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HETEROLOGOUS PRODUCTION OF L-GLUTAMINASE THROUGH SCREENING AND ISOLATION FROM LOCALLY ISOLATED MICROBES

(PENGHASILAN L-GLUTAMINASE HETEROLOGUS MELALUI PENCIRIAN DAN PENGASINGAN DARIPADA MIKROB TERPENCIL TEMPATAN)

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ABSTRACT

L-glutaminase present in living organisms namely human, animals, plants, and microorganisms. L-glutaminase has been identified having potential applications for several industries including pharmaceutical, food, and health care. Industrial sectors nowadays demand natural producing enzyme compared to artificial enzymes due to several safety issues. On top of that, L-glutaminase extracted from microorganisms has more advantages and benefits to fulfil demands from industries. Up until now, less research about bacteria production of L-glutaminase from Malaysia marine environments had been carried out. Hence, the objectives of this research study is to have an in-depth investigation on L-glutaminase production, optimization and characterization of partially-purified L-glutaminase from bacteria of Malaysia marine environment. In this study, the screening of bacteria-producing L-glutaminase had been implemented from three different Pahang Beaches known as Pantai Teluk Cempedak, Pantai Batu Hitam, and Pantai Balok. This was followed by biochemical characterizations and 16S rRNA gene sequencing to identify the identity of bacteria that showed positive results on L-glutaminase production. Additionally, production optimization, enzyme purification and characterization were carried out from the best L-glutaminase producer. The highest enzyme activity recorded was *Kosakonia radicincitans* with 0.103 U/ml followed by *Shigella flexneri* which has the enzyme activity of 0.100 U/ml. Furthermore, two isolates that had the highest enzyme activity were selected for optimization of process parameters through One Factor at A Time method. The parameters involved are temperature, pH, additional of organic and inorganic nitrogen sources, and additional of carbon sources. It was found out that *K. radicincitans* had the highest enzyme activity of 0.3542 U/ml at temperature 37°C, pH 7, additional of beef extract for organic nitrogen source, additional of ammonium chloride for inorganic nitrogen source, and additional of glucose for carbon source. L-glutaminase extracted from *K. radicincitans* was further partially purified and characterized. It had molecular weight of 70 kDA. L-glutaminase was most reactive and stable at temperature of 37ºC and pH 7. The information on production, optimization, and characterization of L-glutaminase are very important in order to understand its characteristics and suitability for industrial usage.

ABSTRAK

L-glutaminase boleh didapati di dalam organisma – organisma termasuklah manusia, binatang, tumbuh-tumbuhan, dan mikroorganisma. L-glutaminase telah dikenalpasti mempunyai peluang aplikasi di dalam sektor perindustrian seperti farmasuetikal, makanan, dan perubatan. Enzim yang dihasilkan secara semulajadi berbanding secara tiruan mendapat permintaan yang tinggi daripada sektor industri pada masa kini kerana beberapa isu keselamatan. Selain itu, L-glutaminase yang diekstrak daripada mikroorganisma mempunyai lebih banyak kelebihan dan kebaikan untuk memunuhi permintaan daripada industri. Kekurangan kajian mengenai bakteria yang menghasilkan L-glutaminase daripada persekitaran marin Malaysia masih kurang sehingga sekarang. Oleh itu, objektif kajian ini adalah untuk membuat penyelidikan dengan lebih mendalam mengenai penghasilan L-glutaminase, pengoptimum dan pencirian separa penulenan L-glutaminase daripada bakteria marin Malaysia. Di dalam kajian ini, saringan bakteria yang menghasilkan L-glutaminase telah dijalankan daripada tiga pantai berbeza di Pahang iaitu Pantai Teluk Cempedak, Pantai Batu Hitam, dan Pantai Balok. Kemudian, karakter biokimia serta 16S rRNA penyusunan gen telah dijalankan untuk mengenal pasti identiti bakteria yang mempunyai keputusan positif terhadap penghasilan L-glutaminase. Tambahan pula, penghasilkan, pengoptimum, penulenan enzim, dan pencirian telah dilakukan terhadap produser terbaik L-glutaminase. Bakteria yang menghasilkan bacaan aktiviti enzim tertinggi adalah *Kosakonia radicincitans* yang mempunyai bacaan sebanyak 0.103 U/ml diikuti *Shigella flexneri* yang mempunyai bacaan sebanyak 0.100 U/ml. Selain itu, dua bakteria yang mempunyai bacaan aktiviti enzim tertinggi telah dipilih untuk menjalani proses pengoptimum parameter melalui kaedah Satu Faktor dalam Satu Masa. Parameter yang terlibat adalah suhu, pH, penambahan organic dan bukan organic sumber nitrogen, dan penambahan sumber karbon. *K. radicincitans* telah didapati mempunyai aktiviti enzim tertinggi iaitu 0.3542 U/ml pada suhu 37°C, pH 7, penambahan ekstrak daging lembu sebagai sumber nitrogen organik, penambahan ammonium klorida sebagai sumber nitrogen bukan organik, dan penambahan glukosa sebagai sumber karbon. Kemudian, separa penulenan L-glutaminase yang diekstrak daripada *K. radicincitans* telah dijalankan dan dipercirikan. Berat molekularnya adalah 70 kDA. L-glutaminase yang paling aktif dan stabil adalah pada suhu 37°C dan pH 7. Ilmu mengenai penghasilan, pengoptimum, dan pencirian L-glutaminase adalah sangat penting untuk memahami ciri-ciri L-glutaminase dan kesesuaiannya untuk diaplikasikan dalam sektor industri.

TABLE OF CONTENTS

LIST OF TABLES **viii**

LIST OF FIGURES **ix**

LIST OF SYMBOLS **xi**

LIST OF ABBREVIATIONS **xii**

CHAPTER 1 INTRODUCTION **1**

1.1 Background of study 1

1.2 Problem Statement 2

1.3 Research objectives 3

1.4 Scope of study 3

CHAPTER 2 LITERATURE REVIEW **4**

2.1 Marine as a local source of enzyme 4

2.2 L-glutaminase in general 4

2.3 L-glutaminase producer 6

2.3.1 L-glutaminase from mammals 6
2.3.2 L-glutaminase from bacteria 7
2.3.3 L-glutaminase from fungi 8
2.3.4 L-glutaminase from actinomycetes 9

2.4 Marine source of L-glutaminase 9

2.5 L-glutaminase Production and Optimization 11

2.6 L-glutaminase Purification 13

2.7 Application of L-glutaminase 14

2.7.1 Application in food industry 14
2.7.2 Therapeutic Application 15
2.7.3 L-glutaminase as a biosensor 16
2.7.4 Manufacture of fine chemicals from L-glutaminase 16

CHAPTER 3 RESEARCH METHODOLOGY **17**

3.1 Samples collection and preparation 17

3.1.1 Method of Serial Dilution 17
3.1.2 Preparation of Minimal Glutamine Agar Media 18

3.2 Screening and isolation of Bacterial L-glutaminase Producers 19

3.2.1 Primary screening 19
3.2.2 Secondary screening 19

3.3 Inoculum and Culture broth preparation 20

3.4 Biochemical Characterization of the Microbes 21

3.4.1 Endospore staining 21
3.4.2 Catalase test 21
3.4.3 Oxidase test 21

3.5 16S rRNA Gene Sequence Analysis 21

3.6 Sequence analysis and phylogenetic tree construction 22

3.6.1 Nucleotide sequence analysis 22
3.6.2 Amino acid alignment and phylogenetic analysis 23

3.7 Assay of L-glutaminase 23

3.7.1 Method for standard curve of enzyme analysis 23

3.8 Optimization of process parameters 24

3.8.1 Incubation temperature 24
3.8.2 Additional of organic nitrogen sources 25
3.8.3 Additional of inorganic nitrogen sources 25
3.8.4 Additional of carbon sources 25
3.8.5 Initial pH values of the medium 26
3.8.6 Experiment with all parameters at its optimal level 26

3.9 Partial purification of L-glutaminase 26

3.10 Determination of protein concentration 26

3.10.1 SDS-PAGE analysis 26

	3.10.2 Bradford assay	27
3.11	Bacteria Growth Curve	27
3.12	Characterization of partially purified L-glutaminase	28
	3.12.1 Incubation temperature	28
	3.12.2 Temperature stability	28
	3.12.3 Ph	28
	3.12.4 pH Stability	29
CHAPTER 4	**RESULTS AND DISCUSSIONS**	**30**
4.1	Primary screening of L-glutaminase producers from collected samples	30
4.2	Secondary screening of L-glutaminase producers from positive cultures	32
4.3	Biochemical characterization of the microorganisms	35
	4.3.1 Endospore staining	35
	4.3.2 Catalase test	35
	4.3.3 Oxidase test	36
4.4	16S rRNA Gene Sequence Analysis	36
	4.4.1 Nucleotide sequence analysis	37
4.5	L-Glutaminase Assay Results	41
4.6	Optimization of process parameters	43
	4.6.1 Temperature	43
	4.6.2 Organic Nitrogen Sources	45
	4.6.3 Additional of Inorganic Nitrogen	46
	4.6.4 Carbon sources	48
	4.6.5 pH	49
	4.6.6 Experiment with all parameters at its optimal level	51
4.7	Partial purification of L-glutaminase	51
4.8	Determination of protein concentration	53
	4.8.1 SDS-PAGE and Bradford Analysis	53
4.9	Bacteria Growth Curve	55
4.10	Characterization of partially purified L-glutaminase	56

4.10.1 Temperature 56
4.10.2 Temperature Stability 57
4.10.3 pH 58
4.10.4 pH stability 59

CHAPTER 5 CONCLUSION **61**

5.1 Conclusion 61

5.2 Future studies 62

REFERENCES **63**

APPENDICES **64**

LIST OF TABLES

Table No.	Title	Page
Table 2.1	Sources of L-glutaminase from mammals and microorganisms	15
Table 2.2	Sources of L-glutaminase from marine bacteria, fungi, and yeast	20
Table 2.3	Method of L-glutaminase production from different type of microorganisms	23
Table 3.1	A set of universal primers used in colony PCR	34
Table 4.1	Species identified for sequences of 12 isolates obtained from 16S rRNA gene sequencing using BLAST database from NCBI	50
Table 4.2	Absorbance at 450 nm of wavelength and enzyme activity of 12 positive isolates from Lg1 to Lg12	55
Table 4.3	Ammonium sulphate precipitation of L-glutaminase	67
Table 4.4	Bradford analysis for crude and partially purified L-glutaminase	69

LIST OF FIGURES

Figure No.	Title	Page
Figure 4.1	Primary screening of samples collected from Pantai Teluk Cempedak (a and b) and Pantai Batu Hitam (c and d)	44
Figure 4.2	Patch-streak technique used for primary screening on positive isolates of L-glutaminase production	45
Figure 4.3	Secondary screening of L-glutaminase producers collected from primary screening	47
Figure 4.4	Results of electrophoresis that showed DNA bands after purification	52
Figure 4.5	A phylogenetic tree of isolates based on 16S rRNA gene sequence. The zika virus strain was used as an out-group to root the tree.	53
Figure 4.6	Enzyme activity (U/ml) of 12 successful positive isolates. Lg8 known *as K. radicincitans* has the highest enzyme activity which is 0.103 ± 0.001 U/ml followed by Lg11 identified as *S. flexneri* which has the enzyme activity of 0.100 ± 0.002 U/ml	56
Figure 4.7	Enzyme activity of L-glutaminase from K. radicincitans (A) and *S.flexneri* (B) at five different incubation temperatures	58
Figure 4.8	Enzyme activities of L-glutaminase from *K. radicincitans* (A) and *S. flexneri* (B) in culture media containing different type of organic nitrogen sources.	59
Figure 4.9	Enzyme activities of L-glutaminase from *K. radicincitans* (A) and *S. flexneri* (B) in culture media containing different type of inorganic nitrogen sources	61
Figure 4.10	Enzymatic activity of L-glutaminase extracted from *K. radicincitans* (A) and from *S. flexneri* (B) in culture media containing different type of carbon sources	63
Figure 4.11	Enzyme activity of L-glutaminase extracted from *K. radicincitans* (A) and *S. flexneri* (B) in different pH environment	64
Figure 4.12	SDS – PAGE of partially purified L-glutaminase, M: protein molecular mass marker, Lane 1: sample of crude enzyme and Lane 2: partially purified L-glutaminase supernatant	68

Figure 4.13	Time course of L-glutaminase production by *K. radicincitans* at optimized conditions	70
Figure 4.14	Enzymatic activity of partially purified L-glutaminase at five different incubation temperature	71
Figure 4.15	Enzymatic activity of partially purified L-glutaminase at five different incubation temperature	73
Figure 4.16	Enzymatic activity of partially purified enzyme at five different pH	74
Figure 4.17	Enzymatic activity of partially purified enzyme at five different pH stability	75

LIST OF SYMBOLS

%	Percentage
ºC	Celcius
M	Molarity
mL	Mililitre
ml/min	Millilitre per min
g	Gram
g/l	Gram per litre
V	Voltage
µl	Microliter
F	Forward
R	Reverse
mM	Milimolar
µM	Micromolar
nm	Nanometers
µmol	Micromole
U/ml	Unit per millilitre
w/w	Weight per weight
v/v	Volume per volume
µm	Micrometer
mg	Miligram
kDA	Kilo-Dalton
mg/ml	Milligram per mililitre

LIST OF ABBREVIATIONS

sp.	Species
DEAE	Diethylaminoethyl cellulose
rpm	Rotation per minute
N	North
E	East
LB	Luria Bertani
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
T_m	Melting point
DNA	Deoxyribonucleic acid
NCBI	National Center for Biotechnology Info
BLAST	Basic local alignment search tool
MEGA7	Molecular evolutionary genetic analysis 7 software
NJ	Neighbour-joining
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV	Ultraviolet
ATP	Adenosine triphosphate
OFAT	One factor at a time method
NH_4Cl	Ammonium chloride
$NaNO_3$	Sodium nitrate
NH_4SO_4	Ammonium sulphate
KNO_3	Potassium nitrate

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Biotechnology is a field that uses living organisms to enhance human health and environment. It applies both scientific and engineering principles to treat materials using biological agents in order to produce desired products and services. Enzymes, also popularly known as biological catalyst is one of the most vital tools for biotechnology. The major advantage of applying enzyme rather than chemical agent as an industrial catalyst is due to their specificity, precision, efficacy, convenience and economy.

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) is an enzyme that has the ability to catalyzes L-glutamine to L-glutamic acid and ammonia (Kiruthika & Saraswathy, 2013). Over the years, L-glutaminase is popularly known and believed to have potential applications in both food and pharmaceutical industries. L-glutaminase is proposed as an important therapeutic agent for cancer therapy especially for acute lymphocytic leukemia (Abdallah, Amer, & Habeeb, 2012). L-glutaminase is being regarded as anticarcinogenic along with the inability of lymphatic tumor cell to synthesize glutamine that causes glutamine depletion compared to normal cells. Furthermore, this enzyme also has attracted huge attention in food industries as food flavouring agent. It controls the tastefulness of fermented condiments such as soy sauce by increasing the glutamic acid content which widely acclaimed as flavour-enhancing amino acid (Weingand-Ziadé, Gerber-Décombaz, & Affolter, 2003).

The marine biosphere is one of the habitat that is densely populated by microorganisms and less characterized. Halophiles that produce halotolerance enzymes provide an alternative for therapeutic application. In addition, sea water has huge potential for offering biomolecules such as enzymes that could have no or less side effects when used in therapeutic purpose. This could be due to its characteristics which is saline in nature and chemically close to human blood plasma (Kiruthika & Saraswathy, 2013). Halotolerance enzyme could also be benefited by food industry as large amounts of salts are used in preparation of certain types of traditionally fermented foods.

1.2 Problem Statement

In industrial sectors, there are demands for natural producing enzyme with high productivity as there are a lot of safety issues about the artificial enzymes and chemical catalysts. This microbial L-glutaminase could be one of the enzyme that can fulfilled the demand since it is extracted from biological means which produce naturally. L-glutaminase can also be derived from plant and animal tissues. However, the production of natural flavours by direct extraction form plants is also subject to various problems. These raw materials often contain low concentrations of the desired compounds, making the extraction expensive. Moreover, their use depends on uncontrollable factors such as weather conditions and plant diseases. Direct extraction from plants showed more disadvantages compared to advantages hence more investigations were carried out to search for other strategies to produce natural flavours (Longo & Sanromán, 2006). Therefore, researches are trying to extract L-glutaminase enzyme from microorganisms including bacteria, yeast, and fungi (Sajitha, Vasuki, Suja, Kokilam, & Gopinath, 2013). In addition, bacteria is preferred for L-glutaminase producers owing to their uncomplicated processing and handling techniques and have simple requirements regarding their growth and cultivation as well as cheaper production cost (A Sabu, 2003).

Screening of L-glutaminase from marine bacteria had been reported by Iyer and Singhal (2009) and Katikala et al. (2009). Meanwhile, production of L-glutaminase from marine *Actinomycetes* and *Streptomyces* had been presented by Balagurunathan et al. (2010) and Krishnakumar et al. (2011), respectively. Sabu and Chandrasekaran (1999) also reported production of L-glutaminase from marine fungi. However, information on L-glutaminase production from Malaysia marine environment is still unknown up to now. Thus, in this study microorganisms producing L-glutaminase from Malaysia marine environment will be revealed,

conditions for L-glutaminase production will be optimized and their partially purified L-glutaminase enzyme will be characterized.

1.3 Objectives

The objectives of this research are:

1) To screening and identify L-glutaminase producing bacterial from marine environment.
2) To optimize L-glutaminase production through One Factor at A Time Method (OFAT).
3) To characterize partially purified L-glutaminase isolated from marine environment.

1.4 Scope of Study

The scope of the research study includes:

1) Identification of L-glutaminase producing bacterial isolates from marine environment through biochemical characterization, 16S rRNA gene sequencing and L-glutaminase enzymatic assay.

2) Optimization of L-glutaminase production through OFAT method with five different parameters such as incubation temperature, pH, additional carbon sources, additional organic nitrogen sources, and additional inorganic nitrogen sources.

3) Purification of L-glutaminase via ammonium sulphate precipitation and dialysis approach. Characterization of partially purified L-glutaminase enzyme using four different parameters such as incubation temperature, temperature stability, pH, and pH stability.

CHAPTER 2

LITERATURE REVIEW

2.1 Marine as a local source of enzyme

Marine is a huge source of undiscovered and less characterized microorganisms. It is a habitat of different type of microorganisms that have different properties and characteristics that make them adapted to their specific habitat environments. Their different characters and physiological factors from terrestrial microorganisms might cause them to produce enzyme with different characteristics functions. Marine microorganisms might have different range of enzymatic activities. Marine has an enormous resource of novel compounds as one half of the total global biodiversity comprised of marine species (Barrow & Shahidi, 2007)

Marine unfavarouble conditions such as low temperature, high salinity, high pressure, and low intensity of light are still bearable for adapted heterotrophic bacteria to endure these types of environment. Different specific characteristics that they have cause them able to adapt in those types of environment. Besides that, marine offers an enormous source of functional materials such as minerals, antioxidants, vitamins, enzymes, polyunsaturated fatty acids, and bioactive compounds (Kim, Ravichandran, Khan, & Kim, 2008). Marine-derived bioactive peptides for example have potential in reducing disease risk and thus promote better human health and well-being (Shahidi & Zhong, 2008).

2.2 L-glutaminase in general

L-glutaminase is an amidase enzyme that catalyse L-glutamine to glutamic acid and ammonia. Amidase generally helps to catalyse the hydrolysis of amide bonds. L-glutaminase is ubiquitously present in organisms including microorganisms. It can also be found in animal,

plant (Vijayan, Swapna, Haridas, & Sabu, 2017) and human (Heini, Gebhardt, Brecht, & Mecke, 1987). The summary of L-glutaminase producing organisms are listed in Table 2.1.

Table 2.1 Sources of L-glutaminase from mammals and microorganisms

Sources of Glutaminase	**References**
Pseudomonas aureofaciens	(Imada, Igarasi, Nakahama, & Isono)
Cryptococcus albidus	Fukushima and Motai (1990)
Aspergillus oryzae	Yano, Ito, Tomita, and Kumagai (1988)
Escherichia coli	Prusiner, Davis, and Stadtman (1976)
Proteus morganii	McIlwain (1948)
Providencia sp.	Iyer and Singhal (2009)
Vibrio sp.	Durai, Selvaraj, Manikkam, and Ramasamy (2014)
Candida scottii	Imada et al. (1973)
Actinomucorelegans	Han, Ma, Rombouts, and Nout (2003)
Streptomyces sp.	Krishnakumar et al. (2011)
Trichoderma koningii	Sayed (2009)
Sporomyces sp	Sabu (2000)
Candida sp	Sabu (2000)
Human	(Martín-Rufián et al., 2012)
Rat	(Martín-Rufián et al., 2012)
Chicken	(Olalla et al., 2002)
Monkey	(Olalla et al., 2002)
Cow	(Olalla et al., 2002)

Rabbit	(Olalla et al., 2002)

2.3 L-glutaminase producer

2.3.1 L-glutaminase from mammals

L-glutaminase is present in most mammalian tissues (Campos-Sandoval et al., 2015). L-glutaminase plays a significant role in energy and nitrogen metabolism of mammalian cells (Márquez, de la Oliva, Matés, Segura, & Alonso, 2006). This mammalian L-glutaminase needs inorganic phosphate for physiological processes to occur including hepatic ureagenesis, neurotransmitter glutamate synthesis in brain and renal ammoniagenesis (Curthoys & Watford, 1995).However, recently L-glutaminase has been discovered to have a degree of complexity in terms of its pattern of expression and functional regulation (Campos-Sandoval et al., 2015). On top of that, liver-type glutaminase in rat for instance was believed to be present in liver mitochondria of adult rats (Smith & Watford, 1990). Meanwhile, kidney-type of glutaminase from rat is presence in kidney, lymphocytes, foetal lever, brain, and small intestine (Curthoys & Watford, 1995). Both kidney-type and liver-type glutaminase are different in terms of immunological and molecular characteristics (Kovacevic & McGivan, 1983). A research study have shown that relative abundance of kidney-type and liver-type glutaminase is species specific (Martín-Rufián et al., 2012). In mammalian brain and liver of mouse, rat, and human, liver-type and kidney-type glutaminase transcripts are co-expressed (Martín-Rufián et al., 2012) however co-expression of those transcripts are not happen in chicken (Olalla et al., 2002). Furthermore, inhibition of those enzymes by the end-product glutamate is a key to differentiate between liver-type glutaminase which is not inhibited by glutamate and kidney-type glutaminase which is strongly inhibited (Márquez et al., 2006).

L-glutaminase is said to be a multifaceted protein due to recent discovery of novel extramitochondrial localizations, the presence of multiple transcripts for mammalian glutaminase genes, the existence of signature sequences and protein motifs on its sequence, and the identification of potential protein interacting partners (Márquez et al., 2006). Hence, L-glutaminase in mammals are not just involve in generation of glutamate from glutamine but it also involves in other functions. For instance, liver-type glutaminase of human is happened to be present in leukocytes where it is responsible for bactericidal action through a glutamine-dependent mechanism of superoxide production (Márquez et al., 2006). Besides that,

glutaminase genes are said to have evolved from common ancestral gene through gene duplication and divergent evolution which has been previously happened in rat genes (Porter, Ibrahim, Taylor, & Curthoys, 2002).

In vertebrate central nervous system, glutamate is the major excitatory neurotransmitter (Collingridge & Lester, 1989). Hence, glutaminase is the main glutamine utilizing enzyme (Kvamme, 1984) of the brain and an essential contributor to neurotransmitter pools of glutamate (Nicklas, Zeevalk, & Hyndman, 1987). Traditionally, glutaminase was suggested to be expressed primarily in neuronal cells but not in astrocytes (Laake et al., 1999). However, recently a study has proved that glutaminase is also expressed in astrocytes in three different levels which are mRNA, protein, and enzymatic activity (Cardona et al., 2015).

