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Bioprospects of Coastal Ecosystem and Sustainable Resource Management

This book is the comprehensive collection of research-based data from the studies on coastal ecosystems of Malaysia (especially from the east coast of peninsular Malaysia). The book consists of nine chapters addressing the issues related to (but not limited to) the bio-prospect potential such as screening of actinomycetes from the coastal ecosystem, microbial bioprospecting using 'omics' approach, importance of integrated Multi-trophic Aquaculture, biotic diversity and shoreline erosion in coastal ecosystem. We are optimistic to say that the in-depth knowledge and scientific insights shared in this book will contribute towards sustainable development goals holistically and in particular on SDG 13,14 and 15.

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BIOPROSPECTS OF COASTAL ECOSYSTEM AND SUSTAINABLE RESOURCE MANAGEMENT

Akbar John
Zaima Azira Zainal Abidin
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TABLE OF CONTENT

Preface	i
Foreword 1	ii
Foreword 2	iii
Chapter 1: Seasonal Variations of Fish Diversity and Species Richness at the Coastal Water, Pekan, Pahang, Malaysia	1-16
Chapter 2: Study of Glucose-6-Phosphate Dehydrogenase Activity Assay in Mangrove Streptomyces for Actinohordin and Undercylprodigiosin Production	17-24
Chapter 3: Cultivation vs the ‘omics’ approach for microbial bioprospecting in the 21st century: Coastal environment in Malaysia	25-34
Chapter 4: Open Water Integrated Multi-Trophic Aquaculture (IMTA) in Coastal Ecosystem: The Status and Prospects in Malaysia	35-41
Chapter 5: Antioxidant Properties of *Nerita articulata* from Estuarine Mangrove Kuantan, Pahang Malaysia	42-51
Chapter 6: Heavy metal resistant bacteria from marine sediment of Pantai Balok, Pahang, Malaysia	52-59
Chapter 7: Salinity Tolerance and Growth Performance of Asian Seabass (*Lates calcarifer*) Juveniles	60-67
Chapter 8: Review: Actinomycetes Diversity and Biosynthetic Capabilities of East Coast of Peninsular Malaysia Coastal Water	68-79
Chapter 9: Climate Change and Coastal Defense in Malaysia: A Review	80-94

Study of Glucose-6-Phosphate Dehydrogenase Activity Assay in Mangrove Streptomyces for Actinohordin and Undercylprodigiosin Production

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ABSTRACT
This study evaluates the potential of using glucose-6-phosphate dehydrogenase activity assay for Actinohordin and Undecylprodigiosin productions from mangrove Streptomyces. Previously, there were several methods used to screen antimicrobial activities such as agar spot test and disc diffusion assay, but those are lengthy screening methods and time consuming. Thus, to overcome the limitations plate-based assay is suggested to enable rapid screening on secondary metabolite production of numerous samples at one time. The development of plate-based assay was performed by optimizing glucose-6-phosphate dehydrogenase activity assay. This coupled assay was based on the production of dihydronicotinamide-adenine dinucleotide phosphate (NADPH) whereby a right combination of nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G6P) were refined. The production of NADPH was measured at absorbance of 340 nm where reduced cofactor NADPH are absorbed readily at this wavelength. Sample with different concentrations of crude lysate was subjected to various substrates concentration to obtain the best activity curve. Even though elucidating clear patterns is speculative, it is believed that some improvements or optimizations of this study could offer promising knowledge which can serve as useful reference in future.

Keywords: *Actinohordin, Dihydronicotinamide-Adenine Dinucleotide Phosphate, Nicotinamide Adenine Dinucleotide and Undecylprodigiosin.*

INTRODUCTION
Actinomycetes are gram-positive filamentous bacteria that produce aerial hypae and differentiate into chains of spores (Kämpfer, 2015; Barka *et. al.*, 2016). They can be found in soil, freshwater and marine environments. They produced various useful compounds known as secondary metabolites with important applications such as antibiotics tetracycline, erythromycin, vancomycin and streptomycin (Weber *et al,* 2015). During the past thirty years, researchers have shown an increased interest towards antibiotics-producing bacteria as they give many benefits in human medicine as well as in commercial production.

