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ISOLATION, OPTIMIZATION AND RECOVERY OF ASTAXANTHIN FROM SHRIMP SHELL WASTES OF LOCALLY ISOLATED AEROMONAS HYDROPHILA (STEINER)

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ISOLATION, OPTIMIZATION AND RECOVERY OF ASTAXANTHIN FROM SHRIMP SHELL WASTES OF LOCALLY ISOLATED *Aeromonas hydrophila* (Steiner)

By

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ISOLATION, OPTIMIZATION AND RECOVERY OF ASTAXANTHIN FROM SHRIMP SHELL WASTES OF LOCALLY-ISOLATED *Aeromonas hydrophila* (Steiner)

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September 2016

Chairman : Muskhazli Mustafa, PhD
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Astaxanthin is a red pigment naturally produced by microalgae. Being part of the shrimp diet, this pigment was accumulated in their body, shells and eggs. The total amount of shell wastes discarded during processing could reach up to 40-50% of its body weight. Previously, astaxanthin was recovered from shrimp shells using lactic acid bacteria fermentation. This method promises a good return but has higher maintenance. Meanwhile aerobic bacteria fermentation has less maintenance and was less explored in this context. Astaxanthin in its free form is readily oxidised while astaxanthin in crustaceans appears to be in complexes (carotenoprotein, carotenolipoprotein and chitinocarotenoids) which is less prone to oxidation. The current aim was to recover astaxanthin from shrimp shell wastes through aerobic bacteria fermentation. In order to obtain astaxanthin from shrimp wastes, chitinase and protease were crucial to dechitinize and deproteinize the pigment from its stable complex structures. In this study, the first objective was to isolate potential shrimp shell degrading bacteria from shrimp shells. Bacteria were isolated from shrimp shells and screened using shrimp crab shell powder. A total of 19 isolates producing chitinase and protease were obtained. Potential isolates were then compared among each other using shrimp shell waste powder (SSWP) to seek for an optimum enzyme (chitianse and protease) producing isolate. The selected isolate was identified as *Aeromonas hydrophila*. Naturally, shrimp shells are calcified and may hinder further recovery of astaxanthin. Hence, the addition of cell disruptions was aimed to loosen the complex structure of shrimp shells. A dual effect "shell disruption" method was adopted where non-chemical shell disruption was applied on the wastes as pre-treatment followed by microbial enzyme disruption as the second. The amount of astaxanthin recorded after the application of shell disruptions was comparable between treatments of control (non-pretreated SSWP fermented with microbial enzyme) and autolysis (pre-treated SSWP with autolysis and microbial enzyme). The control treatment has resulted in 2.3 ± 0.1179µg/ml of astaxanthin recovery, while combined autolysis treatment and microbial enzyme yielded 2.11 ± 0.0961µg/ml. Since both treatments gave similar yield and were not significantly different, single shell disruption (control treatment) was sufficient to produce a good recovery. In order to enhance enzyme production and astaxanthin recovery, the culture media and conditions were also optimized. Optimization of the culture media and conditions was carried out using Response Surface Methodology analysis (RSM). The media was screened with various media

supplements (nitrogen, inorganic salts and carbon sources) and concentrations (1, 3, 5, 7, 9% w/v) before optimizing with RSM. An optimum media containing 3% MSG, 1% glucose, pH 7.0 and 30 °C with a constant supply of 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, and 9% SSWP was used to culture *A. hydrophila*. In comparison, by using the optimum culture astaxanthin recovery, chitinase and protease activity has increase up to 38.4%, 30% and 36% respectively as compared to un-optimized media. To achieve the main goal of this investigation, carotenoids were purified with thin layer chromatography (TLC) and the presence of astaxanthin was confirmed using high pressure liquid chromatography (HPLC). Carotenoids were obtained after bacteria fermentation on SSWP under optimized conditions and were soxhlet extracted with acetone and concentrated before subjecting to TLC. The best mobile phase in separating the pigments was hexane: acetone (3:1 v/v). The potential band for astaxanthin was obtained from TLC at R_f value of 0.33. This band was re-extracted in acetone and subjected to confirmation using HPLC with a reference standard. The presence of astaxanthin was confirmed at retention time of 15.5, 16.4, 17.4, and 18.3 minutes. In conclusion, astaxanthin can be recovered from shrimp shell waste with aerobic fermentation of *A. hydrophila*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGASINGAN, PENGOPTIMUMAN DAN PEMEROLEHAN SEMULA ASTAXANTHIN DARIPADA SISA KULIT UDANG OLEH ISOLAT-TEMPATAN *Aeromonas hydrophila* (Steiner)

