การพัฒนาการผลิตไวโอลาซีนจาก *Pseudoalteromonas luteoviolacea* และโพรดิจิโอซินจาก *Pseudoalteromonas rubra* โดยใช้เรซินดูดซับ

นางสาววาสนา มู่สา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

PRODUCTION DEVELOPMENT OF VIOLACEIN FROM *Pseudoalteromonas luteoviolacea* AND PRODIGIOSIN FROM *Pseudoalteromonas rubra* USING ADSORBENT RESINS

Miss Vassana Musa

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2014

Thesis Title	PRODUCTION DEVELOPMENT OF VIOLACEIN FROM *Pseudoalteromonas luteoviolacea* AND PRODIGIOSIN FROM *Pseudoalteromonas rubra* USING ADSORBENT RESINS
By	Miss Vassana Musa
Field of Study	Biotechnology
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วาสนา มู่สา : การพัฒนาการผลิตไวโอลาซีนจาก *Pseudoalteromonas luteoviolacea* และโพรดิจิโอซินจาก *Pseudoalteromonas rubra* โดยใช้เรซินดูดซับ (PRODUCTION DEVELOPMENT OF VIOLACEIN FROM *Pseudoalteromonas luteoviolacea* AND PRODIGIOSIN FROM *Pseudoalteromonas rubra* USING ADSORBENT RESINS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ปรีชา ภูวไพรศิริศาล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ชุติวรรณ เดชสกุลวัฒนา, หน้า.

ไวโอลาซีนเป็นสารรงควัตถุที่มีสีม่วง ผลิตโดยแบคทีเรียหลากหลายสายพันธุ์ มีฤทธิ์ทางชีวภาพหลากหลาย ในปัจจุบันมีความสำคัญในทางด้านเภสัชกรรมและผลิตภัณฑ์เครื่องสำอาง ส่วนโพรดิจิโอซีนเป็นสารสีแดงมีโครงสร้างหลักเป็น pyrrole ต่อกัน 3 วง ผลิตโดยแบคทีเรียหลายสายพันธุ์ โดยเฉพาะจีนัส Seratia โพรดิจิโอซีนมีฤทธิ์ทางชีวภาพในวงกว้าง แต่พบว่ากระบวนการผลิตสารสีทั้งสองตัวเกิดปัญหาคือผลผลิตที่ได้มีปริมาณต่ำ และการแยกผลิตภัณฑ์จากกระบวนการหมักและการทำให้บริสุทธิ์ก็มีปัญหาเช่นกัน ในงานวิจัยครั้งนี้เป็นการใช้เรซินดูดซับสำหรับการเก็บเกี่ยวและทำให้บริสุทธิ์ของสารไวโอลาซีนและโพรดิจิโอซีน ซึ่งได้จากการหมักของเชื้อ *Pseudoalteromonas luteoviolacea* และ *Pseudoalteromonas rubra* โดยใช้เรซินดูดซับ 5 ชนิด ได้แก่ HP20, XAD4, XAD7, XAD16 และ XAD1180 สำหรับช่วยเพิ่มประสิทธิภาพการผลิตสารทั้งสองตัว จากการศึกษาพบว่าการใช้เรซินดูดซับชนิด XAD1180 แบบโปรยหว่านช่วยเพิ่มผลผลิตไวโอลาซีนได้สูงสุด เมื่อเติมในปริมาณ 2% w/v หลังจากเพาะเลี้ยงไป 16 ชั่วโมง โดยเพิ่มผลผลิตได้ 2 เท่า และให้ผลผลิตได้สูงถึง 3.3 $g.L^{-1}$ เชื้อ *P. luteoviolacea* เจริญได้ดีในช่วงพีเอช 7.6-8.6 อุณหภูมิ 4-30 องศาเซลเซียส รวมทั้งเรซินดูดซับชนิด XAD1180 สามารถนำกลับมาใช้ซ้ำได้ถึง 3 ครั้ง

ในส่วนของการใช้เรซินดูดซับเพิ่มเพิ่มผลผลิตของโพรดิจิโอซีน พบว่าการใช้เรซินดูดซับชนิด HP20 เมื่อเติมในปริมาณ 1% w/v หลังจากเพาะเลี้ยงไป 16 ชั่วโมง โดยเพิ่มผลผลิตเพิ่มขึ้น 1.6 เท่า และให้ผลผลิตได้สูงถึง 8.5 $g.L^{-1}$ รวมทั้งเรซินดูดซับชนิด HP20 สามารถนำกลับมาใช้ซ้ำได้ถึง 4 ครั้ง โดยเชื้อ *P. rubra* เจริญได้ดีในช่วงพีเอช 6.0-7.6 อุณหภูมิ 28-30 องศาเซลเซียส

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5273851323 : MAJOR BIOTECHNOLOGY
KEYWORDS: VIOLACEIN / PRODIGIOSIN / ADSORBENT RESIN / PSEUDOALTEROMONAS SP. / VASSANA MUSA

VASSANA MUSA: PRODUCTION DEVELOPMENT OF VIOLACEIN FROM *Pseudoalteromonas luteoviolacea* AND PRODIGIOSIN FROM *Pseudoalteromonas rubra* USING ADSORBENT RESINS. ADVISOR: ASSOC. PROF.PREECHA PHUWAPRAISIRISAN, Ph.D., CO-ADVISOR: CHUTIWAN DECHSAKULWATANA, Ph.D., pp.

Violacein is a bacterial violet pigment produced by several species of bacteria that currently important for pharmaceutical and cosmatics applications. The other pigment, prodigiosin is red pigment alkaloid comprising conjugated three pyrrole residues. It is produced by several strains of bacteria, particularly genus Serratia. Prodigiosin and other related alkaloids exhibit a broad range of biological activities. The problem of fermentation process for violacein and prodigiosin production in large scale were low productivity and the use of large amount of organic solvent for extraction and isolation process. This research, to use adsorbent resin for the recovery and purification of violacein and prodigiosin from fermentation broth of *Pseudoalteromonas luteoviolacea* and *Pseudoalteromonas rubra*, respectively. Of five different adsorbent resins, namely HP20, XAD4, XAD7, XAD16 and XAD1180 were evaluated for efficiency to enhance violacein and prodigiosin production. Dispersion of XAD1180 enhanced the highest yield, particularly when 2% $w.v^{-1}$ XAD1180 was added to medium after 16 h of cultivation. This method could produce 2-fold increment in violacein production when compared with conventional fermentation and maximum yield of violacein was up to 3.3 $g.L^{-1}$. And It was found that *P. rubra* grew well at pH and temperature ranges of 7.6-8.6 and 4-30 °C, respectively. In addition, the regenerated XAD 1180 could be reused additional three times without significant drop in violacein yield.

Furthermore research for Production development of prodigiosin using adsorbent resins, we have developed a practical and easy approach that could be applied to improve prodigiosin production. We demonstrated that addition of 1 %w/v of Diaion HP20 could increase total prodigiosin up to 1.6 folds, compared to control. Prodigiosin was highest (8.58 g/L) produced in the culture broth containing 1 %w/v of Diaion HP20. However, the increased amount of resin did not significantly alter production yield but gradual declines in total pigment were observed. And It was found that *P. rubra* grew well at pH and temperature ranges of 6.0-7.6 and 28-30 °C, respectively. HP20 used in the experiment are expected to be regenerated and retain its adsorption capacity. HP20 was also reused up to four times without a significant drop in the product yield.

Field of Study: Biotechnology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This research was supported by grant from THE 90th ANNIVERSARY OF CHULALONGKORN UNIVERSITY FUND (Ratchadaphiseksomphot Endowment fund) and partial financially supported by the Research Grant and also bacterial samples of Burapha University through National Research Council of Thailand. I would like to express my sincere gratitude to my advisor Associate Professor Preecha Puwapraisirisan for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study. Besides my advisor, I would like to thank of my co-advisor Dr. Chutiwan Dechsakulwatana, Associate Professor Dr. Vuthichai parasuk, Assistant Professor Dr. Onruthai Pinyakong, Associate Professor Chanpen Chanchao and Assistant Professor Dr. Wimolpun Rungprom for their encouragement, insightful comments, and hard questions.

I thank my fellow labmates in Natural Products Research Unit (NPRU) Department of Chemistry, Faculty of Science, Chulalongkorn University for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun.

In addition, I would like to thank to Miss Supattra Chinakool and, who is Ph.D student at Program of Biotecnology, Faculty of Science, Chulalongkorn University for her helpful and suggestions.

Last but not the least, I would like to thank my family: my parents, for giving birth to me at the first place and supporting me spiritually throughout my life.

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CHAPTER I

INTRODUCTION

In recent year, marine organisms produce many of the pharmaceutically active natural compounds (drug). Marine sponges are sessile invertibrates that have developed effective strategies to protect themselves against viruses, bacteria and eukaryotic predators [1]. One of these defense mechanisms is production of secondary metabolites.

Marine invertebrates are a diverse group having habitats in all ocean ecosystems, ranging from intertidal zone to the deep sea environments. Animals belong to marine invertebrates are composed of different taxonomic groups, which can be classified into several major phyla, namely, Porifera (sponges), cnidarians (corals, sea anemones, hydrozoans, jellyfish), annelida (Polychaetes, marine worms), bryozoa (moss animals or sea mats), mollusca (oysters, abalones, clams, mussels, squids, cuttlefish, octopuses), arthropoda (horseshoe, lobsters, crabs, shrimps, prawns, crayfish), and echinodermata (sea stars, sea cucumbers, sea urchin). To date majority of marine natural products have been identified from marine invertebrates of which sponges predominate. Sponges are well known as abundant source bioactive compound that could be used to treat many human diseases [2]. Spongers from phylum Porifera are the most primitive, evolutionarily ancient metazoan

animals. A serious obstacle to the ultimate development of most marine natural products that are currently undergoing clinical trials or are in preclinical evaluation is the problem of supply. The concentrations of many highly active compounds in marine invertebrates are often minute, accounting for less than a millionth of the wet weight. It is clear that large amounts of biomass of these invertebrates can never be harvested from nature without risking extinction of the respective species. Sponge's harbour a rich diversity of microorganisms in their tissues and in some case constitute up to 40% of the biomass, e.g. the Mediterranean sponge Aplysina aerophoba. Numerous natural products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that microorganisms are the true source of these metabolites or are intricately involved in their biosynthesis (table 1.1). Thus an alternative strategy targeting the microorganisms associated with sponges for the screening of bioactive natural products may prove to be an effective approach to circumvent the associated difficulties of dealing with the organism itself [3].

In Thailand, the research of Chutiwan Dejsakulwattana and co-worker from Burapa University in year 1999 reported the isolation of 468 strains of marine sponge associated bacteria collected from Gulf of Thailand. They discovered one interesting strain, the important bioactive compound that violacein and prodigiosin that produce by bacteria genus of Pseudoalteromonas.

Table 1.1 Secondary metabolite of marine sponges associated microorganism [4]

Group of organisms	Compound class	Bioactivity
Fungi	polyketide, cyclohexane, terpenes, amino acid derivatives	cytotoxic, antioxidant, antimicrobial, kinase inhibitors
Bacteria	proteins, peptides, propionic acid, indole, PAFA	antimicrobial
Cyanobacteria	ether, chlorinated metabolites, pigments	
Actinobacteria	amino acid, polyketides, glycosides, methyl esters	Anti-inflammation, antimicrobial, antioxidant, hypochloesterolemic
Actinomycetes	carotenoids	antioxidant
Yeast	indole derivatives	Redical scavengers

Violacein (Figure1.1) is a purple pigment from various bacteria [5] that exhibits multiple biological activities [6], which is produced by marine bacteria isolated from sponge.

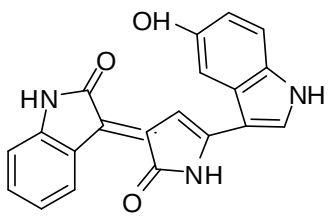


Figure 1.1 Chemical structure of violacein

Violacein appeared as an important pharmaceutical for many infectious diseases such as leishmaniasis, trypanosomiasis, and malaria, besides its major potential as an anticancer agent. Violacein has been shown potential economical importance for industrial such as use for applications in cosmetics and textiles, as well as in the agro industry [7]. There are many reported about optimization method for increasing violacein production (Table 1.2).