2.3.1.1 Glutaminase in human beings

Glutaminase found in human beings have central role in tissues which were then purified and characterized (Heini et al., 1987). Recently, glutaminase genes are found to be co-expressed in some tissues and in many cell types (Campos-Sandoval et al., 2015). Two isozymes of glutaminase can be found within human body which are kidney-type and liver-type (Watford, 1993). Isozymes can be clarified as enzymes that catalyzes the same chemical reactions however they consist of different sequence of amino acids. There are two genes that encode for glutaminase isoforms which located in different chromosomes (Aledo, Gómez-Fabre, Olalla, & Márquez, 2000). One gene encodes kidney-type isozyme and located on chromosome 2 while the other gene encodes for liver-type isozyme and located on chromosome 12 (Aledo et al., 2000). Human kidney-type isozyme gene spans 82kb (Márquez et al., 2006) and split into 19 exons (Porter et al., 2002). Meanwhile, liver-type glutaminase gene has a length more than 18kb and splits into 18 exons (Pérez-Gómez et al., 2003).

Furthermore, kidney-type and liver-type of glutaminase have considerable degree of sequence similarity (Márquez et al., 2006). However, the difference between kidney-type and liver-type genes are number of exons presence in their genes and difference in coding sequence of both genes which are located at exon 1 and 18. Exon 1 between both genes only shares approximately about 62.5% similarity where it codes 129 amino acids for kidney-type glutaminase and only 61 amino acids for liver-type glutaminase. In addition, exon 1 also encodes for signals for mitochondrial targeting and translocation processes (Shapiro, Farrell, Srinivasan, & Curthoys, 1991). Likewise, exon 18 of those genes only share about 29.4%

similarity which codes for C-terminal region of both kidney-type and liver-type protein. On top of that, exons 3 to 17 of both kidney-type and liver-type glutaminase mRNA transcripts have the same length and show high sequences similarity (Márquez et al., 2006) (Gomez-Fabre et al., 2000).

A research study has found out that liver-type of glutaminase in human is not just expressed in the liver but it is also being expressed in the brain (Huang et al., 2011) , pancreas and cells of immune system (Pérez-Gómez et al., 2005) . Meanwhile, kidney-type of glutaminase can be found in kidney, brain, and intestine. Level and activity of kidney-type of glutaminase increases in kidney due to its response of metabolic acidosis (Vijayan et al., 2017). Metabolic acidosis is a phenomenon where there is excessive quantities of acid inside the human body due to kidney is not removing acid accordingly from the body. On the other hand, liver-type kidney increase in amount and its activities due to consuming high protein diets, diabetic, and starvation (Vijayan et al., 2017)

In human brain, the distribution of liver-type and kidney-type glutaminase transcripts was investigated where mRNA encoding liver-type glutaminase was present in all region of brain examined. The highest distribution of liver-type glutaminase mRNA was detected at cerebral cortex while its least distribution was at medulla and spinal cord (Olalla et al., 2002). A similar pattern of distribution was also found for kidney-type glutaminase mRNA.

2.3.2 L-glutaminase from bacteria

L-glutaminase can also be found in bacteria such as *Escherichia coli*, *Lactobacillus reuteri*, and *Saccharomyces cerevisiae*. Unlike L-glutaminase from human, L-glutaminase of *L. reuteri* has its optimum enzymatic activity at temperature of 40ºC instead of 37 ºC (Jeon, Lee, & So, 2009). Besides that, a research study has stated that *E. coli* has two different types of glutaminase which can be classified as glutaminase A and glutaminase B (Campos-Sandoval et al., 2007). Another research study identified that L-glutaminase extracted from *S. cerevisiae* also has two different forms of L-glutaminase which are glutaminase A and glutaminase B. Glutaminase A can be found in a free form while glutaminase B is in the membrane-bound form (Soberón & González, 1987). Both are said to be different in terms of pH optimum, thermostability, and sensitivity to pyruvate (Soberón & González, 1987). L-glutaminase produce from different type of microorganisms seems to have different psychological properties which might be adapted to the needs of each specific of those microorganisms.

2.3.3 L-glutaminase from fungi

L-glutaminase can also be extracted from fungi. L-glutaminase can be extracted mainly from *Aspergillus* and *Trichoderma* sp. (El-Sayed, 2009). Production of L-glutaminase from fungi such as *Verticillium malthousei* was carried out through submerged fermentation (Imada et al., 1973). Moreover, extraction of L-glutaminase form fungi can also be carried through solid-state fermentation. These include Beauveria sp. (A Sabu, Chandrasekaran, & Pandey, 2000), Aspergillus oryzae (Yano et al., 1988), and *Trichoderma koningii* (El-Sayed, 2009). A research study reported by El-Sayed (2009) stated that different agro-industrial by-products had been used as solid substrates for instance ground nut residues, corn steep, rice hulls, cotton seed residues, and wheat bran. After optimization, they found out that L-glutaminase production of solid culture of *T. koningii* was increased by 2.2 fold compared to the submerged cultures (El-Sayed, 2009).

2.3.4 L-glutaminase from actinomycetes

Actinomycetes are a group of heterotrophic prokaryotes having hyphae at some stage of their growth which usually referred as filamentous prokaryotes (Binod et al., 2017). Furthermore, actinomycetes were usually gram-positive bacteria that have high G and C content in their DNA (Binod et al., 2017). Example of actinomycete is *Streptomyces avermitilis*. A research study conducted by Abdallah, Amer, and Habeeb (2013) had explained in details on optimized conditions and characterization of purified L-glutaminase from *S. avermitilis*. Divya Teja, Sri Devi, and Harsha (2014) have reported in their research study that several marine actinomycetes have produced L-glutaminase with their maximum activity.

2.4 Marine source of L-glutaminase

A few researches have been conducted that showed production of L-glutaminase from marine microorganisms including *Aerobacter aerogenes* (Tempest, Meers, & Brown, 1970), *Streptomyces griseus* (Muthuvelayudham & Viruthagiri, 2013), *Pseudomonas aeruginosa* (Ohshima, Yamamoto, & Soda, 1976) , and *Streptomyces enissocaesilis* (Mousumi & Dayanand, 2013). A research reported by A Sabu, Keerthi, Kumar, and Chandrasekaran (2000) stated that mostly microorganisms identified to produce L-glutaminase were isolated from soil

except only a few reports from marine microorganisms. Below are the list of L-glutaminase producing marine microorganisms.

Table 2.2 Sources of L-glutaminase from marine bacteria, fungi, and yeast

Microorganisms	**Sources of L-glutaminase**	**References**
Bacteria	*Providencia* sp.	Iyer and Singhal (2009)
	Vibrio sp.	Durai et al. (2014)
	Pseudomonas sp	Kumar (2003)
	Micrococcus luteus	Moriguchi et al. (1994)
Fungi	*Beauveria* sp.	Sabu et al. (2000)
Yeast	*Zygosaccharomyces rouxii*	(Kashyap, Sabu, Pandey, Szakacs, & Soccol, 2002)

A research study conducted by (Iyer & Singhal, 2009) reported that *Providencia* sp. was a Gram-negative coccobacilli that produce the highest production of glutaminase compared to other isolates. The glutaminase produced was 71.23 U/l extracellularly. Furthermore, *Providencia* sp was identified through 16S rRNA sequencing and biochemical identification tests. It was reported to be isolated from breathing roots of weeds from western seacoast of Maharashtra, India. Over 400 different bacterial strains had been collected and isolated from marine niches covering 300 km of seacoast of Maharashtra, India including muddy clay from mangroves, sand from clear beaches, sea floors exposed by sea tides, seaweeds, fish and crab scales, and others. Maximum production of L-glutaminase by *Providencia* sp. could be obtained through optimized media containing glucose, urea, methionine, succinic acid, ammonim sulphate, and yeast extract at 28 hours of incubation at 25 °C. The maximum enzyme activity obtained was 119.00 ±0.12 U/l.

Meanwhile, *Beauveria* sp. is a type of marine fungus that was reported to produce L-glutaminase by A Sabu, Keerthi, et al. (2000). *Beauveria* sp. was isolated from marine sediment of Cochin, a southwest India's coastal Kerala state. It was fermented through solid state fermentation using polystyrene as an inert support. After optimization of process parameters,

maximum production of L-glutaminase from *Beauveria* sp. was 49.89 U/ml after 96 hour of incubation at pH 9, temperature of 27°C, using seawater based medium supplemented with glucose as carbon source and L-glutamine as substrate (A Sabu, Keerthi, et al., 2000).

On the other hand, *Zygosaccharomyces rouxii* is a saline tolerant yeast that produce extracellular L-glutaminase through solid-state fermentation using agro-industrial substrates such as wheat bran and sesamum oil cake (Kashyap et al., 2002). After optimization process, maximum production of L-glutaminase using sesamum oil cake as agro-industrial substrate could be determined with value of 11.61 U/gds (units per gram dry substrate). The solid-state fermentation was carried out at 30 of incubation temperature, 48 hours of incubation period, 2 ml of 48 hours old inoculum, and 64.2% initial moisture content of substrate. Apart from that, additional of organic and inorganic nitrogen sources into fermentation medium had no benefit for ezyme production (Kashyap et al., 2002).

2.5 L-glutaminase Production and Optimization

L-glutaminase has received significant attentions due to its application in food and pharmaceutical industries. Production of L-glutaminase from microorganism can be either in solid state fermentation or submerged fermentation. For example, production of L-glutaminase from fungus such as *B. bassiana* has been carried out in solid state fermentation (Abdulhameed Sabu, Kumar, & Chandrasekaran, 2002). Furthermore, another research reported that L-glutaminase production from fungus scientifically known as *Trichoderma koningii* showed high yield production under solid-state fermentation when agro-industrial residues were used as substrates (El-Sayed, 2009). On the other hand, method used for commercial production of L-glutaminase is usually submerged fermentation (Vijayan et al., 2017).

Submerged fermentation is a method where microbial cells are cultivated in liquid medium for production of desired metabolites under controlled conditions. There are a few reasons on why submerged fermentation is being used which are components of culture medium such as carbon, nitrogen, vitamins, and minerals are common and inexpensive, also those components can be supplied in uniform quantities.

Meanwhile, solid-state fermentation is a method where microorganisms are cultured on solid or semisolid substrates and occurs on inert solid support. There are a few advantages on usage of solid-state fermentation which are simple, lower waste water output and capital but high product yield, and its practicability to use various agro-industrial residues like oil cakes

and wheat brans (Vijayan et al., 2017). Besides, examples that can used for inert supports are polyurethane foam and polystyrene beads. Table 2.3 showed a few examples of microorganisms producing L-glutaminase from submerged fermentation and solid-state fermentation.

Table 2.3 Method of L-glutaminase production from different type of microorganisms

Microorganisms	**Method of production**	**References**
Zygosaccharomyces rouxii	Solid-state fermentation	Kashyap et al. (2002)
Beauveria bassiana	Solid-state fermentation	Abdulhameed Sabu et al. (2002)
Aspergillus oryzae	Solid-state fermentation	Yano et al. (1988)
Serratia marcescens	Submerged fermentation	Kumar, Muthuvelayudham, and Viruthagiri (2013)
Pseudomonas aeruginosa	Submerged fermentation	Ohshima et al. (1976)
Streptomyces enissocaesilis	Submerged fermentation	Mousumi and Dayanand (2013)

Most researchers were interested to investigate the optimum conditions for maximum production of L-glutaminase. For instance, several physico-chemical parameters had been studied for optimization process such as incubation temperature, additional of carbon sources, additional of organic and inorganic nitrogen sources, and the effect of addition of L-glutamine. L-glutaminase extracted from *Zygosaccharomyces rouxii* for example, had the optimum temperature of 30ºC to have the highest enzyme production (Kashyap et al., 2002). Other results showed that maximum production of L-glutaminase extracted from *Zygosaccharomyces rouxii* could be obtained with addition of glucose as carbon source (Kashyap et al., 2002). On the other hand, additional of organic and inorganic nitrogen source and also additional of L-glutamine did not gave any useful impact on yeast culture and thus showed no utility for enzyme production (Kashyap et al., 2002).

Optimization of parameters for maximum production of L-glutaminase was also investigated for fungi producer. Several parameters had also been studied for optimization

process for *Beauveria sp*. Those parameters were incubation temperature, pH, additional of carbon sources, additional of nitrogen sources, and additional of sodium chloride concentration. Results of optimization process for *Beauveria sp* recorded by A Sabu, Keerthi, et al. (2000) stated that at all of these parameters including incubation temperature of 27, pH 6, glucose as carbon source, malt extract as organic nitrogen source, and potassium nitrate as inorganic nitrogen source showed maximum increment of L-glutaminase production individually. Meanwhile, additional of sodium chloride concentration cause a decrement in enzyme production. This showed that *Beauveria sp* is not halophilic but it could be a halotolerant instead and a commensal organism of the marine environment (A Sabu, Keerthi, et al., 2000). After optimization of these parameters, an experiment had been conducted with all of these parameters were included at its optimal level.

2.6 L-glutaminase purification

According to research carried out by Tork, Aly, and Elsemin (2018), purification of L-glutaminase was carried out using 80% ammonium sulphate precipitation. Then, the dialysis was done against phosphate buffer (pH 5.8) at 4°C for one to two days. After that, the enzyme was purified using DEAE cellulose ion exchange chromatography and Sephadex G-100 gel filtration (Tork et al., 2018).

Another research carried out by Bazaraa, Alian, El-Shimi, and Mohamed (2016) reported that purification of L-glutaminase had been done through fractionation by ammonium sulphate at different saturation levels which were 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%. Known volume of crude enzyme was treated with those level of ammonium sulphate overnight at 5°C and centrifuges at 8000 rpm at 20 minutes. Then, the precipitate was dialyzed in a cellulose bag overnight against sodium phosphate buffer (pH 7, 0.1M) at 5°C with mild agitation. The sodium phosphate buffer was changed every 4 hours. After that, about 1 ml of the dialyzed enzyme solution was added to a Sephadex G-75 column that had been previously equilibrated with sodium phosphate buffer and eluted with the same buffer (Bazaraa et al., 2016).

Abdallah et al. (2012) in his research study had shown that purification of L-glutaminase was done through ammonium sulphate precipitation. Crude enzyme filtrate was slowly added with solid ammonium sulphate with gentle stirring until 40% saturation was achieved. The mixture was allowed to stand overnight at 4°C (Abdallah et al., 2012). It was centrifuged at 4°C for 20 minutes at 10000 rpm. The obtained supernatant was then subjected

to addition of ammonium sulphate until it reached to the concentration of 50% saturation. It was centrifuged by following the same conditions before. After centrifuged, the resulting supernatant was further added with ammonium sulphate to bring 80% saturation. Enzyme precipitate gained from each saturation was dissolved in minimal volume of 0.01M phosphate buffer (pH 8) and dialyzed against the same buffer at 4°C for 48 to 72 hours. The phosphate buffer were changed accordingly.

The L-glutaminase solution from 80% saturation of ammonium sulphate precipitation was loaded into DEAE-cellulose chromatography column. It was previously equilibrated with 0.02 M Tris HCl buffer (pH 8.0). The column was washed with approximately four to five bed volumes of 0.02 M Tris HCl buffer (pH 8.0). Meanwhile, the bound protein was eluted with discontinuous gradient of NaCl prepared in the same buffer at a flow rate of 0.5 ml/min (Abdallah et al., 2012).

2.7 Application of L-glutaminase

2.7.1 Application in food industry

L-glutaminase has a very important application in food industry. It helps to increase or enhance food flavours by becoming flavour- and aroma-enhancing agent (Löliger, 2000). Its mechanism of action is increasing the glutamic acid content in fermented foods thus allowing enhancement of food flavours. L-glutaminase has been widely used in fermented foods such as soy sauce, sufu, and miso to enhance its aroma and taste (Sarada, 2013). Furthermore, a research by Kijima and Suzuki (2007) reported that addition of gamma-glutamyltranspeptidase as a glutaminase in fermentation mixture cause an improvement towards the umami taste of soy sauce.

On top of that, heat-stable and salt-tolerant L-glutaminase make it so suitable to be used in food industry (Yoshimune, Yamashita, Masuo, Wakayama, & Moriguchi, 2004). This is because production of foods in food industry involve high temperature and salt content which cause L-glutaminase to withstand those challenges in order to be fully functional. A research study has stated that salt-tolerant L-glutaminase extracted from *Stenotrophomonas maltophilia* is extensively used by fermenters for their Japanese soy sauce fermentation due to its significant advantages in the production of glutamic acid over the other microbial glutaminases (Wakayama et al., 2005). Another research study has also stated that L-glutaminase with high

productivity increase the glutamic acid content in soy sauce production which then enhance the quality and taste of the soy sauce (Yamamoto & Hirooka, 1974).

2.7.2 Therapeutic Application

L-glutaminase also has the potential as a candidate for cancer therapy. It has a property of anti-cancer agent specifically known as anti-lymphocytic leukaemia because of its anti-leukemic activities (Roberts & McGregor, 1991). Cancer cells need energy to proliferate and survive on daily basis. They depend on energy source from L-glutamine which acts as a precursor molecule for nucleotide and protein synthesis. Fortunately, presence of L-glutaminase could inhibit cancer cells metabolism by breaking down L-glutamine to glutamic acid and ammonia. This cause deprivation of energy source followed by selective death of cancers cells (Sarada, 2013).

For example, a research study by Reda (2015) stated that L-glutaminase extracted from *Streptomyces canaries* has anti-cancer efficiency. The anti-cancer property of this L-glutaminase was tested and evaluated against five different types of cell lines which are Hep-G2, RAW264.7, HCT-116, MCF7, and HeLa. Reda (2015) in his research reported that L-glutaminase showed high efficiency as anti-cancer agent against HeLa and Hep-G2 cell lines. Meanwhile, the L-glutaminase showed moderate efficiency against RAW264.7 and HCT-116 cell lines. However, L-glutaminase extracted from *S. canaries* did not showed any efficiency against MCF7 cell line as there was growth of MCF7 cells. Hence, L-glutaminase from *S. canaries* gives promising value as a candidate for cancer therapy. On the other hand, another research on application of L-glutaminase as anti-cancer agent had also been conducted. PLNSN, Siddalingeshwara, Karthic, Pramod, and Vishwanatha (2014) on their research study also reported that L-glutaminase from *Aspergillus niger* has anti-tumor property. It is proved when the L-glutaminase has high efficiency against human breast cancer cell lines MCF-7 using MTT assay.

Apart from that, L-glutaminase also has a potential application in HIV therapy. Sarada (2013) in his research study reported that L-glutaminase causes depletion of glutamine levels in serum and tissue for prolonged periods and therefore reduce the reverse transcriptase activity of Human Immunodeficiency Virus (HIV). Furthermore, a research study also showed that L-glutaminase from *Pseudomonas* sp. stopped further replication of HIV virus inside infected

cells when being administered into the infected cells (Roberts, MacAllister, Sethuraman, & Freeman, 2001). The enzyme inhibits DNA biosynthesis and formation of tumour inside the affected cells.

2.7.3 L-glutaminase as a biosensor

Unissa, Sudhakar, Reddy, and Sravanthi (2014) in their research study have stated that currently, L-glutaminase has been used as biosensor in forms of free enzyme or immobilized on membranes to monitor glutamine and glutamate levels of fluids. Not only that, L-glutaminase is also used as biosensors to detect glutamine levels in human and pharmaceutical formulations. A research reported by Botrè et al. (1993) stated that they have developed a biosensor that use L-glutaminase to detect glutamine and glutamate levels in pharmaceutical formulations. Furthermore, L-glutaminase has been used as a biosensor to monitor glutamine level particularly for hybridoma and mammalian cell culture (Sarada, 2013). Besides that, Villarta, Palleschi, Suleiman, and Guilbault (1992) had explained in their research that an amperometric enzymes electrode probe added with membrane immobilized L-glutaminase could determine serum glutamine level in humans.

2.7.4 Manufacture of fine chemicals from L-glutaminase

Japanese green tea has a unique taste-enhancing amino acids present in it. The amino acids are also the major components presence. The amino acids are known as theanine which scientifically known as gamma-l-glutamyl ethylamide. Unissa et al. (2014) in their research study stated that theanine has its own unique advantages including the ability to suppress stimulation by caffeine, act as antihypertensive agents, and improve effects of antitumor agents which then lead to more research and developmental activities of physiological roles of theanine. Meanwhile, a strategy has developed for production of theanine using both glutamate and ethylamine through reactions between bacterial glutaminases and baker's yeast (Tachiki et al., 1998).

CHAPTER 3

METHODOLOGY

3.1 Samples collection and preparation

Water and soil samples were collected from three different beaches in Pahang, Malaysia known as Pantai Teluk Cempedak (3.8120° N, 103.3726° E), Pantai Batu Hitam (103.36349° N, 3.88207° E) and Pantai Balok (3.9207° N, 103.3699° E). All of the samples were collected in sterile screw capped tubes and were used as sources to isolate microorganisms producing L-glutaminase. About 10g of each of the samples were diluted with 50 ml of sterile water. The suspension was shaken using a rotary shaker for 30 minutes and kept aside to settle the soil matter. About one ml of the suspension was serially diluted three times with sterile water. Then, one ml of the final dilution was spread plated in sterile petri dish containing minimal glutamine agar (2.0g glucose, 0.5g KCl, 0.5g $MgSO_4.7H_2O$, 1.0g KH2PO4, 0.1g $FeSO_4.7H_2O$, 0.1g $ZnSO_4.7H_2O$, 0.5g NaCl, 20g Agar, 10g L-glutamine, and 2.5 % of phenol red solution) and incubated for 24 hours at 37°C. Water samples were also serially diluted three times and plates as above individually.

3.1.1 Method of Serial Dilution

A serial dilution is a process to dilute a solution repeatedly in order to reduce the concentration of a substance in a solution. The objective of serial dilution method is concentration estimation of the unknown sample by counting the number of colonies cultured

from serial dilutions of the unknown sample and then used the measured counts to calculate the unknown concentration (Ben-David & Davidson, 2014).

For example, three times serial dilution requires the sample to be diluted for three times. Four test tubes were needed. The first test tube labelled as US which stands for undiluted sample. Then, the other three test tubes should contained 9 ml of sterile distilled water in each of the test tubes. The second, third, and fourth test tubes were labelled as 1:10, 1:100, and 1:1000 respectively. A total of 1 ml from undiluted sample was transferred to the second test tube containing 9 ml of sterile distilled water and mixed thoroughly. Hence, there was 1 ml of undiluted sample in 9 ml of sterile distilled water inside a second test tube. Therefore, the undiluted sample was being diluted by a factor of 10. After that, for second serial dilution, 1 ml of the unknown sample from test tube 1:10 was drawn into third test tube labelled as 1:100 containing 9 ml of sterile distilled water. The solution was mixed thoroughly. The solution inside test tube 1: 100 had been diluted by a dilution factor of 100. Finally, for third serial dilution, 1 ml of solution from test tube 1:100 was taken and put inside a fourth test tube labelled as 1:1000 containing 9 ml of sterile distilled water. The solution was mixed thoroughly. Hence, the solution was being diluted by a dilution factor of 1000. 1 ml of the solution in test tube 1:1000 was being used to be cultured in petri dish containing minimal glutamine agar media.

3.1.2 Preparation of Minimal Glutamine Agar Media.

Minimal glutamine agar medium is consisted of 2.0g/l of glucose, 0.5g/l of KCl, 0.5g/l of $MgSO_4.7H_2O$, 1.0g/l of KH2PO4, 0.1g/l of $FeSO_4.7H_2O$, 0.1g/l of $ZnSO_4.7H_2O$, 0.5g/l of NaCl, 20g/l of Agar, 10g/l of L-glutamine, and 2.5 % of phenol red solution. The medium was sterilized by autoclaving at 121ºC for 20 minutes. After sterilization and being cooled down, the medium was poured about three quarters inside petri dishes and let harden in a laminar flow chamber that provide a sterile environment. After that, all of the petri dishes containing the medium were sealed and kept in a chiller with a temperature of 4 ºC until further use. Shelf life for the medium is up to several months.

3.2 Screening and isolation of Bacterial L-glutaminase Producers

3.2.1 Primary screening

Bacteria that showed growth on minimal glutamine agar medium with a colour change from yellow to pink was chosen for primary screening. The positive responses showed by those bacterial colonies were selected to be sub cultured using patch streak technique. Those positive colonies were picked using sterile inoculating loop and patch streaked on the minimal glutamine agar media. The results were observed after 48 hours of incubation at 37°C.

