MICROBIAL DIVERSITY AND BACTERIAL BIOCATALYTIC ACTIVITIES IN PITCHER FLUID OF *Nepenthes* sp.

CHAN XIN YUE

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

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CHAN XIN YUE

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FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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MICROBIAL DIVERSITY AND BACTERIAL BIOCATALYTIC ACTIVITIES IN PITCHER FLUID OF *Nepenthes* sp.

ABSTRACT

Carnivorous pitcher plant, *Nepenthes*, develops pitcher-shaped pitfall trap, which is equipped with enzymatic fluid for prey digestion, as an alternative nutrient acquisition strategy. Despite of the antimicrobial compounds and pitcher fluid acidification by *Nepenthes* plant, the pitcher fluids host a range of micro- and macro-organisms. In this study, metagenome samples were extracted from the pitcher fluids of wild *Nepenthes albomarginata*, *Nepenthes gracilis* and *Nepenthes sanguinea*, and *Nepenthes hookeriana* planted in nursery. Metagenomic samples and bacterial 16S rRNA amplicons were subjected to next generation sequencing. Analysis on the bacterial 16S rRNA sequences showed that Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria and Firmicutes were the dominant bacteria phyla in pitcher fluid. Highly acidic pitcher fluids were dominated by alphaproteobacterial genus *Acidocella* and *Acidisoma*. Meanwhile, more diverse bacterial communities were observed in low acidity pitcher fluids. The bacterial community assemblage was strongly influenced by the pitcher fluid acidity. Besides, eukaryotic community namely insects, arachnids, fungi and protista were found in the pitcher fluids based on eukaryotic 18S rRNA genes extracted from metagenomic data. The viral community was predominated by Dasheen mosaic virus, followed by Vicia cryptic virus or Bombyx mori nucleopolyhedrovirus. To my knowledge, this is the first report on the microbial community composition in the pitcher fluid of *N. sanguinea*. From the metagenomic data, the detection of genes coding for the key enzymes in chitin, *N*-acetylglucosamine and protein hydrolysis suggested the contribution of inhabitant in *Nepenthes* pitcher fluid in the catabolism of

insect exoskeleton, which is mainly composed of chitin and protein extracellular matrix. On the other hand, allantoin was suggested as an alternative nitrogen source for the pitcher fluid's microbial community pitcher fluid when preferred nitrogen is depleted. The ammonia generated during insect and allantoin degradation might be assimilated by glutamine synthetase for glutamine synthesis. Furthermore, fermentation of xylose by bacteria in pitcher fluid was also suggested as the key enzymes in xylose isomerase pathway were found in the metagenome data. The bacterial biocatalytic activities were tested using 18 bacteria isolates from pitcher fluid. Among the isolates, positive results in chitinolytic (β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase), proteolytic, xylanolytic, cellulolytic or amylolytic activity tests were observed from 11 strains. In addition, 13 putative GH18 chitinase genes and 2 putative GH19 chitinase genes were determined from the genome of *Klebsiella*, *Pseudomonas*, *Serratia* and *Bacillus* strains isolated from the pitcher fluids. Insect degradation ability of the isolated strains was tested on sterilized *Drosophila melanogaster*. Although insect disintegration was not observed in this test, the contribution of bacteria in insect degradation cannot be ruled out as colonization of bacteria on *D. melanogaster* were observed. *D. melanogaster* with bloated abdomen was found in the medium inoculated with bacteria indicated the bacteria putrefaction activity. Besides, colonization of bacteria on insect was more successful in experiment using multiple bacteria strains. The synergistic interactions between pitcher fluid inhabitants and *Nepenthes* plant were believed to enhance their survival in nutrient limiting environment.

Keywords: *Nepenthes* pitcher fluid acidity, inhabitant in *Nepenthes* pitcher fluid, *Acidocella*, chitinolytic enzymes and insect degradation

DIVERSITI MIKROB DAN AKTIVITI BIOKATALITIK DALAM CECAIR PERIUK KERA *Nepenthes* sp.

ABSTRAK

Tumbuhan karnivorous *Nepenthes*, menghasilkan perangkap berbentuk keran, yang diisikan dengan cecair berenzim untuk pencernaan mangsa, sebagai strategi pemerolehan nutrient alternatif. Walaupun sebatian-sebatian antimikrob dan pengasidan cecair periuk kera dihasilkan oleh tumbuhan *Nepenthes*, cecair perik kera adalan habitat untuk pelbagai organism mikro dan makro. Dalam kajian ini, sampel metagenom telah disarikan daripada cecair yang dikumpulkan daripada periuk kera *Nepenthes albomaginata*, *N. gracilis* dan *N. sanguinea* yang bertumbuh di habitat semulajadi dan juga *Nepenthes hookeriana* yang ditanam di tapak semaian. Sampel-sampel metagenom dan amplicon 16S rRNA bakteria telah dijujukan melalui penjujukan generasi akan dating. Analisi pada jujukan 16S rRNA bakteria menunjukkan bahawa Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria dan Firmicutes adalah bakteria phyla dominan dalam cecair *Nepenthes*. Alphaproteobacteria genus *Acidocella* atau *Acidisoma* adalah bakteria yang paling banyak dalam cecair *Nepenthes* yang berkeasidan tinggi. Sementara itu, komuniti bakteria yang lebih pelbagai telah diperhatikan dalam cecair *Nepenthes* yang berkeasidan rendah. Komunity bakteria dalam cecair *Nepenthes* amat dipengaruhi oled keasidan cecair. Selain itu, analisi eukariotik 18S rRNA yang disarikan daripada data metagenomik menunjukkan bahawa komuniti eukariotik dalam cecair *Nepenthes* mengliputi serangga, araknida, kulat dan protista. Komuniti virus didominasi oleh virus Dasheen mosaic virus, diikuti dengan Vicia cryptic atau Bombyx mori nucleopolyhedrovirus. Setakat ini, ini adalah laporan pertama mengenai komposisi komuniti mikrob dalam cecair *N. sanguinea*. Dari data metagenomik, gen untuk enzim

dalam hidrolisis kitin, *N*-asetilglucosamine dan protein mencadangkan sumbangan organisma dalam cecair *Nepenthes* pada katabolism exoskeleton serangga, yang terbentuk daripada kitin dan protein. Selain itu, allantoin dicadangkan sebagai sumber nitrogen alternatif untuk mikrob dalam cecair *Nepenthes* apabila nitrogen yang dikehendaki habis. Amonia yang dihasilkan semasa degradasi serranga dan allantoin akan diguna oleh glutamin synthetase untuk sintesis glutamin. Tambahan pula, pengenalan enzim utama dalam laluan isomeras xilosa mencadangkan penapain xilosa oleh bakteria dalam cecair *Nepenthes*. Aktiviti biokatalitik oleh 18 bakteria yang dipencilkan daripada cecair *Nepenthes* telah diuji. Antara 18 baka bakteria, 11 baka bakteria menunjukkan aktiviti kitinolytic (β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase), proteolitik, xylanolytic, selulolytik atau amilolitik. Di samping itu, 13 gen kitinase GH18 dan 2 gen kitinase GH19 kitinase ditentukan daripada genom *Klebsiella*, *Pseudomonas*, *Serratia* dan *Bacillus* yang dipencilkan daripada cecair *Nepenthes*. Keupayaan dergadasi serangga *Drosophila melanogaster* oleh bakteria dipencilkan daripada cecair *Nepenthes* diuji dalam kajian ini. Walaupun disintegrasi serangga oleh bakteria tidak berlaku, sumbangan bakteria dalam degradasi serangga tidak dapat dikesampingkan, kerana kolonisasi bakteria pada *D. melanogaster* diperhatikan. Pengembangan abdomen *D. melanogaster* dalam medium yang diinokulasikan dengan bakteria menunjukkan aktiviti degradasi serangga oleh bakteria. Selain itu, kolonisasi bakteria pada serangga lebih berjaya dalam eksperimen yang menggunakan pelbagai baka bakteria. Interaksi sinergistik antara organisma-organisma dalam cecair *Nepenthes* dan tumbuhan *Nepenthes* dipercayai meningkatkan daya hidup mereka dalam persekitaran yang mempunyai nutrient terhad.

Kata Kunci: Keasidan cecair periuk kera, kumpulan organisma dalam cecair *Nepenthes*, *Acidocella*, enzim kitinolytic dan degradasi serangga

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$(NH_4)_2SO_4$	: Ammonium sulfate
%	: Percentage
°C	: Degree Celcius
β	: Beta
µg	: Microgram
µL	: Microlitre
µm	: Micrometre
µM	: Micromolar
ACN	: Acetonitrile
AGE	: Agarose gel electrophoresis
bps	: Base pairs
$CaCl_2.2H_2O$	: Calcium chloride dihyrate
CDS	: Coding DNA sequence
cm	: Centimetre
$CoCl_2.H_2O$	: Cobalt chloride monohydrate
COG	: Clusters of orthologous group
CTAB	: Cetyltrimethylammonium
$CuSO_4.5H_2O$	: Copper sulfate
DAPI	: 4',6'-diamidino-2-phenylindole
DF	: Dilution factor
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphates
dsRNA	: Double-stranded RNA
EDTA	: Ethylenediaminetetraacetic
et al.	: et alia

$FeSO_4.7H_2O$: Iron (II) sulfate heptahydrate

g : Gram

g : Gravitational

GH : Glycoside hydrolase

GO : Gene ontology

GOLD : Genomes OnLine Database

GPS : Global positioning system

H_3BO_3 : Boric acid

HCl : Hydrochloric acid

HMDS : Hexamethyldisilazane

hrs : Hours

K_2HPO_4 : Dipotassium phosphate

$KAl(SO_4)_2.12H_2O$: Aluminium potassium sulfate dodecahydrate

kb : Kilobase

kbps : Kilobase pairs

KH_2PO_4 : Potassium dihydrogen phosphate

L : Litre

LB : Luria-Bertani

LC : Liquid chromatography

m : Metre

M : Molarity

m/z : Mass-to-charge ratio

mA : Miliampere

MALDI-TOF : Matrix-assisted laser desorption ionization time of flight

Mbps : Million base pairs

MCG : Monkey cup garden

MetaPhlAn2 : Metagenomic phylogenetic analysis 2

mg : Miligram

$MgCl_2$: Magnesium chloride

$MgCl_2.7H_2O$: Magnesium chloride heptahydrate

min : Minute

min^{-1} : Per minute

mL : Mililitre

mL^{-1} : Per mililitre

mM : Milimolar

$MnSO_4.H_2O$: Manganese sulfate monohydrate

MS : Mass spectrometry

mU : Miliunit

$Na_2MoO_4.2H_2O$: Sodium molybdate

NaCl : Sodium chloride

NaH_2PO_4 : Sodium phosphate monobasic

NaOH : Sodium hydroxide

NCBI : National Center for Biotechnology Information

ng : Nanogram

NGS : Next generation sequencing

nm : Nanometer

NMDS : Non-metric multidimensional scaling

nmole : Nanomole

nr : Non-redundant

OD : Optical density

OTU : Operational taxonomic unit

PBS : Phosphate buffer saline

PCR : Polymerase chain reaction

PERMANOVA : Permutational multivariate analysis of variance

pH : Potential of hydrogen

pM : Picomolar

PR : Pathogenesis-related

psi : Pounds per square inch

QIIME : Quantitative Insights Into Microbial Ecology

qTOF : Quadrupole time of flight

RDP : Ribosomal Database Project

rRNA : Ribosomal ribonucleic acid

RSB : Resuspension buffer

S : Svedberg

SDS : Sodium dodecyl sulfate

sec : Second

SEM : Scanning electron microscope

SMRT : Single-molecule real time

SRA : Sequence Read Archive

ssRNA : Singled-stranded RNA

TBE : Tris-borate ethylenediaminetetraacetic

TFA : Trifluoroacetic acid

Tris-HCl : Tris-hydrochloride

U : Unit

UPGMA : Unweighted pair group method with arithmetic mean

V : Volt

v/v : Volumn per volumn

w/v : Weight per volumn

X : Time(s)

$ZnSO_4.7H_2O$: Zinc sulfate heptahydrate

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Nepenthes, a tropical carnivorous pitcher plant, typically grow at sunny, moist but nutrient deficient habitats (Clarke & Moran, 2016; Givnish et al., 1984; Pavlovič et al., 2007). In order to survive on nutrient deficient soil, *Nepenthes* plants develop insectivorous behavior as an alternative nutrient acquisition strategy (Ellison & Gotelli, 2001; Givnish et al., 1984).

Nepenthes pitcher forms at the end of tendril via leaf epiasidiation (Owen & Lennon, 1999). It plays an important role in preys (mainly arthropods) attraction, capturing, killing, digestion and nutrient absorption (Bauer et al., 2008; Gaume & Forterre, 2007; Moran, 1996; Owen et al., 1999). The slippery wet peristome and pitcher's inner wall cause the prey to slip into the pitcher fluid (Bohn & Federle, 2004; Scholz et al., 2010; Wang & Zhou, 2016). The hydrolytic enzymes in the pitcher fluid digest the drowned insects and the nutrients are absorbed from the pitcher fluid through the plant's multicellular glands (Clarke, 1997; Phillipps & Lamb, 1996; Thornhill, 2008).

Nepenthes pitcher fluid is hypothesised as a harsh living environment for bacteria, due to high acidity, lacking of macro-nutrient, presence of antimicrobial compounds and presence of pathogenesis-related (PR) proteins (Buch et al., 2014; Buch et al., 2013; Hatano & Hamada, 2008; Mithöfer, 2011). Contrary to the hypothesis, recent studies have confirmed that *Nepenthes* pitcher fluid contains complex microbial community (Chan et al., 2016; Chou et al., 2014; Kanokratana et al., 2016; Sickel et al., 2016; Takeuchi et al., 2015). Recent study on the microbial community in the pitcher fluids of *Nepenthes ampullaria*, *Nepenthes andamana*, *Nepenthes gracilis*, *Nepenthes mirabilis*

var. *globosa*, *Nepenthes mirabilis* var. *mirabilis*, *Nepenthes smilesii*, and *Nepenthes suratensis* by Kanokratana et al. (2016) shows that the bacteria phyla distribution is strongly influence by pitcher fluid acidity. However, the report on microbial community in *Nepenthes* pitcher fluid is limited and the virus community remains unknown.

The microbial community in *Nepenthes* pitcher fluid is suggested to play an important role in *Nepenthes* prey degradation. Former studies have detected bacterial protease, caseinase, lipase, β-$_D$-glucosidase, and β-$_D$-glucosaminidase in *Nepenthes* pitcher fluid (Adlassnig et al., 2010; Hepburn, 1918; Higashi et al., 1993; Morohoshi et al., 2011; Takeuchi et al., 2011). However, the identity of the bacteria, which is responsible for the secretion of the mentioned hydrolytic enzymes, is not reported. To date, the bacterial hydrolytic enzymes in *Nepenthes* pitcher fluid is not fully discovered and the insect degradation efficacy of the biocatalytic bacteria in the *Nepenthes* pitcher fluid is rarely tested.

1.2 Research Objectives

This thesis concentrated on **three** research objectives:

1. To study the bacteria, eukaryote and virus communities, and the gene coding enzymes for nutrient recycling in the pitcher fluid of *Nepenthes* sp..
2. To study the biocatalytic activities and insect degradation efficacy of the bacteria isolated from the pitcher fluid of *Nepenthes* sp..
3. To study the *N*-acetylglucosamine and the fluid condition in opened and closed *Nepenthes* pitchers.

CHAPTER 2: LITERATURE REVIEW

2.1 An Introduction of *Nepenthes* Plant

Carnivorous pitcher plant genus *Nepenthes* is locally known as "monkey cup" plant due to the fact that monkeys have been observed drinking rainwater from the *Nepenthes* pitcher. *Nepenthes* is named by Swedish botanist, Carl Linnaeus after the drug of grief relief in reference to a Greek mythology passage in the fourth book of Homer's Odyssey (Arata, 2004; Phillipps & Lamb, 1996). In this mythos, Helena, the daughter of Zeus thrown the drug (*Nepenthe*) into wine to calm her guest, making them relax and forget their pass suffering (Arata, 2004).

Nepenthes is a dioecious plant (Jebb, 1991). It typically scrambled amongst adjacent vegetation (Jebb, 1991; Moran & Clarke, 2010). The habitat of *Nepenthes* is restricted to sunny and moist environment with nutrient-depleted soil (Givnish et al., 1984). The nutrient-depleted soil type includes, sand and peat deposits, granite, limestone and sandstone surfaces, ultramafic substrate, heavily leached volcanic soil and lands that are nutrient-deficient by human activities (Clarke & Moran, 2016; Jebb & Cheek, 1997). The natural habitats of *Nepenthes* include riverbank, abrupt, opened or rocky ridges, sandstone cliff, montane forest and mossy forest (Jebb & Cheek, 1997).

Nepenthes plant produces two assimilatory organs, the photosynthesis active leaf blades and the pitcher-shape pitfall traps (hereafter refer as pitcher), in order to sustain the plant growth and development on nutrient-depleted soil (Mithöfer, 2011; Osunkoya et al., 2008). *Nepenthes* pitcher is a highly specialized organ which is responsible to attracts, kills, digests, and absorbs nutrient from its prey (Adamec, 2010). These

characteristics fit the definition of carnivorous plant, which is proposed by Givnish et al. (1984).

> First, it must be able to absorb nutrients from dead animals juxtaposed to its surfaces, and thereby obtain some increment to fitness in terms of increased growth, chance of survival, pollen production or seed set. Second, the plant must have unequivocal adaptation or resource allocation whose primary result is the active attraction, capture, and/or digestion of prey. (Givinish, 1983, p. 480).

2.2 Distribution of *Nepenthes* Plant

The distribution of *Nepenthes* plant ranges from northern Australia throughout Southeast Asia to southern China and eastward in Madagascar (Clarke & Moran, 2016; Jebb & Cheek, 1997; Moran & Clarke, 2010; Thornhill et al., 2008). It is predominantly distributed within Southeast Asia and the species richness is highest in Borneo Island and Sumatra (Clarke, 1997; Clarke & Moran, 2016; Moran & Clarke, 2010; Robinson et al., 2009). Small number of *Nepenthes* species are found in western Pacific island (Jebb & Cheek, 1997; Moran & Clarke, 2010).

The factors that shape the distribution patterns of *Nepenthes* species are the climate, seedling dispersal methods, type of substrate and vicariances. The distribution of *Nepenthes* species and their trapping strategies are remarkably correlated to the climate or microclimate at its habitat (Clarke & Moran, 2016). *Nepenthes* species, which relies on epicuticular wax for prey capturing, inhabit in areas with more seasonal climate and has higher range of toleration on its living habitat (Clarke & Moran, 2016; Moran et al., 2013). On the other hand, *Nepenthes* species, which employed large peristome or

viscoelastic pitcher fluid as prey captures mechanism, is restricted to perhumid environment (Clarke & Moran, 2016; Moran et al., 2013).

2.3 Taxonomy of the Genus *Nepenthes*

Nepenthes is the most species rich carnivorous pitcher plant genus (Moran & Clarke, 2010). It belongs to the order *Caryophyllalles*, monotypic family *Nepenthaceae* (Bhau et al., 2009; Moran & Clarke, 2010). To date, more than 150 *Nepenthes* species are identified (Christenhusz & Byng, 2016).

Phylogenetic analysis on the molecular markers (nrITS1-5.8S-nrITS2 region and *trnK* gene) of 15 *Nepenthes* species shows that *Nepenthes* species inhabit at higher altitude experienced rapid and recent radiation (Schwallier et al., 2016). In contrary, *Nepenthes* species inhabits at lower altitude evolve more gradually, leading to more molecular divergence (Schwallier et al., 2016). The natural and cultivated interspecific hybridization between *Nepenthes* species have also contribute to the growing list of *Nepenthes* species.

2.3.1 *Nepenthes sanguinea*

The epithet of *N. sanguinea* originates from a Latin word "*sanguineus*", meaning bloody red. It describes the deep red coloured pitcher of *N. sanguinea*. *N. sanguinea* is widely distributed in the mountains and hills of Peninsula Malaysia (Clarke, 2002; Jebb & Cheek, 1997; Shivas, 1984; Tsuchihashi, 2013). It usually grows at environment with opened and disturbed vegetation at altitudes 1000 m above sea level (Clarke, 2002; Jebb & Cheek, 1997; Shivas, 1984). In Peninsula Malaysia, the presence of *N. sanguinea* in Fraser's Hill, Cameron Highlands, Genting Highland, Maxwell Hill, Gunung Tahan and

Gunung Ledang are documented (Clarke, 2002; Hopkins et al., 1990; Shivas, 1984; Tsuchihashi, 2013).

N. sanguinea shows pitcher dimorphism (Tsuchihashi, 2013). Both upper and lower pitchers have flattened and ribbed peristome, and ovate lid with large glands at the lower surface (Shivas, 1984). The upper pitchers are cylindrical infundibular throughout and have broader flattened peristome (flattened up to 15 cm), while the lower pitchers have ovoid base and narrower peristome (flattened up to 2 cm) (Clarke, 2002; Shivas, 1984; Tsuchihashi, 2013). The pitcher's size is usually range from 10 cm to 30 cm in height, but may occasionally exceed 40 cm (Clarke, 2002; Shivas, 1984). The pitcher's colour varies from pure green through green background with red spots or patches to completely deep red (Clarke, 2002; Shivas, 1984). Wax layer is found on the pitcher's wall but viscoelastic characteristic is absent from the pitcher fluid of *N. sanguinea* (Moran et al., 2013).

2.3.2 *Nepenthes albomarginata*

The epithet of *N. albomarginata* originates from Latin words "*albus*" meaning white and "*marginatus*" meaning edges (Phillipps & Lamb, 1996). It describes the unique white trichome under the pitcher's peristome (Phillipps & Lamb, 1996). *N. albomarginata* is distributed in Peninsula Malaysia (Penang Hill, Gunung Jerai, Bukit Larut, Gunung Jasar, Gunung Ledang), Borneo, and west Sumatra (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Jebb & Cheek, 1997; Phillipps & Lamb, 1996). Kerangas forest, moss forest, peat swamp forest, coastal localities, coastal rocky ridge, coastal sandy podsol soil, opened vegetation on sandstone or lime stone, summit vegetation on lower hills are documented as the habitats of *N. albomarginata* (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Jebb & Cheek, 1997; Phillipps & Lamb, 1996).

N. albomarginata produces narrow cylindrical upper pitchers and ovoid based lower pitchers (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004). The pitchers are generally 15 cm in length (Clarke, 2002; Phillipps & Lamb, 1996; Shivas, 1984). The colours of the pitchers vary from pure green, through green background with purple spots to pure purple or pinky-red (Clarke, 2002; Phillipps & Lamb, 1996; Shivas, 1984). Penang Hill's population often produces larger infundibuliform upper pitchers and richly coloured lower pitchers (Clarke, 2002; Phillipps & Lamb, 1996; Shivas, 1984). Wax layer is found on the pitcher's wall but viscoelastic characteristic is absent from the pitcher fluid of *N. albomarginata* (Moran et al., 2013).

Studies have revealed the prey (termite) specialization behavior of *N. albomarginata* (Gaume et al., 2016; Merbach et al., 2002). *N. albomarginata* uses the edible white trichome ring under the peristome to lure and trap large amount of termites (Merbach et al., 2002). Thousands of termites can be caught in single pitcher when the pitcher is activated (Merbach et al., 2002). The termites derived nitrogen contributed 53.8% of the total foliar nitrogen in *N. albomarginata* plant (Moran et al., 2001).

2.3.3 *Nepenthes gracilis*

The epithet of *N. gracilis* originates from a Latin word "*gracilis*", meaning slender (Phillipps & Lamb, 1996). It describes the slender leaves and pitchers of *N. gracilis* plant (Phillipps & Lamb, 1996). *N. gracilis* is widely distributed in Borneo, Peninsular Malaysia, Singapore, Sulawesi, Sumatra and Thailand (Adam et al., 1992; Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Jebb & Cheek, 1997; Phillipps & Lamb, 1996; Shivas, 1984). It is the commonest lowland *Nepenthes* species in Sunda Islands and Peninsula Malaysia (Clarke, 1997; Clarke & Lee, 2004; Phillipps & Lamb, 1996; Shivas, 1984). The common habitat of *N. gracilis* includes peat swamps forest, rain

forest, kerangas forest, area disturbed by human activities, roadside, forest clearing area, coastal sandhills and wet, open areas (Clarke, 1997; Clarke, 2002; Jebb & Cheek, 1997; Shivas, 1984).

N. gracilis produces small (up to 20 cm) and cylindrical pitchers with narrow peristome and round lid (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Moran, et al., 1999). The upper pitchers of *N. gracilis* are cylindrical infundibular throughout, while the lower pitchers of *N. gracilis* have ovoid base (Clarke, 1997; Phillipps & Lamb, 1996; Shivas, 1984). A pair of fringed wings is observed on the front of the lower pitchers (Clarke, 1997; Clarke, 2002). Wings are absence on upper pitcher (Clarke, 1997; Clarke, 2002). The colour of the pitchers varies from light green through light green with purple spots, to purely dark purple (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Shivas, 1984). Wax layer is found on the pitcher's wall but viscoelastic characteristic is absent from the pitcher fluid of *N. gracilis* (Moran et al., 2013; Phillipps & Lamb, 1996).

2.3.4 *Nepenthes hookeriana*

N. hookeriana is named after Sir Joseph Dalton Hooker. It is a natural hybrid between *N. ampullaria* and *Nepenthes rafflesiana* (Clarke, 1997; Shivas, 1984). *N. hookeriana* is distributed in Peninsular Malaysia, Borneo, Singapore, Sumatra (Adam et al., 1992; Clarke, 1997; Jebb & Cheek, 1997; Phillipps & Lamb, 1996; Shivas, 1984). The natural habitat of *N. hookeriana* includes, swampy areas, dense heath forest, open sandy area, low secondary scrub, drier exposed sites and opened and disturbed areas (Clarke, 1997; Jebb & Cheek, 1997; Phillipps & Lamb, 1996; Shivas, 1984). The population of *N. hookeriana* can only be found near the population of its parental species (Jebb & Cheek, 1997; Phillipps & Lamb, 1996).

The structure of *N*. *hookeriana* pitchers resembled some characteristics of each parent species (Jebb & Cheek, 1997; Shivas, 1984). The lid of *N*. *hookeriana* pitcher is larger than that of *N*. *ampullaria* but smaller and narrower than that of *N*. *rafflesiana* (Clarke, 1997; Phillipps & Lamb, 1996). The peristome of *N*. *hookeriana* pitchers are expanded (but not overhang the outer side of the rim) and toothed on the inner edge (Clarke, 1997; Shivas, 1984). The upper pitcher is cylindrical infundibular throughout which is thinner and taller than the upper pitcher of *N*. *ampullaria*, but is more ovoid than the upper pitchers of *N*. *rafflesiana* (Clarke, 1997; Phillipps & Lamb, 1996; Shivas, 1984). The round pot like shape and ovoid base of *N*. *hookeriana* lower pitchers resembled the characteristic of the lower pitchers produces by *N*. *ampullaria* plant (Jebb & Cheek, 1997). A pair of broad wings is observed on the front of *N*. *hookeriana* lower pitchers (Jebb & Cheek, 1997). The colour of the pitchers varies from purely green or ivory white to green background with red spots (Jebb & Cheek, 1997; Phillipps & Lamb, 1996; Shivas, 1984).

2.4 Characteristics of the *Nepenthes* Pitcher

Nepenthes pitcher develops at the end of the tendril (Mithöfer, 2011; Owen & Lennon, 1999). The tendril of *Nepenthes* plant emerges from leaf apex and forms a small, flat bud at its tip (Clarke, 1997; Clarke & Moran, 2016). When the bud is physiologically activated, it swells and elongates into pitcher-shaped trap (Clarke, 1997; Owen & Lennon, 1999). The duration of pitcher development depends on *Nepenthes* species, pitcher size and pitcher type (upper or lower pitcher) (Clarke, 1997).

At developmental stage, the interlocking epidermal cells on peristome and lid, together with the interwoven trichomes, tightly seal the lid to the orifice of *Nepenthes* pitcher (Owen & Lennon, 1999). The peristome is folded inwards and sealed inside the pitcher during the pitcher development (Owen & Lennon, 1999). Before the pitcher opens, *Nepenthes* plant secretes fluid and enzymes into the digestive zone at the bottom of the pitcher (Clarke, 1997; Jentsch, 1972; Phillipps & Lamb, 1996; Shivas, 1984). When the pitcher is closed to maturity, the cells located at the peristome upper surface expand and push the peristome tip outward, causing it to curl around the outside of the pitcher orifice, leading to the disconnection of pitcher's lid from the pitcher orifice and the exposure of cylindrical tube to the environment (Owen & Lennon, 1999).

In general, lid, peristome and pitcher (Figure 2.1) are the main components of a *Nepenthes* pitcher (Clarke & Lee, 2004). The morphology variety of *Nepenthes* pitcher is the main feature used in *Nepenthes* species identification. Majority of the *Nepenthes* species has a broad and flat lid which is connected to the pitcher at the neck of the peristome (Clarke, 2002; Phillipps & Lamb, 1996). The structure and position of the lid covers the pitcher's orifice, preventing the dilution of nutrient by the overflowed rainwater (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Phillipps & Lamb, 1996). Exceptional case is observed on the pitcher of *N. ampullaria*. The lid of *N. ampullaria* points away from the pitcher, allowing rainwater to enter the pitcher freely (Clarke, 1997). The colour of the lid and the nectar production on the lid play an important role in prey attraction (Bauer et al., 2012b; Clarke, 1997; Shivas, 1984). A structure known as spur is often observed at the connecting point of the lid and pitcher (Clarke, 1997; Clarke, 2002). However, the function of the spur is yet to be discovered.

The peristome of *Nepenthes* pitcher (Figure 2.1) is a ridge of hardened tissue that surrounded the rim of pitcher's orifice (Clarke & Lee, 2004). The *Nepenthes* peristome plays an important role in prey attraction and trapping prey. The inner edge of the peristome is packed with extrafloral nectaries for insect attraction (Clarke, 1997; Clarke & Lee, 2004). The combination of nectar hygroscopic effect, peristome anisotropic surface topography and peristome hydrophilicity, create a fully wettable peristome surface (Bauer & Federle, 2009; Corsaro et al., 2007; Hsu et al., 2015). The wetted peristome surface extensively reduces the insect foot attachment ability, causing the insect to aquaplanes and slips into the pitcher (Bauer et al., 2012b; Bohn & Federle, 2004; Bauer & Federle, 2009). The over-hanged rim of the peristome forms a barrier to prevent the escape of insect which has successfully enter the pitcher and accessed to the nectar gland located at the inner side of the peristome (Bohn & Federle, 2004; Clarke & Lee, 2004).