Table 1.2 Violacein production from various microorganisms [7]

Strain	Comments	Yields
Psychotropic bacterium RT102	*Closed to J. lividum*	20°C, 30 h, pH 6.0, 3.5 $g.L^{-1}$
Janthinobacterium lividum strain DSM1522		25°C, 0.016 $g.L^{-1}$
J. lividum S9601		No data were found
Marine sediment bacterium *Pseudoalteromonas* sp.		25°C, 96 h, 0.052 $g.L^{-1}$
Psychotropic bacterium, XT1	*Closed to J. lividum*	15°C; pH 8.0, 0.8 $g.L^{-1}$
Chromobacterium violaceum	Formerly *Bacillus violaceum*	27°C, 144 h, pH 6.2, 0.025$g.L^{-1}$
Alteromonas luteoviolacea	Marine bacteria	22°C, 72 h, 0.002$g.L^{-1}$
C. violaceum B78	From Amazon River, Manaus, Brazil	28°C, 24 h, pH 7.2 Liquid fermentation, 3.4 $g.L^{-1}$
C. violaceum ATCC 553		30°C, pH 7.2, 0.002 $g.L^{-1}$
C. violaceum CCT 3496		30°C,36 h, pH 7.2, 0.43 $g.L^{-1}$
Pseudoalteromoonas luteoviolacea	From sponge *A. carvernosa*	20°C,240 h, 0.013 $g.L^{-1}$
Duganella sp. B2	From China	25°C,40 h, pH 8.4, 1.62 $g.L^{-1}$
C. violaceum CCT 3496		30°C, 24 h, Semisolid fermentation, 2.0 $g.L^{-1}$
C. violaceum B78	From Amazon River, Manaus, Brazil	32°C, 36 h, pH 7.8 Semisolid fermentation, 2.6 $g.L^{-1}$

Prodigiosin (Figure1.2) is a red pigment alkaloid comprising conjugated three pyrrole residues. It is produced by several strains of bacteria, particularly genus *Serratia*. Prodigiosin and other related alkaloids exhibit a broad range of biological activities such as inducing apoptosis in many different human cancer cell lines with little effect on nonmalignant cells, inhibiting a wide variety of bacteria and fungi as well as cation binding ability [8].

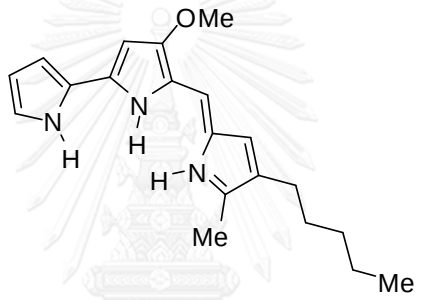


Figure 1.2 Structures of prodigiosin

Violacein and prodigiosin were produced in the stationary phase of fermentation; however the productivity typically drops due to toxicity of violacein and prodigiosin towards producer cell. This problem would be circumvented by adding adsorbent resin to trap violacein and prodigiosin from broth, thus extending production period. The successes of this approach have been exemplified by improved productivity of red pigment excreted from *Serratia* sp. on adding HP20 resin [9].

Adsorbent resins have frequently been used to recover pigments, organic acid, peptides, proteins, nucleic acid and other products from plant extracts to removal of phenolic and nitrophenol compound from wastewater.They are usually made of styrene-divinylbenzene (SDVB) or acrylic polymers, which are durable, easy to regenerate and have a high adsorption capacity. Furthermore, the resins are rather inexpensive and their application for food contact has been approved by the Food and Drug Administration. The use of adsorbent resin in violacein and prodigiosin production not only enhanced product yield but also facilitated isolation of violacein from aqueous media. Our approach also serves as an environmentally friendly method for cultivation of bacteria producing bioactive metabolite. Therefore, the aims of this research are as follow:

The aims of this research

1. To determine the appropriate type of resin adsorption capacity to absorb. And the ability to release the violacein and prodigiosin pigment which produced by marine bacteria *Pseudoalteromonas luteoviolacea* and *Pseudoalteromonas rubra*, respectively.

2. To study the effectiveness of the recovery pigments using absorbent resin compared with using classical and tedious liquid-liquid extraction.

3. To study for development efficiency fermention system of violacein and prodigiosin production by using absorbent resin and to apply these in industrial scale in the future.

CHAPTER II

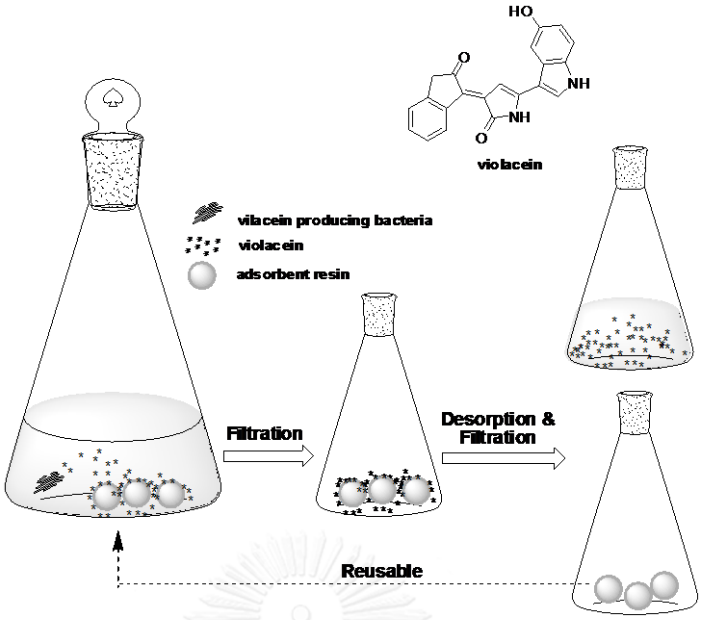
Production development of violacein from *Pseudoalteromonas luteoviolacea* using adsorbent resins

2.1 Introduction

Violacein is a violet pigment produced by various strain of Gram-negative bacteria such as *Chromobacterium violaceum* [7, 10], *Janthinobacterium lividum* [11] and *Pseudoalteromonas* sp [12]. It comprises indole moieties that are biosynthesized from coupling reaction of two tryptophans [13]. Violacein possesses broad spectrum biological activities such as antibacterial, antitrypanocidal, antifungal, antitumoral, antiparasitary, antiviral, antiprotozoal, antioxidant, antiulcerogenic, Immunomodulatory and antinociceptive [7]. To date, violacein has potential applications, especially in the fields of medicine, cosmetics, and food [7]. Therefore, bulk supply of violacein to precede such applications is inevitably required. There have been several investigations [5, 14, 15] aiming at optimizing cultureconditions such as temperature, agitation and culture media to suitable for target bacteria. However, product yield has not been significantly improved due in part to bacterial susceptibility toward violacein. Of violacein-producing strains, the genus *Chromobacterium* is likely to generate the pigment in relatively high yield, approximately 2.0-3.4 g/L [16-18]. However, large scale production of violacein by

culturing *Chromobacterium* raises safety concern since it has been suspected as pathogenic bacteria causing skin lesions, sepsis, and liver abscesses that may be fatal [19].

To address aforementioned problems, we have an idea to introduce the approach that can be applied to all strain of violacein-producing bacteria, without complicated optimization. Adding adsorbent resin into culture media, in order to remove violacein released into the broth, is a potential method of choice. Once cell mortality is reduced, the log phase of bacteria is extended and results in improved quantity violacein generated. Moreover, violacein adsorbed on the resin can be easily extracted by washing with methanol or ethanol. This methodology not only enhances violacein yield per batch but also facilitates isolation of violacein from aqueous media without using classical and tedious liquid-liquid extraction. In the present study, we applied this method (Scheme 1) to promote violacein production of marine sponge-associated bacterium *P. luteoviolacea* which isolated and identified by Institute of Marine Science, Burapa University and Optimized conditions for bacterial growth in the presence of adsorbent resins were elaborated.



Scheme 2.1 Schematic representation of how adsorbent resins promote violacein production and facilitates isolation and purification.

2.2 Experimental

2.2.1 Microorganism and culture medium

Pseudoalteromonas luteoviolacea was isolated from marine sponge which collected off Chonburi province, northern Gulf of Thailand, in March 2010 by Institute of Marine Science, Burapa University. Bacterial authentication was made, at Thailand Institute of Scientific and Technological Research, by analysis of 16S rDNA of amino acid sequence [20], and stored at 4°C until further use.

P. luteoviolacea was maintained on an agar slant of modified Zobell agar (MZA) containing peptone (1 g) yeast extract(1 g), phytone (0.5 g), sodium thiosulphate (0.2 g), sodium sulfite (0.05 g), Fe-citrate (1 ml) and agar (15 g) in sea

water (900 ml) and distilled water (100 ml). The initial pH of the medium was adjusted to 7.6 before sterilization.

2.2.2 Growth curve and violacein production

Fermentations were carried out in 250 ml Erlenmeyer flasks, each containing 50 ml of the culture medium and operating at room temperature (29±1^{O}C), for 48 h. and the pH was not controlled. The culture broth was sample at the initial time and every 12 h. The 0.1 mL of culture broth was diluted in sterile sea water before spreading a culture broth on MZA surface. The other portion of culture broth was diluted (1 mL), and violacein concentration were measured by using UV-VIS spectrophotometer (Hewlett Packard G1103A, Germany) at 570 nm.

The growth curve and violacein production were obtained by plotting the logarithm data colony forming unit per milliliter (CFU.mL^{-1}) and violacein production (mg.mL^{-1}) versus the time of culture broth.

2.2.3 Adsorbent resins

Resins XAD4, XAD7, XAD16, XAD1180 and HP20 (Amberlite Sigma-Aldrich Corp. USA) having different characteristics as adsorbent were used in this study (Table 2.1).

Prior to use, these resins were soaked in distilled water and methanol for 24 h then drying in hot air at 65°C after the resins were sterilized by autoclaving (Hirayama manufacturing Corp. Japan HVE-50) at 121°C for 15min.

Table 2.1 Typical physical properties of adsorbent resins

Resin type	Functional group	Pore radian (A^{o})	Surface area ($m^2.g^{-1}$)	Porosity ($mL.g^{-1}$)	Particle size (mm)	Density ($g.mL^{-1}$)
HP 20	SDVB	260	500	1.30	0.25-0.85	1.01
XAD 4	SDVB	50	725	0.98	0.24-0.84	1.08
XAD 7	Acrylic	90	450	1.14	0.24-0.84	1.24
XAD 16	SDVB	100	600	1.82	0.24-0.84	1.08
XAD 1180	SDVB	300	800	1.68	0.24-0.84	1.04

2.2.4 Analytical procedures

1) Analyses and measurement

In this study, the actual concentration of violacein was calculated from the standard curve obtained using purified violacein which to measure the absorbance of violacein at various concentration by UV spectrometer at 570 nm.

2) Evaluation of adsorbent resin efficiency

The efficiency of adsorbent resins were evaluated from 3 parameter including adsorbtioncapacity (Q_a):

$$Q_a = \frac{V_0(C_0 - C_e)}{W} \quad (1)$$

Where V_0 is the total volume (ml), C_0 is initial concentration of violacein ($mg.ml^{-1}$), C_e is final concentration of the violacein ($mg_{violacein}.ml^{-1}$) and W is the weight of adsorbent resin (g).

Desorption capacity (Q_d):

$$Q_d = \frac{V_d C_d}{W} \quad (2)$$

Where V_d is the total volume of solution after elution with methanol (ml) and C_d is concentration of the violacein ($mg_{violacein}.ml^{-1}$).

Desorption ratio:

$$\text{desorption ratio} = \frac{V_d C_d}{V_0(C_0 - C_e)} \quad (3)$$

2.2.5 Fermentation with adsorbent resins

All of fermentations were studied in Erlenmeyer flasks with various type of adsorbent resins (XAD4, XAD7, XAD16,XAD1180 and HP20). They were carried out in a 2L Erlenmeyer flask with 1L working volumn and fermentation at 29 ± 1^{0}C for 48 h with an inoculum size of 10% (v/v) on an orbital shaker at 100 rpm. After 48 h, adsorbent resins were dispersed and fermentation was carried out until 72 h. Adsorbent resins were separated using plankton net (100 μm), and the obtained aliquot was centrifuged (Hettich EBA-20, Germany) at 8500 rpm for 15 min and filtered by filter paper (45 μm), yielding bacterial cell and medium. After that, cell was added the solution of methanol : dichloromethane (2:1) to break cell with ultrasonic (Bandelin UW2200, United instrument Co., Ltd.). Then cell residue was removed by centrifugation at 6000 rpm, 10 min. and the solution from the cell was evaporated to obtain crude extract. While, The medium was separated and extracted with ethyl acetate with ratio 1:5 between ethyl acetate and medium in separation funnel. The ethyl acetate layer was contained violacein which remove ethyl acetate by evaporation in reduced pressure. Finally, the resin was desorbed by using methanol, then evaporated to obtain crude extract. After that, Crude extract from adsorbent resin, cell and medium were measured by using UV-VIS spectrophotometer at 570 nm. The production of violacein was calculated from the standard curve (appendix) obtained using purified violacein.

