosis of TTX intoxication since the concentration of TTX in plasma/serum would decrease to an undetectable level after 24 hours due to the rapid excretion of TTX to urine (O'Leary *et al.,* 2004). TTX can be detected in urine for up to 5 days after ingestion and it is suggested that the measurement of TTX in urine samples collected in 24 hours is the most sensitive test for the patient (Oda et al., 1989). Also, urinary excretion of TTX is in parallel with the patient's clinical recovery.

The method of measurement of TTX in serum and urine was done by several researchers including the use of immunoaffinity chromatography for the extraction of TTX from urine followed by post column fluorescent detection using HPLC (Kawatsu *et al*., 1999). However, the monoclonal antibody specific for TTX, which is not commercially available, is difficult to obtain and expensive. Other researcher have developed the method of using LC-MS (Tsai Y.H. *et al.*, 2006), gas chromatography-mass spectrometry (GC/MS) (Kurono *et al*., 2001), HPLC with post column modification using fluorescent detector (O'Leary M.A. *et al*., 2004) for the detection of TTX in serum and urine. However, these methods had limited applications as they involved either lengthy and laborious procedures or expensive and sophisticated equipment.

1.8.3 TTX poisoning treatment

There is no known antidote to TTX at present. Treatments are only supportive and symptomatic including mechanical respiration for breathing difficulty, administration of activated carbon to remove the unabsorbed toxin, administration of atropine to treat bradycardia. Life threatening effects are unlikely to happen after 24 hours, intensive care monitoring the cardiovascular and respiratory function is critical in early diagnosis (Lange, 1990).

1.9 Objectives

This project was undertaken with the following objectives:

1) To study the background information of puffer fish in Hong Kong
2) To develop biological and chemical methods for the detection of TTX
3) To develop different methods for the extraction and purification of TTX from different media
4) To develop method for the rapid extraction and detection of TTX in urine for clinical diagnosis
5) To screen TTX producing bacteria for the production of TTX by fermentation
6) To optimize the fermentation condition for the growth and production of TTX by TTX producing bacteria
7) To investigate and identify important compound or chemical for the production of TTX by TTX producing bacteria

2 Materials

2.1 Puffer fish and Standard TTX

Two batches of puffer fish collected during January, 2006 & March, 2007 and the puffer fish species used in the study were *Takifugu alboplumbeus, Takifugu niphobles Chelonodon patoca and Torafugu* (Toxic species from Japan water and non-toxic species from a fish farm in Japan).

Standard TTX was purchased from Sigma, USA.

2.2 General Buffer and Bacterial culture medium

a) General Buffer

Phosphate buffered Saline (PBS) was prepared with 0.2g/L KCl, 0.2g/L KH_2PO_4, 8g/L NaCl and 1.44g/L Na_2HPO_4. Homogenizing buffer for total protein extraction was prepared with 250mM Sucrose, 7.5mM Na_2HPO_4, 5mM EGTA, 5mM EDTA (pH=7.4) and 1mM PMSF

b) Bacterial culture medium

Luria-Bertani (LB) Medium was prepared with 10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.0. LB Agar was prepared with LB Broth with 10g/L Agar powder.

Ocean Research Institute (ORI) Medium was prepared with 2g/L Bacto-yeast extract (Difco), 2g/L protease peptone No. 3 (Difco), 1g/L phytone peptone (BBL), 1g/L sodium sulphite, 0.4g/L sodium thiosulphate, 0.08g/L iron citrate, pH 7.6. (Lee *et al.*, 2000). E.coli/Coliform Count Plate was purchased from (3M, USA).

2.3 Detection of TTX

a) Tissue culture assay

The cell line used in tissue culture assay was Neuroblastoma cell line Neuro-2A (ATCC, CCL131). The tissue culture medium used for the cultivation of neuroblastoma cell line was Minimum Essential Medium (MEM) (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA) with 10% Fetal bovine serum (FBS) (Gibco, USA). Trypsin/EDTA solution (0.5/0.2%) (Gibco, USA)

The reagent used in tissue culture assay included the two sodium channel opener drugs veratridine (Sigma, USA) and ouabain (Sigma, USA). (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl) -2H-tetrazolium (MTS) (Promega, USA) with 10% phenazine methosulfate (PMS) (Aldrich, USA) were used for the quantification of survival cells.

b) HPLC with Fluorescent detector (HPLC-FLD) & HPLC with UV detector (HPLC-UVD)

The HPLC system used in this study was (Agilent model 6890, USA) and the column used was a reverse phase C18 column (Platinum EPS C18, Alltech, USA) with guard column (All-Guard^{TM} cartridge system, Alltech, USA). A Column Heater (Waters, USA) was used to maintain a constant temperature of column during the experiment.

For the HPLC-FLD, a post column deriviterizer with fluorescent detector (Agilent, USA) was used for the measurement of TTX. The mobile phase used in the HPLC-FLD system was 0.005M 1-heptanesulfonic acid sodium salt monohydrate (HSA) [HPLC grade] (International Laboratory, USA), 3% Acetonitrile [HPLC grade] and 3M sodium

hydroxide was used in the post column system for the reaction of TTX into C-8 compound with fluoresecent signal.

For HPLC-UVD, a Diode Array Detector (Agilent, USA) was used with two different mobile phase were studied. Mobile phase A (Yan, 2005) contained 0.04% phosphoric acid [HPLC grade] (International Laboratory, USA) and Mobile phase B contained 4.8 mM HSA, 41.8 mM sodium dihydrogen phosphate anhydrous and 10% methanol, adjusted to pH 3.0 with phosphoric acid

c) Mouse Bioassay

In the mouse bioassay, Institute of Cancer Research (ICR) mice, both sex, between 15-25g was used for TTX measurement and they were supplied by the Central Animal Unit of the Hong Kong Polytechnic University.

d) TTX purification and detection in urine

For the spiked urine experiment, blank urine without TTX was collected from colleague and the urine samples of TTX poisoned patients were collected from Queen Mary Hospital. For solid phase extraction of TTX, C18 Sep-Pak Plus cartridge (Waters, USA) and Weak cation exchange cartridge (HiTrap Column, GE Healthcare) were used and the organic solvent and chemicals used in the solid phase extraction were HPLC grade. Roche Modular System (Roche Diagnostics, Indianapolis, IN) was used for the measurement of urinary creatinine of the patient.

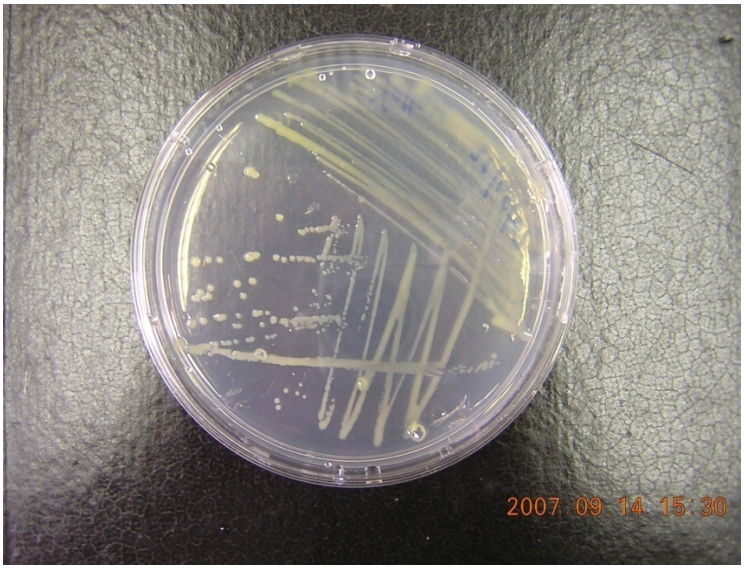
2.4 Bacterial Production of TTX

a) **Bactarial strain**

Four bactarial strains including *Microbacterium arabinogalactanolyticum* (M. arabinogalactanolyticum), *Serratia marcescens (S. marcescens), Pseudomonas putida (P.putida)* (Provided by Dr C.F. Yu isolated in Japan), *Bacillus-cereus (B.Cereus*) (Screened from puffer fish) and *Raoultella-terrigea (R.terrigea*) (Screened from puffer fish) were used in this study.

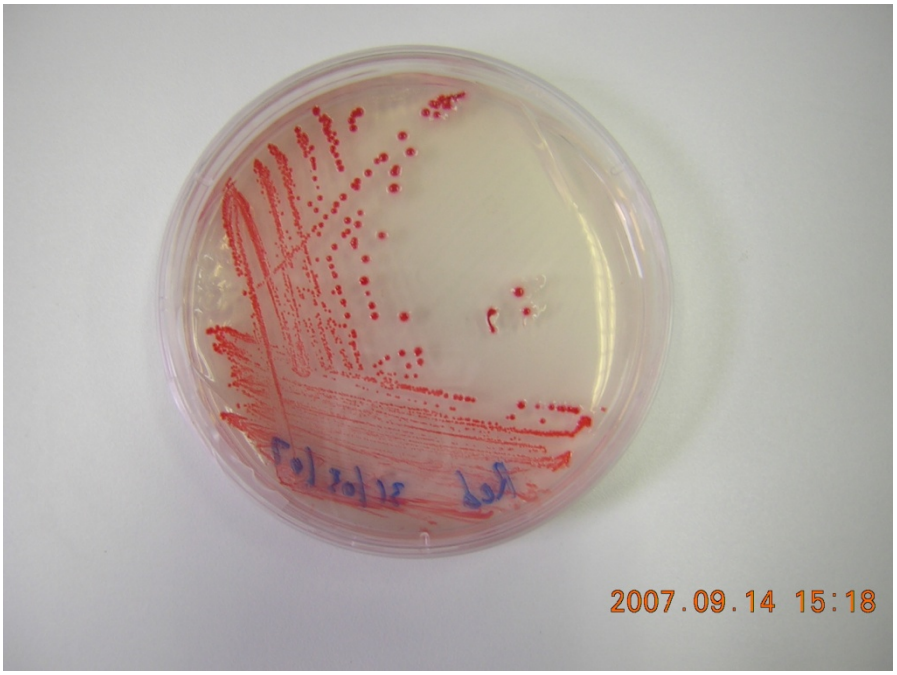
M. arabinogalactanolyticum is Gram-positive, non acid-fast, nutritionally exacting and chemoorganotrophic. It is aerobic but also has a weak anaerobic growth. It has no sporulation but with the ability to produce yellow pigment in colonies.

Figure 2.1 Overview of *M. arabinogalactanolyticum*



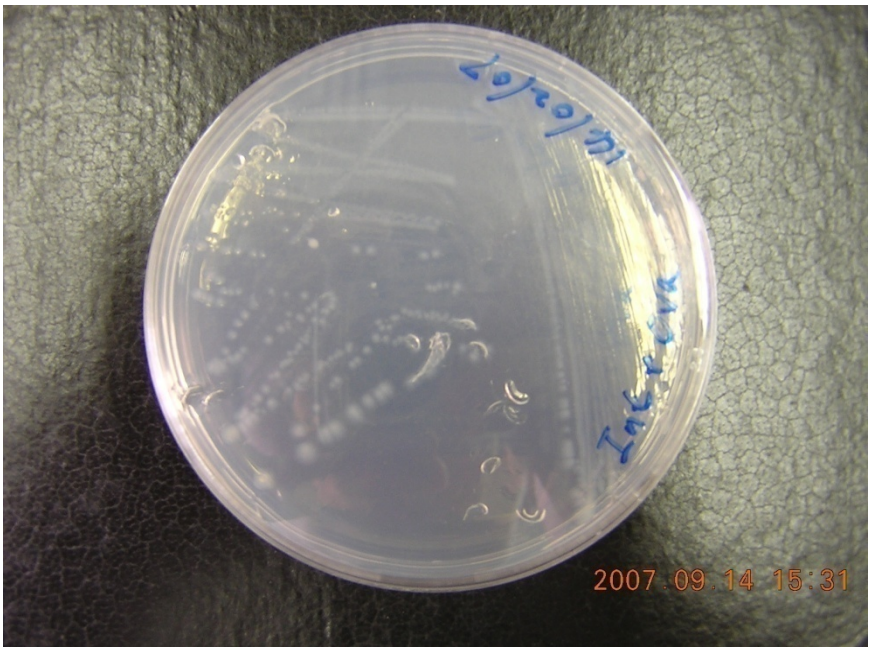
S. marcescens is a Gram-negative, straight rod, non-sporing, non acid-fast and facultatively anaerobic bacterium. This strain is motile due to the presence of peritrichous flagella. The temperature growth range is between 10^{o}C and 36^{o}C. *S. marcescens* produce prodigiosin which is a red, nondiffusible pigment which can be used as antifungal, immunosuppressive and anticancer drug (Giri *et al*., 2004).

Figure 2.2 Overview of *S. marcescens*



P.putida is a Gram-negative, rod shped, non-sporing, motile with one or more polar flagella. It has aerobic metabolism which is known as saprophytic soil bacterium. *P.putida*, unlike other pseudomonads, is non-pathogenic which is used in bioremediation (Raghavan *et al*., 1999).

Figure 2.3 Overview of *P.putida*



b) Fermentation of TTX producing bacteria

For the shake flask study, Orbital Shaker (Innova 2300, New Brunswick scientific) in warm room (30°C, 37°C) was used and a 15L computer controlled stainless fermenter (B.Braun, Germany) was used for the fermenter study.

c) Measurement of fermentation parameter

Spectrohpotometer (Spectronic® 20 Genesys™) was used for optical density measurement and pH meter was used for the measurement of pH. Freeze dryer (LABCONCO, Freezezone 6®) was used for the removal of water in the bacterial samples for cell dry weight measurement.

The reagents used in total carbohydrate measurement was a Phenol reagent containing 5g phenol in 100ml ddH_2O with concentrated sulphuric acid

d) Purification of TTX in bacterial culture medium

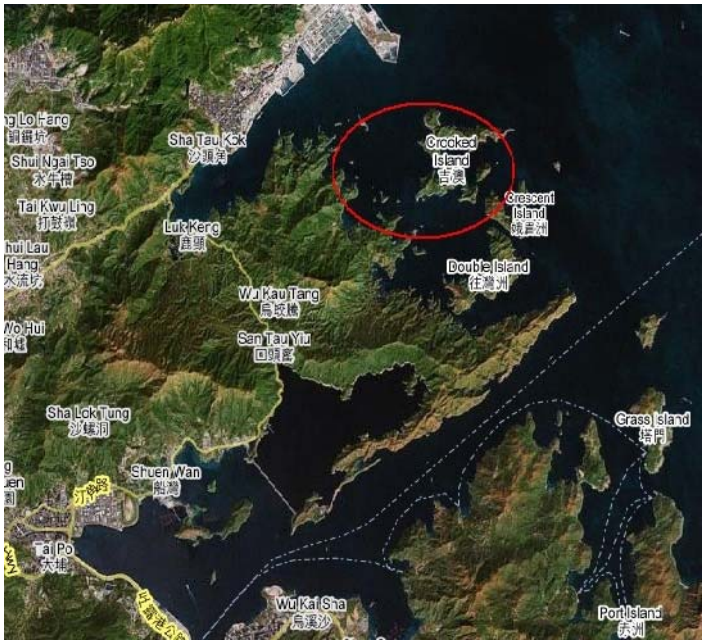
The use of acid washed activated carbon (Sigma, USA) was to remove pigment and contaminant in the bacterial samples. In the solid phase extraction of bacterial samples, C18 Sep-Pak Plus cartridge (Waters, USA) and a weak cation exchange column (HiTrap Column, GE Healthcare) were used. For further or large scale purification, self packed column with Weak ion-exchange resin (BioRad) was used. Rotary evaporator (Hiedolph Laborata 4000, Germany) was used to reduce the volume of samples.

3 Methods

3.1 Puffer fish background study

Puffer fish was collected in the coastal water near Crooked Island by a local fisherman. Two batches of puffer fish were collected in January, 2006 and March, 2007. Caught puffer fishes were kept alive in the net cage until transport to the laboratory.

Figure 3.1 Map of the collection of puffer fish near Crooked Island in Hong Kong



3.1.1 Dissection and collection of Puffer fish

Puffer fishes collected were stored in ice during transportation or kept at -20°C in the laboratory before dissection. The puffer fishes were dissected in the earliest time to avoid contamination and kept the freshness. The species of puffer fishes were firstly identified

by comparing the color, the pattern on the skin and the special features on the puffer fish body according to the reference document (Yu, 2003).

Some basic parameters were measured including the length and the weight of each puffer fish. Puffer fish was dissected using sterilized laboratory scissors and forceps aseptically. Gender of the puffer fishes were identified according to their sex organ. Puffer fish organs including gonad (ovary or testis), liver, intestine, skin and flesh were dissected and weighed separately. Food content in the intestine was collected if it was present for toxicity analysis.

3.1.2 Bacterial count of puffer fish organs

Total bacterial count and *E.coli* count were performed to study the bacterial number in different organs of puffer fish. Bacteria were extracted from the puffer fish organs (ovary, testis, liver, intestine, flesh, skin) by adding 10ml of PBS to 2g of organs. The tissue extract was blended for 1 minute using homogenizer in ice bath. Tissue residue and fat were removed using filter in stomacher bag. Tissue extract were subjected to serial dilutions (10^{-1} and 10^{-2}) using PBS and the bacterial solutions were ready for bacterial count.

For total bacterial count, LB agar was used as the growing medium. 100ul of the bacterial solution was added to the Petri dish using spread plate method and the number of single colony was counted after incubated at 30°C for 24 hours.

For *E.coli* count, 3M E.coli Petrifilm was used. 100ul of bacterial solution was added to the Petrifilm and incubated at 30°C for 24 hours. Colony surrounded with blue color ring and a tiny air bubble on its surface was identified as *E.coli*.

3.1.3 Toxicity of puffer fish organs

Standard method for the extraction of TTX in puffer fish organs was used to measure the toxicity of puffer fish organs. Briefly, 10g of puffer fish organ (Ovary, Testis, liver, intestine, flesh and skin) was cut into small pieces. 50ml of 0.1% acetic acid was added to the tissue and extracted in 90ºC water bath for 10 minutes. After the heating process, samples were centrifuged at 4000rpm for 30 minutes. The supernatant was collected and stored at -20ºC, which was ready for the mouse bioassay or further purify using C-18 cartridge and weak ion-exchange cartridge for HPLC-UVD analysis.

3.1.4 Screening of TTX producing bacteria

Three targeted organs in puffer fish including liver, ovary and intestine were used to screen TTX producing bacteria. Bacteria were extracted using PBS with homogenization similar to 3.1.2. Two different growing agars were used including LB agar and ORI agar with 10g/L NaCl. After homogenization, the extract were filtered with stomacher filter, 100ul bacterial solution was added to two different agars using streak plate method respectively. The Petri dishes were incubated at two different temperatures (30ºC & 37ºC) for 24 to 72 hours. Colonies were divided into types according to colony characteristics, isolated single colony was picked and subjected to streak plate procedure for a few more times to ensure a single bacterial strain was isolated. Isolated strain was fermented using shake flask method and measured with mouse bioassay as mentioned in section 3.4.1.1 and 3.2.4 respectively, to check whether it was toxic and its toxicity. Bacteria species identified as toxic were subjected to MIDI identification system for species identification.

3.1.5 Preparation of puffer fish extract

Since it was suspected that the ovary of puffer fish contained substances which could trigger the production of TTX by bacteria, the puffer fish ovary extract was prepared as supplement in the fermentation broth to study the effects of puffer fish extract on the growth of TTX producing bacteria and the production of TTX.

The puffer fish ovary extract was prepared by adding 3ml PBS per gram of puffer fish organ tissue and homogenized for 3-5 minutes in ice bath. Puffer fish extract was deep frozen at -80^{o}C until fermentation.

3.1.6 Extraction of total protein from puffer fish ovary

Provided that the puffer fish ovary contained substances which could trigger the production of TTX using bacteria, protein fraction in the puffer fish ovary was isolated to study the effect of protein in the puffer fish to the bacterial production of TTX.

Total protein was extracted from ovary by adding 3ml/g homogenizing buffer to ovary and homogenize for 3-5 minutes in ice bath. The ovary extract was filtered through cheese cloth to remove fat and tissue residue.

Dialysis tubing (10k Dalton) was prepared by heating the dialysis tubing (40ml of 0.5M EDTA + 60ml ddH_2O) in boiling water bath for 10 minutes. The dialysis tubing was washed in 100ml ddH_2O in boiling water bath for another 10mins. Afterward, it was rinsed with cold ddH_20 five times followed with PBS which was dialysis buffer. The ovary extract was loaded into the dialysis tubing and soaked into dialysis buffer with stirring in a 4^{o}C cold room. Dialysis was performed for 3 days and the dialysis buffer was

changed every day. The puffer fish extracts after dialysis were stored in 4ºC until fermentation using fermenter in section 3.4.2.

3.2 Detection of TTX

3.2.1 Tissue culture assay

Neuroblastoma cell line was grown in EMEM with 1% penicillin-streptomycin and supplemented with 10% FBS as culture medium, kept in 5% CO_2 incubator at 37°C. Culture medium was changed every 2 days and subculture was done when high cell density was observed. Cells were subcultured by trysin/EDTA solution (0.5/0.2%) and resuspended into culture medium.

For the tissue culture assay, each well of 96 wells plate was inoculated with 10^5 cells with 200 µl culture medium. After 4 hours of incubation for cells to attach on the culture plate, culture medium was removed and replaced by culture medium with 0.02mM veratridine and 0.2mM ouabain. 10ul of testing samples were added at the same time and triplicate wells were done for each sample. For each of the toxicity testing sample, it was filtered with 0.2µm syringe filter. Afterward, the 96 wells plate was incubated in a CO_2 incubator for 18 hours at 37°C followed by MTS (Promega) assay for the determination of cell survival. MTS mixture was prepared freshly (MTS:PMS = 9:1) and mixed with the culture medium in a ratio of 1:9 followed by 3hrs incubation at 37°C. During the MTS assay, it was done in the absence of light since the MTS reagent is light sensitive. Finally, the absorbance of the 96 wells plate was measured using microplate reader at 490nm. By comparing to the standard curve produced by standard TTX, the concentration of TTX in the sample could be obtained.

3.2.2 HPLC-FLD

The HPLC-FLD was a HPLC system (Agilent 1100, USA) with a post column deriviterizer followed by a fluorescent detector. The mobile phase used was 0.005M HSA with 3% ACN and the flow rate was 0.5ml/min. The column used was a reverse phase C18 column with a guard column. The post column system was connected to 3M NaOH with a pumping rate of 0.3ml/min. The temperature of the post column system was kept at 110°C to allow complete reaction between TTX and NaOH for the detection of C9 base presented in the samples. Standard curve of TTX was prepared by measuring the fluorescent signal of standard TTX at concentration from 0.1ug/ml to 100ug/ml. By comparing with the standard curve, TTX concentration in the samples could be determined.

3.2.3 HPLC-UVD

The HPLC system was (Agilent 1100, USA) with a UV detector. The column used was reverse phase C18 column with guard column and the temperature was kept at 40ºC with a column heater. Two mobile phases were used named as mobile phase A and mobile phase B.

Mobile phase A was developed by our colleagues (Yan, *et al.* 2005) and it was a mobile phase with 0.04% phosphoric acid. The detection wavelength was 195nm and the flow rate was 0.5ml/min. Mobile phase B was contained HSA, NaH_2PO_4, 10% MeOH, pH 3.0. The detection wavelength was 197nm and the flow rate was 0.6ml/min.

TTX standard curve was determined by known concentration of TTX from 10 – 50000 ng/mL and the serial dilution of TTX was done by using the mobile phase B for dilution.

3.2.4 Mouse bioassay

Healthy mice (ICR strain, both sex) which weighed between 15-25g were used in the mouse bioassay. Toxin samples used in the mouse bioassay are purified by 0.2um syringe filter before the injection. A group of 4 mice were chosen randomly and 1ml of toxin sample was injected intraperitoneally into the mice. This should be noted that the toxin sample was injected into the peritoneal cavity without leaking outside the body. Mice with leaking of toxin samples should be discarded. The death time of mice is obtained by the time between the injection of toxin and the last gasping breath of the mice. The median death time of the mice should not be shorter than 4 minutes. Samples with median death time shorter than 4 minutes required subsequent dilution (dilution factor) to meet the requirement of the toxicity conversion table.

By using the death time of mice and comparing to the toxicity conversion table (Appendix 1), the mouse unit (MU) can be obtained. This could be corrected by the weight correction table (Appendix 2) to the corrected mouse unit (CMU) and multiplied by the dilution factor. By comparing the specific toxicity of pure TTX (5000MU/mg), the toxicity and purity of the samples could be determined.

3.3 TTX purification and detection in urine

TTX-spiked urine samples were prepared by mixing fresh and normal urine donated from our colleagues with standard TTX (10 to 500 ng/mL). The spiked samples were stored at -80°C before solid phase extraction described below and followed with the HPLC-UVD analysis in section 3.2.3.

3.3.1 Solid phase extraction (SPE) of TTX followed with HPLC-UVD measurement

Sample for SPE was centrifuged at 1800 x g for 20 minutes to remove precipitates. The supernatant of each sample was passed through a pre-conditioned C18 cartridge with a syringe at a flow rate of 1 mL/min. Each C18 cartridge was pre-conditioned with (1) methanol (2 mL), (2) 0.2M HCl in 20% methanol (3 mL), and (3) water (5 mL), which was then sucked dry under vacuum before application.