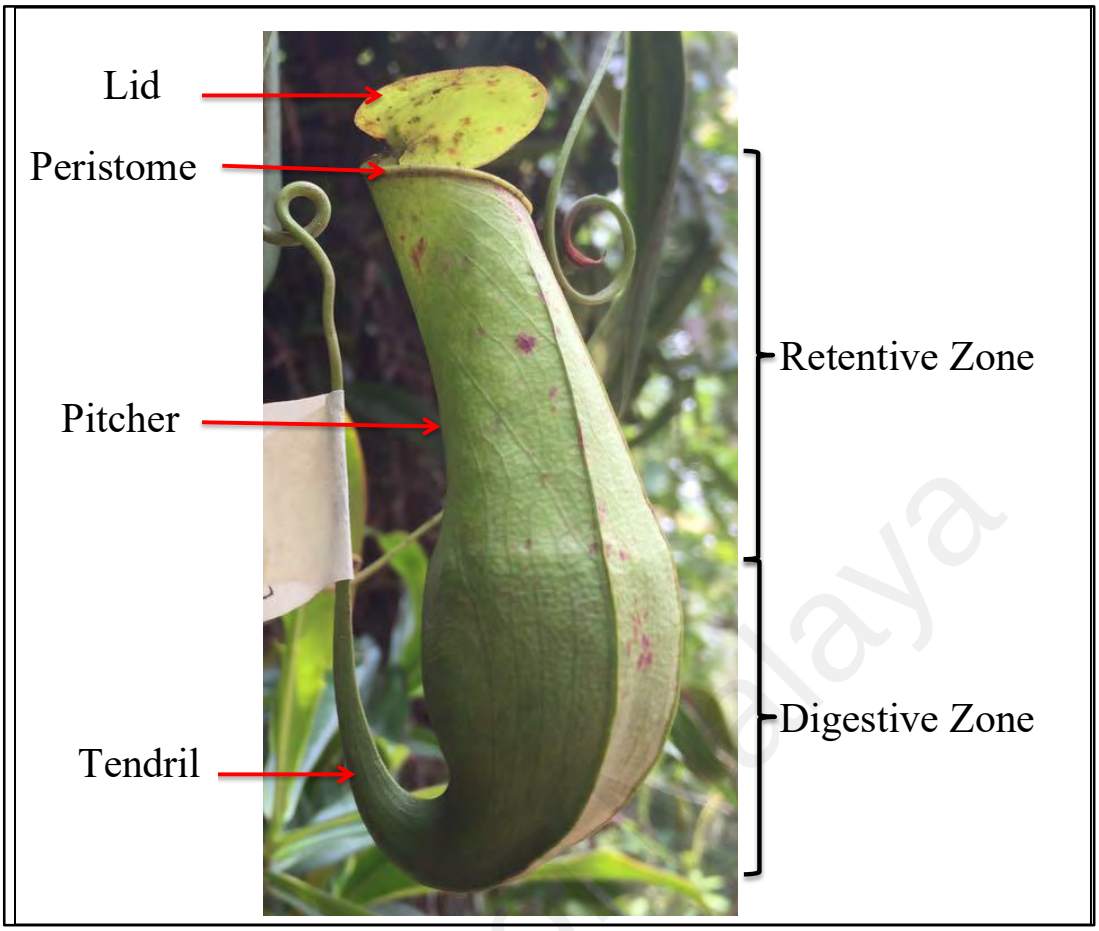


Figure 2.1: The structure of a *Nepenthes* pitcher

A *Nepenthes* pitcher can be divided into retentive zone and digestive zone (Figure 2.1) (Clarke, 1997; Clarke 2002). The downward-directed lunate cells form by the modified stomata cells are scattered in the retentive zone (Owen & Lennon, 1999; Wang et al., 2009). The epidermal and lunate cells on the inner wall of the retentive zone are coated with intracuticular wax and epicuticular wax crystal (Clarke & Lee, 2004; Gaume et al., 2002; Owen & Lennon, 1999; Riedel et al., 2003). The intracuticular wax microstructure roughness and hydrophobicity along with the epicuticular wax crystal create an extremely slippery surface to insect, hence efficiently retained insect in the pitcher (Scholz et al., 2010; Wang & Zhou, 2016). On the other hand, the inner wall of *Nepenthes* pitcher's digestive zone is covered with multicellular glands and the bottom of the digestive zone contains acidic pitcher fluid (Clarke, 2002; Clarke & Lee, 2004; Gorb et al., 2004; Owen & Lennon, 1999; Phillipps & Lamb,

1996). Insects are retained at the *Nepenthes* pitcher retention zone by the viscoelastic picther fluid, wax crystal on the multicellular glands, multicellular glands secretion and multicellular glands microstructure, which affect the insect's mobility (Bauer et al., 2011; Gaume & Forterre, 2007; Gaume et al., 2002; Gorb et al., 2004; Moran et al., 1999; Wang et al., 2009). Besides, the multicellular glands in *Nepenthes* pitcher also secrete acidic pitcher fluid and hydrolytic enzymes into the pitcher for organic material (prey carcass, plant detritus and mammal excrement) catabolism (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Clarke et al., 2009; Phillipps & Lamb, 1996; Shivas, 1984; Wells et al., 2011). Nutrients from the digested preys are absorbed and distributed to the other parts of *Nepenthes* plant through the multicellular glands connected to the vascular bundles (Owen & Lennon, 1999; Phillipps & Lamb, 1996; Shivas, 1984; Thornhill et al., 2008).

The immature plants and short stems produce "lower" or "terrestrial" pitchers while the climbing stems produce "upper' or "aerial" pitchers (Clarke, 2002; Moran, 1996; Phillipps & Lamb, 1996). Both pitcher types are produced by *Nepenthes* plant at different stages of its life cycles (Clarke, 2002). The lower pitcher is generally ovoid and richly coloured (Clarke & Lee, 2004; Moran, 1996). It is larger than the upper pitcher, thus, allowing it to carry larger volume of pitcher fluid (Clarke & Lee, 2004). The lower pitcher typically rests on the ground to relieve the plant of their weight (Clarke & Lee, 2004). A pair of fringe "wings" is observed at the front of the lower pitcher in most *Nepenthes* species (Clarke, 1997; Shivas, 1984). The upper pitcher is generally funnel-shaped and less colourful compared to the lower pitcher (Clarke, 1997; Clarke & Lee, 2004; Shivas, 1984). It has smaller size and lighter weight, thus, allowing the main stem to climb higher and hooked on to the adjacent vegetation at its tendril (Clarke, 1997; Clarke, 2002; Moran, 1996). Reduced or no wing is observed on the

upper pitcher (Clarke, 2002; Clarke & Lee, 2004; Jebb & Cheek, 1997). The colour spectra of the *Nepenthes* pitcher ranges from light green to dark purple (Phillipps & Lamb, 1996). The morphology of upper pitcher and lower pitcher of some *Nepenthes* species can be very distinctive (pitcher dimorphism) and occasionally causes difficulty in species identification (Clarke, 2002; Clarke & Moran, 1994; Moran, 1996; Shivas, 1984).

2.5 *Nepenthes* Pitfall Trap

The pitchers of *Nepenthes* are equipped with complex prey attraction, entrapment, retention, and assimilation features. Prey and mutualistic organisms are attracted to *Nepenthes* pitchers by *Nepenthes* extrafloral nectar, pitcher colourization, visualization cues (light, ultraviolet patterns and visible wavebands), and fragrance from pitcher fluid (Bauer et al., 2008; Di Giusto et al., 2010; Di Giusto et al., 2008; Kurup et al., 2013; Moran, 1996; Moran et al., 2012; Plachno, 2007; Schaefer & Ruxton, 2008). The prey trapping effiency is enhanced when rainwater and air humidity aid in creating a slippery surface on the peristome and pitcher inner wall (Bauer et al., 2008; Bauer et al., 2009; Bauer et al., 2011; Bauer et al., 2012b). The pitchers of *N. gracilis* are reported to employ the impact of rainwater for 'flicking' its prey at the lower lid surface into the pitcher (Bauer et al., 2012b).

The wet surface of pitcher peristome, waxy layers on the pitcher inner wall and viscoelastic pitcher fluid are the key components for insects aquaplaning and retaining insect in *Nepenthes* pitcher. Previous studies have found that *Nepenthes* species with large peristome and viscoelastic pitcher fluid often has waxless or reduced wax coverage on its pitcher wall and vice-versa (Bauer et al., 2012a; Benz et al., 2012; Bonhomme et al., 2011b; Clarke & Moran, 2016; Moran et al., 2013). The distribution

of *Nepenthes* plant with 'wet' syndrome trapping mechanism (large peristome, waxless or reduced wax layer on pitcher wall and viscoelastic pitcher fluid) in perhumid environment and 'dry' syndrome trapping mechanism (small perisome, waxy pitcher wall and non-viscoelastic pitcher fluid) in less humid environment are observed by Moran et al. (2013). Researchers suggest that the evolutionary force has resulted diverse trapping strategies among *Nepenthes* species under different ecological condition, in order to maximize the plant nutrient acquisition at lower cost (Bauer et al., 2012a; Bonhomme et al., 2011b; Gaume et al., 2016; Moran et al., 2013).

2.6 Prey Spectrum of *Nepenthes*

In general, *Nepenthes* plants are not very selective about their prey spectrum (Merbach et al., 2002). The prey spectrum of *Nepenthes* is subjected to *Nepenthes* species, living environment, prey availability, prey trapping strategies and the pitcher's position (Adam, 1997; Bonhomme et al., 2011b; Chin et al., 2014; Gaume et al., 2016). The main prey assemblages in *Nepenthes* pitchers are ants, termites and flying insects (Adam, 1997; Chin et al., 2014; Clarke, 1997; Di Giusto et al., 2008; Gaume et al., 2016; Kato et al., 1993; Moran, 1996; Rembold et al., 2010). Highland *Nepenthes* species (species that grow at elevation 1000 m above sea level and higher) generally catches higher ratio of flying insects while lowland *Nepenthes* species (species that grow below 1000 m above sea level) catches more creeping insect (Adam, 1997; Bonhomme et al., 2011b; Davey, 2007; Shivas, 1984). The upper pitcher has wider prey spectrum and captures more flying insects compared to the lower pitcher due to the difference in prey trapping strategies (Adam, 1997; Clarke, 1997; Di Giusto et al., 2008; Moran, 1996; Rembold et al., 2010).

A few *Nepenthes* species have adapted to less insectivorous "diet". The pitchers of *Nepenthes hemslayana* offer quality roosting site to Hardwicke's woolly bat, while the pitchers of *Nepenthes rajah*, *Nepenthes macrophylla* and *Nepenthes lowii* feed tree shrews and summit rats with extrafloral nectars in exchange of their feaces as the main nitrogen source (Clarke et al., 2009; Clarke et al., 2010; Grafe et al., 2011; Greenwood et al., 2011; Schöner et al., 2013; Schöner et al., 2015; Schöner et al., 2017;). On the other hand, the pitchers of *N. ampullaria* accumulate and digest plant detritus as main nutrient source (Adlassnig et al., 2010; Moran et al., 2003; Pavlovicet al., 2011).

2.7 The Importance of Prey to *Nepenthes*

The digested organic materials (arthropod based prey, mammalian feaces and plant detritus) in *Nepenthes* pitcher fluid are the main nitrogen sources for *Nepenthes* plant. Previous studies show that insect based prey contributed 77.0%, 68.1%, 61.5% and 53.8 % of the total foliar nitrogen in *Nepenthes bicalcarata* without symbiotic ants, *N. rafflesiana*, *N. mirabilis* and *N. albomarginata*, respectively (Moran et al., 2001; Scharmann et al., 2013; Schulze et al., 1997). Bat and tree shrew feaces contributed 33.8% and up to 100% of the total foliar nitrogen in *N. hemsleyana* and *N. lowii*, respectively. The leaf litter contributes 35.7% to 54.8% of the total foliar nitrogen in *N. ampullaria* (Grafe et al., 2011; Moran et al., 2003; Pavlovic et al., 2011).

Addition of prey into the *Nepenthes* pitcher fluid enhanced the photosynthetic rate of *Nepenthes* leaf laminae (He & Zain, 2012; Pavlovič et al., 2009; Pavlovic et al., 2011; Schöner et al., 2017). The improvement of *Nepenthes* plant photosynthetic rate by prey nutrient could enhance the growth and reproduction of *Nepenthes* plant, thus, enhancing the plant survivability in nutrient poor habitats (Pavlovič et al., 2009). Smaller pitchers and less pitcher production are observed on prey-deprived *N. rafflesiana* plant,

suggesting the importance of prey derived nutrient on the growth of *Nepenthes* plant (Moran & Moran, 1998). Besides, the increment in photosynthetic rate is postulated to play an important role in triggering the root nutrient uptake event (Adamec, 2013). However, these hypotheses are not experimentally tested.

2.8 *Nepenthes* Pitcher Fluid

2.8.1 Acidity

Nepenthes pitcher fluid is generally acidic and the pH value is subjected to *Nepenthes* species, age of the pitcher, number of prey and types of prey in the pitcher fluid (An et al., 2002a; Clarke, 1997; Clarke & Lee, 2004; Higashi et al., 1993; Higgins, 2001). The acidity of the pitcher fluid ranges from very acidic (pH1.9) to neutral (pH7) but rarely alkaline (pH8) (Clarke, 1997; Clarke, 2002; Clarke & Lee, 2004; Kanokratana et al., 2016; Shivas, 1984; Takeuchi et al., 2015). In general, the pitcher fluid is less acidic in unopened pitcher. The acidity of pitcher fluid increases significantly within 24 hours after the pitcher opened, and gradually decreases after the plummet (An et al., 2001; An et al., 2002a; Higashi et al., 1993; Morrissey, 1955). The acidic characteristic of pitcher fluid enhances the prey retention and prey killing efficiency as well as providing an optimal condition for plant enzymatic activities (Bazile et al., 2015; Eilenberg & Zilberstein, 2008; Michelet & Boutry, 1995; Moran, Hawkins et al., 2010; Shivas, 1984). The pH of pitcher fluid in old pitchers which do not catch insect are reported to be closed to neutral (An et al., 2001).

Although the feeding mechanism of *Nepenthes* is not fully understood, it was postulated that the pitcher fluid acidification is caused by the prey digestion (Eilenberg & Zilberstein, 2008; Higashi et al., 1993). Prey catabolism by enzymatic activities released ammonium, which is the plant preferred nitrogen source into the *Nepenthes* pitcher fluid (Higashi et al., 1993; Schulze et al., 1999; Schulze et al., 1997). Subsequently, the ammonium in the pitcher fluid induce the secretion of protease for further prey digestion (Higashi et al., 1993). The ammonium in the pitcher fluid also induce the expression of plasma-membrane H^+-ATPase gene, *NaPHA*, of the head cells in the digestive glands (An et al., 2001; Higashi et al., 1993; Schulze et al., 1999). The influx of H^+ secreted by plasma-membrane proton pumps increase the pitcher fluid acidity and create a proton gradient across the plasma membrane which drives the uptake of solute from the pitcher fluid (Adlassnig et al., 2010; An et al., 2001; Higashi et al., 1993; Michelet & Boutry, 1995). The acidity of the pitcher fluid returned to the initial condition after the absorption of ammonium by the plant which causes the reduction in the concentration of H^+ (Higashi et al., 1993).

2.8.2 Mineral Nutrients and Secondary Metabolites

In general, the *Nepenthes* pitcher fluid resembles potassium chloride solution supplemented with calcium, magnesium, sodium, sulfate, malte, citrate, oxalate and traces of other elements (Adlassnig et al., 2010; Buch et al., 2013; Nemček et al., 1966; Smith, 1893). The concentration of nitrogen and phosphate are low in the fluid of unopened *Nepenthes* pitcher (Buch et al., 2013). Secondary metabolites analysis have identified napthoquinones and its derivaties (droserone, 5-*O*-methyldroserone, plumbagin and 7-methyl-juglone) in *Nepenthes* pitcher fluid (Buch et al., 2013; Eilenberg et al., 2009; Raj et al., 2011). The napthoquinones and its derivaties may

serve as the protective agents for the *Nepenthes* plant against pathogen invasions (Buch et al., 2013; Eilenberg et al., 2009; Raj et al., 2011).

2.8.3 Plant Hydrolytic Enzymes

Nepenthes plant secretes hydrolytic enzymes into the pitcher fluid before the pitcher opens (Clarke, 1997). Biochemical studies have detected protease, exopeptidase, nuclease, phosphoamidase, phosphatase, lipase, chitinase and esterase activities in *Nepenthes* pitcher fluid (An et al., 2002a; An et al., 2002b; Athauda et al., 1998; Buch et al., 2015; Chin et al., 2007; Eilenberg et al., 2006; Ekanayaka, 2009; Hepburn, 1918; Higashi et al., 1993; Ishisaki et al., 2012; Kadek et al., 2014; Nakayama & Amagase, 1968; Nishimura et al., 2014; Rottloff et al., 2011; Stephenson & Hogan, 2006; Takeuchi et al., 2011; Tökés et al., 1974). Recent proteome analysis has identified more than 30 proteins, including hydrolytic enzymes, PR proteins, and signal transduction related proteins from *Nepenthes* pitcher fluid, suggesting a complex carnivory mechanism in the pitcher of *Nepenthes* (Lee et al., 2016; Mithöfer, 2011; Rottloff et al., 2016).

Proteolytic activity was detected in the fluid of both opened and unopened *Nepenthes* pitchers (Hepburn, 1918). Nepenthesin, an aspartic proteinase found in *Nepenthes* pitcher fluid is named and characterized by Nakayama and Amagase (Nakayama & Amagase, 1968). Biochemical studies showed that the enzymatic activity of nepenthesin 1 and nepenthesin 2 were stable over wide range of pH (from pH3 to pH10) and the pH optima for nepenthesin enzymatic activity range from pH2 to pH3 (An et al., 2002b; Athauda et al., 1998; Athauda et al., 2004; Buch et al., 2015; Kadek et al., 2014; Nakayama & Amagase, 1968; Stephenson & Hogan, 2006; Takahashi et al., 2009). The presence of 12 cysteine residues which forms 6 disulphide bonds in the protein

sequence of Nepenthesin 1 resulted its stablilty at wide range of temperature (up to 60 °C) (Athauda et al., 1998; Lee et al., 2016; Rottloff et al., 2016). In addition to nepenthesin 1 and nepenthesin 2, recent extensive proteome analysis have also catalogued other proteases, including aspartic proteases (nepenthesin 3, nepenthesin 4 and nepenthesin 5), prolyl endoproteases (neprosin 1 and neprosin 2) and serine carboxypeptidases which are associated to protein metabolism in the *Nepenthes* pitcher fluid (Athauda et al., 2004; Lee et al., 2016; Rottloff et al., 2016).

The chitinases in *Nepenthes* pitcher fluid contribute to the insect exoskeleton digestion (Eilenberg et al., 2006; Ishisaki et al., 2012; Ishisaki et al., 2011; Rottloff et al., 2011). Class I, class III and class IV plant endochitinases were found in the *Nepenthes* pitcher fluid (Eilenberg et al., 2006; Honda et al., 2008; Ishisaki et al., 2012; Ishisaki et al., 2011; Lee et al., 2016; Rottloff et al., 2011). Biochemical studies showed that class III and class IV chitinases were active in acidic environment (Ishisaki et al., 2012; Ishisaki et al., 2011; Rottloff et al., 2011). The pH optima for class III and class IV chitinases enzymatic activities are pH3 to pH4 and pH5.5, respectively (Ishisaki et al., 2012; Ishisaki et al., 2011; Rottloff et al., 2011). As the chitinases in *Nepenthes* pitcher fluid hydrolysed different substrates, it is suggested that *Nepenthes* utilises two steps insect degradation mechanism in order to enhance its insect degradation efficiency (Rottloff et al., 2016). The long chitin polymers on the insect exoskeletons are fragmented into chito-oligosaccharides by class III endochitinase (Ishisaki et al., 2012; Rottloff et al., 2016; Rottloff et al., 2011). Subsequently, the chito-oligosaccharides are further hydrolyzed by class I chitinase, class IV chitinase and *N*-acetylglucosaminidases which are secreted by *Nepenthes* plant or the micro-organisms in the pitcher fluid (Eilenberg et al., 2006; Ishisaki et al., 2012; Ishisaki et al., 2011; Rottloff et al., 2016; Takeuchi et al., 2011).

2.8.4 Antimicrobial Compounds and Defensive Proteins

Nepenthes pitcher fluid shows antifungal properties against plant and human pathogenic fungi (Eilenberg et al., 2009; Mithöfer, 2011; Yolanda et al., 2015). Two napthoquinones, which are the droserone and 5-*O*-methyldroserone, are found in the pitcher fluid of *Nepenthes khasiana* after chitin induction (Eilenberg et al., 2009; Raj et al., 2011). However, droserone and 5-*O*-methyldroserone which are detected in the pitcher fluid of *N. khasiana* are not detected in the pitcher fluid of *N. ventricosa* (Buch et al., 2013). Instead, plumbagin and its derivative 7-methyl-juglone are detected in the fluid of *Nepenthes ventricosa* opened pitcher (Buch et al., 2013). In contrary, plumbagin is not detected in the pitcher fluid of *N. khasiana* but found in the root of *N. khasiana* (Raj et al., 2011). In the study by Buch et al. (2013) the presence of plumbagin and its derivative 7-methyl-juglone in the pitcher fluid of *N. ventricosa* are suspected to be the causative agent for the death of bacteria innoculated into the pitcher fluid. However, the antimicrobial effect of napthoquinones on the bacteria inhabitated in *Nepenthes* pitcher fluid are not experimentally tested.

PR proteins which are secreted by plant to defense themselves from the attack of pathogens, such as bacteria and fungi, are found in the *Nepenthes* pitcher fluid (Buch et al., 2014; Buch et al., 2013; Hatano & Hamada, 2008). The PR-1 found in the pitcher fluid of *N. mirabilis* shows antibacterial property (Buch et al., 2014; Mithöfer, 2011). Besides, based on the enzymes's biochemical properties, various hydrolytic enzymes involved in prey catabolism in the *Nepenthes* pitcher fluid were classified as PR proteins (Mithöfer, 2011). These include 1,3-β-glucanase (PR-2), class I and class IV chitinases (PR-3), class III chitinase (PR-8), nepenthesin 1 and nepenthesin 2 (PR-7), cystein protease (PR-7) and ribonuclease (PR-10) (Athauda et al., 2004; Buch et al., 2013; Eilenberg et al., 2006; Mithöfer, 2011; Rottloff et al., 2011; Stephenson & Hogan,

2006). This suggested the dual functions of *Nepenthes* hydrolytic enzymes which are responsible for prey catabolism as well as protecting the plant or pitcher from pathogenic invasion (Mithöfer, 2011). Thaumatin-like protein which is putatively antimicrobial is also found in the pitcher fluid (Buch et al., 2013; Hatano & Hamada, 2008; Mithöfer, 2011).

2.9 Visitors and Inquilines of *Nepenthes* Pitcher Fluid

Nepenthes plant does not kill all living organism that enters the pitcher and pitcher fluid. For example, the unique swimming pattern of *Camponotus schmitzi* ant not only prevent the ants from drowning, but also allowed the worker ants to regularly dive and swim into the pitcher fluid (Bohn et al., 2012). *Nepenthes* pitcher fluid harbours wide variety of organisms, including bacteria, fungus, metazoa, algae, nematode, midge, mite, tick, dipteran larvae, ant, fly, frog, crab spider and lizard (Adlassnig et al., 2010; Beaver, 1979; Bonhomme et al., 2011a; Chua & Lim, 2012; Clarke & Kitching, 1995; Fashing, 2002; Fashing, 2008; Grafe & Kohout, 2013; Lam et al., 2017; Lee et al., 2014; Mogi, 2004; Mogi & Yong, 1992; Pocock, 1898; Ratsirarson & Silander, 1996). *Nepenthes* plant maintains mutualistic relationship with majority of its inquiline. It provides habitation, food and protection to its inquilines; in return, the inquilines clean the pitcher, enhance pitcher prey capture efficiency, assist on the necromass digestion, protect the plant from herbivore, and control the bacterial population in the *Nepenthes* pitcher fluid (Bonhomme et al., 2011a; Lam et al., 2017; Merbach et al., 2007; Pocock, 1898; Takeuchi et al., 2011; Thornham et al., 2012). The inquilines form complex food web in *Nepenthes* pitcher fluid where the necromass in the pitcher fluid serves as food source for microorganism and saprophages, while the microorganisms and saprophages are preyed by aquatic and terresterial predator (Clarke, 1997; Cresswell, 2000; Phillipps & Lamb, 1996; Ratsirarson & Silander, 1996; Sota et al., 1998).

2.10 Microbial Community in *Nepenthes* Pitcher Fluid

Bacterial isolation from *Nepenthes* pitcher's fluid was documented a century ago. The fluid of 12 unopened *Nepenthes* pitchers were plated on nutrient agar plates and incubated at 37 °C for 4 days (Hepburn, 1918). No bacterial colony was observed on the agar plates at the end of the incubation leads to the conclusion that the pitcher fluid in unopened pitcher is sterile (Hepburn, 1918). The result was supported by Buch et al. (2013) study, where bacterial 16S rDNA genes were not amplified from the fluid of unopened *Nepenthes* pitcher (Buch et al., 2013). In contrary, bacteria were found in the fluid of *Nepenthes alata* unopened pitcher by Sota et al. (1998). This result was supported by recent researches on different *Nepenthes* species (*N. ampullaria*, *N. gracilis*, *N. mirabilis*, *N. andamana*, *N. suratensis*) (Chou et al., 2014; Kanokratana et al., 2016; Takeuchi et al., 2015). The possibility of contamination was ruled out by the authors as the bacteria presence in the fluid of unopened *Nepenthes* pitcher were also detected in the fluid of opened pitcher (Chou et al., 2014). The improvement on DNA extraction methodologies and gene amplification sensitivity can be credited for the succession of bacteria detection from the unopened pitcher's fluid in the recent researches.

The bacterial cell density in the fluid of unopened pitchers ranges from 9.63×10^5 cells/mL to 1.6×10^7 cells/mL (Sota et al., 1998; Takeuchi et al., 2015). Bacterial composition studies through the sequencing of bacterial 16S rRNA gene clonal libraries and next generation sequencing (NGS) approaches have shown that Proteobacteria is the predominant phylum in the fluid of unopened pitchers (Chou et al., 2014; Kanokratana et al., 2016; Takeuchi et al., 2015). The study by Takeuchi et al. (2015) showed that the fluid of *N. ampullaria* unopened pitchers contained high proportion of Alphaproteobacteria, *Rhodobacteraceae*, *Methylobacteriaceae* and *Sphingomonadaceae*

which represented the typical phyllosphere microbiota (Takeuchi et al., 2015). The isolation of endophytes from the *Nepenthes* leaf and pitcher as well as the detection of endophytic bacteria (for example, *Sphingomonas*) from metagenomic data have raised a hypothesis that endophyte or phyllosphere bacteria are the sources of "native" bacteria in *Nepenthes* pitcher fluid (Bhore et al., 2013; Chou et al., 2014; Takeuchi et al., 2015). However, this hypothesis is not tested.

Despite of the *Nepenthes* pitcher fluid acidity and the presence of antimicrobial compounds, bacteria and yeast were detected and isolated from the fluid of *Nepenthes* opened pitcher (Hepburn, 1918; Hidaya, 2015; Higashi et al., 1993; Shivas & Brown, 1989). The results of acridine orange or 4',6'-diamidino-2-phenylindole (DAPI) cell counting methods showed that bacterial density in the fluid of opened pitchers range from 2.09×10^5 cells/mL to 4.83×10^9 cells/mL (Sota et al., 1998; Takeuchi et al., 2015; Takeuchi et al., 2011). The bacterial cell density increases with pitcher age but does not show significant correlation with the fluid acidity and bacterial diversity indexes (Sota et al., 1998; Takeuchi et al., 2015; Takeuchi et al., 2011).

The bacterial community in *Nepenthes* pitcher fluid was less diverse than the environmental samples (soil and ocean) and termite gut, and similar to the phyllosphere of *Arabidopsis* and mouse gut, but more diverse than human gut (Sickel et al., 2016; Takeuchi et al., 2015). The bacterial composition in *Nepenthes* pitcher fluid was similar to the bacterial composition on its phyllosphere and in the pitcher of *Sarracenia* (North American carnivorous pitcher plant) (Takeuchi et al., 2015). Although the *Nepenthes* pitcher shares certain degrees of functional analogue (food assimilation and contains acidic fluid) with eukaryotic digestive organ, the microbial assemblage of *Nepenthes*

pitcher fluid has very low similarity with the microbial community in the guts of human, mouse and termite (Takeuchi et al., 2015).

In general, Proteobacteria was the predominant bacteria phyla in the pitcher fluid of the reported *Nepenthes* species (*N. albomarginata*, *N. ampullaria*, *N. andamana*, *N. gracilis*, *N. hemsleyana*, *N. hirsuta*, *N. mirabilis* var *globosa*, *N. mirabilis* var *mirabilis*, *N. mirabilis* var *echinostoma* , *N. rafflesiana*, *N. smilesii* and *N. suratensis*) (Chou et al., 2014; Kanokratana et al., 2016; Sickel et al., 2016; Takeuchi et al., 2015). The relative abundance of Alphaproteobacterial genus *Acidocella* was extremely high (>50% of the bacterial community) in the highly acidic *Nepenthes* pitcher fluid (pH2) (Kanokratana et al., 2016; Sickel et al., 2016). Even though the *Nepenthes* pitcher fluid is typically acidic, the relative abundance of acidophilic bacteria (except *Acidocella*) are generally low in *Nepenthes* pitcher fluid (Kanokratana et al., 2016; Sickel et al., 2016). Considerable amount of *Bacteroidetes* and *Actinobacteria* were also detected in the fluid of opened pitcher (Kanokratana et al., 2016; Sickel et al., 2016; Takeuchi et al., 2015).

The bacterial composition in *Nepenthes* pitcher fluid could be shaped by the living habitat of *Nepenthes* plant, *Nepenthes* species, pitcher age, pitcher type (upper or lower pitchers), types of prey captured, plant secretion and pitcher fluid acidity (Chou et al., 2014; Kanokratana et al., 2016; Sickel et al., 2016; Takeuchi et al., 2015). Study by Kanokratana et al. (2016) showed that the bacterial diversity was higher in less acidic pitcher fluid of *N. ampullaria*, *N. andamana*, *N. gracilis*, *N. mirabilis* var *globosa*, *N. mirabilis* var *mirabilis*, *N. smilesii* and *N. suratensis*. No correlation was found between bacterial composition and the sampling location and *Nepenthes* species (Kanokratana et al., 2016; Takeuchi et al., 2015). To date, majority of the factors mentioned are not

experimentally tested. Hence, the influence of the biotic and abiotic factors on bacterial community compostition is not fully discovered.

2.11 Hydrolytic Enzyme Produces by Bacteria in *Nepenthes* Pitcher Fluid

Bacterial protease activity was detected from *Nepenthes* pitcher fluid and bacteria isolated from the pitcher fluid of *Nepenthes hybrida* (Higashi et al., 1993; Lüttge, 1964). The bacterial protease and caseinase activities degrade amino acid into ammonium cation which is a preferred nitrogen species of *Nepenthes* plant (Adlassnig et al., 2010; Higashi et al., 1993; Lüttge, 1964). The proteolytic activities in the pitcher fluid of *N. hybrida* is optimum at acidic (pH3) condition, and the proteolytic activities of it's bacterial inquiline is optimum at neutral and alkaline (pH7 to pH9) condition (Higashi et al., 1993). The differences in pH optima between bacterial and plant proteases have suggested the cooperation between the organisms by acting on different substrate and provides different nutrient to their collaborators.

Two new bacterial lipases, Lip1 and Lip2, were identified through lipase activity screening of a clonal library created from *Nepenthes* microbial community. The pH optima of Lip1 and Lip2 activities are pH5 and pH6, respectively (Morohoshi et al., 2011). In addition to lipase activity, Lip2 also showed esterase activity, which hydrolysed *p*-nitrophenyl palmitate and *p*-nitrophenyl butyrate (Morohoshi et al., 2011). Phylogenetic analysised using the amino acid sequence of Lip1 and Lip2 showed that Lip1 was clustered into lipase family IV and Lip2 belongs to a novel lipase family, hitherto (Morohoshi et al., 2011).

To date, bacterial protease, caseinase, lipase, putative bacterial β-$_D$-glucosidase and putative bacterial β-$_D$-glucosaminidase activities were detected from *Nepenthes* pitcher fluid and the culture medium of bacterial isolated from the *Nepenthes* pitcher fluid (Adlassnig et al., 2010; Hepburn, 1918; Higashi et al., 1993; Morohoshi et al., 2011; Takeuchi et al., 2011).

2.12 Bacterial 16S rRNA Gene

The 16S rRNA gene encodes for a component of the bacterial small subunit ribosome (Huang et al., 2009b). The bacterial 16S rRNA genes was used as an universal molecular marker for bacterial phylogenetic relationship studies, new species identification and bacterial diversity studies (Case et al., 2007; Janda & Abbott, 2007; Srinivasan et al., 2015; Wang & Qian, 2009; Weisburg et al., 1991; Woo et al., 2008). It is chosen as the molecular tool for bacterial identification because (i) there is at least a copy of 16S rRNA gene in almost all bacterial genome; (ii) the sequence diversity due to the random changes in nucleotide sequence during evolution and (iii) the length of 16S rRNA gene sequence (approximately 1500 bps) is sufficent for informatics purposes (Clarridge, 2004; Janda & Abbott, 2007; Patel, 2001; Woese, 1987; Woese & Fox, 1977).

Bacterial 16S rRNA gene contains 9 hypervariable regions (V1 to V9) (Chakravorty et al., 2007). The variants of 16S rRNA hypervariable regions are results of different evolutionary rate across bacteria species (Peer et al., 1996). These variants provide considerable amount of sequence diversity for species identification (Chakravorty et al., 2007; Peer et al., 1996; Wang & Qian, 2009). Assessments on bacterial 16S rRNA hypervariable regions suggest that V1 through V6 regions (especially V3 and V6 regions) are suitable for distinguishing bacterial up to species level, except closely

related *Enterobacteriaceae* (Cai et al., 2013; Chakravorty et al., 2007; Claesson et al., 2010; Kim et al., 2011; Mizrahi-Man et al., 2013; Yang et al., 2016). Hypervariable regions V7, V8 and V9 are less suitable for bacterial identification (Chakravorty et al., 2007; Yang et al., 2016).

Bacterial 16S rRNA gene was widely adopted in bacterial identification since the invention of polymerase chain reaction (PCR) and Sanger sequencing (Bartlett & Stirling, 2003; Janda & Abbott, 2007; Patel, 2001; Wang & Qian, 2009; Woo et al., 2008). Primers for bacterial 16S rRNA genes amplification were designed using the conserved regions flanking the hypervariable region(s) (Chakravorty et al., 2007; Clarridge, 2004; Wang & Qian, 2009). This method had increased the efficiency and accuracy of bacterial identification, enabled rapid identification of fastidious bacterial and aided in identifying bacterial that does not fit into any recognized biochemical profiles (Janda & Abbott, 2007; Lagier et al., 2015; O'Neill et al., 1992).

Bacterial 16S rRNA gene was also applied in metagenome projects for microbial ecological studies (Handelsman, 2004; Rondon et al., 2000; Wang & Qian, 2009). Since the invention of NGS, sequencing of massive amount of 16S rRNA gene sequences has opened the gateway for comprehensive microbial ecological studies (Bartram et al., 2011; Burns et al., 2016; Kim et al., 2011; Mwaikono et al., 2016; Połka et al., 2015; Tamaki et al., 2011). However, only selected hypervariable regions are amplified and sequenced due to the limitation of the NGS platforms. These 16S rRNA gene sequences are compared to the database for microbial community composition profiling. To date, multiple bacterial 16S rRNA gene databases, for example SILVA, Ribosomal Database Project (RDP), National Center for Biotechnology Information (NCBI) 16S Microbial, Greengenes, EzTaxon and Genomes OnLine Database (GOLD) are built from the

deposited data (Chun et al., 2007; DeSantis et al., 2006; Liolios et al., 2007; Maidak et al., 1997; Quast et al., 2012).