2.2.6 Violacein production on addition of adsorbent resins at various times

The effect of various time to produce of violacein by all culturing flasks was added 2% w/v of adsorbent resins at 0, 8, 16, 24 and 36 h after inoculation. Each flask cultures were then growth for 48 h at 29±1 °C with shaking (150 rpm). After 48 h the violacein was measured as previously described.

2.2.7 Effect of the amount of adsorbent resin on violacein production

The bacterial culture was grown in MZB under the same conditions in experiments 2.2.6. The Adsorbent resin was added at the concentration of 0, 2, 4, 8, 10, 15 and 20% w/v. After 48 h the violacein was measured as previously described.

2.2.8 Effect of temperature and initial pH of culture medium on violacein production

The cultures with above optimized conditions in three different temperatures (4, room temperature (29±1) and 37 °C) and pHs (6.0, 7.6 and 8.6). Then the violacein was measured as previously described.

2.2.9 Molecular identification

To confirm the marine bacteria species, the selected *Pseudoalteromonas* sp. RAD 6-3DP and *Pseudoalteromonas* sp. CC 10-5R which isolated and identified by Chutiwan Dechsakulwatana, Institute of Marine Science, Burapa Univesity. DNA was by using DNA extraction kit (Qiagen, Germany) and 16S

rDNA was amplified by using a pair of primer, BF1 (5′-GAGTTTGATCATGGCTCAG-3′) and BR1 (5′-AAGTCGTAACAAGGTAACCG-3′). PCR conditions were set as follows: 5 min initial denaturation at 94°C; followed by 30 cycles at 94°C for 1 min, at 58 °C for 1 min, at 72 °C for 2 min and a final extension step at 72 °C for 5 min [5]. The amplication of 16S rDNA was confirmed by running the product in 1% agar rose gel electrophoresis in 1 x TAE buffer. The PCR product of 16S rDNA of the isolated was sequenced and further comparison was made with previously available sequence in National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) [21].

2.3 The Result and Discussion

2.3.1 The profile of violacein production from *P. luteoviolacea*

Prior to introducing adsorbent resin into culture media of violacein producing bacterium *P. luteoviolacea*, times courses for growth of the target bacteria and violacein production was investigated. In Figure 2.1, bacterial growth (solid line) reached a stationary phase by 24 h and gradually declined after 48 h, at which maximum viable cell count was determined. Violacein (dash line) was first detectable, using UV-Vis spectrometer at 570 nm, after 6-7 h of inoculation, while elevated increase in the pigment was observed after 12 h. Noteworthy, violacein concentration remained unchanged after accessing its maxima in stationary phase (24 h), suggesting that cell growth was directly inhibited by high concentration of

violacein. The observed result is common among antibiotic and toxic producing microorganisms.

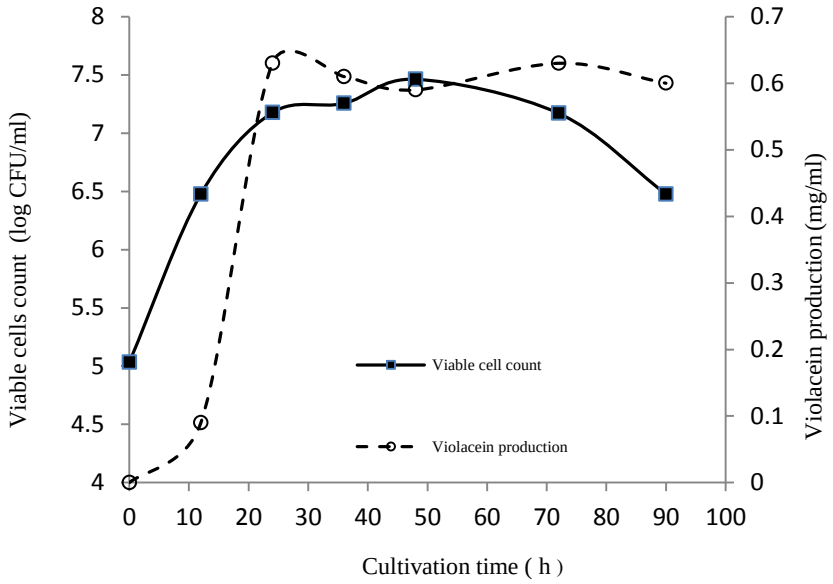


Figure 2.1 Time courses for growth of *P. luteoviolacea* and the production of violacein

To address this problem and promote violacein production, extension of bacterial log phase is essentially required. In principle, if a large excess of violacein is depleted from culture media, the bacteria can longer tolerate and continuous producing new violacein. Adding adsorbent resin into the media is a potential method of choice accounting for the above require, in addition to its advantage such as environmentally friendly, reusability and no toxicity toward bacterial cells. Five adsorbent resins (XAD4, XAD7, XAd16, XAD1180 and HP20), typically used in

laboratory for removal of organic compounds from aqueous media were examined for adsorptive property toward violacein.

All of them, except for XAD7, are polystyrene divinylbenzene (SDVB) based resins varying in surface and particle properties [22-24]. Three parameters, namely adsorption capacity (Q_a), desorption capacity (Q_d) and desorption ratio, were evaluated and the result are shown in Table 2.2 All adsorbent resins showed comparable capacities both in terms of adsorption (3.74-5.14 mg/g) and desorption (0.29-0.67 mg/g). However, significantly high desorption ratio observed in XAD7 (13.04) and XAD1180 (16.84) suggested their potential for applying in culture media. In the present study, due to its highest desorption ratio, XAD1180 was selected for further investigation in hope that it would provide high percent recovery of violacein after resin washing.

Table 2.2 Adsorption capacity, desorption ability and desorption ratio of violacein on various resins

Adsorbent resin	Adsorption capacity (mg/g_{resin})	Desorption cability (mg/g_{resin})	Desorption ratio
XAD4	4.15	0.29	6.99
XAD7	5.14	0.67	13.04
XAD16	4.60	0.33	7.17
XAD1180	3.74	0.63	16.84
HP20	4.55	0.37	8.13

To verify our assumption that adsorbent resins, particularly XAD1180, would enhance violacein production, fermentation of *P. luteoviolacea* in the presence of tested resins was also performed. XAD1180 proved to increase total highest yield of 0.19 g/L or 35% compared to the control (figure 2.2). This result was possibly due to the pore radian of XAD1180 (300^0A), which was the largest in this experiment, and larger surface are (800 m^2/g). However, the addition of HP-20 did not help to improve total violacein production compared to with control. This result could be explained by the lowest density of HP-20 (1.01 g/mL), thus preventing the contact of resin to medium broth. From these results and previous experiment, XAD1180 was proved to be most efficient resin in improved production of violacein in this research.

This study is the first report of using adsorbent resin in fermentation process for violacein production. XAD 1180 was also used to adsorb crocin, a carotenoid from *Gardenia jasminoides*. It showed promising adsorption capacity and selectivity towards crocin, in addition to HP20, HPD100A and AB8. Yang suggested that adsorption mechanism on XAD1180 surface processed through physical rather than chemical mean, therefore facilitating desorption process and enhancing product recovery [25].

More strikingly, violacein adsorbed on XAD1180 resin accounted for 60% of the entire amount, rendering feasibility in obtaining it by just soaking the resin in methanol or ethanol rather than applying conventional and tedious liquid-liquid extraction.

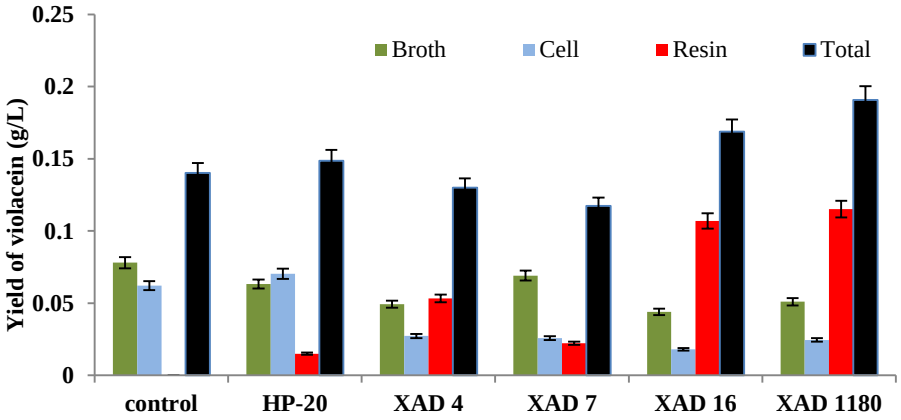


Figure 2.2 Violacein production in the batch fermentation with various adsorbent resins

With XAD1180 as the most effective adsorbent resin in hand, we subsequent explore when the culture is suitably added. The sterilized XAD1180 (2%w/v) was added to medium culture broth at 0, 8, 16, 24 and 36 h after inoculation, and all cultures were carried out until 48 h. Total violacein production was increased by 15-47%, compare with control, when resin was added into medium broth at 0 to 24 h. Interestingly, the highest violacein production (47%) was detected when resin was added at 16 h, which is the late log phase or early production phase of bacterial growth. However, violacein gradually dropped after 16 h resin addition, and the lowest production was detected when resin was added at 36 h, which is lower than that of control (figure 2.3) A similar result was also observed by Kim and coworkers [9] when prodigiosin production was maximized by adding HP-20 resin within production phase (10 h) of *Serratia* sp. KH-95.

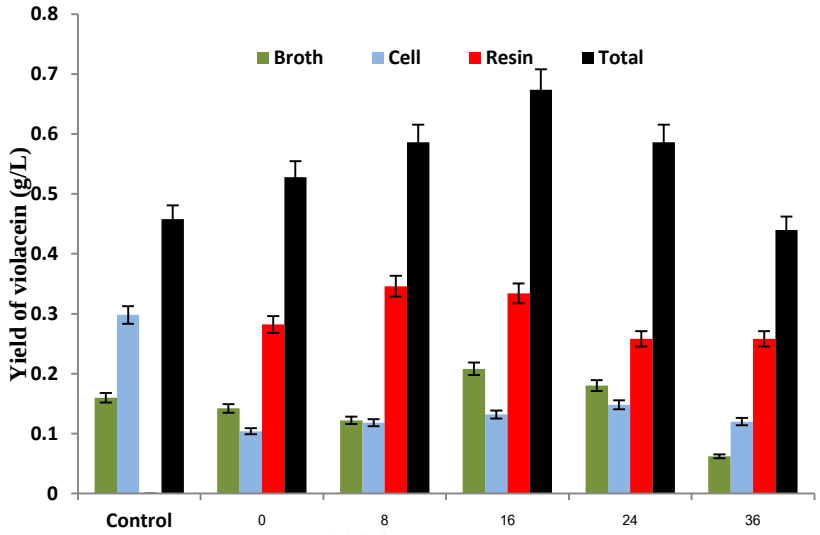


Figure 2.3 Violacein production on addition of XAD 1180 at various times

We subsequently investigated violacein yield is whether proportional to the amount of XAD1180 added. Additional five sets of bacterial cultures in the presence of higher concentrations (2, 4, 8, 10, 15 and 20%w/v) of XAD1180 were performed. The addition of 4%w/v XAD1180 resulted in comparable amount of violacein generated as 2%w/v XAD1180 (figure 2.4) However, the culture having 8-20%w/v XAD1180 generated violacein with approximately 25-56% drop in amount. This observation could be rationalized by the fact that excess resin may block oxygen dissolved into medium broth and absorb some nutrients essential for bacterial growth. Therefore, we would apply 2%w/v XAD1180 for further studies due to the reasons of effective productivity and economic.

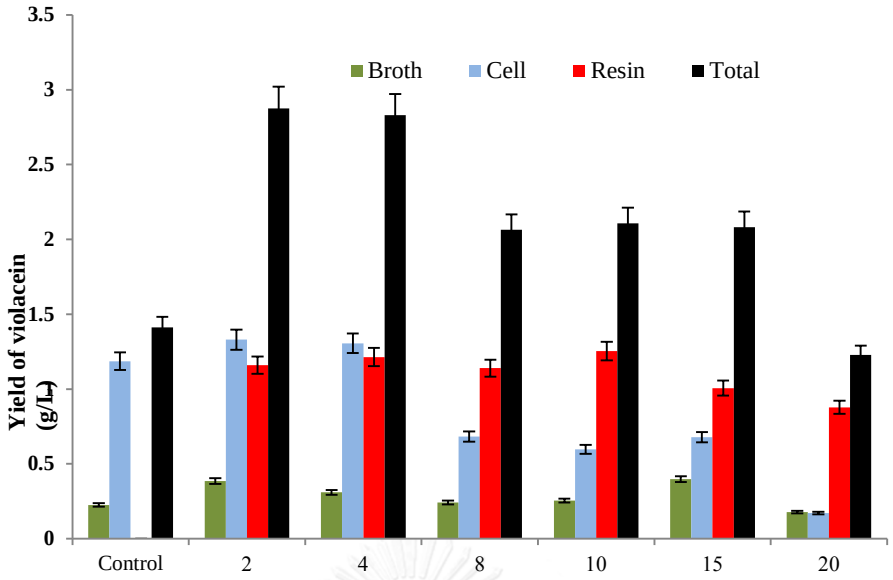


Figure 2.4 Violacein production from batch culture of *P. luteoviolacea* when addition of XAD 1180 in various content

Finally, we also examined the effect of temperature and initial pH of culture medium toward violacein production. The cultures with above optimized conditions in three different temperatures (25, room temperature (29±1) and 37 °C) and pHs (6.0, 7.6 and 8.6) were carried out. Figure 2.5 demonstrated that under optimized condition, *P. luteoviolacea* grew well at pH and temperature ranges of 7.6-8.6 and 4-30 °C, respectively. However, under human temperature (37°C) and all pH tested, the bacteria failed to produce violacein as relatively low concentration of the target compound was detected. The Results may be Low temperature was helping to cell growth and pigment production.