The eluant of the C18 cartridge was loaded onto a prepacked (1ml) weak ion exchange column HiTrap Column (GE Healthcare) at a flow rate of 0.5ml/min which was conditioned with (1) water (5 mL), (2) methanol (2 mL), (3) water (5 mL) and the pH was adjusted to 7.5-8.0 with 0.2M HCl or 0.2M NH_3. The column was then sucked dry under vacuum before sample application and, once the sample (0.5 – 1 mL) was loaded inside the column, it was washed with (1) water (5 mL), (2) acetonitrile (2 mL), (3) methanol (2 mL), and (4) water (5 mL) at a flow rate of 0.5ml/min. Eventually, TTX in samples was eluted at a flow rate of 0.5 mL/min with the same buffer solution used as the mobile phase B in (refer to Section 2.3). The eluate was collected in 1 mL per fraction (2-3 fractions) which was subject to subsequent HPLC analyses in section 3.2.4.

3.3.2 Case study of TTX poisoning in Hong Kong

3.3.2.1 Background information of the TTX poisoning case

There was a poisoning case happening in December 2007 in Hong Kong involved four mainland Chinese seamen, aged between 34 and 40, who had consumed puffer fish freshly caught from the sea when their boat was stationed in Hong Kong waters.

All of them experienced nausea, dizziness, perioral numbness and paraesthesia over the limbs shortly after consuming the dishes of puffer fish. Two of them also developed generalized muscle weakness and impending respiratory muscle paralysis requiring mechanical ventilation and being nursed in the intensive care unit (ICU) shortly after their arrival at the emergency room of Queen Mary Hospital.

3.3.2.2 Urine samples collected from TTX poisoning patients

Spot urine samples were collected from the four patients in hospital within 24 hours after ingestion of puffer fish, ranging from 7 – 11 hours. No puffer fish remains were available to our laboratory. Urine (4 – 5 mL) aliquots were stored at -80°C before the HPLC analysis.

3.3.2.3 Determination of creatinine in urine samples

The creatinine measurement in urine was done by Queen Mary Hospital. Creatinine concentration in the spot urine samples of TTX poisoned patient was determined by a kinetic colorimetric assay based on the modified Jaffé method (Junge *et al*., 2004). The assay was automated on the Roche Modular System (Roche Diagnostics, Indianapolis, IN), with an analytical range between 360 and 57500 μmol/L.

3.4 Bacterial production of TTX

In this project, three species of bacteria including *M.arabinogalactanolyticum*, *S.marcescens and P. putida* were studied. Bacteria species used in fermentation were either kept in the agar plate at 4°C or in frozen stock at -80°C. Both methods in keeping the bacterial stock were prepared by growing a single strain confirmed bacterial specie either from picking a single colony on an agar plate or from frozen stock, in a 250ml shake flask with 80ml sterilized ORI or LB medium. The bacteria were cultivated in a shaker with 250rpm at 30°C for 16 hours. For bacteria kept in the agar plate, 1ml of bacterial solution was transferred aseptically to the agar plate followed with spread plate method and kept at 4°C until further fermentation. The agar plate was subcultured every month to ensure the stability of the culture. For bacteria kept in the frozen stock, 500ul of bacterial solution was mixed with 500ul 50% glycerol in a frozen stock vial. The vials were deep frozen at -80°C until further fermentation.

3.4.1 Optimization of fermentation condition for TTX producing bacteria

3.4.1.1 General procedure for fermentation using shake flask

Frozen stock was thawed and cultivated in sterilized ORI medium for 16 hours as seed. 1% of seed culture was inoculated into sterilized production medium in a shake flask for fermentation. The pH and optical density (OD) of the fermentation medium was monitored during fermentation at specific time point. pH was measured using pH meter and OD was monitored by spectrophotometer (Spectronic ® 20 Genesys ^{TM}) at 600nm using the corresponding culture medium as blank.

3.4.1.2 Optimization parameter in fermentation condition

a) Aerobic and anaerobic condition

From the literature information, *M. arabinogalactanolyticum* and *S. marcescens* can grow at either aerobic or anaerobic condition. The TTX biosynthetic pathway may be related to the growth of bacteria and availability of oxygen. Hence, the relationship between oxygen availability and TTX-producing abilities of bacteria was studied by growing bacteria at aerobic and anaerobic conditions.

In aerobic condition, seed culture was prepared as indicated in section 3.4.1.1 and 1% (3ml) was inoculated into 300ml ORI medium with 0.5% NaCl inside a 1 liter shake flask and shaken at 250rpm at 30^{o}C throughout the fermentation period. In anaerobic condition, 1% of bacteria solution was inoculated into 500ml reagent bottles containing 500ml of ORI medium. The bacterial cultures were put into 30^{o}C incubator without shaking and the medium was turned upside down every day to mix the medium, but to minimize the oxygen diffusion into the medium. OD and pH were monitored during the fermentation period.

b) Effect of sodium chloride in ORI medium on bacterial growth

Since it was found by our colleagues that several species of bacteria isolated from puffer fish did not show much variation in the growth in the medium with or without the presence of sodium chloride, the effects of sodium chloride on the growth of the bacteria species in this study were investigated. Sea water collected from Ocean Park, artificial sea water prepared by artificial sea salt purchased from the market, 5g/L and 10g/L NaCl

solution were added to the ORI medium to study the effect of salt on the growth of bacteria.

c) Carbon source enrichment and modification

The ORI medium used in the cultivation of TTX producing bacteria only contained 0.08g/L iron citrate as carbon source and it was suspected that the carbon source in the medium was limited which may alter the optimal growth of the bacteria. Also, it was found that the pH rose higher than 8 during fermentation using original ORI medium which inhibited the growth of bacteria. The rise in pH may be caused by the ammonia related metabolite produced during the consumption of nitrogen source in the medium while the consumed carbon source in the modified medium would produced acidic metabolite which could lower the pH and promote cell growth.

Two carbon source, glucose and sucrose with different concentration (5,10g/L) in addition to the ORI medium were studied with respect to its changes in pH and OD during fermentation.

d) Addition of arginine and puffer fish organ extract in the fermentation medium

Complex molecules could be formed from simple starting molecules during the biosynthetic pathway of bacteria. Also, L-arginine was proposed by the scientists as a starting material of TTX from its similarity in structure to certain part of TTX. In this study, different concentrations of arginine in addition to ORI medium were used to study the effect of arginine in the biosynthesis of TTX.

Since the production of TTX by TTX producing bacteria was lower compared to the amount of TTX accumulated in the puffer fish, the compounds specifically present in the puffer fish organ were suspected to trigger or involved in the biosynthetic pathway for the production of TTX. Puffer fish ovary extract prepared according to section 3.1.5 in addition to ORI medium was used to study the effect of puffer fish organ extract on the production of TTX.

3.4.2 Fermentation of TTX producing bacteria using fermenter

Three bacteria species including *P. putida, M. arabinogalactanolyticum* and *S.marcescens* were cultivated using fermenter. The formulas of the culture medium used in different batches were listed as follows:

P.putida

Batch 1: ORI with 20g/L sucrose

Batch 2: ORI with 20g/L sucrose + 3ml/L Ovary extract

Batch 3: ORI with 20g/L sucrose + 3ml/L Total protein of ovary extract

Fermentation was performed using 15L computer controlled stainless fermenter (B.Braun, Germany) and the growing condition was monitored and kept controlled by the computer of the fermenter. Briefly, seed culture was prepared as described in section 3.4.1.1 and 1% volume of seed culture to the total volume of the culture medium was inoculated. The temperature of the fermenter was kept at 30°C and the pH was at 7.6 with the addition of either acetic acid or ammonia by the computer controlled pump. The partial pressure of oxygen (PO_2) was kept not less than 20% by changing the stirrer

speed. The stirrer speed was kept at 300rpm when the PO_2 was higher than 20%. 5ml, 10% antifoam was added at the start of fermentation and further antifoam was added whenever it is necessary to prevent foaming during fermentation. The fermentation was run for 48-60 hours. 1L bacterial culture was collected at specific time point for different measurements including **a)** Total carbohydrate measurement, **b)** OD, **c)** Cell dry weight and **d)** TTX measurement.

a) Total carbohydrate measurement

This assay was performed to monitor the amount of carbohydrate consumed during fermentation and its relationship with the growth and accumulation of TTX. Phenol sulphuric method was used to measure the carbohydrate in the bacterial broth. Briefly, bacterial broth samples were centrifuged at 4000rpm for 30 minutes and the supernatant was subjected to the assay. The supernatant was serial diluted with ddH20 to fall in the range within 100mg/L carbohydrate in the samples. 1ml diluted supernatant was mixed with 1ml phenol reagent in a 25ml test tube using vortex mixer. 5ml of concentrated sulphuric acid was added slowly to the test tube to turn all non-reducing sugar, including sucrose, into reducing sugar. The mixture was kept at room temperature for 25 minutes to allow the reaction between phenol and carbohydrate to form yellowish orange compound. Afterward, the contents were mixed by vortex and allowed to stand for 5 minutes for bubbles to disperse. The OD of the content was measured at 488nm using 1ml ddH_20 instead of samples prepared by the above method as blank.

Standard curve was prepared with known concentration of sucrose from (0-80mg/L) for the total carbohydrate assay and the concentration of carbohydrate in the samples could be determined from the standard curve and the dilution factor.

b) OD

Refers to section 3.4.1.1

c) Cell dry weight measurement

1L bacterial broth was centrifuged at 8000rpm for 30 minutes. The cell pellet was washed with ddH20 once and then centrifuged at 8000rpm for 30 minutes again. The cells were frozen at -20C overnight and put in the freeze drier until all the water content was removed. The cell dry weight was measured using electronic balance.

d) TTX measurement

Refer to section 3.4.3

3.4.3 Extraction and purification of TTX from bacterial culture

The bacterial culture broth collected was extracted by adjusting the pH to 3.5 using acetic acid following a heating process in 90°C or above water bath for 10 minutes. The extracted bacterial broth was centrifuged at 8000rpm for 30 minutes. The cell pellet was discarded for cell dry weight measurement and the pH of the supernatant was adjusted to 5 using ammonia solution. The supernatant was reduced in volume to around 10ml using rotary evaporation. The reduced volume supernatant was ready for mouse bioassay.

For further analysis using other TTX detection methods, the supernatant was purified with activated carbon treatment (ACT). The activated charcoal was acid washed and the pH was adjusted around 7 before the treatment. The activated carbon was filled in filter assembly for suction filtration with filter paper no.1. The supernatant sample was filtered through the activated carbon and the filtrate was filtered several times to ensure the TTX was bound to the activated carbon. The TTX-bound activated carbon would be washed with distilled water for several times before elution. The TTX was eluted with 500ml of 1% acetic acid in 20% aqueous ethanol (Yu, 2003) as elution buffer. Also, the elution buffer was washed through the filter assembly several times to maximize the elution of TTX from the activated carbon. The eluant pH was adjusted to 5 and concentrated to around 10ml using rotary evaporator. The concentrated samples were further purified by using SPE as in section 3.3.1 for qualitative measurement of TTX by using HPLC-UV as in section 3.2.3.

4. Result

4.1 Puffer fish background study

4.1.1 Dissection and collection of Puffer fish

Two batches of puffer fish were collected during the study. The first batch (Batch A) was collected in January, 2006 and the second batch (Batch B) in March, 2007. Batch A was generally used in the anatomical study of puffer fish and organ extract preparation for subsequent bacterial TTX production study. Batch B was used for internal organ toxicity studies, bacterial count and screening for TTX producing bacteria in the puffer fish internal organs.

Tables 4.1 and 4.3 show the general background information of the puffer fish collected in this study. In Batch A, 20 puffer fish belonging to the *T. alboplumbeus* were collected. 10 were male and 10 were female. Table 4.2 shows the average weight and length of the puffer fish in batch A. The average gonad weight percentage is the average percentage of (Gonad weight / Total weight of puffer fish) x 100%. Samples A9 and A20 were not counted since they were mature puffer fishes with a higher gonad weight percentage. The gonad weight percentage of samples A9 and A20 were 13.4% and 29.2% respectively.

Figures 4.1 and 4.2 showed the overview of *T. alboplumbeus* and *Chelonodon patoca* respectively. Special pattern, spots and a lateral line were found on the skin of the puffer fish and were the characteristic morphology used for species identification. Figure 4.2 shows a *C. patoca* specimen whose stomach was inflated for self protective purpose even it was dead.

Also, in Figure 4.3, the anatomy of a dissected female *T. alboplumbeus* is shown. It can be seen that the size of the puffer fish ovary and liver were larger compared to other fish species.

Table 4.1 Background information of Batch A puffer fish collected in Jan 2006

Species: *T. alboplumbeus*

Sample	Length (cm)	Weight (g)	Sex	Puffer fish organ weight (g)		
				Gonad	Liver	Intestine
A1	13.0	43.3	F	1.6	1.8	1.5
A2	13.5	52.0	M	3.9	3.0	1.3
A3	12.5	53.0	M	3.4	2.0	0.9
A4	13.5	63.8	M	3.6	2.7	1.7
A5	13.5	63.9	F	4.4	9.6	2.4
A6	15.0	65.6	M	4.1	2.3	1.7
A7	14.5	70.6	M	3.9	3.2	1.6
A8	15.0	78.7	M	4.2	3.5	1.8
A9	16.0	85.9	F	11.5	3.7	3.9
A10	15.0	92.1	M	5.8	8.8	2.0
A11	17.0	98.9	F	3.6	4.4	3.6
A12	18.0	99.9	M	4.6	8.0	2.5
A13	17.5	100.4	F	7.1	5.1	1.6
A14	18.0	108.8	F	4.0	N.A.	4.9
A15	18.0	123.8	F	6.4	7.8	4.0
A16	18.5	132.1	M	6.3	7.3	3.5
A17	19.5	146.9	M	9.8	12.5	4.3
A18	19.5	178.5	F	8.9	12.1	6.2
A19	22.0	212.0	F	7.6	16.7	4.6
A20	20.0	253.0	F	74	8.9	5.1

Species: *C. patoca*

Sample	Length (cm)	Weight (g)	Sex	Puffer fish organ (g)		
				Gonad	Liver	Intestine
A21	14.0	25.0	M	N.A.	0.9	0.9
A22	14.5	85.3	M	0.8	7.1	2.6
A23	16.0	112.0	M	1.4	6.1	2.6
A24	17.0	151.0	M	1.5	15.6	4.5
A25	17.0	166.1	M	2.1	10.9	8.5

N.A. = Not available

Table 4.2 Average length, weight and the gonad weight percentage of puffer fish batch A

Puffer fish species	Averaged length (cm)		Averaged weight (g)		Gonad weight % **	
	Male	Female	Male	Female	Male	Female*
Takifugu species	17.0	17.5	85.4	126.7	5.90%	4.83%
Chelonodon patoca	15.7	N.A.	107.9	N.A	4.44%	N.A.

* Except sample A9 and A20

** Gonad weight % = Gonad weight / Total weight of puffer fish

Table 4.3 Background information of batch B puffer fish collected in Mar 2007

Species: *T. alboplumbeus and T. niphobles*

Sample	Length (cm)	Weight (g)	Sex	Puffer fish organ weight (g)		
				Gonad	Liver	Intestine
B1	18.0	118.0	M	N.A.	5.3	5.3
B2	19.0	120.0	M	N.A.	4.7	5.4
B3	19.0	121.0	M	N.A.	3.64	3.1
B4	19.0	125.0	M	N.A.	N.A.	4.2
B5	19.5	209	F	19.2	N.A.	6.5

Species: *Chelonodon patoca*

Sample	Length (cm)	Weight (g)	Sex	Puffer fish organ weight (g)		
				Gonad	Liver	Intestine
B6	17.5	140	N.D.	3.6	13.9	3.3
B7	17.5	155	N.D.	2.5	12.6	3.6

N.D. = Sex of puffer fish could not be determined due to the immature sex organ

Figure 4.1 Overview of *Takifugu alboplumbeus*

Figure 4.2 Overview of *Chelonodon patoca*

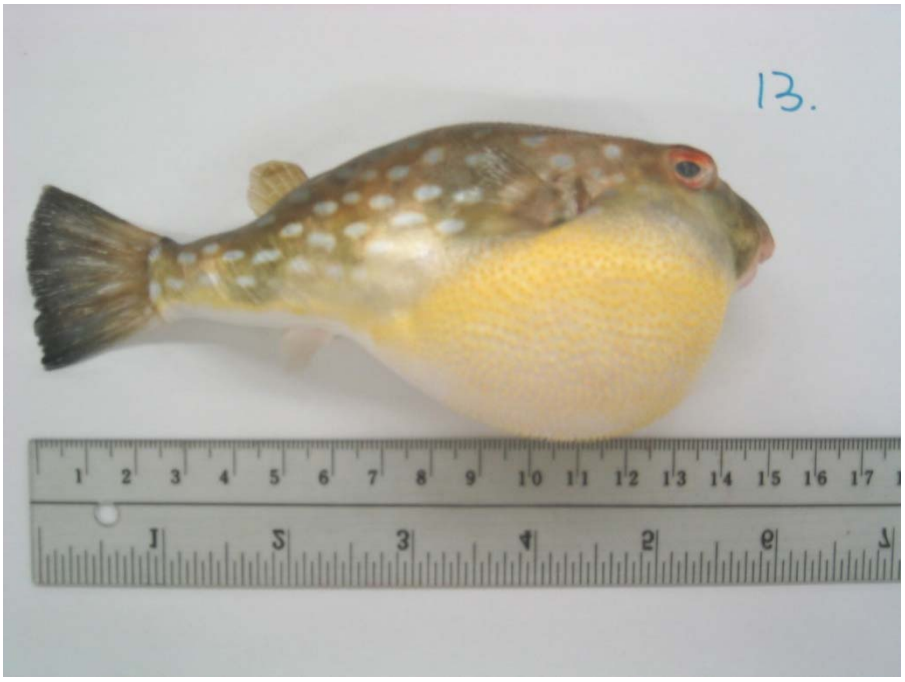
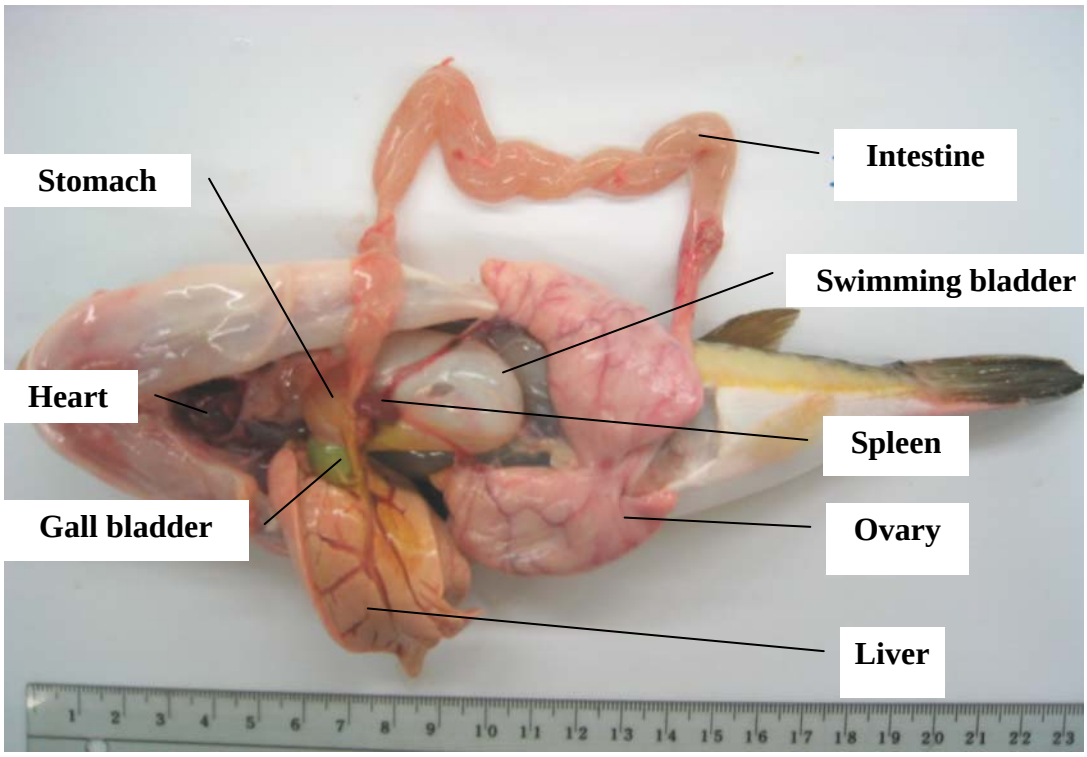


Figure 4.3 Anatomy of a dissected female *Takifugu alboplumbeus*



4.1.2 Bacterial count of puffer fish organs

The E. coli count was done by 3M Petri film and there was no E. coli found in the puffer fish organs. The total bacterial count was done by the spread plate method with serial dilution and the detailed result is shown in Appendix 3. Bacteria were found in all the tested organs and the number of bacteria in the gut showed a large variation from 5 to 29400 CFU. The total bacteria count in the ovary, liver and testis were found to be below 100 CFU.

Table 4.4 E.coli count and total bacterial count of puffer fish organs

Sample	Puffer fish organ	E.coli count	Total bacterial count (CFU)
B1	gut	0	5
B2	gut	0	29400
B3	gut	0	17
B4	gut	0	480
B5	gut	0	46
B6	gut	0	61
B5	Ovary	0	10
B2	Liver	0	38
B3	Liver	0	0
B6	Liver	0	7
B1	Testis	0	34

4.1.3 Toxicity of puffer fish organs

The organs of four species of puffer fish including *T. alboplumbeus, T. niphobles, C.patoca* and wild and cultured *Torafugu "Takifugu rubripes"*, were studied and a summary is shown in Table 4.5. The test on *Torafugu* samples was performed during a visit to the Nagasaki University, Japan. The toxic *Torafugu* sample was puffer fish caught in waters near Nagasaki and the non-toxic *Torafugu* sample was bought from a fish farm in Japan.

All the ovary samples were found to be toxic except the non-toxic *Torafugu* ovary sample which was not performed and the ovary samples in *T. niphobles* was the most toxic with 356ug/g TTX measured using mouse bioassay. The testis sample was found to be non-toxic. All the liver samples were found to be toxic, with the *T. niphobles* liver being the most toxic. However, the intestine, skin and muscle samples of *T. niphobles* were found to be non-toxic. The intestine samples of *T.alboplumbeus* and *C.patoca* were found to be

toxic and it was found that the food residue remained in the intestine of *C.patoca* contained 1.3 ug/g of TTX. The skin sample of *C. patoca* contained 86.4ug/g TTX, which was also highly toxic and the muscle sample of *C. patoca* was the only muscle sample found to be toxic.

Table 4.5 Toxicity of puffer fish organs from different puffer fish species

Puffer fish species	**Toxicity of Puffer fish organ (ug/g)**						
	Ovary	Testis	Liver	Intestine	Intestine content	Skin	Muscle
Takifugu alboplumbeus	47.9	0	13.5	3.0	N.A.	N.A.	0
Takifugu niphobles	356	N.A.	121	0	N.A.	0	0
Chelonodon patoca	Gonad: 6.0		5.0	28.5	1.3	86.4	6.4
Torafugu (Toxic)	11.4	11.4	2.2	N.A.	N.A.	N.A.	N.A.
Torafugu (Non-toxic)	N.A.	N.A.	0.5	N.A.	N.A.	N.A.	N.A.

N.A. = Not available

4.1.4 Screening of TTX producing bacteria

LB and ORI medium were used to screen potential TTX producing bacteria from the puffer fish organs. Around 20 species of bacterial colonies were isolated from the agar plates. Isolated strains were subjected to shake flask fermentation for 3-7 days in different temperatures and culture media (LB and ORI). It was found that about 10 out of 20 isolated strains were poorly grown in shake flask fermentation and were thus discarded for further analysis. Four species were found to be toxic. Three toxic samples including LBgutB01, LBLiver01 and ORILiverB were subjected to identification using MIDI system. LBgutB01 and LBLiver01 were found to be the same species belonging to

R.terrigena while ORILiverB was identified as *B.cereus*. The similarity index was above 0.8 which showed a good library comparison.

Table 4.6 MIDI bacteria identification and the toxicity of potential TTX producing bacteria

Sample name	Toxicity (MU/L)	Bacterial species	Similarity index
LBgutB01	454	*Raoultella-terrigena*	0.906
LBLiver01	426	*Raoultella-terrigena*	0.900
ORILiverB	52	*Bacillus-cereus-GC subgroup B*	0.809
LB ovary	38	N.I.	N.D.

N.I. = Not Identified

According to the MIDI System manual, strains with a similarity of **0.600 or higher** is considered **good library comparisons**.

If the similarity index is **between 0.400 and 0.600**, it may be a **good match** but an atypical strain.