3.2.2 Secondary screening

A total of 12 out of 28 colonies patches gave a strong colour change from yellow to pink on minimal glutamine agar media. Hence, those 12 isolates were selected for secondary screening by using streak plate technique. Each of those selected isolates was streaked on a sterile petri dish containing minimal glutamine agar media individually. Those petri plates were incubated at 37°C for 24 hours. After that, a single colony from each of the 12 isolates was cultured individually in nutrient Luria Bertani (LB) broth and left overnight. Bacterial stock was made by using those bacterial culture and glycerol stock and stored in -80°C freezer until further use.

3.2.2.1 Preparation of Bacterial Stock solution

In order to prepare bacterial stock, a fresh bacterial culture and glycerol stock were prepared. Firstly, 1 l of LB broth with pH 7.0 need to be prepared that contains 1% NaCl, 1% Tryptone, and 0.5% Yeast Extract. 10g NaCl, 10g tryptone, and 5g yeast extract were dissolved in 950 ml of distilled water. pH of the solution was adjusted to 7.4 with NaOH and then brought the volume up to 1 litre. The solution was autoclaved at 121ºC for 20 minutes and let the medium cool to approximately 55 ºC. The broth then can be kept inside a chiller with a temperature of 4ºC.

After that, preparation of 10 ml of bacterial culture could be proceed by pouring 10 ml of LB broth into 50 ml falcon tube. Growth of bacteria need to be in 1:5 ratio. Sterile inoculating loop was used to touch on single colony by making sure that the inoculating loop does not touch any other single colony. Then, the inoculating loop was soaked into LB broth. Ensure that the wall of falcon tube was not touched by the inoculating loop. All of these steps were conducted inside a laminar flow. The falcon tube was incubated inside a shaker incubator at

37ºC, 200 rpm, for 16 hours. A control tube contains only LB broth. After 16 hours, the falcon tube was kept inside a chiller at 4 ºC. Bacterial culture can be kept inside a chiller for about 2 weeks.

After fresh bacterial stock has been prepared, preparation of 1.0 ml bacterial stock could be performed. Initially, amount of 50% glycerol stock needed to make 15% of bacterial culture diluted was calculated by using formula $M_1V_1 = M_2V_2$. Hence, amount of 50% of glycerol stock needed was 0.3 ml while the amount of fresh bacterial culture needed was 0.7 ml to give a total of 1.0 ml. Those glycerol stock and bacterial culture were mixed inside a sterile 2.0 ml centrifuge tube in a laminar flow. Then, the centrifuge tubes were kept inside a -80 ºC freezer.

3.3 Inoculum and Culture broth preparation

Each of the positive isolates were grown on minimal glutamine agar media using streak plate technique (Appendix A) and incubated at 37°C for 16 hours. Besides, sterile minimal glutamine broth media was also prepared and sterilized by autoclaving at 121°C for 20 minutes. A total of 5 ml of sterile minimal glutamine broth was transferred into 50ml falcon tube. By using sterile inoculating loop, one loop of full cells from well grown plates of each of the positive isolates were transferred into 50 ml falcon tube containing 5 ml of sterile minimal glutamine broth individually. Then, those inoculated falcon tubes were shaken using a rotary shaker at 150 rpm and incubated at 37°C for 16 hours. Thereafter, a haemocytometer and trypan blue solution which act as a cell stain were used to measure the cell concentration of the inoculum.

Each volume taken from inoculum of 12 isolates was measured individually to have cell concentration of 2×10^5 cells before being cultured in a 250 ml conical flask containing 50 ml of sterile minimal glutamine broth. Those inoculated conical flasks were kept on rotary shaker at 150 rpm and incubated at 37ºC for 24 hours. After that, all of the 12 isolates culture were transferred into 50 ml falcon tubes individually and refrigerated centrifuge at 6000 rpm at 4 ºC for 20 minutes. The supernatant is a source of crude enzyme and was used further for enzymatic assay analysis. Procedure of using haemocytometer and cell count were further explained in Appendix B.

3.4 Biochemical Characterization of the Microbes

Biochemical characterizations were conducted on positive isolates including endospore staining, catalase test, and oxidase test.

3.4.1 Endospore staining

Endospore staining was used to resolve highly resistant spores of some microorganisms within their vegetative cells. By following the Schaeffer and Fulton (1933) method , malachite green which acts as a primary stain was added on top of the heat fixed bacterial smear and pass through over a steam bath for a few minutes. Then, safranin was used to counterstain and the cells appeared red or pink in colour. The slide was observed under microscope to see the presence of endospore.

3.4.2 Catalase test

Liquid culture stocks of unknown bacteria isolates were prepared. Then, two test areas can be made on each slide so that two samples can be analysed simultaneously. A total of 20 µl of each of the liquid culture stock was transferred onto the test area. A few drop of hydrogen peroxide was then being added onto the test area. The slide was incubated at room temperature for 20 seconds however, no longer than 2 minutes. All unknown bacterial culture isolates were tested and their results were recorded.

3.4.3 Oxidase Test

For oxidase test, 20µl of each of liquid culture stock was transferred onto the center of oxidase test slide. A few drop of 1.0% of Kovac's reagent was added on the slide. The test slide was then incubated at room temperature for 20 seconds and no longer than 2 minutes. A blue or purple colour indicates that the bacterium is oxidase positive while no colour change indicates oxidase negative (Mac Faddin, 1976).

3.5 16S rRNA Gene Sequence Analysis

All of the positive isolates were selected for 16S rRNA gene sequencing. 16S rRNA gene sequencing is used as a reference tool in order to identify identity of bacterial isolates. 16S rRNA gene was amplified through colony PCR reaction using PCR amplification kit (Qiagen). Primers used in this reaction were a set of universal primers as mentioned in a table below.

Table 3.1 A set of universal primers used in colony PCR

Primer	Tm (°C)	GC content (%)	Sequence of primer
8F	54.3	50.0	5’ AGA GTT TGA TCC TGG CTC AG 3’
U1492R	49.4	42.1	5’ GGT TAC CTT GTT ACG ACT T 3’

A PCR master mix used was TopTaq Master Mix Kit from Qiagen. The PCR master mix consisted of 25 µl of TopTAq master mix used for each reaction which contained 1.25 units TopTaq DNA polymerase, 1x PCR buffer containing 1.5 mM $MgCl_2$, and 200 µM of each dNTP. Also, 15 µl of RNase-free water, 5 µl of 1x CoralLoad concentrate, 0.3 µl of forward primer (8F), and 0.3 µl of reverse primer (U1492R) were used in one PCR reaction. The reaction mixture was vortexed by ensuring no bubbles formation and followed by a brief spin in a micro centrifuge.

Thereafter, PCR product approximately about 10 to 11 µl was loaded in 0.8% agarose gel (100 V, 30 minutes). The PCR product contained 2 µl of loading dye, 7 µl of distilled water, 1 to 1.5 µl of PCR sample, and 0.5 µl of Diamond nucleic acid dye. This mixture was being pipetted up and down for three to four times before being loaded into 0.8% agarose gel. In addition, about 0.5 µl of molecular-weight size marker which is also can be referred as DNA ladder was mixed with 0.5 µl Diamond nucleic acid dye and pipetted up and down. The DNA ladder was loaded into agarose gel and usually will be loaded into the first well of agarose gel. Then, electrophoresis process was carried out at 100V for 30 minutes. The DNA bands were being visualized under UV transluminator.

After that, PCR products were being purified using PCR purification kit from Analytik Jena AG, conducted based on the protocol enclosed by the manufacturer. Then, amplicons were subjected for sequencing service.

3.6 Sequence analysis and phylogenetic tree construction

3.6.1 Nucleotide sequence analysis

A BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) from the National Center for Biotechnology Information (NCBI) was used to execute a nucleotide sequence homology

search for 16S rRNA gene sequences for all of the 12 isolates. Identities of bacteria were identified.

3.6.2 Amino acid alignment and phylogenetic analysis

A multiple sequence alignment and phylogenetic tree were carried out and constructed using Molecular Evolutionary Genetic Analysis software (MEGA7). A phylogenetic tree for all of the 12 positive isolates was performed in order to investigate the evolutionary relationships of 16S rRNA genes presence in different kind of microorganisms. NCBI Blast Nucleotide service was used to search for 16S rRNA gene sequences used for the phylogenetic tree construction. A total of 16 sequences of 16S rRNA genes were downloaded from gene bank and aligned using ClustalW. Based on the alignments, a Neighbour-Joining (NJ) tree was constructed to examine distances among those sequences. The tree topology was then analysed using bootstrapping. Zika virus strain was used as an out-group to root the tree.

3.7 Assay of L-glutaminase

L-glutaminase was assayed according to protocol of Imada *et al* (1973). L-glutamine is used as substrate and the product which is ammonia released from the reaction was measured using Nessler's reagent. Reaction mixture was prepared in 1.0 ml containing an aliquot of 0.1 ml enzyme preparation, 0.1 ml of 0.04M L-glutamine, and 0.8 ml of phosphate buffer (0.1M, pH 7.0). The reaction mixture was then being incubated at 37°C for 30 minutes and stopped by the addition of 0.25 ml of 1.5M Trichloroacetic acid. Enzyme and substrate blanks were prepared and used as controls. After that, 0.1 ml of reaction mixture was added to 3.7 ml of distilled water and then addition of 0.2 ml of Nessler's reagent into the solution. The mixture was incubated at room temperature for 20 minutes for colour development. The absorbance was measured at 450 nm using visible spectrophotometer. One International Unit of glutaminase activity was defined as amount of enzyme that liberates 1μmol of ammonia per minute under optimal assay conditions. Assays were conducted in triplicate and mean of L-glutaminase activity was expressed as international unit per ml (U/ml).

3.7.1 Method for standard curve of enzyme analysis

Ammonium chloride with molecular weight of 53.49 g/mol was used to make different concentration of ammonium chloride solution. This was needed to carry out an experiment for enzyme analysis standard curve. By using this formula which is Mass (g) = Concentration (M)

x Volume (L) x Molecular Weight, different concentration of ammonium chloride solution could be prepared. The different concentrations of ammonium chloride solution used were $5.0x10^{-6}$,$1.0x10^{-5}$,$1.5x10^{-5}$,$2.0x10^{-5}$,$2.5x10^{-5}$,$3.0x10^{-5}$, $3.5x10^{-5}$, $4.0x10^{-5}$, $4.5x10^{-5}$ and $5.0x\ 10^{-5}$. Each of the concentration of ammonium chloride solution was assayed through enzyme analysis. A total amount of 0.1 ml of each of the concentration of ammonium chloride solution was added to the 3.7 ml of distilled water individually. Then, those solutions were added with 0.2ml of Nessler's reagent. Those mixtures were then incubated at room temperature for 20 minutes for colour development. After that, the absorbance of each of the solution was measured using visible spectrophotometer at 450 nm. All of the absorbance for each concentration of the solution was recorded. A standard graph was plotted as the standard for computation of the concentration of ammonia and shown in Appendix C.

3.8 Optimization of process parameters.

L-glutaminase production can be maximized through optimization of various parameters under favourable conditions. A total of 2 isolates that produce the most for L-glutaminase production through enzyme assay will be selected to undergo optimization process. In this study, five parameters were examined to determine the optimum conditions for those two selected isolates which are Lg8 and Lg11 to produce L-glutaminase. The parameters under study including incubation temperature, additional organic nitrogen, additional inorganic nitrogen sources, additional carbon sources, and initial pH values of the medium. After optimisation of each parameter, the optimal result for each parameter will then be included in the next study. Enzyme assay will be carried out for every parameter in order to find out the enzymatic activity. All experiments are conducted in triplicate and mean values are recorded.

3.8.1 Incubation temperature

First of all, inoculum and culture broth for these two selected isolates, Lg8 and Lg11 were prepared as mentioned in page 4. The difference was that different incubation temperature had been applied which was 31, 34, 37, 40, and 43°C for each of the isolates. As crude enzyme for those two isolates have been obtained, a process known as enzyme analysis had been conducted for these enzymes extracted from Lg8 and Lg11. The culture was incubated and agitated at 150 rpm for 24 hours. Methods on how enzyme analysis should be conducted had been explained in section 3.7. By using a spectrophotometer, average absorbance at 450 nm

results were obtained for each isolate and their triplicates based on different incubation temperatures. A graph of average absorbance at 450 nm at different incubation temperature for each isolates was illustrated. Based on the absorbance result from enzyme analysis, enzymatic activity of Lg8 and Lg11 can be calculated.

3.8.2 Additional of organic nitrogen sources

In this study, three different organic nitrogen sources which are peptone, yeast, and beef extract were added individually to the production media which is minimal glutamine broth media for the culture preparation. Each of the organic nitrogen sources (1% w/v) was added into production media. The composition of these medium is listed in Appendix D. The culture was incubated at 37 ℃ and agitated at 150 rpm for 24 hours. The activity of secreted L-glutaminase was assayed using enzyme assay described in section 3.7.

3.8.3 Additional of inorganic nitrogen sources

Isolates Lg8 and Lg11 was also cultured in production medium with additional of inorganic nitrogen sources. Those inorganic nitrogen sources are ammonium sulphate, ammonium chloride, sodium nitrate, and potassium nitrate with the addition of 1% w/v into the production medium individually. The composition of these medium is listed in Appendix E. The culture was incubated and agitated at 150 rpm for 24 hours. The activity of secreted L-glutaminase was subsequently assayed by using enzyme assay described in section 3.7.

3.8.4 Additional of carbon sources

Three different types of carbon sources were added into the production medium at 1% w/v individually. Lg8 and Lg11 were cultured in the production medium added with different type of carbon sources namely glucose, maltose, and sucrose. The composition of these medium are listed in Appendix F. The culture was incubated and agitated at 150 rpm for 24 hours. Enzymatic activity of L-glutaminase secreted by these two isolates was assayed through enzyme assay as described in section 3.7.

3.8.5 Initial pH values of the medium

Isolate Lg8 and Lg11 were cultured in different pH of production medium which were pH 5, pH 6, pH 7, and pH 8. The culture was incubated at 37ºC and agitated at 150 rpm for 24 hours. The L-glutaminase activity was assayed using enzyme assay as described in section 3.7.

3.8.6 Experiment with all parameters at its optimal level.

After optimization of each parameter, an experiment had been conducted that include all the parameters which are incubation temperature, additional organic nitrogen, additional inorganic nitrogen sources, additional carbon sources, and initial pH values of the medium at its optimal level. The experiment had been carried out for both isolates (Lg8 and Lg11) individually. The culture was incubated and agitated at 150 rpm for 24 hours. The L-glutaminase activity was assayed using enzyme assay as described in section 3.7.

3.9 Partial purification of L-glutaminase

Isolate that produce the highest L-glutaminase production was chosen to partially purified through ammonium sulphate precipitation approach and dialysis. Different saturation levels of ammonium sulphate which are 40%, 50%, and 60% were successively treated with known volume of crude enzyme filtrate overnight at 4ºC. Then, those samples were centrifuged at 10000 rpm for 10 minutes at 4ºC using refrigerated centrifuge. The precipitates were then being dialyzed in a cellulose bag against phosphate buffer (0.01M, pH7) under mild agitation at 4ºC overnight. The phosphate buffer was changed at 2 hours interval. The content of cellulose bag was centrifuged again at 10000 rpm and 4ºC for 10 minutes. The supernatant was collected and analysed for glutaminase activity and protein content.

3.10 Determination of protein concentration

3.10.1 SDS-PAGE analysis

SDS-PAGE analysis is a type of technique that is used to separate proteins based on their molecular weight. This analysis is performed to determine the molecular weight of partially purified enzyme and to confirm that the enzyme was being purified. Protein samples were mixed with SDS-PAGE sample buffer and then followed by boiling approximately about 95 ºC for 10 minutes. The mixture was centrifuged before loading into SDS-PAGE.

The polyacrylamide gel was comprised of two parts which are separating gel for separation of the proteins and stacking gel for concentration of the protein samples. Procedures of preparing those two types of gel were described in details in Appendix G. The 12% (v/v) of separating gel was mixed well and poured between two plates and overlaid with approximately about 1ml of 70% isopropanol for about 30 to 40 minutes. This will allow polymerization to occur and keep the gel surface flat. A distinct interface appeared between the interface of gel and isopropanol after the gel had polymerized. After that, the isopropanol was removed by using filter papers and 5% (v/v) stacking gel was prepared. The 5% stacking gel was poured on top of the separating gel and a comb was inserted on top of the stacking gel. This is to form wells for protein samples loading and to prevent bubbles formation. The stacking gel was allowed to polymerize for one hour. Once polymerized, the gel was inserted into electrophoresis tank. The tank was then being filled with 1X Tris-glycine electrophoresis buffer.

The comb was removed from the stacking gel. The wells formed were loaded with protein marker and protein samples. Electrophoresis was carried out at a constant voltage of 100V at room temperature for two to three hours or until the dye at the bottom of the separating gel. Lastly, the gel was stained with Coomasie Brilliant Blue R-250 staining solution for about two hours and destained with destaining solution for about two hours. All of the solutions used in SDS-PAGE analysis were included in Appendix G.

3.10.2 Bradford assay

Bradford assay was used to determine protein concentration in a solution. Bovine serum albumin (BSA) was used as a protein standard or as a reference protein. Protein concentration in a sample was determined by dispending 1 ml of each sample into labelled test tubes. 1 ml of distilled water was used as blank. A total of 1 ml of 1X Bradford reagent was added into each of the test tubes. The reaction mixtures were mixed well and incubated about 10 minutes at room temperature. After that, the absorbance of each sample was measured using a UV spectrophotometer at a wavelength of 595 nm. A standard curve was plotted by taking the absorbance of BSA standard at 595 nm versus its concentration as shown in Appendix H.

3.11 Bacteria Growth Curve

A time course experiment had been conducted in order to monitor growth of the isolate and its enzyme production. This experiment was carried out under optimized conditions of each

process parameter for a total period of 28 hours with sampling every 2 hours. Optimized minimal glutamine media broth with pH 7 was prepared with additional of glucose as carbon source, beef extract as additional organic nitrogen source, and ammonium chloride as additional inorganic nitrogen source. Inoculum of isolate Lg8 was being cultured in a 250 ml sterile conical flask containing 50 ml optimized minimal glutamine media broth. The inoculated conical flask was kept on rotary shaker at 150 rpm and incubated at 37ºC for 28 hours. Every 2 hours, about 1.0 ml of isolate culture were transferred into 2 ml of sterile eppendorf to check the optical density at 600 nm using spectrophotometer.

3.12 Characterization of partially purified L-glutaminase

After crude L-glutaminase enzyme was partially purified by precipitating the crude enzyme using ammonium sulphate and dialysis, the partial purified L-glutaminase was characterized with four different parameters. The enzyme activity will then be determined through enzyme assay. Moreover, this partially purified enzyme was characterized based on incubation temperature, temperature stability, effect of pH by using different buffer systems, and lastly, pH stability. All experiments were conducted in triplicate and mean values were recorded.

3.12.1 Incubation temperature

In this study, the partially purified enzyme was incubated at five different incubation temperature respectively which was 31, 34, 37, 40, and 43°C for 30 minutes. The enzymatic activity of L-glutaminase was being assayed through enzyme analysis as described in section 3.7 after each of the incubation temperature.

3.12.2 Temperature stability

The partially purified enzyme was pre-incubated at five different temperatures for 30 minutes with the absence of substrate. Those five different temperatures was 31, 34, 37, 40, and 43°C. After that, the enzymatic activity of L-glutaminase was being assayed through enzyme analysis as described in section 3.7 after each of the incubation temperature.

3.12.3 pH

The effect of pH on partially purified L-glutaminase was determined at 37°C by carrying out enzyme reaction at different pH level ranging from pH 4 to pH 8. Appropriate

buffers were used namely citrate-phosphate buffer (pH 4 to 6) and potassium phosphate buffer (pH 6 to 8) following the procedure of L-glutaminase assay. The procedure of buffer preparation is described in Appendix I. The enzymatic activity of the partially purified enzyme was analysed through the enzyme assay as described in section 3.7.

3.12.4 pH Stability

The pH stability of the enzyme was investigated further by pre-incubation of the enzyme solutions without substrates at different pH level ranging from pH 4 to pH 8 using appropriate buffer systems for 30 minutes. After that, the reaction mixture was then subjected to the L-glutaminase assay as described in section 3.7.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Primary screening of L-glutaminase producers from collected samples

For primary screening, isolation of bacteria from samples was carried out by using spread plate method with three time serial dilution. A total of 15 samples were taken from both soil and sea water of three beaches in Pahang, Malaysia known as Pantai Teluk Cempedak, Pantai Batu Hitam, and Pantai Balok. However, only three sea water samples from Pantai Teluk Cempedak and one sea water sample from Pantai Batu Hitam showed positive growth on selective medium specifically known as minimal glutamine agar medium. Figure 1 shows primary screening of isolated samples with the resulting colonies. Unfortunately, another 11 samples showed negative results as there were no growth with pink zones on minimal glutamine agar medium.

Extracellular L-glutaminase production by bacteria colony causes colour change of the minimal medium from yellow to pink (Katikala et al., 2009). pH of the medium was altered when there was production of L-glutaminase. This happened due to mechanism of a reaction where disintegration of amide bond in L-glutamine occurred and ammonia production happened which causes colour change of the medium from yellow to pink in colour. Selective media is a type of medium that allows a researcher to grow certain type of bacteria that only capable of utilizing components of the medium. Minimal glutamine medium is one of the examples of selective media that only allow growth of bacteria producing L-glutaminase. In addition, this medium contained phenol red, a very pH sensitive dye indicator. Phenol red will cause the medium turns yellow in neutral and acidic conditions while pink in alkaline condition. Reaction that occur due to production of L-glutaminase causes accumulation of ammonia in

the medium that eventually change pH of the medium into alkaline condition causing formation of pink zone around bacterial colony (Alian, Bazaraa, El-Shimi, & Mohamed, 2015).

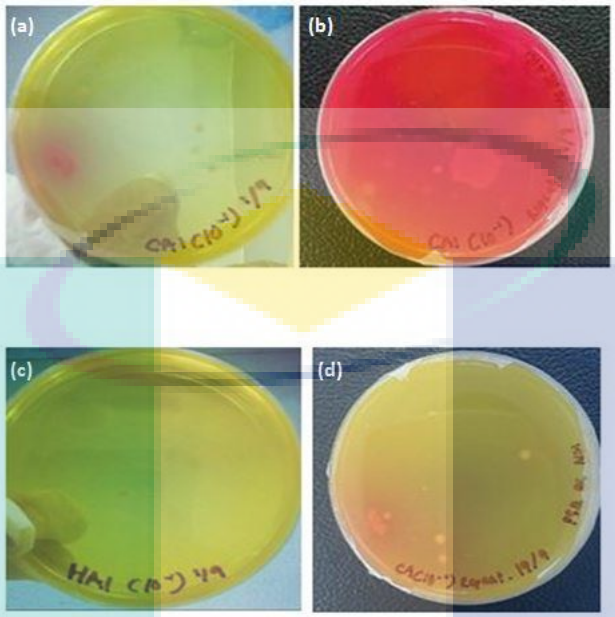

Figure 4.1 Primary screening of samples collected from Pantai Teluk Cempedak (a and b) and Pantai Batu Hitam (c and d).

Production of L-glutaminase by the samples grown on minimal glutamine agar medium is an example of extracellular enzyme. Extracellular enzyme can be described as the enzyme that is secreted and functions outside the host cell while intracellular enzyme is secreted and functions within the host cell. Most of microorganisms such as bacteria, yeast, and fungi are tested positive for their extracellular enzyme productivity (Arima, Sakamoto, Araki, & Tamura, 1972). A research study had shown that production of extracellular L-glutaminase enzyme is 2.6 to 6.8 times greater than the production of intracellular enzyme by certain strains such as *Pseudomonas fluorescens*, *Vibrio cholera*, and *Vibrio costicola* (Renu & Chandrasekaran, 1992). In terms of research and industrial applications, extracellular enzyme is more advantageous compared to intracellular enzyme. This is because extracellular enzyme can be produced abundantly in culture broth under normal conditions and it can be purified economically (Renu & Chandrasekaran, 1992).