2.13 Bacterial Identification by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Biotyper

MALDI-TOF MS deploys short nitrogen laser pulse for soft ionization mechanism to ionized large molecules (Lagier et al., 2015; Sandle, 2015). The mass-to-charge ratio of the ion is measured based on the velocity of the ion under vacuum condition (Hillenkamp et al., 1991; Sandle, 2015). The combination of MALDI-TOF MS technology and MALDI BioTyper software is applied in viral, bacterial, yeast and fungal identification (Fenselau & Demirev, 2001; Lagier et al., 2015; Lay, 2001; Sogawa et al., 2011).

The advantages of bacterial identification using MALDI-TOF MS BioTyper which includes rapid turnover, high accuracy, simple protocol and low operating cost had lead to the wide adoption of this bacterial identification technique in the current decade (Ferreira et al., 2010; Lagier et al., 2015; Maier et al., 2006). The bacterial whole cell protein fingerprint signature is generated by MALDI-TOF MS and compared with the reference spectra database in MALDI BioTyper software for bacterial identification (Fenselau & Demirev, 2001; Sogawa et al., 2011; Veloo et al., 2011). However, the size of spectra database in MALDI BioTyper software, resolution for closely related bacteria species, interferences cause by the presence of spores, sample-to-matrix ratio or culture medium are the challenges for bacterial identification using MALDI-TOF MS BioTyper (Fenselau & Demirev, 2001; Lagier et al., 2015; Pavlovic et al., 2012; Sandle, 2015; Werno et al., 2012).

2.14 Next Generation Sequencing (NGS)

NGS also known as high throughput sequencing, is a technology breakthrough in sequencing era which enables the parellel sequencing of massive amount of nucleotide sequences at lower cost (cost per base) and shorter time (Behjati & Tarpey, 2013; Marguerat et al., 2008; Metzker, 2010; Vincent et al., 2017). The rapid maturation of NGS on DNA sequencing technologies have revolutionized biological, epigenetics, clinical, microbiology and microbiome studies (Ansorge, 2009; MacLean et al., 2009; Shendure & Ji, 2008; Dijk et al., 2014; Vincent et al., 2017).

The 454 pyrosequencing system by Roche was the first commercially available NGS sequencer in the market (Ansorge, 2009; Margulies et al., 2006; Shendure & Ji, 2008). It deploys pyrosequencing technology which enable continuous DNA strand synthesis (also known as "sequencing by synthesis") (Mardis, 2013). The long read length (up to 1 kbps) has made 454 FLX system one of the most commonly used system in microbial diversity research (Bevilacqua et al., 2014; Cañas et al., 2014; Shokralla et al., 2012). Nonetheless, 454 FLX pyrosequencer is completely phase out at 2016 by Roche when other NGS platforms outperformed the system. On the other hand, Illumina is one of the leading companies in the NGS industry. Illumina NGS platforms deployed cyclic sequencing by synthesis principles (Liu et al., 2012; Mardis, 2008; Shendure & Ji, 2008). The fluorescence signals produce during nucleotide incorporation are captured by the CCD camera and process for basecalling (Reuter et al., 2015). To date, the massive output and low error rate are the advantages of the Illumina NGS platforms. Other flagship NGS platforms include Ion Torrent™ by ThermoFisher Scientific which employed semiconductor chip based sequencing and PacBio System by Pacific Biosciences which employed single-molecule real time (SMRT) sequencing technology

(Eid et al., 2009; Metzker, 2010; Rhoads & Au, 2015; Merriman et al., 2012; Quail et al., 2012; Van Dijk et al., 2014).

2.15 History and Application of Metagenomic

The prefix "meta-" indicates the collection of similar but not identical objects and genomic refers to the study of genomes (Handelsman, 2004; Tringe & Rubin, 2005). Hence, metagenome refers to the total genetic material retrieved from sampled environment and metagenomic refers to the genomic study on metagenome sample (Handelsman, 2004; Marchesi & Ravel, 2015; Tringe & Rubin, 2005). The termed metagenome was first used by Jo Handelsman and colleagues in 1998, in their publication which described the functional analysis of the collective genome from soil microflora (Handelsman et al., 1998).

Microbiology studies using pure cultures often hit a bottleneck where large proportion of bacteria were viable but not readily culturable in the laboratory (Gilbert & Dupont, 2011; Handelsman, 2004; Hugenholtz & Tyson, 2008; Kell et al., 1998; Pace, 1997; Torsvik et al., 1990; Torsvik & Øvreås, 2002; Tringe & Rubin, 2005). The bacterial counts obtained from viable plate count and microscopic counting could differed by several magnitude (Kogure et al., 1979; Staley & Konopka, 1985; Torsvik et al., 1996). Investigations on bacterial diversity in soil, marine and aquatic environments showed that less than 1% of bacteria was readily cultivable in the laboratory (Amann et al., 1995; Connon & Giovannoni, 2002; Handelsman et al., 1998; Hugenholtz, 2002; Staley & Konopka, 1985; Torsvik et al., 1996). Metagenomic, which utilizes culture independent approaches, overcomed this barrier and enabled the genomics studies of both cultivable and uncultivated microorganisms (Schloss & Handelsman, 2005).

Recent metagenome studies are divided into targeted and shotgun metagenomic approaches (Gabor et al., 2007; Gilbert & Dupont, 2011; Sleator et al., 2008). Present studies by targeted metagenomic approach involved the amplification of gene of interest from metagenome DNA samples, followed by amplicons sequencing and data analysis (Gabor et al., 2007; Gilbert & Dupont, 2011; Sleator et al., 2008; Suenaga, 2012). The bacterial diversity studies using 16S rRNA gene is an example of targeted metagenomic (Suenaga, 2012). To date, targeted metagenomic using 16S rRNA gene have characterized the microbial community in wide range of environments, for example human and animal guts, carnivorous plants, extreme environments, soil and aquatic environments (Chan et al., 2016; D'Argenio & Salvatore, 2015; Dave et al., 2012; Hou et al., 2013; Kanokratana et al., 2016; Kim et al., 2014; Koopman & Carstens, 2011; Korehi et al., 2014; Oulas et al., 2015; Shaufi et al., 2015; Shelomi et al., 2013; Shokralla et al., 2012; Sickel et al., 2016; Sleator et al., 2008).

On the other hand, shotgun metagenomic involved the directed sequencing of libraries created from metagenome samples (Gilbert & Dupont, 2011; Guo et al., 2016; Sharpton, 2014). Unlike targeted metagenome which only reveals the gene of interest, shotgun metagenome provides an insight to all gene composition in the sampled community (Gilbert & Dupont, 2011; Sharpton, 2014). Thus, microbial community study using shotgun metagenomic approach reveals both microbial assemblage and the genes presences in the metagenome sample (Sharpton, 2014). However, deeper sequencing coverage and higher sequencing cost are required in order to generate sufficient data for the shotgun metagenome data analysis compared to targeted metagenome (Peabody et al., 2015; Prakash & Taylor, 2012; Sharpton, 2014). In addition, higher computing power is needed for the data analysis (Scholz et al., 2012; Sharpton, 2014).

CHAPTER 3: METHODOLOGY

3.1 Materials

3.1.1 Sterilization

Buffers, culture media, agar base and ultrapure water were sterilized by autoclaving at 121 °C, 15 psi for 15 min using autoclave machine (HVE-50 Hirayama, Japan) before used. Glass utensils, gauzes and toothpick were sterilized by autoclaving at 121 °C, 15 psi for 20 min using autoclave machine (HVE-50 Hirayama, Japan) before used. Skim milk was filtered sterilized using syringe filter (Sartorius, Germany)(pore size of 0.22 μm) before use.

3.1.2 Chemicals and Solvents

Chemicals used in this project were purchased from Sigma, USA; Merck, Germany; Amresco, USA; BDH Ltd. UK and BD Difco™ Laboratories, USA. Bacteriological agar was purchased from Scharlau, Spain; Merck, Germany; BDH Ltd. UK and BD Difco™ Laboratories. Solvents used in this project were purchased from Fisher Scientific, UK and Merck, Germany.

3.1.3 Buffers

3.1.3.1 Lysis Buffer

Cell lysis buffer for DNA extraction contains 0.1 M Tris-hydrochloride (Tris-HCl) (15.76 g/L), 0.1 M ethylenediaminetetraacetic (EDTA) (29.22 g/L), 0.1 M dipotassium phosphate (K_2HPO_4) (17.42 g/L), 1.5 M sodium chloride (NaCl) (87.66 g/L), 0.035 M sodium dodecyl sulfate (SDS) (10.00 g/L) and 0.027 M cetyltrimethylammonium bromide (CTAB) (10.00 g/L) in 1 L distilled water. The pH of the lysis buffer was adjusted to pH8 using 1 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH)

before autoclave. The lysis buffer was sterilized by autoclaving at 121 °C, 15 psi for 15 min using autoclave machine (HVE-50 Hirayama, Japan).

3.1.3.2 Tris-Borate Ethylenediaminetetraacetic (TBE) Buffer

Five times concentrated TBE buffer contains 0.01 M EDTA (2.92 g/L), 0.45 M of boric acid (H_3BO_3) (27.50 g/L) and 0.45 M Tris-base (54.00 g/L) in 1 L distilled water. The pH of TBE buffer was adjusted to pH8.5 using 1 M HCl and 1 M NaOH before autoclave. The 5 times concentrated TBE buffer was sterilized by autoclaving at 121 °C, 15 psi for 15 min. Five times concentrated TBE buffer was diluted to 1 time TBE buffer at the ratio of 4 volumns of sterilized distilled water to 1 volumn of 5 times concentrated TBE buffer.

3.1.3.3 Phosphate Buffered Saline (PBS)

One time PBS contains 1.9 mM sodium phosphate monobasic (NaH_2PO_4) (0.23 g/L), 8 mM sodium phosphate dibasic (Na_2HPO_4) (1.15 g/L) and 0.15 M NaCl (8.76 g/L) in 1 L distilled water. The pH of the PBS was adjusted to pH6.5 using 1 M HCl and 1 M NaOH before autoclave. The PBS was sterilized by autoclaving at 121 °C, 15 psi for 15 min.

3.1.3.4 Phosphate Buffer

The 0.2 M phosphate buffer was prepared for scanning electron microscope (SEM) sample preparation. The 0.2 M phosphate buffer contains 0.14 M Na_2HPO_4 (20.44 g/L) and 0.056 M NaH_2PO_4 (6.72 g/L) in 1 L distilled water. Phosphate buffer was adjusted to pH7.2 using 1 M HCl and 1 M NaOH before autoclave. Phosphate buffer was sterilized by autoclaving at 121 °C, 15 psi for 15 min. The 0.2 M phosphate buffer was diluted to 0.1 M phosphate buffer using 1 volume of ultrapure water before used.

3.1.3.5 Elution Buffer

DNA was eluted using Buffer EB purchased from Qiagen (Qiagen, USA).

3.1.4 Culture Media

3.1.4.1 Luria Bertani (LB) Medium

LB medium contains 1.0% (w/v) NaCl, 1.0% (w/v) peptone and 0.5% (w/v) yeast extract in 1 L distilled water. Bacteriological agar 1.5% (w/v) was used as the solidifying agent. The pH of the LB medium was adjusted to pH6.5 using 1 M HCl and 1 M NaOH before autoclave. LB medium was sterilized by autoclaving at 121 °C, 15 psi for 15 min.

3.1.4.2 Skim Milk Agar

Skim milk agar for proteolytic assay contains 0.05% (w/v) yeast extract, 0.1% (w/v) tryptone, 1.0% (w/v) NaCl, and 1.5% (v/v) skim milk in 1 L distilled water. Bacteriological agar 1.5% (w/v) was used as the solidifying agent. Medium without skim milk was sterilized by autoclaving at 121 °C, 15 psi for 15 min. Filter sterilized skim milk was added into the autoclaved medium before used.

3.1.4.3 Starch Agar

Starch agar contains 0.2% (w/v) yeast extract, 0.5% (w/v) tryptone, 0.5% (w/v) NaCl and 0.5% (w/v) soluble starch in 1 L distilled water. Bacteriological agar 1.5% (w/v) was used as the solidifying agent. Starch agar was sterilized by autoclaving at 121 °C, 15 psi for 15 min.

3.1.4.4 Cellulose Agar

Bacterial cellulolytic activity was screened using cellulose agar consisted of basal salt medium supplied with 0.15% (w/v) microcrystalline cellulose. The cellulose agar was adjusted to pH7.0 using 1 M HCl and 1 M NaOH before autoclave. Basal salt medium contains 0.01% (w/v) NaCl, 0.01% (w/v) ammonium sulfate ($(NH_4)_2SO_4$), 0.001% (w/v) calcium chloride dihydrate ($CaCl_2.2H_2O$), 0.0001% (w/v) magnesium chloride heptahydrate ($MgCl_2.7H_2O$), 0.0001% (w/v) zinc sulfate heptahydrate ($ZnSO_4.7H_2O$), 0.0001% (w/v) cobalt chloride monohydrate ($CoCl_2.H_2O$), 0.0005% (w/v) manganese sulfate monohydrate ($MnSO_4.H_2O$), 0.002% (w/v) Iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$), 0.001% (w/v) H_3BO_3, 0.0001% (w/v) aluminium potassium sulfate dodecahydrate ($KAl(SO_4)_2.12H_2O$), 0.0001% (w/v) sodium molybdate ($Na_2MoO_4.2H_2O$), 0.0001% (w/v) copper sulfate ($CuSO_4.5H_2O$), 0.05% (w/v) K_2HPO_4, 0.01% (w/v) potassium dihydrogen phosphate (KH_2PO_4), and 0.2% (w/v) gelatin in 1 L distilled water. Cellulose agar was stained red using 0.02% (w/v) congo red. Bacteriological agar 1.5% (w/v) was used as the solidifying agent. Starch agar was sterilized by autoclaving at 121 °C, 15 psi for 15 min.

3.2 Methods

3.2.1 Sample Collection

Pitcher fluids of *N. sanguinea* were collected from the opened pitchers of wild *N. sanguinea* plants at Cameron Highlands, Pahang, Peninsular Malaysia and Fraser's Hill, Pahang, Peninsular Malaysia. The pitcher fluids of *N. sanguinea* plants were sampled at different season and sites (Table 3.1). Pitcher fluid of *N. hookeriana* was collected from the opened pitcher of *N. hookeriana* plant that grows in a shaded plant nursery at D' Paradise Park, Malacca, Peninsular Malaysia. The *N. hookeriana* plant in the plant nursery was exposed to local environment. Pitcher fluids of *N. albomarginata* were

collected from the opened pitchers of 2 *N. albomarginata* that were native to Penang Hill, Penang Island, Peninsular Malaysia, in Monkey Cup Garden (MCG). The *N. albomarginata* plants were grow adjacent to each other in the opened-air garden and exposed to local environment. Pitcher fluids of *N. gracilis* were collected from the opened pitchers of wild *N. gracilis* plants at University of Malaya, Kuala Lumpur, Peninsular Malaysia. The identity of the *Nepenthes* plants were identified based on the pitcher's morphologies.

Pitcher fluids were poured into the sterile glass bottles or sterile polypropylene tubes. The samples were transported to laboratory within 24 hours after collection. The pH value of the pitcher fluids were measured using pH strips, The pH value, volume of each *Nepenthes* pitcher fluid, *Nepenthes* species and plant individual that carry the pitcher (hereafter refer as "plant"), sampling site's GPS coordination, sampling locations, sampling dates and the altitude of sampling sites were recorded (Table 3.1).

Table 3.1: *Nepenthes* sample metadata. The records of sampling site, geographical information, *Nepenthes* plant species and pitcher fluid information.

Sample	GPS Coordinates	Location	Altitude (m)	Fluid pH	Fluid Volume (mL)	Plant	Sampling Date	*Nepenthes* species
H1	04°31'19.1"N, 101°22'55.6"E	Cameron Highlands	1,970	6	35	G	24th May 2010	*N. sanguinea*
LK	02°24'58.9"N, 102°07'49.4"E	D' Paradise Park	29	5	40	H	6th July 2010	*N. hookeriana*
C2	04°31'28.0"N, 101°23'20.1"E	Cameron Highlands	1,770	3	20	C	22nd Nov 2011	*N. sanguinea*
C4	04°31'28.0"N, 101°23'20.1"E	Cameron Highlands	1,770	3	5	C	22nd Nov 2011	*N. sanguinea*
FR1	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	5	25	E	26th Feb 2012	*N. sanguinea*
FR2	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	5	25	E	26th Feb 2012	*N. sanguinea*
FR3	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	7	7	E	26th Feb 2012	*N. sanguinea*
FR4	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	5	50	E	26th Feb 2012	*N. sanguinea*
FR5	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	2	10	E	26th Feb 2012	*N. sanguinea*
FR7	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	7	10	E	26th Feb 2012	*N. sanguinea*
FR8	03°42'53.2"N, 101°44'25.1"E	Fraser's Hill	1,222	7	15	E	26th Feb 2012	*N. sanguinea*
N1	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	2	5	F	17th Jun 2012	*N. sanguinea*
N2	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	2	4	F	17th Jun 2012	*N. sanguinea*
N3	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	1	3	F	17th Jun 2012	*N. sanguinea*
N4	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	1	7	F	17th Jun 2012	*N. sanguinea*
N5	03°42'53.2"N, 101°44'24.7"E	Fraser's Hill	1,212	2	3	F	17th Jun 2012	*N. sanguinea*
N7	03°43'07.4"N, 101°44'43.6"E	Fraser's Hill	1,179	3	3	F	17th Jun 2012	*N. sanguinea*
FH1	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	3	17	D	16th Mar 2013	*N. sanguinea*
FH2	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	5	10	D	16th Mar 2013	*N. sanguinea*
FH3	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	5	6	D	16th Mar 2013	*N. sanguinea*
FH6	03°42'53.5"N, 101°44'25.1"E	Fraser's Hill	1,219	3	10	D	16th Mar 2013	*N. sanguinea*
BB1	05°25'00.5"N, 100°15'39.4"E	Penang Hill	733	4	12	A	5th May 2013	*N. albomarginata*
BB2	05°25'00.5"N, 100°15'39.4"E	Penang Hill	733	2	6	A	5th May 2013	*N. albomarginata*
BB3	05°25'00.5"N, 100°15'39.4"E	Penang Hill	733	4	8	A	5th May 2013	*N. albomarginata*
BB4	05°25'00.5"N, 100°15'39.4"E	Penang Hill	733	5	16	A	5th May 2013	*N. albomarginata*
BB5	05°25'00.5"N, 100°15'39.4"E	Penang Hill	733	4	7	A	5th May 2013	*N. albomarginata*
BB6	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	10	B	5th May 2013	*N. albomarginata*
BB7	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	12	B	5th May 2013	*N. albomarginata*
BB9	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	10	B	5th May 2013	*N. albomarginata*

Table 3.1, continued.

Sample	GPS Coordinates	Location	Altitude (m)	Fluid pH	Fluid Volume (mL)	Plant	Sampling Date	*Nepenthes* species
BB10	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	14	B	5th May 2013	*N. albomarginata*
BB11	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	25	B	5th May 2013	*N. albomarginata*
BB12	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	17	B	5th May 2013	*N. albomarginata*
BB13	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	4	B	5th May 2013	*N. albomarginata*
BB14	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	22	B	5th May 2013	*N. albomarginata*
BB15	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	20	B	5th May 2013	*N. albomarginata*
BB16	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	40	B	5th May 2013	*N. albomarginata*
BB17	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	15	B	5th May 2013	*N. albomarginata*
BB18	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	3	22	B	5th May 2013	*N. albomarginata*
BB19	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	9	B	5th May 2013	*N. albomarginata*
BB20	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	5	50	B	5th May 2013	*N. albomarginata*
BB21	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	25	B	5th May 2013	*N. albomarginata*
BB22	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	25	B	5th May 2013	*N. albomarginata*
BB23	05°25'01.5"N, 100°15'40.1"E	Penang Hill	746	6	23	B	5th May 2013	*N. albomarginata*
BKT5.2	03°7'49.0"N, 101°39'05.0"E	University of Malaya	81	3	5	I	1st Sept 2016	*N. gracilis*
BKT6.2	03°7'49.0"N, 101°39'05.0"E	University of Malaya	81	4	15	I	1st Sept 2016	*N. gracilis*
BKT9	03°7'49.0"N, 101°39'06.0"E	University of Malaya	80	3	3	I	24th Aug 2016	*N. gracilis*
BKT19	03°7'49.0"N, 101°39'08.0"E	University of Malaya	90	2	3	I	25th Aug 2016	*N. gracilis*

3.2.2 DNA Extraction from Pitcher Fluids

Large particles includes insect's carcass and plant debris were filtered from *Nepenthes* pitcher fluids using sterile gauze. Cells were pelleted from *Nepenthes* pitcher fluid by centrifuged at 14,000 × *g* for 10 min. Total genomic DNA was extracted from pelleted cells using modified CTAB DNA extraction method (Manjula et al., 2011; Murray & Thompson, 1980; Zhou et al., 1996). One milliliter of lysis buffer was added to the pelleted cells and homogenised by vortexing. Cells were enzymatically lysed using 100 µg/mL lysozyme (Sigma, USA), incubated at 37 °C for 30 min. Subsequently, the DNA was treated using 40 µg/mL Proteinase K (Qiagen, Germany) and incubated at 56 °C for 30 min. RNA was removed from the metagenome samples using 1 µg/mL RNase A (Qiagen, Germany) and incubated at 37 °C for 60 min.

Samples H1 and LK were purified using phenol/chloroform/isoamyl alcohol mixture (25:24:1). Washed DNA was precipitated using 70% (v/v) isopropanol. The precipitated DNA was washed twice using 70% (v/v) ethanol (Wilson, 1987). DNA pellet was evaporated to dryness at room temperature and rehydrated using 35 µL Buffer EB. The DNA samples were kept in -20 °C.

Samples (except samples H1 and LK) were purified using DNA clean and concentrator™ kit (Zymo Research, USA) as described by manufacture. Two volumes of DNA Binding Buffer was mixed with 1 volumn of DNA sample. The solution was transferred into Zymo-Spin™ Column and centrifuged at 15,000 × *g* for 30 sec. Subsequently, DNA was washed twice-using 200 µL DNA wash buffer. The purified DNA was eluted using 50 µL Buffer EB. The DNA samples were kept in -20 °C. Samples that yielded at least 1 µg of pure DNA were preceded to shotgun metagenome sequencing.

3.2.3 DNA Quantity and Quality Assessment

Purified DNA was quantified using dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, USA) on Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) (Chan et al., 2013). The purity of the DNA was assessed using and NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) (Chan et al., 2013). Acceptable DNA sample has $A_{260/280}$ ratio of 1.8 to 2.0 and $A_{260/230}$ ratio of 2.0 to 2.3. The DNA intactness and size of the amplicons were assessed by agarose gel electrophoresis (AGE) as described in section 3.2.4.

3.2.4 Agarose Gel Electrophoresis (AGE)

AGE was performed using 1.0% (w/v) agarose gel. Agarose gel was submerged in 1LisX TBE buffer. The AGE was performed at condition: 80 V, 400 mA and 40 min. The agarose gel was pre-stained using 1X nucleic acid stain (Lonza, USA). The stained agarose gel was viewed under the ultraviolet illuminator (UVP, USA). The size of the DNA was estimated by referring to 1 kb DNA ladder (Fermentas, USA).

3.2.5 Amplification of Bacterial 16S rRNA Genes

3.2.5.1 454 GS-FLX Sequencing Library

Bacterial 16S rRNA genes were amplified from metagenome samples H1 and LK using i-Taq DNA Polymerase PCR reagents (iNtRON Biotechnology, Korea). Each PCR reaction mix contains 1X PCR buffer, 800 µM dNTP mix, 20 mM magnesium chloride ($MgCl_2$), 10 µM forward primer, 10 µM reverse primer, 1 unit taq-polymerase and 1 ng genomic DNA. Each reaction mix was topped up to 15 µL using sterilized ultrapure water (Mili-Q Merck, USA). The forward primer and reverse primer used for bacterial 16S rRNA gene amplification were listed in Table 3.2. Primers were purchased from Integrated DNA Technologies.

The bacterial 16S rRNA genes were amplified at PCR condition of one cycle pre-denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C to 65 °C for 20 sec, and elongation at 72 °C for 30 sec followed by one cycle of final extension at 72 °C for 7 min. The gradient annealing temperature ranged from 55 °C to 65 °C with 1 °C interval. The amplicons were quantified as described in section 3.2.3. The sizes of the amplicons were assessed using AGE as described in section 3.2.4. The sequencing libraries were sequenced using 454 GS-FLX Titanium platform as described in section 3.2.9.1.

Table 3.2: Primers used in bacterial 16S rRNA gene amplification for NGS using 454 GS-FLX system.

Name	Primer Sequence	Reference
MIDI_530'F	ACGAGTGCGTGTGCCAGCMGCNGCGG	(Chan et al., 2016)
MIDI_1100mod'R	ACGAGTGCGTGGGTTNCGNTCGTTRC	(Chan et al., 2016)
MID2_530'F	ACGCTCGACAGTGCCAGCMGCNGCGG	Designed for this study
MID2_1100mod'R	ACGCTCGACAGGGTTNCGNTCGTTRC	Designed for this study

* 454 GS-GLX system MID barcode sequences were written as underlined capital letters.
* Primer sequences for bacterial 16S rRNA gene V3 to V6 hypervariable regions amplification was written as capital letter without underline.

3.2.5.2 MiSeq Sequencing Library

Bacterial 16S rRNA genes were amplified from samples (except samples H1, LK, FR5, N1, N3, N4, N5, BKT5.2, BKT6.2, BKT9 and BKT19) using KAPA HiFi HotStart ReadyMixPCRkit (KAPA Biosystem, USA). Each PCR reaction mix contains 1X KAPA Hifi Hotstart Ready Mix (KAPA Biosystem, USA), 0.3 μM forward primer, 0.3 μM reverse primer and 10 ng metagenome DNA. Each reaction mix was top up to 25 μL using sterilized ultrapure water. The forward primer and reverse primers used for bacterial 16S rRNA gene amplification were as listed in Table 3.3. Primers were purchased from Integrated DNA Technologies. Adapter sequences and indexes were added to the bacterial 16S rRNA amplicons during the gene amplification.

Bacterial 16S rRNA genes were amplified under the PCR condition of one cycle pre-denaturation at 95 °C for 5 min, 20 cycles of denaturation at 98 °C for 20 sec, annealing at 63 °C for 15 sec, and elongation at 72 °C for 5 sec followed by one cycle of final extension at 72 °C for 5 min. The amplicons (hereafter refer as MiSeq amplicon sequencing libraries) were purified using QIAquick gel extraction kit (Qiagen, Germany) as described in section 3.2.6. The size distribution and quantity of the MiSeq amplicon sequencing libraries were assessed as described in section 3.2.7. The MiSeq amplicon sequencing libraries were sequenced as described in section 3.2.9.2.

3.2.5.3 Bacterial 16S rRNA Gene Amplification from Bacterial Genome

Bacterial 16S rRNA gene was amplified from the bacterial genomic DNA using i-Taq DNA Polymerase PCR reagents (iNtRON Biotechnology, Korea). Each PCR reaction mix contains 1X PCR buffer, 800 µM dNTP mix, 20 mM $MgCl_2$, 10 µM forward primer, 10 µM reverse primer, 1 unit taq-polymerase and 1 ng genomic DNA. Each reaction mix was topped up to 15 µL using sterilized ultrapure water (Mili-Q Merck, USA). Primers (27F and 1525R) and internal primers (338F, 515F and 1174F) used in Sanger sequencing of bacterial 16S rRNA gene were listed in Table 3.4. Primers were purchased from Integrated DNA Technologies.

The PCR was performed at the following condition: 1 cycle of denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 63 °C for 30 sec and extension at 72 °C for 90 sec, with 1 cycle of final extension at 72 °C for 7 min. The amplicon was purified from agarose gel as described in section 3.2.6. Purified DNA was quantified as described in section 3.2.3. The sizes of the amplicons were assessed using AGE as described in section 3.2.4. The purified amplicon was sent to 1st Base Laboratories Sdn. Bhd. for Sanger sequencing service.

Table 3.3: Primers used in bacterial 16S rDNA gene amplification for NGS using MiSeq platform.

Name	Primer Sequence	Reference
V3_F	aatcatacggcgaccaccgagatctACACTCTTTCCCTACACG ACGCTCTTCCGATCTNNNNCCTACGGGAGGCAG CAG	(Bartram et al., 2011)
V3_1R	caagcagaagacggcatacgagat**CGTGAT**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_2R	caagcagaagacggcatacgagat**ACATCGG**TGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_3R	caagcagaagacggcatacgagat**GCCTAAG**TGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_4R	caagcagaagacggcatacgagat**TGGTCA**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_5R	caagcagaagacggcatacgagat**CACTGTG**TGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_6R	caagcagaagacggcatacgagat**ATTGGC**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_7R	caagcagaagacggcatacgagat**GATCTG**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_8R	caagcagaagacggcatacgagat**TCAAGT**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_9R	caagcagaagacggcatacgagat**CTGATCG**TGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_10R	caagcagaagacggcatacgagat**AAGCTA**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)
V3_11R	caagcagaagacggcatacgagat**GTAGCC**GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCTATTACCGCGGC TGCTGG	(Bartram et al., 2011)

* Adapter sequences for MiSeq platform were written as lower case letters without underline.
* Barcode sequences for sample multiplexing were written as bolded capital letters without underline.
* MiSeq sequencing primer binding sites were written as capital letters without underline.
* Primer sequences for bacterial 16S rRNA V3 hypervariable region amplification was written as underlined capital letters.

Table 3.4: Primers for bacterial 16S rRNA gene amplification and sequencing.

Name	Primer Sequence	Reference
27F	AGA GTT TGA TCM TGG CTC AG	(Chong et al., 2012)
338F	ACT CCT ACG GGA GGC AGC AG	(Zoetendal et al., 1998)
515F	GTG CCA GCM GCC GCG GTA A	(Budsberg et al., 2003)
1174F	GAG GAA GGT GGG GAT GAC GT	(Geerlings et al., 2001)
1525R	AAG GAG GTG WTC CAR CC	(Chong et al., 2012)

3.2.6 Amplicon Purification

Amplicon with desired size was purified from 1.0% (w/v) agarose gel after AGE using QIAquick PCR Purification Kit (Qiagen, Germany) according to manufacturer's instruction. In brief, 3 gel volumes of Buffer QG was added to the agarose gel slice and incubated at 50 °C for 10 min or until the agarose gel slice was fully dissolved. One gel volume of isopropanol was added into the solution. The solution was transferred into QIAquick spin column and centrifuged at 17,900 × *g* for 1 min. The flow through was discarded. Subsequently, 500 µL of Buffer QG was pipetted into QIAquick spin column and centrifuged at 17,900 × *g* for 1 min. DNA was washed using 750 µL Buffer PE and centrifuged at 17,900 × *g* for 1 min. Purified DNA was eluted using 50 µL of Buffer EB and kept at -20 °C.

3.2.7 Illumina Next Generation Sequencing Library Assessment

The size distributions of the purified sequencing libraries were assessed using High Sensitivity DNA kit (Agilent, USA) on Bioanalyzer 2100 (Agilent, USA).

The purified sequencing libraries were quantified using KAPA Library Quantification kit (KAPABiosystems, USA) on Eco™ Real-Time PCR system (Illumina, USA). DNA libraries were diluted 10,000 fold using Buffer EB supplied with 0.1 % (v/v) Tween 20. A master mix containing 1X KAPA SYBR FAST qPCR Master Mix and 1X Primer Mix was prepared using sterilized ultrapure water. For each reaction, 4 µL of DNA sample or a DNA standards (20 pM, 2 pM, 0.2 pM, 0.02 pM, 0.002 pM or 0.0002 pM) was added into 16 µL master mix solution. Each reaction was loaded into a well in the 48-well plate. The qPCR was performed at the following cycling protocol; one cycle pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30

sec and annealing at 60 °C for 45 sec, followed by melting curve analysis at 65 °C to 95 °C.

3.2.8 Shotgun Metagenomic Next Generation Sequencing Library Preparation

Shotgun metagenomics NGS libraries were prepared for samples FR5, N1, N3, N4, N5, BKT5.2, BKT6.2, BKT9 and BKT19 using TruSeq DNA PCR-Free library preparation kit (Illumina, USA). The NGS libraries were prepared according to low sample protocol. In brief, metagenome DNA (52.5 µL) were sheared using Covaris M220 (Thermo Fisher Scientific, USA). The fragmented DNA was cleaned using 80 µL Sample Purification Beads and eluted using 50 µL Buffer EB. The cleaned DNA was subjected to end-repair using 40 µL End Repair Mix 2 and incubated at 30 °C for 30 min. Subsequently, the end-repaired DNA was subjected to size selection using Sample Purification Beads. Large DNA fragments were removed from the end-repaired DNA using 160 µL diluted Sample Purification Beads. The Sample Purification Beads was diluted using PCR grade water with 1:1 dilution ratio. Small DNA fragments were removed from the end-repaired DNA using 30 µL of undiluted Sample Purification Beads. The 3' end of the DNA was adenylated using 12.5 µL A-Tailing Mix and incubated at 37 °C for 30 min, followed by incubations at 70 °C for 5 min and 4 °C for 5 min. Subsequently, 2.5 µL Ligation Mix 2 and 2.5 µL DNA Adapter Index were added to adenylated DNA. The solution was incubated at 30 °C for 10 min for adapter ligation. The adapter ligated DNA was cleaned using 42.5 µL Sample Purification Beads and eluted using 50 µL Buffer EB. The DNA was cleaned again using 50 µL Sample Purification Beads and eluted using 20 µL Buffer EB.