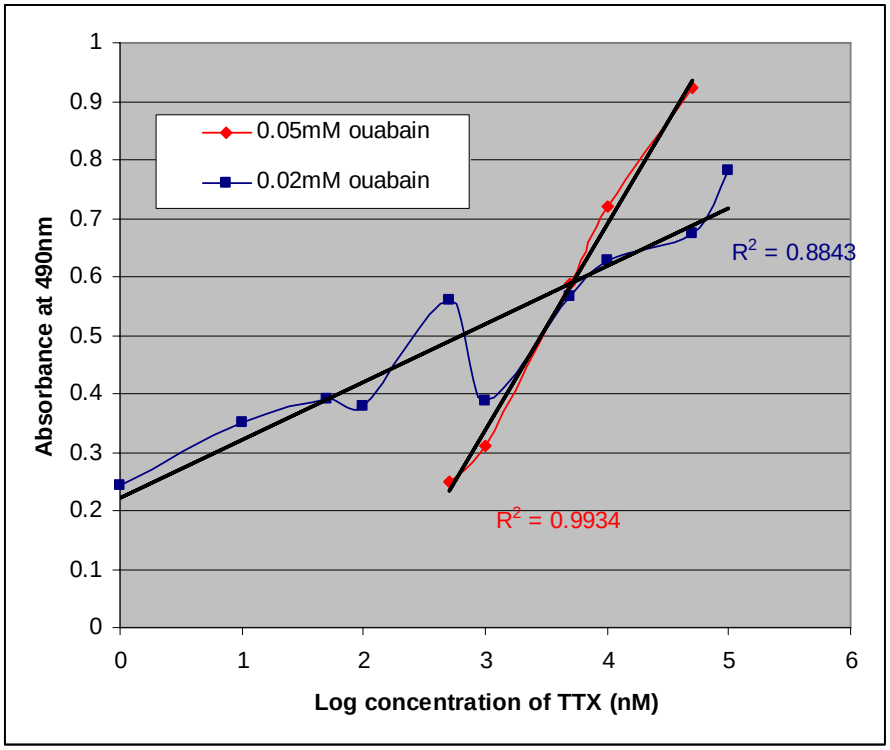
Values **lower than 0.400** suggest that the system does not have the species in the database, but indicate the **most closely related species**.

4.2 Detection of TTX

4.2.1 Tissue culture assay

Figure 4.4 shows the standard curve of TTX using 0.02mM and 0.05mM ouabain in different TTX concentration (1-10000nM). Standard curve of TTX using 0.02mM and 0.05mM ouabain, and showed the R^2 values of 0.8843 and 0.9959 respectively. The absorbance showed larger fluctuation at high concentration range of TTX in using 0.02mM ouabain while it showed a good linear relationship even at log concentration 0nM which equals to 1nM of TTX. This showed that using 0.02mM ouabain could work in lower concentration range as compared to 0.05mM. (Appendix 4)

Figure 4.4 Standard curve of TTX using 0.02mM and 0.05mM ouabain with log concentration of TTX from 0 nM to 5 nM



4.2.2 HPLC-FLD

TTX standard with concentration from 0.01 to 100ug/ml was used to determine the sensitivity of the HPLC system using a fluorescent detector. Standard curve of TTX using the HPLC-fluorescent detector was shown in Figure 4.5 with R^2 = 0.9995. Figure 4.6 showed the HPLC chromatogram of standard TTX measured with a fluorescent detector and the TTX peak was shown at retention time = 12.2 minutes.

Figure 4.5 Standard curve of TTX using HPLC-FLD

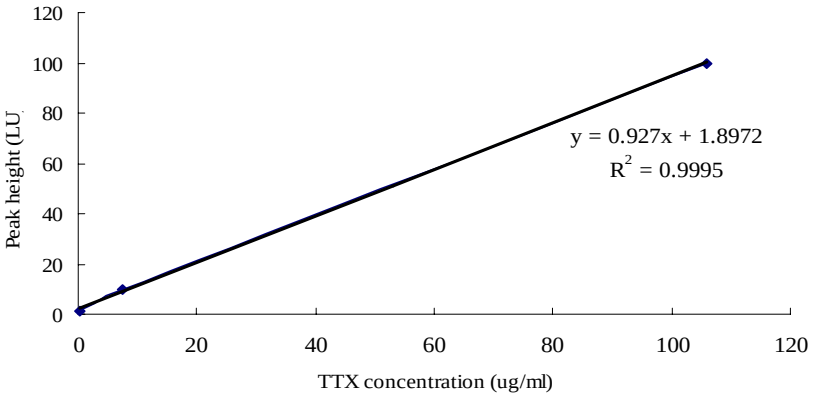
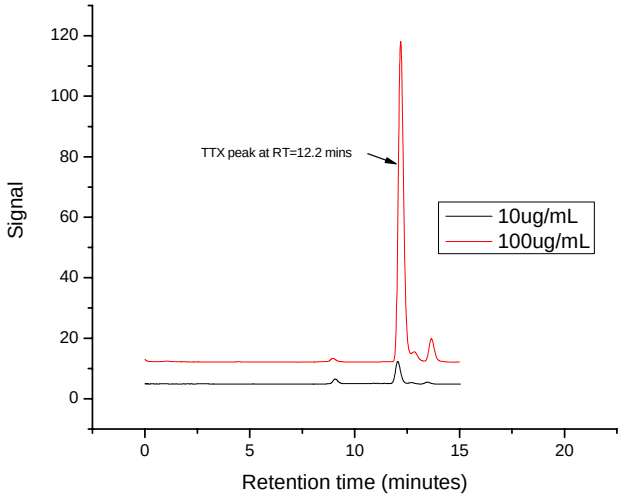


Figure 4.6 HPLC chromatogram of TTX using HPLC-FLD



4.2.3 HPLC-UVD

HPLC-UVD chromatograms using mobile phase A monitored at 195nm with 10ug/ml and 1ug/ml TTX are shown in Figure 4.7 and Figure 4.8 respectively. The detection limit of TTX using mobile phase A was from 1 to 10ug/ml and the retention time of TTX was 3.0 minutes. The baseline of TTX chromatogram in 1ug/ml using mobile phase A was in a wavy pattern which affected the accuracy for the determination of TTX concentration and confined the detection limit of the HPLC-UVD system in TTX measurement.

The retention time of TTX using mobile phase B at 197nm was 6.1 minutes (Figure 4.9) and the detection limit was 10ng/ml. The standard curve of TTX using mobile phase B showed a good relationship from 1.7 to 4.5 ng/ml in log concentration with R^2 value = 0.9993 (Figure 4.10). The stability of the HPLC system using mobile phase B was studied by intra-day and inter-day HPLC measurement study as shown in Table 4.7. The HPLC-UV intra-day stability study resulted in an averaged coefficient of variation (CV) of 0.22% and 0.53%, in retention time and signal area respectively. For the inter-day stability study, three consecutive days HPLC measurement were done and the averaged CV were less than 3% for the variation in both the retention time and the signal area. (Appendix 5)

Figure 4.7 HPLC Chromatogram of 10µg/ml TTX standard measured by HPLC-UVD using mobile phase A at 195nm

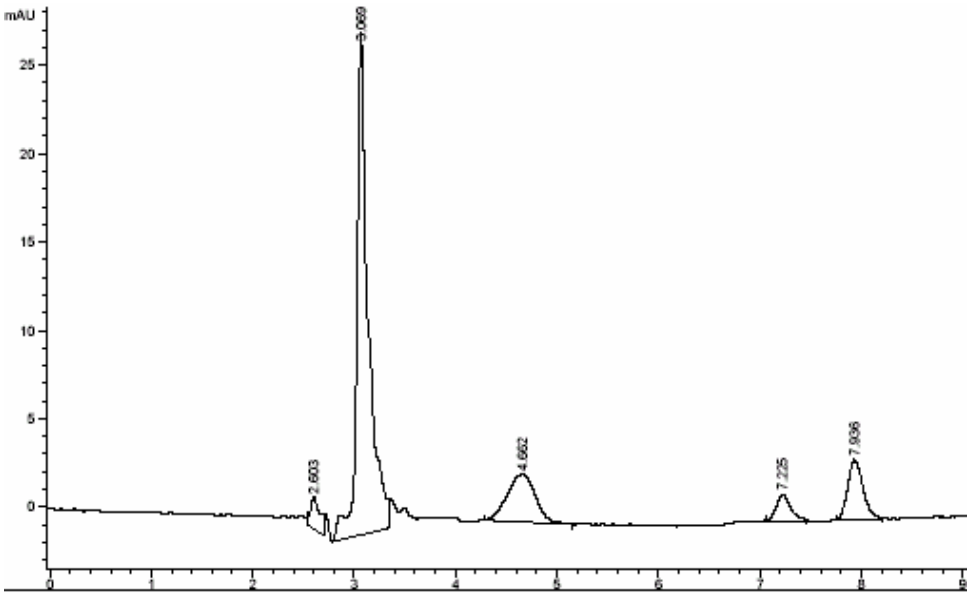


Figure 4.8 HPLC Chromatograms of 1µg/ml TTX standard measured by HPLC-UVD using mobile phase A at 195nm

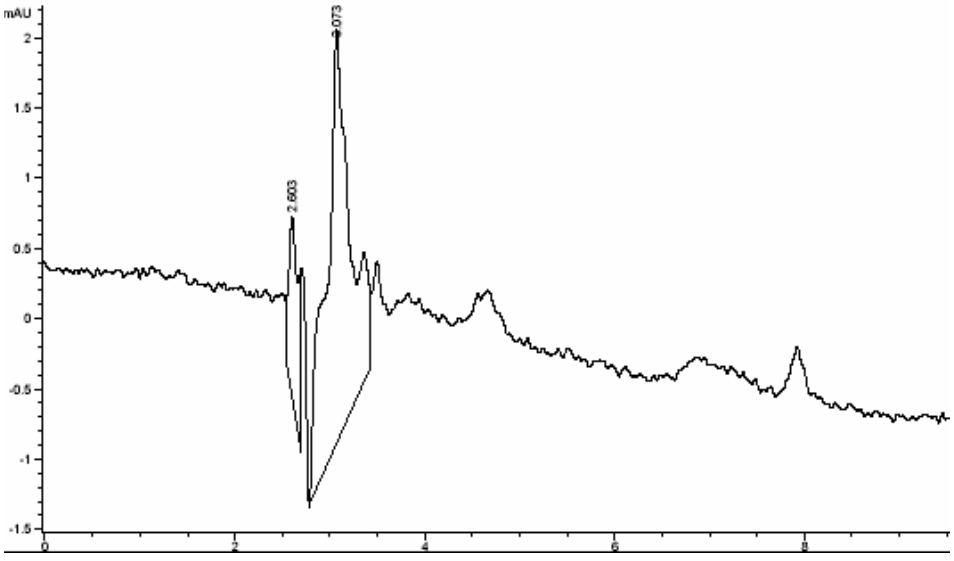


Figure 4.9 **HPLC Chromatograms of 1 to 25µg/ml TTX standard measured by HPLC-UVD using mobile phase B at 197nm**

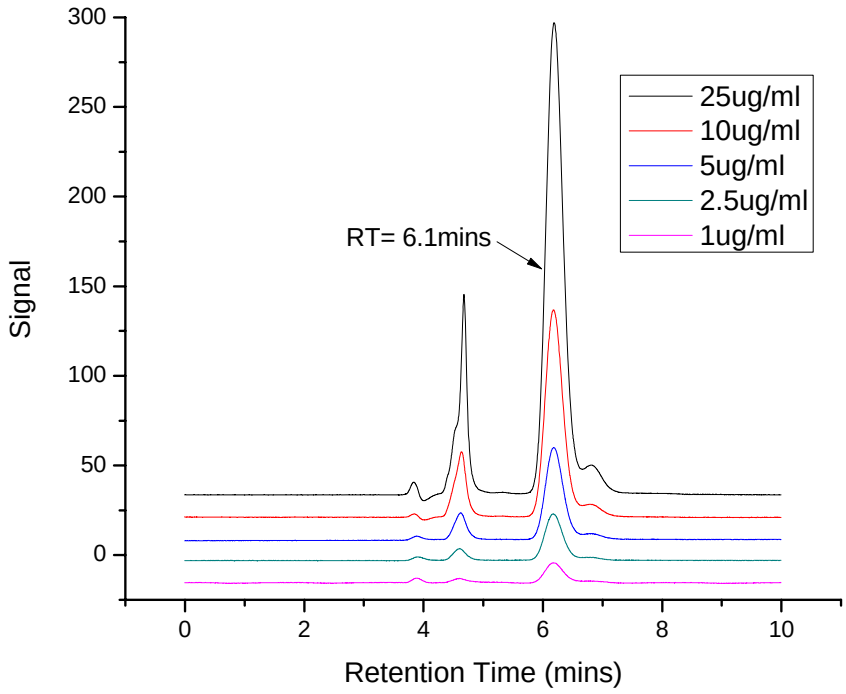


Figure 4.10 **Standard curve of TTX measured with HPLC-UVD at 197nm using mobile phase B**

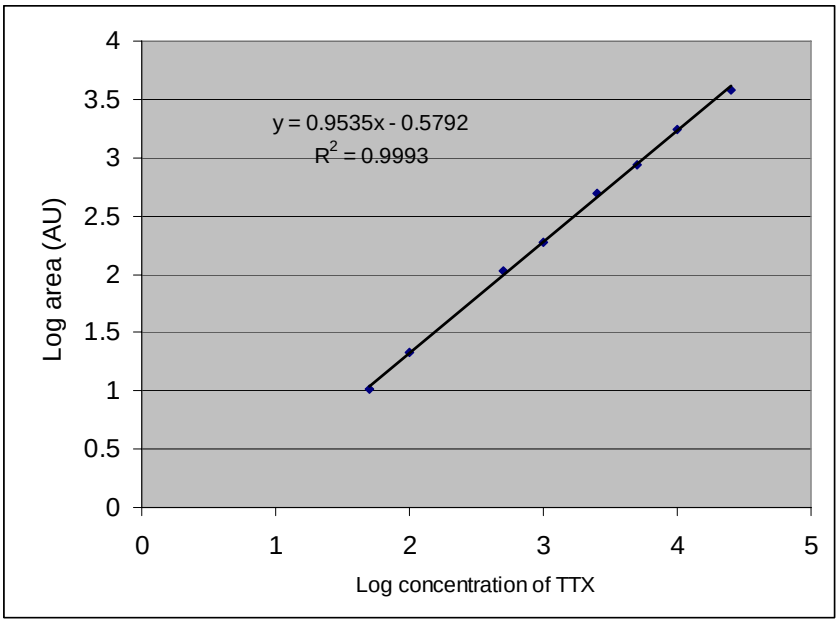


Table 4.7 Intra-day and inter-day stability measurement of HPLC-UVD system

TTX concentration	Intra-day		Inter-day	
	Retention time[1]	CV*(%)[2]	Retention time[1]	CV*(%)[2]
25ug	6.201±0.021	0.34	6.213±0.016	0.26
10ug	6.192±0.018	0.29	6.216±0.042	0.68
5ug	6.188±0.013	0.20	6.206±0.037	0.60
2.5ug	6.186±0.013	0.21	6.197±0.035	0.57
1ug	6.181±0.006	0.10	6.191±0.032	0.51
0.5ug	6.188±0.012	0.19	6.189±0.034	0.55
0.25ug	6.181±0.013	0.20	6.161±0.042	0.69
0.1ug	6.173±0.013	0.21	6.165±0.024	0.38
Average	/	**0.22**	/	**1.67**
	Signal Area[1]	CV* (%)[2]	Signal Area[1]	CV* (%)[2]
25ug	5125±100.2	1.96	5330±177.6	3.33
10ug	2295±19.1	0.83	2336±42.4	1.81
5ug	1022±12.1	1.18	1039±17.7	1.70
2.5ug	519±1.5	0.29	528±14.4	2.73
1ug	233±2.1	0.89	241±7.0	2.88
0.5ug	96.7±2.1	2.17	99.1±2.6	2.58
0.25ug	51.6±0.4	0.86	54.7±1.7	3.13
0.1ug	24.7±1.3	5.16	24.8±0.5	2.03
Average	/	**0.53**	/	**2.53**

* CV = Coefficient of variation
[1]expressed as mean value ± standard deviation
[2]coefficients of variation = standard deviation / mean value of recovery
Intra-day measurement: (n=3); Inter day-measurement: (3 consecutive days)

4.3 TTX purification and detection in urine

4.3.1 Solid phase extraction (SPE) of TTX in urine samples

The standard curve for the TTX-spiked urine samples was constructed by plotting the TTX peak areas against the amount of TTX added to 1mL normal urine. The standard curve was linear in the range of 10 to 500ng/mL (Figure 4.11; $y = 0.20566x – 0.6283$; $r^2 = 0.9976$) and the detection limit of TTX in urine was 10 ng/mL (Appendix 6). The loading capacity of the SPE cartridges used in this study became saturated when the TTX concentration in urine was above 500ng/mL. The percentage recovery using SPE of TTX in urine from the TTX spiked samples ranged from 82.7% to 94.9% in the range of 10 to 500ng/mL and the average recovery was 90.3 ± 4.0% with the average coefficients of variation 5.0 ± 2.5% (Table 4.8)

Figure 4.11 Calibration curve of TTX-spiked urine

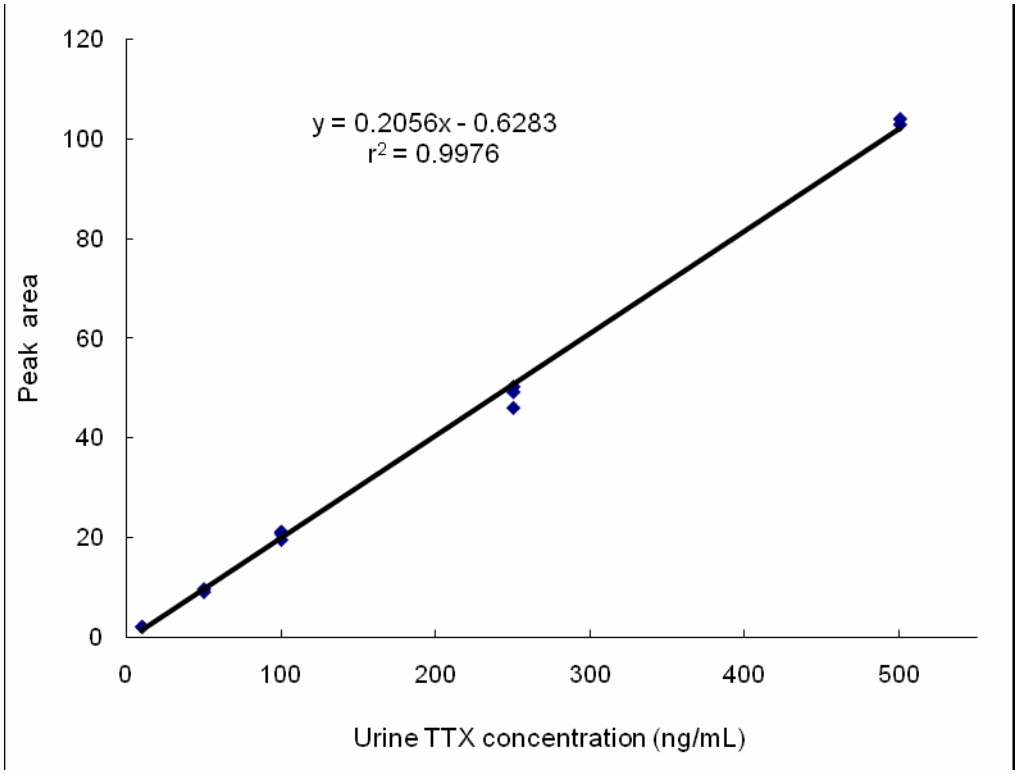


Table 4.8 TTX recovery in TTX-spiked urine samples

	TTX Concentration (ng/ml)	Recovery[1] (%)	CV[2] (%)
Urine samples	500	91.0 ± 8.4	9.2
	250	83.8 ± 2.8	3.3
	100	91.0 ± 4.4	4.8
	50	94.9 ± 4.5	4.7
	10	90.9 ± 2.8	3.1
	average recovery / CV	90.3 ± 4.0	5.0 ± 2.5[1]

[1]expressed as mean value ± standard deviation
[2]coefficients of variation = standard deviation / mean value of recovery

4.3.2 Case study of TTX poisoning in Hong Kong

By comparing the TTX peak areas between the standard TTX curve and TTX-spiked urine calibration curve, the amount of TTX in four patients' urine samples was determined in the range of 35.1 to 144.0 ng/mL, which were converted to 5.9 to 45.8 ng/μmol creatinine when UCC was made. Table 4.9 shows the concentration of TTX detected in the patients' urine samples, the creatinine-adjusted TTX levels and their clinical manifestations at presentation to the hospital.

The HPLC chromatograms of SPE cartridge washed with water and organic solvents did not show any TTX peaks. In Figure 4.12, chromatograms of standard TTX, TTX-spiked urine sample, patient's urine sample and normal urine sample were placed together, showing the retention time (RT) of their respective TTX peaks at 5.7 minutes and there was no interfering peak present near the TTX retention time in urine blank chromatogram. The RT of TTX was 5.7 minutes in the case study which was different from the RT = 6.1 minutes reported in section 4.2.3 because the HPLC-UV mobile phase was under development during the case study. Hence, the RT showed variation and the HPLC chromatogram of standard TTX in Figure 4.11 shows a negative peak which was not present in Figure 4.9.

Table 4.10 is a brief summary of recently published TTX poisoning cases, which shows the concentration of TTX in urine and serum specimens of the poisoned patients determined with different detection methods.

Table 4.9 TTX concentration in the patients' urine samples and their clinical features

Patient	Age	Sex	Urine TTX (ng/mL)	Urine Creatinine (μmol/L)	Urine TTX (ng/μmol creatinine)	Clinical Features
Pt#1 WJ	34	M	144	12428	11.6	Dizziness, perioral numbness, paraesthesia over the limbs and mild weakness of four limbs; self-induced vomiting for a few times, hospitalized for 2 days
Pt#2 TZ	40	M	63.9	1395	45.8	Perioral numbness, paraesthesia over the limbs, dropping of saliva, marked generalized decrease muscle power, reduced reflexes and muscle tone over the four limbs; assisted ventilation and ICU care for 1 day, discharged on day 5
Pt#3 GC	39	M	50.4	2148	23.1	Dizziness, nausea and repeated vomiting, paraesthesia and profound weakness of the four limbs, drooping of saliva, assisted ventilation and ICU care for 1 day and discharged on day 5
Pt#4 ZJ	34	M	35.1	5992	5.9	Dizziness, paraesthesia over the four limbs, perioral numbness, mild muscle weakness, self-induced vomiting once, hospitalized for 2 days

Figure 4.12 **HPLC chromatograms of standard TTX (2500ng/mL)(A), TTX-spiked urine (250ng/mL) (B), patient's urine (Pt#2 TZ) (C) and blank urine (D)**

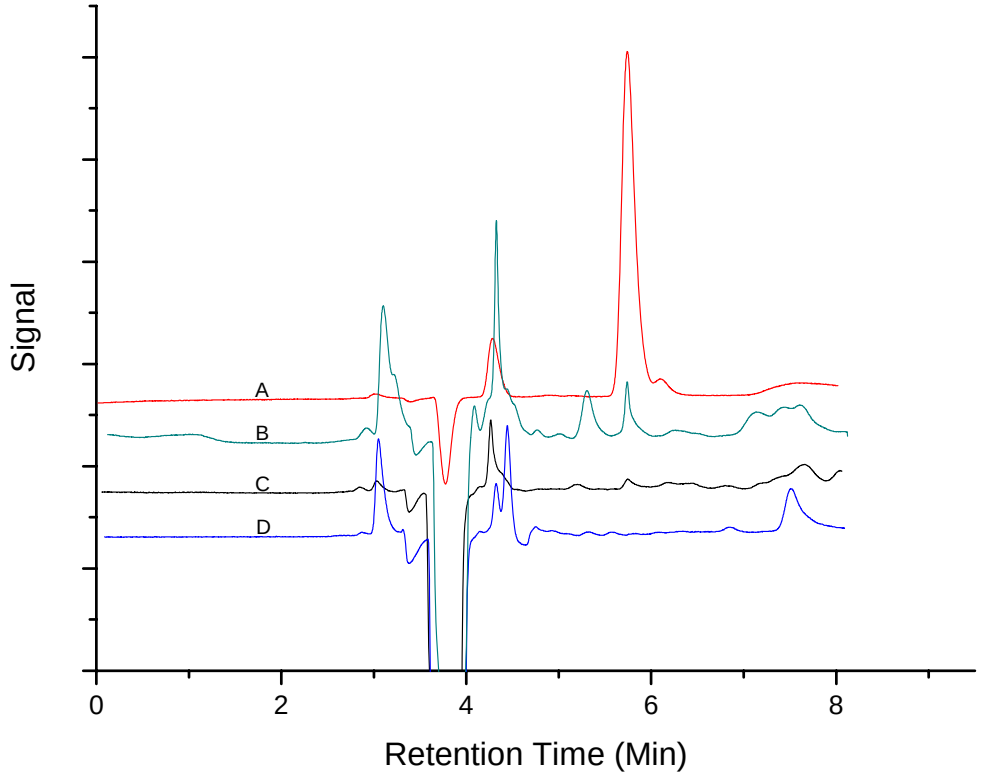


Table 4.10 A summary of the amount of TTX detected in urine and plasma/serum of patients with TTX poisoning in recent years

