RGC Ref. No.:
UGC/FDS25/M03/15
(please insert ref. above)

RESEARCH GRANTS COUNCIL
COMPETITIVE RESEARCH FUNDING SCHEMES FOR THE LOCAL SELF-FINANCING DEGREE SECTOR

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report
(for completed projects only)

Submission Deadlines:
1. *Auditor's report with unspent balance, if any: within **six** months of the approved project completion date.*
2. *Completion report: within **12** months of the approved project completion date.*

Part A: The Project and Investigator(s)

1. Project Title

Fermentative Production of Tetrodotoxin (TTX) from Marine Microorganisms for Pharmaceutical and Medical Applications

2. Investigator(s) And Academic Department(s) / Unit(s) Involved

Research Team	Name / Post	Unit / Department / Institution
Principal Investigator	Chua Hong/Dean	REO/Technological and Higher Education Institute of Hong Kong
Co-Investigator(s)	Peter Hoi Fu Yu/Professor	
Others	Yu Chun Fai/Assistant Professor	Environmental Science/United International College

3. Project Duration

	Original	Revised	Date of RGC / Institution Approval *(must be quoted)*
Project Start Date	1 January 2016	N/A	N/A
Project Completion Date	31 December 2017	30 Jun 2018	30 October 2017
Duration *(in month)*	24	30	30 October 2017
Deadline for Submission of Completion Report	31 December 2018	30 Jun 2019	30 October 2017

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. To develop a more standard and reliable process for the extraction and purification of TTX.
2. To optimize TTX production to about 1 mg/L by combining the innovative fermentation method and the developed extraction and purification method.
3. To elucidate the mechanisms of TTX biosynthetic pathways, which may provide a better understanding in the formulation and planning the optimal culture media and conditions.
4. To develop and prepare an Eliza kit for the detection of tetrodotoxin.

5.2 Revised objectives

Date of approval from the RGC: No revision

Reasons for the change:

1.

2.

3. ....

5.3 Realisation of the objectives
(Maximum 1 page; please state how and to what extent the project objectives have been achieved; give reasons for under-achievements and outline attempts to overcome problems, if any)

Objective 1. To develop a more standard and reliable process for the extraction and purification of TTX.
Conducted a more comprehensive update literature search on tetrodotoxin, production by different types of living organisms, pharmaceutical and medical application and usage, bacteria production of TTX, and development of antibodies against TTX.

Conducted preliminary fermentation of TTX-producing bacteria with shake flasks

Novel species of non-sporing, non-acid-fast and chemoorganotrophic organisms capable of generating tetrodotoxin (TTX, a powerful non-protein neurotoxin) were isolated and purified from the poisonous marine puffer fish gathered in Hong Kong waters.

This objective is 90% achieved.

Objective 2. To optimize TTX production to about 1 mg/L by combining the innovative fermentation method and the developed extraction and purification method.

Microbacterium arabinogalactanolyticum, *Serratia marcescens* and *Vibrio alginolyticus* are commonly dispersed in soil, wastewater or marine environments, respectively. Each bacterial isolate (500 ml broth medium grown in darkness at 25°C for 10 days without aeration) are found to generate a toxicity of 75.4 to 108.3 mouse units (MU) after extraction and purification.

This project is 80% achieved.

Objective 3. To elucidate the mechanisms of TTX biosynthetic pathways, which may provide a better understanding in the formulation and planning the optimal culture media and conditions.

Established collaboration research with Dr. J. Y. Wu and Dr Chen Sheng of the Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University (since they do have better equipped fermenters.
Worked with the RA- Crystal to get fish samples from Zuhai; discussed project w Co-I Dr Yu C F in UIC.
Conducted some fermentation with Co-Investigator Dr. Yu Chun Fai at his laboratory inUIC in Zhuhai.

This objective is 80% achieved.

Objective 4. To develop and prepare an Eliza kit for the detection of tetrodotoxin.
Visited the School of Food Science of the South China Agriculture University to start a collaboration project on the production of ELIZA kit for TTX

visited Prof. Lei of South China Agriculture U in Guangzhou on TTX antibody- Elisa Rapid test kit
We have previously corresponded (2016) with, and visited the antibody researchers (Prof. Hongtao and Prof. Zhong Qinping) of the College of Food Science, South China Agriculture University) who previously had collaborated with us on the production of TTX-antibody in my previous RGC grant (at HKPU) in 2016, and they are enthusiastic and supportive in our proposed collaboration in the production of ELIZA kit for TTX

This objective is 80% achieved.