From results suggested that the violacein production is dependent on temperature and initial pH of culture medium. There was the resulted show that the *P. luteoviolacea* need an alkaline condition and the suitable temperature range of 4-30°C to produce the violacein.

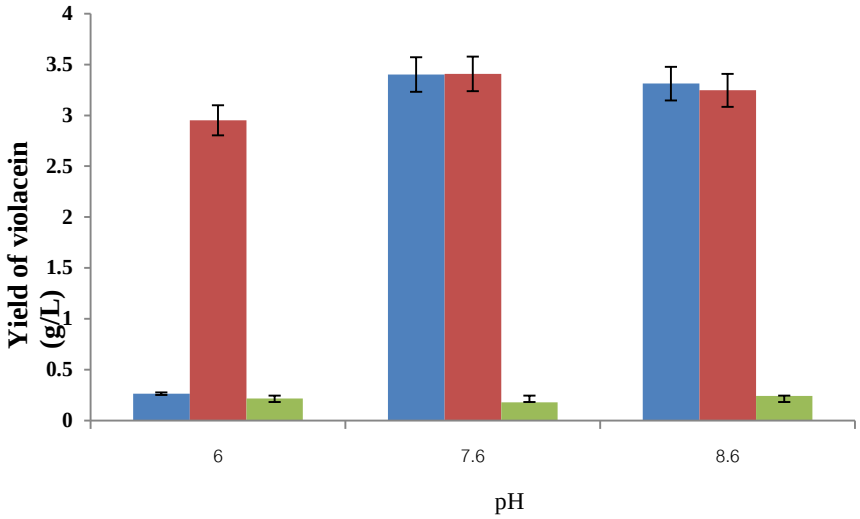


Figure 2.5 Production of violacein production from batch culture of *P. luteoviolacea* under differences pH (6, 7.6, 8.6) and temperature (4°C, RT, 37 °C)

With optimized conditions in hand, we further compared the effect of adding XAD1180 on viable cell, pH of culture and violacein production with control. XAD1180 clearly extend log phase of bacterial cell approximately 25 h while viable cell count is larger than that of control (figure 2.6A). In addition, the amount of resin added in the culture did not affect pH of medium broth (Figure 2.6B). Under the optimized conditions, production of violacein in the presence of the resin was

significantly higher than that of control at 96 h of inoculation (figure 2.6C). As environmentally friendly concern, XAD1180 used in the experiment are expected to be regenerated and retain its adsorption capacity. XAD1180 was also reused up to three times without a significant drop in the product yield (figure 2.7). After 48 h fermentation of the first run, the surface of XAD1180 was rinsed and examined under SEM. *P. luteoviolacea* could attach the resin surface but did not produce biofilm essentially for its growth (Figure 2.8).

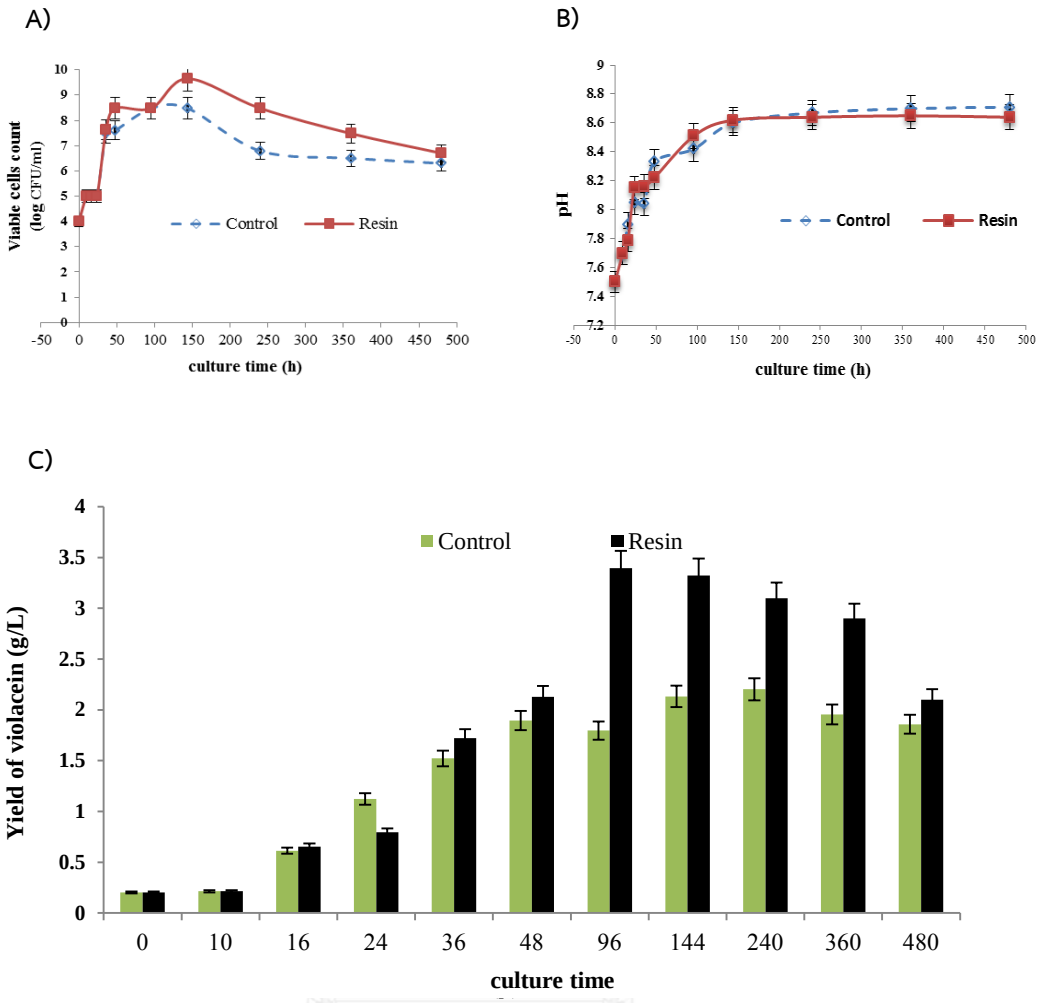


Figure 2.6 Production of violacein by *P. luteoviolacea* in flask with XAD1180 and without XAD 1180 (control) (C) without pH control at room temperature (29±1), viable cell count (A), and pH (B).

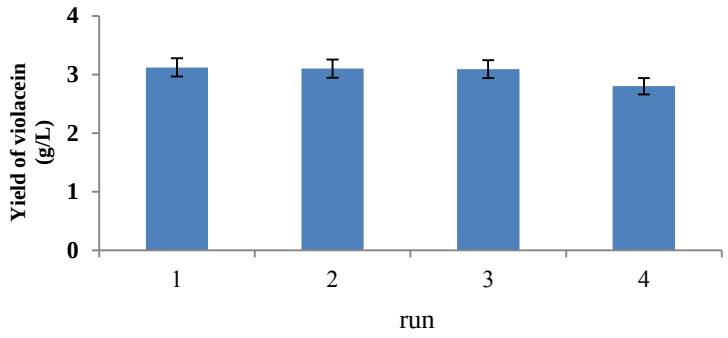


Figure 2.7 Violacein production when addition of XAD 1180 that catalyst turnover

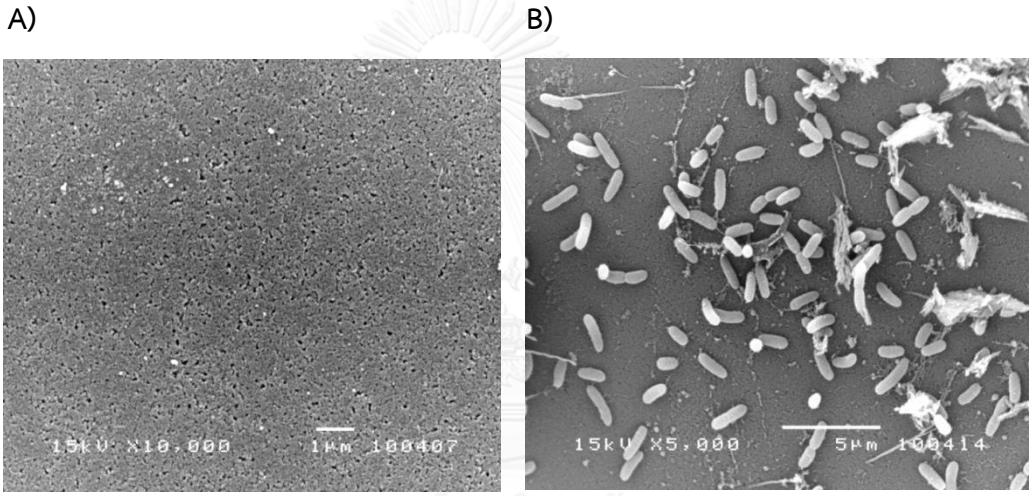


Figure 2.8 SEM of *P. luteoviolacea* bacteria attached to XAD1180 bead on fermentation for violacein production for 48 h. (Surface of XAD1180; A) x 10,000 *P. luteoviolacea* bacteria attached to XAD1180 ; B) x 5,000

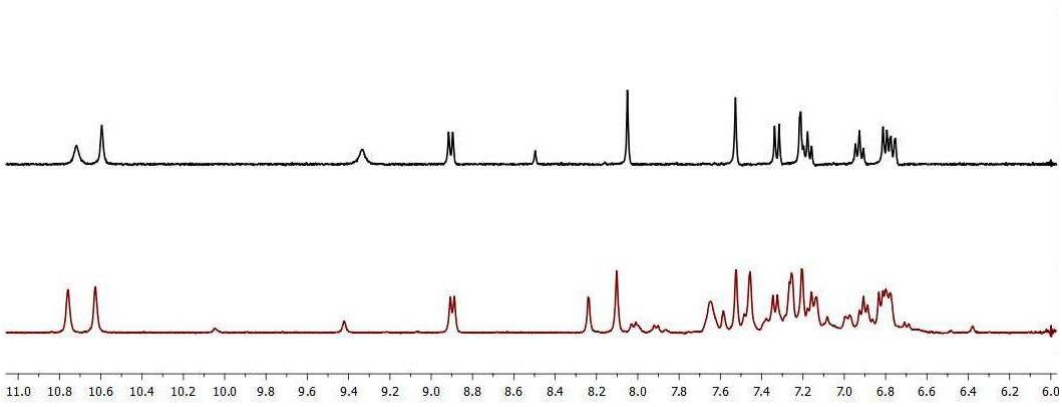


Figure 2.9 ^{1}H NMR spectra (400 MHz) of purified violacein (top) and violet pigment obtained upon eluted from XAD1180 (bottom). Both samples were prepared at concentration of 20 mg/mL.

In Figure 2.9 Comparing NMR spectra between purified violacein (top) and violet pigment obtained upon eluted from XAD1180 (bottom) it show that this method leads to highly purified violacein and easy extracted by washing with methanol or ethanol. This methodology not only enhances violacein yield per batch but also facilitates isolation of violacein from aqueous media [26].

Molecular identification

For the molecular confirmation, It was found that the sequence of *Pseudoalteromonas* sp. RAD 6-3 DP approximated size 1,375 bp exhibited the highest level of the homology (99% identity) with *P. luteoviolacea.* (Appendix B)

In conclusion, the use of the XAD 1180 resulted in improved production of violacein, suggesting considerable possibilities for improving the pigment productivity if applied to industrial scale fermentation.