Previously, the antimicrobial activities of secondary metabolites were assessed either by covering an isolation plate with indicator organism or agar-spot test where it has been used to detect antagonistic activity between bacteria (Kun, 2003). However, these methods have major limitations where potential contamination of selected colonies with indicator organisms could occur. In addition, they are lengthy screening methods as only one indicator organism can be applied to each isolation plate at a time. Apart from that, HPLC is also one of the options of screening methods, yet time consuming (Ethiraj *et al.,* 2011).

Nonetheless, secondary metabolites are typically produced at a very low amount in nature. Thus, many researches have been done previously to study the metabolic network of central carbon metabolism, precursors and cofactors required in synthesizing secondary metabolites to improve the product yield (Fan *et al.,* 2016). It is found that the amounts of precursors for secondary metabolite production required from primary metabolism gradually becomes limited as the product yield increases. Therefore, it is necessary to

supply an adequate number of precursors which is generally provided by catabolism of carbon substrates to obtain high yield of secondary metabolites.

Thus, to optimize the enzyme assay, a study was designed to induce the production of two secondary metabolic compounds, actinohordin (ACT) and undecylprodigiosin (RED) by targeting the pentose phosphate pathway (PPP) of *Streptomyces*. This is performed by promoting the conversion of the first enzyme of the pathway, which is glucose-6-phosphate dehydrogenase (G6PDH) by finding the best ratio combination of its substrates; glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide (NAD). This is to ensure G6PDH enzymes are supplied with adequate amounts of substrate in order to maximize the production of NADPH prior catalyzing the second metabolic pathway which in concert will enhance the antibiotic production as suggested by Gunarson *et al.,* (2004). Essentially, NADPH is the reducing agent used in the process of making secondary metabolites.

ACTINOMYCETES

The name actinomycetes was derived from Greek word "aktis" which means a ray and "mykes" which refers to fungus. This name was given by looking at their morphology where they possess characteristics of both bacteria and fungi (Das *et al.,* 2008) but yet, they are categorized into bacteria kingdom (Madigan *et al.,* 2009). They contain DNA rich in G+C at about 57-75% (Lo *et al.,* 2002) which are phylogenetically related from evidence of 16s ribosomal cataloguing and DNA: rRNA pairing studies by Goodfellow & Williams (1983). They are characterized by a complex life cycle, as described by phylum Actinobacteria, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria (Ventura *et al*., 2007).

Actinomycetes are commonly found in both terrestrial and aquatic ecosystems which mainly in soil. They play an important role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants, animals and fungal materials resulting in production of many extracellular enzymes which are conductive to crop production (Chaudhary *et al.,* 2013). In addition, actinomycetes also give major effects in biological buffering of soils, biological control of environments by nitrogen fixation and degradation of high molecular weight compounds like hydrocarbons in the polluted soil. Thus, these microorganisms play vital roles in maintaining our ecosystems.

Above all, actinomycetes are valuable bacteria which are commonly known due to their ability to produce secondary metabolites. Berdy (2005) reported that 10000 out of 23000 bioactive secondary metabolites produced by microorganisms originate from actinomycetes bacteria, representing 45 % of all bioactive microbes discovered. Among various genera of actinomycetes, the major producers of commercially bioactive compounds are *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora and Actinoplanes* (Solanki *et al.,* 2008).

***Streptomycetes coelicolor* A3 (2)**

Streptomycetes species are aerobic and gram-positive bacteria that show filamentous growth from a single spore. A network of branched filaments called as a substrate mycelium will be formed when their filaments grow through tip extension and branching (Dyson, 2011). They are widely recognized as they are the major producer and have produced a total of 7600 compounds (Berdy, 2005). As a result, *streptomycetes* have become the primary antibiotic-producing actinomycetes exploited by the pharmaceutical industry.

Streptomyces coelicolor A 3(2), is the best-known strain of secondary metabolite producer of streptomycetes. According to Zhu *et al.,* (2014), many secondary metabolites have been discovered from this strain such as actinohodin (ACT), undecylprodigiosin (RED), calcium-dependent antibiotic (Cda), and the plasmid-encoded methylenomycin (Mmy). Besides, *S. coelicolor* genome sequence still revealed many previously unidentified biosynthetic gene clusters including one for a likely antibiotic called cryptic polyketide (Cpk) even after 50 years of research on it. A sequence study on antibiotic gene clusters and the

complete genome of *S. coelicolor* revealed that such microorganisms are probably capable of producing a greater number of secondary metabolites (Higginbotham & Murphy, 2010).