Prior to size distribution assessment using Bioanalyzer 2100 (Agilent, USA), 2 µL of sequencing libraries were diluted with 10 µL PCR grade water. The size distribution and quantity of the sequencing libraries were assessed as described in section 3.2.7.

3.2.9 Next Generation Sequencing (NGS)

3.2.9.1 454 GS-FLX

The bacterial 16S rRNA amplicons from samples H1 and LK were subjected to NGS using 454 GS-FLX Titanium platform (Roche, USA) by service provider, 1st Base Laboratory Sdn. Bhd..

3.2.9.2 MiSeq

NGS sequencing libraries were diluted to 4 nM with Buffer EB supplied with 0.1% (v/v) Tween 20. Equal volume of diluted sequencing libraries were pooled in a clean microcentrifuge tube. The pooled sequencing library was diluted to 2 nM with Buffer EB supplied with 0.1% (v/v) Tween 20. The pooled sequencing library was further diluted with buffer HT1 and loaded into MiSeq reagent cartridge. The NGS was performed on MiSeq platform (Illumina, USA).

3.2.9.3 HiSeq

Samples FR5, N1, N3, N4 and N5 were sent to ScienceVision Sdn. Bhd. for NGS using HiSeq 2000 platform (Illumina, USA).

Normalised NGS sequencing libraries of samples BKT5.2, BKT6.2, BKT9 and BKT19 were clustered into the 8-channel flow cell using cBot System (Illumina, USA). The 8-channel flow cell was loaded onto HiSeq 2500 platform. NGS was performed on high-output mode using HiSeq 2500 platform (Illumina, USA).

3.2.10 Metagenomic Raw Reads Quality Filtering

3.2.10.1 Amplicons from 454 GS-FLX

The quality of NGS raw reads were assessed using FastQC (Schmieder & Edwards, 2011). Quality trimming was performed using CLC Genomic workbench (version 7.5). Raw reads were trimmed at Phred Score Q20. DNA sequence shorter than 36 bps and ambiguous nucleotide (N) were discarded.

3.2.10.2 Amplicons from MiSeq

The quality of NGS raw reads were assessed using FastQC (Schmieder & Edwards, 2011). Paired-end reads generated by MiSeq were joined using fastq-join on Quantitative Insights Into Microbial Ecology (QIIME) (version 1.9.1) pipeline (Caporaso et al., 2010b). The overlapped region of the joined paired-end reads was at least 90% similarity. Quality trimming was performed using CLC Genomic Workbench (version 7.5). Nucleotide with Phred score lower than 30 and ambiguous nucleotide (N) were trimmed. DNA sequence shorter than 10 bps was discarded.

3.2.10.3 Shotgun Metagenomic Raw Reads

The qualities of NGS raw data for samples (samples FR5, N1, N3, N4, N5, BKT5.2, BKT 6.2, BKT9 and BKT19) were assessed using FastQC (Schmieder & Edwards, 2011). The raw data were trimmed using Trimmomatic (version 0.36) (Bolger et al., 2014). Sequences with Phred score lower than 33 and read length shorter than 36 bps were discarded from the dataset. The quality assessed paired-end reads and singled-end reads were assembled using Velvet (version 1.2.10) (Zerbino & Birney, 2008). The trimmed reads were assembled into contigs with at least 10X coverage and the read lengths of the contigs were at least 200 bps.

3.2.11 Chimeric Sequence Removal from Bacterial 16S rRNA Genes Sequences

Bacterial 16S rRNA gene sequences were binned from quality assessed paired-end reads in shotgun metagenomic samples (samples FR5, N1, N3, N4, N5, BKT5.2, BKT6.2, BKT9 and BKT19) using SortMeRNA (version 2.1) (Kopylova et al., 2012). Silva bacterial 16S rRNA sequence representative database (release 119) was used as reference database to bin bacterial 16S rRNA gene sequences from the shotgun metagenome samples.

Bacterial 16S rRNA sequences from all metagenomic samples were concatenated into an input file for QIIME using QIIME script "add_qiime_labels.py". Chimeric sequences were identified from samples using QIIME script "identify_chimeric_seqs.py" and USEARCH (version 7.0) (Edgar, 2015). The chimeric sequences were removed from samples using QIIME script "filter_fasta.py".

3.2.12 Bacterial Community Composition Analysis

Operational taxonomic unit (OTU) clustering, taxonomy assignment, phylogenetic tree construction were performed using QIIME (version 1.9.1) pipeline (Caporaso et al., 2010b). Non-chimeric sequences were clustered into OTU by QIIME open reference OTU picking protocols using QIIME script "pick_open_reference_otus.py" and UCLUST algorithm at 97.0% sequences similarity (Caporaso et al., 2010b; Edgar, 2010). Taxonomy was assigned to the representative sequences using Greengenes database (version August 2013). Representative sequences were aligned against Greengenes (version August 2013) core set with PyNAST (Caporaso et al., 2010a). Phylogenetic tree was constructed using Fast Tree tools on QIIME pipeline (Caporaso et al., 2010b; Price et al., 2009). Bacterial community composition was summarized using QIIME script "summarize_taxa_through_plots.py". OTU with relative abundance

less than 1% in the total dataset were filtered from the OTU table. The heatmap of bacterial relative abundance profile was plotted using Hclust2 (https://bitbucket.org/nsegata/hclust2). Bray-Curtis dissimilarity distance function was used for sample hierarchical clustering.

3.2.13 Sample Rarefaction and Alpha Diversity Analysis

OTU table rarefaction, alpha diversity analysis, and generation of rarefaction plots were performed using QIIME (version 1.9.1) pipeline (Caporaso et al., 2010b). OTU table rarefaction, alpha diversity metrics and alpha rarefaction plots were computed using QIIME script "alpha_rarefaction.py". OTU tables were rarefied to 46,435 sequences per sample prior to alpha diversity and beta diversity analysis. Percentage of the total bacterial species represented in the subsampled data was estimated using Good's coverage index. Chao1, observed OTUs, PD whole tree (phylogenetic diversity), Simpson's Index of Diversity (1-D) and Shannon were the alpha diversity indices used in bacterial species richness and diversity estimation.

3.2.14 Statistical Analysis

Beta diversity analysis was performed using Bray-Curtis dissimilarity, unweighted (qualitative measure) and weighted (quantitative measure) UniFrac matrices. Mantel statistic based on Pearson's product-moment correlation (9999 permutations) was used to study the correlation significance of microbial community dissimilarities and the pH of *Nepenthes* pitcher fluid. Permutational multivariate analysis of variance (PERMANOVA) (9999 permutations) was used to study the correlation significance of microbial diversity dissimilarities and a) the sampling location or b) the plants individual. P_{value} less than 0.05 were accepted as significant correlation between microbial diversity dissimilarity and the factor tested. Non-metric multidimensional

scaling (NMDS) analysis was performed on Bray-Curtis dissimilarity, unweighted UniFrac and weighted UniFrac distance matrices. NMDS and statistical tests were performed using R, VEGAN package (Dixon, 2003). NMDS plots by pH, sampling location and plant individual were constructed using R, VEGAN package (Dixon, 2003).

3.2.15 Eukaryote Composition

Eukaryote 18S rRNA gene sequences were binned from quality assessed reads of shotgun metagenomic samples FR5, N1, N3, N4, N5, BKT5.2, BKT6.2, BKT9 and BKT19 using SortMeRNA (version 2.1) (Kopylova et al., 2012). Silva eukaryote 18S rRNA gene sequence representative database (release 119) was used as reference database to bin eukaryote 18S rRNA gene sequences from shotgun metagenomic samples.

Eukaryotic 18S rRNA gene sequences were clustered into OTU by QIIME close reference OTU picking protocols using QIIME script "pick_closed_reference_otus.py" and UCLUST algorithm at 97% sequences similarity (Caporaso et al., 2010b; Edgar, 2010). Silva eukaryote 18S rRNA gene sequence representative database (release 128) was used as reference database for OTU picking. Taxonomy was assigned to the representative sequences using Silva 18S rRNA gene database (release 128). Eukaryote community composition was summarized using QIIME script "summarize_taxa_through_plots.py". OTU with relative abundance less than 0.5% in the total dataset were filtered from the OTU table. The heatmap of eukaryotic relative abundance profile was plotted using Hclust2 (https://bitbucket.org/nsegata/hclust2). Bray-Curtis dissimilarity distance function was used for sample hierarchical clustering.

3.2.16 Viral Community Composition

Virus community composition in *Nepenthes* pitcher fluid was characterized using metagenomic phylogenetic analysis 2 (MetaPhlAn2) (version 2.6.0) (Truong et al., 2015). The heatmap of virus relative abundance profile was plotted using Hclust2 (https://bitbucket.org/nsegata/hclust2). Bray-Curtis dissimilarity distance function was used for sample hierarchical clustering.

3.2.17 Shotgun Metagenomic Analysis

The contigs were subjected to gene prediction using MetaGeneMark (version 3.38) (Zhu et al., 2010). The predicted gene sequences were annotated using NCBI non-redundant (nr) database. The alignment was performed using Diamond (version 0.9.17) (Buchfink et al., 2015). Functional analyses were performed using MEGAN and InterPro2GO, SEED and eggNOG (version 6.10.8) databases (Huson et al., 2007). UPGMA (unweighted pair group method with arithmetic mean) tree was constructed using Bray-Curtis distance metrics by taxonomy profiles of the samples. Taxonomy profiles of the samples were extracted from the BLAST result of predicted gene sequences against NCBI-nr database. Data were normalized, compared and visualized using MEGAN (version 6.10.8) (Huson et al., 2007).

3.2.18 Bacterial Isolation

One hundred microlitre of pitcher fluid from sample H1 was spread on 3 LB agar plates and incubated at 28 °C for a day. Bacterial colonies with different morphologies were purified on LB agar plates. The bacterial cultures were maintained on LB medium (pH6.5) at 28 °C.

3.2.19 Scanning Electron Microscope (SEM)

Bacterial sample for observation using SEM was prepared using hexamethyldisilazane (HMDS) method described in user manual. Prior to SEM sample preparation, 25.0% (w/v) glutaraldehyde was diluted to 5.0% (v/v) using 0.1 M phosphate buffer (pH7.2). Bacterial cell was pelleted by centrifuged at 1,500 × *g* for 15 min. Cell pellet was resuspended in 5.0% (v/v) glutaraldehyde (fixation agent). The mixture was incubated at room temperature for at least 2 hrs. Cell was pelleted by centrifuged at 1,500 × *g* for 15 min and washed using 0.1 M phosphate buffer (pH7.2). The washed cell was subjected to post-fixation for 1 hr at room temperature in 1.0% (w/v) osmium tetroxide. The post-fixed cell was pelleted by centrifuged at 1,500 × *g* for 15 min and washed using distilled water. The post-fixed cell was subjected to serial dehydration process using 50.0% (v/v) ethanol, 75.0% (v/v) ethanol, 95.0% (v/v) ethanol, absolute ethanol and HMDS. At the end of dehydration process, HMDS was slowly decanted from the tube. Cell pellet was dried in dessicator at room temperature. Dried cell was mounted onto SEM specimen stub using doubled sided sticky tape. Specimen was coated with gold and the morphology of selected bacteria was observed using tabletop SEM (TM3030) (Hitachi, Germany).

3.2.20 Gram Stain

A drop of bacterial culture was smeared and heat fixed on glass slide. The smeared bacterial cell was flooded with crystal violet for 1 min and gently rinsed with water. Next, the bacterial cell was flooded with Gram's iodine for 1 min and gently rinsed with water. The stained cell was decolourized using acetone for 10 sec and immediately rinsed with water. Lastly, the bacterial cell was counter stained using safranin for 45 sec and gently rinsed with water. Gram stained slide was observed at 100X magnification with oil immersion, using light microscope.

3.2.21 Bacterial Genomic DNA Extraction and Purification

Prior to cell lysis, 1 mL of the overnight bacterial culture was pelleted by centrifuging for 1 min at 14,000 × *g*. The supernatant was discarded.

Genomic DNA was extracted and purified from bacteria cells as described by QIAamp DNA mini kit (Qiagen, Germany) manufacture user manual. Cell pellet was resuspended in 180 µL Buffer ATL. Cell pellet from Gram-positive bacterial cell culture was supplied with 20 mg/mL lysozyme and lysed by overnight incubation at 37 °C. Subsequently, 20 µl Proteinase K (20 µg/µL) and 200 µL Buffer AL was added to the solution. Proteinase K treatment was performed at 56 °C for 30 min. An additional incubation at 95 °C for 15 min was applied on samples from Gram-positive bacterial. RNase treatment was carried out using 4 µL RNase A (100 µg/µL) and incubated at room temperature for 2 min followed by incubation at 70 °C for 10 min. DNA was precipitated using 200 µL absolute ethanol. The mixture was loaded into QIAamp Spin Column and centrifuged at 6000 × *g* for 1 min. The DNA was washed using 500 µL Buffer AW1. Purified DNA was eluted from QIAamp Spin Column using 50 µL of Buffer EB. The quality and quantity of the DNA was assessed and measured as described in section 3.2.3. DNA was kept in -20 °C.

3.2.22 Bacterial Identification

3.2.22.1 Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

Standard solvent was prepared using 50.0% (v/v) Acetonitrile (ACN), 2.5% (v/v) trifluoroacetic acid (TFA), and 47.5% (v/v) distilled water. MALDI TOF MS matrix solution was prepared by adding 250 µL of standard solvent into the matrix crystals (Bruker, Germany) and vortex until the matrix crystal was fully dissolved.

Sample preparation for bacterial identification using MALDI-TOF MS was performed as described by direct transfer sample preparation procedure in the manufacture user manual. Minute amount of bacterial cell was picked from single bacterial colony using sterilized toothpick. Bacterial cell was smeared as thin film on a spot of MALDI-TOF MS target plate (MSP 96 target polished steel BC). Subsequently, 1 µL of MALDI-TOF MS matrix was overlaid on the bacterial cell and air-dried in fume hood at room temperature. The MALDI-TOF MS target plate was loaded into MALDI-TOF MS instrument (Bruker, Germany). MALDI-TOF MS was equipped with Bruker FlexControl software version 3.3 and Bruker MALDI Biotyper Real Time Classification (RTC) version 3.1. Bacterial cell protein was measured using MALDI-TOF MS. The spectrum generated was compared to the reference database for bacteria identification. Results with the log value (score) equal or higher than 2 indicates high confidence identification.

3.2.22.2 Bacterial 16S rRNA Gene Phylogenetic Analysis

Bacterial 16S rRNA gene was amplified from bacterial genomic DNA as described in section 3.2.5.3. Phylogenetic analysis was performed using MEGA (version 6) (Tamura et al., 2013). Bacterial 16S rRNA gene sequences were aligned using Cluster W algorithm. Taxonomy information was assigned to bacterial 16S rRNA gene sequences by comparing the sequences against NCBI 16S microbial blast database using BLAST. Phylogenetic trees were constructed using neighbor joining method. Bootstrap value was expressed as percentages of 1000 replicates in the phylogenetic tree. The nucleotide sequences of the 16S rRNA genes were deposited into GenBank.

3.2.23 Biocatalytic Activity Test

3.2.23.1 Proteolytic Activity

Bacterial proteolytic activity was screened using modified skim milk agar (5.0% (w/v)) (section 3.1.5.2) (Jo et al., 2008). Bacterial was cultured on skim milk agar and incubated at 28 °C for 24 hrs. Bacterial proteolytic activity resulted a clear halo around bacterial colony on the opaque agar.

3.2.23.2 Amylolytic Activity

Bacterial amylolytic activity was screened using modified starch agar (0.5% (w/v)) (section 3.1.4.3) (Hobson & Mann, 1955). Bacterial was cultured on starch agar and incubated at 28 °C for 24 hrs. The starch agar was flooded with iodine. Dark blue colour was observed on the starch agar plate with iodine due to the formation of iodine-starch complex. Starch utilization by bacterial amylolytic activity resulted yellow halo around bacterial colony on the dark blue background.

3.2.23.3 Cellulolytic Activity

Bacterial cellulolytic activity was tested using 0.15% (w/v) microcrystalline cellulose agar (section 3.1.4.4). Bacterial was cultured on starch agar and incubated at 28 °C for 120 hrs. Bacterial cellulolytic activity discolourized congo red forming light yellow halo around bacterial colony on the red background (Jo et al., 2010).

3.2.23.4 Chitinolytic Assay

Chitin utilization by bacterial β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase were measured using chitinase assay kit (Sigma, USA) as described by manufacture user manual. Substrate for β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase assays were 4-nitrophenyl *N*-acetyl-β-$_D$-glucosaminide, 4-nitrophenyl *N*,*N*'-diacetyl-β-$_D$-chitobioside and 4-nitrophenyl β-$_D$-*N*,*N*',*N*''-triacetylchitotriose, respectively. Chitinase from *Trichoderma viride* was used as positive control (hereafter refer as chitinase control enzyme). Prior to loading, chitinase control enzyme was diluted to 10 µg/mL using PBS (pH6.5) and substrates were adjusted to 1 mg/mL using assay buffer. Standard solution was prepared by diluting 10 mM p-nitrophenol solution to 0.05 mM using Stop solution. The volume of blank, standard solution, positive control, and sample loaded into each well of the microtitre plate were summarized in Table 3.5. Microtitre plate was incubated at 28 °C (bacterial isolated from *Nepenthes* pitcher fluid) or 37 °C (*E. coli* transformant with chitinase gene) for 24 hrs. Stop solution (200 µL) was added into blank, positive control and samples at 24 hrs. The yellow tint formed was measured at the absorption 405 nm using Infinite® M200 (Tecan, Switzerland). The amount of chito-oligosaccharides utilized by bacteria and chitinase control enzyme were calculated using the formula in Figure 3.1.

Table 3.5: Composition of blank, standard solution, positive control and sample loaded into each well of microtitre plate for β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase assays.

Composition	Substrate (1 mg/mL)	Sample	Standard
Blank	100 µL	N/A	N/A
Standard solution	N/A	N/A	300 µL
Positive Control	90 µL	10 µL of (10 µg/mL chitinase control enzyme)	N/A
Sample	90 µL	10 µL of bacterial culture	N/A

* N/A : Not available

$$\text{Units/ml} = \frac{(A_{405}\ \text{sample} - A_{405}\ \text{blank}) \times 0.05 \times 0.3 \times DF}{A_{405}\ \text{standard} \times \text{time} \times V_{enz}}$$

Legend

A_{405} sample	: absorbance reading of the sample at 405 nm
A_{405} blank	: absorbance reading of the blank at 405 nm
0.05	: µmole of p-nitrophenol in the standard solution
0.3 (mL)	: final volume of each well after addition of Stop Solution
DF	: dilution factor of chitinase enzyme or sample
A_{405} standard	: absorbance reading of the standard solution at 405 nm
Time	: time of incubation (min)

Figure 3.1: Formula for chito-oligosaccharides utilization calculation

3.2.23.5 Xylanolytic Assay

Bacterial xylanolytic assay was screened using EnzChek® Ultra Xylanse Assay kit (Thermo Fisher Scientific, USA) as described as manufacture user manual. Xylanase substrate (synthetic hemicellulose polysaccharides tagged with fluorescence dye) was diluted to 50 µg/mL using 1 time reaction buffer. Substrate was protected from light in the entire process. Xylanase from *Trichoderma viride* (Sigma, USA) was diluted to 10 U/mL and served as xylanase stock. Xylanase stock was diluted to S1: 200 mU/mL, S2: 100 mU/mL, S3: 50 mU/mL, S4: 25 mU/mL and S5: 0 mU/mL and used as xylanase standard in this assay. Bacterial cell pellet was pelleted from 1 mL overnight bacteria culture by centrifuging at 14,000 × *g* for 1 min. Cell pellet was resuspended using 100 µL of 1 time reaction buffer. Standard, sample and negative control were loaded into 96 well microtitre plate as listed in Table 3.6. The assay was incubated at 25 °C. The assay was measured at excitation 360 nm and emission 465 nm using Tecan® M200 Pro (Tecan, Switzerland) at 10 min interval for 4 hrs.

Table 3.6: Composition of blank, standard solution, positive control and sample loaded into each well of microtitre plate for xylanase assay.

Composition	Substrate (50 µg/mL)	Sample	Reaction Buffer
Negative Control	50 µL	N/A	50 µL
Standard	50 µL	50 µL of (xylanase)	N/A
Sample	50 µL	50 µL	N/A

* N/A : Not available

3.2.24 Bacterial Genome Sequencing Library Preparation

NGS library for bacterial genome sequencing was prepared using Nextera™ DNA sample preparation kit (Illumina, USA). Bacterial genomic DNA (50 µg) was fragmented using DNA tagmentation enzyme (Nextera transposome) at 55 °C for 5 min. Adapter sequences were added to the DNA template during DNA tagmentation. Tagmented DNA was purified using Zymo Research DNA clean and concentrator (Zymo Research, USA). Zymo DNA binding buffer (180 µL) was added to the tagmented DNA and mixed well. The solution was loaded into Zymo-Spin™ column and centrifuged at 1,300 × *g* for 2 min. DNA was washed twice using 300 µL wash buffer. Purified DNA (hereafter referred as NGS sequencing library) was eluted with 25 µL resuspension buffer (RSB). Subsequently, 5 µL of selected indexes were added to the NGS sequencing library through limited cycle PCR. The limit cycle PCR was performed at the following condition: 1 cycle of 72 °C for 3 min, 1 cycle of 98 °C for 30 sec, 5 cycles of 98 °C for 10 sec, 63 °C for 30 sec and 72 °C for 3 min. The PCR product was purified using 30 µL AMPure XP beads (Beckman Coulter, USA). The DNA-beads complex was washed twice using 80.0% (v/v) ethanol on a magnetic stand. NGS library was eluted from dried DNA-beads using 32.5 µL of RSB. The size distribution and concentration of the NGS sequencing library were assessed as described in section 3.2.7. The concentration of the NGS library was also measured using Qubit 2.0 Fluorometer as described in section 3.2.3.

3.2.25 Raw Read Assessment, Genome Assembly, Gene Prediction and Gene Annotation

Quality of bacterial genome NGS raw reads were assessed using FastQC (Schmieder & Edwards, 2011). Paired-end reads were imported into CLC Genomic Workbench (version 6) for quality trimming. Nucleotide with Phred score lower than Q30 and ambiguous nucleotide were trimmed using CLC Genomic Workbench (version 6). Bacterial genome was subjected to *de novo* assembly using CLC Genomic Workbench (version 6). Contigs with at least 30X coverage and 200 bps were exported for further analysis. Gene prediction was carried out using Prodigal (version 2.6) (Hyatt et al., 2010). Bacterial genome was annotated by comparing against NCBI-nr database using BLASTX (Acland et al., 2013). The draft genomes were deposited into DDBJ/EMBL/GenBank.

3.2.26 Chitinase Gene Study

Nucleotide and amino acid sequences of putative bacterial chitinase gene with e-value 0.0 (based on BLAST result) were manually extracted from the bacterial genome. Chitinase gene was clustered with its closely related gene from NCBI-nr database by phylogenetic analysis using MEGA (version 6.06) (Tamura et al., 2013).

3.2.27 Cloning of Chitinase Gene

Putative chitinase genes were sent to service provider, GenScript for gene synthesis service. The synthesized putative chitinase coding gene was inserted into pET-23(+) expression vector. The vector was transformed into *Escherichia coli* BL21. The transformant was subjected to chitinolytic assay as described in section 3.2.23.4.

3.2.28 Insect Degradation Test

Nepenthes pitcher fluid was autoclaved at 121 °C for 15 min using autoclave machine (HVE-50 Hirayama, Japan) before used. Wild type *Drosophila melanogaster* was killed and placed into 1X PBS (pH6.5) before autoclaved at 121 °C for 15 min using autoclave machine (HVE-50 Hirayama, Japan). Negative controls for insect degradation test were prepared by adding 3 *D. melanogaster* into 15 mL of 1X PBS, (pH6.5), 15 mL of autoclaved *Nepenthes* pitcher fluid and 15 mL of filtered sterilized *Nepenthes* pitcher fluid. Each negative control was loaded into a well in a 6-well plate. Overnight culture of *Bacillus* sp. strain H1a, *P. aeruginosa* strain H1l and *S. marcescens* strain H1q was adjusted to OD_{600}. Eighty microlitre of OD adjusted bacteria culture was seeded into 15 mL autoclaved *Nepenthes* pitcher fluid supplied with 3 *D. melanogaster.* The mixture was loaded into a well in a 6-well plate. The experimental setup in each well of the 6-well plate was as listed in Table 3.7. The reactions in the 6-well plates were incubated at 25 °C. The degradation test was observed using stereomicroscope M165C (Leica, Germany) for a week (negative controls and culture with only one bacterial strain) or 8 days (mixed culture).

Table 3.7: Negative control and samples loaded into each well of insect degradation test.

Composition	*Nepenthes* Pitcher Fluid or PBS	Number of *D. melanogaster*	Bacteria
Negative Control	15mL	3	N/A
Sample	15mL	3	80 µL of one bacterial strain
Mixed Culture	15mL	3	80 µL of all bacterial strains

* N/A : Not available

3.2.29 Sampling of *Nepenthes* Pitcher Fluid for *N*-acetylglucosamine detection

Nepenthes pitcher fluids were collected from the opened pitchers and unopened pitchers of *N. gracilis* plant in University of Malaya, Kuala Lumpur, Malaysia (03°7'49.0"N, 101°39'05.0"E). The pitcher condition, pH value of the pitcher fluid, volume of pitcher fluid collected and pitcher fluid condition were recorded. The pitcher fluids conditions were recorded as brown and turbid, slightly brown and clear (Figure 3.2).

Nepenthes pitcher fluids were centrifuged at 14,000 × *g* for 1 min. The fluid was transferred into sterile polypropylene tubes and the pellet was discarded. The pitcher fluid was filtered using cellulose acetate filter membrane (0.22-µm pore size) to remove microorganism and larvae. Pitcher fluids were dried using Vacumn Concentrator Plus (Eppendorf, Germany).

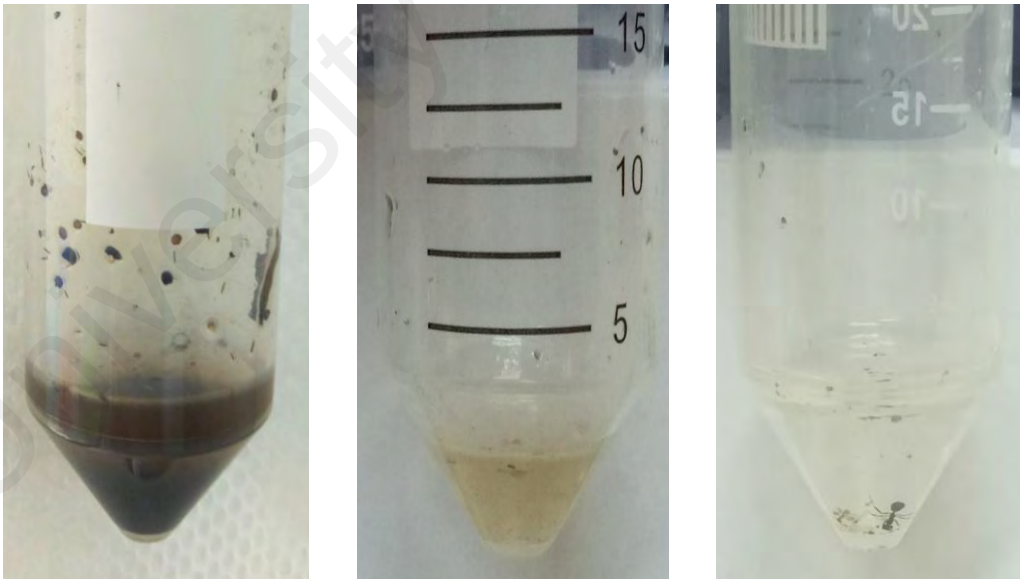


Figure 3.2: The *Nepenthes* pitcher fluid in (a) brown and turbid, (b) slightly brown and (c) clear conditions

3.2.30 *N*-acetylglucosamine Detection

Dehydrated pitcher fluid samples were reconstituted using methanol: water (ratio 1:1). External standard, 0.01 g/mL *N*-acetylglucosamine (Sigma, USA) was prepared using methanol: water (ratio 1:1). The *N*-acetylglucosamine detection was performed using LCMS-quadrupole Time of Flight (qTOF) maXis II™ MS (Bruker, USA). The compound separation was performed using Acclaim™ RSLC PolarAdvantage II liquid chromatography column (Thermo Fisher Scientific, USA) with a flow rate of 0.3 ml/min. Mobile phases used in liquid chromatography were distilled water supplied with 0.1% (v/v) formic acid (hereafter refer as "Solvent A") and acetonitrile supplied with 0.1% (v/v) formic acid (hereafter refer as "Solvent B"). Liquid chromatography was performed at gradient condition as listed in Table 3.8. The chromatogram generated by LCMS-qTOF and spectra extracted from chromatogram were analyzed using Compass DataAnalysis (version 4.3) for *N*-acetylglucosamine detection.

Table 3.8: Gradient conditions for compounds separation using liquid chromatography.

Retention, min	Flow, mL/min	Solvent A[a], %	Solvent B[b], %
0.0	0.3	95.0	5.0
2.0	0.3	95.0	5.0
9.0	0.3	50.0	50.0
15.0	0.3	10.0	90.0
17.0	0.3	10.0	90.0
19.0	0.3	95.0	5.0
21.0	0.3	95.0	5.0

a. Solvent A : Distilled water supplied with 0.1% (v/v) formic acid.
b. Solvent B : Acetonitrile supplied with 0.1% (v/v) formic acid.

CHAPTER 4: RESULTS

4.1 Metagenome Sample Metadata

Nepenthes pitcher fluids for microbial community study were collected from 47 opened pitchers of *N. sanguinea*, *N. hookeriana, N. albomarginata* and *N. gracilis*. Forty-four pitcher fluids sampled were acidic (pH1 to pH6) and 3 samples were neutral (pH7) (Table 3.1). The neutral pitcher fluid samples were collected from the same *N. sanguinea* plant on the same day at Fraser's Hill (Table 3.1, samples FR3, FR7 and FR8). Among the acidic samples, 20 samples were highly acidic (pH1 to pH4) and 24 samples were less acidic (pH5 and pH6).

4.2 Amplicon Next Generation Sequencing Output and Quality Assessment

The NGS using 454 GS-FLX generated 585,046 and 664,276 raw reads for samples H1 and LK, respectively (Table 4.1). After quality trimming and removing chimeric sequences, 536,993 and 607,783 sequences of samples H1 and LK, respectively (Table 4.1), were preceded to downstream analysis. The averages read length of trimmed sequences were 353 bps and 367 bps for samples H1 and LK, respectively (Table 4.1). The amplicon raw reads were deposited in NCBI sequence read archive (SRA). The SRA accession number of samples H1 and LK are SRR916131 and SRR916130, respectively.

At least 500,000 paired-end reads were generated by NGS using MiSeq. Sample FR2 has the lowest number of reads (578,912 sequences) and sample BB19 has the highest read count (2,532,972 sequences) among the samples (Table 4.1). The average read length of unfiltered sequences ranges from 173 bps to 190 bps (Table 4.1). After quality trimming and removing chimeric sequences, 572,260 to 2,523,331 sequences subjected

to sample were preceded to downstream analysis (Table 4.1). The average read length of unfiltered sequences ranges from 170 bps to 185 bps (Table 4.1). The amplicon raw reads were deposited in NCBI SRA. The SRA accession number of this study is SRP092873.

A total of 200,000 to 760,000 bacterial 16S rRNA gene sequences were binned from the trimmed shotgun metagenomic data (Table 4.2). The average read length of bacterial 16S rRNA genes sequence binned from samples N1, N3, N4, N5 and FR5 were 99 bps and the average read length of samples BKT5.2, BKT6.2, BKT9 and BKT19 were 120 bps (Table 4.2). The metagenomic raw reads were deposited in NCBI SRA. The SRA accession number of the studies were listed in Table 4.2.

Table 4.1: Sequencing platforms, read counts and average read length of amplicons raw reads and quality filtered reads, and SRA accession number of the study.