CHAPTER III

Production development of prodigiosin from *Pseudoalteromonas rubra* using adsorbent resins

3.1 Introduction

Prodigiosin is a red pigment alkaloid comprising conjugated three pyrrole residues (Figure 3.1). It is produced by several strains of bacteria, particularly genus *Serratia*. Prodigiosin and other related alkaloids exhibit a broad range of biological activities such as inducing apoptosis in many different human cancer cell lines with little effect on nonmalignant cells, inhibiting a wide variety of bacteria and fungi as well as cation binding ability [8].

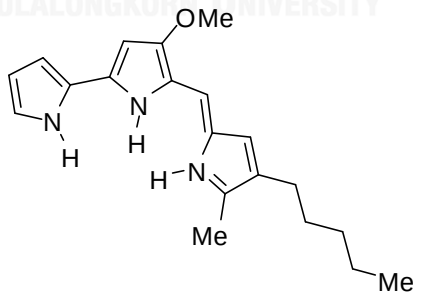
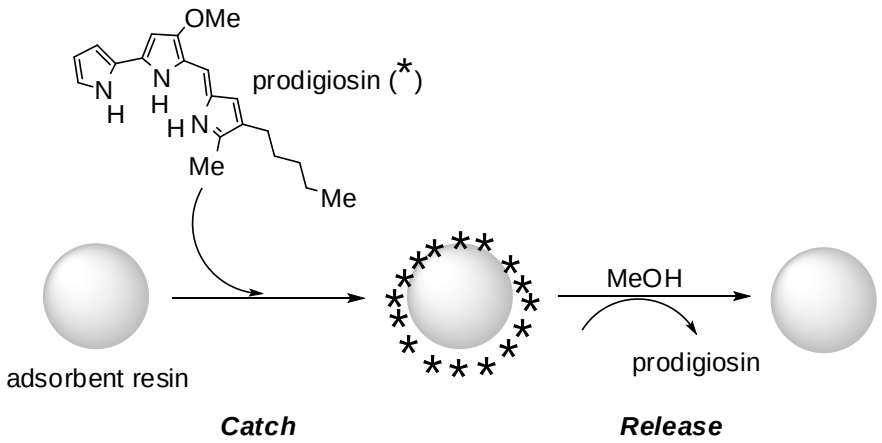


Figure 3.1 Structures of prodigiosin

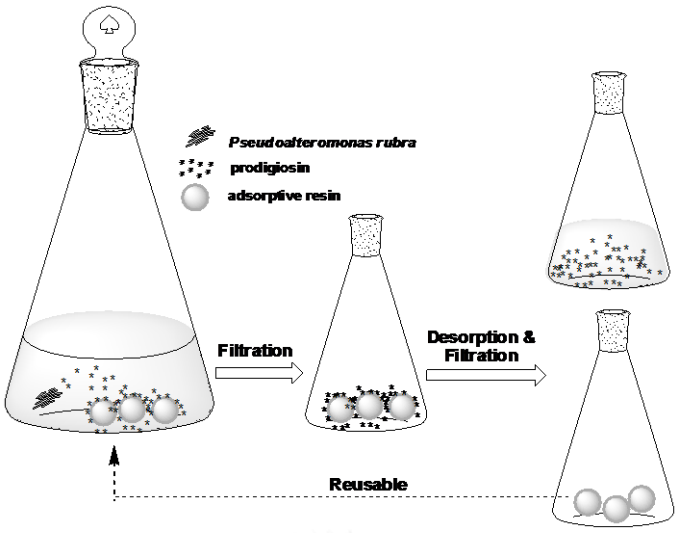
More interestingly, prodigiosin revealed immunosuppressive activity at doses that are nontoxic. Their efficacy use has been supported by the in vivo experiments on various animal models [27, 28]. In addition, prodigiosin showed synergstic effect on the immune system, when administrated simultaneously with cycloporin® or FK507®, which are clinically immunosuppressive agents for the treatment of patient after organ transplantation. Due to promising applications in clinical trials, an urgent need of prodigiosin in bulk supplied is inevitable. Although prodigiosin can be obtained by synthetic and fermentation methodology [29], the later approach is likely to suitable for commercial supply.

Various approaches have been investigated in order to produce prodigiosin in high yield. However, there is no a general approach that can be easily applied for fermentation of different strains, and isolation of the newly generated prodigiosin from the media is tedious if conventional liquid-liquid extraction is applied [30-32]. In addition, all fermentations usually encountered early stationary phase, in which production rate is not increased or perhaps gradually dropped. This observation could be elaborated by the innate toxicity of prodigiosin toward various microbes, even though prodigiosin-producing strain.



Scheme 3.1 Schematic representation of "catch and release" approach applied in this study.

To address aforementioned problems, we have an idea to introduce the approach that can be applied to all strains of prodigiosin-producing bacteria, without complicated optimization. The catch and release approach using adsorbent resin seems to fit the requirement. This methodology is based on the fact that the target prodigiosin is adsorbed ("Catch") on adsorbent resin, which is neutral aromatic based resin. After filtration from broth media, prodigiosin adsorbed on resin can be simply desorbed ("Release") by soaking the adsorbed resins in alcohol (Scheme 1). In this communication, we described the simple methodology that can be applied for production and isolation of prodigiosin.



Scheme 3.2 Schematic representation of how adsorbent resins promote prodigiosin production and facilitates isolation and purification.

3.2 Experimental

All experimental procedure are as described in chapter II, but for this experiment to replace with marine bacteria *Pseudoalteromonas rubra*

3.3 The Result and Discussion

3.3.1 The profile of violacein production from marine bacteria *P. rubra*

Time courses for growth of *P. rubra* and production of Prodigiosin are shown in Figure 1. Bacterial growth reached a stationary phase by 48 h and the growth was decline after 48 h. Maximum viable cell count was measured at 48 h. This result indicate that cell growth was inhibited by high concentration of prodigiosin.

In this study, *P. rubra*, a prodigiosin-producing, marine bacteria was employed. Initially, time course for the growth of *P. rubra* in standard marine broth was examined. Apparently, bacterial growth reached a stationary phase by 48 h and thereafter gradually declined (Figure 3.2). This observation was consistent with maximum amount of prodigiosin released from the bacteria. Therefore, quantitative determination of prodigiosin in all experiments, by colorimetric method (λ_{max} 535 nm), was carried out after 48 h of inoculation.

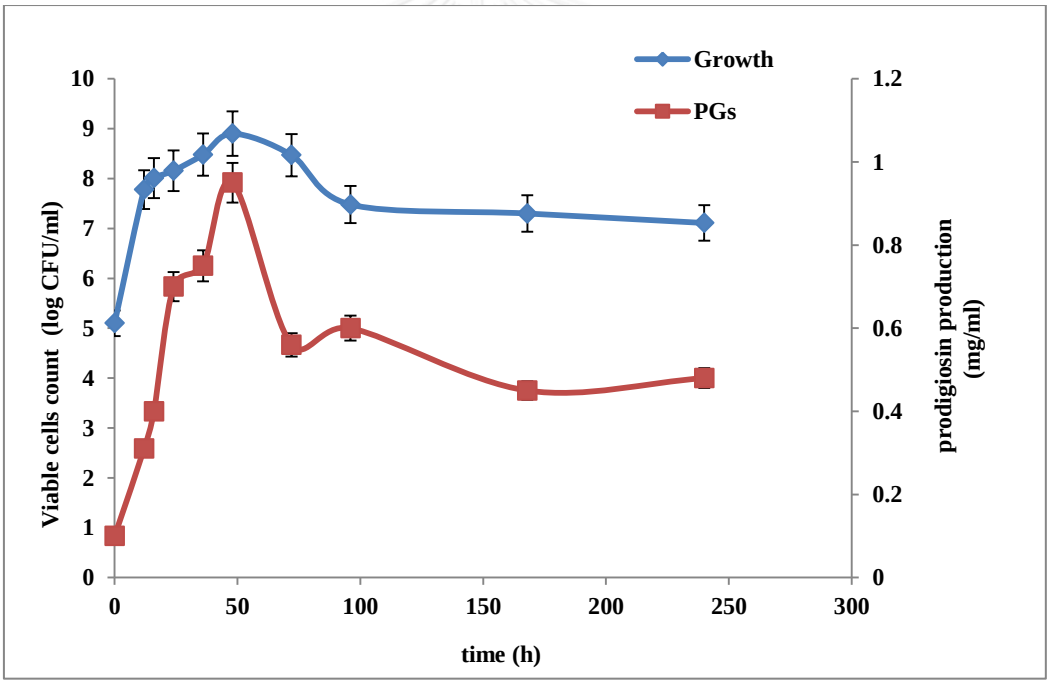


Figure 3.2 Time courses for growth of *Pseudoalteromonas rubra*and the production of prodigiosin

All of them, except for XAD7, are polystyrene divinylbenzene (SDVB) based resins varying in surface and particle properties [22, 23, 33]. Three parameters, namely adsorption capacity (Q_a), desorption ability (Q_d) and desorption ratio, were

evaluated and the result are shown in Table 1. All adsorbent resins showed comparable capacities both in terms of adsorption and desorption. However, significantly high desorption ratio observed in HP20 (27.5) suggested their potential for applying in culture media. In the present study, due to its highest desorption ratio, HP20 was selected for further investigation in hope that it would provide high percent recovery of prodigiosin after resin washing.

Table 3.1 Adsorption capacity, desorption ability and desorption ratio of prodigiosin on various resins

Adsorbent resin	Adsorption capacity ($mg_{Prodigiosin}/g_{resin}$)	Desorption ability ($mg_{Prodigiosin}/g_{resin}$)	Desorption ratio
XAD4	9.12	0.45	5.0
XAD7	10.23	2.10	20.5
XAD16	9.89	0.63	6.4
XAD1180	8.89	1.01	11.4
HP20	16.21	4.45	27.5

We further screened five different adsorbent resins (Scheme 1), which included XAD4, XAD7, XAD16, XAD1180 and Diaion HP20, typically used in laboratory for removal of organic compounds from aqueous media were examined for adsorptive property toward prodigiosin. It's were tested to prodigiosin adsorption efficiently. For their absorption behavior (adsorption capacity, desorption ability and

desorption ratio). Of resins examined, Diaion HP20 demonstrated highest adsorption capacity, desorption ability and desorption ratio (Table 3.1). With effective adsorbent resin in hand, we subsequently investigated total prodigiosin produced by *P. rubra* in the presence and absence of adsorbent resins. Batch fermentation of *P. rubra* in the presence of Diaion HP20 (1 %w/v) produced highest total prodigiosin (Figure 3.2), which was 28% higher than that produced by fermentation without resin added (control). Although prodigiosin detected on resin were slightly lower than that found in bacterial cell, this problem could be solved by pretreatment with surfactant or ultrasonic-assisted extraction [34].

3.3.2 Prodigiosin production with various adsorbent resins

In order to investigate the relationship between resin type and prodigiosin production, culture broth (1L) was added with five difference adsorbent resins (XAD4, XAD7, XAD16, XAD1180 and HP20). After 48 h inoculation, prodigiosin production in cell, resin and medium was determine using the method previously described.

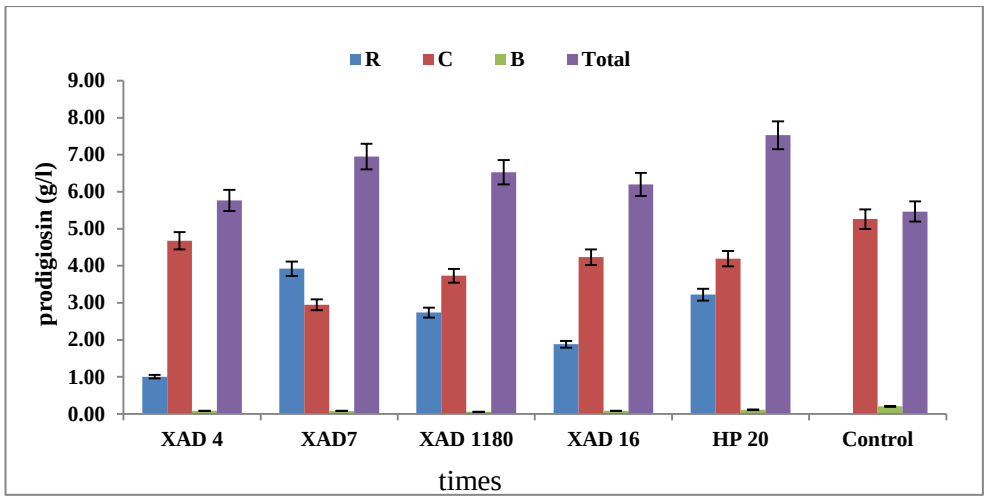


Figure 3.3 Prodigiosin production in the batch fermentation with various adsorbent resins

To verify our assumption that adsorbent resins, particularly HP20, would enhance prodigiosin production, fermentation of *P. rubra* in the presence of tested resins was also performed. HP20 proved to increase total highest yield of 7.53 g/L or 28% compared to the control as shown in the figure 3.3 and suggests that the desorption process is very fast and easy because of HP 20 reaches to equilibrium after 30 only [24].