Isolates with positive results spread plate method were sub-cultured by using patch-streak technique on minimal glutamine agar and incubated at 37 ºC for 24 hours. This was being done in order to obtain pure isolates from positive bacterial colonies. Most of positive bacterial colonies gave positive results. However, there were also some colonies showed

negative results, where there was no colour changes observed. Figure 4.2 showed the primary screening of positive isolates that produce L-glutaminase using patch-streak technique.

Figure 4.2 Patch-streak technique used for primary screening on positive isolates of L-glutaminase production.

4.2 Secondary screening of L-glutaminase producers from positive cultures.

Secondary screening to obtain L-glutaminase producer was carried out for all positive bacterial colonies. These bacterial colonies were labelled as LG1, LG2, LG3, LG4, LG5, LG6, LG7, LG8, LG9, LG10, LG11, LG12, LG13, LG14, LG15, LG16, and LG 17. They were preserved by mixing culture broth with glycerol stock (Sambrook, Fritsch, & Maniatis, 1989) and stored stable in the freezer at -80 ºC until further use.

Secondary screening enable a researcher to obtain microorganisms having the desired products and thus eliminating those lacking this potential (Sukesh, 2010). Secondary screening and analysis was carried out for all of the positive colonies from primary screening using streak-plate method. A total of 17 colonies were subjected for secondary screening as shown in Figure 4.3. The possible L-glutaminase producers were sub-cultured on minimal glutamine agar medium for isolation of pure isolates. All of the isolates showed positive results with variation in red zone formation except LG 15.

A total of 12 isolates known as LG1 until LG12 showed strong colour change on the minimal media whereas isolate LG13, LG14, LG16, and LG17 exhibited less intensity of pink zone. However, in secondary screening, LG15 showed yellow colour of the media which means there was no colour change. This indicates that L-glutamine was not being utilize by LG15 as

a carbon source and hence can acts as a control. Apparently, researchers found out that the elections of industrially dominant L-glutaminase strains were accomplished based on steady colour modification in the L-glutamine inclusive nutrient media and their number of colonies (Prakash, Poorani, Anantharaman, & Balasubramaniam, 2009). Therefore, the screening study showed that 12 out of 17 isolates which were LG1 to LG 12 gave positive results with strong colour change of minimal glutamine media compared to others. However, LG13, LG14, LG16, and LG17 showed less intensity of colour change from yellow to pink in colour on minimal glutamine media and hence these were not chosen for further studies. Those 12 positive isolates of LG1 to LG12 were proceed for biochemical characterizations, 16S rRNA, enzymatic analysis, optimization, partial purification, and characterization.

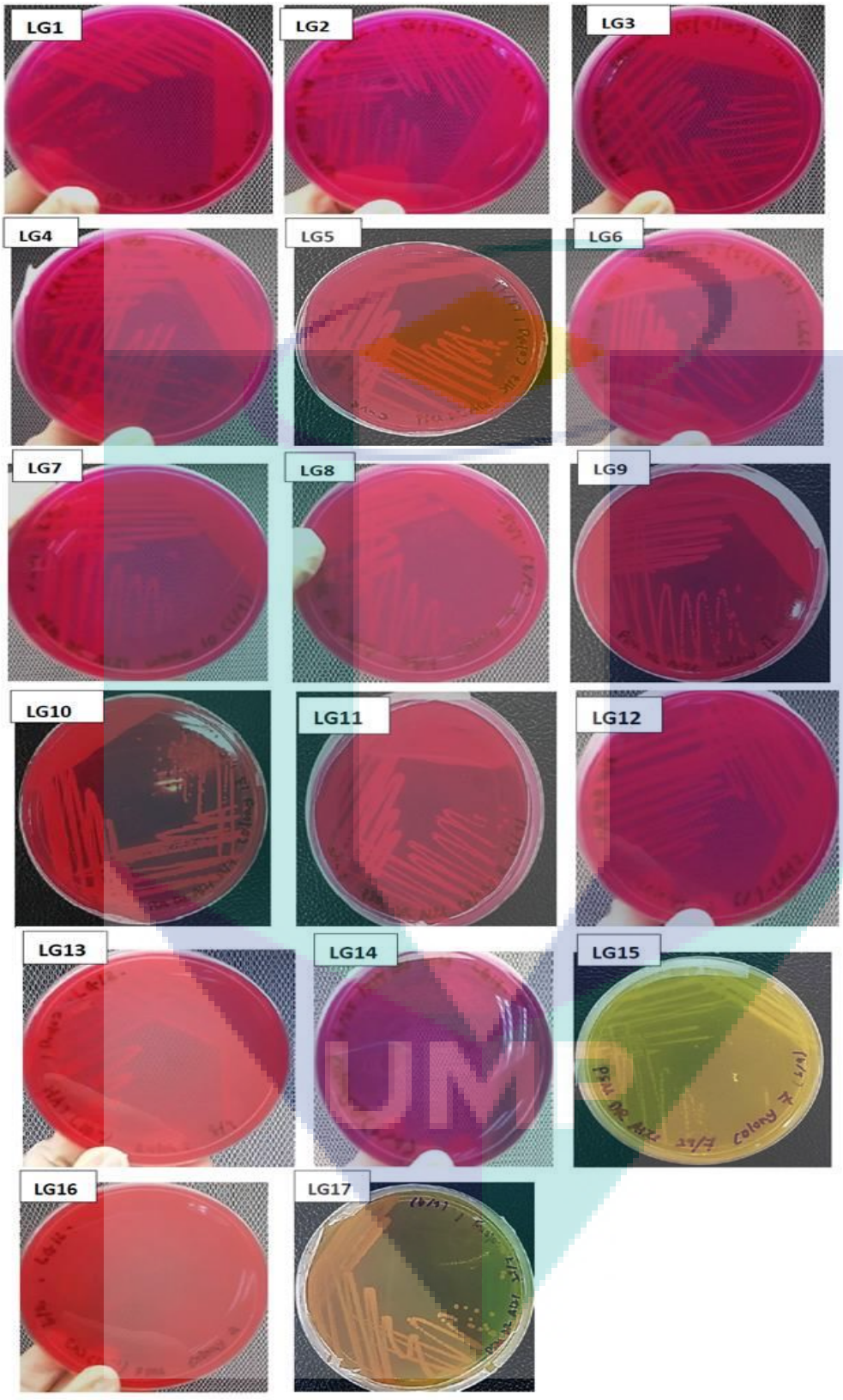

Figure 4.3 Secondary screening of L-glutaminase producers collected from primary screening

4.3 Biochemical characterization of the microorganisms.

4.3.1 Endospore staining

Spores enable bacteria to survive in hostile conditions. This is because spores are invulnerable to heat, dehydration, chemicals, and radiation. Bacteria capable to form endospores approximately in 6 to 8 hours after being exposed to adverse conditions (Krishnapriya & Babu, 2015). Spores contain a considerable amount of calcium and other particular elements such as manganese (Setlow, 2006). All of the 12 isolates were subjected to endospore staining and the results revealed that those were non-spore forming bacteria. This is because no green colour under the microscope was detected which means there was no endospores presence. Apart from that, the isolates are believed to have vegetative cells as the isolates were stained red in colour. Vegetative cell is the normally-growing cell that forms endospores however the spores are metabolically inactive and dehydrated. Spores can remain viable for years. When spores are exposed to favourable conditions, they can germinate into vegetative cells within 90 minutes (Krishnapriya & Babu, 2015). Apart from that, spore is a type of cell that can survive for extended period of time, with little or without nutrient and yet is capable to return to life if there is availability of nutrients (Setlow, 2006). Through this endospore staining test, we can conclude that all of the 12 isolates are non-spore forming bacteria.

4.3.2 Catalase Test

Catalase test is a type of test that helps to detect the presence of catalase enzyme in organisms. Based on the tests that had been carried out, all of the isolates showed negative response as there were no bubbles produced. The result of catalase test is positive if effervescence of oxygen occurs. The absence of catalase enzyme is indicated by no formation of bubbles. This showed that those bacteria were unable to breakdown hydrogen peroxide. Hydrogen peroxide is one of the oxidative end products of aerobic carbohydrate metabolism. From this test results, we can conclude that all of the 12 isolates are facultative anaerobic. Facultative anaerobic enables microorganisms to carry out aerobic respiration when oxygen is present and those microorganisms are capable of switching its respiration system to carry out anaerobic respiration if oxygen is absent. In addition, the metabolism of anaerobic bacteria can be visualized as an open system where there will be a continuous input and output of matter and energy (Thauer, Jungermann, & Decker, 1977). Furthermore, a new research has found out

that stress hormones such as norepinephrine and epinephrine can give effect to the growth of anaerobic bacteria either increase, decrease, or no growth at all (Boyanova, 2017).

4.3.3 Oxidase test

Oxidase test is used to identify the existence of bacterial cytochrome oxidase enzyme. Based on the oxidase test results, all of the isolates showed negative results as there were no dark purple colour appeared. Negative result was indicated as there was no colour change to dark purple which means the absence of cytochrome oxidase enzyme. For a positive result, there will be a development of dark purple colour which indicates the presence of oxidase. Presence of this enzyme causes the cytochrome oxidase dye to be oxidized to indophenols blue while in its reduced stage, it will be colourless. Hence, isolates of Lg1 to Lg12 can be categorized as oxidase negative.

4.4 16S rRNA Gene Sequence Analysis

Biochemical characterization is an approach that can be used to identify bacterial species. However, bacterial species could be confirmed through 16S rRNA gene sequence analysis. This is due to ability of 16S rRNA gene sequence analysis to identify bacterial species through its genetics. 16S rRNA gene sequence analysis is by far the most common housekeeping genetic marker used to study bacterial phylogeny and taxonomy. This is due to some reasons which are the function of 16S rRNA gene is unchanged overtime, this gene is present in almost all bacteria, and the number of bases for 16S rRNA gene is big enough for informatics purposes (Janda & Abbott, 2007). A total number of 12 positive isolates namely Lg1 to Lg12 were chosen to be analysed through 16S rRNA gene sequencing. 16S rRNA gene fragments of the isolates were amplified using universal primers of forward and reverse named as 8F and U1492R, respectively. Fully sequences gained were analysed online through BLASTN for nucleotide similarity against 16S rRNA database. BLAST program is so useful as it can find short matches between two sequences and enable to start alignments from these spots (Ye, McGinnis, & Madden, 2006).

All of the isolates were undergo colony PCR reaction using PCR amplification kit. Results from the reactions which was the PCR products which was DNA fragments were used as samples to run electrophoresis. Electrophoresis is a laboratory technique used to separate macromolecules such as DNA, RNA, or proteins based on their size and charge with usage of current through agarose gel. The samples will travel through agarose gel from negative charge

to positive charge. After electrophoresis gel was visualized under UV transluminator and DNA bands happened to be present, PCR products were then subjected to be purified using PCR purification kit. After that, the samples were undergo electrophoresis again in order to make sure that the desired DNA bands were present.

4.4.1 Nucleotide sequence analysis

After purification of PCR samples, those samples were sent to First BASE Malaysia Sdn Bhd for sequencing services. Identities of all 12 isolates were identified using a BLAST database from the National Center for Biotechnology Information (NCBI). This platform was used to execute nucleotide sequences homology of those 12 isolates from its database. Sequences and query coverage of those 12 isolates were tabulated and shown in Appendix J.

16S rRNA gene sequencing and BLAST database had helped in revealing species of those 12 isolates. It was revealed that Lg1, Lg3, Lg4, Lg6, Lg8, Lg9, Lg10, and Lg12 turned out to be the same species of bacteria known as *Kosakonia radicincitans.* All of them have a BLAST identity with a range of 86% to 99% as shown in Table 4.1.

Table 4.1 Species identified for sequences of 12 isolates obtained from 16S rRNA gene sequencing using BLAST database from NCBI.

Isolates	Species	BLAST Identity
Lg1	*Kosakonia radicincitans*	97%
Lg2	*Kosakonia oryzae*	95%
Lg3	*Kosakonia radicincitans*	94%
Lg4	*Kosakonia radicincitans*	99%
Lg5	*Kosakonia oryzae*	86%
Lg6	*Kosakonia radicincitans*	95%
Lg7	*Pseudomonas aeruginosa*	99%

Lg8	*Kosakonia radicincitans*	99%
Lg9	*Kosakonia radicincitans*	94%
Lg10	*Kosakonia radicincitans*	99%
Lg11	*Shigella flexneri*	95%
Lg12	*Kosakonia radicincitans*	97%

Kosakonia is a type of genus that is formerly known as Enterobacter. It has a complicated history on classification of its strains with several species had been transfers in and out of the genus over the past 20 years (Brady, Cleenwerck, Venter, Coutinho, & De Vos, 2013). For instance, *Enterobacter agglomerans* which was classified in genus Enterobacter was transferred to novel genus Pantoea back in 1989 (Ewing & Fife, 1972). Several *Enterobacter* species known as *Enterobacter kobei*, *Enterobacter nimipressuralis*, *Enterobacter asburiae*, and *Enterobacter cloacae* were belong to a group known as '*E. cloacae* complex' which was classified based on phenotypic and genotypic relatedness (Hoffmann et al., 2005). Recently, a research study has shown that *Enterobacter* can be reclassified into five different genera based on phylogenetic analyses of the concatenated nucleotide sequence (Brady et al., 2013). Genus of *Enterobacter* that consists of 19 species is known to be one of the largest genera within the family of *Enterobacteriaceae (Brady et al., 2013).*

Meanwhile, Lg2 and Lg5 were identified as *Kosakonia oryzae* with 95% and 86% BLAST identity respectively. Both *K. radicincitans* and *K. oryzae* came from the same genus which was Kosakonia. However, they were both different in terms of species which defines an organism to its simplest level. *K. radicincitans* are rod-shaped cells with measurement about 0.8 to 1.2 µm in length and 1.0 to 1.6 µm in width (Kämpfer, Ruppel, & Remus, 2005). Meanwhile, *K. oryzae* forms straight or slightly curved rod cells with measurement of 0.8 to 1.1 µm in length and 1.0 to 1.6 µm in width (Peng et al., 2009).

Both species were happened to be facultatively anaerobic. Facultative anaerobic is the way of how organisms respire. Facultative anaerobe organisms capable of producing adenosine triphosphate (ATP), a type of high-energy molecule by anaerobic respiration or fermentation without the presence of oxygen. However, the energy production efficiency is very low but rate

of glucose utilization is high. With the presence of oxygen, those organisms capable to switch from anaerobic respiration to aerobic respiration with high yield of ATP production. This phenomena is called the Pasteur Effect. A study reported by Pasteur explained that glucose utilization by yeast is rapid in the presence of oxygen compared to the rate of glucose utilization in the absence of oxygen (Racker, 1974). In 1926, this phenomenon was called the "Pasteur reaction" by Warburg and later it changed to 'Pasteur Effect' (Racker, 1974). The action of mechanism of the Pasteur effect happens at the cellular level where there is changes in the concentration of adenine nucleotides and inorganic phosphate (Schmidt & Kamp, 1996).

On the other hand, Lg7 turned out to be classified in a genus of Pseudomonas. It was identified as *Pseudomonas aeruginosa* with a BLAST identity of 99%. Lg11 was found out to be *Shigella flexneri* with identity of 95%. Both *P. aeruginosa* and *S. flexneri* are gram-negative bacteria (Hancock, 1998). Generally, bacteria can be divided into gram-negative and gram-positive bacteria. A gram stain test can be used to differentiate this two type of bacteria. Bacteria that retained purple is said to be gram-positive bacteria while bacteria that stained red is classified as gram-negative bacteria. This staining is due to difference in chemical and structural make up of their cell walls (Beveridge, 2001). Gram- positive bacteria has a very thick and relatively impermeable cell wall that consist of very thick peptidoglycan. Meanwhile, gram-negative bacteria has a thin peptidoglycan that are sandwiched between outer and inner bacterial membrane (Beveridge, 2001).

Phylogenetic tree also known as evolutionary tree is a branching diagram that shows evolutionary relationships between biological species. Process that are involved in identification of prokaryotic isolates using 16S rRNA gene sequence include similarity search against public domain nucleotide databases, sequence retrieval of type strains with validity published names, calculation of pairwise nucleotide similarities values between sequences of the isolate and phylogenetically neighbouring type strains and finally phylogenetic analysis (Chun et al., 2007). Figure 4.5 showed a phylogenetic tree of four identified species from 12 isolates based on 16S rRNA gene sequence.

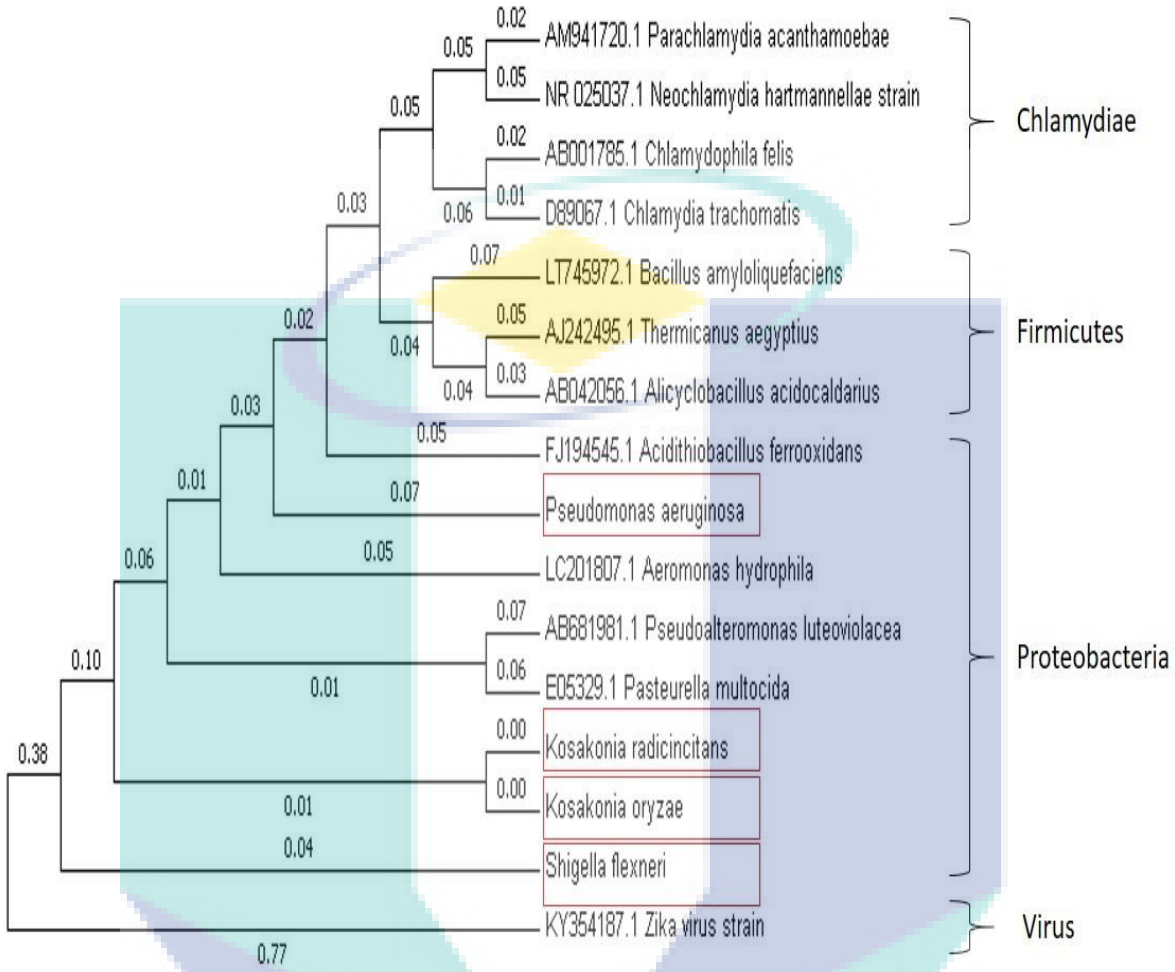

Figure 4.5 A phylogenetic tree of isolates based on 16S rRNA gene sequence. The zika virus strain was used as an out-group to root the tree.

The analysis summarized in Figure 4.5 showed that 16S rRNA gene of four identified species from 12 isolates were *P. aeroginosa, K. radicincitans, K. oryzae,* and *S. flexneri*. All of them were clustered in the *Proteobacteria* group. The phylum of *Proteobacteria* has the largest phylogenetic composition that consists of 116 validated bacterial families (Shin, Whon, & Bae, 2015). *Proteobacteria* is abundant in marine and terrestrial environments (Giovannoni et al., 2005) *Proteobacteria* can be further divided into five different classes which are *Alphaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Gammaproteobacteria,* and *Zetaproteobacteria*. *K. radicincitans, K. oryzae, P. aeroginosa,* and *S. flexneri* happened to be in *Gammaproteobacteria* class.

In addition, all of them belong in the same scientific classification from kingdom lower down to family which was Enterobacteriaceae except *P. aeroginosa*. *P. aeroginosa* was in the same class with the others. However, it belonged to different order, family genus, and species which was *Pseudomonadales*, *Pseudomonadaceae*, *Pseudomonas*, and *P. aeruginosa* respectively. Meanwhile, *S. flexneri* was classified in different genus and species from *K. radicincitans* and *K. oryzae*. It belonged to genus of *Shigella* and species of *S. flexneri*.

K. radicincitans and *K. oryzae* were happened to be sister groups. Both of them came from the common recent ancestor, which was then form a clade of *K. radicincitans* and *K. oryzae*. *S. flexneri* was sister group to clade of *K. radicincitans* and *K. oryzae*. A research study had stated that phylogenetic analysis of the 16S rRNA gene sequence of *K. oryzae* is closely related to *K. radicincitans* with 98.9% similarity (Peng et al., 2009). In addition, bootstrap values which is an index of phylogenetic accuracy are higher outside the tree while lower inside the tree (Kawase et al., 2004).

4.5 L-Glutaminase Assay Analysis.

A total of 12 successful positive isolates were subjected for L-glutaminase assay individually to measure their enzymatic activity. Quantitative estimation of L-glutaminase activity by those isolates was being done using Nesslerization process and followed by the absorbance reading at 450 nm (Karim & Thalij, 2016). The standard curve for enzyme assay was plotted and shown in Appendix C. Furthermore, after those 12 isolates were assayed, the absorbance readings and enzyme activities of those 12 positive isolates were recorded and tabulated.

Table 4.2 Absorbance at 450 nm of wavelength and enzyme activity of 12 positive isolates from Lg1 to Lg12

Samples	Absorbance at 450 nm wavelength	Enzyme activity ± SE (U/ml)
Lg1	0.061	0.074 ± 0.010
Lg2	0.073	0.095 ± 0.002
Lg3	0.054	0.062 ± 0.006

Lg4	0.055	0.064 ± 0.008
Lg5	0.058	0.069 ± 0.011
Lg6	0.059	0.071 ± 0.004
Lg7	0.069	0.088 ± 0.002
Lg8	0.078	0.103 ± 0.001
Lg9	0.065	0.081 ± 0.007
Lg10	0.070	0.089 ± 0.017
Lg11	0.076	0.100 ± 0.002
Lg12	0.071	0.091 ± 0.000

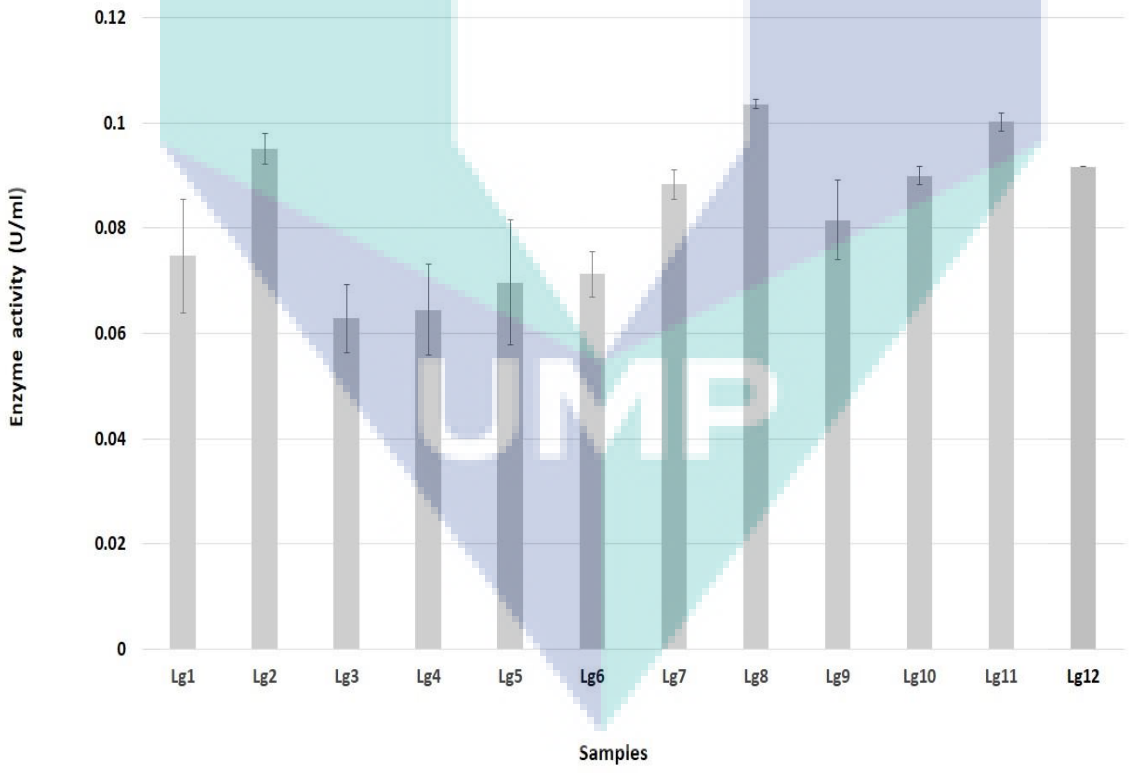

Figure 4.6 Enzyme activity (U/ml) of 12 successful positive isolates. Lg8 known *as K. radicincitans* has the highest enzyme activity which is 0.103 ± 0.001 U/ml followed by Lg11 identified as *S. flexneri* which has the enzyme activity of 0.100 ± 0.002 U/ml.