Sample	Sequencing Platform	Numbers of Raw Reads	Average Length of Unfiltered Read, bps	Number of Sequences After Filter	Average Length of Filtered Reads, bps	SRA Accession Number
H1	454 GS-FLX	585,046	386	536,993	352	SRR916131
LK	454 GS-FLX	664,276	426	607,783	367	SRR916130
C2	Miseq	1,416,289[a]	173[b]	1,411,807	172	SRP092873
C4	Miseq	1,835,106[a]	176[b]	1,831,710	175	SRP092873
FH1	Miseq	782,929[a]	174[b]	781,615	172	SRP092873
FH2	Miseq	1,211,028[a]	179[b]	1,203,652	177	SRP092873
FH3	Miseq	774,395[a]	181[b]	769,301	176	SRP092873
FH6	Miseq	1,238,749[a]	175[b]	1,234,966	173	SRP092873
FR1	Miseq	1,429,964[a]	179[b]	1,420,889	176	SRP092873
FR2	Miseq	578,912[a]	180[b]	572,260	174	SRP092873
FR3	Miseq	1,497,593[a]	181[b]	1,488,507	179	SRP092873
FR4	Miseq	892,313[a]	180[b]	886,693	174	SRP092873
FR7	Miseq	1,912,303[a]	190[b]	1,892,377	187	SRP092873
FR8	Miseq	752,197[a]	188[b]	745,875	183	SRP092873
N2	Miseq	1,017,074[a]	173[b]	1,015,177	171	SRP092873
N7	Miseq	1,058,222[a]	180[b]	1,055,389	178	SRP092873
BB1	Miseq	1,418,415[a]	175[b]	1,414,569	173	SRP092873
BB2	Miseq	1,557,344[a]	173[b]	1,520,264	171	SRP092873
BB3	Miseq	907,838[a]	186[b]	901,307	184	SRP092873
BB4	Miseq	1,002,082[a]	176[b]	1,000,166	175	SRP092873
BB5	Miseq	1,253,255[a]	188[b]	1,242,370	185	SRP092873
BB6	Miseq	1,543,515[a]	175[b]	1,536,493	173	SRP092873
BB7	Miseq	1,178,278[a]	190[b]	1,172,780	187	SRP092873
BB9	Miseq	920,947[a]	186[b]	919,170	184	SRP092873
BB10	Miseq	1,512,852[a]	177[b]	1,502,820	177	SRP092873
BB11	Miseq	2,328,964[a]	181[b]	2,321,197	179	SRP092873
BB12	Miseq	1,595,335[a]	181[b]	1,587,202	177	SRP092873

Table 4.1, continued.

Sample	Sequencing Platform	Numbers of Raw Reads	Average Length of Unfiltered Read, bps	Number of Sequences After Filter	Average Length of Filtered Reads, bps	SRA Accession Number
BB13	Miseq	1,075,920[a]	181[b]	1,074,567	179	SRP092873
BB14	Miseq	2,340,678[a]	181[b]	2,327,478	179	SRP092873
BB15	Miseq	1,162,901[a]	177[b]	1,160,042	174	SRP092873
BB16	Miseq	1,322,101[a]	177[b]	1,317,397	174	SRP092873
BB17	Miseq	2,360,549[a]	185[b]	2,350,578	181	SRP092873
BB18	Miseq	1,163,309[a]	183[b]	1,156,902	180	SRP092873
BB19	Miseq	2,532,972[a]	184[b]	2,523,331	181	SRP092873
BB20	Miseq	1,465,480[a]	179[b]	1,460,314	177	SRP092873
BB21	Miseq	1,857,935[a]	190[b]	1,840,391	187	SRP092873
BB22	Miseq	1,109,334[a]	183[b]	1,103,044	180	SRP092873
BB23	Miseq	1,040,948[a]	177[b]	1,037,732	175	SRP092873

a. Number of joined paired-end raw reads. Paired-end reads were generated using MiSeq System.
b. Average length of joined paired-end reads.

Table 4.2: Number of bacterial 16S rRNA sequences binned from filtered shotgun metagenome data, read count of non-chimeric bacterial 16S rRNA sequences and the SRA accession number of the study.

Sample	Number of 16S rRNA Binned	Average Length of Unfiltered Reads, bps	Number of non-chimeric Sequence	Average Length of Filtered Reads, bps	SRA Accession Number
N1	534,159	99	534,159	99	SRP131938
N3	513,863	99	513,861	99	SRP131938
N4	759,529	99	759,527	99	SRP131938
N5	473,608	99	473,605	99	SRP131938
FR5	362,597	90	362,592	90	SRP131938
BKT5.2	435,306	120	435,301	120	SRP131938
BKT6.2	293,996	119	293,989	119	SRP131938
BKT9	243,374	120	243,364	120	SRP131938
BKT19	243,039	120	243,034	120	SRP131938

4.3 Composition of Dominant Bacterial in Bacterial Community of *Nepenthes* Pitcher Fluid

In general, Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria and Firmicutes were the dominant bacterial phyla in the bacterial community of the *Nepenthes* pitcher fluids, sampled for this study (Figure 4.1). Based on the bacterial communities composition at the genus level, the samples were clustered into high acidity (pH1 to pH4) samples (dark green cluster in Figure 4.2) and low acidity (pH5 to pH7) samples (light green cluster in Figure 4.2). Sample clustering by metagenome mode (targeted or shotgun metagenomics) was not observed (Figure 4.2). The hierarchical clustering was performed using Bray-Curtis dissimilarity distance function.

Proteobacteria was the predominant bacterial phylum in most of the samples (41 samples out of 47 samples) (Figure 4.1). The relative abundance of Proteobacteria was at least 50% in the bacterial communities of the *Nepenthes* pitcher fluid samples, except samples H1 (46.4%), LK (20.5%), FR3 (37.1%), FR7 (25.2%), FR8 (19.9%), BB13 (32.1%), and BB15 (29.4%) (Figure 4.1 and Appendix A). The relative abundance of

Proteobacteria was remarkably high (at least 90%) in samples C2 (99.0%), C4 (99.1%), N1 (99.3%), N2 (98.5%), N3 (99.4%), N4 (99.0%), N5 (98.3%), N7 (99.9%), BB2 (99.3%), BB4 (95.8%), BB5 (92.5%), BB6 (93.2%), BB7 (92.5%), BB9 (91.7%), BKT5.2 (98.9%), BKT6.2 (96.3%), and BKT9 (95.2%) (Figure 4.1 and Appendix A).

Within Proteobacteria, the genus *Acidocella* was the predominant bacterial genus in highly acidic (pH1 to pH4) samples except samples N7 and BB18 (Figure 4.2). The relative abundance of *Acidocella* was remarkably high (at least 90%) in bacterial community of samples C2 (98.2%), N1 (92.0%), N2 (99.5%), N3 (92.6%), N4 (92.2%), N5 (92.2%), BB2 (99.2%), BKT5.2 (92.4%), BKT9 (90.6%) (Figure 4.2 and Appendix B). The relative abundance of *Acidocella* in low acidity (pH5 to pH7) samples ranges from moderate (10% to 40%) to relatively low (9.9% to 1%) (Figure 4.2 and Appendix B). The composition of *Acidocella* was less significant (less than 1%) in low acidity (pH5 to pH7) samples H1 (0.0%), LK (0.0%), N7 (0.1%), BB4 (0.1%), BB11 (0.1%), BB12 (0.5%), BB15 (0.1%), BB16 (0.1%), BB18 (0.1%), BB20 (0.1%) and BB21 (0.8%) (Figure 4.2 and Appendix B).

The relative abundance of Proteobacterial genus *Acidisoma* was remarkably high (at least 90%) in the bacterial community of sample N7 (99.4%) (Figure 4.2). In contrary, the relative abundance of *Acidisoma* was relatively low (9.9% to 1%) in samples FR4 (5.3%), N1 (1.3%), N3 (1.6%), N4 (1.7%), N5 (1.5%), BKT5.2 (1.3%), BKT9 (1.0%), and BKT19 (1.2%) (Appendix B). The composition of *Acidisoma* was less significant (less than 1%) in all samples with low acidity (pH5 to pH7) except sample FR4 (5.3%) (Figure 4.2 and Appendix B).

Proteobacteria order Ellin 329 was one of the predominant bacterial order in the bacterial community of the low acidity (pH5 to pH7) samples (Figure 4.2). The relative abundance of unclassified Ellin was remarkably high (at least 90%) in the bacterial community of sample BB4 (93.6%) (Figure 4.2). The composition of unclassified Ellin was significant (at least 1%) in low acidic (pH5 to pH7) samples except samples H1 (0.1%), LK (0.6%), FR7 (0.4%), FR8 (0.3%), BB9 (0.6%) and BB13 (0.2%), (Figure 4.2 and Appendix B). The relative abundance of Proteobacteria order Ellin 329 was also significant (at least 1%) in the bacterial community of samples collected from *N. albomarginata* plant (samples with "BB" initial) except samples BB1 (0.2%), BB2 (0.0%), BB3 (0.0%), BB5 (0.3%), BB9 (0.6%), BB13 (0.2%) (Figure 4.2 and Appendix B). In contrary, the relative abundance of unclassified Ellin was less significant (less than 1%) in the bacterial community of samples collected from the pitcher of *N. sanguinea* (except samples FH2 (17.5%), FH3 (3.8%), FR1 (35.2%), FR2 (13.1%), FR3 (5.7%) and FR4 (15.0%)), *N. gracilis* (samples with "BKT" initial) and *N. hookeriana* (sample LK) (Figure 4.2).

Other Proteobacterial with relative abundance more than 1% in the total dataset were *Kaistia*, *Burkholderia*, *Cupriavidus*, unclassified Rhizobiales, unclassified *Bradyrhizobiaceae*, unclassified *Acetobacteraceae*, unclassified *Sphingomonadaceae* and unclassified *Xanthomonadaceae* (Figure 4.2). In general, the relative abundance of *Kaistia*, unclassified Rhizobiales and unclassified *Bradyrhizobiaceae* were higher in low acidity (pH5 to pH7) samples collected from *N. sanguinea* plant (samples with "H", "C", "FH", "FR" and "N" initial) and *N. albomarginata* plant (samples with "BB" initial) (Figure 4.2). The relative abundance of *Cupriavidus*, *Burkholderia* and unclassified *Sphingomonadaceae* were higher in samples BB7, BB9 and BB17 (Figure 4.2). The relative abundance of unclassified *Acetobacteraceae* was at least 10% in the

bacterial community of samples BB6 (21.5%), BB16 (32.1), BB18 (32.9%) and BB19 (10.3%) (Figure 4.2 and Appendix B). Unclassified *Xanthomonadaceae* was found in pitcher fluid samples collected from *N. albomarginata* plant (samples with "BB" initial) except samples BB2 (0.2%), BB4 (0.2%), BB12 (0.8%), BB15 (0.2%), BB16 (0.7%), BB20 (0.1%). The relative abundance of unclassified *Xanthomonadaceae* was at least 10% in the bacterial community of samples FH3 (23.1), BB3 (55.4%), BB5 (48.0%), BB13 (18.9%) and BKT6.2 (27.7%).

Bacterial phyla Actinobacteria accounted at least 10% in the bacterial communities of 23 samples (Figure 4.1). The relative abundance of Actinobacteria was significantly higher (at least 50%) in the bacterial communities of samples FR3 (51.4%), BB13 (67.6%) and BB15 (70.1%) (Figure 4.1). However, the relative abundance of Actinobacteria is remarkably low (less than 1%) in the bacterial communities of samples LK (0.4%), C2 (0.1%), C4 (0.2%), N1 (0.0%), N2 (0.8%), N3 (0.0%), N4 (0.4%), N5 (0.5%), N7 (0.1%), BB2 (0.1%), and BKT5.2 (0.3%) (Appendix A).

Actinobacteria family *Microbacteriaceae* was one of the predominant bacterial compositions in the bacterial community of the samples with low acidity (pH5 to pH7) (Figure 4.2). In general, the relative abundance of unclassified *Microbacteriaceae* was significant (at least 1%) in the bacterial community of low acidity (pH5 to pH7) samples collected from *N. albomarginata* plant (samples with "BB" initial) and *N. sanguinea* plant (samples with "H", "C", "FH", "FR" and "N" initial) (Figure 4.2). It accounted more than 50% in the bacterial community of samples BB15 (71.5%) and BB13 (59.4%) (Figure 4.2 and Appendix C). The relative abundance of unclassified *Microbacteriaceae* was remarkably low (less than 1%) in highly acidity (pH1 to pH4) samples (Figure 4.2).

Significant portion of (relative abundance of at least 1%) Actinobacteria genus *Curtobacterium*, *Microbacterium*, and *Mycobacterium* were found in samples collected from *N. sanguinea* plant in Fraser's Hill at 26th Feb 2012 (samples FR1, FR2, FR3, FR4, FR5, FR7, and FR8) (Figure 4.2). The relative abundance of *Curtobacterium* was also significant (at least 1%) in the bacterial community of samples FH2 (1.7%), BB10 (3.4%), BB13 (6.1%), BB16 (4.7%), BB21 (1.3%) and BB23 (7.5%) (Figure 4.2 and Appendix B). Meanwhile, relative abundance of *Microbacterium* was also significant (at least 1%) in samples FH3 (2.1%) and BB17 (9.0%) (Figure 4.2 and Appendix C). The relative abundance of *Mycobacterium* was higher in samples FH1 (28.7%), FH2 (24.6%), FH3 (17.0%), and FH6 (37.3%), which were collected from the opened pitcher of *N. sanguinea* plant in Fraser's Hill at 16th March 2013 (Figure 4.2 and Appendix C).

In general, the relative abundance of Bacteroidetes was higher in *Nepenthes* pitcher fluid samples with low acidity (pH5 to pH7) (Figure 4.1). The relative abundance of Bacteroidetes was significantly higher in samples FR7 (63.5%) and FR8 (61.3%) compared to the other samples collected for this study (Figure 4.1 and Appendix A). The pH value of samples FR7 and FR8 were 7, and these neutral *Nepenthes* pitcher fluid were collected from the pitcher of *N. sanguinea* plant in Fraser's Hill. In contrary, the relative abundance of Bacteroidetes was remarkably low (less than 1%) in the bacterial communities of highly acidity (pH1 to pH4) samples (Figure 4.1).

Within Bacteroidetes, the relative abundance of family *Sphingobacteriaceae* was significant in samples H1 (22.2%), FH2 (11.6%), FH3 (5.4%), FR2 (2.7%), FR4 (1.0%), FR7 (3.5%), BB4 (2.0%), BB6 (3.6%), BB16 (1.6%), BB18 (8.1%), BB19 (3.9%), and BB22 (31.3%) (Figure 4.2 and Appendix D). Bacteroidetes genus *Sediminibacterium* was the predominant bacterial genera in the bacterial community of samples LK and BB21 (Figure 4.2). Besides, the relative abundance of *Sediminibacterium* was significant (at least 1%) in samples LK (36.9%), BB11 (2.0%), BB12 (3.0%), BB16 (6.0%), BB19 (1.8%), BB20 (2.1%) and BB21 (63%) (Figure 4.2 and Appendix D).

The unclassified *Chitinophagaceae* (family within Bacteroidetes) was found in low acidity (pH5 to pH7) samples except samples BB9 (0.1%), BB13 (0.0%) and BB15 (0.1%) (Figure 4.2 and Appendix D). The unclassified *Chitinophagaceae* was the predominant bacterial family in the bacterial community of samples H1, FR4, FR7, FR8 and BB11 (Figure 4.2). The relative abundance of unclassified *Chitinophagaceae* was highest in sample FR8 (54.9%) (Figure 4.2). The relative abundance of unclassified *Chitinophagaceae* was less than 1% in the bacterial community of highly acidic (pH1 to pH4) samples, except sample BB18 (20.6%) (Figure 4.2 and Appendix D).

The relative abundance of Acidobacteria and Firmicutes in the bacterial communities of pitcher fluid was lower compared to other dominant phyla (Proteobacteria, Actinobacteria and Bacteroidetes) (Figure 4.1). At least 50% of the samples have significantly low amount (less than 1%) of Acidobacteria and Firmicutes in their bacterial community (Figure 4.1 and Appendix A). The relative abundance of Acidobacteria was the highest in the bacterial community of sample BB18 (6.5%) followed by samples BB9 (4.6%), BB7 (3.2%), BB22 (2.7%), BKT19 (2.7%) and FR4 (2.3%) (Figure 4.1 and Appendix A). The relative abundance of Firmicutes was the

highest in the bacterial community of sample FR4 (4.6%) followed by samples BB10 (4.5%), BB21 (4.0%), FR7 (3.8%), FR2 (3.6%) and FR3 (3.5%) (Appendix A).

The relative abundance of other bacterial taxa (bacteria that were not classified under phyla Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria and Firmicutes) was less than 1% in all samples collected for this study, except samples H1 (16.1%), LK (41.4%), FR5 (3.8%), BKT6.2 (1.3%) and BKT19 (1.7%), (Figure 4.1 and Appendix A). The relative abundance of other bacterial taxa in samples LK (41.4%) and H1 (16.1%) were higher compared to the other samples in this study (Figure 4.1 and Appendix A).

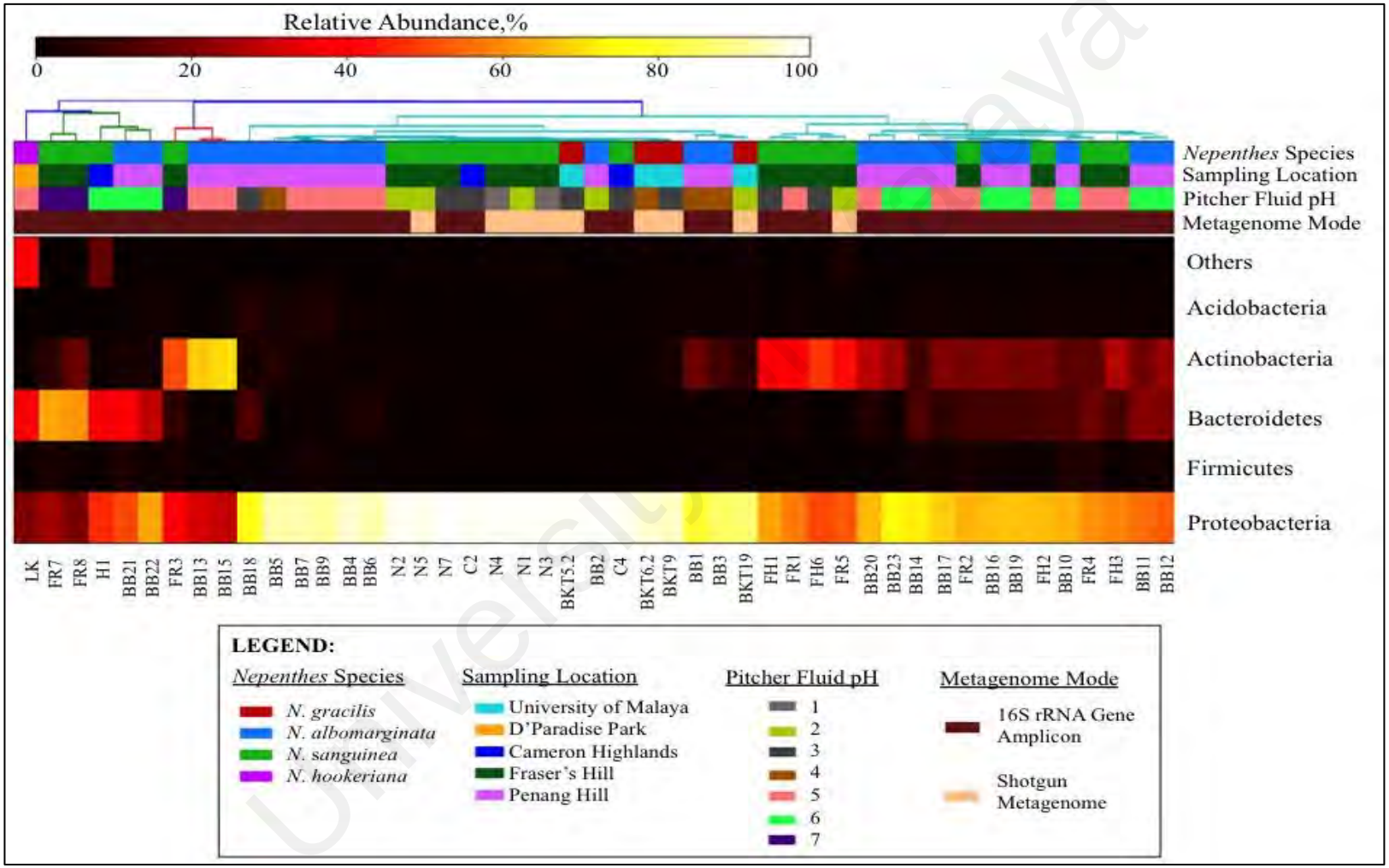


Figure 4.1: Bacterial community compositions in *Nepenthes* pitcher fluid at phylum level. Bacterial phyla with less than 1% relative abundance in total dataset were not included in the heatmap

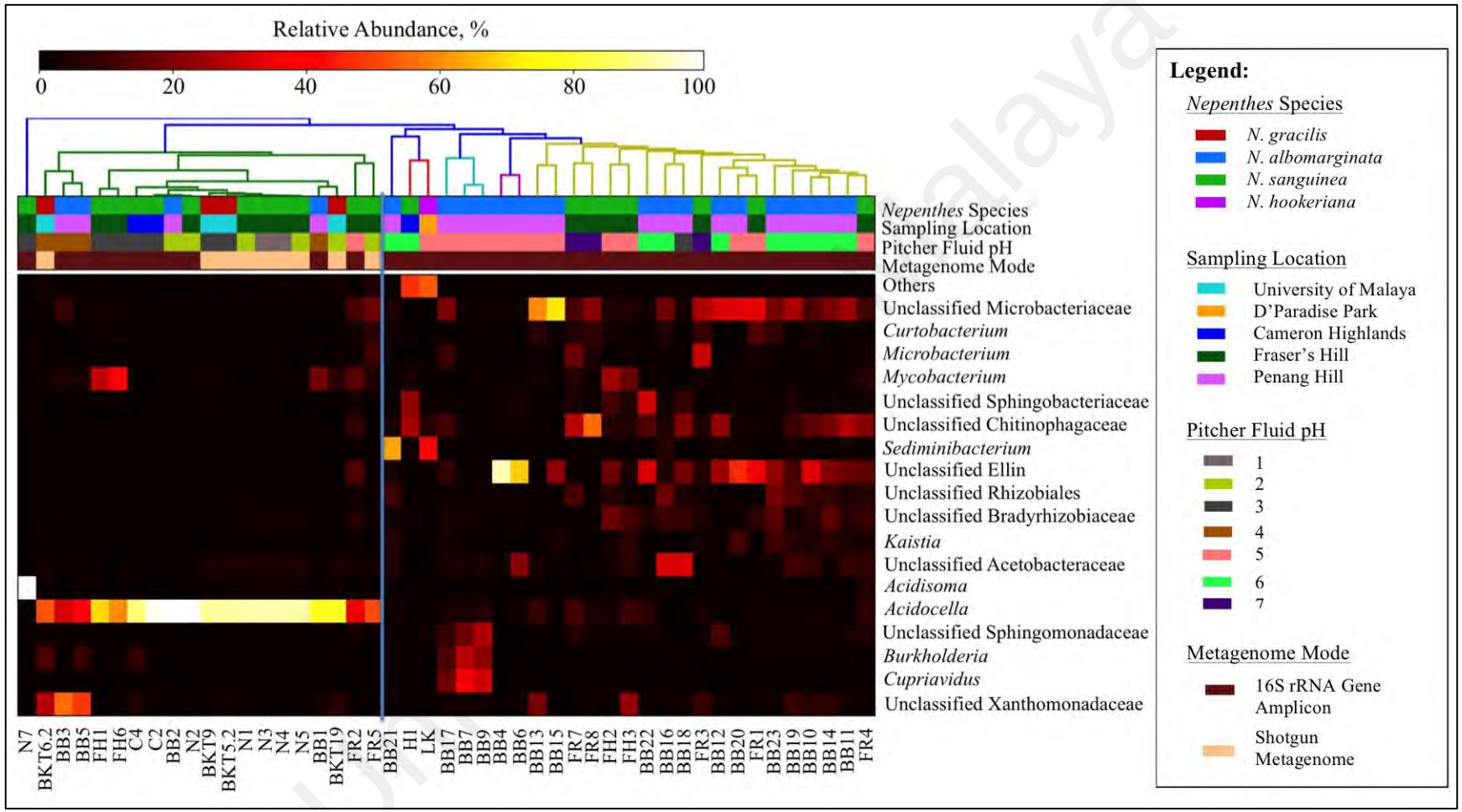


Figure 4.2: Bacterial community compositions in *Nepenthes* pitcher fluid at genus level. Blue line indicates the separation of high acidity (pH1 to pH4) samples cluster and low acidity (pH5 to pH7) samples cluster. Bacterial genera with less than 1% relative abundance in total dataset were not included in the heatmap

4.4 Bacterial Diversity in *Nepenthes* Pitcher Fluid

The Good's coverage of the rarified samples was at least 0.98 except samples H1 (0.839) and LK (0.814) (Table 4.3). It indicates that at least 98% of the bacterial species was represented in the subsampled data except samples H1 and LK. Based on Good's coverage estimator, 46,435 sequences per sample were sufficient to capture the alpha diversity of the bacterial community in the *Nepenthes* pitcher fluid samples except samples H1 and LK. Besides, rarefaction curve plotted based on alpha diversity metrics Chao1 (Figure 4.3a), observed OTUs (Figure 4.3b), PD whole tree (Figure 4.3c), Shannon (Figure 4.3d) and Simpson's index of diversity (Figure 4.3f) reaches plateau indicated the adequateness of subsampled data (46,435 sequences per sample) in capturing the alpha diversity of the bacterial community in *Nepenthes* pitcher fluid samples except samples H1 and LK. Subsampling with higher number of reads was needed for samples H1 and LK, in order to perform better bacterial community diversity studies.

In general, alpha diversity metrics, observed OTU, Chao1 and PD whole tree showed that the OTU richness, species richness and phylogenetic diversity were higher in low acidity (pH5 to pH7) samples compared to high acidity (pH1 to pH4) samples (Figure 4.3a-c). An exception was observed from sample BB18 with pH value 3 (Table 4.3 and Figure 4.3a-c). The OTU richness, species richness and phylogenetic diversity of sample BB18 were remarkably higher in this high acidity sample compared to other samples collected in this study.

The OTU richness, species richness and phylogenetic diversity of samples LK (pH5), H1 (pH6), FR2 (pH5), and FR4 (pH5) were higher compared to the other samples in this study (Figure 4.3a-c). The observed OTU, Chao1 and PD whole tree (phylogenetic diversity) showed that the OTU richness, species richness and phylogenetic diversity in sample LK was highest among the samples collected for this study (Figure 4.3a-c, Table 4.3). Based on Shannon (Figure 4.3d) and Simpson's index of diversity (Figure 4.3e), the bacterial diversity in sample H1 was highest among the samples.

On the other hand, the Chao1 estimator showed that the species richness was lowest in sample N4 (Table 4.3 and Figure 4.3a), while Shannon (Table 4.3) and Simpson's diversity (Table 4.3) indices showed that the species richness was lowest sample C2. The observed OTU metric and PD whole tree showed that the bacterial OTU richness and phylogenetic diversity was lowest in sample BB2 (Table 4.3 and Figure 4.3b-c).

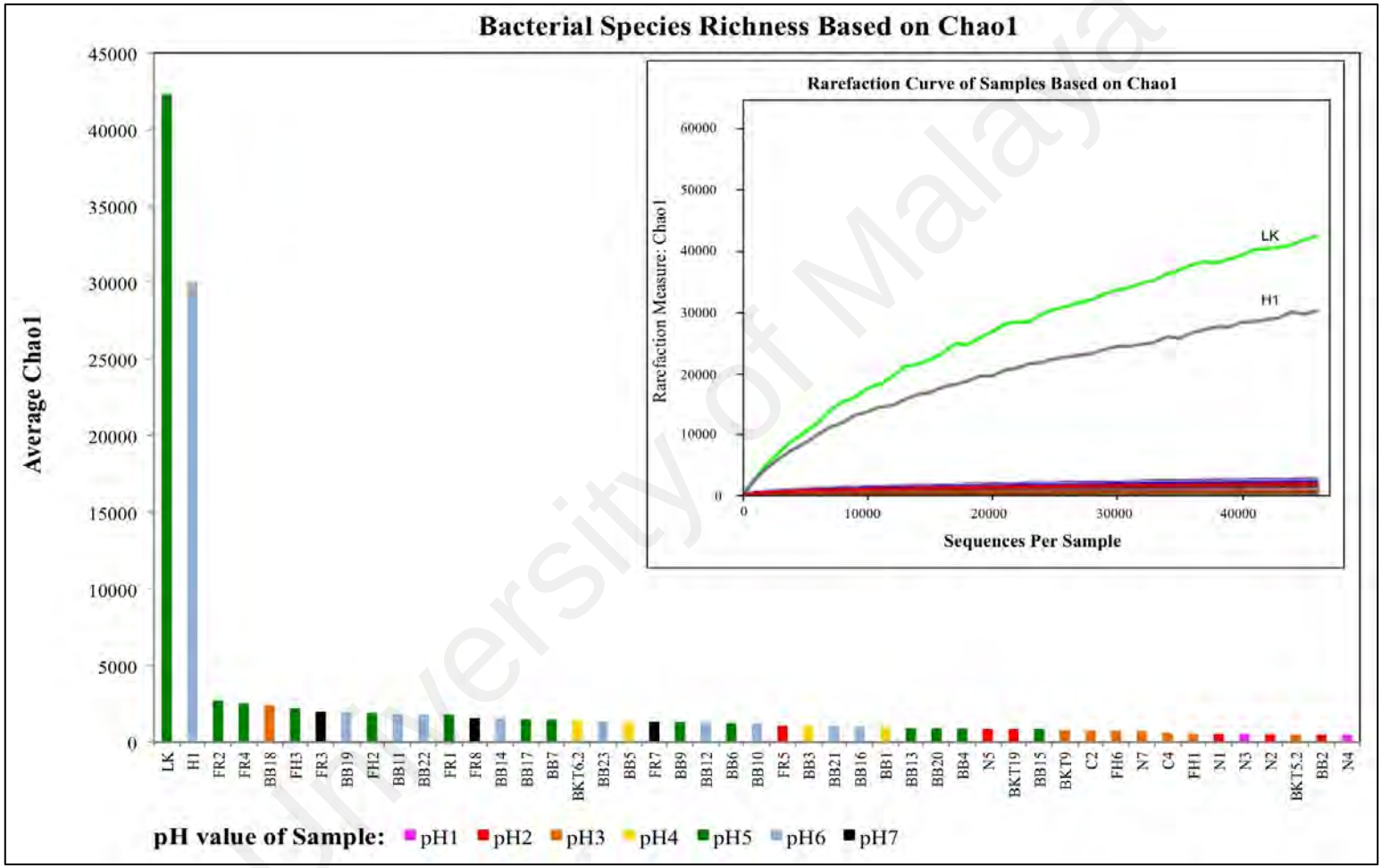
Table 4.3: Mean value of Good's coverage estimator, observed OTU, Chao1, Shannon, Simpson and PD whole tree alpha diversity indices.

Samples	Fluid pH	Location	*Nepenthes* Species	Good's Coverage	Observed OTUs	Chao1	Shannon	Simpson[a]	PD Whole Tree
H1	6	Cameron Highlands	*N. sanguinea*	0.839	10,377.000	30,073.547	10.096	0.994	524.120
LK	5	D' Paradise Park	*N. hookeriana*	0.814	10,898.200	42,274.613	9.420	0.985	631.711
C2	3	Cameron Highlands	*N. sanguinea*	0.996	339.800	758.598	1.177	0.263	35.688
C4	3	Cameron Highlands	*N. sanguinea*	0.997	311.000	633.143	2.132	0.513	29.318
FR1	5	Fraser's Hill	*N. sanguinea*	0.991	849.500	1,797.610	4.650	0.878	63.995
FR2	5	Fraser's Hill	*N. sanguinea*	0.985	1,523.600	2,715.666	6.830	0.967	117.220
FR3	7	Fraser's Hill	*N. sanguinea*	0.989	1,072.000	1,987.090	5.697	0.937	85.619
FR4	5	Fraser's Hill	*N. sanguinea*	0.987	1,468.300	2,524.484	6.954	0.976	110.837
FR5	2	Fraser's Hill	*N. sanguinea*	0.994	544.400	1,055.830	4.661	0.888	58.492
FR7	7	Fraser's Hill	*N. sanguinea*	0.993	762.700	1,320.997	5.430	0.950	56.241
FR8	7	Fraser's Hill	*N. sanguinea*	0.991	914.300	1,572.867	5.636	0.949	68.551
N1	2	Fraser's Hill	*N. sanguinea*	0.997	271.900	518.723	2.692	0.641	30.735
N2	2	Fraser's Hill	*N. sanguinea*	0.997	229.000	494.362	1.248	0.305	27.254
N3	1	Fraser's Hill	*N. sanguinea*	0.997	249.700	517.585	2.378	0.610	27.585
N4	1	Fraser's Hill	*N. sanguinea*	0.997	228.500	459.338	2.315	0.601	25.927
N5	2	Fraser's Hill	*N. sanguinea*	0.995	442.100	853.001	2.710	0.639	54.753
N7	3	Fraser's Hill	*N. sanguinea*	0.996	261.400	694.527	1.674	0.512	27.040
FH1	3	Fraser's Hill	*N. sanguinea*	0.997	245.400	526.987	2.501	0.660	24.238
FH2	5	Fraser's Hill	*N. sanguinea*	0.990	1,145.600	1,891.045	6.161	0.958	86.405
FH3	5	Fraser's Hill	*N. sanguinea*	0.988	1,302.400	2,196.766	6.361	0.960	96.368
FH6	3	Fraser's Hill	*N. sanguinea*	0.996	370.000	723.945	2.888	0.751	35.971
BB1	4	Penang Hill	*N. albomarginata*	0.995	489.800	953.166	2.383	0.543	51.791
BB2	2	Penang Hill	*N. albomarginata*	0.998	200.300	467.017	1.506	0.477	22.816
BB3	4	Penang Hill	*N. albomarginata*	0.995	504.800	1,052.925	3.225	0.752	53.017
BB4	5	Penang Hill	*N. albomarginata*	0.995	441.400	887.866	2.004	0.408	44.383
BB5	4	Penang Hill	*N. albomarginata*	0.993	647.600	1338.417	4.272	0.855	46.461
BB6	5	Penang Hill	*N. albomarginata*	0.993	662.300	1245.629	3.145	0.659	62.352

Table 4.3, continued.