Oleh

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Astaxanthin adalah pigmen merah semulajadi yang dihasilkan oleh mikroalga. Sebagai sebahagian daripada diet udang, pigmen ini sering terkumpul di dalam badan, kulit dan telur. Jumlah kulit udang yang dibuang semasa pemprosesan boleh mencecah sehingga 40-50% daripada beratnya. Sebelum ini, pemulihan semula astaxanthin daripada kulit udang adalah secara penapaian dengan bakteria asid laktik. Kaedah ini menjanjikan hasil yang lumayan, tetapi mempunyai kos penyelenggaraannya tinggi. Sementara itu, penapaian bakteria secara aerobik yang kurang penyelenggaraan kurang diterokai. Astaxanthin dalam struktur bebas adalah lebih cenderung untuk dioksidakan manakala astaxanthin yang wujud dalam krustacea merupakan astaxanthin dalam struktur kompleks (karotenoprotein, karotenolipoprotein dan khitinokarotenoid) yang kurang cenderung pada pengoksidaan. Tujuan utama penyelidikan ini adalah untuk menulenkan astaxanthin daripada sisa kulit udang melalui cara penapaian bakteria aerobic. Dalam usaha untuk mendapatkan astaxanthin daripada sisa udang, kitinase dan protease adalah penting proses pembebasan khitin dan protein daripada struktur pigmen yang stabil dan kompleks. Dalam kajian ini, objektif pertama adalah untuk mengasingkan bakteria yang berpotensi untuk degradasi sisa kulit udang daripada kulit udang. Bakteria yang berjaya diasingkan daripada kulit udang disaring menggunakan serbuk kulit ketam. Sebanyak 19 isolat yang menghasilkan kitinase dan protease telah diperolehi. Isolat yang berpotensi dibandingkan antara satu sama lain dengan menggunakan serbuk sisa kulit udang (SSWP) bagi mendapatkan isolat yang paling optimum dalam penghasilan enzim (kitinase dan protease). Isolat yang terpilih telah dikenal pasti sebagai *Aeromonas hydrophila*. Secara semulajadi, kulit udang mengalami proses kalsifikasi yang menyukarkan bagi mendapatkan lebih astaxanthin. Oleh itu, penambahan kaedah gangguan sel bertujuan untuk melonggarkan struktur kompleks kulit udang. Kaedah Dwi kesan 'ganguan kulit' digunakan dimana 'gangguan kulit' tanpa bahan kimia dikenakan pada sisa-sisa sebagai pra-rawatan dan diikuti oleh gangguan enzim mikrob dijadikan fasa kedua. Jumlah astaxanthin direkodkan selepas gangguan kulit adalah setanding antara rawatan kawalan (tiada pra-rawatan penapaian SSWP dengan enzim mikrob) dan autolisis (pra-rawatan SSWP dengan autolisis dan enzim mikrob). Rawatan kawalan telah memberikan perolehan astaxanthin sebanyak 2.3 ± 0.1179μg/ml manakala gabungan rawatan autolisis dan enzim mikrob menghasilkan 2.11 ± 0.0961μg/ml. Oleh kerana, kedua-dua rawatan memberikan hasil statiistik yang tidak signifikasi, gangguan secara tunggal (rawatan