Incident Time	Location	Patients involved	Source of Poisoning	Detection method[1]	TTX conc. in urine	TTX conc. in plasma/serum	Sample collection after ingestion	Patient status[2]	Reference
Nov 2006	Hong Kong	4	Puffer fish	HPLC-UVD	35.1 to 144.0 ng/mL	N/A[3]	Within 24 hours	R(4)	This study
Apr 2001	Taiwan	6	Puffer fish	LC-MS	47 - 344 nM	4.5 – 40.6 nM	N.M.	D(1), R(5)	Tsai et al.,2006
2004	Japan	7	Puffer fish	LC-MS-MS	15 - 150 ng/ml	0.9 - 1.8 ng/ml	N.M.	R(7)	Akaki & Hatano, 2006
Apr 2005	Taiwan	6	Gastropod	LC-MS	169 - 325 ng/ml	<1 - 8 ng/ml	15 hours	D(2), R(4)	Hwang et al.,2005
N.M.	Australia	7	Puffer fish	HPLC-FLD	28.0 - 258 ng/ml	≤ 5	24 hours	R (7)	O'Leary (2004) & Kiernan (2005)
Mar 2001	Japan	1	Puffer fish	HPLC-FLD	290 ng/ml	N/A	1day	R (1)	Takata et al., 2001
				HPLC-FLD	40 ng/ml	N/A	9 days		
1989-1996	Japan	6	TTX-food	HPLC-FLD	6 - 102 ng/ml	N/A	1day	R (6)	Kawatsu et al.,1999
N.M.	Japan	1	Puffer fish	N.M.	N/A	52.3 ng/ml	1day	R (1)	Yamazaki & Shibuya,1995
					N/A	0 ng/ml	3 - 42days		
N.M.	Japan	11	Puffer fish	GC-MS	650 ng/ml (deceased)	93 -320 ng/ml (deceased)	N.M.	D (3)	Fukushima,1991
					15 - 443 ng/ml (recovered)	2.5 -63.9 ng/ml (recovered)	N.M.	R (8)	
N.M.	Japan	1	Puffer fish	GC-MS	Not detected	36.3 ng/ml	1 day	R (1)	Oda et al., 1989
					281ug/day	0	2 day		
					43ug/day	0	3 day		
					16ug/day	0	4 day		
					0	0	5 day		

[1] Detection method: LC-MS-MS liquid chromatography with tandem mass spectrometry; GC-MS gas chromatography-mass spectrometry

[2] Patient status: D = Deceased, R= Recovered, numbers in parentheses denote patients involved

[3] N/A: not available, N.M.: not mentioned

4.4 Bacterial production of TTX

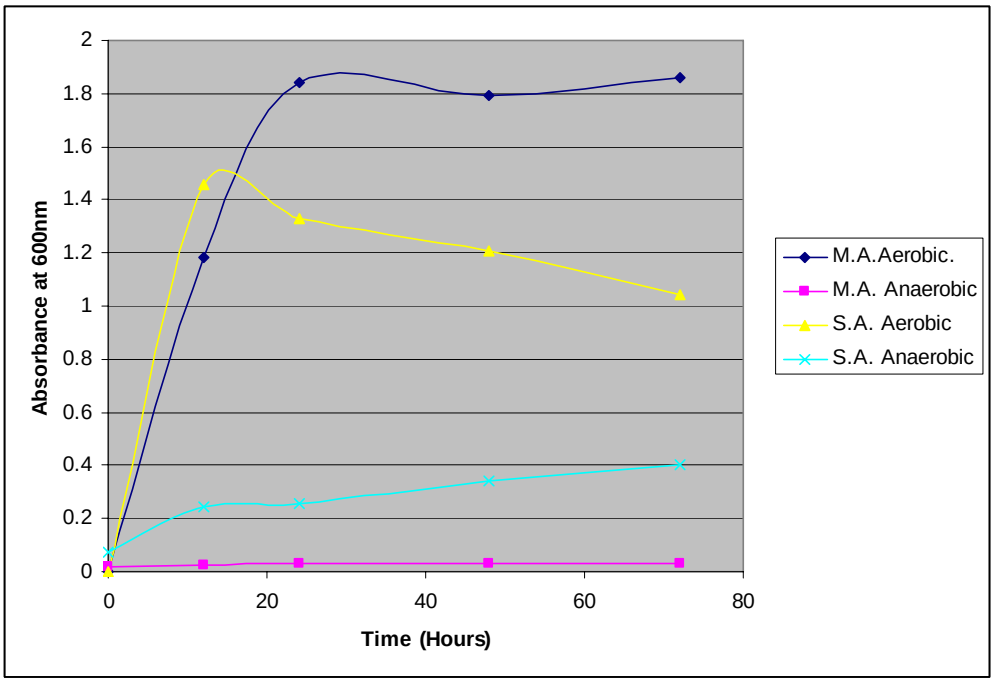
4.4.1 Optimization of fermentation condition for TTX producing bacteria using shake flask method

4.4.1.1 Aerobic and anaerobic condition

Two species of bacteria, *M.arabinogalactanolyticm* and *S.marcescens,* were studied in the effect of oxygen availability on the growth of bacteria. Both species grew much better in aerobic condition than anaerobic condition and the absorbance under aerobic condition was at least 10 times and 3 times higher than in anaerobic condition for *M.arabinogalactanolyticm* and *S.marcescens* respectively. (Figure 4.13) In aerobic condition, *S.marcescen* showed the highest absorbance at 12 hours and the absorbance started to drop after 12 hours. *M.arabinogalactanolyticm* showed the highest absorbance at 24 hours and kept constant until 72 hours. For samples in anaerobic condition, *M.arabinogalactanolyticm* showed a very slow growth in the 72-hours fermentation study while *S.marcescens* showed a continuous growth during the 72-hours.

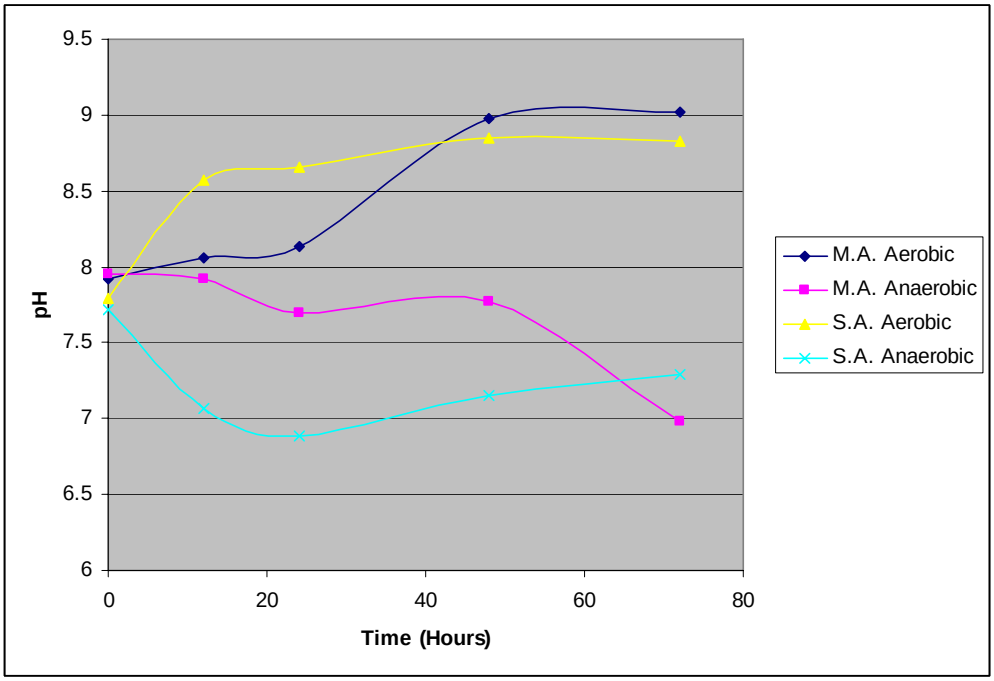
The pH profile was studied at the same time during the 72-hours ferementation study (Figure 4.14). Generally, the pH increased in aerobic condition and decreased in anaerobic condition in both species of bacteria. For *S.marcescens* under anaerobic condition, the pH dropped in the first 24 hours and started to increase after 24 hours until 72 hours. (Appendix 7)

Figure 4.13 Growth curves of *M.arabinogalactanolyticm* and *S.marcescens* in ORI medium under aerobic and anaerobic condition



M.A. = *M.arabinogalactanolyticum*, S.M. = *S.marcescens*

Figure 4.14 pH profiles of *M.arabinogalactanolyticum* and *S.marcescens* under aerobic and anaerobic condition



M.A. = *M.arabinogalactanolyticum*, S.M. = *S.marcescens*

4.4.1.2 Effect of salt in ORI medium on the bacterial growth

Sea water collected from the Ocean Park and synthetic sea water purchased from market were studied along with sodium chloride in different concentrations in *S.marcescens* (Figure 4.15), *M.arabinogalactanolyticum* (Figure 4.16) and *P.putida* (Figure 4.17). (Appendix 8)

For *S.marcescens,* ORI medium with NaCl showed better growth followed with sea water and synthetic sea water. The ORI without any salt grew worse compared to the medium with salt.

In figure 4.16, *M.arabinogalactanolyticum* grown in sea water and synthetic sea water showed the highest grow rate in the first 12 hours and the increase in absorbance leveled off until 72 hours. For the growth in ORI medium without salt, the absorbance continued to increase until 24 hours. The growth in the mediums with NaCl showed the highest absorbance and the absorbance level kept constant for the longest time until 48 hours.

P.putida showed similar growth pattern in culture medium with or without NaCl and the highest absorbance were about the same.

Figure 4.15 **Growth curve of *S.marcescens* with different source of salt and in different salt concentration**

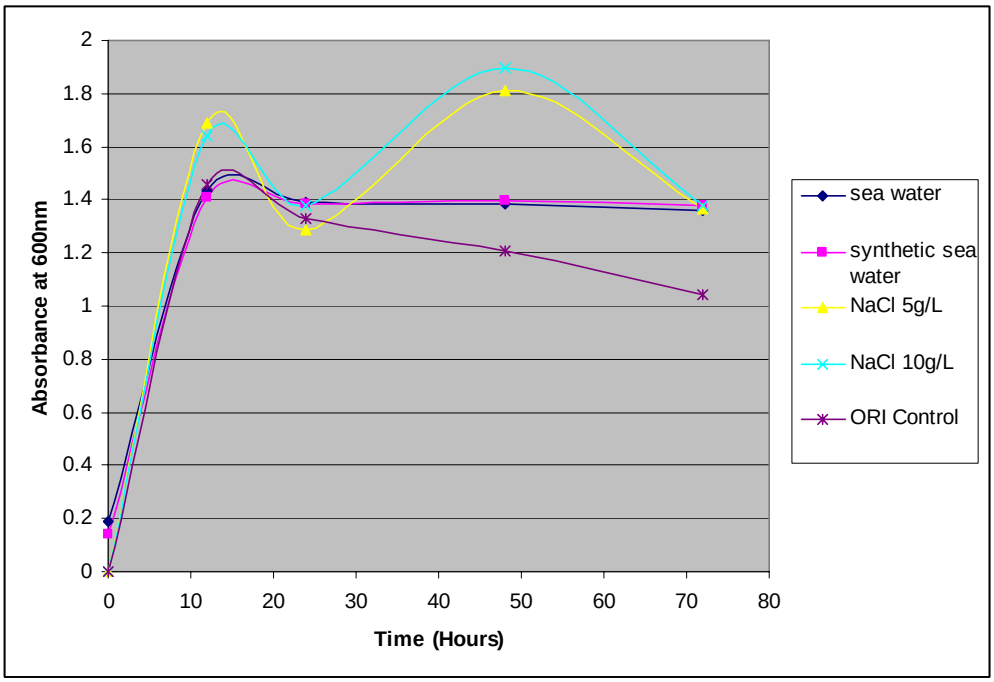


Figure 4.16 **Growth curve of *M.arabinogalactanolyticum* with different source of salt and in different salt concentration**

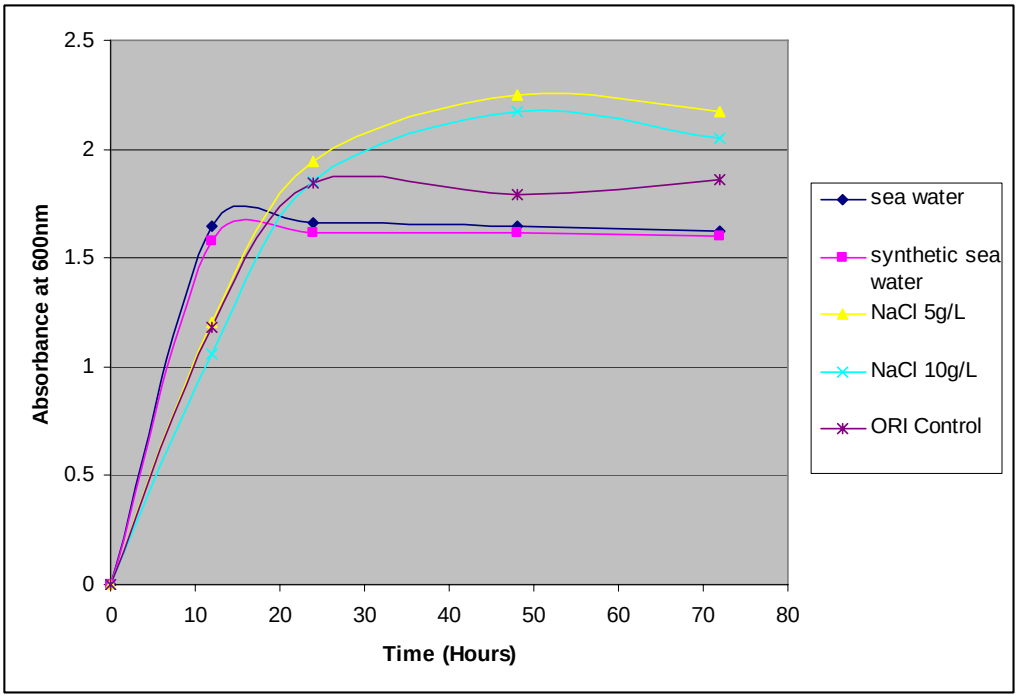
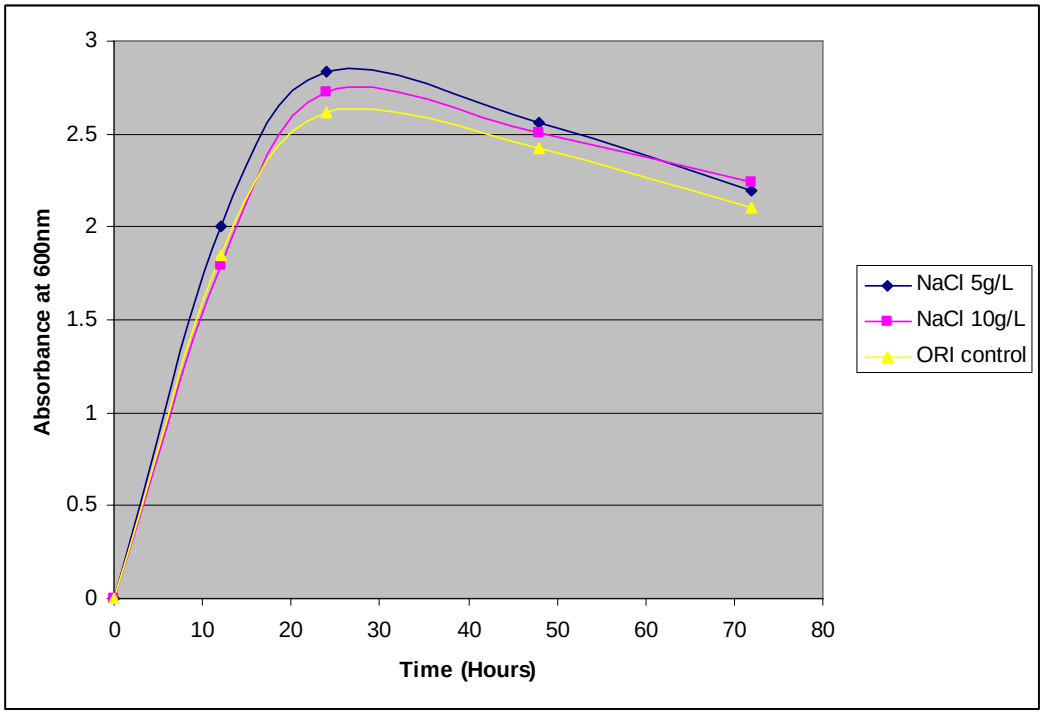


Figure 4.17 Growth curve of *P.putida* in different salt concentration



4.4.1.3 Carbon source enrichment and modification in the culture medium

The growth rate of *S.marcescens* was the highest and reached the maximal absorbance in the first 6 hours for samples with or without the addition of carbon source (Figure 4.18). The sample without an addition of carbon source showed the highest absorbance and the level of absorbance were followed with sucrose at 5g/L, sucrose at 10g/L and glucose at 5g/L. After 6 hours fermentation, the absorbance started to drop in the ORI control until the end of fermentation, whilst it was after 24 hours in sample with an addition of sucrose and glucose.

The growth of *M.arabinogalactanolyticum* showed the highest absorbance in ORI control and the growth maintained in the first 24 hours (Figure 4.19). For the samples with the addition of either sucrose or glucose, the absorbance was significantly lower. The growth

of the sucrose samples were faster and leveled off after 12 hours while the glucose samples continued to grow until 48 hours.

For *P.putida*, the addition of sucrose can enhance the growth of *P.putida* compared with the ORI control while the addition of glucose showed inhibition effect (Figure 4.20). The growth of the ORI control showed the highest absorbance of around 2.6 at 12 hours while the sample with 10g/L sucrose grew continuously over the 72-hour fermentation. (Appendix 9)

The pH of the samples in ORI control for both *M.arabinogalactanolyticum* and *S.marcescens* increased above pH 8 after 24 hours fermentation and the pH value became steady after 24 hours (Figure 4.21). For samples *M.arabinogalactanolyticum* with the addition of glucose at 5g/L and *S.marcescens* with the addition of sucrose at 10g/L, dropped to around pH 5.5 after 24 hours and kept at acidic pH until the end of fermentation. The *M.arabinogalactanolyticum* samples with the addition of sucrose at 10g/L showed a drop in pH under 6 in the first 24 hours and the pH rose again to pH 7.5 after 48 hours and approached pH 8 at 72 hours. (Appendix 10)

Figure 4.18 Growth curve of *S.marcescens* with different carbon source supplements

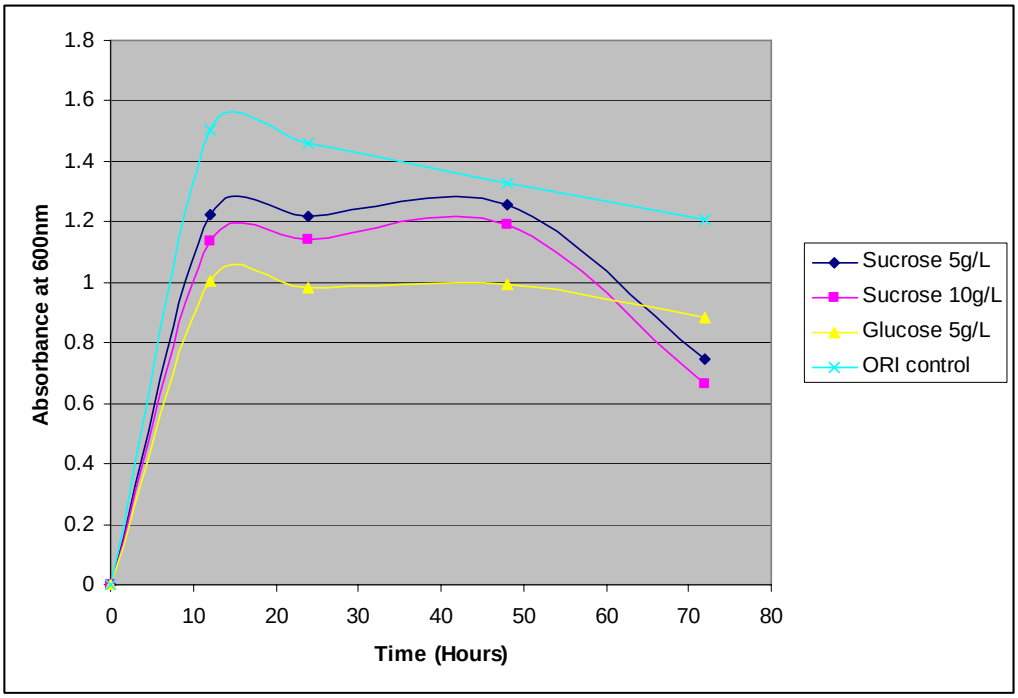


Figure 4.19 Growth curve of *M.arabinogalactanolyticum* with different carbon source supplements

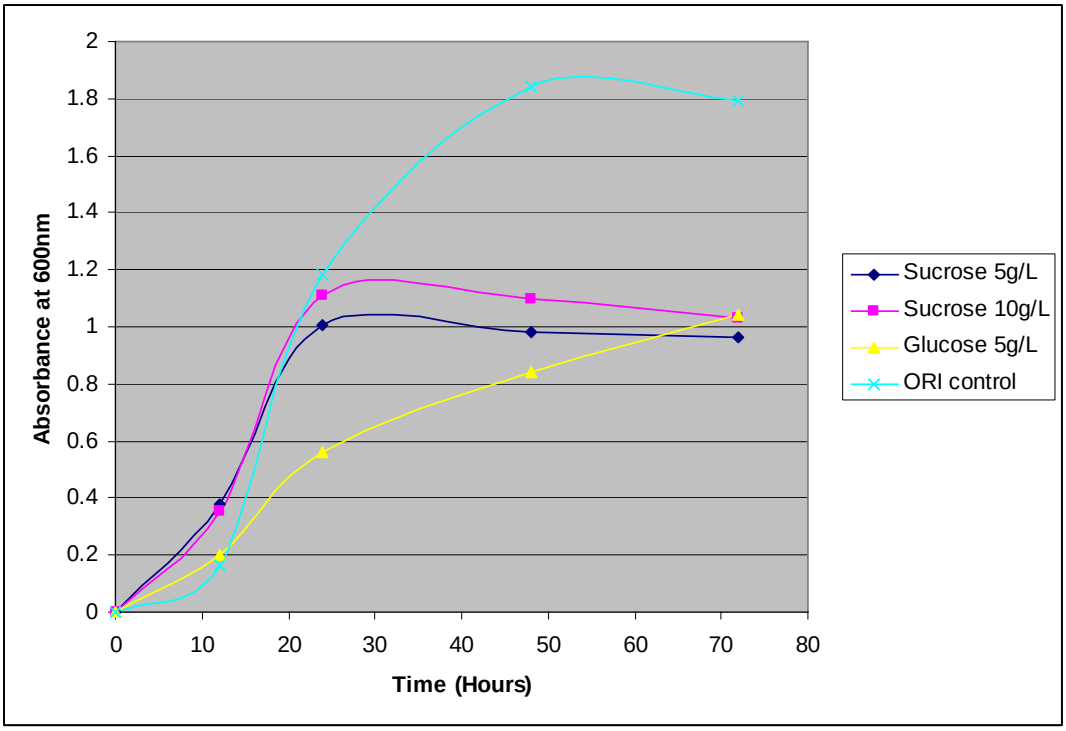


Figure 4.20 Growth curve of *P.putida* with different carbon source supplement

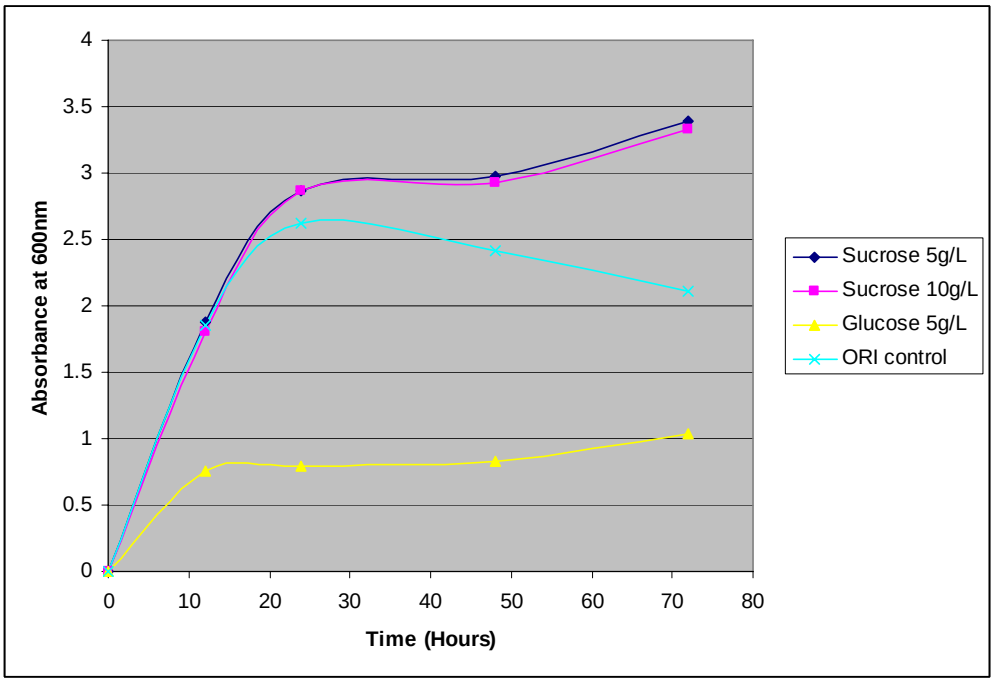
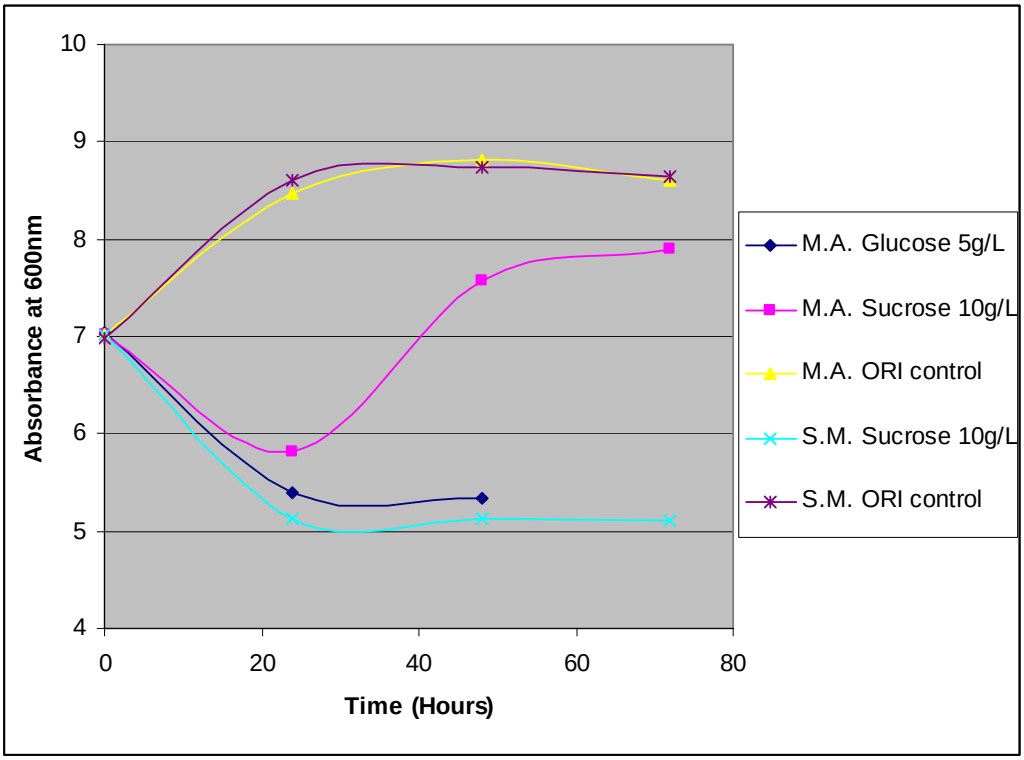