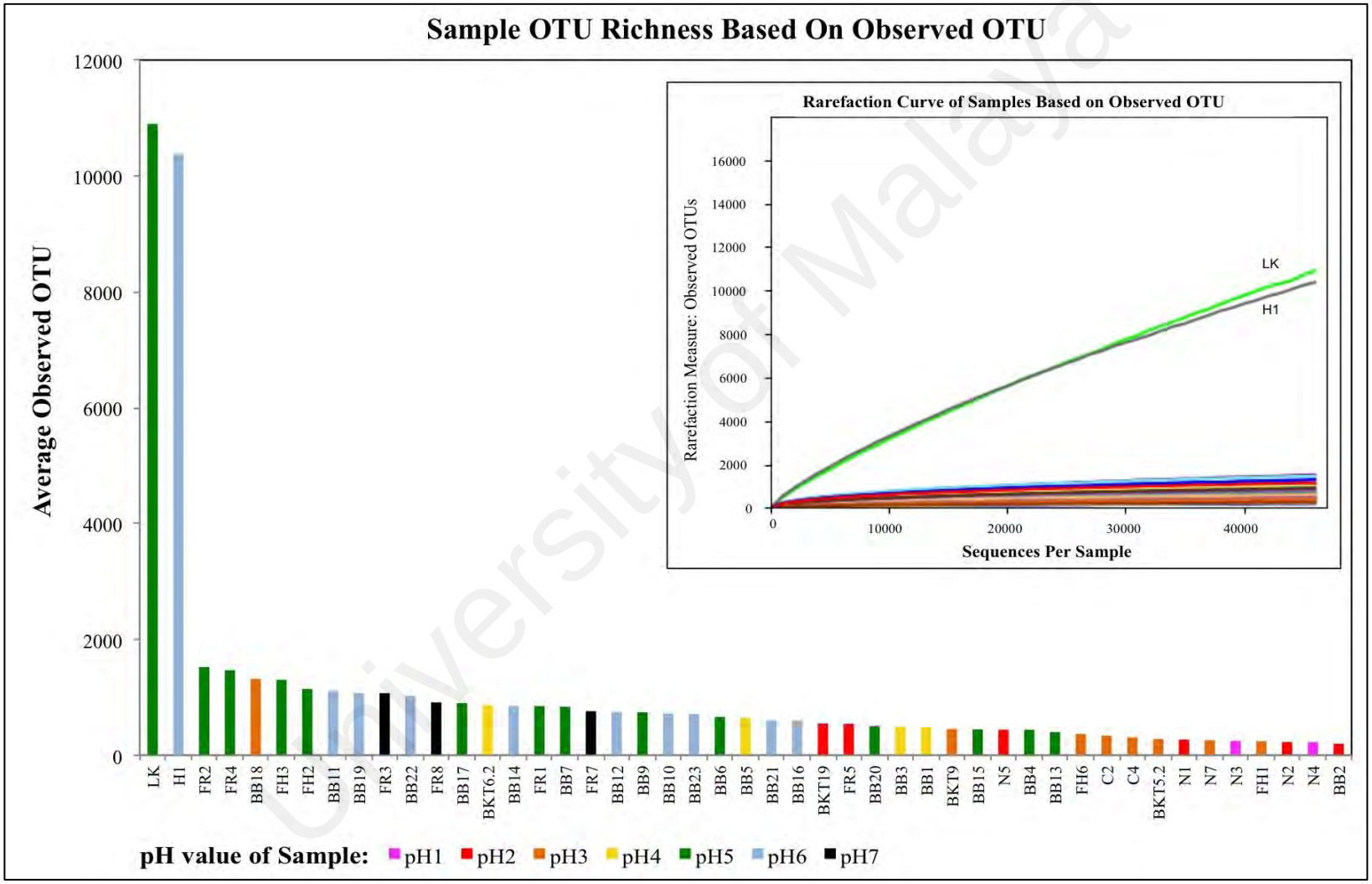
Samples	Fluid pH	Location	*Nepenthes* Species	Good's Coverage	Observed OTUs	Chao1	Shannon	Simpson[1]	PD Whole Tree
BB7	5	Penang Hill	*N. albomarginata*	0.992	838.200	1,472.677	5.260	0.922	63.281
BB9	5	Penang Hill	*N. albomarginata*	0.993	741.900	1,304.142	5.266	0.926	53.616
BB10	6	Penang Hill	*N. albomarginata*	0.994	718.300	1,228.940	4.968	0.903	56.589
BB11	6	Penang Hill	*N. albomarginata*	0.990	1,114.700	1,815.915	6.286	0.965	90.194
BB12	6	Penang Hill	*N. albomarginata*	0.993	750.500	1,272.560	4.983	0.923	64.569
BB13	5	Penang Hill	*N. albomarginata*	0.996	399.800	907.339	3.326	0.740	37.719
BB14	6	Penang Hill	*N. albomarginata*	0.992	853.400	1,562.317	6.033	0.965	65.858
BB15	5	Penang Hill	*N. albomarginata*	0.995	447.700	845.380	3.326	0.820	50.876
BB16	6	Penang Hill	*N. albomarginata*	0.995	602.400	1,012.498	5.205	0.944	48.320
BB17	5	Penang Hill	*N. albomarginata*	0.992	899.900	1,483.584	6.006	0.963	66.871
BB18	3	Penang Hill	*N. albomarginata*	0.987	1,321.200	2,410.794	6.687	0.973	111.843
BB19	6	Penang Hill	*N. albomarginata*	0.990	1,073.400	1,948.647	6.247	0.966	84.470
BB20	5	Penang Hill	*N. albomarginata*	0.995	506.700	902.838	3.987	0.841	53.649
BB21	6	Penang Hill	*N. albomarginata*	0.994	603.300	1,034.745	4.257	0.841	49.769
BB22	6	Penang Hill	*N. albomarginata*	0.990	1,026.200	1,799.309	5.554	0.934	75.694
BB23	6	Penang Hill	*N. albomarginata*	0.993	716.000	1,363.831	4.646	0.904	52.686
BKT5.2	3	University of Malaya	*N. gracilis*	0.997	281.000	484.012	2.875	0.678	31.944
BKT6.2	4	University of Malaya	*N. gracilis*	0.992	867.500	1,468.670	5.193	0.913	77.703
BKT9	3	University of Malaya	*N. gracilis*	0.995	456.800	778.515	3.045	0.694	59.299
BKT19	2	University of Malaya	*N. gracilis*	0.996	549.100	850.383	4.216	0.802	56.483

a. Simpson's Index of Diversity, 1-Dominance was used in this bacterial diversity study.



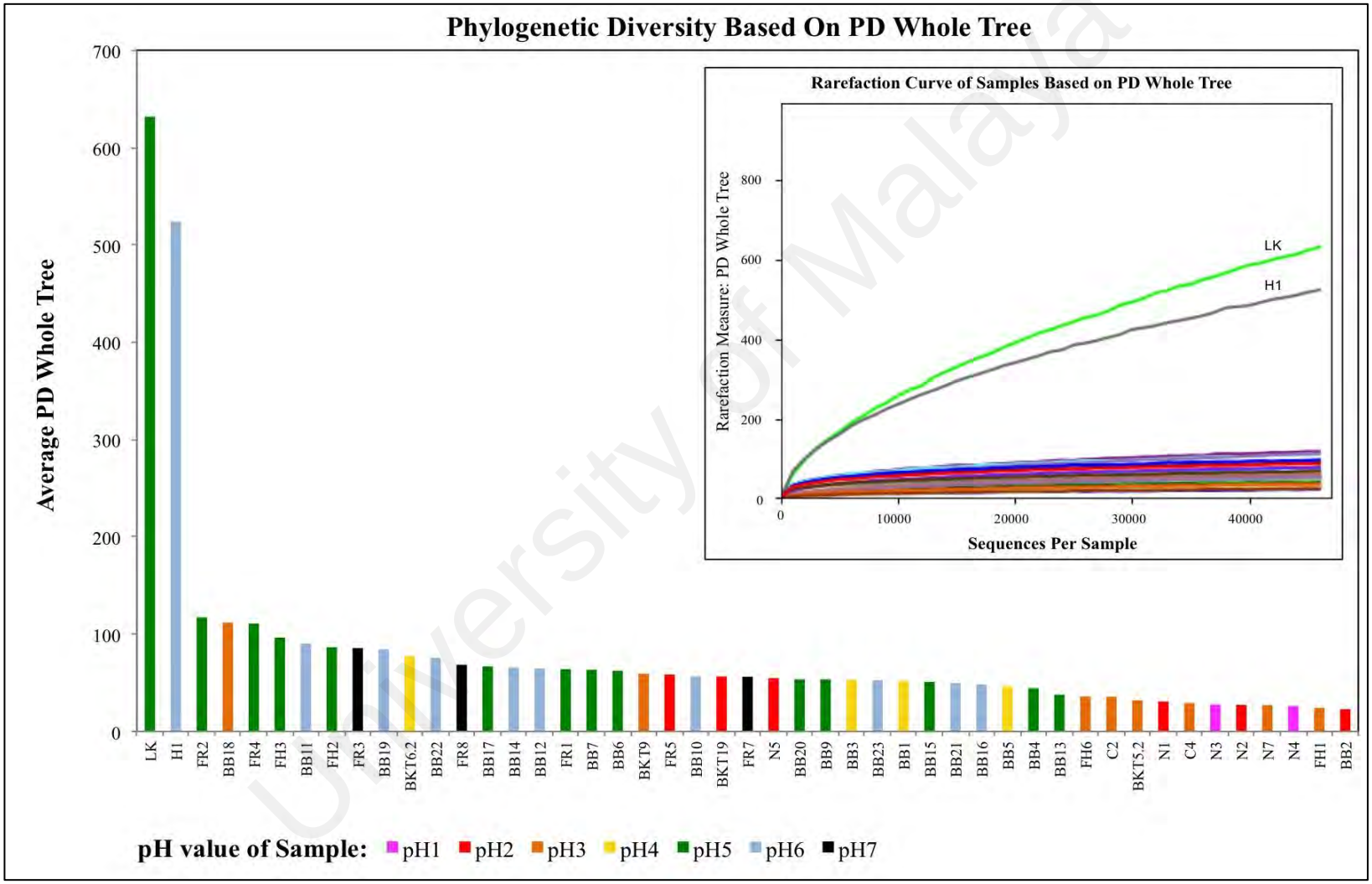
(a)

Figure 4.3: Rarefaction curve and bacterial diversity of rarified samples based on (a) Chao1, (b) observed OTU, (c) PD Whole Tree, (d) Shannon and (e, f) Simpson's Index of diversity. The pH values of the samples were indicated by the color of the bar in bar chart



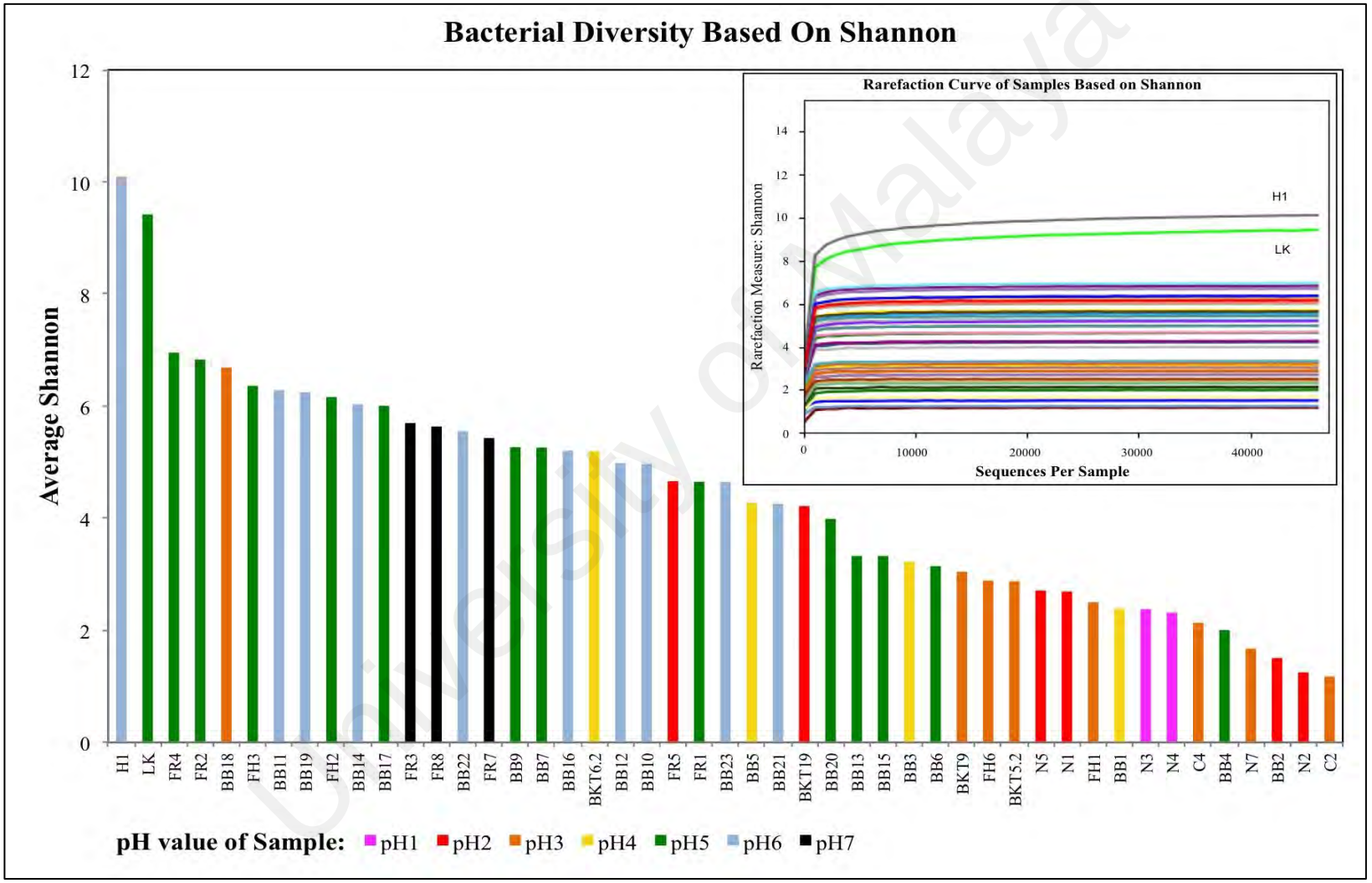
(b)

Figure 4.3, continued.



(c)

Figure 4.3, continued.



(d)

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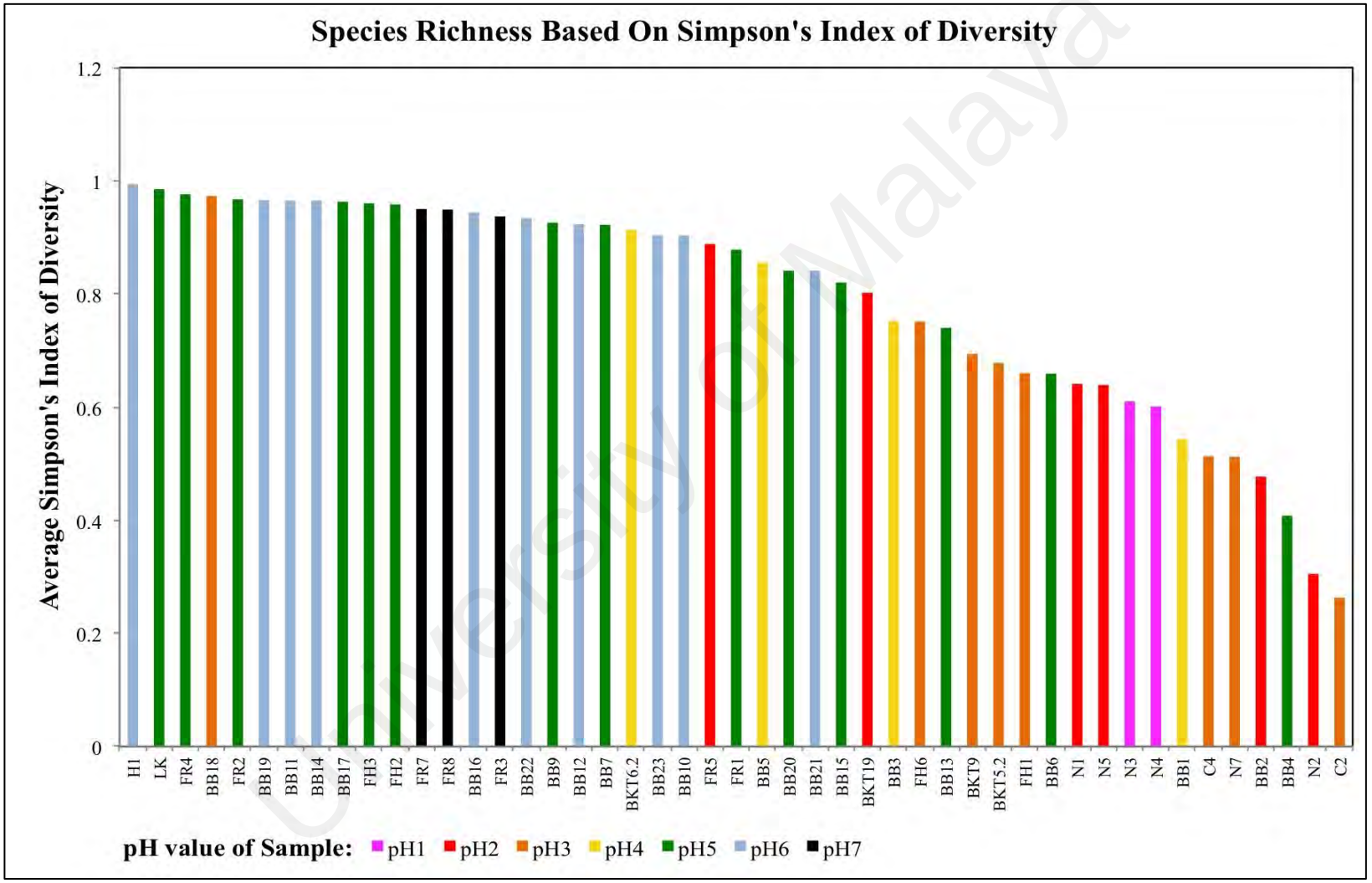

(e)

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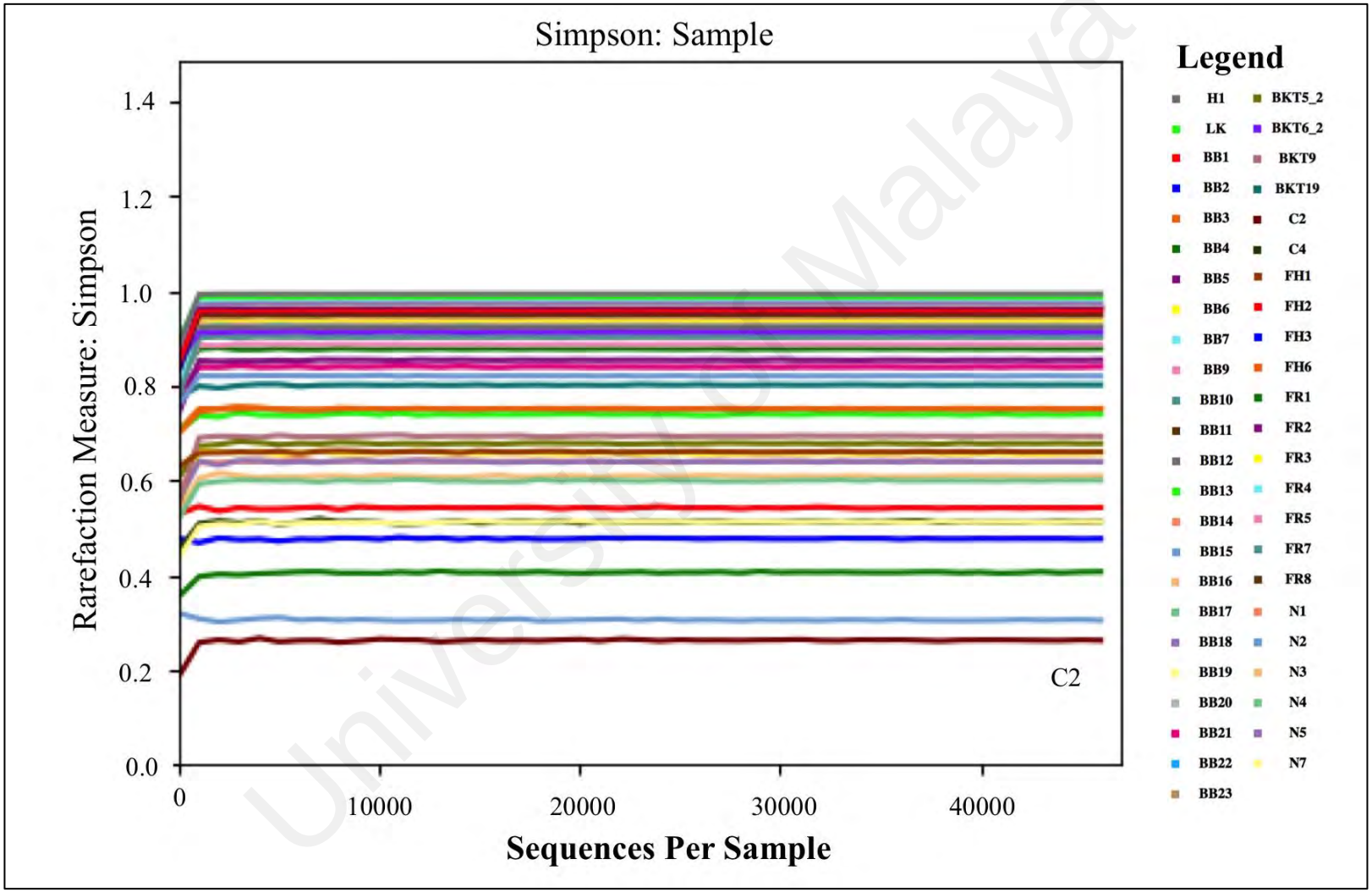


(f)

Figure 4.3, continued.

4.5 Correlation of Bacterial Communities with the Pitcher Fluid pH, Host Plant and Sampling Location

Correlation of bacterial community and the a) pitcher fluid acidity, b) host plant and c) sampling location were studied using samples collected from the pitchers of *N. sanguinea* and *N. albomarginata* plants. Samples H1, LK, FR5, N1, N3, N4, N5, BKT6.2, BKT9 and BKT19 were not included in this study as the NGS libraries were prepared using different protocols, and sequenced using different NGS platforms. Besides, sample collected from *N. hookeriana* (sample LK) plant was also excluded from this study due to low sample size.

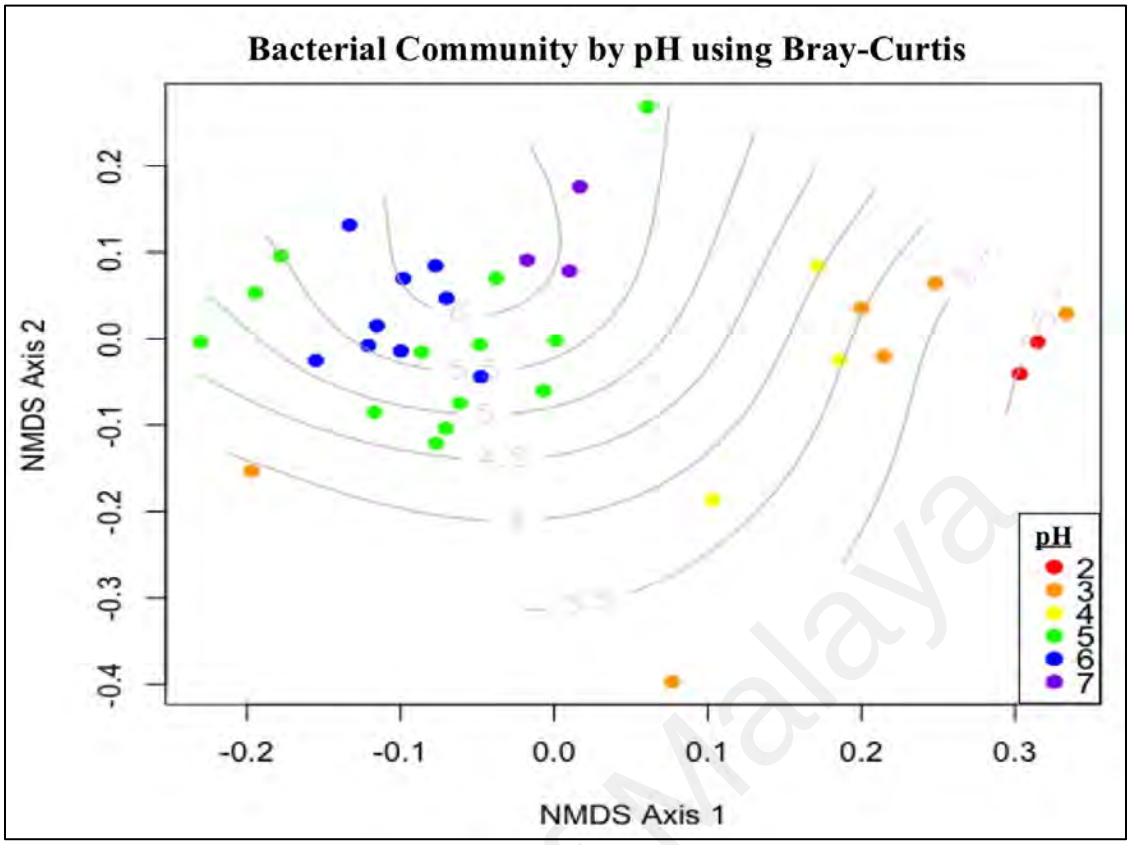
Significant correlation was found between the bacterial communities dissimilarity and the pitcher fluid acidity, the host plant and the sampling location. Based on PERMANOVA on Bray-Curtis, weighted UniFrac and unweighted UniFrac distance matrices, the influence of plant individual (r^2: 0.19, $P_{value} < 0.001$) and sampling location (r^2: 0.12, $P_{value} < 0.001$) on bacterial communities dissimilarity were significant (Table 4.4). However, other factors, including *Nepenthes* plant species, sampling date, sampling sites, and sampling site altitude were included in the analysis. Hence, the strength of influence of the host plant and sampling sites on *Nepenthes* digestive fluid bacterial communities is not conclusive.

Among the factors tested, *Nepenthes* pitcher fluid acidity exerted the strongest influence on the bacterial communities (Table 4.4). Strong positive correlation was found between the bacterial communities dissimilarity and the acidity of the pitcher fluid using Mantel test on Bray-Curtis dissimilarity, weighted Unifrac and unweighted Unifrac metrics (Table 4.4). Clustering of samples by *Nepenthes* fluid pH value was observed in the NMDS plots using Bray-Curtis dissimilarity (Figure 4.4a), weighted

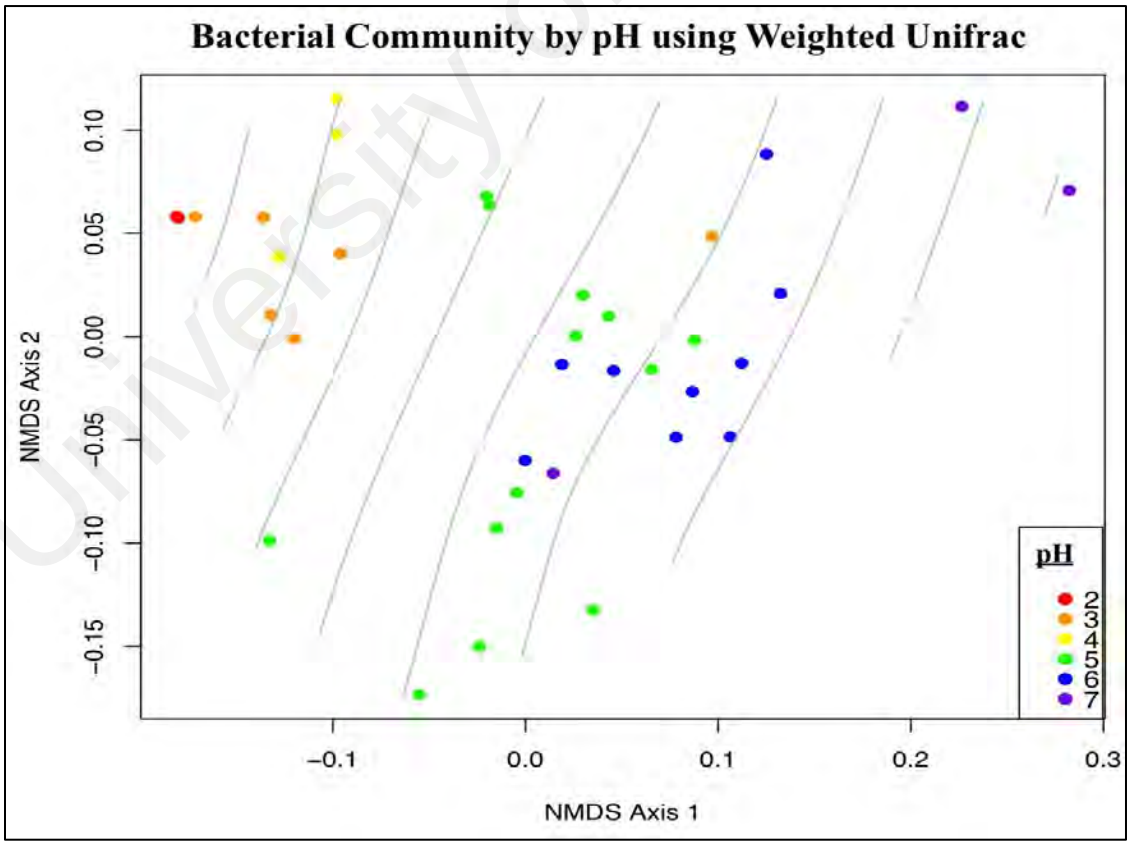
Unifrac (Figure 4.4b) and unweighted Unifrac (Figure 4.4c) metrics. A distinct separation was observed among samples from high acidity (pH2 to pH4) samples and low acidity (pH5 to pH7) samples (Figure 4.4a-c).

N. albomarginata pitcher fluid samples were collected on the same day at the same sampling location (MCG, Penang Hill) (Table 3.1). In contrary, *N. sanguinea* pitcher fluid samples were collected at different seasons and sampling site (Table 3.1). In order to prevent the variation in bacterial community structure caused by the host species, sampling season and sampling location, further investigation was performed on only *N. albomarginata* pitcher fluid samples collected at MCG.

Mantel test on Bray-Curtis dissimilarity, weighted Unifrac and unweighted Unifrac metrics of *N. albomarginata* pitcher fluid samples showed that the bacterial communities dissimilarity were strongly influenced by the pH of *Nepenthes* pitcher fluid (Table 4.4). Clustering of sample by *Nepenthes* fluid pH values were observed in the NMDS plots constructed using Bray-Curtis dissimilarity (Figure 4.4d), weighted Unifrac (Figure 4.4e) and unweighted Unifrac (Figure 4.4f). A distinct separation was observed between samples from high acidity (pH2 to pH4) and low acidity (pH5 to pH6) (Figure 4.4d-f). These results were corroborated with the statistical analysis on both *N. albomarginata* and *N. sanguinea* samples.