kawalan) adalah memadai untuk menghasilkan perolehan yang baik. Dalam usaha untuk meningkatkan pengeluaran enzim dan pemulihan astaxanthin , media kultur dan keadaan telah dioptimumkan. Pengoptimuman kultur media dan penyediaan bakteria telah dilaksanakan dengan menggunakan analisis kaedah response surface (RSM). Kultur media telah disaring dengan pelbagai jenis bahan tambahan (sumber nitrogen, garam inorganic dan karbon) dan kepekatan (1, 3, 5, 7, 9% w/v) sebelum dioptimisasi dengan RSM. Media optima yang mengandungi 3% MSG, 1% glukosa, pH 7,0 dan 30 ° C dengan bekalan malar 0.1% K_2HPO_4 , 0.05% $MgSO_4.7H_2O$, dan 9% SSWP untuk kultur *A. hydrophila.* Sebagai perbandingan, penggunaan kultur media optima telah masing-masing memberikan pemulihan astaxanthin, peningkatan aktiviti enzim kitinase dan protease sebanyak 38.4%, 30% dan 36% berbanding media tidak optima. Bagi mencapai objektif utama dalam kajian ini, karotenoid telah ditulenkan dengan menggunakan kaedah kromatografi lapis tipis (TLC) dan kehadiran astaxanthin disahkan dengan menggunakan kromatografi cecair tekanan tinggi (HPLC). Karotenoid yang diperolehi selepas penapaian dengan bakteria dalam media optimum diekstrak dengan aseton menggunakan kaedah soxhlet dan dipekatkan sebelum digunakan dalam TLC. Campuran heksana : aseton (3: 1 v / v) adalah sistem eluen terbaik untuk pemisahan pigmen. Kehadiran astaxanthin dapat telah ditunjukkan oleh jalur yang terbentuk dari TLC pada nilai R_f 0.33 . Jalur ini kemudiannya diekstrak semula dengan aseton dan digunakan untuk pengesahan astaxanthin menggunakan HPLC dengan perbandingan kepada bahan rujukan piawaian. Astaxanthin disahkan kehadirannya pada sela masa minit ke 15.5, 16.4, 17.4 dan 18.3. Kesimpulannya, astaxanthin dapat diperolehi semula daripada sisa kulit udang melalui penapaian aerobik dengan *A. hydrophila*.

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I certify that a Thesis Examination Committee has met on 6 September 2016 to conduct the final examination of Cheong Jee Yin on her thesis entitled "Isolation, Optimization and Recovery of Astaxanthin from Shrimp Shell Wastes of Locally-Isolated *Aeromonas hydrophila* (Steiner)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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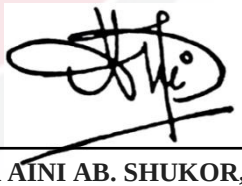

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%	percentage
°C	Degrees Celsius
µg	Microgram
µl	Microlitre
µm	Micrometre
16S/18S	Svedberg units
AMW	Apparent Molecular Weight
ANOVA	Analysis Of Variance
APCI	Atomic Pressure Chemical Ionization
ATCC	American Type Cell Culture
BLAST	Basic Local Alignment Search Tool
BLIS	Bacteriocin-Like Inhibitory Substances
CCD	Central Composite Design
CFU	Colony Forming Units
cm	centimetre
DAD	Diode Array Detector
DMAB	Dimethylamino benzoic acid
DMSO	Dimethyl Sulfoxide
DNA	Deoxyriboneuclotide Acid
EDTA	Ethylenediaminetetraacetic Acid
ESI	Electronic Spray Ionization
FAO	Food And Agriculture Organization Of The United Nations
g	Gram
g	Gravity
GlcNAc	N-acetylglucosamine

h	Hours
HCL	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HSD	Honest Significant Difference
K_2HPO_4	Dipotassium Hydrogen Phosphate
kDa	Kilo Dalton
L	Litre
LCMS	Liquid Chromatography Mass Spectrum
m	Molarity
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time Of Flight
mg	Milligram
$MgSO_4.7H_2O$	Magnesium Sulphate 7-Hydrate
mm	Millimetre
MSA	Minimal Synthetic Agar
MSG	Monosodium Glutamate
MSM	Minimal Synthetic Media
NaCl	Sodium Chloride
NAFLD	Non-Alcoholic Fatty Liver Disease
NCBI	National Center for Biotechnology Information
nm	Nanometre
NMR	Nuclear Magnetic Resonance
OCP	Orange Carotenoid Binding Proteins
OVAT	One Variable At Time
PMSF	Phenylmethylsulfonyl Fluoride
PTFE	Polytetraflouroethylene
CC	Column Chromatography