Figure 4.21 pH profiles of *M.arabinogalactanolyticum* and *S.marcescens* with different carbon source supplement



4.4.1.4 Addition of arginine and puffer fish extract in the ORI medium

The addition of arginine in the ORI medium resulted in a slightly higher absorbance for *S.marcescen* and *P.putida* (Figures 4.22, and 4.24) and there was no significant difference observed in *M.arabinogalactanolyticum.*

For the samples with the addition of puffer fish ovary extract to ORI medium, it was found that the absorbance of all the three species were reduced compared to ORI control and the percentage of reduction in the maximal absorbance for *P.putida* was the highest with around 25%. (Appendix 11)

Figure 4.22 Growth curves of *S.marcescen* with the addition of arginine and puffer fish ovary extract in the ORI medium

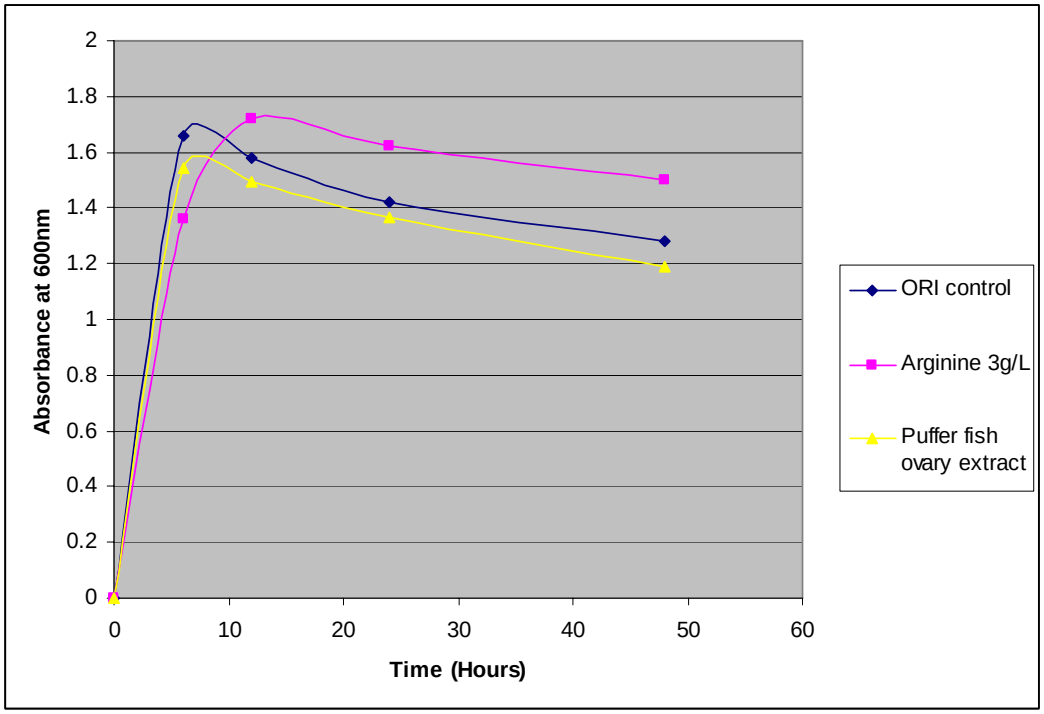


Figure 4.23 Growth curve of *M.arabinogalactanolyticum* with the addition of arginine and puffer fish ovary extract in the ORI medium

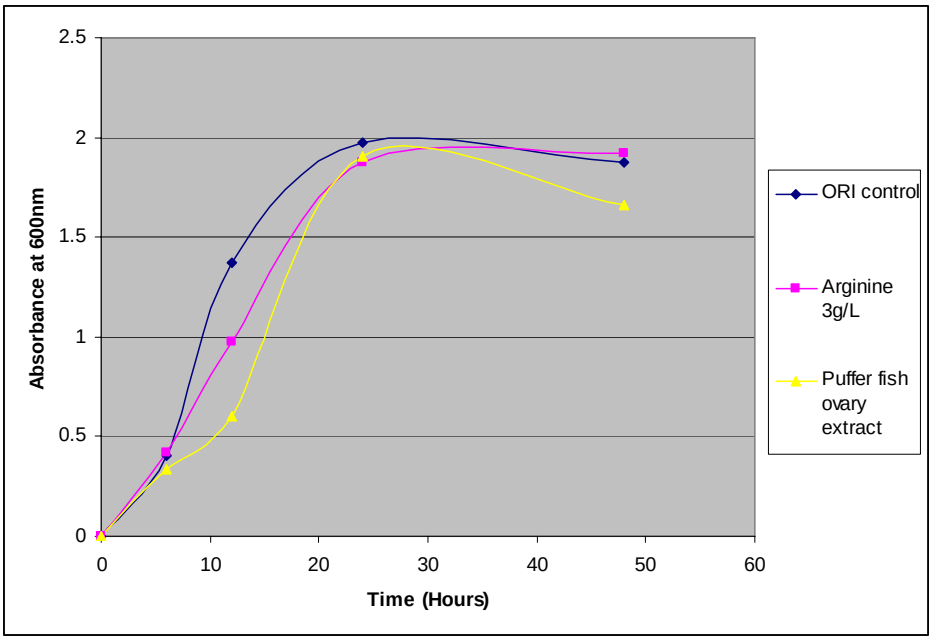
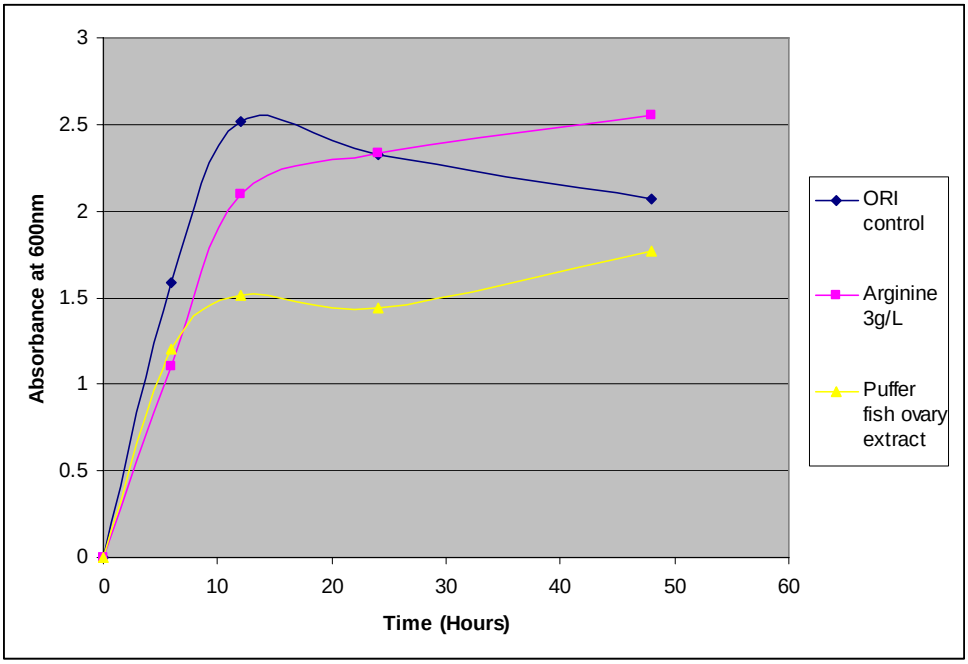


Figure 4.24 Growth curve of *P.putida* with the addition of arginine and puffer fish ovary extract in the ORI medium



4.4.1.5 Comparison of the growth and toxicity of the TTX producing bacteria

The growth of *P.putida* showed the highest maximal absorbance, followed with *M.arabinogalactanolyticum, Bacillus-cereus, S.marcescen* and *Raoultella-terrigena* (Figure 4.25). Except *M.arabinogalactanolyticum* which required 24 hours to achieve maximal absorbance, all the other bacteria species could achieve it within the first 12 hours. (Appendix 12)

The toxicity of the bacteria cultures using shake flask fermentation measurement with mouse bioassay were shown in Table 4.11. *S.marcescen* showed the lowest toxicity among the other bacteria species and the toxicity was 28.5 MU/L at 24 hours fermentation. The toxicity of *S.marcescen* samples decreased to an undetectable range measured by mouse bioassay at 48 hours. For the samples of *M.arabinogalactanolyticum* measured at 48hours and *P.putida* at 24hours, the highest toxicity was 120.0 and 210.8 MU/L, respectively.

For the bacteria species obtained from the screening of TTX producing bacteria study, the toxicity of *Bacillus-cereus* was 52 MU/L. And the other screened bacteria species, *Raoultella-terrigena*, showed a very high toxicity of 454 MU/L, which was the highest among the other species. For bacteria in anaerobic condition, the toxicity of both *S.marcescen* and *M.arabinogalactanolyticum* was too low to be detected using mouse bioassay. Also, the addition of arginine in the culture medium of *P.putida* reduced the amount of toxin produced compared to the sample of ORI control. (Appendix 13)

Figure 4.25 Growth curves of S.marcescen, M.arabinogalactanolyticum, P.putida, Raoultella-terrigena and Bacillus-cereus in shake flask fermentation

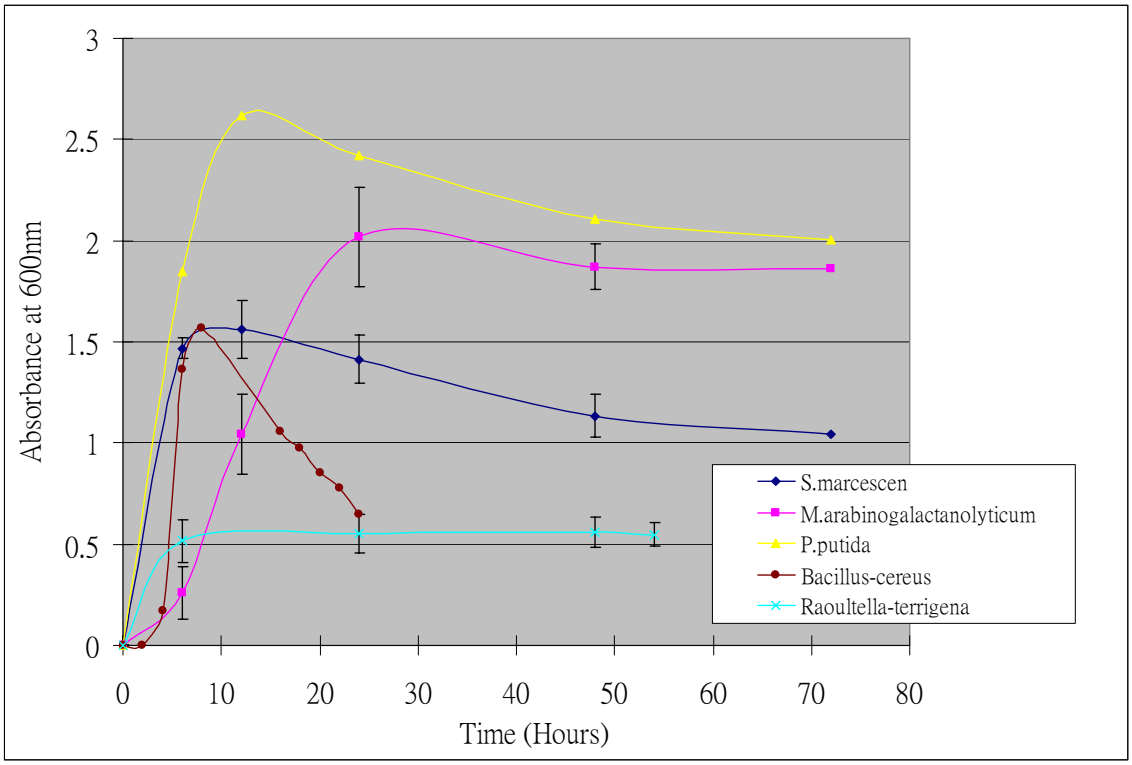


Table 4.11 Toxicity of TTX producing bacteria in shake flask fermentation measured by mouse bioassay

Bacteria species	Culture medium	Time of Fermentation	Toxicity from mouse bioassay	
			MU/L	ug/L
S.marcescen	ORI control	24h	28.5	5.7
		48h	*	*
	Anaerobic condition	7days	*	*
M.arabinogalactanolyticum	ORI control	24h	21.0	4.2
		48h	120.0	84.0
	Anaerobic condition	7days	*	*
P.putida	ORI control	24h	210.8	42.2
		48h	105.0	21.0
	ORI + 3g/L arginine	24h	< 53.0	< 10.6
Raoultella-terrigena	ORI control	48h	454	90.8
Bacillus-cereus	ORI control	48h	52	10.4

***** Cannot be determined using mouse bioassay = < 1MU

4.4.2 Fermentation of TTX producing bacteria using fermenter

In this study, three batches of fermenter were done to investigate the growing pattern of *P.putida* in culture medium with different supplements. Batch 1 used ORI with 20g/L sucrose as culture medium and it was used as a blank control of the other two batches of fermenter (Figure 4.26). Batches 2 and 3 used ORI + 20g/L sucrose medium with the addition of ovary extract and total protein of ovary extract, respectively (Figures 4.27 and 4.28). (Appendix 14)

The TTX accumulation pattern of the three batches of fermenter showed a similar trend. TTX concentration increased within the first 24 hours and achieved the highest concentration. Afterward, the TTX concentration diminished with increasing

fermentation time. The raw data for the measurement with mouse bioassay in the three batches of fermenter were shown in (Appendix 15-17).

For the cell dry weight or absorbance in the fermenter studies, the cell growth showed the highest growth rate in the first 24-30 hours and the cell dry weight kept in a constant level or had a slight decrease after it reached the highest cell dry weight level. The concentration of carbohydrate in the fermenter during fermentation was monitored by measuring the total carbohydrate in the bacterial culture collected in each sampling point and the result was shown in figure 4.27. The cell dry weight and the amount of carbohydrate in the fermenter were out of phase and the cell dry weight ceased to increase and kept constant with the concentration of carbohydrate becoming the lowest at around 30 hours.

The three batches of fermenter showed a similar growth pattern as shown in figure 4.29. Batch 2 with the ovary extract resulted in the highest maximal cell growth, followed by Batch 3 with the protein extract from ovary. Batch 1, the blank control without the addition of supplement had the lowest maximal cell growth.

Since there was TTX in the ovary extract of puffer fish, the initial TTX concentration of Batch 2 (ovary extract) at 0 hour was not zero and this was known as the basal TTX level of the Batch 2 fermenter. The net TTX production in Batch 2 fermenter was calculated as follows:

Net TTX concentration = (TTX concentration measured – Basal TTX concentration)

The TTX accumulation of different batches of fermenter was plotted in figure 4.30. After the subtraction of the basal TTX concentration, the net TTX production in Batch 2 (ovary

extract) fermenter had the highest TTX concentration of 29.8ug/L. For Batch 1 (Blank control) and Batch 3 (Total protein of ovary) fermenter, the highest TTX concentration was 17.8ug/L and 27.3ug/L, respectively. The highest TTX accumulation in Batch 3 fermenter was similar to Batch 2 fermenter and was 50% higher than Batch 1 fermenter (Appendix 18).

The history plot recording the fermenter parameters including pO2, stir speed, with the addition of acid and base was monitored during the whole course of fermentation as shown in Appendix 19.

The growth profile of TTX accumulation of *M.arabinogalactanolyticum* and *S.marcescens* done by our colleagues using fermenter were attached in Appendix 20 and Appendix 21 as reference.

Figure 4.26 The growth profile and TTX accumulation of *P.putida* in ORI medium using fermenter (Batch 1)

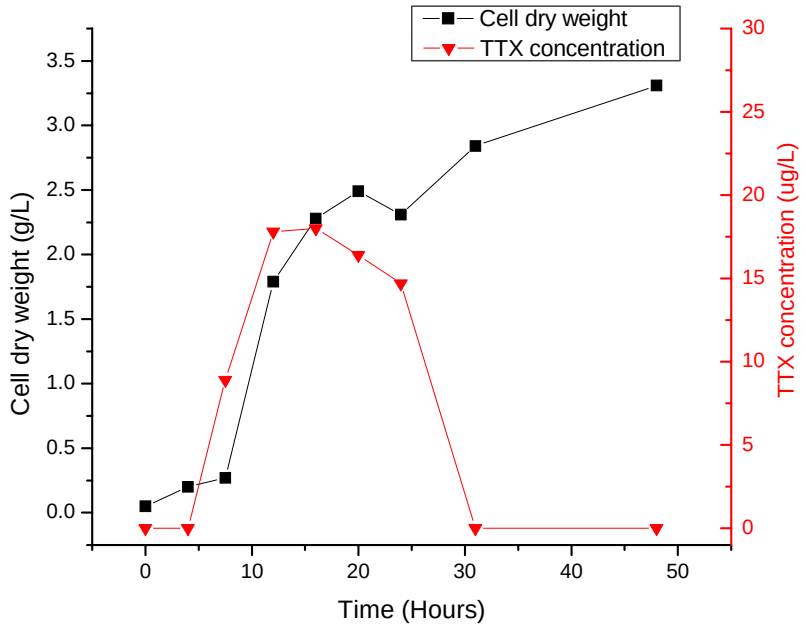


Figure 4.27 **The growth profile and TTX accumulation of *P.putida* in ORI medium with ovary extract using fermenter (Batch 2)**

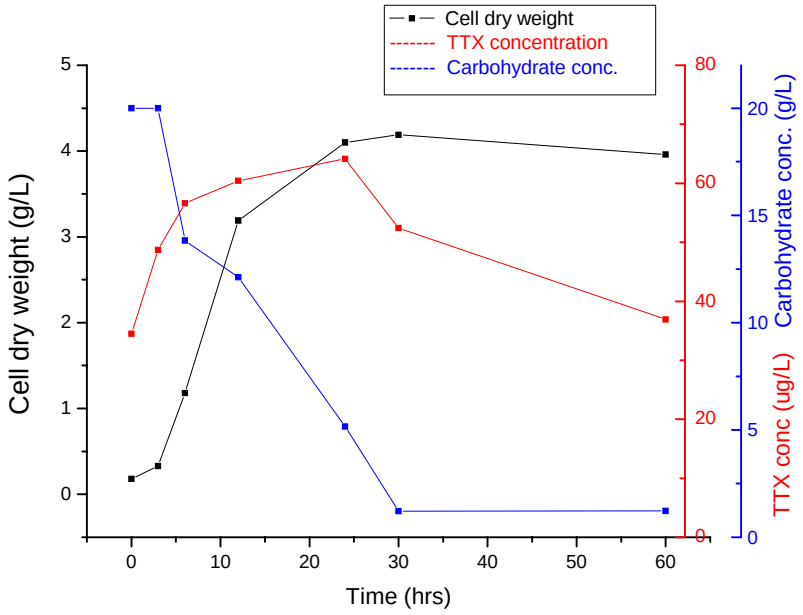


Figure 4.28 **The growth profile and TTX accumulation of *P.putida* in ORI medium with total protein from puffer fish ovary using fermenter (Batch 3)**

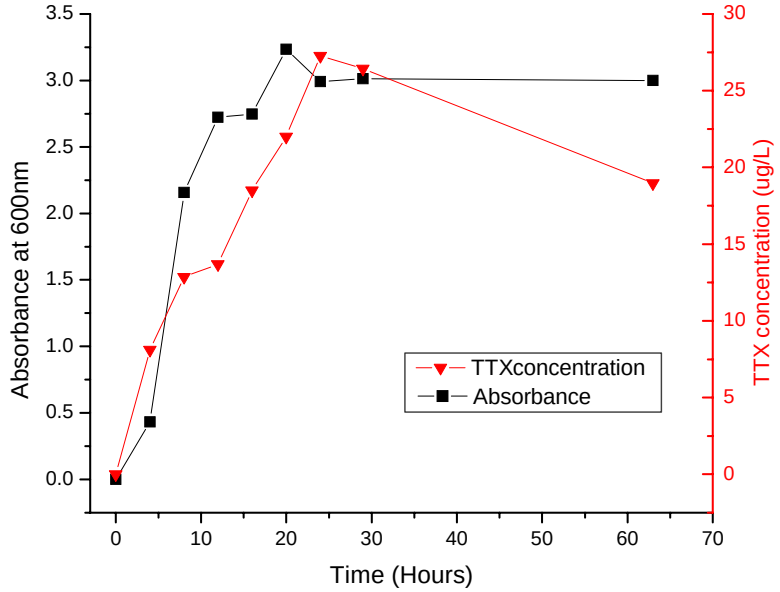


Figure 4.29 **The growth curve of *P.putida* in different culture medium using fermenter**

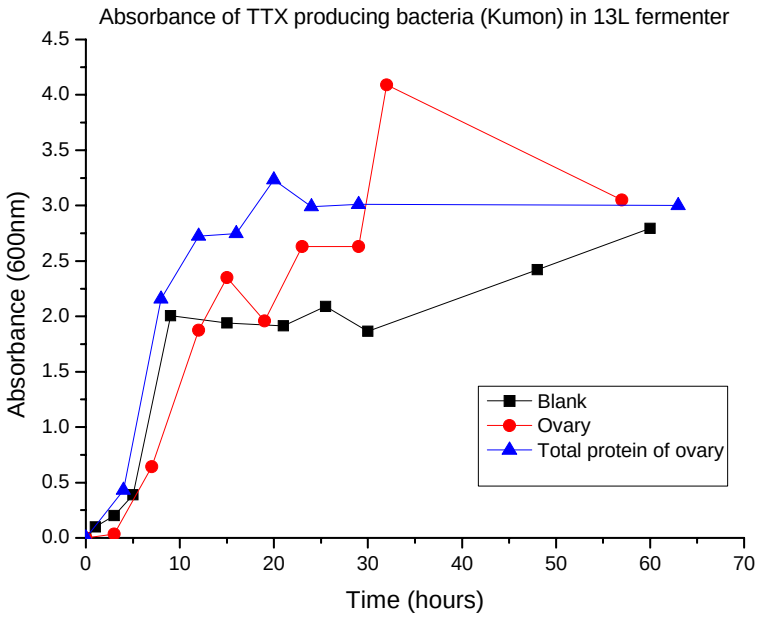
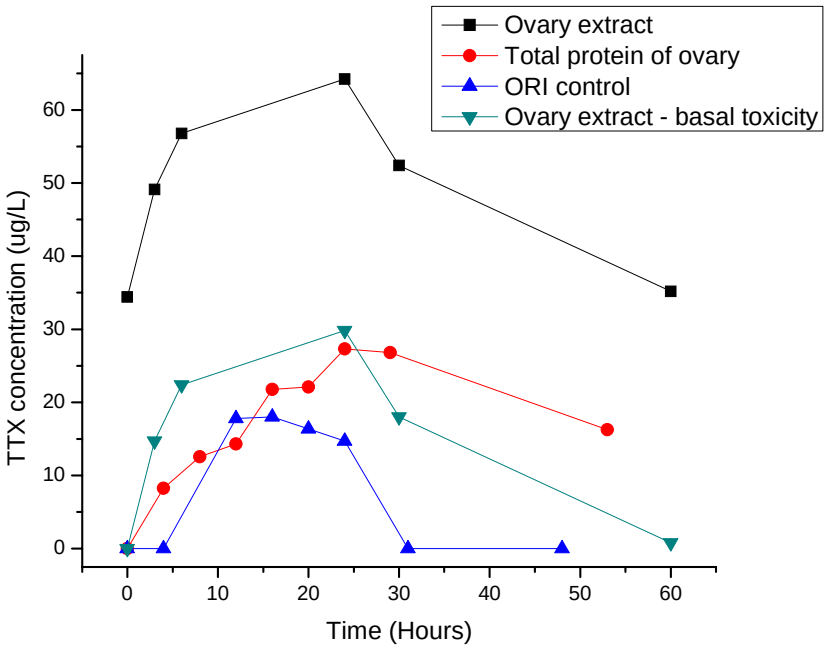


Figure 4.30 **The TTX accumulation profile of *P.putida* in different culture medium using fermenter.**



4.4.3 Extraction and purification of TTX from the bacterial culture

Batch 3 fermenter, with the addition of total protein extraction as supplement, was used for the study in the extraction and purification of TTX from bacterial culture. Sampling points (12, 16 and 20 hours) and (24 and 30 hours) were poured together as Fraction 2 and Fraction 3, respectively. Each fraction was subjected to ACT mentioned in section 3.4.3. After ACT, sample was tested with mouse bioassay again to check the efficiency and recovery of ACT. In order to further confirm the toxicity measured in mouse bioassay was the toxicity arisen from TTX, samples after ACT were further purified with SPE mentioned in section 3.3.1 followed with HPLC-UVD measurement for TTX.