The absorbance at 450nm wavelength by spectrophotometer (Table 4.2) is proportional to the production of end product of the enzyme activity which is ammonia. The higher the absorbance, the higher the production of ammonia. Hence, the greater the enzyme activity is taking place. The enzymatic activities were ranged from 0.062 to 0.103 U/ml as shown in figure 4.6. The maximum activity was recorded for Lg8 which is *K. radicincitans* where it has the enzymatic activity of 0.103 ± 0.001 U/ml followed by Lg11 identified as *S. flexneri* which has the enzyme activity of 0.100 ± 0.002 U/ml. These two isolates were then being chosen for further study. A study of production of L-glutaminase from Enterobacter cloacae has been reported (Nandakumar, Yoshimune, Wakayama, & Moriguchi, 2003). However, no study on L-glutaminase production from *Shigella* species has been reported.

4.6 Optimization of process parameters

Optimization of process parameters are very important in order to provide optimum conditions for bacteria to grow and produce maximum production of interest enzyme with high enzymatic activity. A conventional method known as One Factor at A Time (OFAT) was used for the optimization of parameters. OFAT method is widely used and known for its sensitivity analysis (Qi, Mikhael, & Funnell, 2004). In this research study, two isolates that gave the highest enzymatic activity were choosen for further studies. Those isolates were Lg8 and Lg11 which scientifically known as *K. radicincitans* and *S. flexneri*, respectively. Five different parameters had been manipulated such as temperature, additional of organic nitrogen sources, additional of inorganic nitrogen sources, additional of carbon sources and pH. After optimization of each of these parameters, an experiment had been conducted under all of the optimized conditions for both isolates individually.

4.6.1 Temperature

Figure 4.7 below showed enzymatic activities of L-glutaminase from *K. radicincitans and S.flexneri* respectively at five different incubation temperatures which were 31°C, 34°C, 37°C, 40°C, and 43°C. Based on the figure 4.7(A), *K. radicincitans* has the highest enzymatic activity when being incubated at 37°C followed by 34°C, 40°C, 31°C and 43°C. The highest enzyme activity recorded was 0.1103 ± 0.002 U/ml. On the other hand, figure 4.7(B) showed that enzymatic activity of *S. flexneri* had the highest value when it was being incubated at 37°C. Hence, the optimum temperature for *S. flexneri* was at 37°C where its enzymatic activity reached 0.1025 ± 0.001 U/ml. At 40°C of incubation, enzymatic activity of *S. flexneri* was decrease slightly to 0.0769 ± 0.002 U/ml and its enzymatic activity was further decrease to

0.0629 ± 0.005 U/ml at 34°C of incubation. Enzymatic activity of *S. flexneri* showed larger decrease of its value when being incubated at 31°C and 43°C. The enzymatic values recorded were 0.0377 ± 0.007 U/ml and 0.0255 ± 0.004 U/ml for incubation at 31°C and 43°C respectively.

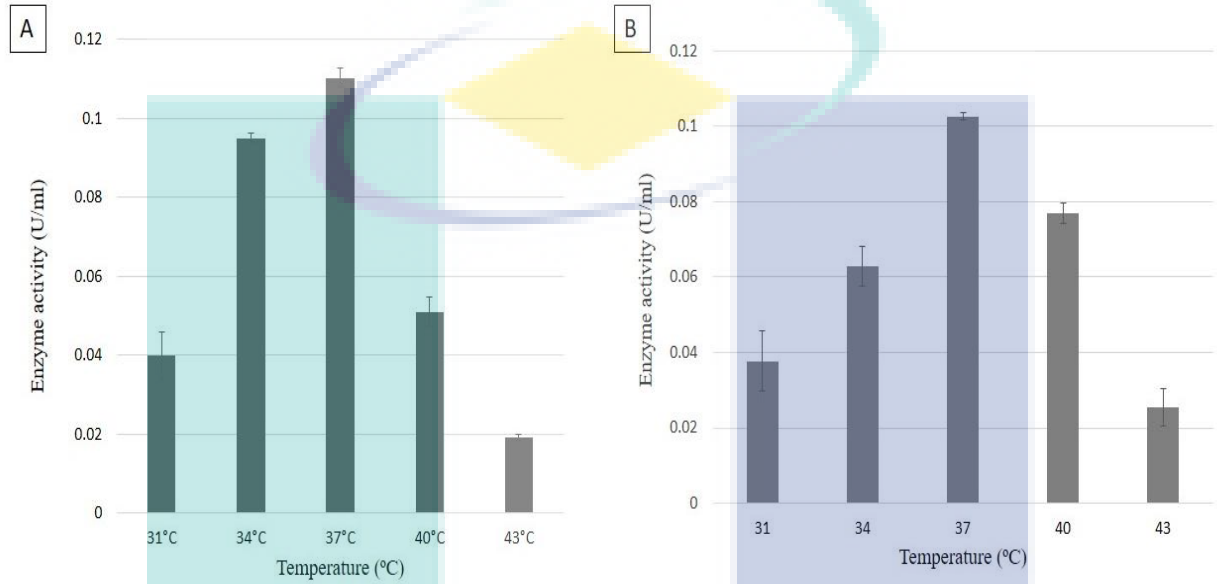

Figure 4.7 Enzyme activity of L-glutaminase from K. radicincitans (A) and *S.flexneri* (B) at five different incubation temperatures.

Both results of enzymatic activities from *K. radicincitans* and *S. flexneri* were parallel with each other. Both results showed that incubation temperature above and below 37°C caused decrement of the enzymatic activities for both *K. radicincitans* and *S. flexneri*. This proved that there were reduction in production of L-glutaminase enzyme by *K. radicincitans* and *S. flexneri*. Hence, incubated temperature definitely influenced the L-glutaminase production by both microorganisms. Therefore, temperature is one of the physiochemical factors that would give impact towards the enzymatic activity of L-glutaminase from *K. radicincitans.*

Optimum temperature for maximum enzymatic activity varies for each species of bacteria. Mesophilic microbes were found to have optimum temperature in a range of 22°C to 37°C for L-glutaminase production (Keerthi, Suresh, Sabu, Rajeevkumar, & Chandrasekaran, 1999). If the incubation temperature is below or above the optimum range then this will leave destructive effects on metabolic activities of microorganisms (Abdallah et al., 2012). In addition, another study had reported that temperatures that were outside the range of optimum temperature either lower or higher showed slower rates of microbial metabolic activities

(Tunga, Banerjee, & Bhattacharyya, 1999). Hence, there will be less production of enzyme in those temperature which will then has lesser enzymatic activity.

4.6.2 Organic Nitrogen Sources

Organic nitrogen sources is one of the nutrients that needed by bacteria in order to grow and survive. In this research study, investigation on which organic nitrogen sources that *K. radicincitans* and *S. flexneri* utilize the most for its production of L-glutaminase is performed. Based on figure 4.8(A), we can see that *K. radicincitans* culture that supplied with additional beef extract has the highest enzymatic activity compared with peptone and yeast extract. The highest enzymatic activity is recorded to be 0.120 ± 0.003 U/ml, this was followed by additional of peptone that has enzymatic activity of 0.098 ± 0.003 U/ml and the least is yeast extract where the enzymatic activity is 0.088 ± 0.003 U/ml.

Meanwhile, *S. flexneri* utilize yeast extract the most for its L-glutaminase production. This is because *S. flexneri* has the highest enzymatic activity when its culture media was supplied with yeast extract. The highest enzymatic activity recorded was 0.1007 ± 0.007 U/ml and followed by 0.0669 ± 0.002 U/ml with the additional of peptone. On the other hand, additional of beef extract gave the least enzymatic value which is 0.0477 ± 0.0004 U/ml.

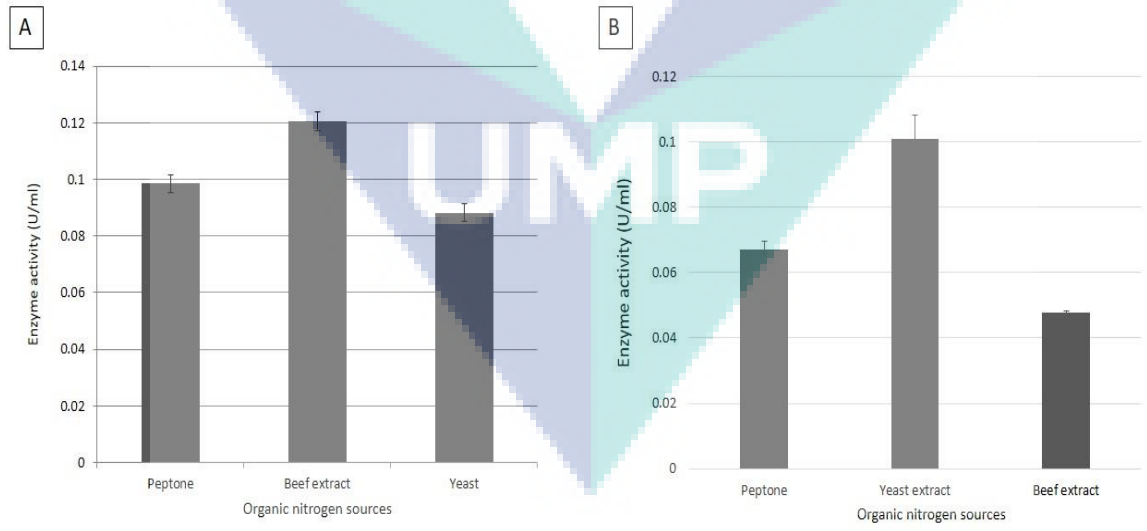

Figure 4.8 Enzyme activities of L-glutaminase from *K. radicincitans* (A) and *S. flexneri* (B) in culture media containing different type of organic nitrogen sources.

A research study has discovered that organisms can consume either organic or inorganic forms of nitrogen as their only source of nitrogen (Wheeler & Kirchman, 1986). Not only that, organisms such as bacteria are well competitive for simple carbohydrates under nitrogen-rich conditions (Meidute, Demoling, & Bååth, 2008). With addition of organic nitrogen sources in culture media such as beef extract, yeast extract or peptone, *K. radicincitans* and *S. flexneri* become well competitive for simple carbohydrate such as glucose which then allow their rapid growth under favored conditions and environment.

Beef extract and yeast extract are both gave the highest impact on L-glutaminase production from *K. radicincitans* and *S. flexneri*, respectively. Both beef and yeast extract are nitrogen rich sources and very suitable for use in microbiological culture media preparation. Beef extract is a dehydrated extract of selected fresh lean beef while yeast extract is an autolysate of yeast cells. Nitrogen sources in culture media can be considered as limiting factor in enzyme production (Abdallah et al., 2012). A research study conducted by Abdallah et al. (2012) stated that additional of nitrogen source which was peptone in culture media gave a maximum L-glutaminase enzymatic activity of *Streptomyces avermitilis*. In addition, peptone was also found to be the most suitable for L-glutaminase production from *Streptomyces labedae*. Different type of bacteria tend to prefer different type of organic nitrogen sources for maximum L-glutaminase production.

4.6.3 Additional of Inorganic Nitrogen

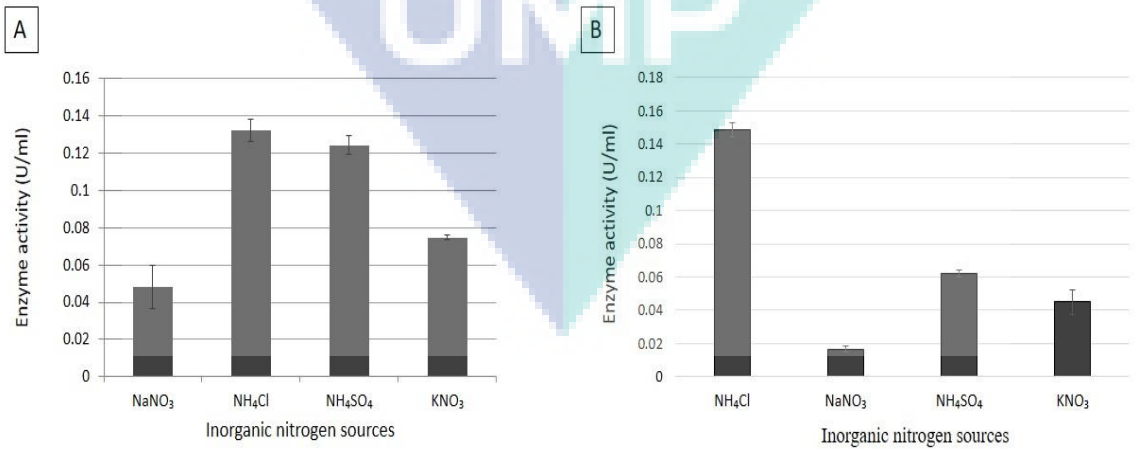

Figure 4.9 Enzyme activities of L-glutaminase from *K. radicincitans* (A) and *S. flexneri* (B) in culture media containing different type of inorganic nitrogen sources.

Results recorded in figure 4.9 showed the influence and the effect of additional inorganic nitrogen sources towards the enzymatic activity. In this research study, additional of NH_4Cl in *K. radicincitans* culture cause the biggest impact on the enzymatic activity of L-glutaminase by *K. radicincitans* compared to other inorganic nitrogen sources such as $NaNO_3$, NH_4SO_4, and KNO_3. It is proved that the addition of NH_4Cl gave a significant influence on L-glutaminase production by *K. radicincitans*.

With the addition of NH_4Cl, a type of inorganic nitrogen source within the *K. radicincitans* culture, the enzymatic activity experience a sudden increase to 0.132 ± 0.005 U/ml. However, the highest enzymatic activity with the addition of organic nitrogen sources is only 0.120 ± 0.003 U/ml, an addition with beef extract. The enzymatic activity of *K. radicincitans* culture with the addition of NH_4SO_4 is 0.124 ± 0.005 U/ml. Production of L-glutaminase enzyme was the least when *K. radicincitans* culture was added with $NaNO_3$. Its enzymatic activity is only 0.048 ± 0.012 U/ml.

Besides that, enzymatic activity of L-glutaminase from *S. flexneri* was the highest when there was additional of NH_4Cl within the *S. flexneri* culture. The highest enzymatic activity recorded was 0.1486 ± 0.004 U/ml. This was followed by addition of NH_4SO_4 within the culture that had the enzymatic activity of 0.0618 ± 0.002 U/ml. The enzymatic activity of L-glutaminase from *S. flexneri* is the least with the additional of $NaNO_3$ in its culture media. The enzymatic activity recorded was only 0.0165 ± 0.001 U/ml. Among three different type of inorganic nitrogen involved, $NaNO_3$ gave the least impact for enzymatic activity of L-glutaminase from both *K. radicincitans* and *S. flexneri*. Meanwhile, L-glutaminase extracted from both microorganisms had the highest enzymatic activity with the additional of NH_4Cl into their culture media.

A study has revealed that ammonium is an important primary nitrogen source for organisms such as heterotrophic bacteria (Wheeler & Kirchman, 1986). A research study reported by stated that amount of inorganic nitrogen uptake by marine heterotrophic bacteria account for approximately 78% and 32% of total ammonium and nitrate uptake, respectively (Fouilland, Gosselin, Rivkin, Vasseur, & Mostajir, 2007). This proof is in line with the experiment result where addition of NH_4Cl within the *K. radicincitans* culture cause the highest enzymatic activity increment to 0.132 ± 0.005 U/ml. Not only that, ammonium also boosted the enzymatic activity of L-glutaminase from *S. flexneri* with the additional of NH_4Cl in its culture medium. Hence, ammonium indirectly increase the production L-glutaminase from

those two different type of microorganisms. Furthermore, bacteria require both glucose and ammonium to stimulate their growth and no effect when supplied singly and hence can be concluded that heterotrophic bacteria require and utilize both substrate (Wheeler & Kirchman, 1986). Nitrogen generally is required by all living organisms and its presence is vast in the atmosphere, hydrosphere, and biosphere of the Earth (Li et al., 2018). Excess of nitrogen such as ammonia can be converted into nitrite and nitrate which then can be released to atmosphere as nitrogen gas through nitrification and denitrification respectively (Li et al., 2018).

4.6.4 Carbon sources

Beside, bacteria culture was also being added with different type of carbon sources individually such as maltose, glucose, and sucrose. Hence, enzymatic activity of L-glutaminase from *K. radicincitans* was measured. From figure 4.10 (A), bacteria culture added with additional glucose has the highest enzymatic activity compared to maltose and sucrose. Its highest enzymatic activity is 0.125 ± 0.002 U/ml followed by additional maltose which has enzymatic activity of 0.086 ± 0.006 U/ml. Additional of sucrose into bacteria culture has the lowest enzymatic activity which is 0.069 ± 0.003 U/ml. Meanwhile, L-glutaminase extracted from *S. flexneri* had the highest enzymatic activity when its culture medium was added with glucose. The enzymatic activity recorded was 0.1516 ± 0.006 U/ml. This was followed with the additional of sucrose with enzymatic activity of 0.0797 ± 0.004 U/ml. Additional of maltose in the culture medium has the lowest enzymatic activity of 0.0532 ± 0.0005 U/ml.

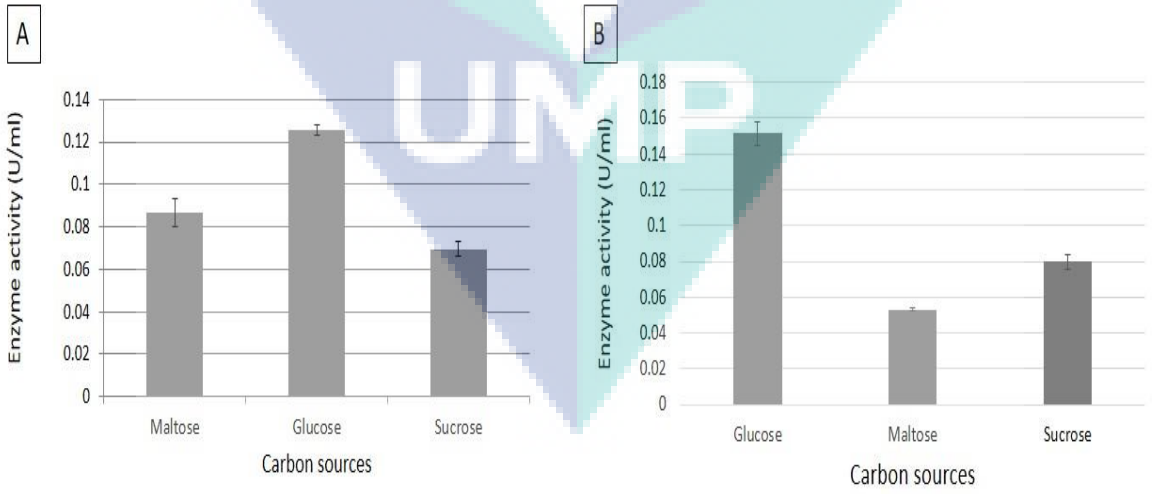

Figure 4.10 Enzymatic activity of L-glutaminase extracted from *K. radicincitans* (A) and from *S. flexneri* (B) in culture media containing different type of carbon sources.

Enzymatic activities of these two microorganisms were optimized with additional of glucose as carbon sources into their culture medium as both had the highest reading of enzymatic activities compared to the other type of carbon sources. Between these three types of carbon sources, additional of sucrose was the least favored for L-glutaminase extracted from *K. radicincitans*. However, additional of maltose in culture medium is the least favored by L-glutaminase from *S. flexneri*

On top of that, another research had found out that glutaminase production of *Trichoderma koningii* can be enhanced by the availability of carbon sources such as glucose and maltose under solid state fermentation (Pallem, Manipati, & Somalanka, 2010). This might be due to easy accessibility of additional carbon sources other than glutamine which cause rapid growth to be accomplished (Keerthi et al., 1999). Easy accessibility of carbon sources other than glutamine such as glucose in the culture medium helped both *K. radicincitans* and *S. flexneri* to undergo rapid growth thus allowing more production of L-glutaminase and hence increase their enzymatic activities.

K. radicincitans and *S. flexneri* need energy for their growth and enzyme production. Hence, glucose is abundantly used to provide energy in a form of molecule known as Adenosine triphosphate (ATP) molecule through glycolytic pathway. Besides that, supplementation of glucose to the wheat bran medium for solid state fermentation of *Zygosaccharomyces rouxii* gave maximum L-glutaminase production (Kashyap et al., 2002). Furthermore, glucose was also the best carbon source for maximum L-glutaminase production extracted from *Streptomyces pratensis* (Tork et al., 2018).

4.6.5 pH

pH is one of the vital factors that gives big impacts towards bacterial development and survival. Some pH might not be suitable for bacterial growth and its development while the other might be the best environment for it to grow and survive at optimum level.

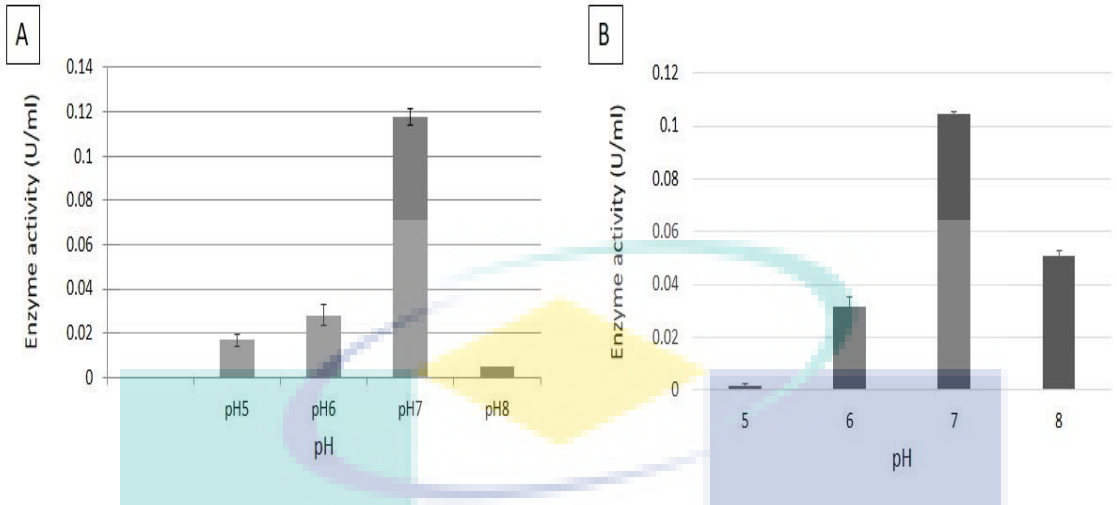

Figure 4.11 Enzyme activity of L-glutaminase extracted from *K. radicincitans* (A) and *S. flexneri* (B) in different pH environment.