5.4 Summary of objectives addressed to date

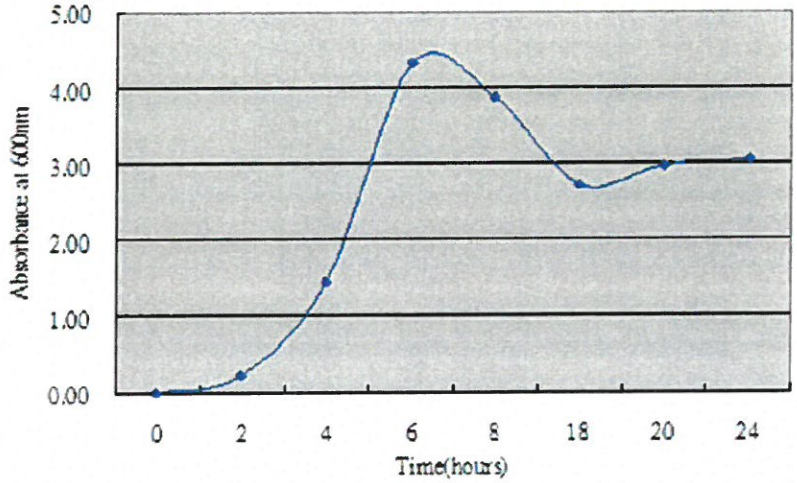
Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. To develop a more standard and reliable process for the extraction and purification of TTX.	√	90
2. To optimize TTX production to about 1 mg/L by combining the innovative fermentation method and the developed extraction and purification method.	√	90
3. To elucidate the mechanisms of TTX biosynthetic pathways, which may provide a better understanding in the formulation and planning the optimal culture media and conditions.	√	80
4. To develop and prepare an Eliza kit for the detection of tetrodotoxin.	√	80

6. Research Outcome

6.1 Major findings and research outcome
(Maximum 1 page; please make reference to Part C where necessary)

In the TTX detection part of this project, liver, intestine, muscle and skin are separated from the puffer fish. The appearance, weight, length, body characteristics, number of rays in dorsal fin, pectoral fin and anal fin of the puffer fish specimen are recorded. Next, the puffer fish specimen is dissected and body parts that are used for obtaining extracts are separated. Photos are taken after dissection. For the liver, intestines, skin and muscles, their weights are recorded and cut into small pieces. After that, the body tissues are put into different bottles and labeled separately. 0.5% acetic acid is added to the body tissues, with a ratio of 2mL/g. The body tissues with acetic acid are then boiled at 100°C for 15 minutes. Finally, filtration is carried out to obtain extracts without puffer fish tissues.

The Vibrio spp. was cultivated in LB medium. The medium contained the vital materials for bacterial growth, including carbon source, amino acids, phosphate, organic acids, vitamins, nitrogen source and inorganic sulfur, which was generally used for growing marine bacteria We are conducting fermentation in 500 and 1000 mL Erlenmeyer flasks. By changing the conditions of the fermentation: different nutrient sources, including carbohydrate content and nitrogen concentration in the culture medium, by variation of the incubation temperature, swirling speed, and various types of additives (including homogenized puffer fish organs) in the culture medium, in order to observe the best growth conditions for the growth of TTX-producing bacteria. The pH value and optical density of the cultured medium were chosen as the parameters for investigating the growth of the bacteria. After the shake flasks cultivation, the optimal bacterial growth conditions were determined. A 15L fermenter was used and totally 8L of LB medium was used with 1%(80ml) seed inoculation. The fermentation process was kept at 30°C, and pH was maintained at 7.3 under the partial pressure of oxygen (PO2) at around 20%.



The Optical density of *Vibrio spp* in LB medium

TTX could then be purified from the bacterial medium by centrifugation at 8000 rpm for 30 min, and purified by running gel filtration and ion-exchange chromatography (Bio-Gel P2 Gel and Bio-Rex 70 (Bio-RadLaboratories)).