Juang and Yeh [24] have to obtained similar results using HP20 for increase prodigiosin from *Serratia marcescen*. One the reason is the ratio of adsorbent resin pore size to the adsorbate molecular size can be used to judge the sites where adsorption occurs. Kawazoe *et al*. [19].

In previous study, It was found that HP 20 to increase the productivity of prodigiosin from *Serratia* sp. KH-95, that taking into consideration the adsorption

capacity and the desorption ability. Desorption ratio of HP 20 is also an important factor for chosen to further study [9].

HP-20 did not help to improve total violacein production compared to with control. This result could be explained by the lowest density of HP-20 (1.01 g/mL), thus preventing the contact of resin to medium broth.

3.3.3 Production of Prodigiosin with adsorbent resin HP20 in various time

To optimize Prodigiosin production in the presence of HP20, time of resin addition was investigated. The sterilized HP20 (2%w/v) was added to medium culture broth at 0, 8, 16, 24,36 and 48 h after inoculation, and all cultures were carried out until 48 h.

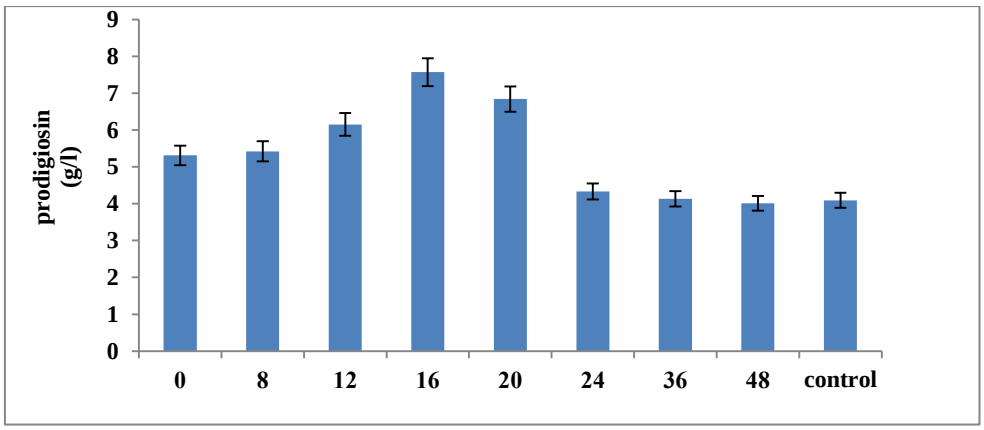


Figure 3.4 Prodigiosin production when addition of HP20 in various times

With HP20 as the most effective adsorbent resin in hand, we subsequent explore when the culture is suitably added. The sterilized HP20 (1%w/v) was added to medium culture broth at 0, 8, 16, 24 and 36 h after inoculation, and all cultures were carried out until 48 h. Total prodigiosin production was increased by 6-85%, compare with control, when resin was added into medium broth at 0 to 24 h. Interestingly, the highest violacein production (85%) was detected when resin was added at 16 h, which is the late log phase or early production phase of bacterial growth. However, violacein gradually dropped after 16 h resin addition, and the lowest production was detected when resin was added at 48 h, which is lower than that of control (figure 3.4). A similar result was also observed by Kim and coworkers (1999), when prodigiosin production was maximized by adding HP-20 resin within production phase (10 h) of *Serratia* sp. KH-95. From this result, resin was added at 16 h (log phase or nearly production phase) is the optimum time to enhance yield of prodigiosin.

3.3.4 The appropriate amount of HP20 on fermentation for prodigiosin production

The different six concentration (1, 2, 4, 5 7.5 and 10 %w/v) of HP20 was added to culture medium broth at 16 h after inoculation, and all of these were incubated until 48 h.

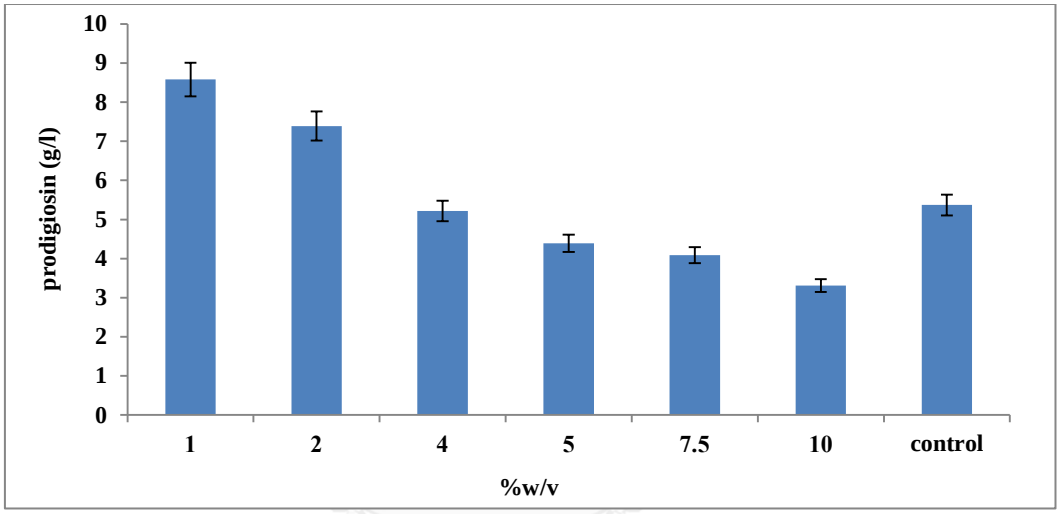


Figure 3.5 Prodigiosin production when addition of HP20 in various content

As the results, to envision the appropriate amount of Diaion HP added to ferment broth, six different ratios of resin and culture broth (1, 2, 3, 4, 5, 7.5 and 10 %w/v) were validated. Prodigiosin was highest (8.58 g/L) produced in the culture broth containing 1 %w/v of Diaion HP (Figure 3.5). However, the increased amount of resin did not significantly alter production yield but gradual declines in total pigment were observed. Due to highest productivity and economic reason, 1 %w/v of Diaion HP was suitable for prodigiosin production, that may be caused by increasing resin

blocking oxygen dissolved in to culture broth, and the essentially constitutive in culture broth has been removed by higher concentration of resin, that is due to the fact that of lowered cell growth and division led to low productivity [35].

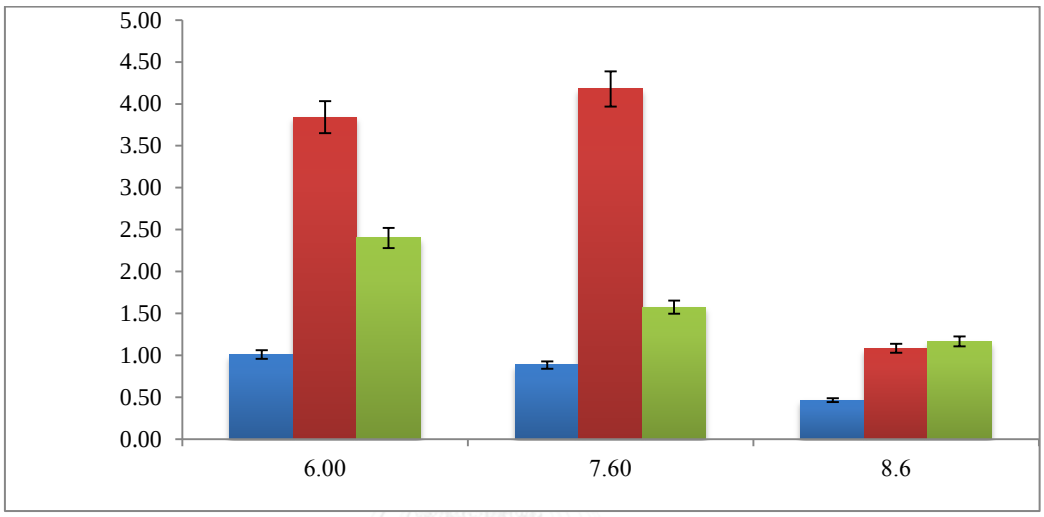


Figure 3.6 Production of prodigiosin production from batch culture of *P. rubra* under differences pH (6, 7.6, 8.6) and temperature (4°C, RT, 37 °C)

3.3.5 Effect of temperature culture and initial pH of culture medium

And nextly, we also examined the effect of temperature and initial pH of culture medium toward prodigiosin production. The cultures with above optimized conditions in three different temperatures (4, room temperature (28-30) and 37 °C) and pHs (6.0, 7.6 and 8.6) were carried out. Figure 5 demonstrated that under optimized condition, *P. rubra* grew well at pH and temperature ranges of 6.0-7.6 and 28-30 °C, respectively. However, under human temperature (37°C) and all pH tested,

the bacteria failed to produce prodigiosin as relatively low concentration of the target compound was detected (figure 3.6).

From results suggested that the prodigiosin production is dependent on temperature and initial pH of culture medium. There was the resulted show that the *P. rubra* need neutral to weak acid condition and the suitable temperature range of 28-30°C to produce the prodigiosin. This is phenomena was also reported by Samrot *et al* [36], who observed that *Serratia marcescen* can produce more prodigiosin at 28^0C at pH 7 and the rate was reduced as the temperature increases. Williams and Quadri [37] reported that no prodigiosin was produced when cultures were incubated at 38°C.

3.3.6 Change in Prodigiosin production and cell growth during various fermentation

With optimized conditions in hand, we further compared the effect of adding HP20 on viable cell, pH of culture and violacein production with control. HP20 clearly extend log phase of bacterial cell approximately 25 h while viable cell count is larger than that of control (figure 3.7A). In addition, the amount of resin added in the culture did not affect pH of medium broth (Figure 3.7B). Under the optimized conditions, production of prodigiosin in the presence of the resin was significantly higher than that of control at 96 h of inoculation (figure 3.7C). As environmentally friendly concern, HP20 used in the experiment are expected to be

regenerated and retain its adsorption capacity. HP20 was also recycled up to four times without a significant drop in the product yield (figure 3.8). After 96 h fermentation of the first run, the surface of HP20 was rinsed and examined under SEM. *P. rubra* could not attach the resin surface t did not produce biofilm essentially for its growth (Figure 3.9).

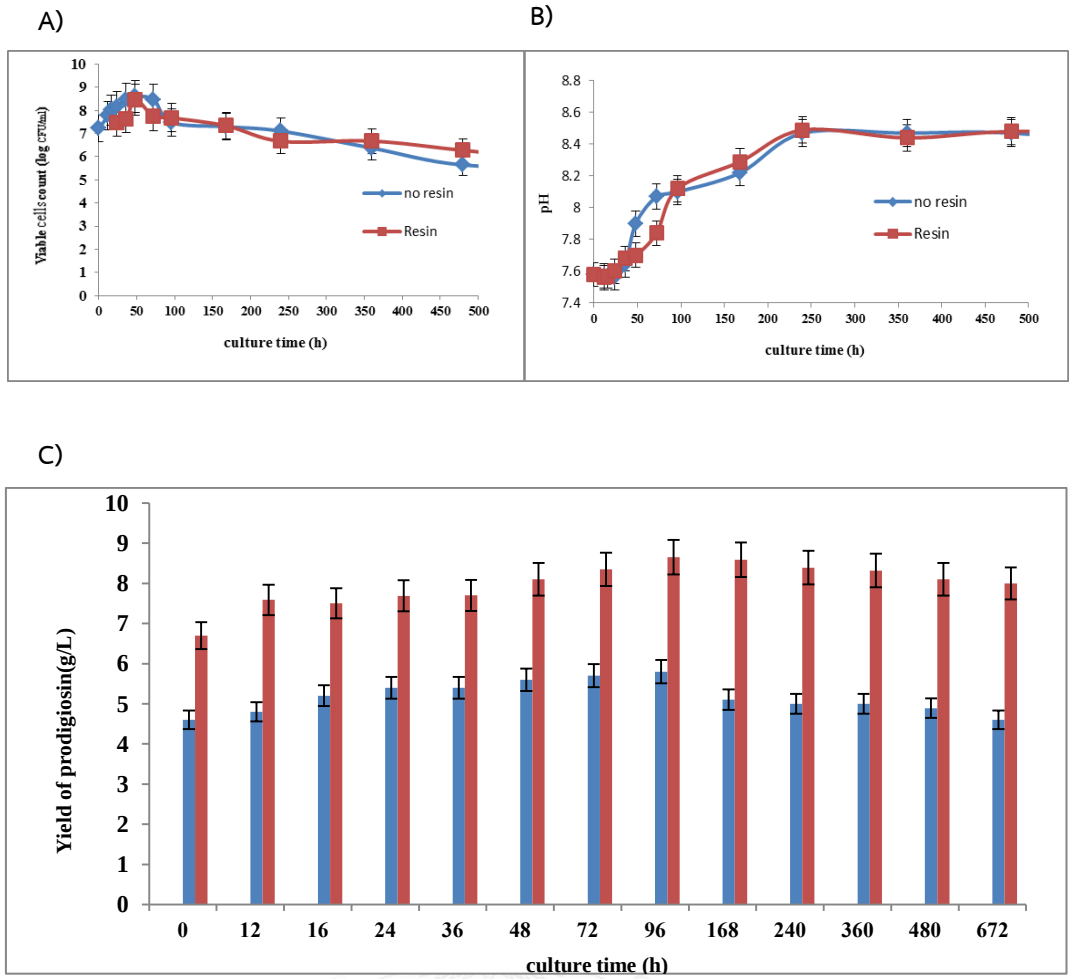


Figure 3.7 Production of prodigiosin by *P. rubra* in flask with HP20 and without HP20 (control) (C) without pH control at room temperature (29±1), viable cell count (A), and pH (B) during various fermentation for 672 h