Figure 4.11 (A) shows the enzymatic activity of *K. radicincitans* at different pH environments such as pH 5, pH 6, pH 7, and pH 8. Enzymatic activity of *K. radicincitans* is the highest at pH 7 which is 0.117 ± 0.004 U/ml and followed by pH 6 that has the enzymatic activity of 0.028 ± 0.002 U/ml. This shows that pH 7 is the optimum pH that is the most suitable environment for *K. radicincitans* growth and survival. This might be due to the balance of ionic strength of *K. radicincitans's* plasma membrane (El-Sayed, 2009). On the other hand, figure 4.11 (B) also shows the enzymatic activity of *S. flexneri* in different pH environment. L-glutaminase extracted from *S. flexneri* showed the highest enzymatic activity at pH 7.The highest enzymatic activity recorded was 0.1045 ± 0.0009 U/ml. This showed that pH 7 is the optimum environment for L-glutaminase production by *S. flexneri*. The enzymatic activity of L-glutaminase from *S. flexneri* at pH 6 was 0.0317 ± 0.003 U/ml while the lowest enzymatic activity recorded was at pH 5 which is 0.0015 ± 0.0007 U/ml.

One of the important factors affecting microbial community is pH (Rousk, Brookes, & Bååth, 2009). A study had discovered that 19 different soils from different areas that have pH range between pH 4 to 8, showed that bacterial growth was increase with higher pH as measured by Leu incorporation (E Bååth, 1998). This fact is actually parallel with the results obtained. The enzymatic activity of L-glutaminase extracted from both *K. radicincitans* and *S. flexneri* showed increment from pH 4 to pH 7. This can be concluded that there were more bacteria growth and thus more L-glutaminase production with increase in value of pH environments. However, as the pH increase to pH 8, the enzymatic activities of both

microorganism showed a bit of decrement. Therefore, this is indirectly explained that pH 7 is the optimum pH for both microorganisms. Furthermore, a research study had shown that bacterial growth increases four times between pH 4 and pH 8 (Rousk et al., 2009). Apart from that, another research study found out that bacterial growth raise about five fold due to soil pH change form pH 4 to pH 7, after treated with lime and ash (Erland Bååth & Arnebrant, 1994). On top of that, a research study conducted by Abdallah et al. (2012) reported that maximum L-glutaminase production by *Streptomyces avermitilis* was recorded at pH 8 while maximum L-glutaminase production by *Streptomyces labedae* was at pH 7.

4.6.6 Experiment with all parameters at its optimal level.

Experiments were being carried out under optimized conditions of temperature, additional of organic and inorganic nitrogen sources, additional of carbon sources, and pH for both *K. radicincitans and S. flexneri*, individually. For *K. radicincitans* the experiment was conducted at 37°C and pH 7 with the additional of ammonium chloride as inorganic nitrogen source, beef extract a type of organic nitrogen sources, and lastly, with addition of glucose a type of carbon source. *K. radicincitans* showed the highest enzymatic activity which is 0.3542 ± 0.006 U/ml.

Meanwhile, the experiment conducted for *S. flexneri* was at 37°C and pH 7 with the additional of ammonium chloride as inorganic nitrogen source, yeast extract a type of organic nitrogen sources, and lastly, with addition of glucose a type of carbon source. *S. flexneri* showed the highest enzymatic activity of 0.3012 ± 0.005 U/ml. As been discussed before, heterotrophic bacteria such as *K. radicincitans and S. flexneri* can easily be stimulated for its growth and survival under favored conditions. One of its favored conditions is high nitrogen sources such as ammonium which then cause it to easily absorbed simple carbohydrates particularly glucose that was highly presence in its culture.

4.7 Partial purification of L-glutaminase

K. radicincitans had been chosen for further studies due to higher enzymatic activity compared to *S. flexneri* when an experiment had been carried out with all parameters at its optimal level. Hence, *K. radicincitans* was used to be proceed for partial purification, SDS-Page, Bradford Analysis, and characterization.

A method known as ammonium sulphate precipitation was chosen to partially purify crude L-glutaminase enzyme. The precipitation was done at three different saturation which was 50, 60, and 70% saturation. Results indicated that maximum specific activity was 9.71 units/mg at 60% saturation followed by 2.47 units/mg and 4.62 units/mg at 50% and 70% saturation, respectively. L-glutaminase enzyme produce by different L-glutaminase producers probably have different molecular characteristics that cause different saturation of ammonium sulphate for purification purpose. A research study proved that L-glutaminase from *Brevundimonas diminuta* needed to use 60% saturation to have maximum specific activity of 21.9 units/mg (Jayabalan et al., 2010). On the other hand, a research study reported that 80% saturation of ammonium sulphate are needed to obtain the highest specific activity of L-glutaminase from *Streptomyces olivochromogenes* (Balagurunathan et al., 2010). Meanwhile, another research study conducted by Abdallah et al. (2013) showed that L-glutaminase from *Streptomyces avermitilis* needed 80% saturation in order to obtain maximum specific activity of 9.7 units/mg. Table 4.3 showed purification table of L-glutaminase from *K. radicincitans*.

Table 4.3 Ammonium sulphate precipitation of L-glutaminase

Purification step	Volume (ml)	Total Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude enzyme	1000	152.00	369.10	2.43	1.00	100.00
$(NH_4)_2SO_4$ (60%)	200	29.53	286.77	9.71	3.99	77.69
Dialysis	5	1.28	0.19	0.15	0.06	0.05

Table 4.3 showed purification steps of L-glutaminase from *K. radicincitans*. The specific activity and purification fold of L-glutaminase enzyme increase up to 9.71 units/mg and 3.99 fold respectively after precipitation with 60% ammonium sulphate saturation. However, the value of those specific activity and purification fold of L-glutaminase decrease

after dialysis. This might be due to protein degradation as the enzyme was dialyzed in a cellulose bag overnight at 4ºC in a chiller. Enzyme has its own range of optimum temperature for it to work effectively. With low temperature, enzyme started to lose their activity and denatured.

4.8 Determination of Protein Concentration

4.8.1 SDS-PAGE and Bradford Analysis

Partial purified L-glutaminase enzyme was assessed by SDS-PAGE. The result for SDS-PAGE are shown in figure 4.12.

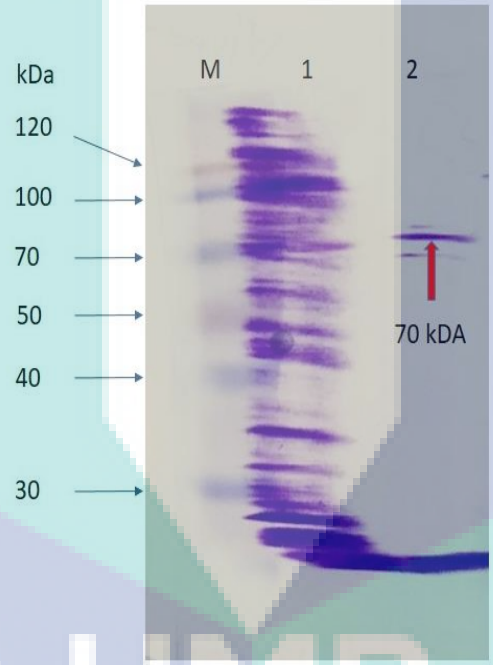

Figure 4.12 SDS – PAGE of partially purified L-glutaminase, M: protein molecular mass marker, Lane 1: sample of crude enzyme and Lane 2: partially purified L-glutaminase supernatant.

Based on figure 4.12, a protein band of 70 kDA was observed. Based on a few researches, L-glutaminase produce by different type of species tend to have different molecular weight. Purified L-glutaminase enzyme from *S. avermitilis* was estimated to have a molecular weight of 50 kDA (Abdallah et al., 2013). Meanwhile, a research reported by Kozlov, Kovalenko, and Mardashev (1972) showed that L-glutaminase enzyme produced by *B. subtilis* has a molecular weight of 55kDA. However, some species of bacteria and fungus produced L-glutaminase enzyme with higher molecular weight. For instance, Jayabalan et al. (2010) in his

research found out that L-glutaminase enzyme produced by *B. diminuta* has a molecular weight of 140kDA. On top of that, *P.aeruginosa* was reported to produce L-glutaminase with a molecular weight of 137 kDA (Nandakumar et al., 2003). A fungus known as *Aspergillus oryzae* was also reported to produce L-glutaminase enzyme with a molecular weight of 82 kDa (Klein, Kaltwasser, & Jahns, 2002). Hence, this can be suggested that molecular weight of L-glutaminase is organism specific due to variation of L-glutaminase molecular weight produced from different microorganisms (Bazaraa et al., 2016).

Partial purified L-glutaminase enzyme was also assessed by Bradford analysis. Table 4.4 showed protein concentration of L-glutaminase before and after being purified.

Table 4.4 Bradford analysis for crude and partially purified L-glutaminase

Crude L-glutaminase			**Partially purified L-glutaminase**		
Trials	Absorbance	Concentration of protein(mg/ml)	Trials	Absorbance	Concentration of protein (mg/ml)
1	0.905	0.155	1	1.341	0.233
2	0.860	0.147	2	1.419	0.247
3	0.938	0.161	3	1.413	0.246

From table 4.4, Bradford analysis results showed that protein concentration of L-glutaminase in partially purified enzyme was higher than the crude enzyme. Protein concentration in partially purified enzyme from first, second, and third trials were 0.233 mg/ml, 0.247 mg/ml, and 0.246 mg/ml respectively. However, based on three trials that had been conducted, crude enzyme contained only 0.155 mg/ml, 0.147 mg/ml, and 0.161 mg/ml of protein concentration. Hence, increment of protein concentration in partially purified enzyme compared to crude enzyme showed that partial purification of crude L-glutaminase is a success. In Bradford analysis, the Coomassie brilliant blue dye bound to the protein and formed a complex that intensely absorb light at 594 nm (Zor & Selinger, 1996). With standard assay condition, ratio of absorbances is strictly linear with protein concentration (Zor & Selinger,

1996). Bradford analysis is widely used because of its rapidity, ease of performance, relative sensitivity, and specificity for proteins (Bradford, 1976).

4.9 Bacteria Growth Curve

Time course experiment had been conducted for 28 hours with sampling every 2 hours. The bacterial growth curve was conducted to indicate growth of bacteria through optical density at 600 nm of wavelength. This time course experiment was conducted at optimized conditions including incubation temperature at 37 ºC, pH 7, ammonium chloride for inorganic nitrogen source, beef extract for organic nitrogen source, and glucose as carbon source.

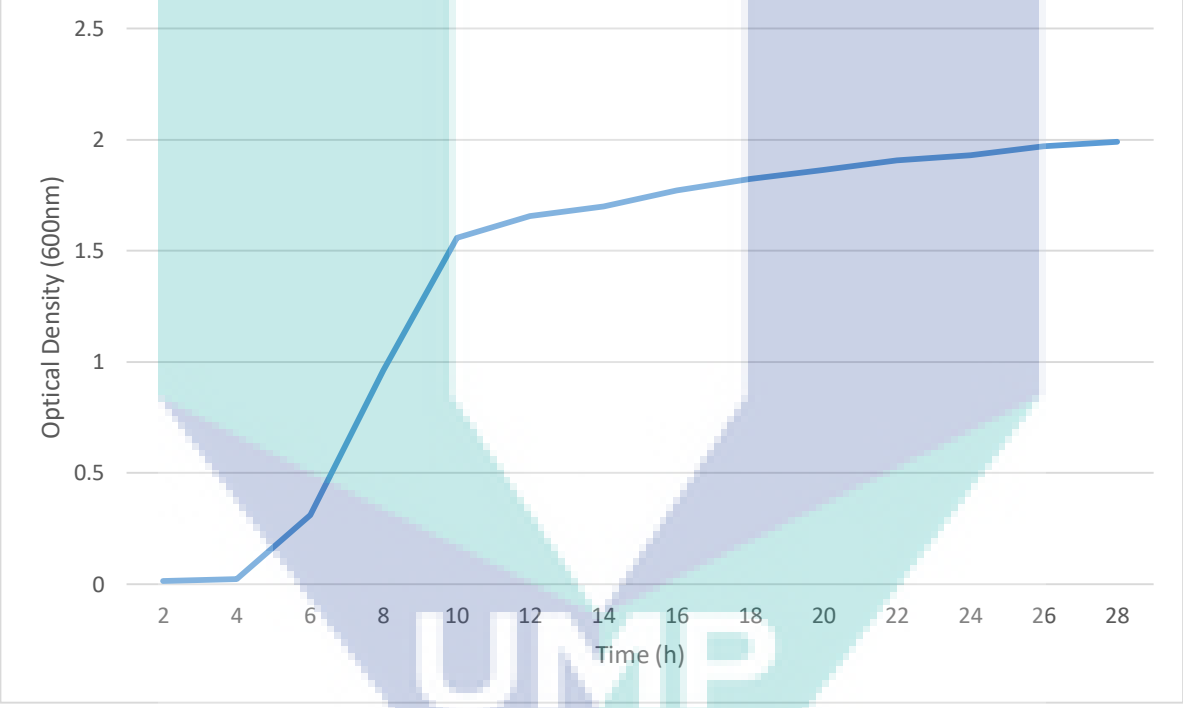

Figure 4.13 Time course of L-glutaminase production by *K. radicincitans* at optimized conditions.

Based on figure 4.13, the bacterial growth graph showed log phase during for the first four hours. At this stage, *K. radicincitans* were in unable to multiply yet as they were trying to adapt themselves to the new environment. The specific growth rate of the bacteria is zero (Buchanan, Whiting, & Damert, 1997). Then, the bacteria undergo exponential phase from the fourth hour to the tenth hour. During this stage, the bacteria continues to grow and divide at a maximum rate. The specific growth rate is assumed to be constant, where the log of cell population increase linearly with time (Buchanan et al., 1997). After tenth hour of incubation,

the bacteria seemed to divide in a slower rate until it reached stationary phase. Stationary phase is a phase where the rate of cell growth equals the rate of cell death. This happened due to growth-limiting factor such as lack of essential nutrients for cells to grow. Mutations also happened at this stage. Bridges, Foster, and Timms (2001) in their research study stated that many mutations happened due to DNA damage in genome of starving bacteria during stationary phase.

4.10 Characterization of partially purified L-glutaminase

Partially purified L-glutaminase of *K. radicincitans* was characterized by four different parameters which are temperature, temperature stability, pH and pH stability.

4.10.1 Temperature

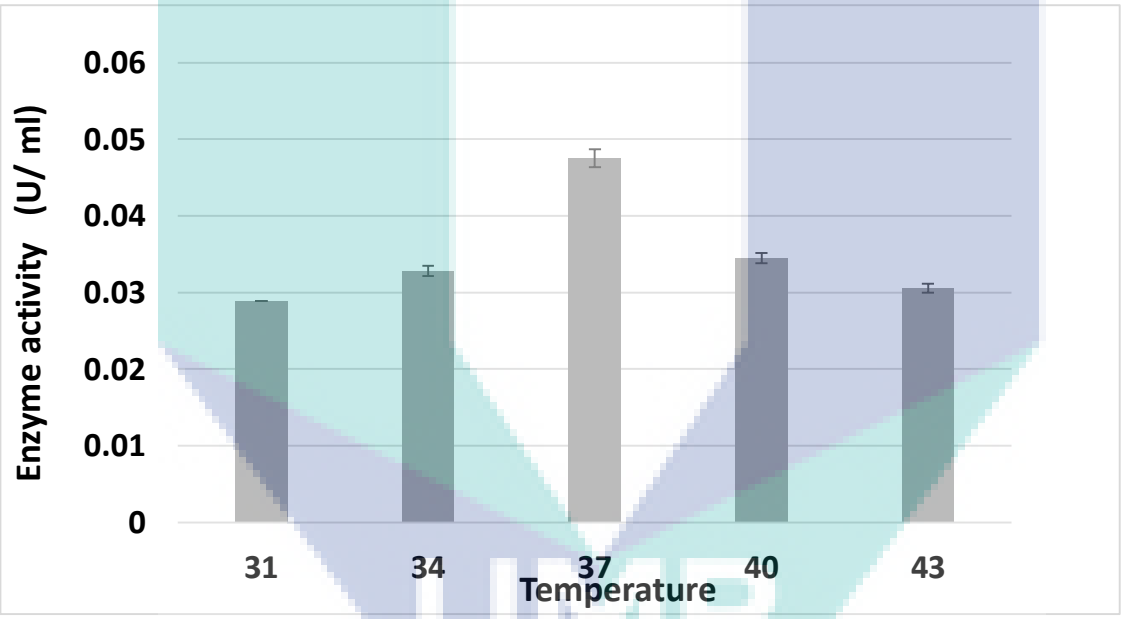

Figure 4.14 Enzymatic activity of partially purified L-glutaminase at five different incubation temperature.

Figure 4.14 showed that the partially purified L-glutaminase of *K. radicincitans* has different enzymatic activity when being incubated at five different temperatures which are 31, 34, 37, 40, and 43ºC. At 37ºC, partial purified enzyme of *K. radicincitans* exhibited the highest value of its enzymatic activity which is 0.047 ± 0.001 U/ml. This is followed by the enzyme's enzymatic activity incubated at 40ºC which has the value of 0.034 ± 0.0006 U/ml. A research study has found out that incubation of L-glutaminase enzyme from *Streptomyces avermitilis* at temperature above 40 ºC will cause it to be inactivated (Abdallah et al., 2012). However, in this case, partially purified L-glutaminase from *K. radicincitans* is still in less active state even

though it is being incubated above 40ºC. At 43ºC incubation, the enzyme still showed its enzymatic activity which has the value of 0.031 ± 0.0005 U/ml. Furthermore, another research conducted by Dura, Flores, and Toldrá (2002) figured out L-glutaminase enzyme from *Debaryomyces* spp has maximum enzymatic activity at 40 ºC. Therefore, different sources of L-glutaminase has their own specific temperature for the optimum enzymatic activity.

4.10.2 Temperature Stability

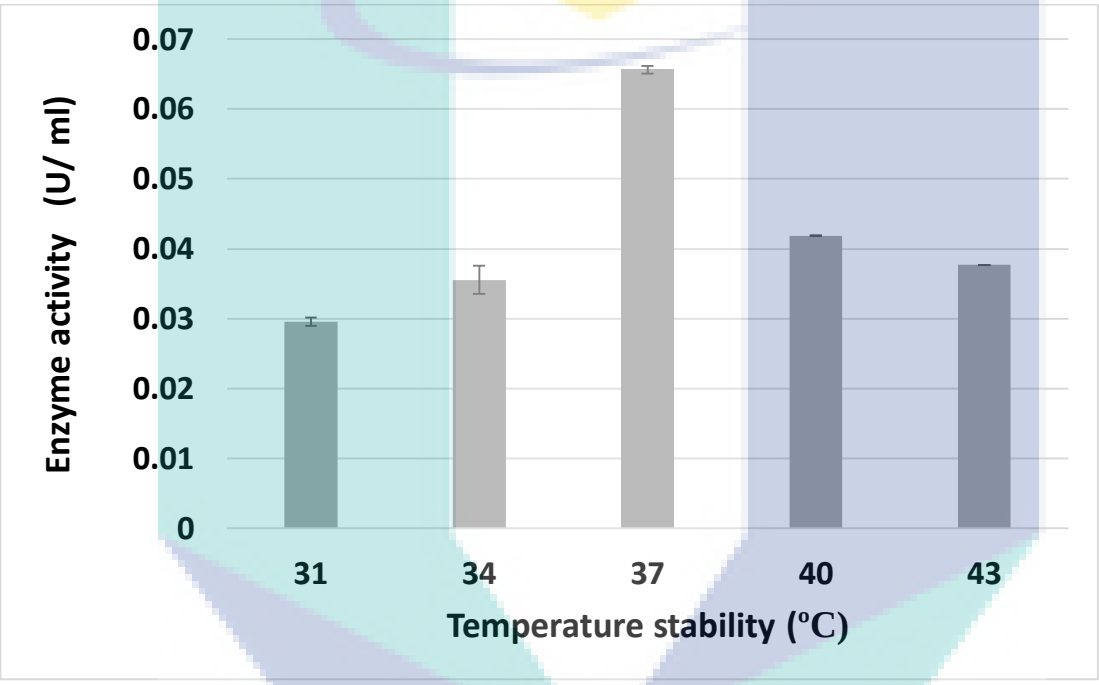

Figure 4.15 Enzymatic activity of partially purified L-glutaminase at five different incubation temperature.

Based on figure 4.15, enzymatic activity of partially purified L-glutaminase are the highest when the enzyme is incubated at 37ºC for 30 minutes before enzyme analysis was carried out. At 37ºC, L-glutaminase was at its maximum stability which then recorded with the highest enzymatic activity compared with other incubation temperatures Its enzymatic activity was 0.065 ± 0.0005 U/ml. Data from a research study has shown that by increasing the incubation temperature over its optimum value, the loss of enzymatic activity will be faster (Bazaraa et al., 2016). This is because the enzyme lost its stability due to conformational changed. Indeed the same situation happened in this research where the experiment result from their research study is parallel with ours.

At 40ºC incubation, the enzymatic activity of L-glutaminase enzyme decrease tremendously from 0.065 ± 0.0005 U/ml at 37ºC incubation to 0.041 ± 0.0000 U/ml. The enzymatic activity of L-glutaminase enzyme kept decreasing when the temperature was increased up to 43 ºC. This phenomenon happened due to nature of enzyme itself. Enzyme is a type of protein that is very sensitive to temperature. By increasing the temperature beyond its limit, the protein's structure will be disrupted and cause enzyme to lost its stability and decrease its shelf life. Hence this will affect its efficiency on enzymatic reaction. Temperature is one of the factors that cause increment of enzymatic activity to a certain point, however increasing temperature could cause enzyme denaturation and losing its activity irreversibly (Daniel & Danson, 2013).

4.10.3 pH

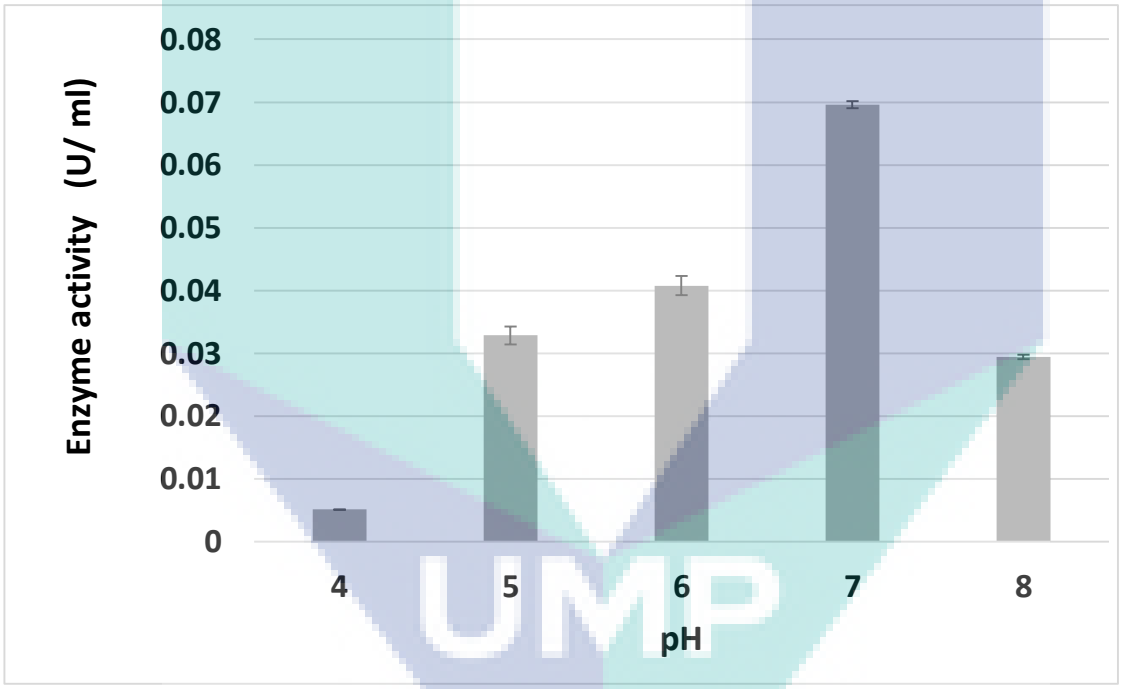

Figure 4.16 Enzymatic activity of partially purified enzyme at five different pH.