In Table 4.12:

Sampling time = Time for the collection of 1L sample

Amount of TTX = Amount of TTX present in 1L sample collected measured by mouse bioassay

Total amount of TTX before ACT = Sum of TTX amount in each fraction

Amount of TTX after ACT = Toxicity measured by mouse bioassay after ACT

Recovery after ACT = Amount of TTX after ACT / Total amount of TTX before ACT x 100%

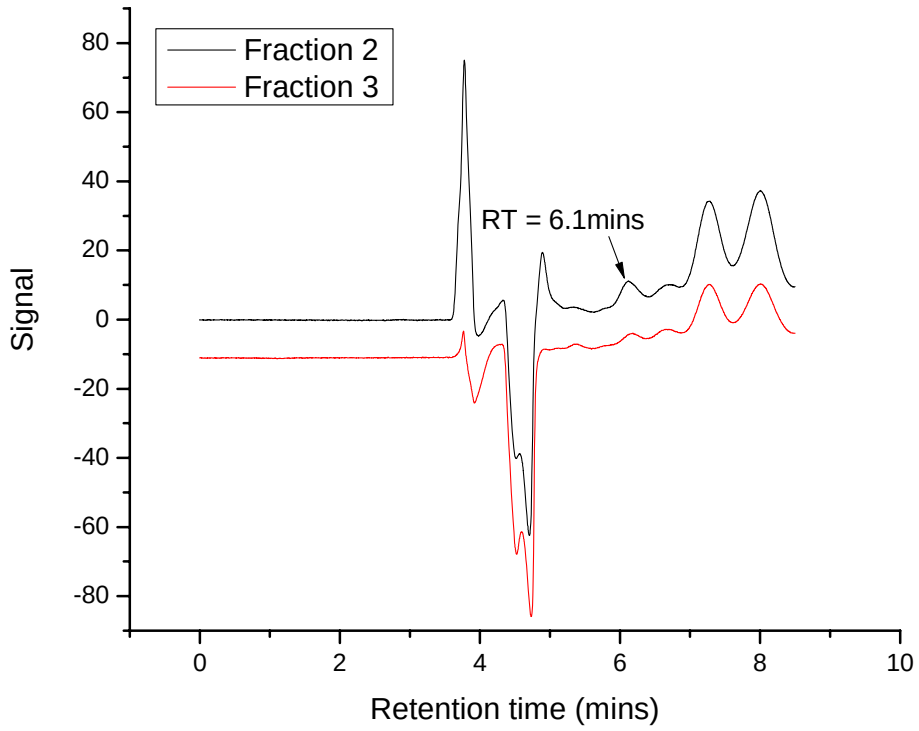
The recovery of TTX with ACT was 49.1% and 32.3% in fractions 2 and 3, respectively, and the mean recovery of ACT was around 40% as shown in Table 4.12. After the purification with ACT, the samples were subjected to SPE for further purification. From the HPLC chromatogram in Figure 4.31, the TTX peak of the bacterial culture sample in Fraction 2 and 3 was found at retention time = 6.1 minutes. Some other impurities peaks or TTX derivatives peaks and negative peak were observed in the chromatogram.

Table 4.12 Recovery of TTX from Batch 3 fermenter bacterial culture with ACT

	Sampling time (hrs)	Amount of TTX (ug)	Total amount of TTX before ACT (ug)	Amount of TTX after ACT (ug)	Recovery after ACT (%)
Fraction 2	12	13.7	(13.7+ 18.5+ 22.0) x 0.75* = 40.65	19.94	49.1
	16	18.5			
	20	22.0			
Fraction 3	24	27.2	(27.2 + 26.4) x 0.75* = 40.2	12.98	32.3
	30	26.4			
				Mean Recovery %	40.7

* 75% of the sample from each fraction were subjected to ACT

Figure 4.31 HPLC chromatogram of TTX purified from Batch 3 fermenter bacterial culture with ACT and SPE



Bactch 3 fermenter with the addition of total protein as supplement:
Fraction 2 = Three 1L sample collected at 12, 16 and 20 hours.
Fraction 3 = Two 1L sample collected at 23 and 29 hours.

5. Discussion

5.1 Puffer fish background study

5.1.1 Dissection and collection of Puffer fish

Three species of puffer fish including *T. alboplumbeus, T. niphobles* and *C. patoca* were collected in this study from Hong Kong waters. They were comprehensively studied by one of our colleagues in a research study on puffer fish in Hong Kong and its relationship with TTX (Yu, 2003). From the puffer fish collected in batch A, it was found that the average length and weight of the female puffer fish was higher than that of the male puffer fish. This may be related to the extensive development of the female sex organ during spawning season. For the male puffer fish and the female puffer fish with immature ovary development, the gonad weight percentage was similar of around 5%, while the female puffer fish with developed ovary during spawning season, the ovary weight percentage could be up to 29.2%.

5.1.2 Bacterial count of puffer fish organs

Total bacterial count was done using LB agar. Bacterial counts were spotted in all the tested organs. The bacteria count of ovary, testis and liver were very low within 50 CFU which suggested that bacterial activity might not be active in these areas. This might caused by the immune system of the puffer fish would act on the foreign bacteria in these organs. However, the bacteria in these organs might have a higher potential to be TTX producing because they had a higher chance to be symbiotic bacteria which exert certain positive functions to the host.

For the total bacterial count in the gut samples, the count showed a large variation and the highest count could be up to 29400 CFU while the lowest count could be 5 CFU only. This suggested that the number of bacteria in puffer fish could vary in a large extent from individual fish even they belonged to the same species. Also, it was expected that the nutrient availability in the gut could affect the number of bacterial count. However, it was reported that many species of marine bacteria were non-culturable, which meant that these marine bacteria could not be grown by using conventional bacterial culture medium. Hence, the gut samples showing a low total bacterial count might not have a small amount of bacteria as those bacteria might be non-culturable since gut sample was not expected to have bacterial count within 10 CFU.

For the detection of *E.coli* count, it was aimed at screening of E.coli which isTTX producing. Since E.coli has a well developed genome database, this allows further studies the gene related to TTX production in bacteria. However, E.coli was not found in the internal organ of puffer fish screened in this study.

5.1.3 Toxicity of puffer fish organs

Batch B puffer fish was used for toxicity measurement and the collection time of Batch B puffer fish was in March which was the time just after the spawning season of *T. alboplumbeus* and *T.niphobles*. It was reported that the toxicity of *T.alboplumbeus* and *T.niphobles* were less toxic in their spawning season. The ovary and liver samples of *T. alboplumbeus* were from moderately toxic (100-1000MU/g) to weakly toxic (10-100MU/g) in the batch B samples. However, the ovary sample of *T.niphobles* was highly toxic, up to 356ug/g (1780MU/g) while the liver sample was moderately toxic (121ug/g

or 605MU/g). The toxicity level of the internal organs obtained in this study were slightly different from the result previously reported (Yu and Yu, 2002).

In *C. patoca* sample, the gonad was weakly toxic only (6ug/g) while the intestine and the skin were more toxic. It was reported that there were succiform cells (also referred to unicellular glands or secretory cells) in the skin section of *C.patoca* containing TTX and proposed to be associated with TTX secretion for defense purpose (Mahmud Y. *et al*., 2003) . This may explain the relatively high toxicity found in the skin tissue of *C.patoca* in Hong Kong, that might accumulate in the skin for secretion when they are in danger. It was noteworthy that the muscle sample of *C.patoca* was the only muscle sample found to be toxic and it was reported that TTX was found to be distributed in muscle fiber in *C.patoca* while the function was still unknown (Mahmud Y. *et al*., 2003). Also, it was found that the intestine of the *C.patoca* sample was moderately toxic (28.5 ug/g) and the food residue collected from the same specimen was also toxic. It was suspected that the toxin in the intestine was from the food or even from the sediment in the sea bed since puffer fish would ingest the food (clam, shellfish, etc) together with the sediment. Clam, shellfish and the deep sea sediment were found to contain TTX and one of the hypotheses of the origin of TTX in puffer fish was acquired from food chain (Noguchi *et al.*, 2006).

In the *Torafugu* samples, the ovary and liver samples of the toxic *Torafugu* were found to be weakly toxic while the liver sample non-toxic *Torafugu* was also found to contain a tiny amount of toxin (0.5ug/g or 2.5MU/g), which was usually classified to be non-toxic to human.

5.1.4 Screening of TTX producing bacteria

Several internal organs of the puffer fish including the gut, liver and ovary were chosen for the screening of TTX producing bacteria. Many species of TTX producing bacteria were identified from different organs of the puffer fish (Yu *et al.,* 2004). In this study, around 20 isolated colonies were screened. However, half of them were not well cultured using shake flask fermentation. This was not unexpected as many marine bacteria were non-culturable by using the conventional fermentation system.

Four isolated colony species were found to be toxic. ORILiverB isolated from the liver of the puffer fish was found to be toxic. It was identified as *Bacillus-cereus-GC subgroup B (B.cereus)* by using MIDI identification system and *Bacillus* spp. was reported to be TTX producing by other researchers (Wu *et al.,* 2005).

LBgutB01 and LBLiver01 were isolated from the gut and the liver of the puffer fish respectively, which showed similar toxicity and were identified as the same species, *Raoultella-terrigena (R.terrigena)*. The toxicity of *R.terrigena* was very high compared to the bacteria species studied by our research group before, but whether it is TTX producing or not requires further confirmation. However, it was interesting that *R.terrigena* were found in both liver and gut of the puffer fish which were two separated organs. Though it is too early to make a conclusion that *R.terrigena* or other TTX producing bacteria would be distributed in different organs of the puffer fish and serve to produce TTX in a high amount that accumulated in the puffer fish, it is worthwhile to do further study as the TTX origin of puffer fish is still a mystery.

5.2 Detection of TTX

5.2.1 Tissue culture assay

The use of tissue culture assay for the detection of TTX has been developed in our laboratory. In this assay, veratrine and ouabain were used as sodium channel opener which resulted in an osmotic imbalance between the neuroblastoma cell and the surrounding medium. Water from the surrounding rushed into the cell which caused cell death while TTX as a sodium channel blocker could reverse the action. By measuring the amount of cell survival with constant amount of sodium channel opener drug added, the concentration of TTX could be determined.

Standard TTX was used to study the dose response effect of TTX to a fixed amount of sodium channel opener added to the neuroblastoma cells. The detection limit of tissue culture assay was found to be extremely low which was in nano molar range and a large amount of samples can be measured at the same time in the 96 wells plate. The detection was highly sensitive compared to other detection methods and it was based on the biological response of sodium channel to TTX which was a direct relationship to the toxicity of TTX. The confirmation of unknown compound as neurotoxin is usually done by both biological assay like mouse bioassay and, chemical assay like HPLC. The development of tissue culture assay provided an alternative to mouse bioassay for the confirmation of neurotoxin which has raised ethical problem concerning the use of mouse bioassay that has drawn much public attention in recent year.

It was found that the use of different concentration of ouabain (0.02mM and 0.05mM) showed different sensitivity in the detection of TTX. The use of 0.02mM ouabain provided a larger detection range and a lower detection limit from 1nM to 100uM while

the use of 0.05mM ouabain has a detection range of around 1uM to 100uM which has a higher R^2 value of 0.9934 compared to 0.8843.

However, the use of tissue culture assay in the detection of TTX in bacterial culture medium was problematic though it was reported that the detection of spiked-TTX in *E.coli* culture medium was feasible. There were several problems regarding to the preparation of samples for the tissue culture assay. First, the samples should be highly purified and it should be ensured that it was bacteria free to avoid the contamination to the neuroblastoma cell. Second, the pH of the samples should be adjusted to the pH of the tissue culture medium. Third, compounds including all the metabolites produced by the bacteria during fermentation might affect the result of the assay. Hence, a suitable blank control is necessary to obtain a more reliable result from the assay and the development of simple purification steps is required for the use of tissue culture assay in the detection of TTX in the bacterial culture medium.

5.2.2 HPLC-FLD

The use of HPLC with post column modification using a fluorescent detector was widely used and accepted in the detection of TTX in different research areas. The use of HPLC-FLD in the detection of TTX analogues was developed and the detection required known confirmed analogue standard which were not available in the market. However, the HPLC-fluorescent system is a destructive measurement through which TTX was converted to fluorescent C-9 compound and it could not be used as preparative HPLC for the purification of TTX. Also, the use of post column modification system involved the addition of sodium hydroxide to react with TTX to C-9 compound which diluted the

sample and lowered the detection limit. Hence, in order to minimize the dilution effect, higher concentration of sodium hydroxide as high as 4M with lower pumping rate from the derivatizer was desirable to enhance the detection sensitivity. However, the high concentration of sodium hydroxide at 100°C was extremely corrosive and would give rise to a maintenance problem of the equipment.

5.2.3 HPLC-UVD

The aim of the development of HPLC-UVD is to provide a relatively simple and more rapid method for the detection of TTX. Also, with the use of UV detection which is non-destructive measurement, provides possible alternatives to develop preparative HPLC for sample purification. The use of phosphoric acid as the mobile phase (mobile phase A) was previously reported by our group (Yan *et al*., 2005) and another research team (Cui *et al*., 2006). Nevertheless, the main limitation of this method was its poor detection limit, which were 1 µg/mL and 0.2 µg/mL respectively in the above two studies. With the modification of the mobile phase of the HPLC system by the addition of an organic modifier (10% MeOH), the addition of HSA salt and sodium dihydrogen phosphate which provided the buffering capacity, the HPLC-UV system offer (1) a lower detection limit, (2) reduced background noise on the baseline, (3) stabilized retention time of TTX and (4) reduced negative peaks in the system during analysis. Also, several wavelengths including 195, 197, 200 and 205nm were studied as the detection wavelength and 197nm gave the best detection in the HPLC-UVD system. Also, apart from the main TTX peak found in the HPLC chromatogram at RT = 6.1 minutes, other peaks which were

suspected to be TTX derivates were also found. However, further confirmation is needed to develop the HPLC-UVD system for the detection of TTX derivatives.

Furthermore, for the purification of TTX using SPE, and dilution of TTX standard, the use of the HPLC mobile phase as an eluant or diluents during sample preparation for HPLC analyses can significantly reduce the noise signal in the chromatograms and therefore enhance the detection limit to down to 10 ng/mL in the samples, which was comparable to HPLC-FLD (5 - 20 ng/mL; O'Leary *et al*., 2004) but less than LC-MS (15.6 nM or 0.32 ng/mL; Tsai *et al*., 2006).

However, different sample purification steps are required by different types of sample for the measurement of TTX. For measurement of TTX of fish tissue sample or urine sample, the rapid purification method by using C-18 cartridge followed with weak ion-exchange cartridge could provide a satisfactory result in the detection of TTX. But, for samples from the bacterial culture, more complex extraction and purification steps were required, including the use of hot acid extraction, activated carbon treatment and weak ion-exchange column purification.

5.3 TTX purification and detection in urine

5.3.1 Solid phase extraction (SPE) of TTX in urine

In the purification of TTX in the urine samples, C-18 SPE cartridge was used to remove hydrophobic substance and the pigment in the urine. The eluate was further purified in the Hitrap weak ion exchange column while the TTX was retained. After a series of washing with different organic solvents and water, TTX was entrapped in the column while most of the contaminant was removed. The elution of TTX was usually done by high ionic strength solvent or acidic solvent. 0.2M HCl and weak acetic acid were tried as eluent, the results were not satisfactory in HPLC with UV detection since high acid peak in the HPLC chromatogram was observed. However, by using HSA mobile phase used in HPLC system as elutant in SPE extraction, TTX could be eluted efficiently. At the same time, the undesirable noise signal and acid peak were removed with an improvement in the detection limit. .

As the maximum loading capacity of the used ion exchange cartridge for urine specimen was only about 500 ng of TTX respectively, diluting samples or using smaller injection volumes would allow for detecting larger amounts of TTX in poisoned patients' urine and plasma .

The use of prepacked SPE cartridges available commercially offered efficient extraction of TTX from patients' urine samples. Also, it had high recovery of TTX and reproducibility in the purification of TTX which is a rapid method suitable for clinical use. The use of HPLC-UVD system for the detection of TTX in the purified urine sample is also suitable for clinical application since HPLC-UVD is commonly found in ordinary laboratories which are an advantage over the use of HPLC-FLD and LC-MS.

Oda *et al.* (1989) firstly reported using GCMS in quantifying TTX in urine and serum. Afterwards, HPLC-FLD with post column modification became used LCMS has been used. And in recent years, to further enhance the detection limit, LCMS have been used for detection of TTX in urine and serum. However, LCMS involve the use of expensive equipment, its clinical application is thus limited.

5.3.2 Case study of TTX poisoning in Hong Kong

Puffer fish poisoning (PFP) in Hong Kong has been documented since 1994 (Sun *et al*., 1994; Lau *et al*., 1995) but the amount of TTX in patients' urine has never been analyzed. In this case study, urine samples were collected in the first 24 hours after entering Queen Mary Hospital and they were used for the detection of TTX with HPLC. From the HPLC result of the TTX concentration without UCC, it was found that it did not correlate well with the degree of TTX poisoning observed from clinical symptoms. It was observed that the highly poisoned patients required immediate assisted ventilation and ICU care in hospital did not give the highest concentration (Table 4.9), and this was probably affected by other factors such as the patients' urinary flow and volume, as well as their bodies' degree of hydration. It is worth noting that the patient with the highest urinary TTX concentration had repeated self-induced vomiting prior to hospital admission whilst the other two more severely poisoned patients had received intravenous fluid hydration upon arrival at the hospital. These findings prompted us to consider using creatinine-adjusted urinary TTX concentrations for diagnosis and assessment of TTX toxicity to eliminate the effect of dehydration and variation in urinary output as creatinine is excreted from the human body at a relatively stable rate which is only slightly influenced by physical

exercise and stress and is not influenced by diet, temperature or volume of liquid imbibed (Šperlingová *et al*., 2007). After UCC, the adjusted TTX concentrations in the patients' urine samples collected within 24 hours after ingestion of TTX were highly related to the degree of poisoning, whereas TTX urinary level alone without UCC could be misleading with regard to the severity of TTX poisoning. When the TTX level in urine was more than 20 ng/μmol creatinine (just as the two of the patients in this study), the patient would have profound and generalized muscle weakness and impending respiratory failure from paralysis of respiratory muscles. Death might ensue if mechanical ventilation and other respiratory supportive care were not administered promptly in hospital.

Table 4.10 shows a brief summary of TTX poisoning casess with a quantifying analysis of TTX presence in the urine or serum of victims in recent years. Most of the cases were reported in Japan and related to the consumption of puffer fish. Only the poisoning case reported in Taiwan involved the consumption of Gastropod (Hwang *et al,* 2005). Also, the amount of TTX detected in plasma/serum was very low and always less than that in urine, and it became undetectable usually after 24 hours of ingestion of TTX-containing food

For the patients resulted in death, the TTX concentration ranged from 103 to 650ng/ml in urine were reported. For patient with high TTX concentration like the case reported by Fukushima, 1991, the patient's TTX concentration in urine was 650 ng/ml and patient with such a severe poisoning might probably have died before arriving at hospital. TTX poisoning resulted in death was usually happens within the first 24 hours. However, Moriya et al. (1992) reported a strange poisoning case caused in 2 deaths after the consumption of puffer fish. TTX was not detected in the serum and urine of both victims

while 0.053ug/g TTX was detected in the stomach content (110g in total) of one victim who was dead 4 days after poisoning and the other victim was certified dead before admitted to hospital.

For the recovered patients, the serum TTX concentration collected in the first 24hours ranged from <1 to 63.9ng/ml. Oda *et al*.(1989) and Yamazaki *et al,* (1995) reported the serum TTX concentration dropped to 0 after 24 hours. It was suggested that the TTX in the blood stream was excreted and concentrated in urine within 12-24 hours. The urine TTX concentration collected within the first 24 hours ranged from 15-325ng/ml and TTX could be detected in urine up to 9 days. Continuous day measurements of TTX in the urine samples were reported and TTX could be detected in urine up to 4 days and there 281 µg of TTX was excreted in just the second day of PFP (Oda, *et al*., 1989).

It is estimated that the lethal dosage for an adult is an intake of TTX-containing food equivalent to 2000 µg of TTX (Kawabata, 1978) and a consumption of just a small amount of highly toxic puffer fish, especially the ovary, could reach this fatal level easily. As TTX is excreted from blood stream into urine in a short period of time, measurement of TTX in urine is an effective method to evaluate the severity of the patient being poisoned. At present, there is still no antidote for TTX-poisoning and treatment is only supportive, and rapid renal elimination of TTX from poisoned patients may be one of the alternative treatment methods.

5.4 Bacterial production of TTX

5.4.1 Optimization of fermentation condition for TTX producing bacteria using shake flask

5.4.1.1 Aerobic and anaerobic condition

For the study on the effect of aerobic and anaerobic condition to the growth of TTX producing bacteria, two species of bacteria including *S.marcescens* and *M.arabinogalactanolyticum* were studied. It was found that the growth of both species of bacteria were significantly better in aerobic condition (Figure 4.15). It was expected that the growth rate in aerobic condition was much faster than anaerobic condition since the continuous aeration and orbital shaking could enhance nutrient transfer and the metabolite rate in aerobic respiration was higher. However, the growth of *M.arabinogalactanolyticum* in anaerobic condition was extremely low or even zero growth which suggested that it was not suitable to grow in the absence of oxygen. For *S.marcescens*, it was reported that it is a facultatively anaerobic bacterium which can grow in both aerobic and anaerobic conditions and the result showed that it can grow slowly in anaerobic condition (Kreig, 1986). However, the toxicity of both species was beyond the detection limit of mouse bioassay and could not be detected.

The pH profile showed an upward trend for the bacteria to grow in aerobic condition and a downward trend in anaerobic condition. The increase in pH in aerobic condition is perhaps due to the production of ammonia during the growth of bacteria by the consumption of nitrogen source in the culture medium. The decrease in pH in anaerobic

condition may result from in the acidic metabolite produced during anaerobic respiration, like lactic acid.

5.4.1.2 Presence of salt in ORI medium

The three bacteria species, *S.marcescens*, *M.arabinogalactanolyticum* and *P.putida* were screened from puffer fish collected from marine. It was expected that the sea water and synthetic sea water would be suitable for the growth of these bacteria species. However, it was found that ORI medium with lower concentration of NaCl, compared to sea water (~35g/L NaCl), showed better growth in these bacteria species. Though the *S.marcescens* and *M.arabinogalactanolyticum* were screened from marine organism, they were not suspended in the open marine sea, but living in the host which the salt concentration was not as high as 35g/L (Figure 4.17 and 4.18). For *P.putida*, the presence or absence of salt did not show much interference to the growth and it suggested that the effect of salt on the bacteria was species specific (Figure 4.19).