ACTINORHODIN (ACT) AND UNDECYLPRODIGIOSIN (RED)

S. coelicolor synthesizes two chemically distinct pigments which are generally regarded as secondary metabolites which are actinorhodin (ACT), a diffusible red-blue pH indicator and undecylprodigiosin (RED), a red cell-wall associated compound (Rudd & Hopwood, 1980). During the past thirty years, researchers have shown an increased interest in RED compounds due to their immunosuppressive and anticancer properties in addition to antimicrobial activities. Meanwhile, ACT compound exhibit antibacterial activity against gram-positive cells (Mak, Xu & Nodwell, 2014)

Actinorhodin is an aromatic polyketide synthesized by enzymes encoded in a 22-kb gene cluster. The gene cluster responsible for actinorhodin production contains the biosynthetic enzymes and genes responsible for export of the antibiotic. The actinorhodin biosynthetic cluster also encodes a pathway-specific activator (actII-orf4) that activates the biosynthetic genes. This activator gene is in turn subject to the action of global regulators that can either activate or repress its expression (Craney, Ahmed & Nodwell, 2013). Furthermore, their production occurs using a type II polyketide synthase (PKS). The formation of actinorhodin started as the carbon backbone is produced entirely from fatty acids precursors, acetyl-CoA and malonyl-CoA in primary metabolism.

Meanwhile, undecylprodigiosin is a red pigmented, cell wall-associated antibiotic that belongs to a group of polypyrrole bioactive compounds called prodiginines (Luti & Yonis, 2014) which is directed by a 30-kb gene cluster. Two pathway-specific transcriptional activators involved for the activation of the prodiginine gene are RedZ and RedD. In the pathway, RedZ functions as a direct activator of RedD which then acts on the biosynthetic genes (Craney, Ahmed & Nodwell, 2013).

A study has been conducted with aimed to determine the relationship between secondary metabolite production and growth media composition. As a result, it shows that Act produced mainly in the stationary phase of batch cultures grown with glucose and sodium nitrate as the sources of carbon and nitrogen. Meanwhile, Red accumulated during the exponential phase. The production of both pigments were sensitive to the levels of ammonium and phosphate in the medium (Hobbs *et al.,* 1990).

Besides, several studies have been done on deletion of the coding region of the ppGpp synthetase gene, relA in *Streptomyces celicolor* A3 (2) correspond with antibiotic production. They noted that there is correlation between ppGpp synthetase gene, relA and the onset of undecylprodigiosin (Red) and actinorhodin (Act) production, leading to the suggestion that ppGpp plays a central role in triggering antibiotic synthesis (Chakraburtty *et al.,* 1996).

Studies of batch cultures, some of which were subjected to amino acid starvation, indicated a correlation between ppGpp synthesis and transcription between pathway specific regulatory genes for Red and Act (the two pigmented antibiotics made by the strain). The relA null mutant was grown at the same rate as the parental strains resulting in depletion production of both Act and Red under condition of nitrogen limitation, but appeared to produce normally under other conditions (Chakraburtty, R., & Bibb, M. 1997). This indicates that actinorhodin and undecylprodigiosin cannot be produced due to ppGpp synthetase gene, relA cannot work at its best under amino acid starvation.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH) ASSAY

Previously, many researches had proved that the production of secondary metabolites depends on precursors supplement from primary metabolism. For instance, in 2012, a study was conducted by Wentzel *et al*, to find the relationship between carbon fluxes towards biomass formation and antibiotic production by changing carbon and nitrogen sources or varying initial seeding volumes of cells in cultivation media

(Cheng *et al*., 2013). Both studies had revealed that the reaction related to the amino acid pathway helped in concentrating fluxes towards the biosynthesis of various precursors required for synthesizing secondary metabolites.

Following this, the recent study has been conducted by targeting pentose phosphate pathways to improve the production of secondary metabolites (Actinorhodin and Undecylprodigiosin). As mentioned by Fan *et al.,* (2016), pentose phosphate pathway plays an important role in secondary metabolite production and is considered as the sources of precursors.