Results in figure 4.16 revealed that the optimum pH for enzymatic activity of partially purified L-glutaminase enzyme is pH 7 where its enzymatic activity is 0.069 ± 0.0005 U/ml. This is followed by pH 6 that gave enzymatic value of 0.041 ± 0.002 U/ml. A research study reported by Moriguchi, Sakai, Tateyama, Furuta, and Wakayama (1994) figured out that *Micrococcus luteus* from marine environment has optimum enzymatic activity at pH 7. This showed to us that even though some microorganisms such as *K. radicincitans* and *M. leteus*

came from marine environment which has pH less than seven, but their optimum condition to achieve optimum enzymatic activity is not based on pH of their habitat.

The least enzymatic activity of L-glutaminase is at pH 4 where its enzymatic value is 0.005 ± 0.0005 U/ml. Decrease in enzymatic value of L-glutaminase showed that it has lost its enzymatic activity. pH that is too low or too high can cause enzyme to lost its shape. This is because pH maintains proper conformation of enzyme as its active site contains ionizable groups which need to be in proper ionic form to maintain its conformation (Kaur, Kaur, & Gupta, 2014). Furthermore, a research study reported by Bazaraa et al. (2016) found out that L-glutaminase activity extracted from *Aspergillus oryzae* increase gradually by increasing the pH up to pH 7. However, the enzyme activity decrease when pH was set at pH 8, 9, and 10.

4.10.4 pH stability

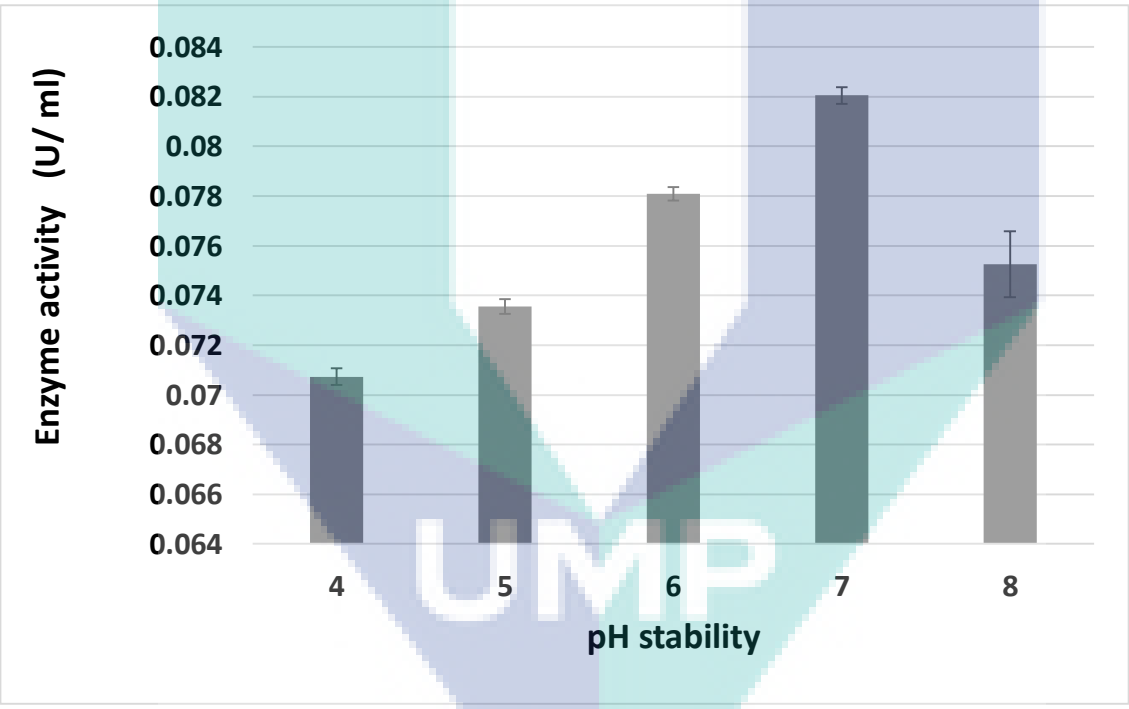

Figure 4.17 Enzymatic activity of partially purified enzyme at five different pH stability.

According to figure 4.17, the enzymatic activity of partially purified L-glutaminase increase up to 0.082 ± 0.0003 U/ml at pH7. The phosphate buffer of pH 7 was incubated for 30 minutes at 37ºC before the enzyme analysis took place. This is followed by pH 6 where its enzymatic value is 0.078 ± 0.0002 U/ml. The lowest stability was obtained with pH 4 that has enzymatic activity of only 0.071 ± 0.0003 U/ml. A research study by Ohshima et al. (1976) reported that the highest stability of L-glutaminase was at pH 7. However, it is undeniable that

different L-glutaminase producers produce L-glutaminase with different optimum pH stability to obtain optimum enzymatic activity. For instance, *Pseudomonas aeruginosa* has the highest stability of L-glutaminase at pH 7.5 (Roberts, Holcenberg, & Dolowy, 1970) while *Debaromyces* sp. obtained highest L-glutaminase activity at pH 8.5 (Dura et al., 2002). Meanwhile, L-glutaminase extracted from *A. oryzae* has the highest stability at pH 7 followed by pH 6 and its lowest stability was noticed at pH 3 and 10 with 35% and 100% loss of activity, respectively (Bazaraa et al., 2016).

CHAPTER 5

CONCLUSION

5.1 Conclusion

As a conclusion, L-glutaminase was present in microorganisms specifically bacteria from Malaysia marine environment. The enzymatic activity of L-glutaminase was quantified for 12 positive isolates. Through 16S rRNA gene sequencing, four different species of bacteria-producing L-glutaminase were revealed which were *K. radicincitans, K. oryzae, P. aeruginosa,* and *S. flexneri.* The highest L-glutaminase production was found to be *K. radicincitans* and followed by *S.flexneri*. L-glutaminase enzyme from both species was optimized with different parameters including temperature, pH, additional of organic and inorganic nitrogen sources, and additional of carbon sources. Results from the optimization process showed that both species shared the same optimum temperature of 37 ºC, pH 7, glucose as carbon source, and ammonium chloride as inorganic nitrogen source but both prefer different type of organic nitrogen source. *K. radicincitans* prefer beef extract as organic nitrogen source meanwhile *S.flexneri* prefer yeast extract. L-glutaminase extracted from *K. radicincitans* was further partially purified and characterized. Through characterization, L-glutaminase was most reactive and stable at 37 ºC and pH 7. The molecular weight of L-glutaminase extracted from *K. radicincitans* were found to be 70 kDA. With this breakthrough, knowledge on production, optimization, and characterization of L-glutaminase is very important in order to give insights on its suitability of fulfilling the industrial demands.

5.2 Future studies

L-glutaminase is a promising enzyme that has great application potentials in many industrial fields such as health care, food, and pharmaceutical. More research need to be done in order to make sure that L-glutaminase extracted especially *K. radicincitans* is very suitable and safe to be used by those industries.

Furthermore, extraction of L-glutaminase from bacteria in bulk is needed for industrial demands. Isolation of gene responsible for L-glutaminase production, cloning, and expression of recombinant glutaminase could be carried out. High production of L-glutaminase is needed in order to fulfil its high demand and usage for industrial purposes in the future.

On the other hand, less research about intracellular L-glutaminase had been carried out compared to extracellular production of L-glutaminase. Intracellular L-glutaminase might has its own unique characteristics that can contribute positively and give benefits to the industries. Besides that, structural analysis of L-glutaminase from *K. radicincitans* is worth an in-depth investigation as protein structure determines its function and product specificity.

REFERENCES

Abdallah, N. A., Amer, S. K., & Habeeb, M. K. (2012). Screening of L-glutaminase produced by actinomycetes isolated from different soils in Egypt. *International Journal of ChemTech Research, 4*(4), 1451-1460.

Abdallah, N. A., Amer, S. K., & Habeeb, M. K. (2013). Production, purification and characterization of L-glutaminase enzyme from Streptomyces avermitilis. *African Journal of Microbiology Research, 7*(14), 1184-1190.

Aledo, J. C., Gómez-Fabre, P. M., Olalla, L., & Márquez, J. (2000). Identification of two human glutaminase loci and tissue-specific expression of the two related genes. *Mammalian genome, 11*(12), 1107-1110.

Alian, A. M., Bazaraa, W. A., El-Shimi, N. M., & Mohamed, R. M. (2015). Optimization of L-glutaminase synthesis by *Aspergillus oryzae* NRRL 32657 in submerged culture. *African Journal of Microbiology Research, 9*(9), 588-597.

Arima, K., Sakamoto, T., Araki, C., & Tamura, G. (1972). Production of extracellular L-asparaginases by microorganisms. *Agricultural and Biological Chemistry, 36*(3), 356-361.

Bååth, E. (1998). Growth rates of bacterial communities in soils at varying pH: a comparison of the thymidine and leucine incorporation techniques. *Microbial Ecology, 36*(3-4), 316-327.

Bååth, E., & Arnebrant, K. (1994). Growth rate and response of bacterial communities to pH in limed and ash treated forest soils. *Soil Biology and Biochemistry, 26*(8), 995-1001.

Balagurunathan, R., Radhakrishnan, M., & Somasundaram, S. (2010). L-glutaminase producing actinomycetes from marine sediments–selective isolation, semi quantitative assay and characterization of potential strain. *Aust J Basic Appl Sci, 4*(5), 698-705.

Barrow, C., & Shahidi, F. (2007). *Marine nutraceuticals and functional foods*: CRC Press.

Bazaraa, W., Alian, A., El-Shimi, N., & Mohamed, R. (2016). Purification and characterization of extracellular glutaminase from Aspergillus oryzae NRRL 32567. *Biocatalysis and agricultural biotechnology, 6*, 76-81.

Ben-David, A., & Davidson, C. E. (2014). Estimation method for serial dilution experiments. *Journal of microbiological methods, 107*, 214-221.

Beveridge, T. J. (2001). Use of the Gram stain in microbiology. *Biotechnic & Histochemistry, 76*(3), 111-118.

Binod, P., Sindhu, R., Madhavan, A., Abraham, A., Mathew, A. K., Beevi, U. S., . . . Pandey, A. (2017). Recent developments in L-glutaminase production and applications–an overview. *Bioresource technology*.

Botrè, F., Botrè, C., Lorenti, G., Mazzei, F., Porcelli, F., & Scibona, G. (1993). Determination of L-glutamate and L-glutamine in pharmaceutical formulations by amperometric L-glutamate oxidase based enzyme sensors. *Journal of pharmaceutical and biomedical analysis, 11*(8), 679-686.

Boyanova, L. (2017). Stress hormone epinephrine (adrenaline) and norepinephrine (noradrenaline) effects on the anaerobic bacteria. *Anaerobe*.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1-2), 248-254.

Brady, C., Cleenwerck, I., Venter, S., Coutinho, T., & De Vos, P. (2013). Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into Lelliottia gen. nov. as Lelliottia nimipressuralis comb. nov. and Lelliottia amnigena comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii, E. radicincitans, E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis, E. helveticus and E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Systematic and applied microbiology, 36*(5), 309-319.

Bridges, B. A., Foster, P. L., & Timms, A. R. (2001). Effect of endogenous carotenoids on "adaptive" mutation in Escherichia coli FC40. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 473*(1), 109-119.

Buchanan, R., Whiting, R., & Damert, W. (1997). When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. *Food microbiology, 14*(4), 313-326.

Campos-Sandoval, J. A., de la Oliva, A. R. L., Lobo, C., Segura, J. A., Matés, J. M., Alonso, F. J., & Márquez, J. (2007). Expression of functional human glutaminase in baculovirus system: Affinity purification, kinetic and molecular characterization. *The international journal of biochemistry & cell biology, 39*(4), 765-773.

Campos-Sandoval, J. A., Martín-Rufián, M., Cardona, C., Lobo, C., Peñalver, A., & Márquez, J. (2015). Glutaminases in brain: multiple isoforms for many purposes. *Neurochemistry international, 88*, 1-5.

Cardona, C., Sánchez-Mejías, E., Dávila, J. C., Martín-Rufián, M., Campos-Sandoval, J. A., Vitorica, J., . . . Norenberg, M. D. (2015). Expression of Gls and Gls2 glutaminase isoforms in astrocytes. *Glia, 63*(3), 365-382.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K., & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA

gene sequences. *International journal of systematic and evolutionary microbiology*, *57*(10), 2259-2261.

Collingridge, G. L., & Lester, R. A. (1989). Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacological reviews*, *41*(2), 143-210.

Curthoys, N. P., & Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. *Annual review of nutrition*, *15*(1), 133-159.

Daniel, R. M., & Danson, M. J. (2013). Temperature and the catalytic activity of enzymes: A fresh understanding. *FEBS letters*, *587*(17), 2738-2743.

Divya Teja, D., Sri Devi, V., & Harsha, N. (2014). Production of L-glutaminase from marine ecosystems and optimal conditions for maximal production by actinomycetes. *International Journal of Advanced Research*, *2*, 485-491.

Dura, M., Flores, M., & Toldrá, F. (2002). Purification and characterisation of a glutaminase from Debaryomyces spp. *International journal of food microbiology*, *76*(1-2), 117-126.

Durai, S., Selvaraj, B., Manikkam, R., & Ramasamy, B. (2014). Production and optimization of L-glutaminase from Vibrio sp. M9 isolated from mahabalipuram marine sediments. *World J. Pharm. Res*, *3*, 2117-2126.

El-Sayed, A. S. (2009). L-glutaminase production by Trichoderma koningii under solid-state fermentation. *Indian journal of microbiology*, *49*(3), 243-250.

Ewing, W., & Fife, M. (1972). Enterobacter agglomerans (Beijerinck) comb. nov.(the herbicola-lathyri bacteria). *International journal of systematic and evolutionary microbiology*, *22*(1), 4-11.

Fouilland, E., Gosselin, M., Rivkin, R., Vasseur, C., & Mostajir, B. (2007). Nitrogen uptake by heterotrophic bacteria and phytoplankton in Arctic surface waters. *Journal of Plankton Research*, *29*(4), 369-376.

Fukushima, Y., & Motai, H. (1990). Continuous conversion of glutamine to glutamate by immobilized glutaminase-producing yeast. *Journal of fermentation and bioengineering*, *69*(3), 189-191.

Giovannoni, S. J., Tripp, H. J., Givan, S., Podar, M., Vergin, K. L., Baptista, D., . . . Noordewier, M. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science*, *309*(5738), 1242-1245.

Gomez-Fabre, P. M., Aledo, J. C., Del Castillo-Olivares, A., Alonso, F. J., De Castro, I. N., Campos, J. A., & Marquez, J. (2000). Molecular cloning, sequencing and expression studies of the human breast cancer cell glutaminase. *Biochemical Journal*, *345*(2), 365-375.

Han, B.-Z., Ma, Y., Rombouts, F. M., & Nout, M. R. (2003). Effects of temperature and relative humidity on growth and enzyme production by Actinomucor elegans and Rhizopus oligosporus during sufu pehtze preparation. *Food Chemistry*, *81*(1), 27-34.

Hancock, R. E. (1998). Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria. *Clinical Infectious Diseases, 27*(Supplement_1), S93-S99.

Heini, H. G., Gebhardt, R., Brecht, A., & Mecke, D. (1987). Purification and characterization of rat liver glutaminase. *European journal of biochemistry, 162*(3), 541-546.

Hoffmann, H., Stindl, S., Ludwig, W., Stumpf, A., Mehlen, A., Heesemann, J., . . . Roggenkamp, A. (2005). Reassignment of Enterobacter dissolvens to Enterobacter cloacae as E. cloacae subspecies dissolvens comb. nov. and emended description of Enterobacter asburiae and Enterobacter kobei. *Systematic and applied microbiology, 28*(3), 196-205.

Huang, Y., Zhao, L., Jia, B., Wu, L., Li, Y., Curthoys, N., & Zheng, J. C. (2011). Glutaminase dysregulation in HIV-1-infected human microglia mediates neurotoxicity: relevant to HIV-1-associated neurocognitive disorders. *Journal of Neuroscience, 31*(42), 15195-15204.

Imada, A., Igarasi, S., Nakahama, K., & Isono, M. (1973). Asparaginase and glutaminase activities of micro-organisms. *Microbiology, 76*(1), 85-99.

Iyer, P. V., & Singhal, R. S. (2009). Screening and selection of marine isolate for L-glutaminase production and media optimization using response surface methodology. *Applied biochemistry and biotechnology, 159*(1), 233-250.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology, 45*(9), 2761-2764.

Jayabalan, R., Jeeva, S., Sasikumar, A., Inbakandan, D., Swaminathan, K., & Yun, S. (2010). Extracellular L-glutaminase production by marine Brevundimonas diminuta MTCC 8486. *International Journal on Applied Bioengineering, 4*(2), 19-24.

Jeon, J.-M., Lee, H.-I., & So, J.-S. (2009). Glutaminase activity of Lactobacillus reuteri KCTC3594 and expression of the activity in other Lactobacillus spp. by introduction of the glutaminase gene. *African Journal of Microbiology Research, 3*(10), 605-609.

Kämpfer, P., Ruppel, S., & Remus, R. (2005). *Enterobacter radicincitans* sp. nov., a plant growth promoting species of the family Enterobacteriaceae. *Systematic and applied microbiology, 28*(3), 213-221.

Karim, G. F., & Thalij, K. M. (2016). Determination of L-Glutaminase Activity by Some Bacterial Species. *Int. J. Curr. Microbiol. App. Sci, 5*(4), 218-225.

Kashyap, P., Sabu, A., Pandey, A., Szakacs, G., & Soccol, C. R. (2002). Extra-cellular L-glutaminase production by Zygosaccharomyces rouxii under solid-state fermentation. *Process Biochemistry, 38*(3), 307-312.

Katikala, P. K., Bobbarala, V., Tadimalla, P., & Guntuku, G. S. (2009). Screening of L-glutaminase producing marine bacterial cultures for extracellular production of L-glutaminase. *International Journal of ChemTech Research, 1*(4), 1232-1235.

Kaur, R., Kaur, N., & Gupta, A. K. (2014). Structural features, substrate specificity, kinetic properties of insect α-amylase and specificity of plant α-amylase inhibitors. *Pesticide biochemistry and physiology*, *116*, 83-93.

Kawase, T., Saito, A., Sato, T., Kanai, R., Fujii, T., Nikaidou, N., . . . Watanabe, T. (2004). Distribution and phylogenetic analysis of family 19 chitinases in *Actinobacteria*. *Applied and Environmental Microbiology*, *70*(2), 1135-1144.

Keerthi, T., Suresh, P., Sabu, A., Rajeevkumar, S., & Chandrasekaran, M. (1999). Extracellular production of L-glutaminase by alkalophilic Beauveria bassiana BTMF S10 isolated from marine sediment. *World Journal of microbiology and Biotechnology*, *15*(6), 751-752.

Kijima, K., & Suzuki, H. (2007). Improving the umami taste of soy sauce by the addition of bacterial γ-glutamyltranspeptidase as a glutaminase to the fermentation mixture. *Enzyme and Microbial Technology*, *41*(1-2), 80-84.

Kim, S.-K., Ravichandran, Y. D., Khan, S. B., & Kim, Y. T. (2008). Prospective of the cosmeceuticals derived from marine organisms. *Biotechnology and Bioprocess Engineering*, *13*(5), 511-523.

Kiruthika, J., & Saraswathy, N. (2013). Production of L-glutaminase and its optimization from a novel marine isolate *Vibrio azureus* JK-79. *African Journal of Biotechnology*, *12*(50), 6944.

Klein, M., Kaltwasser, H., & Jahns, T. (2002). Isolation of a novel, phosphate-activated glutaminase from Bacillus pasteurii. *FEMS microbiology letters*, *206*(1), 63-67.

Kovacevic, Z., & McGivan, J. (1983). Mitochondrial metabolism of glutamine and glutamate and its physiological significance. *Physiological reviews*, *63*(2), 547-605.

Kozlov, E., Kovalenko, N., & Mardashev, S. (1972). Purification and various properties of Basillus sutillis glutaminase. *Biokhimiya*, *37*, 56-64.

Krishnakumar, S., Rajan, R. A., & Ravikumar, S. (2011). Extracellular production of L-Glutaminase by marine alkalophilic Streptomyces sp.-SBU1 isolated from Cape Comorin coast.

Krishnapriya, S., & Babu, D. V. (2015). Isolation and identification of bacteria to improve the strength of concrete. *Microbiological research*, *174*, 48-55.

Kumar, S. S., Muthuvelayudham, R., & Viruthagiri, T. (2013). Production and optimization of L-glutaminase (EC. 3.5. 1.2) by Serratia marcescens using wheat bran under statistical designs. *Journal of Chemical, Biological and Physical Sciences (JCBPS)*, *3*(4), 2601.

Kvamme, E. (1984). Enzymes of cerebral glutamine metabolism *Glutamine metabolism in mammalian tissues* (pp. 32-48): Springer.

Laake, J., Takumi, Y., Eidet, J., Torgner, I. A., Roberg, B., Kvamme, E., & Ottersen, O. (1999). Postembedding immunogold labelling reveals subcellular localization and pathway-specific enrichment of phosphate activated glutaminase in rat cerebellum. *Neuroscience*, *88*(4), 1137-1151.

Li, S., Peng, C., Cheng, T., Wang, C., Guo, L., & Li, D. (2018). Nitrogen-cycling microbial community functional potential and enzyme activities in cultured biofilms with response to inorganic nitrogen availability. *Journal of Environmental Sciences*.

Löliger, J. r. (2000). Function and importance of glutamate for savory foods. *The Journal of nutrition*, *130*(4), 915S-920S.

Longo, M. A., & Sanromán, M. A. (2006). Production of food aroma compounds: microbial and enzymatic methodologies. *Food Technology and Biotechnology*, *44*(3), 335-353.

Mac Faddin, J. F. (1976). *Biochemical tests for identification of medical bacteria*: Williams & Wilkins Co.

Márquez, J., de la Oliva, A. R. L., Matés, J. M., Segura, J. A., & Alonso, F. J. (2006). Glutaminase: a multifaceted protein not only involved in generating glutamate. *Neurochemistry international*, *48*(6-7), 465-471.

Martín-Rufián, M., Tosina, M., Campos-Sandoval, J. A., Manzanares, E., Lobo, C., Segura, J., . . . Márquez, J. (2012). Mammalian glutaminase Gls2 gene encodes two functional alternative transcripts by a surrogate promoter usage mechanism. *PloS one*, *7*(6), e38380.

McIlwain, H. (1948). Synthesis and breakdown of glutamine by various micro-organisms. *Microbiology*, *2*(2), 186-196.

Meidute, S., Demoling, F., & Bååth, E. (2008). Antagonistic and synergistic effects of fungal and bacterial growth in soil after adding different carbon and nitrogen sources. *Soil Biology and Biochemistry*, *40*(9), 2334-2343.

Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y., & Wakayama, M. (1994). Isolation and characterization of salt-tolerant glutaminases from marine Micrococcus luteus K-3. *Journal of fermentation and bioengineering*, *77*(6), 621-625.

Mousumi, D., & Dayanand, A. (2013). Production and antioxidant attribute of l-glutaminase from Streptomyces enissocaesilis DMQ-24. *Int J Latest Res Sci Technol*, *2*(3), 1-9.

Muthuvelayudham, R., & Viruthagiri, T. (2013). Medium optimization for production of L-Glutaminase (EC 3.5. 1.2) by Streptomyces griseus under submerged fermentation. *International Journal of Science and Engineering Applications*, *2*(1), 1-5.

Nandakumar, R., Yoshimune, K., Wakayama, M., & Moriguchi, M. (2003). Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic*, *23*(2-6), 87-100.