5.4.1.3 Carbon source enrichment and modification in the culture medium

In the study of carbon source enrichment, the addition of a suitable carbon source was essential for the growth of bacteria. Since there was only 0.08g/L iron citrate as carbon source in ORI medium, glucose and sucrose were added. Also, the pH of shake flask fermentation could not be adjusted during fermentation and high pH was observed to be an inhibitory factor to the cell growth. The consumption of added carbon source was expected to produce acidic metabolite that can maintain a suitable pH and allowed higher cell growth. However, *S.marcescens* and M.*arabinogalactanolyticum* did not show positive function with the addition of sucrose and glucose (Figure 4.20 and 4.21). For

P.putida, the addition of sucrose could enhance the cell growth while glucose inhibition was also observed (Figure 4.22). Also, the addition of carbon source could lower the pH of the culture medium significantly. Nevertheless, too low pH might inhibit the cell growth as well.

5.4.1.4 Addition of arginine and puffer fish extract in the ORI medium

The addition of special chemical or extract into the culture medium was aimed at triggering the production of TTX. Since it was reported that arginine was proposed as the starting material of the hypothetical biosynthetic pathway of TTX and its derivatives, arginine was added in the culture medium as supplement for TTX production (Shimizu, 1993). Though the addition of arginine did not showed much influence to the growth of bacteria, the TTX production was inhibited which was not desirable. For the addition of puffer fish ovary extract in shake flask fermentation, the toxicity was not determined since the basal toxicity of the ovary extract was not measured. From the toxicity assay in fermenter trial with the addition of ovary extract, it was found that the addition of puffer fish extract could enhance the production of TTX in bacterial culture.

5.4.2 Fermentation of TTX producing bacteria using fermenter

With the use of computer controlled fermenter, static pH could be maintained which was one of the main limiting factors in shake flask fermentation. Also, it could maintain the pO2 of the fermenter higher than a certain limit by adjusting the airflow and stir speed which could ensure sufficient aeration for the growth of bacteria.

From the result of three batches of fermenter, it was observed that the 20g/L sucrose was consumed effectively during fermentation (Figure 4.29) and acidic metabolites were

produced which would result in the addition of base to maintain the pH of the fermenter as shown in (Appendix 19). The base was usually fed during the log phase of fermentation.

Three batches of fermenter with *P.putida* were done with different formulation of culture medium. It was found that the addition of puffer fish ovary extract could enhance the production of TTX. The net TTX production in fermenter with the addition of ovary extract was 70% higher compared to the fermenter in blank ORI medium. It might suggest that there was compound found in puffer fish ovary which was related to the production of TTX, and protein in the puffer fish ovary was suspected. Hence, total protein in the puffer fish was extracted and added as supplement in ORI medium used in fermenter fermentation. It was found that the addition of protein could enhance the production of TTX by 50% compared to blank ORI. This might suggest that the protein in the ovary extract contained important protein which might involve in the biosynthetic pathway of TTX. However, further experiment could be done to confirm the function of puffer fish ovary protein in the biosynthesis of TTX including the use of puffer fish ovary protein from different batches or species of puffer fish for fermenter fermentation study, the identification of protein related to TTX production using protein purification techniques.

From the TTX accumulation profile in the fermenter studies, the TTX production had occurred during the lag phase or the early log phase of fermentation and two possible mechanisms were proposed. First, this might be related to the metabolic pathway of the bacteria which would produce TTX during the period in accommodating the new environment and it might shift when the bacteria entering log phase that cease the

production of TTX. Second, this might be related to the limited amount of precursor or compound required for TTX production in the culture medium. The limited precursor might be consumed after a short period of time and the TTX production was ceased. This might be the reason for Batch 2 (ovary extract) and Batch 3 (total protein extract) fermenter to achieve the highest TTX concentration in a longer period of time for it was suspected that the extract provided supplementary compound required for the production of TTX.

Also, it was observed that TTX would degrade when the cell entered stationary phase or dead phase. It probably because the nutrient in the culture medium was near completely consumed when the cell entered stationary phase or dead phase, in which TTX in the culture medium would be degraded by the bacteria as nutrient for survival.

5.4.3 Extraction and purification of TTX from the bacterial culture

For the extraction of TTX from the bacterial culture, the procedures were simple with hot acetic acid extraction followed with centrifugation to remove cell debris. Afterward, volume reduction was done by rotary evaporation and the samples were ready for mouse bioassay.

For further purification, the TTX samples was subjected to ACT which the activated carbon would retain the TTX and the impurities were removed by repeated washing. After the ACT, all the pigments in the sample were removed and it was ready for SPE. However, it was found that the TTX recovery with ACT was rather low, with 40% only, which limited the accuracy of quantitative measurement of TTX by SPE followed with HPLC-UVD. Hence, the SPE extraction followed with HPLC-UVD was used as

qualitative measurement of TTX to further confirm the presence of TTX in the bacterial culture samples. In the HPLC chromatogram of bacterial culture sample after SPE shown in Figure 4.31, it was found that the combination of ACT and SPE could purify the sample from bacterial culture to the purity adequate for the measurement of TTX by HPLC-UVD. However, it was found that a number of impurities peak and negative peak were present, in which further purification was needed for pure TTX isolation.

6 Conclusion

The project can be divided into four parts: 1) the background study of puffer fish in Hong Kong; 2) TTX detection and purification method; 3)a case study of puffer fish poisoning in Hong Kong; 4) the screening and fermentation of TTX producing bacteria.

In the background study of puffer fish in Hong Kong, *Takifugu aloplumbeus* and *Takifugu niphobles* were the two most commonly found puffer fish in Hong Kong while *Chelonodon patoca* was found occasionally. In the bacterial distribution study, it was found that *E.coli* was not present. The total bacteria count in the gut samples showed large variation among different individual even they belonged to the same species. The bacteria count of other internal organs including the ovary, liver and testis were low, within 50CFU, suggested that the bacterial activity in these organs might be low.

In the second part, four methods of TTX detection including two biological assays and two chemical assays were developed. Tissue culture assay with the use of neuroblastoma cell line provided a very low detection limit of 1nM. However, the use was limited in the detection of bacterial culture sample since highly purified sample was required to provide reliable result. Mouse bioassay was found to be suitable used in the measurement of TTX in the bacterial cultured sample and puffer fish organs sample since simple purification steps were involved. However, the non-specificity and the ethical issues addressed by the use of mouse bioassay triggered the development of alternative methods for TTX detection. The use of HPLC with different detection strategies were studied in this project. The HPLC-FLD system was developed, but the destructive nature of the detection method limited the development of preparative HPLC for TTX and thus it led us for the development of HPLC-UVD. The development of novel HSA buffer system as

mobile phase enhanced the detection limit of HPLC-UV system to 10ng/ml, which was comparable to HPLC-FLD system. Also, the development of SPE system provided a rapid method for TTX purification from urine and bacterial culture sample. The SPE system with HPLC-UV detection provided a simple, efficient and reproducible method for TTX purification and detection for the measurement of TTX.

In the case study of puffer fish poisoning in Hong Kong, the measurement of TTX in the urine sample of the patients demonstrated the effectiveness of the SPE extraction followed with HPLC-UVD. Also, we have developed the use of UCC to the TTX concentration measured from the HPLC which showed better correlation to patient's manifestations and the severity of TTX poisoning.

In the screening and fermentation of TTX producing bacteria, two species of bacteria were found to be toxic and identified as *B.cereus* and *R.terrigena*. The toxicity of *R.terrigena* was high compared to the bacteria species studied by our research group. For the fermentation of TTX producing bacteria, it was found that TTX was accumulated in the lag phase or the early log phase and the high cell growth did not have a direct relationship to the production of TTX. Also, it was found that TTX production was up regulate with the addition of puffer fish ovary extract and the addition of total protein extracted from puffer fish ovary showed similar result. Further experiment has to be done to confirm the function of ovary extract or protein in the ovary extract in the relation to the production of TTX.

Reference

Akaki, K. and Hatano, K. (2006). Determination of tetrodotoxin in puffer-fish tissues, and in serum and urine of intoxicated humans by liquid chromatography with tandem mass spectrometry. *The Food Hygienics Society of Japan* 47 (2), 46 – 50.

Brian L. Cardall, Edmund D. Brodie Jr., Edmund D. Brodie III, Charles T. Hanifin (2004). Secretion and regeneration of tetrodotoxin in the rough-skin newt (Taricha granulosa). *Toxicon*. 44: 933-938.

Cui, J., Shen, X., Gong, Q. and Gu, O. (2006). Determination of Tetrodotoxin by HPLC with ultraviolet detection and fluorescence detection (in Chinese). *Chinese journal of Chromatography* 24 (3), 317.

Denac, H., Mevissen, M. and Scholtysik, G. (2000). Structure, function and pharmacology of voltage-gated sodium channels. *Naunyn-Schmiedeberg's Arch Pharmac,* 362, 453-479.

Do, H.K., Kogure, K., Imada, C., Noguchi, T., Ohwada, K. and Simidu, U. (1991). Tetrodotoxin Production of Actinomycetes Isolated from Marine Sediment. *Jappl*. *Environment*. *Microbiol* 56 (4), 1162-1163.

Do, H.K., Hamsaki, K., Ohwada, K., Simidu, U., Noguchi, T., Shida, Y. and Kogure, K. (1993). Presence of Tetrodotoxin and Tetrodotoxin-Producing Bacteria in Freshwater Sediment. A*ppl. Environment. Microbiol* 59 (11), 3934-3937.

Fuhrman, F.A. (1986). Tetrodotoxin, Tarichatoxin, and Chiriquitoxin: Historical Perspectives. Chapter 1 (pp 1-13) in Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel (Volume 479) (C.Y. Kao and S.R. Levinson editors). New York Academy of Sciences.

Fukushima, S. (1991). Examination of the poisoning level of tetrodotoxin in body fluids. *Japanese Journal of Forensic Toxicology* 9 (2): 126 – 127.

Giri, A.V., Anandkumar, N., Muthukumaran, G. and Pennthur, G. (2004). A novel media for the enhanced cell growth and production of prodigiosin from Serratia marcescens isolated from soil. *BMC Microbiology* 4 (11).

Goto, T., Kishi, Y., Takahashi. S., and Hirata, Y. (1965). Tetrodotoxin. *Tetrahedron* 21, 2509-2088.

Hamasaki, K., Kogure, K., and Ohwada, K. (1996). A biological method for the quantitative measurement of tetrodotoxin (TTX): Tissue culture bioassay in combination with a water-soluble tetrazolium salt. *Toxicon* 34, 490-495.

How, C.K., Chern, C.H., Huang, Y.C., Wang, L.M. and Lee, C.H. (2003). Tetrodotoxin poisoning. *American journal of emergency medicine* 21 (1), 51-54.

Hwang, P.A., Tsai Y.H., Lu, Y.H. and Hwang D.F. (2003). Paralytic toxins in three new gastropod (Olividae) species implicated in food poisoning in southern Taiwan. *Toxicon* 41, 529-533.

Hwang, P.A., Tsai, Y.H., Deng, J.F., Cheng, C.A., Ho, P.H. and Hwang, D.F. (2005). Identification of Tetrodotoxin in a marine gastropod (Nassarius glans) responsible for human morbidity and mortality in Taiwan. *Journal of Food Protection* 68 (8), 1696-1701.

Isbister, G.K., Kiernan, M.C. (2005). Neurotoxic marine poisoning. *Lancet Neurology* 4, 219-228.

Junge, W., Wilke B., Halabi A. and Klein G. (2004). Determination of reference intervals for serum creatinine, creatinine and creatinine clearance with an enzymatic and a modified Jaffé method. *Clinica Chimica Acta* 344, 137 – 148.

Kanchanapongkul, J., Krittayapoositpot, P. (1995). An epidemic of tetrodotoxin poisoning following ingestion of the horseshoe crab Carcinoscorpius Rotundicauda. *The Southeast Asian Journal of Tropical Medicine and Public Health* 26 (2), 364-367.

Kawabata, T. (1978). Tetrodotoxin, in: Food Hygiene Examination Manual, ed. Environment Health Breaue (Japan Food Hygien Association, Tokyo) p. 233

Kawatsu, K., Shibata, T. and Hamano, Y. (1999). Application of immunoaffinity chromatography for detection of tetrodotoxin from urine samples of poisoned patients. *Toxicon* 37, 325-333.

Kishi, Y., Aratani, M., Fukuyama, T., Nakatsudo, F., Goto, T., Inoue, S., Tanino, H., Sugiura, S., and Kakoi, H. (1972). Synthetic studies of on tetrodotoxin and related compounds. III. A stereospecific synthesis of an equivalent of acetylated tetrodamine. *Journal of American Chemcial Society* 94, 9217-19.

Kurono, S., Hattori, H., Suzuki, O., Yamada, T. and Seno, H. (2001). Sensitive analysis of tetrodotoxin in human plasma by solid-phase extractions and gas chromatography/mass spectrometry. *Analytical Letters* 34 (14), 2439 – 2446.

Lange W.R. (1990). Puffer fish poisoning. *American family physician* 42(4),1029-33.

Lau, F.L., Wong, C.K. and Yip, S.H. (1995). Puffer fish poisoning. *Journal of Accident and Emergency Medicine* 12, 214-215.

Lee M.J., Jeong D.Y., Kim W.S., Kim H.D., Kim C.H., Park W.W., Park Y.H., Kim K.S., Kim H.M. and Kim D.S. (2000). A Tetrodotoxin-producing Vibro Strain, LM-1, from the puffer fish Fugu vermicularis radiatus. *Applied and environmental microbiology* 66(4), 1698-1701.

Mahmud, Y., Okada, K., Takatani, T., Kawatsu, K., Hamano, Y., Arakawa, O. and Noguchi, T. (2003). Intra-tissue distribution of tetrodotoxin in two marine puffers Takifugu vermicularis and Chelonodon patoca. *Toxicon* 41,13-18.

Ministry of Health and Welfare. (1991). Japan Standard Methods of Analysis in Food Safety Regulation (Chemistry), Japan Food Hygienic Association, Tokyo. 296-300. (In Japanese).

Moriya, F., Miyaishi, S., Yamamoto, Y. and Ishizu, H. (1992). The use of mass fragmentography for the detection of Tetrodotoxin in human body fluid. *The Japanese journal of legal medicine* 46 (2) 117-120.

Nakamura M., Yasumoto T. (1984). Tetrodotoxin derivatives in puffer fish. *Toxicon*, 23(2), 271-276.

Narahashi, T. (2001). Pharmacology of tetrodotoxin. *Journal of Toxicology-Toxin Reviews* 20(1), 67-84.

Noguchi, T., Ebesu, J.S.M. (2001). Puffer fish poisoning: Epidemiology and treatment. *Journal of toxicology-toxin reviews* 20(1), 1-10.

Noguchi, T., Arakawa, O. and Takatani, T. (2006). TTX accumulation in pufferfish. *Comparative Biochemistry and Physiology*, Part D. 1, 145-152.

O'Leary, M.A., Schneider, J.J. and Isbister, G.K. (2004). Use of high performance liquid chromatography to measure tetrodotoxin in serum and urine of poisoned patients. *Toxicon* 44, 549-553.

Oda, K., Araki, K. Totoki, T. and Shibasaki, H. (1989). Nerve conduction study of human tetrodototoxication. *Neurology* 39(5), 743-745.

Poulsen T., Pedersen N., Pedersen M. and Poulsen H (2005). The snake venom toxin taipoxin confers specific toxicity to smallcell lung cancer cell lines. *Lung Cancer,* 49(2), 326-327.

Prasad, H.S.R., Qi, Z., Srinivasan, K.N., and Gopalakrishnakone, P. (2004). Potential effects of tetrodotoxin exposure to human glial cells postulated using microarray approach. *Toxicon* 44, 597-608.

Raghavan, P.U.M. and Vivekanandan, M. (1999). Bioremediation of oil-spilled sites through seeding of naturally adapted Pseudomonas putida. *International Biodeterioration & Biodegredation* 44(1), 29-32.

Schwartz, D.M., Fields, H.L., Duncan, K.G., Duncan, J.L. and Jones, M.R. (1998). Experimental Study of Tetrodotoxin, a Long-acting Topical Anesthetic. *Am K Ophthalmol* 125, 481-487.

Shimizu, Y. (1986). Chemistry and Biochemistry of Saxitoxin Analogues and Tetrodotoxin. Chapter 3 (pp 24-30) in Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel (Volume 479) (C.Y. Kao and S.R. Levinson editors). New York Academy of Sciences.

Shimizu, Y. and Kotaki Y. (1993). 1-Hydroxy-5,11-dideoxytetrodotoxin, the First N-Hydroxy and Ring-Deoxy Derivative of Tetrodotoxin Found in the Newt *Taricha granulose*. Journal of the American chemical society 115(3), 827-30.

Shui, L.M., Chen, K., Wang, J.Y., Mei, H.Z., Wang, A.Z., Lu, Y.H. and Hwang, D.F. (2002). Tetrodotoxin-associated snail poisoning in zhoushan: A 25-year retrospective analysis. *Journal of Food Protection* 66(1), 110-114.

Šperlingová, I., Dabrowská, L., Stránský, V., Kuèera, J., Tichý, M. (2007). Human urine certified reference material CZ 6010: creatinine and toluene metabolites (hippuric acid and o-cresol) and a benzene metabolite (phenol). *Analytical and Bioanalytical Chemistry* 387 (7), 2419 – 2424.

Sun, K., Wat, J. and So, P. (1994). Puffer fish poisoning. *Anaesthesia and intensive care* 22(3), 307-308.

Takata, K., Yamada, K., Ishikawa and Ogawa, H. (2001). Detection of tetrodotoxin from urine samples of puffer-fish poisoned patient. *Hiroshima Prefectural Kure Regional Community Health Center Research Report* 9, 27 – 30.

Tsai, Y.H., Hwan, D.F., Cheng, C.A., Hwang, C.C. and Deng, J.F. (2006). Determination of tetrodotoxin in human urine and blood using C18 cartridge column, ultrafiltration and LC-MS. *Journal of chromatography B* 832, 75-80.

Ushiyama, H., Kan, K., Shindo, T., Yasuda, K. and Nishijima, M. (2001). Interpretation of food poisoning by natural poisons and chemical substances (1980~1997). *Journal of the food hygienic society of Japan* 42(6), J324-330.

Wu, Z., Yang, Y., Xie, L., Xia, G., Hu, J., Wang, S. and Zhang, R. (2005). Toxicity and distribution of tetrodotoxin-producing bacteria in puffer fish Fugu rubripes collected from the Bohai Sea of China. *Toxicon* 46, 471-476.

Yamazaki, M. and Shibuya, N. (1995). Motor nerve conduction velocity is useful for patients with tetrodotoxin. *Anesthesia and Analgesia* 80 (4), 857.

Yan Q., P.H.F. Yu, and Li H.Z. (2005). Detection of tetrodotoxin and bacterial production by Serratia marcescens. *World J. of Microbiology and Biotechnology* 21(1), 1255-1258.

Yasumoto, T., Yotsu, M., Endo, M., Murata, M. and Naoki, H. (1989). Interspecies distribution and biogenetic origin of tetrodotoxin and its derivatives. *Pure Applied Chemistry* 61, 505-508.

Yasumoto T., Yamashita M.Y. (1996). Chemical and etiological studies on tetrodotoxin and its analogs. *J.Toxicol.-Toxin Reviews* 15(2), 81-90.

Yoshida, S. (1994). Tetrodotoxin-resistant sodium channels. *Cellular and Molecular Neurobiology* 14 (3), 227-244.

Yotsu, M., Endo, A., and Yasumoto, T. (1989). An improved tetrodotoxin analyzer. *Agric. Biol. Chem.* 53, 893-895.

Yotsu-Yamashita M., Sugimoto A. Takai A. and Yasumoto T., 1999. Effects of specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. *J. Pharmacol. Exp. Ther* 289, 1688-96.

Yu C.F., Yu, P.H.F. (2002). The annual toxicological profiles of two common pufferfish, Takifugu niphobles (Jordan and Snyder) and Takifugu alboplumbeus (Richardson), collected along Hong Kong coastal waters. *Toxicon* 40, 313-6.

Yu, C.F. (2003). A comprehensive study of the Hong Kong puffer fishes and their toxins. PhD Thesis, The Hong Kong Polytechnic University, Hong Kong.

Yu C.F., Yu, P.H.F., Chan P.L., Yan Q., Wong P.K. (2004). Two novel species of tetrodotoxin-producing bacteria isolated from toxic marine puffer fishes. *Toxicon* 44, 641-647.

Appendix 1 Conversion table from death time to mouse unit relations in mouse bioassay*

Death time	Mouse units	Death time	Mouse units	Death time	Mouse units
min : sec	MU	min : sec	MU	min : sec	MU
4:00	5.62	6:40	2.53	13:00	1.42
4:05	5.40	6:50	2.46	13:15	1.40
4:10	5.19	7:00	2.39	13:30	1.38
4:15	5.00	7:10	2.33	13:45	1.36
4:20	4.82	7:20	2.27	14:00	1.34
4:25	4.66	7:30	2.22	14:30	1.33
4:30	4.50	7:40	2.17	15:00	1.30
4:35	4.36	7:50	2.12	15:30	1.28
4:40	4.23	8:00	2.08	16:00	1.26
4:45	4.10	8:15	2.01	16:30	1.24
4:50	3.99	8:30	1.96	17:00	1.23
4:55	3.88	8:45	1.91	17:30	1.21
5:00	3.77	9:00	1.86	18:00	1.19
5:05	3.68	9:15	1.81	18:30	1.18
5:10	3.58	9:30	1.77	19:00	1.17
5:15	3.50	9:45	1.74	19:30	1.15
5:20	3.42	10:00	1.70	20:00	1.14
5:25	3.34	10:15	1.67	20:30	1.13
5:30	3.26	10:30	1.64	21:00	1.12
5:35	3.19	10:45	1.61	21:30	1.11
5:40	3.13	11:00	1.58	22:00	1.10
5:45	3.07	11:15	1.56	22:30	1.09
5:50	3.01	11:30	1.53	23:00	1.08
5:55	2.95	11:45	1.51	23:30	1.08
6:00	2.89	12:00	1.49	24;00	1.07
6:10	2.79	12:15	1.47	24;30	1.06
6:20	2.70	12:30	1.45	25;00	1.05
6:30	2.61	12:45	1.43		

*taken from the Japanese Standard Methods of Analysis in Food Safety Regulation (Kawabata, 1978; Ministry of Health and Welfare of Japan, 1991).