R_f	Retention Factor
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RSM	Response Surface Methodology
SCSP	Shrimp Crab Shell Powder
SFE	Supercritical Fluid Extraction
SFO	Single Factor Optimization
SPSS	Statistical Package For Social Science
SSW	Shrimp Shell Waste
SSWP	Shrimp Shell Waste Powder
TLC	Thin Layer Chromatography
USA	United States of America
USD	United States Dollar
UV	Ultraviolet
UV-VIS	Ultraviolet Visible
V	Volume
w	Weight
α	Alpha
β	Beta
λ	Lambda

CHAPTER 1

INTRODUCTION

In the recent years, the demand for food has been increasing drastically. As an example, the aquaculture industries have incredibly grown in the past 50 years (Bostok *et al.,* 2010). The global production of fisheries, crustacean and mollusks according to Fisheries Agricultural Organization has reached 91 million tons as of the year of 2012 with China being the largest producer (FAO, 2014). In food processing industries and human consumptions, an average of 48-56% of crustacean weight is being discarded, which includes antennae, carapaces, head, legs, shells, and tails (Pu *et al*., 2010). A number of valuable recoverable substances such as chitins, carotenoids (astaxanthin) and proteins can be recovered from these wastes. Numerous studies have shown the presence of carotenoid with astaxanthin being the main carotenoid pigment in shrimps (vary with species) (Sachindra *et al*., 2007; Shahidi *et al*., 1998). Being that crustacean wastes that are left to degrade naturally or being used as land filling (Prameela *et al*., 2012) are wasteful and causes environmental pollution; recycling the waste aids in environmental issues and benefits the recovery of various valuable substances especially astaxanthin.

Astaxanthin, (3'3-dihydroxy-β-carotene-4'4-dione) is naturally produced in microalgae such as *Haematococcus pluvialis,* certain yeasts; *Xanthophyllomyces dendrorhous,* (Dore and Cysewski, 2008) and the flower of the plant *Adonis aestivalis* (Cunningham and Gantt, 2011) which was native to Europe. Secondarily, astaxanthin is found in birds, fishes such as salmon, crustaceans such as krills, shrimps/prawn; *Penaeus monodon*, (giant tiger prawn) crayfishes and filter feeders up taken in their dietary supplements (Handayani *et al*., 2008). In certain shrimps, ingested beta-carotene can be converted into astaxanthin by the cell's activity (Latscha, 1990). Astaxanthin, which provides coloration in animals varies according to the environment and its complex structure, where it could be green, purple or blue (Sila *et al*., 2012) and red in cooked crustaceans or when exposed to heat (North, 2002). The complexity of astaxanthin can occur in three different forms in animals which are free molecules, forming complex with protein (carotenoprotein) or lipid (carotenolipoproteins), or sterified (esters) (Higuera-Ciapara *et al*., 2006).

This red pigment became important due to its unique DNA protective properties which can be used as a supplement to boost human health (Yoshida *et al*., 2010). Besides having high antioxidant properties (Capelli and Cysewski, 2007), astaxanthin is used in various industries such as cosmetics (Seki *et al*., 2001), feed additives to provide coloration (Paibulkichakul *et al.,* 2008), and medical researches (Bhuvaneswari *et al.,* 2010). The importance of astaxanthin in today's world has brought us to search for this pigment intensity to meet the demand of various industries from the basics of enhancing algae pigment production till genetically modified microbes to produce natural astaxanthin (Cheng and Tao, 2012). This is because synthetically produced astaxanthin do not give the same effect with natural astaxanthin although it fetches a lower price of 2000 USD/kg (Ni *et al.,* 2008) while naturally produced astaxanthin from algae biomass fetches a higher price at 7000 USD/kg (Li *et al*., 2011a).