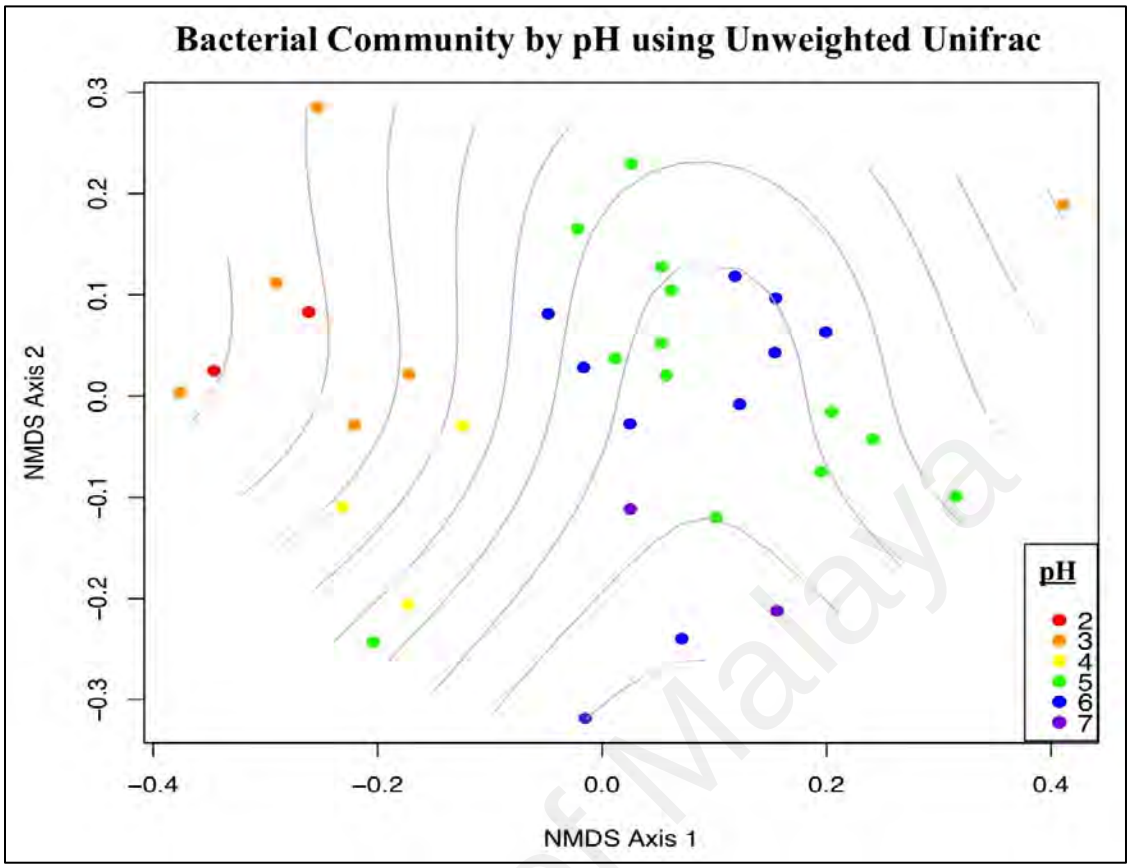


(a)

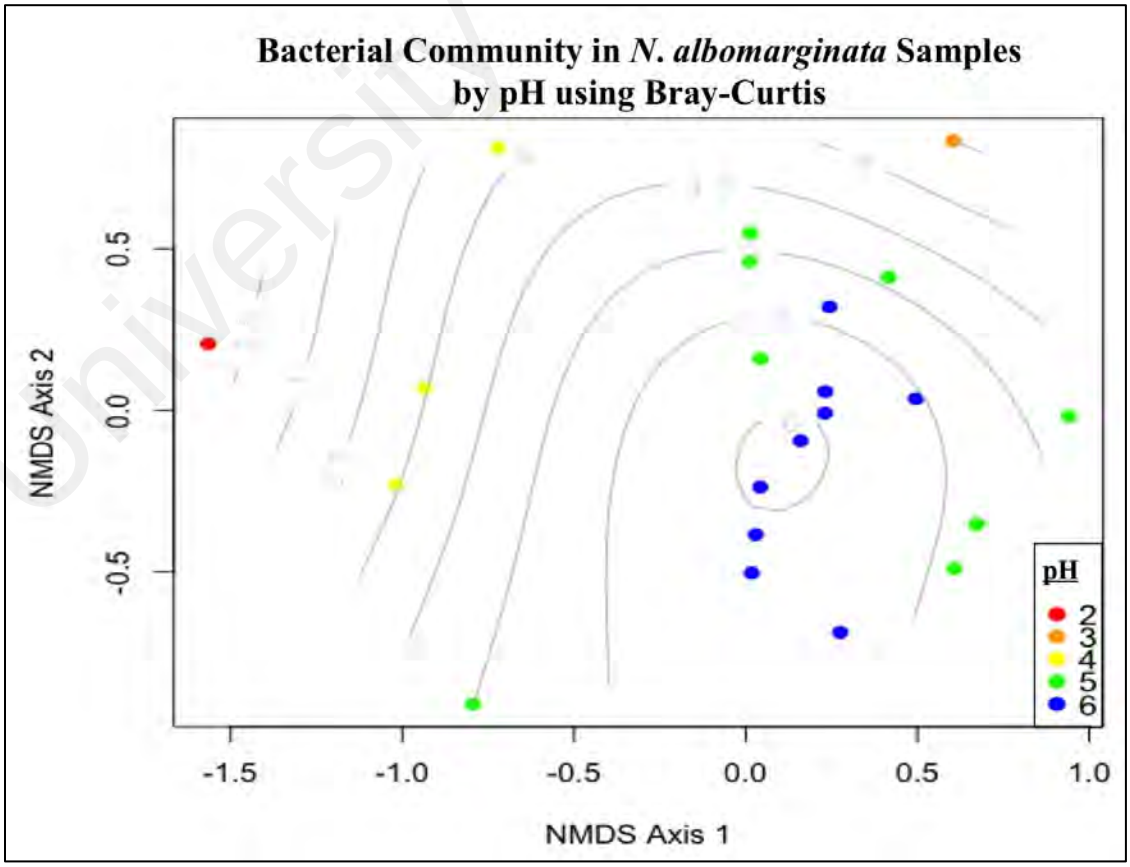


(b)

Figure 4.4: NMDS plots of pitcher fluid samples by pH using (a) Bray-Curtis, (b) weighted Unifrac and (c) unweighted Unifrac metrics. NMDS plots of *N. albomarginata* pitcher fluid samples using (d) Bray-Curtis, (e) weighted Unifrac and (f) unweighted Unifrac metrics

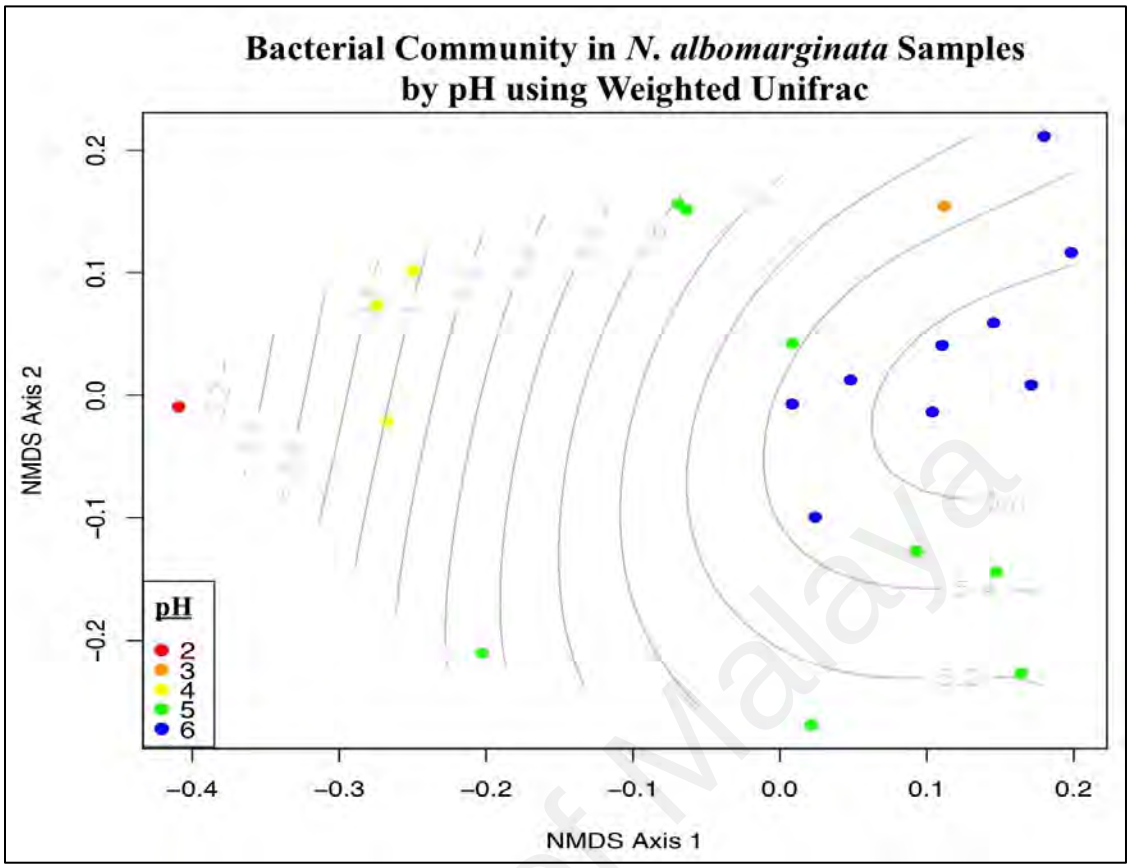


(c)



(d)

Figure 4.4, continued.



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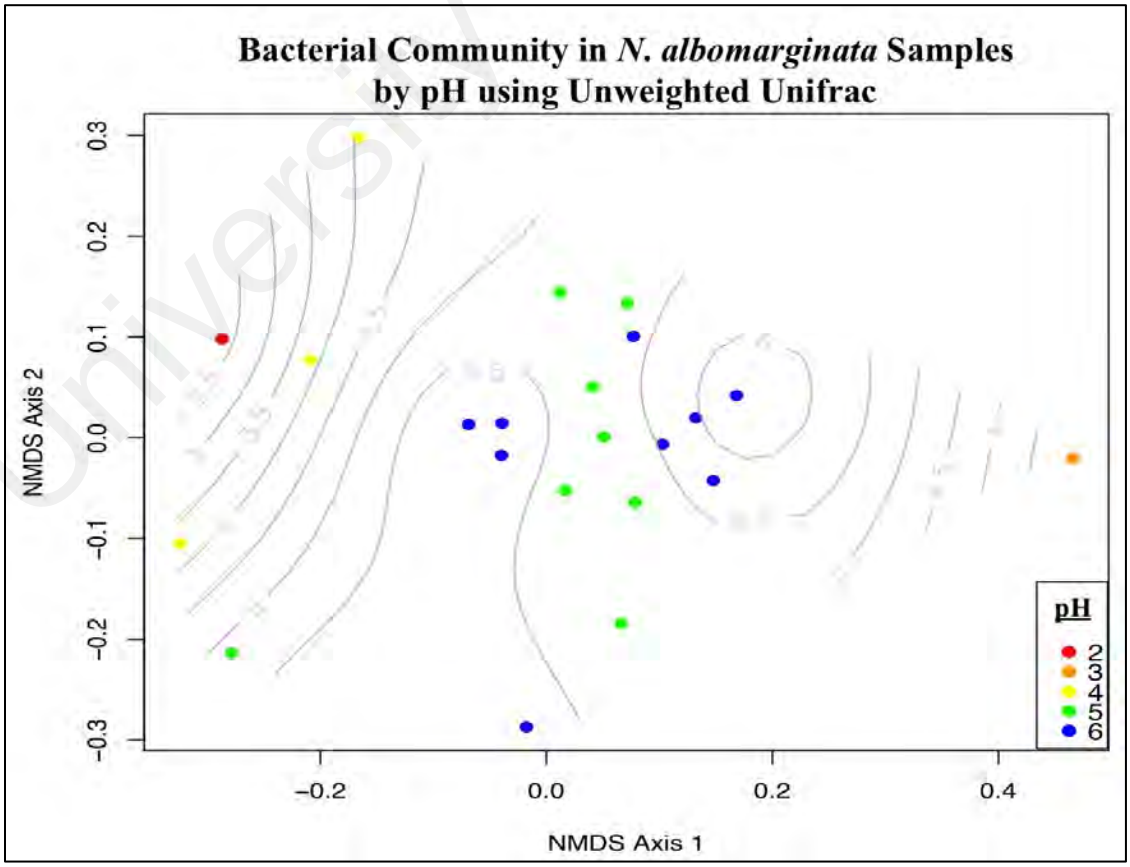


(f)

Figure 4.4, continued.

Table 4.4: Correlation of *Nepenthes* pitcher fluid's bacterial communities dissimilarity and pitcher fluid acidity, plant host and sampling location.

Metrics / Factor	Bray Curtis		Weighted UniFrac		Unweighted UniFrac	
	r^2	P_{value}	r^2	P_{value}	r^2	P_{value}
pH[a]	0.2110	0.0001	0.2929	0.0001	0.2167	0.0001
pH (*N. albomarginata*)[a]	0.1954	0.0001	0.2227	0.0001	0.2104	0.0001
Plant[b]	0.1936	0.0001	0.2212	0.0001	0.1267	0.0001
Location[b]	0.1218	0.0001	0.1058	0.0015	0.0950	0.0001

* P_{value} below alpha = 0.05 indicates significance correlation.
a. Statistical analysis was performed using Mantel test.
b. Statistical analysis was performed using PERMANOVA.

4.6 Eukaryote Community Composition

Composition of eukaryotes in pitcher fluids of *N. sanguinea* and *N. gracilis* were studied using eukaryote 18S rRNA binned from shotgun metagenomic data. Hierarchical clustering by Bray-Curtis dissimilarity distance matrix showed that the samples were clustered by *Nepenthes* species, sampling location and pitcher fluid pH value, except samples N3 and BKT6.2 (Figure 4.5). However, the strength of influence of *Nepenthes* species, sampling location and pitcher fluid pH on the eukaryotic communities remains unknown, as it was not experimentally tested in this study.

The relative abundance of insects belonging to taxa Coleoptera, Diptera, Hemiptera and Hymenoptera were significantly higher in samples collected from the pitcher of *N. sanguinea* plant (samples FR5, N1, N3, N4 and N5) compared to samples collected from the pitcher of *N. gracilis* plant (samples BKT5.2, BKT6.2, BKT9 and BKT19) (Figure 4.5). Coleoptera (beetles) accounted at least 1.7% (up to 6.4%) of the eukaryote community in samples from *N. sanguinea* (Appendix E). However, less than 1% of Coleoptera was found in the samples from *N. gracilis* (Appendix E). The relative abundance of Diptera (flying insects with a pair of wings) and Hymenoptera (flying insects with two pairs of membranous wings, example: wasps; non-flying insects, example: ants) ranges from 3.3% to 69.0% and 3.7% to 17.9%, respectively, in the eukaryotic community of *N. sanguinea* samples (Figure 4.5 and Appendix E). However,

the relative abundance of Diptera and Hymenoptera were less than 10% in the eukaryotic community of *N. gracilis* samples (Figure 4.5 and Appendix E). Diptera and Hymenoptera were not found in sample BKT6.2 (Figure 4.5). Hemiptera (true bugs) formed 3.7% of the eukaryote community in sample N1 (Figure 4.5 and Appendix E). However, the relative abundance of Hemiptera was less than 1% in the eukaryote community of other samples (Figure 4.5 and Appendix E).

In general, the relative abundance of Arachnids subclass Acari (tick and mite) was higher in *N. gracilis* samples compared to *N. sanguinea* samples (Figure 4.5). The relative abundance of Acari was highest in sample BKT9 (19.8%), followed by sample BKT19 (15.8%) (Figure 4.5 and Appendix E). Less than 5% of Acari were found in the eukaryote community of *N. sanguinea* samples. Opiliones (harvester) and *Araneae* (spider) were orders of Arachnids found only in *N. sanguinea* samples (Appendix E). Opiliones was found in samples N1 (1.9%), N4 (2.4%) and N5 (4.5%) while *Araneae* was only found in samples N1 (13.2%) and sample FR5 (1.3%) (Figure 4.5 and Appendix E).

Significant amount of Diplogasterida (worm) was found in *N. gracilis* samples except sample BKT6.2 (Figure 4.5). It formed 45.3%, 39.2%, and 38.0% of the eukaryote community in samples BKT19, BKT5.2 and BKT9, respectively (Figure 4.5 and Appendix E). In contrary, Diplogasterida was not found in *N. sanguinea* samples (Figure 4.5). On the other hand, nematode order Rhabditida (free-living, zooparasitic and phytoparasitic microbivorous roundworm) was only found in *N. sanguinea* samples N1 and N3 (Figure 4.5). The relative abundance Rhabditida was significantly higher in sample N3 (84.7%) (Figure 4.5 and Appendix E). Parasitic nematode order

Trichocephalida was found in sample BKT5.2 (1.0%), N4 (3.5%) and N5 (2.9%) (Figure 4.5 and Appendix E).

The composition and diversity of fungus was remarkably higher in sample N4, N5 and but remarkably low in sample N1 and N3 (Figure 4.5). *Ophiostomataceae* was found in samples BKT5.2 (2.1%), BKT9 (4.0%), BKT19 (2.2%), FR5 (1.3%), N4 (3.3%) and N5 (17.3%) (Figure 4.5 and Appendix E). Sordariomycetes was not found in *N. sanguinea* samples except sample N4 (3.3%) (Figure 4.5). Hypocreales and Barbatosphaeria were only found in samples BKT9 and N5 (Figure 4.5). Entomophthorales and Arthoniomycetes were only found in *N. sanguinea* samples (Figure 4.5). The relative abundance of Entomophthorales was 1.7% in samples FR5 and N1, and 1.5% in sample N4 (Appendix E). The relative abundance of Arthoniomycetes was 1.8% in sample N4 and 2.9% in sample N5 (Appendix E). Fungus order *Aliquandostipitaceae* was only found in samples BKT5.2 (1.7%), N4 (3.9%) and N5 (3.3%) (Figure 4.5 and Appendix E). At least 1% of the fungi in the samples (except samples N1 and N3) were unclassified (Figure 4.5 and Appendix E).

Saccharomycetales (budding yeast) was found in sample BKT5.2 (11.0%), BKT9 (2.0%), FR5 (4.5%), N4 (7.7%) and N5 (5.8%) (Figure 4.5 and Appendix E). Yeast within genus *Candida*, *Dekkera*, *Metschnikowia* and *Meyerozyma* were only found in samples BKT5.2, N4 and N5 (Figure 4.2). The relative abundance of *Candida* was highest in sample N4 (11.4%) followed by samples BKT5.2 (5.6%) and N5 (4.9%) (Figure 4.5 and Appendix E). The relative abundance of *Metschnikowia* and *Meyerozyma* were highest in sample N4 (7.0% and 5.3%, respectively), followed by samples N5 (4.9% and 3.7%, respectively) and BKT5.2 (1.6% and 1.8%, respectively) (Figure 4.5 and Appendix E). *Dekkera* formed 1.2%, 1,5% and 2.9% of the eukaryote

community in samples BKT5.2, N4 and N5, respectively (Appendix E). Tetrapsispora was only found in *N. sanguinea* samples N4 (3.5%) and N5 (2.5%) (Figure 4.5 and Appendix E).

Unicellular eukaryotes, Tetramitia (11.6%), unclassified Ciliophora (62.4%) and unclassified Colopodida (6.9%) were only found in sample BKT6.2 (Figure 4.5 and Appendix E). Besides, unicellular eukaryotes phylum Cercozoa was found in all samples collected from *N. gracilis* plant, but not in samples collected from *N. sanguinea* plant (Figure 4.5). The relative abundance of Cercozoa was highest in sample BKT19 (9.9%) followed by BKT 6.2 (6.2%), BKT5.2 (4.7%) and BKT9 (4.3%) (Appendix E). *Cryptosporidium* (apicomplexan parasite alveolates) was found in *N. gracilis* samples BKT9 (15.4%) and BKT 19 (1.5%) (Figure 4.5 and Appendix E).

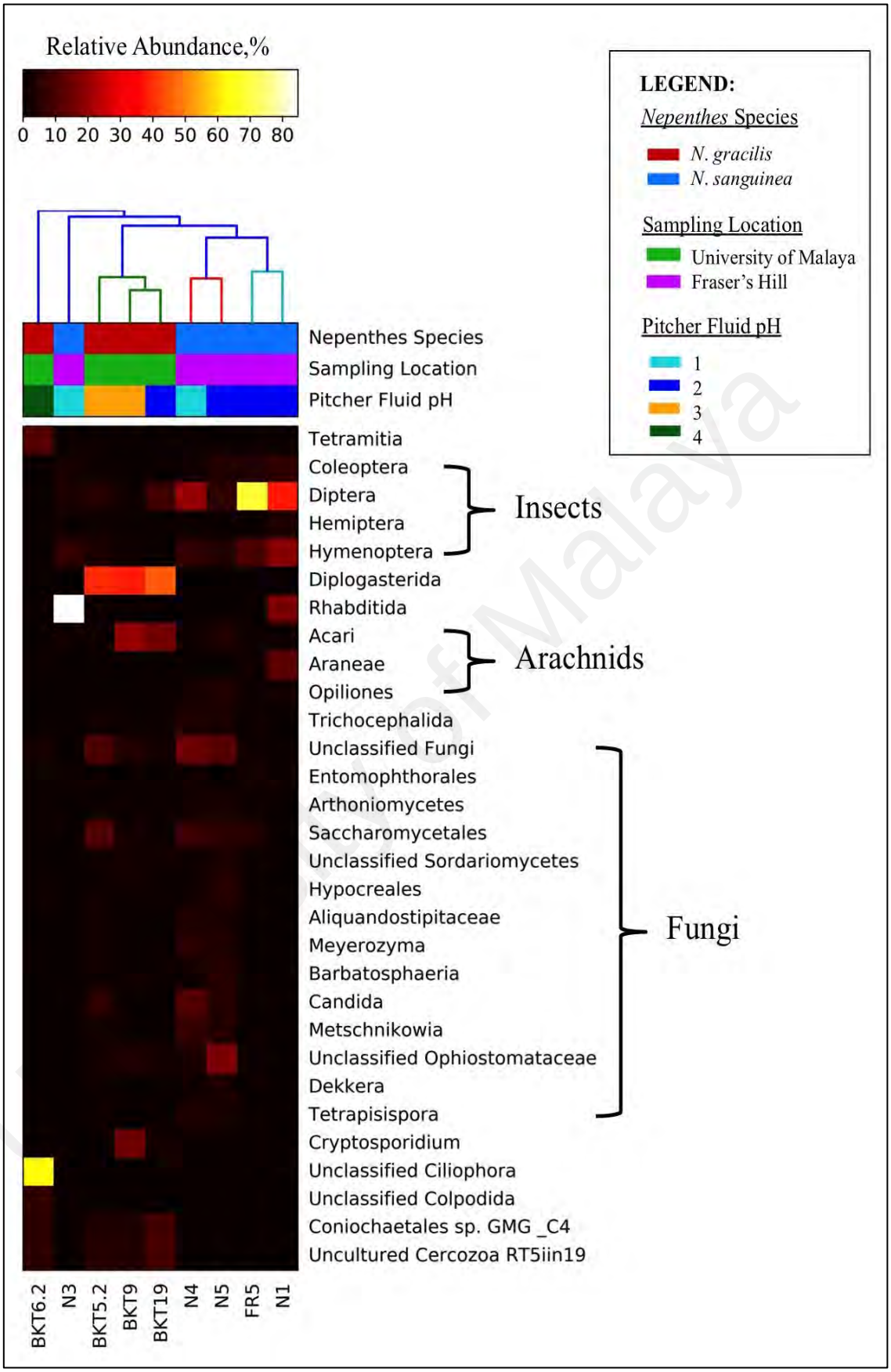


Figure 4.5: Eukaryote compositions in *Nepenthes* pitcher fluid

4.7 Virus Community Composition

Viral community composition of *Nepenthes* pitcher fluid samples was accessed using metagenomic data. Hierarchical clustering based on Bray-Curtis dissimilarity metrics on viral community did not cluster the *Nepenthes* pitcher fluid samples by *Nepenthes* species, sampling location and pitcher fluid pitcher (Figure 4.6).

Dasheen mosaic virus was the dominant virus in the viral community across the samples (Figure 4.6). It was the predominant virus in the viral community of samples BKT5.2 (100.0%), BKT6.2 (73.8%) and FR5 (100.0%) (Figure 4.6 and Appendix F). The viral community of samples BKT5.2 and FR5 were composed of only Dasheen mosaic virus (Figure 4.6). Besides, Bombyx mori nucleopolyhedrovirus was the predominant virus in the viral community of *N. sanguinea* samples N3, N4 and N5 (Figure 4.6). It formed 53.6%, 63.4% and 61.1% of the viral community in samples N3, N4 and N5 (Figure 4.6 and Appendix F). Vicia cryptic virus was predominant in the viral community of samples BKT9, BKT19 and FR5 (Figure 4.6). The relative abundance of Vicia cryptic virus was 57.9%, 53.3% and 84.1% in the viral community of samples BKT9, BKT19 and FR5, respectively (Figure 4.6 and Appendix F).

Alternanthera yellow vein betasatellite and unclassified Bcepmu-like virus were only found in sample BKT6.2 collected from *N. gracilis* (Figure 4.6). The relative abundance of Alternanthera yellow vein betasatellite and unclassified Bcepmu-like virus in the viral community of sample BKT6.2 were 22.0% and 4.2%, respectively (Appendix F). Cyclovirus NGchicken15 NGA 2009, Begomovirus associated DNA III, Malvastrum leaf curl Philippines betasatelite, Vernonia yellow vein Fujian virus betasatelite and Ageratum yellow vein Singapore alphasatellite were only found in sample BKT19 (Figure 4.6). Their relative abundances were less than 4% in the viral community of

sample BKT19 (Appendix F). The relative abundance of unclassified C2-like virus was not significant in sample BKT19 (0.3%) (Appendix F). It was not found in the other samples in this study (Figure 4.6).

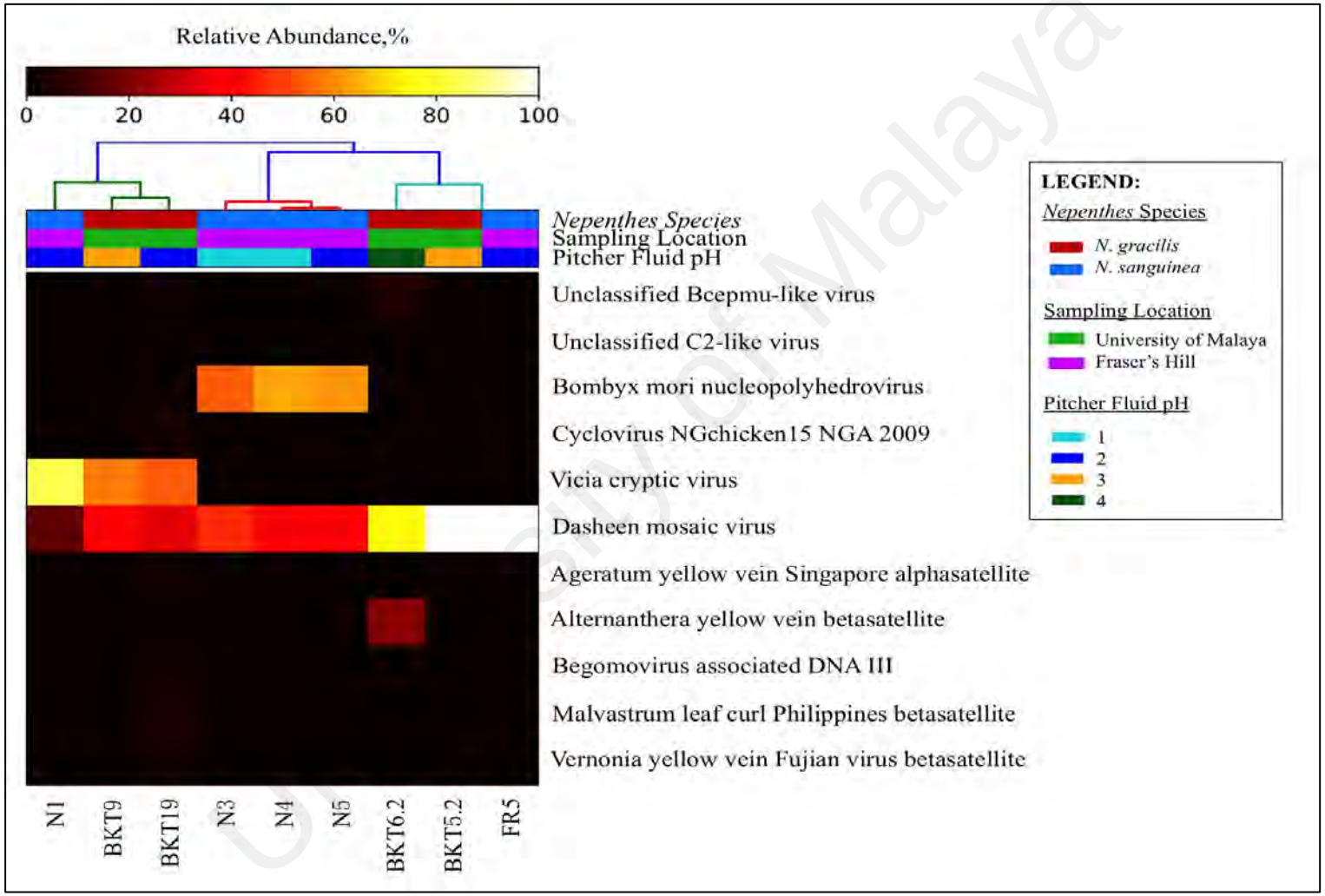


Figure 4.6: Virus community compositions in *Nepenthes* pitcher fluids

4.8 Shotgun Metagenomics

Shotgun metagenomics data were generated from samples of *N. sanguinea* (samples FR5, N1, N3, N4 and N5) and *N. gracilis* (samples BKT5.2, BKT6.2, BKT9 and BKT19) (Table 3.1). In this study, 227,243,404 to 444,773,550 of NGS raw reads were generated using HiSeq platforms (Table 4.5). Among the metagomic samples, the raw read count was lowest for sample BKT9 (227,243,404) and highest for sample N4 (444,773,550) (Table 4.5). After quality trimming at Phred score 33 using Trimmomatic, 219,058,532 to 369,939,880 trimmed reads were proceeded to *de novo* assembly using Velvet (Table 4.5). The numbers of quality sequence was lowest for sample BKT9 (219,058,532) and highest for sample FR5 (369,939,880) (Table 4.5). Gene prediction using MetaGeneMark have found 21,093 to 408,756 coding DNA sequence (CDS) from the metagenomics samples (Table 4.5). Number of CDS was lowest in sample N3 (21,093 CDS) and highest in sample BKT19 (408,756) (Table 4.5). The raw data of these metagenomic studies were deposited in NCBI SRA. The SRA accession number for the metagenomic raw data were SRP131938.

Table 4.5: Numbers of raw reads, quality filtered reads and CDS, The sequencing platforms and SRA accession number of the metagenomic samples.

Sample	Number of Raw Reads	Number of Trimmed Reads	CDS	Sequencing Platform	Accession Number
N1	376,844,084	290,063,023	53,308	Hiseq2000	SRP131938
N3	329,559,331	258,452,467	21,093	Hiseq2000	SRP131938
N4	444,773,550	328,736,754	31,664	Hiseq2000	SRP131938
N5	315,230,324	263,503,712	57,555	Hiseq2000	SRP131938
FR5	435,476,003	369,939,880	49,338	Hiseq2000	SRP131938
BKT5.2	285,100,948	272,784,742	84,191	Hiseq2500	SRP131938
BKT6.2	238,291,144	227,797,970	273,192	Hiseq2500	SRP131938
BKT9	227,243,404	219,058,532	250,000	Hiseq2500	SRP131938
BKT19	290,171,756	280,234,010	408,756	Hiseq2500	SRP131938

Taxonomy profiles were generated from total shotgun metagenomics data. The UPGMA tree constructed using Bray-Curtis distance metrics by samples taxonomy profiles showed that the samples were clustered by *Nepenthes* species except sample BKT5.2 (Figure 4.7). Within *N. sanguinea* samples, the taxonomy similarity was highest with between samples FR5 and N5, followed by samples N4, N3 and N1 (Figure 4.7). On the other hand, *N. gracilis* sample BKT19 has the highest taxonomy similarity with sample BKT19 followed by sample BKT6.2 (Figure 4.7). Sample BKT5.2 collected from *N. gracilis* was clustered with samples collected from *N. sanguinea* instead of samples collected from *N. gracilis* Figure 4.7).