Appendix 2 Weight correction table of mice in mouse bioassay

Weight of mice, g	Mouse units, MU
15.0	0.75
15.5	0.78
16.0	0.80
16.5	0.83
17.0	0.85
17.5	0.88
18.0	0.90
18.5	0.93
19.0	0.95
19.5	0.98
20.0	1.00
20.5	1.03
21.0	1.05
21.5	1.08
22.0	1.10
22.5	1.13
23.0	1.15
23.5	1.18
24.0	1.20
24.5	1.23
25.0	1.25

*Taken from the Japanese Standard Methods of Analysis in Food Safety Regulation

Appendix 3 E.coli count and total bacterial count of puffer fish organs

Sample	Puffer fish organ	E.coli count	Total bacterial count		
		Dilution factor			
		10^0	10^0	10^{-1}	10^{-2}
B1	gut	0	5	2	0
B2	gut	0	TNTC	TNTC	294
B3	gut	0	17	4	0
B4	gut	0	TNTC	48	3
B5	gut	0	46	12	1
B6	gut	0	61	5	0
B5	Ovary	0	3	0	0
	Ovary	0	9	0	0
	Ovary	0	19	0	0
B2	Liver	0	38	0	0
B3	Liver	0	0	0	0
B6	Liver	0	7	0	0
B1	Testis	0	34	0	0

TNTC = Too numerous to count

Appendix 4 Data of TTX standard curve using 0.02mM and 0.05mM ouabain with log concentration of TTX from 0 nM to 5 nM

Log TTX conc. (nM)		5.000	4.699	4.000	3.699	3.000	2.699	2.000	1.699	1.000	0.000
TTX conc. (nM)		100	50	10	5	1	0.5	0.1	0.05	0.01	0.001
0.02nM ouabain	Absorbance at 490nm	0.884	0.656	0.551	0.557	0.36	0.588	0.386	0.352	0.389	0.125
		0.786	0.684	0.62	0.537	0.335	0.502	0.359	0.427	0.304	0.287
		0.731	0.664	0.640	0.510	0.383	0.464	0.388	0.389	0.330	0.279
		0.725	0.693	0.703	0.663	0.47	0.686	0.386	0.389	0.376	0.286
	Average Abs	0.782	0.674	0.629	0.567	0.387	0.560	0.380	0.389	0.350	0.244
0.05nM ouabain	Absorbance at 490nm	0.884	0.656	0.551	0.557	0.36	0.588	0.386	0.352	0.389	0.125
		0.786	0.684	0.62	0.537	0.335	0.502	0.359	0.427	0.304	0.287
		0.731	0.664	0.64	0.51	0.383	0.464	0.388	0.389	0.33	0.279
		0.725	0.693	0.703	0.663	0.47	0.686	0.386	0.389	0.376	0.286
	Average Abs	0.781	0.674	0.629	0.567	0.387	0.560	0.378	0.390	0.350	0.244

Appendix 5 Intra-day and Inter-day stability study of HPLC-UVD system

[TTX] (ug/ml)	Retention Time											
	Intra day						Inter day					
	Tri.1	Tri.2	Tri.3	Avg	SD	CV	Tri. 1	Tri. 2	Tri. 3	Avg	SD	CV
25	6.19	6.19	6.23	6.20	0.02	0.34	6.20	6.21	6.23	6.21	0.02	0.26
10	6.18	6.18	6.21	6.19	0.02	0.29	6.19	6.19	6.27	6.22	0.04	0.68
5	6.17	6.20	6.19	6.19	0.01	0.20	6.19	6.18	6.25	6.21	0.04	0.60
2.5	6.17	6.19	6.19	6.19	0.01	0.21	6.19	6.17	6.24	6.20	0.04	0.57
1	6.17	6.19	6.18	6.18	0.01	0.10	6.18	6.17	6.23	6.19	0.03	0.51
0.5	6.20	6.18	6.19	6.19	0.01	0.19	6.19	6.16	6.22	6.19	0.03	0.55
0.25	6.18	6.17	6.19	6.18	0.01	0.20	6.18	6.11	6.19	6.16	0.04	0.69
0.1	6.19	6.17	6.16	6.17	0.01	0.21	6.17	6.14	6.19	6.17	0.02	0.38
	Average of CV = 0.22						Average CV = 0.53					
	Signal Area											
	Intra day						Inter day					
25	5017	5143	5215	5125	100.22	1.96	5125	5426	5439	5330	177	3.33
10	2274	2301	2311	2295	19.14	0.83	2295	2334	2380	2336	42.4	1.81
5	1010	1034	1024	1022	12.06	1.18	1022	1039	1058	1039	17.6	1.70
2.5	520.0	519.0	517.0	518.6	1.53	0.29	518.6	521.5	544.9	528.3	14.4	2.73
1	234.0	235.0	231.0	233.3	2.08	0.89	233.3	244.1	246.3	241.2	6.96	2.88
0.5	94.40	97.20	98.50	96.70	2.10	2.17	96.70	98.90	101.8	99.13	2.56	2.58
0.25	51.85	51.75	51.04	51.55	0.44	0.86	54.50	53.20	56.60	54.77	1.72	3.13
0.1	25.09	25.77	23.30	24.72	1.28	5.16	24.70	24.30	25.30	24.77	0.50	2.03
	Average CV = 1.67						Average CV= 2.53					

[TTX] = Concentration of TTX; Avg = Average; SD = Standard deviation; CV = Coefficient of variation; Tri = Trial

Appendix 6 Standard curve of TTX spiked urine measured by HPLC-UVD

Spiked standard TTX in urine sample (ng/ml)	Signal Area		
	Trial 1	Trial 2	Trial 3
500	119.00	103.00	104.10
250	50.34	46.10	49.28
100	19.60	21.10	21.20
50	9.12	9.68	7.62
10	2.18	2.09	2.00

Appendix 7 Aerobic and anaerobic growth of M.arabinogalactanolyticum and S.marcescens measured in absorbance and pH

Absorbance at 600nm

Time (Hours)		0	12	24	48	72
M. arabinogalactanolyticum	Aerobic	0	1.185	1.844	1.792	1.86
	Anaerobic	0.016	0.026	0.029	0.028	0.033
S.marcescens	Aerobic	0	1.458	1.329	1.209	1.044
	Anaerobic	0.073	0.241	0.255	0.342	0.403

pH

Time (Hours)		0	12	24	48	72
M. arabinogalactanolyticum	Aerobic	7.92	8.06	8.13	8.98	9.02
	Anaerobic	7.95	7.92	7.7	7.77	6.98
S. marcescens	Aerobic	7.79	8.57	8.66	8.85	8.83
	Anaerobic	7.72	7.07	6.89	7.15	7.29

Appendix 8 Absorbance of *S.marcescens, M. arabinogalactanolyticum* and *P. pituda* with different source of salt and salt concentration

Time (Hours)		0	12	24	48	72
S. marcescens	sea water	0.186	1.43	1.39	1.385	1.36
	synthetic sea water	0.139	1.406	1.384	1.399	1.381
	NaCl 5g/L	0	1.692	1.284	1.812	1.365
	NaCl 10g/L	0	1.638	1.38	1.899	1.377
	ORI Control	0	1.458	1.329	1.209	1.044
M. arabinogalactanolyticum	sea water	0.187	1.645	1.659	1.643	1.624
	synthetic sea water	0.174	1.581	1.616	1.615	1.597
	NaCl 5g/L	0	1.206	1.944	2.248	2.176
	NaCl 10g/L	0	1.059	1.851	2.176	2.048
	ORI Control	0	1.185	1.844	1.792	1.86
P. putida	NaCl 5g/L	0	2	2.836	2.56	2.192
	NaCl 10g/L	0	1.791	2.724	2.508	2.244
	ORI Control	0	1.848	2.62	2.42	2.104

Appendix 9 Absorbance of *S.marcescens, M. arabinogalactanolyticum* and *P. pituda* with different carbon supplement

Time (Hours)		0	12	24	48	72
S. marcescen	Sucrose 5g/L	0	1.224	1.218	1.257	0.747
	Sucrose 10g/L	0	1.137	1.143	1.191	0.666
	Glucose 5g/L	0	1.005	0.981	0.996	0.882
	ORI control	0	1.503	1.458	1.329	1.209
M. arabinogalactanolyticum	Sucrose 5g/L	0	0.378	1.005	0.981	0.963
	Sucrose 10g/L	0	0.352	1.107	1.095	1.029
	Glucose 5g/L	0	0.203	0.564	0.843	1.041
	ORI control	0	0.166	1.185	1.844	1.792
P.putida	Sucrose 5g/L	0	1.872	2.868	2.98	3.395
	Sucrose 10g/L	0	1.8	2.868	2.928	3.33
	Glucose 5g/L	0	0.762	0.798	0.825	1.035
	ORI control	0	1.848	2.62	2.42	2.104

Appendix 10 pH of *M.arabinogalactanolyticum* and *S.marcescens* with carbon source supplement

S.marcescens						
	Time (hours)	Trail 1	Trail 2	Trail 3	Average	Std. dev.
ORI only	0	6.98	6.99	6.99	6.986667	0.005774
	24	8.56	8.45	8.83	8.613333	0.195533
	48	8.66	8.72	8.86	8.746667	0.102632
	72	8.70	8.48	8.75	8.643333	0.143643
Sucrose 10g/L	0	7.02	6.98	7.05	7.02	0.035119
	24	5.12	5.60	5.13	5.12	0.274287
	48	5.12	5.10	5.28	5.12	0.098658
	72	5.11	4.96	5.25	5.11	0.145029
M. arabinogalactanolyticum						
	Time (hours)	Trail 1	Trail 2	Trail 3	Average	Std. dev.
ORI only	0	6.99	6.97	7.07	7.01	0.052915
	24	8.43	8.44	8.55	8.47	0.066583
	48	8.85	8.80	8.81	8.82	0.026458
	72	8.47	8.58	8.78	8.61	0.157162
Sucrose 10g/L	0	7.01	7.00	/	7.01	0.007071
	24	5.84	5.8	/	5.82	0.028284
	48	7.89	7.28	/	7.58	0.431335
	72	8.22	7.58	/	7.90	0.452548
Glucose 5g/L	0	7.03	7.03	7.03	7.03	0
	24	5.45	5.35	5.40	5.40	0.05
	48	5.31	5.37	5.34	5.34	0.03

Appendix 11 Absorbance of *S.marcescens*, *M. arabinogalactanolyticum* and *P. pituda* with addition of arginine and puffer fish ovary extract as supplement

Time (Hours)		0	6	12	24	48
S. marcescen	ORI control	0	1.661	1.579	1.423	1.280
	Arginine 3g/L	0	1.360	1.719	1.624	1.497
	Puffer fish ovary extract	0	1.542	1.493	1.366	1.188
M. arabinogalactanolyticum	ORI control	0	0.403	1.666	1.185	1.792
	Arginine 3g/L	0	0.417	0.972	1.877	1.919
	Puffer fish ovary extract	0	0.334	0.603	1.909	1.658
P.putida	ORI control	0	1.585	2.515	2.325	2.07
	Arginine 3g/L	0	1.101	2.094	2.334	2.552
	Puffer fish ovary extract	0	1.200	1.515	1.437	1.768

Appendix 12 Growth curves of S.marcescen, M.arabinogalactanolyticum, P.putida, Raoultella-terrigena and Bacillus-cereus in shake flask fermentation

Time (Hours)	**0**	**6**	**12**	**24**	**48**	**72**	
S.marcescen	0	1.467	1.559	1.415	1.135	1.044	
M.arabinogalactanolyticum	0	0.258	1.044	2.017	1.871	1.860	
P.putida	0	1.848	2.620	2.420	2.104	2.004	
Time (Hours)	**0**	**6**	**24**	**48**	**54**		
Raoultella-terrigena	0	0.517	0.550	0.559	0.546		
Time (Hours)	**0**	**4**	**6**	**8**	**16**	**20**	**24**
Bacillus cereus	0	0.170	1.365	1.570	1.055	0.850	0.650

Appendix 13 Result of mouse bioassay for the toxicity of TTX producing bacteria in shake flask fermentation

Bacterial species; [Fermentation time]	**Weight of mouse**	**Time of Death**	**Calculation of toxicity in Mouse bioassay**	**Toxicity (MU/L)**	**Toxicity (ug/L)**
S.marcescen; [24hours]	23.5g	04:30	4.5 x 1 x 1.18 x 5.4	28.8	5.76
S.marcescen; [48 hours]	20g	>25 mins	Cannot be measureed	/	/
M.arabinogalactanolyticum; [24 hours]	25g	04:02	5.62 x 4 x 1.25 x 7.5	210.8	42.16
M.arabinogalactanolyticum; [48 hours]	25g	04:00	5.62 x 2 x 1.25 x 10.5	147.5	29.5
P.putida; [24 hours]	23g	12:48	1.43 x 2 x 1.15 x 6	21.4	4.28
P.putida; [48 hours]	21g	04:00	5.62 x 3 x 1.05 x 6.8	120.3	24.06

Calculation of Toxicity = (MU from time of death in mouse correction table*) x (Dilution factor) x (Weight correction factor**) x (Volume of samples after volume reduction by rotary evaporator)

* (Refers to Appendix 1)

** (Refers to Appendix 2)

Appendix 14 Summary of the results from the fermentation of TTX producing bacteria using fermenter

Batch 1 ORI blank control

Sample collection time (Hours	Cell dry weight (g/L)	Absorbance at 600nm	Toxicity from mouse bioassay (MU/L)	Toxicity from mouse bioassay (ug/L)
0	0.05	0	0	0
4	0.2	0.006	0	0
7.5	0.27	0.182	0	0
12	1.79	2.427	88.98	15.1
16	2.28	2.715	89.75	17.2
20	2.49	2.76	82	18.6
24	2.31	3.23	73.6	14.7

Batch 2 ORI with Ovary extract as supplement

Sample collection time (Hours)	Cell dry weight (g/L)	Carbohydrate concentration (g/L)	Toxicity (ug/L)
0	0.18	20	34.4
3	0.33	20	48.07
6	1.18	13.829	56.6
24	4.1	0.5154	64.13
30	4.19	0.1516	52.4
60	3.96	0.123	36.9

Batch 3 ORI with Total protein extract as supplement

Sample collection time (Hours)	Absorbance at 600nm	Toxicity (ug/L)
0	0	0
4	0.432	7.68
8	2.157	12.76
12	2.724	13.7
16	2.748	20.5
20	3.234	22.28
24	2.99	27.27
29	3.012	28.67
53	3	20.46

Appendix 15 Result of the TTX concentration in Batch 1 fermenter with ORI medium only as blank control measured by Mouse bioassay

Sample collection time (hours)	Weight of mouse	Time of Death	Calculation of toxicity in Mouse bioassay	Toxicity (MU/L)	Toxicity (ug/L)	Average Toxicity (ug/L)
0	25.0	>25:00	/	CND	CND	CND
0	25.0	>25:00	/	CND	CND	
0	23.0	>25:00	/	CND	CND	
4	25.0	>25:00	/	CND	CND	CND
4	22.5	>25:00	/	CND	CND	
12	22.5	10:46	1.61x1x1.13x46	83.7	16.7	15.1
12	24.0	10:15	1.2x1x1.2x46	66.2	13.2	
12	21.0	10:48	1.61x1x1.05x46	77.8	15.6	
16	22.0	17:45	1.1x2x1.05x37	85.5	17.1	17.2
16	22.5	25:00:00	1.13x2x1.05x37	87.8	17.6	
16	20.5	7:25	2.22x1x1.03x37	84.6	16.9	
20	19.0	9:35	1.7x1x1.05x47	85.9	16.8	18.6
20	21.0	9:50	1.81x1x1.2x47	102.1	20.4	
24	19.5	12:00	1.49x1x0.98x51	74.5	14.9	14.7
24	23.5	16:25	1.18x1x1.24x51	74.6	14.9	
24	21.0	14:05	1.05x1x1.34x51	71.8	14.4	
31	23.5	>25:00	/	CND	CND	CND
48	25.0	>25:00	/	CND	CND	CND
48	25.0	>25:00	/	CND	CND	

CND = Cannot be determined using mouse bioassay or < 1MU

Calculation of Toxicity = (MU from time of death in mouse correction table*) x (Dilution factor) x (Weight correction factor**) x (Volume of samples after volume reduction by rotary evaporator)

* (Refers to Appendix 1)

** (Refers to Appendix 2)

Appendix 16 Result of the TTX concentration in Batch 2 fermenter with the addition of ovary extract measured by Mouse bioassay

Sample collection time (hours)	Weight of mouse	Time of Death	Calculation of toxicity in Mouse bioassay	Toxicity (MU/L)	Toxicity (ug/L)	Average Toxicity (ug/L)
0	18	05:00	3.77x10x0.9x9.1	308.7	61.74	34.5
0	18.5	16:00	1.26x10x0.93x9.1	106.6	21.32	
0	19	18:00	1.19x10x0.95x9.1	102.9	20.58	
3	18	06:50	2.46x10x0.9x9.5	210.3	42.06	48.7
3	17.5	06:50	2.46x10x0.88x9.5	205.7	41.14	
3	17.5	05:00	3.77x10x0.88x9.5	315.2	63.04	
6	17.5	05:30	3.26x10x0.88x11.7	335.6	67.12	56.6
6	19	07:32	2.22x10x0.95x11.7	246.8	49.36	
6	18.5	06:45	2.46x10x0.93x11.7	267.7	53.54	
24	25	09:00	1.86x10x1.25x13.3	309.2	61.84	64.13
24	23.5	07:33	2.22x10x1.18x13.3	348.4	69.68	
24	24.5	09:02	1.86x10x1.23x13.3	304.3	60.86	
60	23.5	07:55	2.08x10x1.18x5.4	132.5	26.5	36.9
60	20.5	06:38	2.53x10x1.03x5.4	140.7	28.14	
60	20	04:08	5.19x10x1x5.4	280.3	56.06	

CND = Cannot be determined using mouse bioassay or < 1MU

Calculation of Toxicity = (MU from time of death in mouse correction table*) x (Dilution factor) x (Weight correction factor**) x (Volume of samples after volume reduction by rotary evaporator)

* (Refers to Appendix 1)

** (Refers to Appendix 2)

Appendix 17 Result of the TTX concentration in Batch 3 fermenter with the addition of total protein extract measured by Mouse bioassay

Sample collection time (hours)	Weight of mouse	Time of Death	Calculation of toxicity in Mouse bioassay	Toxicity (MU/L)	Toxicity (ug/L)	Average Toxicity (ug/L)
0	17	>25mins	/	CND	CND	CND
0	18.5	>25mins	/	CND	CND	CND
0	19	>25mins	/	CND	CND	CND
4	17.5	18:15	1.18x1x0.88x37	32.56	6.51	7.68
4	18	18:30	1.18x1x0.9x37	39.29	7.86	
4	20	18:40	1.17x1x1x37	43.29	8.66	
8	18.5	9:20	1.77x1x.093x40	65.84	13.17	12.76
8	20.5	13:45	1.36x1x1.03x40	56.03	11.21	
8	22	11:00	1.58x1x1.1x40	69.52	13.90	
12	18	7:40	2.17x1x.0.9x32.5	63.45	12.69	13.7
12	20.5	7:00	2.39x1x1.03x32.5	80	16.00	
12	19	8:20	2.01x1x0.95x32.5	62.06	12.41	
16	20	8:10	2.08x2x1.03x26.5	113.55	22.71	20.5
16	18	8:00	2.08x2x0.9x26.5	99.21	19.84	
16	17.5	11:00	1.58x2x0.88x26.5	73.69	14.74	
16	21	7:30	2.22x2x1.05x26.5	123.54	24.71	
20	20.5	8:40	1.91x2x1.03x31	121.97	24.39	22.28
20	21	10:20	1.67x2x1.05x31	108.71	21.74	
20	19.5	10:00	1.7x2x0.98x31	103.3	20.66	
20	16	4:30	4.5x1x0.8x31	111.6	22.32	
24	20	11:15	1.56x2x1x39.5	123.24	24.65	27.27
24	20	4:30	4.5x1x1x39.5	177.75	35.55	
24	18.5	12:20	1.47x2x0.93x39.5	108	21.60	
29	16.5	5:05	3.68x2x0.83x34.5	210.75	42.15	28.67
29	18.5	9:10	1.81x2x0.93x34.5	116.15	23.23	
29	18.5	8:25	1.96x2x0.93x34.5	125.77	25.15	
29	20.5	10:10	1.7x2x1.03x34.5	120.81	24.16	
53	19.5	8:30	1.96x1x0.98x62.5	133.39	26.68	20.46
53	17.5	12:00	1.49x1x0.88x62.5	78.04	15.61	
53	21	15:00	1.3x1x1.05x62.5	94.79	18.96	

CND = Cannot be determined using mouse bioassay or < 1MU

Calculation of Toxicity = (MU from time of death in mouse correction table*) x (Dilution factor) x (Weight correction factor**) x (Volume of samples after volume reduction by rotary evaporator)

* (Refers to Appendix 1)

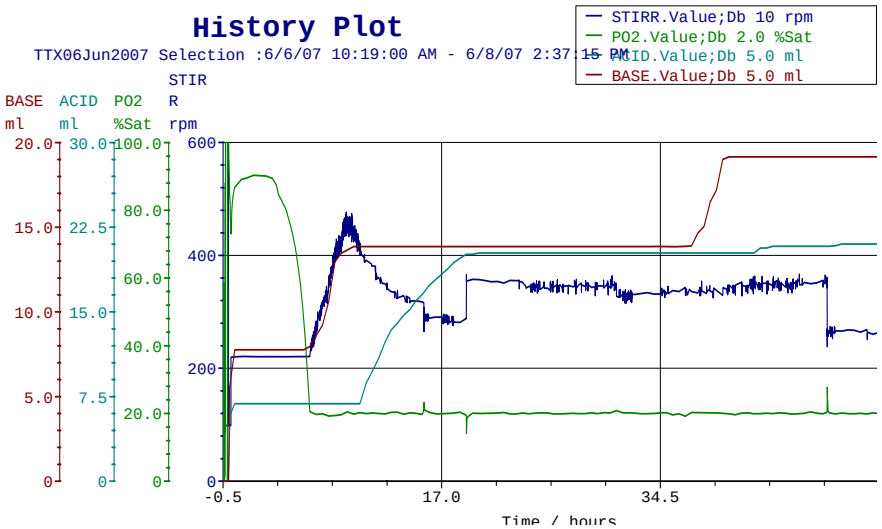
** (Refers to Appendix 2)

Appendix 18 Summary of the TTX accumulation profile of *P.putida* in different culture medium using fermenter

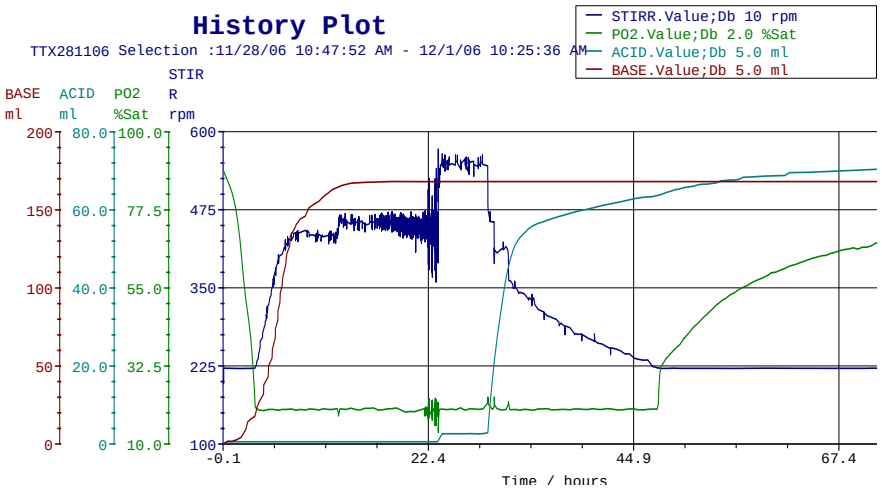
Batch 1 (ORI blank control)			Batch 2 (Puffer fish ovary extract)				Batch 3 (Total Protein extract)		
Time (Hours)	Abs at 600nm	Toxicity (ug/L)	Time (Hours)	Abs at 600nm	Toxicity (ug/L)	Net toxicity	Time (Hours)	Abs at 600nm	Toxicity (ug/L)
0	0	0	0	0	34.4	0	0	0	0
4	0.01	0	3	0.03	48.1	13.7	4	0.43	7.68
7.5	0.18	0	6	0.64	56.6	22.2	8	2.16	12.76
12	2.43	15.1	12	1.88	/	29.7	12	2.72	13.70
16	2.72	17.2	19	1.96	/	18	16	2.75	20.50
20	2.76	18.6	24	2.63	64.1	2.5	20	3.23	22.28
24	3.23	14.7	30	4.09	52.4		24	2.99	27.27
31	3.29	0	60	3.05	36.9		29	3.01	28.67
48	3.41	0					53	3.00	20.46

Appendix 19 History Plot of fermenter studies

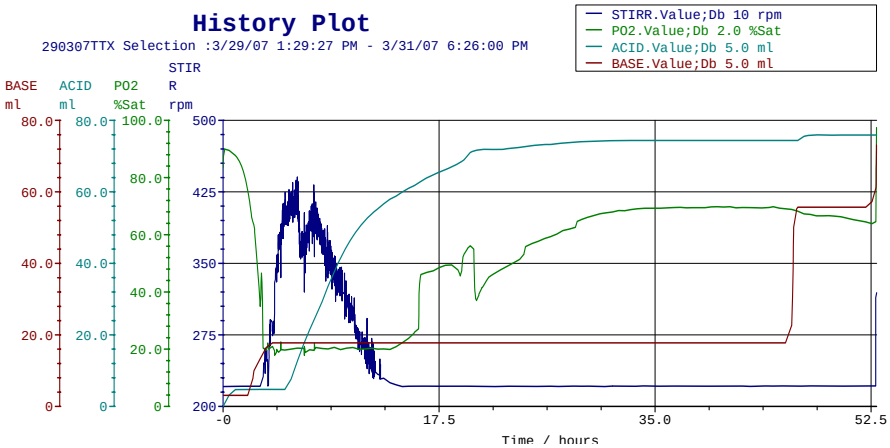
Batch 1 (ORI blank control)



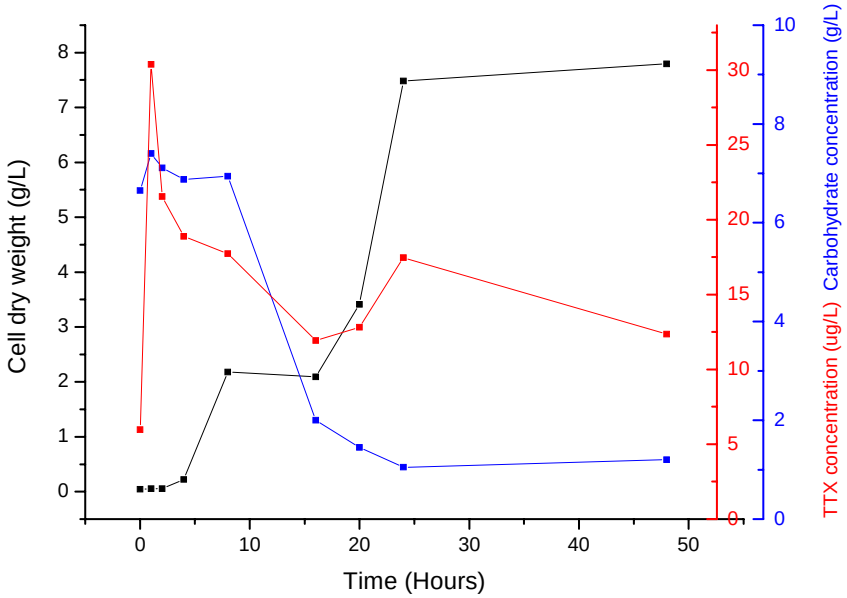
Batch 2 (Puffer fish ovary extract)



Batch 3: (Total protein extract)



Appendix 20 The growth profile and TTX accumulation of *S.marcescens* in ORI medium with ovary extract using fermenter



Appendix 21 The growth profile and TTX accumulation of *M.arabinogalac-tanolyticum* in ORI medium with ovary extract using fermenter