3.3.7 Catalyst turnover of adsorbent resin

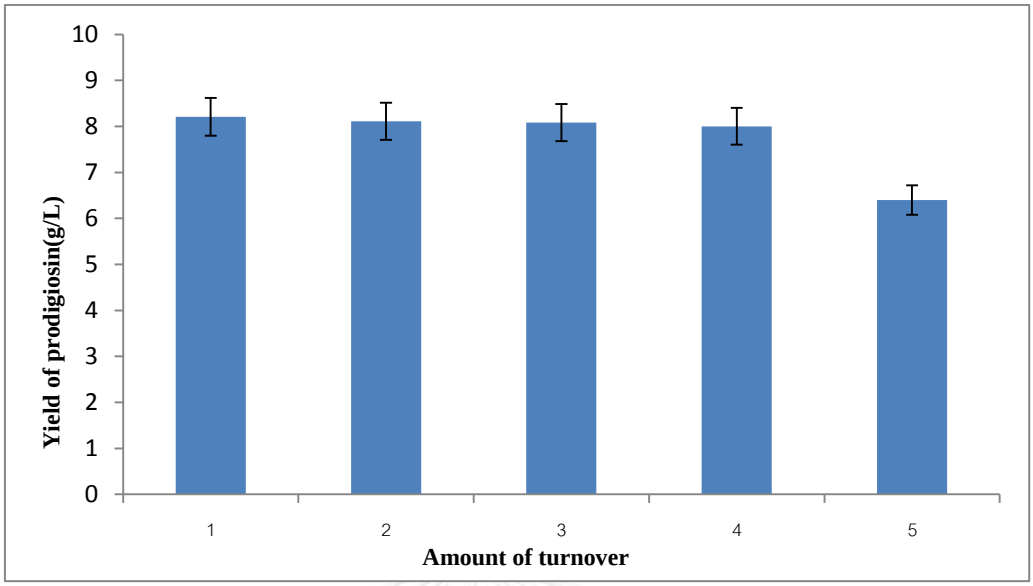


Figure 3.8 Prodigiosin production when addition of HP20 that catalyst turnover

3.3.8 SEM of *P. rubra* bacteria attached to an adsorbent resin bead

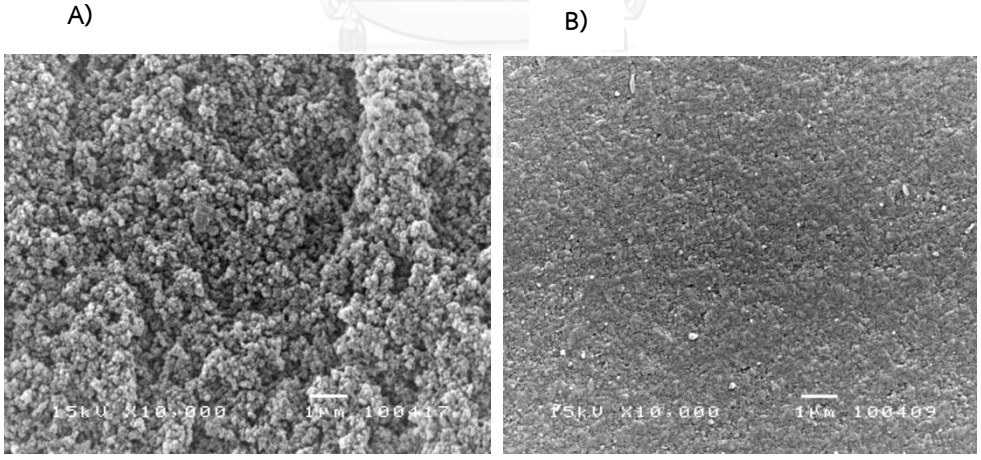


Figure 3.9 SEM of *P. rubra* bacteria attached to HP20 bead on fermentation for prodigiosin production for 48 h. (Surface of HP20; A) *P. rubra* bacteria were not attached to HP20; B) (x10,000)

3.4 Molecular identification

Molecular identification

For the molecular confirmation, It was found that the sequence of *Pseudoalteromonas* sp. CC 10-5R approximated size 1,365 bp exhibited the highest level of the homology (99% identity) with *Pseudoalteromonas rubra* (Appendix B).

In conclusion, the use of the HP 20 resulted in improved production of prodigisin, suggesting considerable possibilities for improving the pigment productivity if applied to industrial scale fermentation.

CHAPTER IV

CONCLUSION

As a part research for Production development of violacein and prodigiosin from *Pseudoalteromonas luteoviolacea* using adsorbent resins which as shown follow :

Marine sponge-associated bacteria are of considerable current interest as a new and promising source of biologically active compounds. They produce a variety of metabolites, some of which can be used for drug development. Violacein is a violet indole alkaloid produced by *Pseudoalteromonas luteoviolacea* which are isolated from the marine sponge, have attracted much attention in recent literature due to its pharmacological properties. Violacein has several kinds of bioactivity, including the broad spectrum antibacterial, strong bactericidal, antitumor, antiviral, antioxidant and antiprotozoan activities and violacein can also be used as a biological dye and need for large scale supply, effective production has been developed. However, the development has been limited due to end-product inhibition of violacein. In this study, five different adsorbent resins, namely HP20, XAD4, XAD7, XAD16 and XAD1180 were evaluated for efficiency to enhance violacein production from marine bacterium *Pseudoalteromonas luteoviolacea*. Dispersion of XAD1180 enhanced the highest yield, particularly when 2% $w.v^{-1}$ XAD1180 was added

to medium after 16 h of cultivation. This method could produce 2-fold increment in violacein production when compared with conventional fermentation and maximum yield of violacein was up to 3.285 g.L^{-1}. In addition, the regenerated XAD 1180 could be reused additional three times without significant drop in violacein yield.

From a part research for Production development of prodigiosin from marine bacteria *Pseudoalteromonas rubra* using adsorbent resins, we have developed a practical and easy approach that could be applied to improve prodigiosin production. We demonstrated that addition of 1 %w/v of Diaion HP20 could increase total prodigiosin up to 1.6 folds, compared to control. Our present methodology not only enhanced prodigiosin yield but facilitated its isolation from culture media by soaking the adsorbed resin in methanol, which was much easier than traditional liquid-liquid extraction. In addition, our proposed method could be employed to all strains of prodigiosin-producing bacteria without complicated optimization such as temperature, pH of media and agitation. It can be seen from this research that. To envision the appropriate amount of Diaion HP added to ferment broth, six different ratios of resin and culture broth (1, 2, 3, 4, 5, 7.5 and 10 %w/v) were validated. Prodigiosin was highest (8.58 g/L) produced in the culture broth containing 1 %w/v of Diaion HP20. However, the increased amount of resin did not significantly alter production yield but gradual declines in total pigment were observed. Due to highest productivity and economic reason, 1 %w/v of Diaion HP was suitable for prodigiosin

production. And It was found that *Pseudoalteromonas rubra*grew well at pH and temperature ranges of 7.6-8.6 and 25-30 °C, respectively. In addition, As environmentally friendly concern, HP20 used in the experiment are expected to be regenerated and retain its adsorption capacity. HP20 was also recycled up to four times without a significant drop in the product yield.

These two strains were molecular level identified as *Pseudoalteromonas luteoviolacea* and *Pseudoalteromonas rubra*

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APPENDIX

APPENDIX A

Culturing Marine bacteria

1. Modified marine Zobell broth

Peptone	1.0 g
Yeast extraxt	1.0 g
Phytone	0.5 g
Sodium thiosulphate	0.2 g
Sodium sulphate	0.05 g
Fe-citrate	1.0 mL

These component were added in 1.0 L of mixer solution between sea water (90% v/v) and distilled water (10% v/v), and adjust pH to 7.6. The medium was sterilized by autoclave at 121 $^{\circ}$C for 15 min.

2. Modified marine Zobell agar

Peptone	1.0 g
Yeast extraxt	1.0 g
Phytone	0.5 g
Sodium thiosulphate	0.2 g
Sodium sulphate	0.05 g
Fe-citrate	1.0 mL
Agar	15.0 g

These component were added in 1.0 L of mixer solution between sea water (90% v/v) and distilled water (10% v/v), Agar 15.0 g was melted into the medium and then the medium was adjust pH to 7.6. The medium was sterilized by autoclave at 121 °C for 15 min.

Preparing of adsorbent resin

Adsorbent resin was immersed into distilled water for overnight, and the adsorbent resin was soaked in 95% (v/v) methanol. The solvent was removed by micropipette, and the adsorbent resin was dried using hot air oven at 50-55 °C until the resin dried.

Calibration curve of violacein and prodigiosin

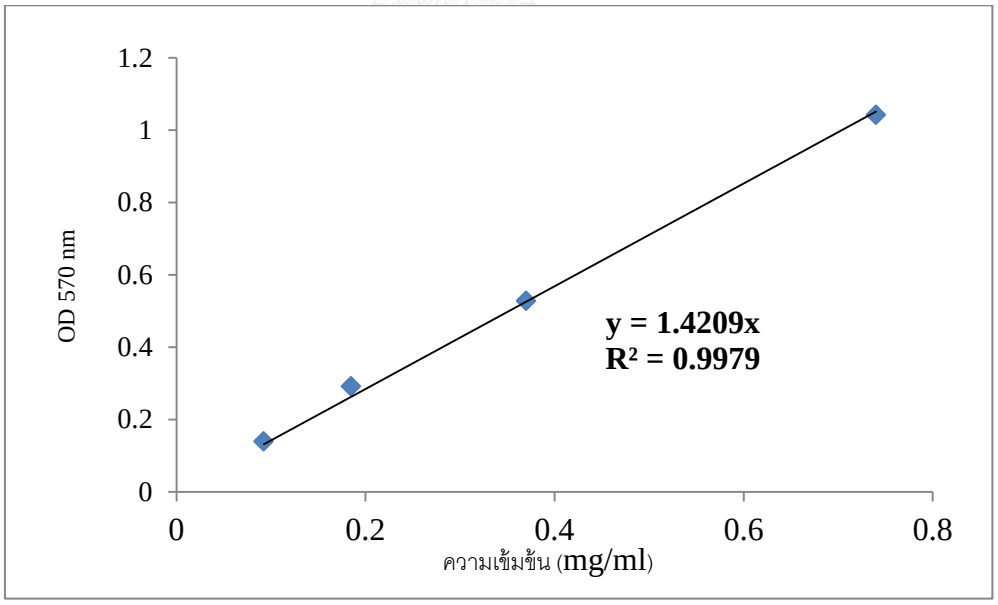

Figure 1 Calibration curve of violacein

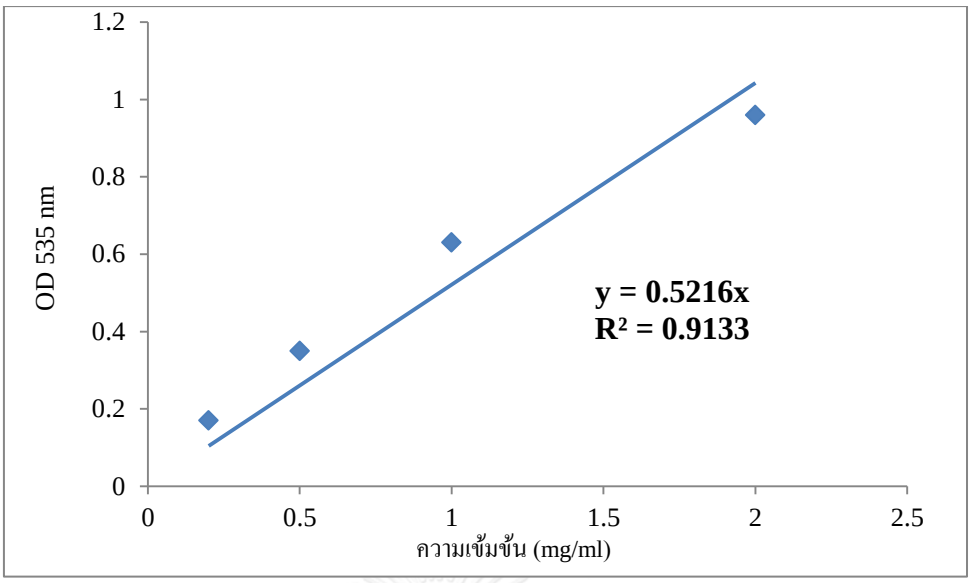

Figure 2 Calibration curve of prodigiosin

APPENDIX B

Nucleotide sequence of *Pseudoalteromonas* sp.