G6PDH + G6P + NAD → 6-phospho-D-glucono-1,5-lactone + NADPH

This is performed by maximizing the conversion of the first enzyme of the pathway, glucose-6-phosphate dehydrogenase (G6PDH) by supplying an adequate number of substrates which are glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide (NAD) to improve production of NADPH. As per suggested by Gunarson, Eliasson & Nielsen (2004), NADPH plays an important role in enhancement of secondary metabolites. NADPH is the reducing agent used in the process of making secondary metabolites, and the pentose phosphate pathway is one of the most important NADPH-producing pathways. The first enzyme of the pathway, glucose-6-phosphate dehydrogenase (G6PDH) is generally considered as an exclusive NADPH producer.

MATERIALS AND METHODS

BACTERIAL STRAINS

Streptomyces sp. K2-11 were taken from laboratory collections (Research Lab 3, Kulliyyah Science, IIUM Kuantan) which were isolated from mangrove sediment of Tanjung, Lumpur, Kuantan, Pahang.
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PREPARATION OF MEDIA

Nitrogen limiting SMMS medium

As much as 2 g of Difco casamino acids, TES buffer (5.68Gl^{-1}) and Bacto agar were dissolved in distilled water. Then the pH was adjusted to 7.2 using 10 M NaOH prior autoclaving. The media with the following ingredients were added with specific amount: NaH_2PO_4 + $K_2H_2PO_4$ (50 Mm each, 10 mL per litre of culture), $MgSO_4.7H_2O$ (1 M, 5 mL per litre of culture), glucose (50% w.v, 18 mL per litre of culture). The trace elements that contain o.1 gL^{-1} each of $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CaCl_2.6H_2O$ and NaCl. The solution was stored at 4^0C in a refrigerator.

CULTURING of *Actinomycetes*

All of the bacterial strains were grown on nitrogen limiting SMMS medium. The samples were incubated at 28^0C, agitated at 120 rpm for fourteen days.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY

Preparation of Extracts

The method was performed according to the protocol by Borodina *et al.,* (2008). Cells used for activity assays were harvested after 67 h of growth in 200 ml of defined medium in a 1-liter flask equipped with a stainless-steel spiral. Cells were harvested by centrifugation and resuspended in buffer containing 50 mM TES, pH 7.2, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM $(NH_4)_2SO_4$, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). Lysozyme (add in the concentration) was used to break the cells.

G6PDH activity Assay

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) assays are based on the production of NADPH and were performed according to the protocol of Lessie and Wyk, (1972) and modified by Butler *et al.,* (2002). Both the consumption of NADH and the production of NADPH were measured spectrophotometrically at 340 nm. The crude lysates were applied to the G6PDH activity assay using supplied substrates (G6P and NAD). The assay was performed in a 96-well plate for two minutes enabling simultaneous analysis of a large number of samples.

G6PDH + G6P + NADP → 6-phospho-D-glucono-1,5-lactone + NADPH

RESULTS AND DISCUSSION

EXTRACTS PREPARATION

Five genera of Actinomycetes which are *Streptomyces, Micromonospora, Nocardia, Nocardiopsis* and *Rodhococcus* were taken from laboratory collections. These microbes have been identified and known to produce antimicrobial activity. All of the isolates were grown on nitrogen limiting SMMS medium. However, due to time constraint, only *Streptomycetes* was chosen to be assayed for secondary metabolite production. The *Streptomycetes* was grown on SMMS plate for five days and was subcultured into SMMS broth for another three days according to the protocol by Borodina *et al.,* (2008). Then, the cells were harvested by using centrifugation and resuspended in a buffer and then repeated for three times. This is to make sure the 90 % of cells was lysed and released the protein. Phenylmethylsulfonyl fluoride which is known as a serine protease inhibitor was included in the buffer to prevent the protein degradation.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAYS

The crude lysates were applied to the G6PDH activity assay using supplied substrates (G6P and NADP). The assay was performed in a 96-well plate that enabling simultaneous analysis of a large number of samples. The reaction was monitored by measuring the absorbance at 340 nm for two minutes and reduced cofactor, NADPH were absorbed readily at this wavelength.