The high price of natural astaxanthin renders a need to search for alternative ways to feed these needing industries. An alternative way is to recycle crustacean wastes as it contains natural astaxanthin. Previously recycling crustacean wastes was conducted using strong acids and alkali under high temperature to increase the degradation rate (Roberts *et al*., 2008). This traditional method is indeed efficient in recovering substance such as chitin but making it almost impossible to recover other substrates such as proteins, lipids and carotenoids (Healy *et al.,* 2003). Furthermore, the current method is expensive, non-environmental friendly (Rao *et al.,* 2002) and requires extra desalting before disposal due to high concentration of salts formed during neutralization (Wang *et al.,* 2006a). With the recent research advancement, managing crustacean waste by chemicals was less preferred due to the complications arise and not eco-friendly. Enzymatic degradation was more favorable compared to chemically treat as the former was more environmentally friendly (Paul *et al.,* 2015).

In the past two decades, alternative researches on managing crustacean wastes has been more focused on the use of enzymes to degrade crustacean waste. Proteolytic enzymes such as alcalases and trypsin (Synowiecki and Al-Khateeb., 2000), peptidases (Duarte de Holanda and Netto, 2006), and Triton X-100 (Mizani and Aminlari, 2007) have been used for hydrolysis on crustacean wastes. Enzymatic hydrolysis is certainly much more eco-friendly than the use of chemicals. However, commercial enzymes are costly to obtain and thus making it unsuitable for cost efficient studies (Prameela *et al.,* 2012).

Microbial fermentations were found to be the most flexible, eco-friendly, and economically viable method (Bashkar *et al*., 2007). Continuous supply of enzymes is certain with microorganism fermentation technique; unlike the usage of commercial enzymes where the enzyme capability has its limit (Synowiecki and Al-Khateeb, 2000). Instead of enzymatic hydrolysis which only lasts a few hours and may have incomplete hydrolysis, the continuous production of enzyme in microbial fermentation gives a more complete hydrolysis which gives a higher yield of recovery in the compound of interest (Jo *et al.,* 2008). However, microbial fermentation usually takes a longer completion time as compared to enzymatic hydrolysis.

In the interest of deproteinizing and demineralizing crustacean wastes for astaxanthin, lactic acid fermentation has shown great success in recovering the pigment (Khanafari *et al*., 2007). Lactic acid producing microbes has an advantage in demineralizing crustacean wastes due to the production of lactic acid during fermentation (Pacheco *et al.,* 2009). The acid has the ability to dissolve the calcium present in the shells causing demineralization and deproteinization to occur (Xu *et al*., 2008). However, this might seems to be a disadvantage in non-lactic acid producing microbes. Yet, non-lactic acid producing microbial fermentation has shown similar results in deproteinizing shrimp and crab wastes (Giyose *et al*., 2010; Oh *et al.,* 2007). Being that astaxanthin is usually present in shrimp exoskeleton as carotenoproteins, carotenolipoproteins or chitinocaroenoids which is a stable form (Sachindra, 2003). Deproteinization is indispensably required to remove the protein-astaxanthin bond. Possessing other enzymes or properties for non-lactic acid bacteria add an advantage to the fermentation system; for instance chitinase for dechitinization. Not only that, enzymes from the current bacteria might have similar potential with lactic acid fermentation as they

showed similar deproteinizing results. In terms of cost, lactic acid bacteria are more costly to be maintained than aerobic bacteria.

Until now, the knowledge on aerobic bacteria fermentation on recovery of astaxanthin from crustacean wastes is lacking. Aerobic fermentation of crustacean wastes is mainly focused on the recovery of chitins while the recovery of astaxanthin was less exploited. The only knowledge of astaxanthin recovery from shrimp shell wastes were by fermentations with lactic acid producing bacteria. Given that bacteria are ubiquitous in our environment (Earl *et al.*, 2008), they are cheap and easy to obtain. Therefore, this provides a route for exploration on the possible recovery of astaxanthin by aerobic bacteria fermentation. Moreover, the maintenance of microbes is less costly and environmentally friendly. The addition of optimization to the microbe culturing media may aid in enhancing its potential in astaxanthin recovery. In optimization studies conducted by Nisha and Divakaran, (2014), enzyme activity was significantly increased which eventually increases the activity of crustacean waste degradation. Meanwhile crustaceans having their carapaces calcified (Finlay *et al.,* 2009) may cause difficulties to be broken down. Since the current study lack of natural acid production during fermentation, therefore the addition of cell disruption may aid in decalcification or demineralization of these shell wastes rendering bond loosening. Traditionally, cell disruptions were conducted on algae/yeast (Foncesca *et al.,* 2011) and plant cells to assist inner cell substance extraction by breaking down the cell wall.