>66_67_FR

1 GCAGTCGAGC GGAACGAGAA TAGCTTGCTA TTCGGCGTCG AGCGGCGGAC GGGTGAGTAA
61 TGCTTGGGAA CGTGCCGTAA GGTGGGGGAC AACCATTGGA AACGATGGCT AATACCGCAT
121 AATGTCTACG GACCAAAGGG GGCTTCGGCT CTCGCCTTAT GATCGGCCCA AGTGGGATTA
181 GCTAGTTGGT AAGGTAATGG CTTACCAAGG CGACGATCCC TAGCTGGTTT GAGAGGATGA
241 TCAGCCACAC TGGAACTGAG ACACGGTCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA
301 TTGCACAATG GGCGCAAGCC TGATGCAGCC ATGCCGCGTG TGTGAAGAAG GCCTTAGGGT
361 TGTAAAGCAC TTTCAGTAAG GAGGAAAGGT TAGTAGTTAA TACCTGCTAG CTGTGACGTT
421 ACTTACAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC GCGGTAATAC GGAGGGTGCG
481 AGCGTTAATC GGAATTACTG GGCGTAAAGC GTACGCAGGC GGTTTGTTAA GCGAGATGTG
541 AAAGCCCCGG GCTCAACCTG GGAACTGCAT TTCGAACTGG CAAACTAGAG TGTGATAGAG
601 GGTGGTAGAA TTTCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGAAGGAA TACCGATGGC
661 GAAGGCAGCC ACCTGGGTCA ACACTGACGC TCATGTACGA AAGCGTGGGG AGCAAACAGG
721 ATTAGATACC CTGGTAGTCC ACGCCGTAAA CGATGTCTAC TAGGAGCTGGG GTCTTCGGAC
781 AACTTTTCCA AAGCTAACGC ATTAAGTAGA CCGCCTGGGG AGTACGGCCG CAAGGTTAAA
841 ACTCAAATGA ATTGACGGGG GCCCGCACAA GCGGTGGAGC ATGTGGTTTA ATTCGATGCA
901 ACGCGAAGAA CCTTACCTAC ACTTGACATA CAGAGAACTT ACTAGAGATA GTTTGGTGCC
961 TTCGGGAACT CTGATACAGG TGCTGCATGG CTGTCGTCAG CTCGTGTTGT GAGATGTTGG
1021 GTTAAGTCCC GCAACGAGCG CAACCCCTAT CCTTAGTTGC CAGCGATTCG GTCGGGAACT
1081 CTAAGGAGAC TGCCGGTGAT AAACCGGAGG AAGGTGGGGA CGACGTCAAG TCATCATGGC
1141 CCTTACGTGT AGGGCTACAC ACGTGCTACA ATGGCAGATA CAGAGTGCTG CGAACTTGCG
1201 AGAGTAAGCG AATCACTTAA AGTCTGTCGT AGTCCGGATT GGAGTCTGCA ACTCGACTCC
1261 ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGAATGCCG CGGTGAATAC GTTCCCGGGC
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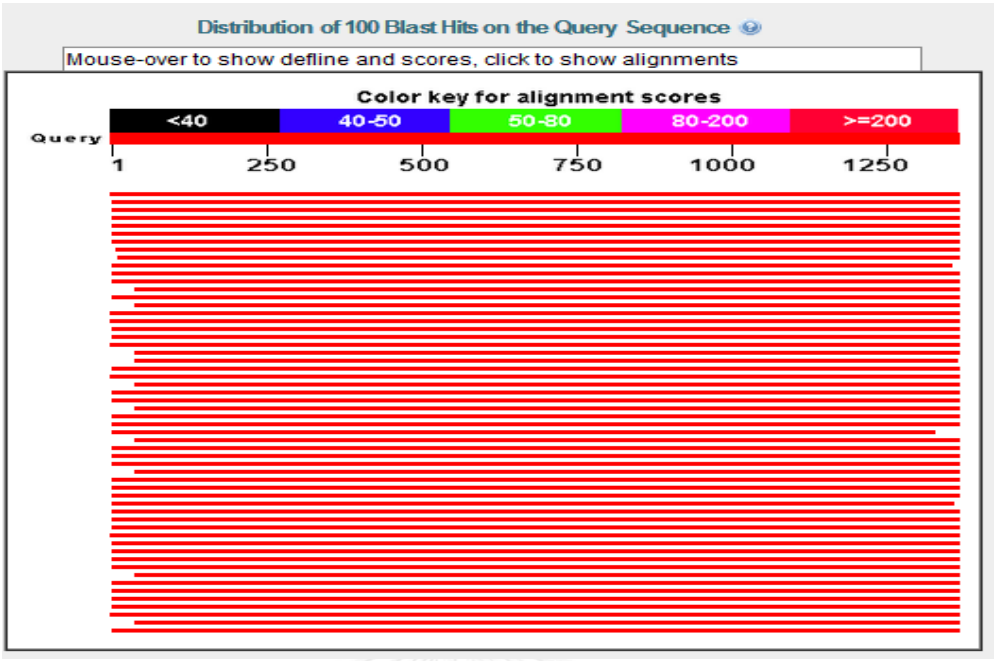

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
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Pseudoalteromonas luteoviolacea strain S4060 16S ribosomal RNA gene, partial sequence	2527	2527	99%	0.0	99%	FJ457238.1
Pseudoalteromonas luteoviolacea strain NBRC 103183 16S ribosomal RNA gene, partial sequence	2521	2521	99%	0.0	99%	NR_114237.1
Pseudoalteromonas luteoviolacea strain B1P 16S ribosomal RNA gene, partial sequence	2521	2521	99%	0.0	99%	HQ439502.1
Pseudoalteromonas sp. PaD1.15b 16S ribosomal RNA gene, partial sequence	2521	2521	99%	0.0	99%	GQ391980.1

Figure 3 Nucleotide sequence of *Pseudoalteromonas* sp. RAD 6-3 DP

>64_65_FR

1 CATTTCTAGC TTGCTAGAAG ATGACGAGCG GCGGACGGGT GAGTAATGCT TGGGAACATG
61 CCTTGAGGTG GGGGACAACC ATTGGAAACG ATGGCTAATA CCGCATAATG TCTACGGACC
121 AAAGGGGGCT TCGGCTCTCG CCTTTAGATT GGCCCAAGTG GGATTAGCTA GTTGGTAAGG
181 TAACGGCTTA CCAAGGCGAC GATCCCTAGC TGGTTTGAGA GGATGATCAG CCACACTGGA
241 ACTGAGACAC GGTCCAGACT CCTACGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGCG
301 CAAGCCTGAT GCAGCCATGC CGCGTGTGTG AAGAAGGCCT TCGGGTTGTA AAGCACTTTC
361 AGTCAGGAGG AAAGGTTAGT AGTTAATACC TGCTAGCTGT GACGTTACTG ACAGAAGAAG
421 CACCGGCTAA CTCCGTGCCA GCAGCCGCGG TAATACGGAG GGTGCGAGCG TTAATCGGAA
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601 AGGTGTAGCG GTGAAATGCG TAGAGATCTG AAGGAATACC GATGGCGAAG GCAGCCACCT
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901 TTACCTACAC TTGACATACA GAGAACTTAC CAGAGATGGT TTGGTGCCTT CGGGAACTCT
961 GATACAGGTG CTGCATGGCT GTCGTCAGCT CGTGTTGTGA GATGTTGGGT TAAGTCCCGC
1021 AACGAGCGCA ACCCTTATCC TTAGTTGCCA GCGATTCGGT CGGGAACTCT AAGGAGACTG
1081 CCGGTGATAA ACCGGAGGAA GGTGGGGACG ACGTCAAGTC ATCATGGCCC T TACGTGTAG
1141 GGCTACACAC GTGCTACAAT GGCATATACA GAGTGCTGCG AACTAGCGAT AGTAAGCGAA
1201 TCACTTAAAG TATGTCGTAG TCCGGATTGG AGTCTGCAAC TCGACTCCAT GAAGTCGGAA
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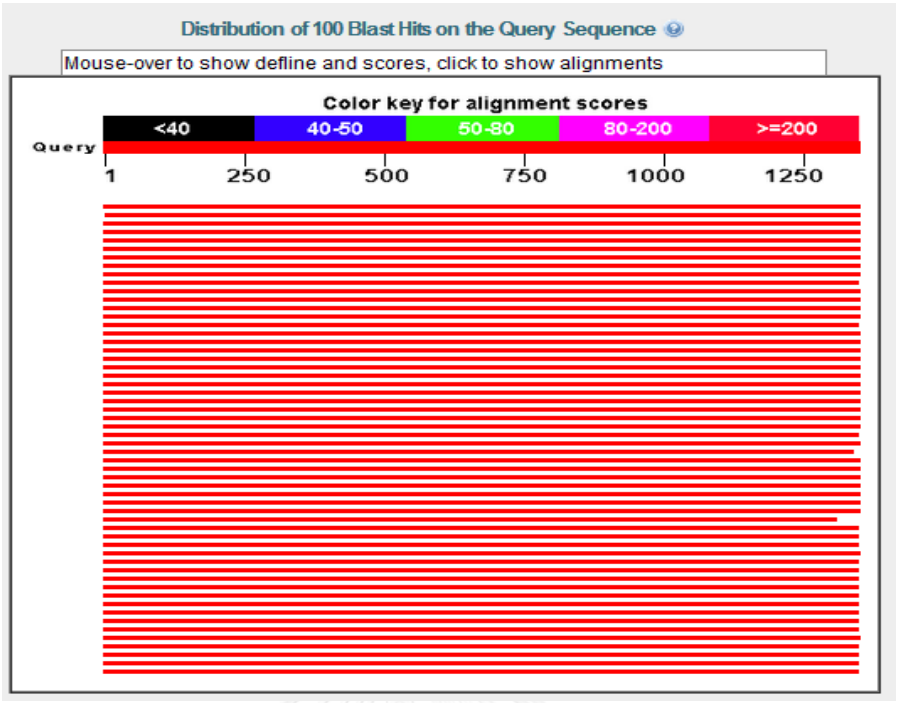

https://blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudoalteromonas rubra strain PC13b 16S ribosomal RNA gene, partial sequence	2510	2510	100%	0.0	99%	HQ439548.1
Pseudoalteromonas rubra strain G31a 16S ribosomal RNA gene, partial sequence	2510	2510	99%	0.0	99%	HQ439547.1
Pseudoalteromonas rubra strain PC13d 16S ribosomal RNA gene, partial sequence	2507	2507	100%	0.0	99%	HQ439541.1
Pseudoalteromonas rubra strain PC13c 16S ribosomal RNA gene, partial sequence	2507	2507	100%	0.0	99%	HQ439540.1
Pseudoalteromonas rubra strain B1M 16S ribosomal RNA gene, partial sequence	2503	2503	100%	0.0	99%	HQ439549.1

Figure 4 Nucleotide sequence of *Pseudoalteromonas* sp. CC 10-5R

VITA

Ms. Vassana Musa was born in Songkhla province, Thailand. She graduate high school from Hadyaiwittayalai. In 1992, he graduate from Prince of Songkla University with a Bachelor of Science in Biology. He received Master of Science in Biotechnology from Prince of Songkla University in 1999, and then studies the doctoral degree at the Chulalongkorn University. She has been working as lecturer at Rajabhat Songkhla University. During the study, she was awarded research funds from THE 90th ANNIVERSARY OF CHULALONGKORN UNIVERSITY FUND (Ratchadaphiseksomphot Endowment fund).

Publication related with this thesis

Musa, V., Dechsakulwatana, C. and Phuwapraisirisan, P. Toward practical, easy and high yield production of violacein produced by marine sponge-associated bacteria. 14th International Symposium on Marine Natural Products/8th European Conference on Marine Natural Products. La Toja Island, Garlicia, Spain.