The reaction rates measured at different substrates and protein concentration were shown in Figure 4.1. In order to obtain the best activity curve for the given condition, seven samples of different crude lysates concentrations were prepared (100 µL, 50 µL, 25 µL, 12.5 µL, 6.25 µL, 3.125 µL, and 1.5625 µL). Then, all the samples were subjected to various substrate concentrations to screen for the best enzyme activity. In this study, eight substrate concentrations were chosen to be tested with different enzyme concentrations (2 µM, 5 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM and 60 µM). The results show that the rate of reaction of various substrate concentrations were increased as the enzyme concentration increased. Reaction with 20 µM of substrate has the highest enzyme activity. Meanwhile, the least enzyme activity was shown in reaction with 50 µM of substrate for all enzyme concentration tested.

Figure 4.1 shows that at higher concentrations of crude lysates specifically 100 µM, 50 µM and 25 µM, the reaction was not stable when subjected to lower concentration of substrates (2 µM, 5 µM, 10 µM, 20 µM). However, the reactions started to increase at substrate concentration 30 µM to 60 µM. These conditions were contradicted with the reaction shown by lower concentrations of crude lysates (12.5 µM, 6.25 µM, 3.125 µM and 1.5625 µM) where the reaction increased slightly at lower concentration of substrates and decreased with in the presence of high concentration of substrate. Hence, it can be seen that higher enzyme and substrate concentration will increase the activity whereas lower concentration of enzyme with higher concentration of substrate will reduce the activity.

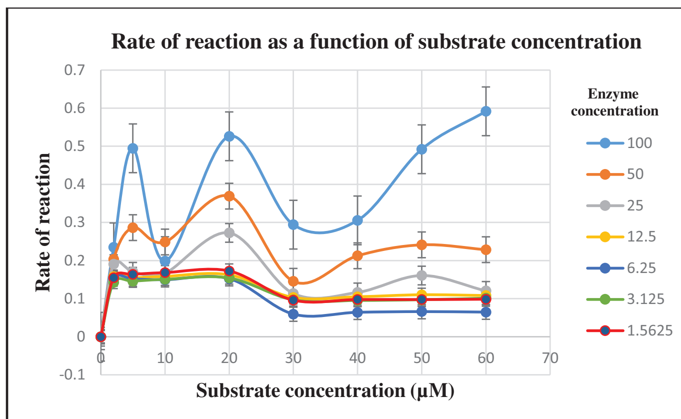

Fig. 4.1: Measurement of enzyme activities from crude lysates produced at wavelength 340 nm with different substrate concentrations. All readings have been normalized with control

Overall, it can be concluded that the enzyme activity works at its best with increasing enzyme's concentrations as well as substrate. However, a better assay could be conducted by using a purified enzyme. According to Sharma and Chand, (2012), purified protein exhibits better activity readings compared to crude enzymes. This might be due to protein impurities present in the reaction which may interfere with the absorbance readings.

According to Bisswanger (2014), there are several factors that may affect the assay other than pH, temperature and ionic strength. For example, the actual concentrations of all assay components. This may contribute to the deviations from the optimum conditions of the protein which causes a reduction of the activity. For instance, enzyme reactions dependent on ATP need Mg^{2+} as essential counter ions. The assay mixture will become limiting if only ATP without Mg^{2+} were added even in sufficient concentration especially if complexing compounds like inorganic phosphates or EDTA are present. In this study, this also could be considered as a contributing factor on the fluctuated readings. This physicochemical property of G6PDH enzymes needs further study for a better assay condition.

CONCLUSION

This preliminary attempt to optimize glucose-6-phosphate dehydrogenase activity assay was encouraging. Even though glucose-6-phosphate dehydrogenase activity assay was not fully optimized, there is some knowledge that we can still perceive out of this project. One of the knowledge was this enzyme is an allosteric which does not obey the Michealis –Menten kinetics due to the presence of multiple binding sites. It is believed, with improvement of certain factors like using purer enzymes, the study could offer more promising results. In addition, this protein has higher potential towards secondary metabolite production through the formation of NADPH as G6PDH is generally considered as NADPH producer through pentose phosphate pathway (PPP). Nevertheless, an intense research on the physical and physicochemical properties of G6PDH should be conducted for a better understanding of the whole enzymatic reaction.

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