Therefore, the aim of the present study is to recover astaxanthin from shrimp shells through fermentation with aerobic bacteria which produces chitinase and protease. Both of these enzymes are required due to the presence of carotenoproteins and interlinks between chitins and proteins (Wang *et al*., 2007). In addition, shrimp shells are calcified and the selected bacterium may not produce natural acids to act as decalcifying agent. Therefore, the selection of a few basic cell disruption techniques may aid the process of astaxanthin extraction by removing minerals and possible calcium. Cell disruption techniques were chosen based on its success in extracting astaxanthin from algae and yeast cells (Xiao *et al*., 2008; Mendes-Pinto *et al.,* 2001). Unfortunately, its effectiveness on shrimp shells was yet to be determined as the shells were not living cells. So far there were not many records on optimizing both enzyme productions (namely chitinase and protease) in aid of astaxanthin recovery in addition to aerobic microbial fermentation. Due to that non-lactic acid fermenting bacteria are chosen for this study, both enzymes are crucial in deproteinization and dechitinization of the wastes. Although the knowledge on astaxanthin purification from shrimp shells has been well known, but the purification of pigment resulted from aerobic microbial fermentations on shrimp shell wastes was yet to be fully explored. With the current knowledge and technology, purification of astaxanthin from microbial fermentation possesses certain challenges in obtaining and maximizing the yield of the pigment.

Astaxanthin exists as a complex carotenoid structure in shrimps and is less likely to be oxidized. By using a green approach, this research focuses on releasing intact astaxanthin where the ultimate goal is to recover astaxanthin from shrimp shell wastes using aerobic bacteria. In order to achieve this, isolations were carried out to search for suitable deproteinizing and dechitinizing bacteria. The bacteria obtained may not be lactic acid producing and therefore requires external shell disruptions to further remove calcium and minerals present in the wastes. Selected shell disruption methods were

non-chemical based which cause less harm to the environment. Deproteinization and dechitinization are crucial in obtaining astaxanthin hence, optimization of the culture media and conditions were investigated to increase enzyme activities. An increase in enzyme activity leads to an increase in the amount of recoverable astaxanthin. This pigment will be extracted and purified with the common chromatography techniques (Thin Layer Chromatography and High Performance Liquid Chromatography) to confirm its presence through this aerobic fermentation system.

In order to achieve the present goal, four objectives were laid out as follows:

1. To isolate and identify bacteria from shrimp shell waste areas that produce maximum chitinase and protease
2. To evaluate the effectiveness of shell disruptions on astaxanthin recovery
3. To optimize growth media, pH, and temperature for optimal enzyme production and astaxanthin recovery
4. To quantify and validate astaxanthin recovered from the present microbial approach.

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BIODATA OF STUDENT

Born in the heart of Malaysia on November 5th, 1988; Cheong Jee Yin obtained her primary and secondary education from SK Convent Bukit Nanas Kuala Lumpur. Upon completing her secondary studies, she was offered a 3 years course by Universiti Putra Malaysia to further her studies in the field of Biology. During her final year of study in UPM, she was registered under Assoc. Prof. Dr. Muskhazli Mustafa to complete a final year project entitled "Deproteinization of crustacean shells wastes by *Bacillus subtilis* 14893". In 2011, she obtained a Bachelor's Honours Degree in Biology.

Upon graduation she was offered a Master's degree by the same undergraduate lecturer which she accepted the offer. In 2013, she successfully converted her Master degree studies into Doctoral degree. Within her years of study she has published two journal articles in her work field. Working on similar background of research, microbes and astaxanthin has been her passion of research.

LIST OF PUBLICATIONS

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