Nicklas, W., Zeevalk, G., & Hyndman, A. (1987). Interactions between neurons and glia in glutamate/glutamine compartmentation: Portland Press Limited.

Ohshima, M., Yamamoto, T., & Soda, K. (1976). Further characterization of glutaminase isozymes from Pseudomonas aeruginosa. *Agricultural and Biological Chemistry*, *40*(11), 2251-2256.

Olalla, L. a., Gutiérrez, A., Campos, J. A., Khan, Z. U., Alonso, F. J., Segura, J. A., . . . Aledo, J. C. (2002). Nuclear localization of L-type glutaminase in mammalian brain. *Journal of Biological Chemistry, 277*(41), 38939-38944.

Pallem, C., Manipati, S., & Somalanka, S. R. (2010). Process optimization of L-glutaminase production by Trichoderma koningii under solid state fermentation (SSF).

Peng, G., Zhang, W., Luo, H., Xie, H., Lai, W., & Tan, Z. (2009). Enterobacter oryzae sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species Oryza latifolia. *International journal of systematic and evolutionary microbiology, 59*(7), 1650-1655.

Pérez-Gómez, C., Campos-Sandoval, J. A., Alonso, F. J., Segura, J. A., Manzanares, E., Pedro, R.-S., . . . José, M. (2005). Co-expression of glutaminase K and L isoenzymes in human tumour cells. *Biochemical Journal, 386*(3), 535-542.

Pérez-Gómez, C., José, M., Gómez-Fabre, P. M., del Castillo-Olivares, A., Alonso, F. J., & Márquez, J. (2003). Genomic organization and transcriptional analysis of the human L-glutaminase gene. *Biochemical Journal, 370*(3), 771-784.

PLNSN, S. D., Siddalingeshwara, K., Karthic, J., Pramod, T., & Vishwanatha, T. (2014). Antitumour property l-glutaminase on from Aspergillus oryzae through submergrd fermentation. *Int. J. Curr. Microbiol. App. Sci, 3*(3), 819-823.

Porter, L. D., Ibrahim, H., Taylor, L., & Curthoys, N. P. (2002). Complexity and species variation of the kidney-type glutaminase gene. *Physiological genomics, 9*(3), 157-166.

Prakash, P. J., Poorani, E., Anantharaman, P., & Balasubramaniam, T. (2009). L-glutaminase production and the growth of marine bacteria. *Res J Microbiol, 4*, 168-172.

Prusiner, S., Davis, J., & Stadtman, E. (1976). Regulation of glutaminase B in Escherichia coli. I. Purification, properties, and cold lability. *Journal of Biological Chemistry, 251*(11), 3447-3456.

Qi, L., Mikhael, C. S., & Funnell, W. R. J. (2004). *Application of the Taguchi method to sensitivity analysis of a middle-ear finite-element model*. Paper presented at the Proc. 28th Ann. Conf. Can. Med. Biol. Eng. Soc.

Racker, E. (1974). History of the Pasteur effect and its pathobiology. *Molecular and cellular biochemistry, 5*(1-2), 17-23.

Reda, F. M. (2015). Kinetic properties of Streptomyces canarius L-Glutaminase and its anticancer efficiency. *Brazilian Journal of Microbiology, 46*(4), 957-968.

Renu, S., & Chandrasekaran, M. (1992). Extracellular L-glutaminase production by marine bacteria. *Biotechnology letters, 14*(6), 471-474.

Roberts, J., Holcenberg, J. S., & Dolowy, W. C. (1970). Antineoplastic activity of highly purified bacterial glutaminases. *Nature, 227*(5263), 1136.

Roberts, J., MacAllister, T. W., Sethuraman, N., & Freeman, A. G. (2001). Genetically engineered glutaminase and its use in antiviral and anticancer therapy: Google Patents.

Roberts, J., & McGregor, W. G. (1991). Inhibition of mouse retroviral disease by bioactive glutaminase-asparaginase. *Journal of general virology, 72*(2), 299-305.

Rousk, J., Brookes, P. C., & Bååth, E. (2009). Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Applied and Environmental Microbiology, 75*(6), 1589-1596.

Sabu, A. (2003). Sources, properties and applications of microbial therapeutic enzymes.

Sabu, A., & Chandrasekaran, M. (1999). *L-glutaminase production by marine fungi*. Cochin University of Science and Technology.

Sabu, A., Chandrasekaran, M., & Pandey, A. (2000). Biopotential of microbial glutaminases. *Chimica oggi, 18*(11-12), 21-25.

Sabu, A., Keerthi, T., Kumar, S. R., & Chandrasekaran, M. (2000). L-Glutaminase production by marine Beauveria sp. under solid state fermentation. *Process Biochemistry, 35*(7), 705-710.

Sabu, A., Kumar, S. R., & Chandrasekaran, M. (2002). Continuous production of extracellular L-glutaminase by Ca-alginate-immobilized marine Beauveria bassiana BTMF S-10 in packed-bed reactor. *Applied biochemistry and biotechnology, 102*(1-6), 71-79.

Sajitha, N., Vasuki, S., Suja, M., Kokilam, G., & Gopinath, M. (2013). Screening of L-glutaminase from Sea Weed Endophytic Fungi. *International Research Journal of Pharmaceutical and Applied Sciences*, 206-209.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*: Cold spring harbor laboratory press.

Sarada, K. (2013). Production and applications of L-glutaminase using fermentation technology. *Asia Pac J Res, 1*, 1.

Schaeffer, A. B., & Fulton, M. D. (1933). A simplified method of staining endospores. *Science, 77*(1990), 194-194.

Schmidt, H., & Kamp, G. (1996). The Pasteur effect in facultative anaerobic metazoa. *Experientia, 52*(5), 440-448.

Setlow, P. (2006). Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. *Journal of applied microbiology, 101*(3), 514-525.

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International, 91*(4), 914-931.

Shapiro, R. A., Farrell, L., Srinivasan, M., & Curthoys, N. (1991). Isolation, characterization, and in vitro expression of a cDNA that encodes the kidney isoenzyme of the mitochondrial glutaminase. *Journal of Biological Chemistry, 266*(28), 18792-18796.

Shin, N.-R., Whon, T. W., & Bae, J.-W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends in biotechnology, 33*(9), 496-503.

Smith, E., & Watford, M. (1990). Molecular cloning of a cDNA for rat hepatic glutaminase. Sequence similarity to kidney-type glutaminase. *Journal of Biological Chemistry, 265*(18), 10631-10636.

Soberón, M., & González, A. (1987). Physiological role of glutaminase activity in Saccharomyces cerevisiae. *Microbiology, 133*(1), 1-8.

Sukesh, K. (2010). *An Introduction to Industrial Microbiology*: S. Chand Publishing.

Tachiki, T., Yamada, T., Mizuno, K., Ueda, M., Shiode, J.-i., & Fukami, H. (1998). γ-Glutamyl transfer reactions by glutaminase from Pseudomonas nitroreducens IFO 12694 and their application for the syntheses of theanine and γ-glutamylmethylamide. *Bioscience, biotechnology, and biochemistry, 62*(7), 1279-1283.

Tempest, D., Meers, J., & Brown, C. (1970). Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route. *Biochemical Journal, 117*(2), 405.

Thauer, R. K., Jungermann, K., & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological reviews, 41*(1), 100.

Tork, S. E., Aly, M. M., & Elsemin, O. (2018). A new l-glutaminase from Streptomyces pratensis NRC 10: Gene identification, enzyme purification, and characterization. *International journal of biological macromolecules, 113*, 550-557.

Tunga, R., Banerjee, R., & Bhattacharyya, B. C. (1999). Optimization of n variable biological experiments by evolutionary operation-factorial design technique. *Journal of bioscience and bioengineering, 87*(2), 224-230.

Unissa, R., Sudhakar, M., Reddy, A. S. K., & Sravanthi, K. N. (2014). A review on biochemical and therapeutic aspects of glutaminase. *Int J Pharm Sci Res, 5*(11), 4617-4634.

Vijayan, N., Swapna, T., Haridas, M., & Sabu, A. (2017). Therapeutic enzymes: L-Glutaminase *Current Developments in Biotechnology and Bioengineering* (pp. 233-248): Elsevier.

Villarta, R. L., Palleschi, G., Suleiman, A., & Guilbault, G. G. (1992). Determination of glutamine in serum using an amperometric enzyme electrode. *Electroanalysis, 4*(1), 27-31.

Wakayama, M., Yamagata, T., Kamemura, A., Bootim, N., Yano, S., Tachiki, T., . . . Moriguchi, M. (2005). Characterization of salt-tolerant glutaminase from Stenotrophomonas maltophilia NYW-81 and its application in Japanese soy sauce fermentation. *Journal of Industrial Microbiology and Biotechnology, 32*(9), 383-390.

Watford, M. (1993). Hepatic glutaminase expression: relationship to kidney-type glutaminase and to the urea cycle. *The FASEB journal, 7*(15), 1468-1474.

Weingand-Ziadé, A., Gerber-Décombaz, C., & Affolter, M. (2003). Functional characterization of a salt-and thermotolerant glutaminase from Lactobacillus rhamnosus. *Enzyme and Microbial Technology, 32*(7), 862-867.

Wheeler, P. A., & Kirchman, D. L. (1986). Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnology and Oceanography, 31*(5), 998-1009.

Yamamoto, S., & Hirooka, H. (1974). Production of glutaminase by Aspergillus sojae. *Journal of Fermentation Technology (Japan)*.

Yano, T., Ito, M., Tomita, K., & Kumagai, H. (1988). Purification and properties of glutaminase from Aspergillus oryzae. *Journal of Fermentation technology, 66*(2), 137-143.

Ye, J., McGinnis, S., & Madden, T. L. (2006). BLAST: improvements for better sequence analysis. *Nucleic acids research, 34*(suppl_2), W6-W9.

Yoshimune, K., Yamashita, R., Masuo, N., Wakayama, M., & Moriguchi, M. (2004). Digestion by serine proteases enhances salt tolerance of glutaminase in the marine bacterium Micrococcus luteus K-3. *Extremophiles, 8*(6), 441-446.

Zor, T., & Selinger, Z. (1996). Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Analytical biochemistry, 236*(2), 302-308.

APPENDIXES

Appendix A: Streak plate technique

Streak plate technique is used for the isolation of pure culture of bacteria or colonies from mixed populations by simple mechanical separation. This technique was being done in a laminar flow in order to have a sterile environment. Inoculating loop was sterilized using a bunsen burner by putting the loop into the flame until it was red hot and allowed it to cool. Then, the sterile inoculating loop was used to pick an isolated single colony from agar plate culture and spread it over a quarter of the minimal glutamine agar plate which was the first quadrant.

The inoculating loop was streaked gently over the first quadrant by moving back and forth. After that, the inoculating loop was flamed again and allowed to cool down. The sterile inoculating loop was used to extend the streak at the second quadrant by starting at the edge of first quadrant. The inoculating loop was sterilized again by putting the loop into the flame and allowed to cool down. The sterile inoculating loop was used to extend the streak into the third quadrant and finally into the forth quadrant. The inoculating loop was flamed again and let it cool down.

Appendix B: Procedure of using haemocytometer and its cell count.

1. Procedure of using haemocytometer

A) Prepare the haemocytometer and its coverslip by cleaning it with alcohol before use. Moisten the haemocytometer's coverslip with water and affix to the hemocytometer.

B) Prepare a uniform cell suspension by pipetting up and down 5 to 7 times using a sterile pipette tip in a sterile environment.

C) Take out 50 ul of cell suspension and put it into 1.5ml eppendorf tube. Add 50 ul of Trypan Blue solution into the eppendorf tube. Mix gently.

D) Take about 10 ul of Trypan Blue-treated cell suspension and load into the hemocytometer. This is done by touching the micropipette tip at the edge of cover slip and fill the haemocytomer's chamber allowing the cell suspension to be drawn out by capillary action.

E) By using a microscope, view the cells at 100x magnification. There will be a grid of 9 squares and focus the microscope on the one of 4 outer squares in the grid as shown below.

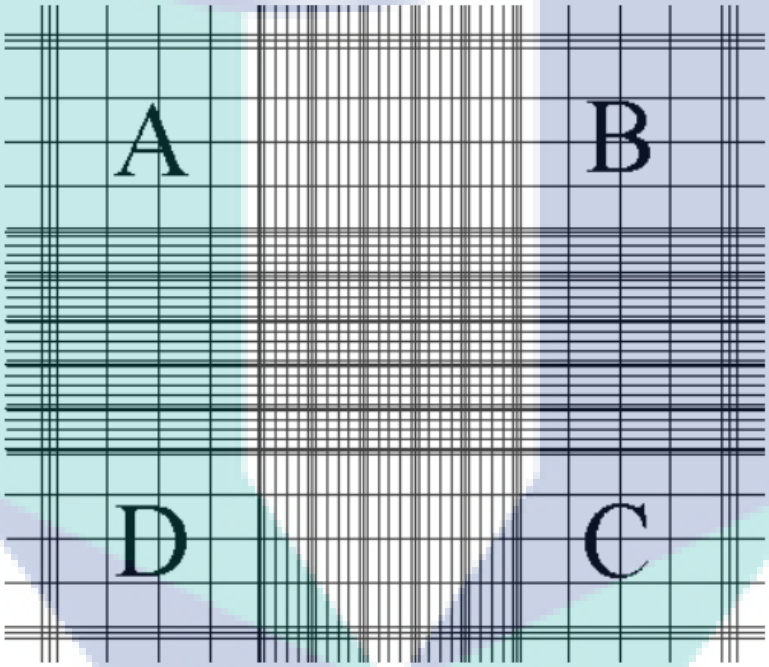

Figure B1: Four outer squares in the grid labelled A, B, C, and D that should be focused on calculating the number of cells.

F) Count the live unstained and dead stained cells in those 4 outer squares. Employ a system where cells are only be counted if it is either within the square or on the right-hand boundary or on the bottom boundary line.

G) Count the other outer squares using the same guidelines and carry on counting until all 4 squares are counted.

2. Cell count using a hemocytometer

A) Total viable cells = 44

B) Total non-viable cells = 2

C) Percentage of viable cells:

$$\frac{Number\ of\ viable\ cells}{Total\ number\ of\ cells}\ x\ 100$$

$$= \frac{44}{46}\ x\ 100$$

= 95.7%

D) Average number of cells per square:

$$\frac{Viable\ cells}{Number\ of\ squares} = \frac{44}{4} = 11$$

E) Dilution factor:

$$\frac{Final\ volume}{Volume\ of\ cells} = \frac{50\ ul\ of\ cells + 50\ ul\ of\ trypan\ Blue}{50\ ul\ of\ cells} = 2$$

F) Concentration of cells (Viable cells/ml):

Average number of cells per square x Dilution factor x 10^4

= 11 x 2 x 10^4

= 220 000 cells/ml

= 2.20 x 10^5 cells/ml.

G) Total cells:

Concentration of cells (cells/ml) x Original Volume of sample

= 220 000 cells/ml x 5 ml

= 1.10 x 10^6 cells

Appendix C: Standard graph for enzyme assay

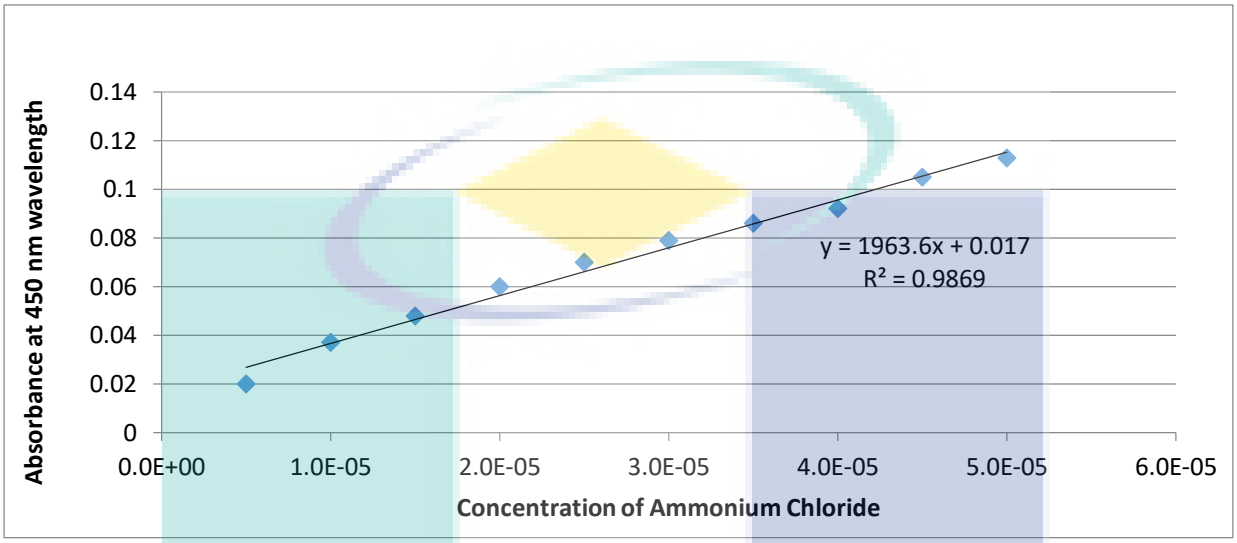

Figure C1: A standard curve of ammonium chloride concentration that was used in quantification of ammonium chloride produced for L-glutaminase assay.

Appendix D: Additional of organic nitrogen sources

Minimal Glutamine Media with additional of organic nitrogen sources 500 ml

Ingredients	Mass (g) / Volume (ml)
Glucose	1.0 g
Mg_2SO_4	0.25 g
KCl	0.25 g
KH_2PO_4	0.5 g
$FeSO_4$	0.05 g
$ZnSO_4$	0.05 g
NACl	0.25 g
L-glutamine	5.0 g
1% w/v of Beef extract / Yeast extract / Peptone	5.0 g
Distilled water	500 ml

Appendix E: Additional of inorganic nitrogen sources

Minimal Glutamine Media with additional of inorganic nitrogen sources 500 ml

Ingredients	**Mass (g) / Volume (ml)**
Glucose	1.0 g
Mg_2SO_4	0.25 g
KCl	0.25 g
KH_2PO_4	0.5 g
$FeSO_4$	0.05 g
$ZnSO_4$	0.05 g
NACl	0.25 g
L-glutamine	5.0 g
1% w/v of Ammonium chloride / Ammonium sulphate / Potassium nitrate / Sodium nitrate	5.0 g
Distilled water	500 ml

Appendix F: Additional of carbon sources

Minimal Glutamine Media with additional of carbon sources 500 ml

Ingredients	**Mass (g) / Volume (ml)**
Glucose	1.0 g
Mg_2SO_4	0.25 g
KCl	0.25 g
KH_2PO_4	0.5 g
$FeSO_4$	0.05 g
$ZnSO_4$	0.05 g
NACl	0.25 g
L-glutamine	5.0 g
1% w/v of Glucose / Maltose / Sucrose	5.0 g
Distilled water	500 ml

Appendix G: Working Solutions for Sds-Page

1. 12% Separation gel **10 ml**

dH_2O	3.30 ml
30% acrylamide mixture	4.00 ml
1.5M Tris-HCl (pH 8.8)	2.50 ml
10% SDS	0.10 ml
10% APS	0.10 ml
TEMED	0.01 ml

2. 4% Stacking gel **5 ml**

dH_2O	2.60 ml
30% acrylamide mixture	1.00 ml
0.5M Tris-HCl (pH 6)	1.25 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

3. 30% Acrylamide mixture **100 ml**

Acrylamide	30 g
Bis-acrylamide	0.8 g
dH_2O	up to 100 ml

- Store solution in amber colour bottle , in the dark, at 4ºC

4. 10% Ammonium persulfate (APS) **2 ml**

Ammonium persulfate	0.2 g
dH_2O	up to 2.0 ml

5. 10% SDS **2 ml**

	SDS	0.2 g	
	dH_2O	up to 2.0 ml	
6.	**10X Electrophoresis buffer**		**100 ml**
	Tris Base	3.0 g	
	Glycine	14.4 g	
	SDS	1.0 g	
	dH_2O	up to 100 ml	
7.	**2X SDS Sample Buffer**		**10 ml**
	1 M Tris-HCl (pH 6.8)	1.0 ml	
	Glycerol	2.0 ml	
	10% SDS	4.0 ml	
	β-mercaptoethanol	2.5 ml	
	1% Bromophonol blue	500 µl	
	dH_2O	up to 10 ml	
8.	**Staining solution**		**1L**
	Coomassie Blue R-250	1.0 g	
	Ethanol	450 ml	
	Glacial acetic acid	100 ml	
	dH_2O	450 ml	
9.	**Destaining solution**		**1L**
	Glacial acetic acid	100 ml	
	Ethanol	100 ml	
	dH_2O	800 ml	

Appendix H: Standard curve for Bradford assay

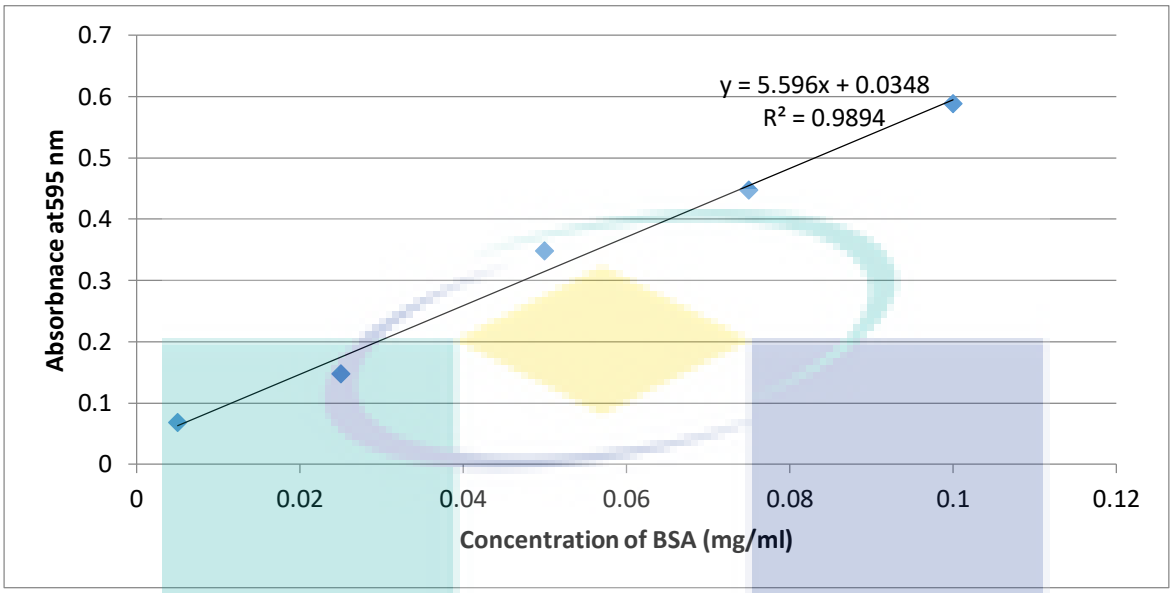

Figure H1: Protein standard curve of BSA which was used in protein quatification.

Appendix I: Procedure of buffer preparation

1. 0.1 M Citrate Buffer (pH 4 – pH 6)

Solution A: 0.1 M citric acid 19.21 g/l

Solution B: 0.1 M sodium citrate dehydrate 29.40 g/l

Desired pH	Solution A (ml)	Solution B (ml)
4.0	33.00	17.00
5.0	20.50	29.50
6.0	9.50	41.50

Table I1: Preparation of Citrate Buffer

Note: Based on table I1, for the desired pH, mix the indicated volumes of Solution A and Solution B, then diluted with distilled water up to 100 ml.

2. 0.1 M Potassium Phosphate Buffer (pH 6 – pH 8)

Solution A: 0.2 M potassium phosphate monobasic 27.8 g/l

Solution B: 0.2 M potassium phosphate dibasic 53.6 g/l

Desired pH	**Solution A (ml)**	**Solution B (ml)**
6.0	92.00	8.00
7.0	39.00	61.00
8.0	5.300	94.70

Table I2: Preparation of Sodium Phosphate Buffer

Note: Based on table I2, for the desired pH, mix the indicated volumes of Solution A and Solution B, then diluted with distilled water up to 200 ml.