The TTX concentration of 4 fractions after Bio-Rex ion exchange chromatography

	Height	Area	Concentration (μ g/ml)
REX-1 RT5.7	4.47	16.86	4.60
REX-1 RT5.9	8.56	30.01	8.18
REX-2	2.92	10.81	2.95
REX-3	9.38	33.85	9.23

Mouse bioassay
Sample (Healthy white mice, 15-25g in both sexes, ICRstrain) extract is injected into mouse to find out the poisoning contents.

	Dead/Total	Dead time
TTX 5MU/ml (1μg/ml)	**0/5**	-
TTX 10MU/ml (2μg/ml)	**0/5**	-
STD TTX 5MU/ml (1μg/ml)	**2/2**	**3min 50Sec(5.62MUá)**
		3min 56Sec(5.62MUá)

※ **STD TTX: TTX of tocris,Inc.**

※ **Death time 4min=5.62MU**

6.2 Potential for further development of the research and the proposed course of action *(Maximum half a page)*

To study the application of TTX in curing various cancer pain
To investigate the accumulation by organ cell to increase the production yield.
To investigate the recovery of TTX vis bacteria culture
To study the medicine to rescue the TTX toxicity

7. Layman's Summary

(Describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Tetrodotoxin (TTX) is a deadly non-protein neurotoxin and a well-known sodium channel blocker, but it is also a potent analgesic and an effective non-opioid (non-addictive) local anesthetic with a quick onset time. TTX is now being explored as a novel pharmaceutical drug. Clinical trials of using TTX as a pain killer for cancer patients and a withdrawal formulation for heroin addicts are undergoing. Published reports show that TTX can suppress prostate cancer cells from metastasizing. Thus, the development of TTX as a novel marine bioactive drug has promising application potential and imminent significance. To date, numerous experiment evidence points out that TTX is synthesized by the puffer fish-associated microbes before accumulating in the fish. However, the in vitro production of TTX by the isolated TTX producing bacteria has been limited to only nanogram level. Hence, TTX production is likely relying on a mixed microbial community, in which some strains might be non-cultivable by traditional methods. The study investigated production of TTX by the consortium of the intestinal microbiota of a toxic puffer fish species, *Takifugu ocellatus* in a fermentation approach. TTX was isolated and purified from the fermentation broth by resin chromatography, which was detected by mouse bioassay and LC-MS/MS.

Part C: Research Output

8. Peer-Reviewed Journal Publication(s) Arising Directly From This Research Project
(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(denote the corresponding author with an asterisk*)*	Title and Journal / Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
Year of Publication	Year of Acceptance *(For paper accepted but not yet published)*	Under Review	Under Preparation *(optional)*						
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

9. Recognized International Conference(s) In Which Paper(s) Related To This Research Project Was / Were Delivered
(Please attach a copy of each conference abstract)

Month / Year / Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
May 2017	Isolation and purification of Tetrodotoxin from a marine bacteria *Takifugu ocellatus*	3rd Fisheries and Aquaculture Conference	2017	Yes	Yes	Yes

10. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning
(Please elaborate)

_____

_____

11. Student(s) Trained

(Please attach a copy of the title page of the thesis)

Name	Degree Registered for	Date of Registration	Date of Thesis Submission / Graduation
FOO Chun Hei	BSc (Hons)	1 Sep 2014	1 May 2018
TAM Wing Shan	BSc (Hons)	1 Sep 2014	1 May 2018
TANG Yuet Ching	BSc (Hons)	1 Sep 2014	1 May 2018

12. Other Impact

(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, teaching enhancement, etc.)

Prof. Amandio Vieira, Simon Fraser University, Canada: research collaboration
Prof. Yan Qun, Dept. ofFood Science, Jiangnan University, China: research collaboration

13. Public Access Of Completion Report

(Please specify the information, if any, that cannot be provided for public access and give the reasons.)

Information that Cannot Be Provided for Public Access	Reasons
NIL	

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FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report - Attachment
(for completed projects only)

RGC Ref. No.:	UGC/FDS25/M03/15
Principal Investigator:	Prof. CHUA Hong
Project Title:	Fermentative Production of Tetrodotoxin (TTX) from Marine Microorganisms for Pharmaceutical and Medical Applications

Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (Please specify)
No. of outputs arising directly from this research project [or conference]	N/A	1	N/A	N/A	N/A