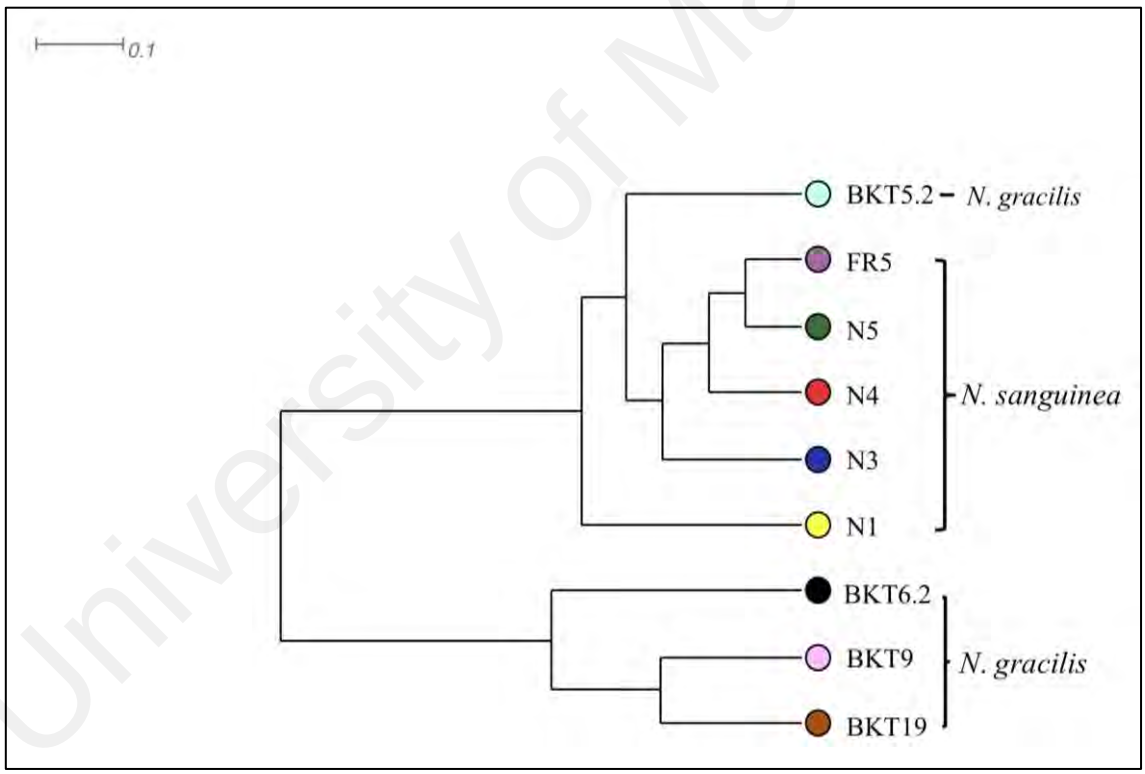


Figure 4.7: UPGMA tree constructed using Bray-Curtis distance metrics by sample taxonomy profile. Samples were clustered by *Nepenthes* species except sample BKT5.2

4.9 Genes Functions using SEED Database

Annotated gene sequences from shotgun metagenomic samples were classified into 43 subsystems based on their functional roles using SEED database (Figure 4.8). Fifteen percent of the total gene sequences were not classified using SEED database. Pairwise comparison showed that SEED subsystem profiles between samples were highly similar and positively correlated (Appendix G). Majority of the gene sequences were classified into subsystems for amino acids and derivatives metabolism, carbohydrates metabolism, cofactors, vitamins, prosthetic groups and pigments metabolism, protein metabolism, fatty acids, lipids and isoprenoids metabolism, cell wall and capsule formation, DNA and RNA metabolism, stress responses, virulence and respiration (Figure 4.8). Within carbohydrates metabolism subsystem, further study was performed on the genes involved in chitin, *N*-acetylglucosamine and xylose utilization. Besides, peptidase-coding genes in protein metabolism subsystem was also assessed.

Further study on chitin, chitobiose, and *N*-acetylglucosamine utilization subsystem showed that gene coded for chitinase, the main enzyme in chitin catabolism, was found in all samples (Figure 4.9a). Chitobiose and *N*-acetylglucosamine, which are the products of chitin catabolism, were further degraded in chitobiose and *N*-acetylglucosamine utilization pathways. α-*N*-acetylglucosaminidase, which degrades chitobiose into *N*-acetylglucosamine, was found in all *N. gracilis* samples except sample BKT6.2 (Figure 4.9a). In contrary, α-*N*-acetylglucosaminidase-coding gene was not found in *N. sanguinea* samples except sample FR5 (Figure 4.9a). Gene coded for *N*,*N'*-diacetylchitobiose utilization operon protein, YdiC was only found in sample N1 (Figure 4.9a). The genes coded for the main enzymes *N*-acetylglucosamine kinase 2 repressor, ORF and kinases (ROK) family, *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and glucosamine-6-phosphate

deaminase [isomerizing] which were responsible for *N*-acetylglucosamine utilization, were found in samples BKT5.2, BKT6.2, BKT19, FR5, N1 and N5 (Figure 4.9a). Besides, genes coded for sugar-binding protein, permease proteins 1 and 2 in *N*-acetyl-D-glucosamine ABC transport system, *N*-acetylglucosamine-regulated TonB-dependent outer membrane receptor, *N*-acetylglucosamine-regulated outer membrane porin and *N*-acetylglucosamine transporter, NagP, which were responsible for *N*-acetylglucosamine transportation were detected (Figure 4.9a). Other gene involved in *N*-acetylglucosamine utilization includes genes coded for *N*-acetylglucosamine-specific IIB component in carbohydrate phosphotransferase (PTS) system, *N*-acetylglucosamine-6-phosphate-responsive transcriptional repressor, NagC, and putative transcriptional regulator of *N*-acetylglucosamine utilization in LacI (Figure 4.9a).

In xylose utilization subsystem, α-xylosidase-coding gene was found in *N. gracilis* samples, while β-xylosidase-coding gene was found in both samples from *N. gracilis* and *N. sanguinea* (Figure 4.9b). In xylose utilization, α-xylosidase and β-xylosidase hydrolysed the α and β glycosidic linkage of xylan to produces xylose. Besides, genes coded for xylose isomerase and xylulose kinase, were found in the all samples (Figure 4.9b). In xylose utilization through isomerase pathway, xylose isomerase catalyzed the inter-conversion of D-xylose and D-xylulose (Karhumaa et al., 2007). The xylulose was further degraded into D-xylulose-5-phosphate by xylulose kinase (Karhumaa et al., 2007; Wenger et al., 2010). Alternatively, xylose can be utilized to 2-ketoglutarate by Weimberg pathway using D-xylose dehydrogenase, xylonolactonase, xylonate dehydratase and 2-ketoglutaric semialdehyde dehydrogenase. Xylonolactonase and xylonate dehydratase-coding gene was found in sample FR5 and sample BKT6.2, respectively (Figure 4.9b). The gene coded for 2-ketoglutaric semialdehyde dehydrogenase was found in all samples (Figure 4.9b). In addition, genes coded for

periplasmic xylose-binding protein, XylF and permease protein, XylH in xylose ABC transporter system, D-xylose transport ATP-binding protein, XylG and D-xylose proton-symporter, XylE, were detected in this study (Figure 4.9b). Putative xylulose kinase and xylose-responsive transcription regulator (ROK family) genes were only found in sample N3 and sample N5, respectively (Figure 4.9b). Endo-1,4-β-xylanase A precursor was only found in sample BKT6.2 (Figure 4.9b).

In subsystem for protein metabolism, gene coded aminopeptidase (proline iminopeptidase) and metallocarboxypeptidase (D-alanyl-D-alanine carboxypeptidase) were presence in all samples (Figure 4.10). Dipeptidases-coding genes (aminoacyl-histidine dipeptidase (Peptidase D), alpha-aspartyl dipeptidase peptidase E and Xaa-Pro dipeptidase PepQ) were found in sample N1 (Figure 4.10). Besides sample N1, alpha-aspartyl dipeptidase peptidase E coding gene was also found in sample BKT19 (Figure 4.10).

On top of the dominant subsystems, predicted gene sequences were also classified into subsystems for central metabolism, secondary metabolism and the metabolism of aromatic compounds, nucleosides, nucleotides, sulfur, phosphorus, nitrogen, potassium, iron, thiamin, and nucleotide sugars (Figure 4.8). Genes responsible for membrane transportation processes, metabolite damage and reparation, regulation and cell signaling, cell division and cell cycle, motility and chemotaxis, iron acquisition, dormancy and sporulation, photosynthesis, autotrophy, phages, prophages transposable elements and plasmids, virulence, disease and defense mechanisms were found in all samples (Figure 4.8). Functional genes in subsystem for nitrogen metabolism was divided into subsystems for cyanate hydrolysis, dissimilatory nitrite reductase, nitrate and nitrite ammonification, nitric oxide synthase, nitrogen fixation with NifL,

nitrosative stress, allantoin utilization and ammonia assimilation (Appendix H). Further study was performed on enzymes for allantoin utilization pathway and ammonia assimilation in subsystem for nitrogen metabolism.

Genes coded for main enzymes (allantoinase, allantoate amidohydrolase, ureidoglycolate hydrolase and ureidoglycolate dehydrogenase) in allantoin utilization were found in the samples (Figure 4.11a). The allantoin degradation process turned allantoin into urea and glyoxylate, which were further degraded into ammonia and phosphoglycerates, respectively. Allantoin permease for allantoin cross membrane transportation was found in samples FR5 and N1 (Figure 4.11a). On the other hand, gene coded for main enzymes (glyoxylate carboligase, 2-hydroxy-3-oxopropionate reducatase and glycerate kinase) in glyoxylate degradation, were found in the samples (Figure 4.11a). Allophanate hydrolase and urea carboxylase, which were the main enzymes in urea degradation, were found in all samples (Figure 4.11a).

Further study on ammonia assimilation subsystem has found genes coding glutamine synthetase and glutamate synthase in all samples (Figure 4.11b). In ammonia assimilation, glutamine synthetase utilizes ammonia and glutamate to generate glutamine, which is an amino acid for protein biosynthesis. The gene coding for glutamine synthetase type I was found in all samples, while gene coding for glutamine synthetase type III, GluN was found in samples BKT19 and N4 (Figure 4.11b). On the other hand, the glutamine and oxoglutarate was converted into glutamate and supplied into ammonia assimilation pathway by glutamate synthase. Gene coding for large and small chain of NADPH-dependent glutamate synthase and ferredoxin-dependent glutamate synthase were found in all samples (Figure 4.11b). Putative gene for Glx chain of NADPH-dependent glutamate synthase was found in samples BKT5.2 and

BKT6.2 (Figure 4.11b). Besides, gene coding glutamine amidotransferase protein, GlxB, which removed ammonia from glutamine, was found in sample BKT6.2 (Figure 4.11b). Gene coded for nitrogen regulatory protein PII was found in all samples, while nitrogen regulation protein NR(I) was found in samples BKT6.2, BKT19, FR5 and N1 (Figure 4.11b). Gene coded for ammonium transporter, glutamate-ammonia-ligase adenylyltransferase were found in all samples (Figure 4.11b).

Lastly, genes coded for enzymes and protein in subsystems arabinoase sensor and transport module were only found in samples FR5, N1 and N5, while genes responsible for general stress responses and stationary phase responses were only found in samples BKT5.2, BKT19, FR5 and N1 (Figure 4.8). The genes responsible for plastidial (cyanobacterial) electron transport system were only found in samples BKT5.2, N1 and N5 (Figure 4.8). On the other hand, genes classified in subsystems for plants mitochondrial electron transport system, cell walls and outer surfaces formation and glucosinolates metabolism were found in all samples (Figure 4.8).

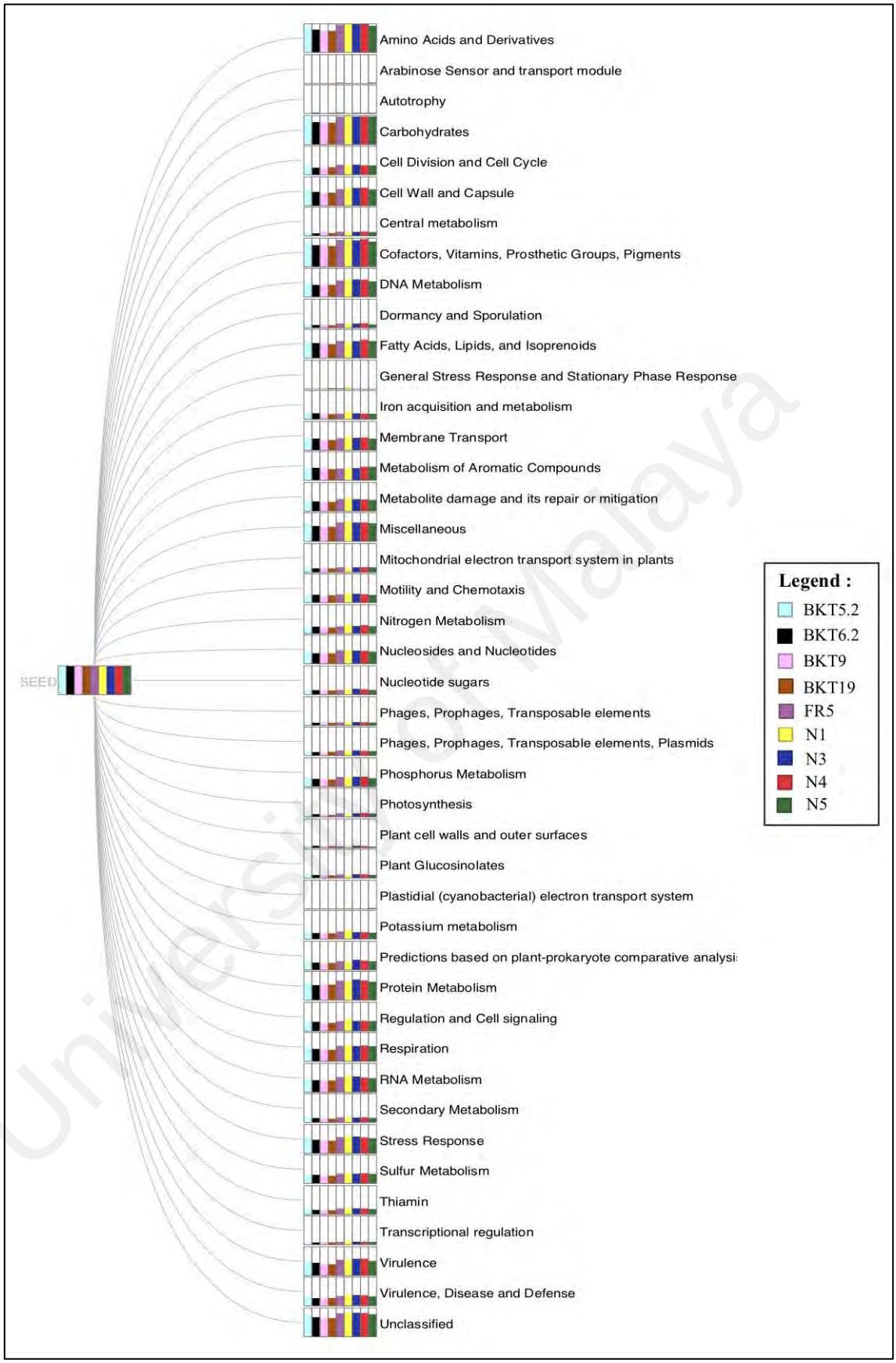
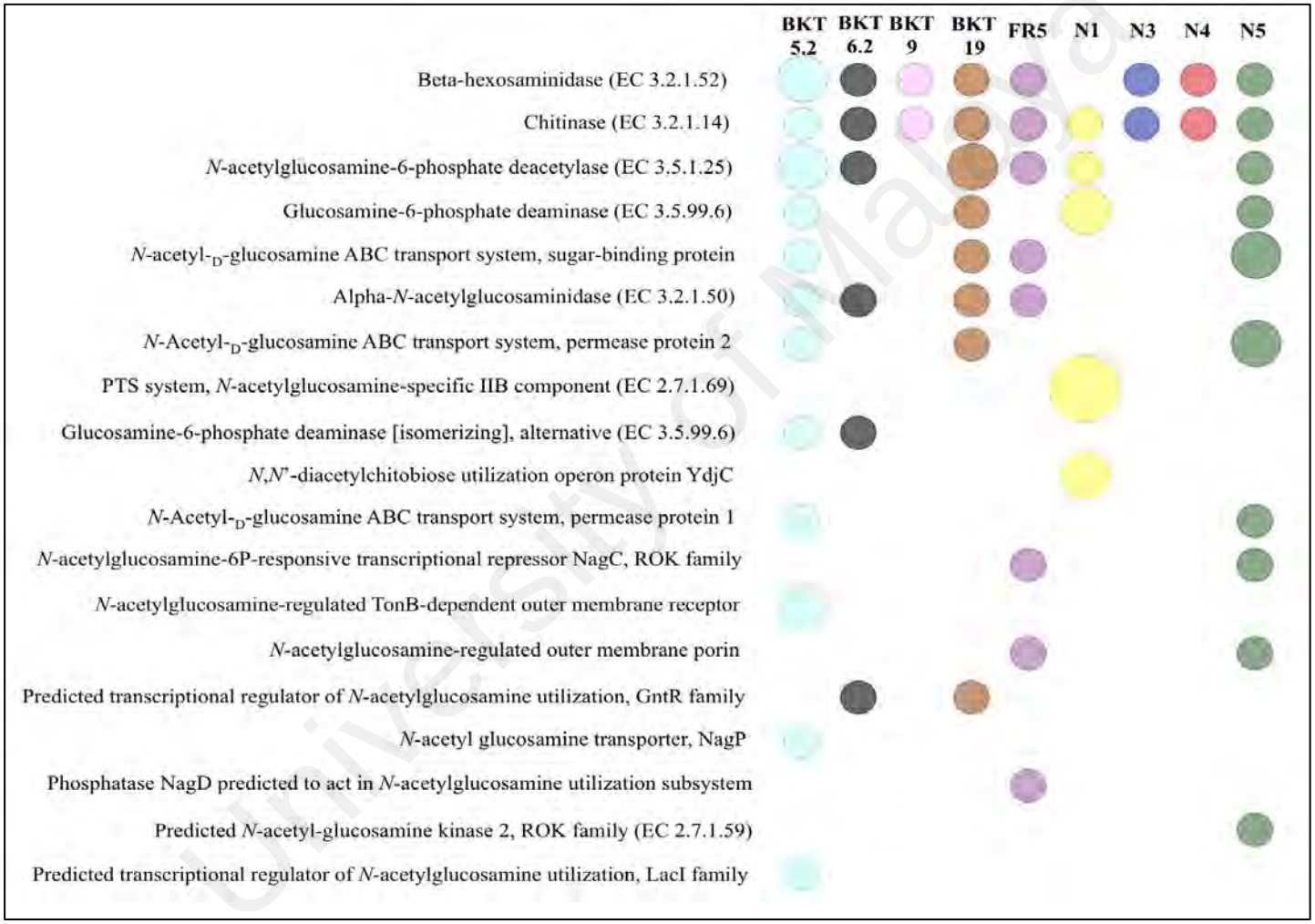
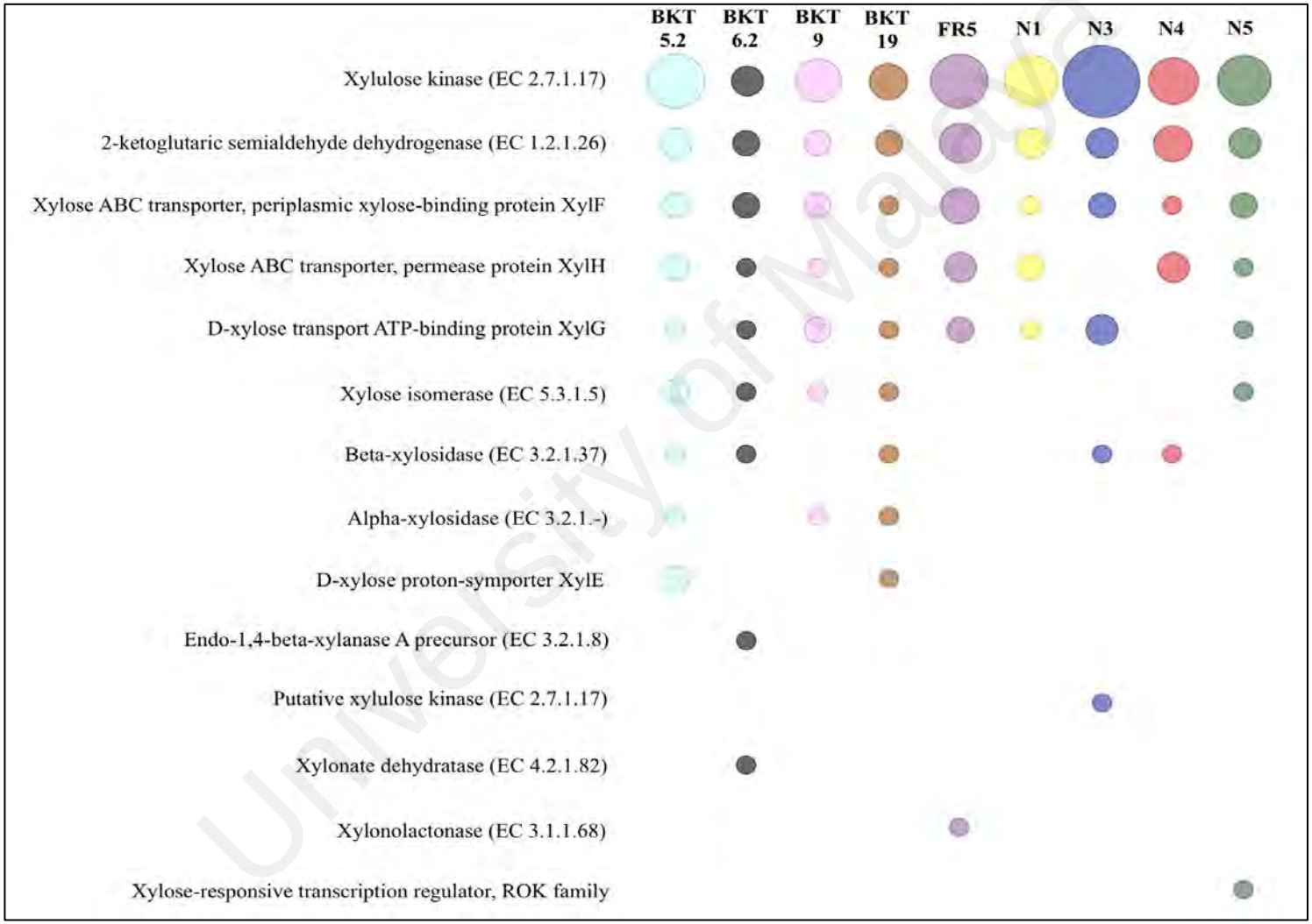


Figure 4.8: Subsystems of annotated gene sequences based on SEED database. Annotated gene sequence was assigned to subsystem based on its functional roles



(a)

Figure 4.9: Subsystem for carbohydrate utilization. Enzymes for (a) chitin and *N*-acetylglucosamine utilization and (b) xylose utilization. The abundance of the genes in the samples was indicated by the size of the circle



(b)

Figure 4.9, continued.

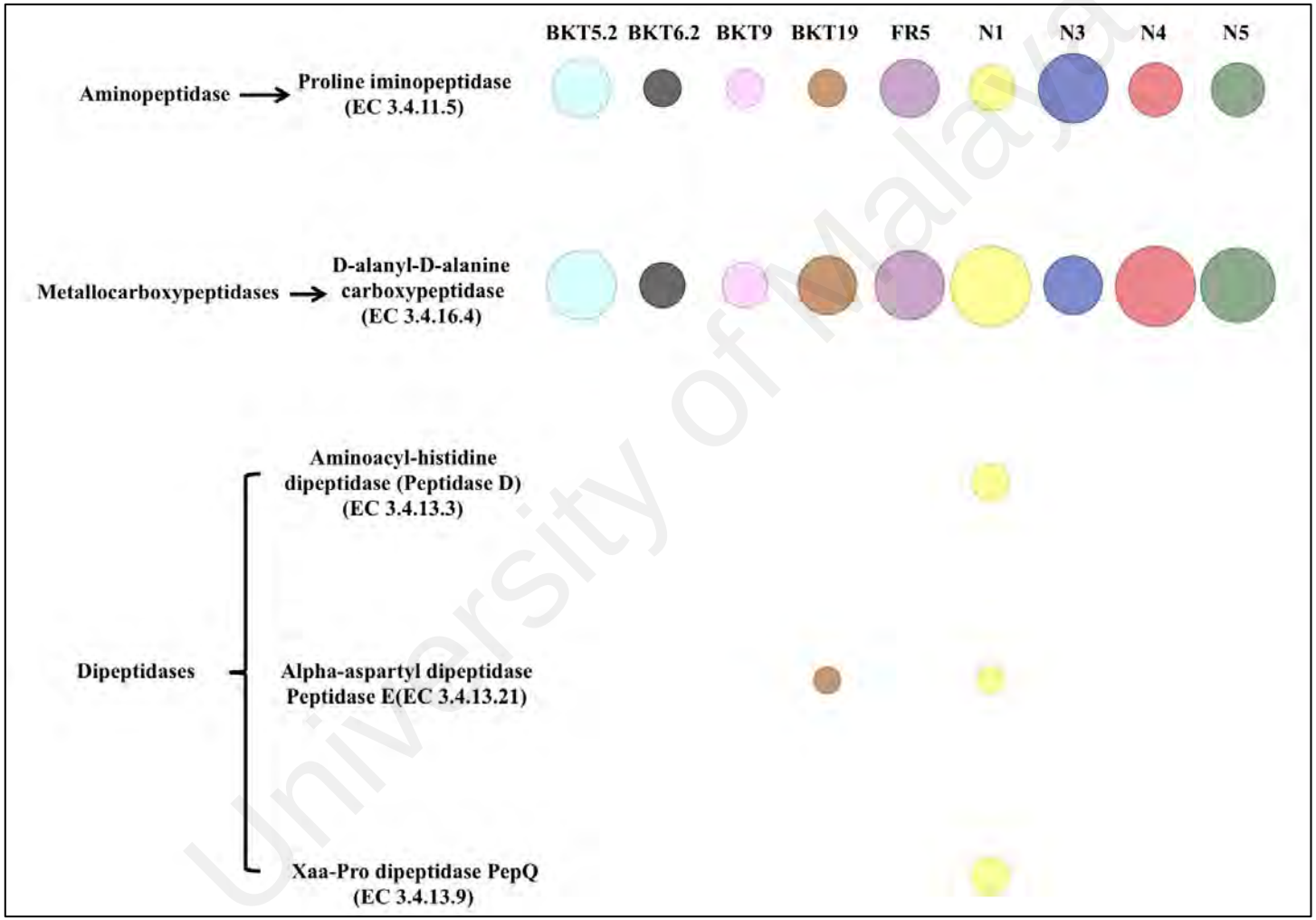
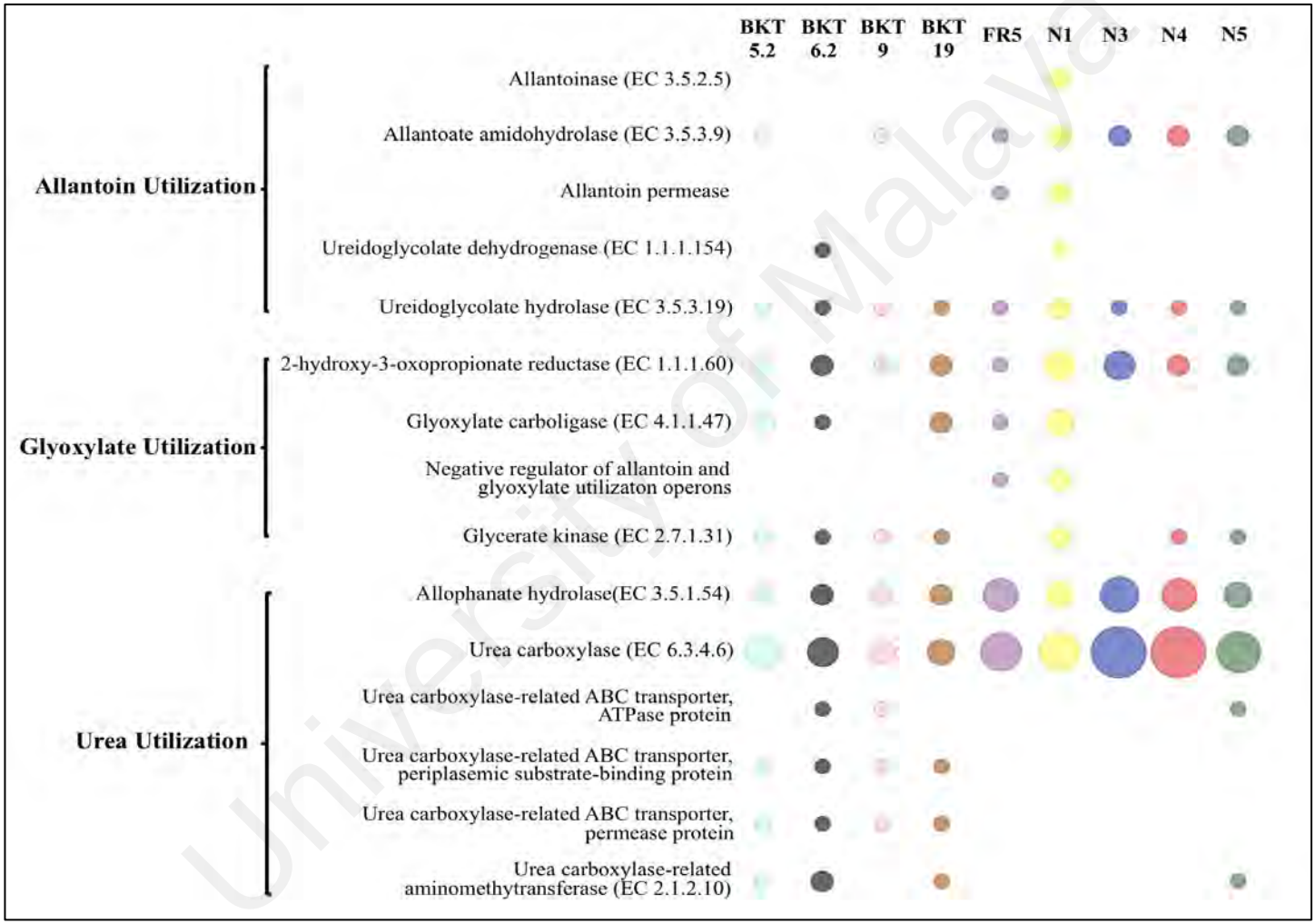
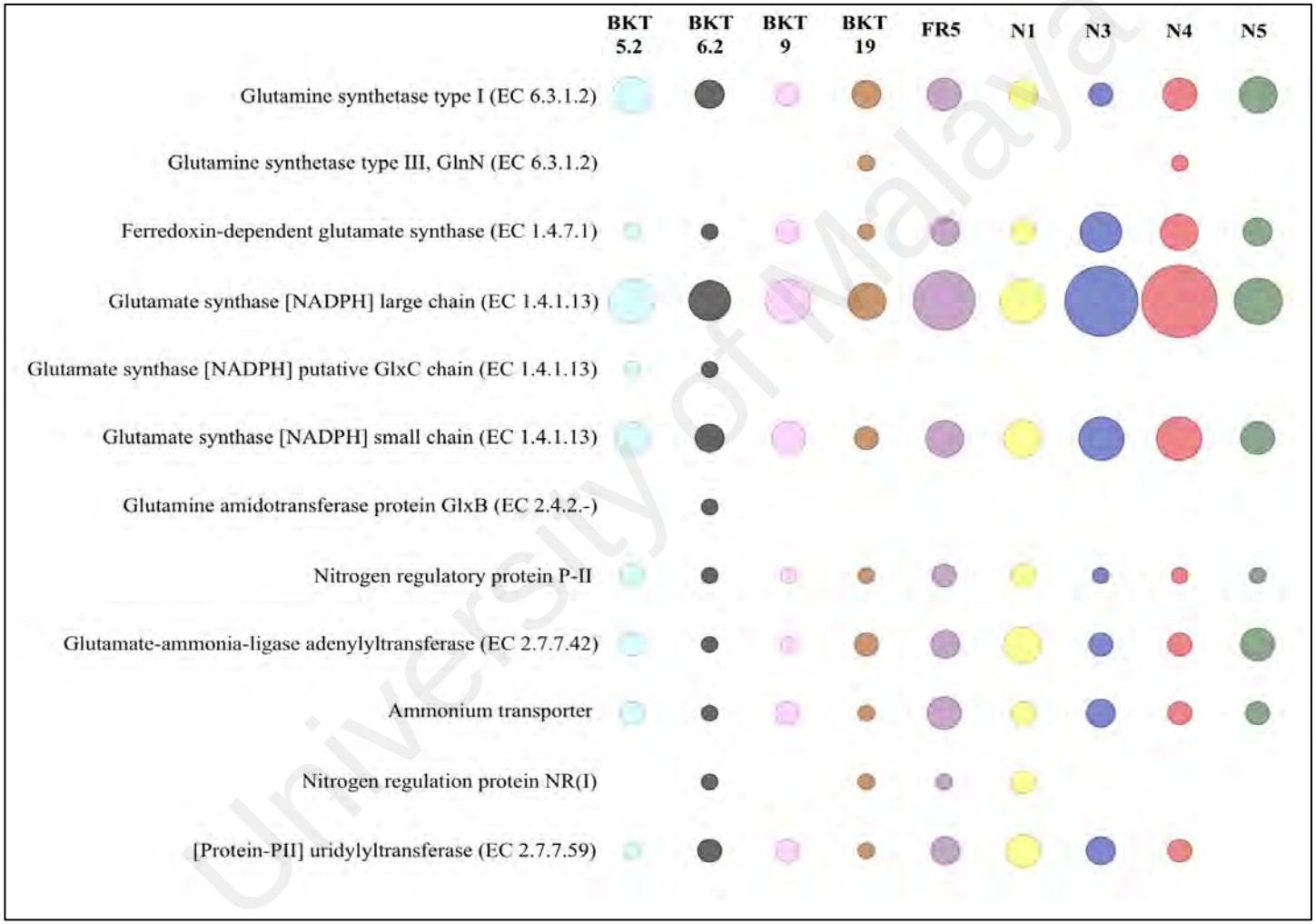


Figure 4.10: Aminopeptidase, metallocarboxypeptidase and dipeptidases from subsystem for protein metabolism. The abundance of the genes in the samples was indicated by the size of the circle



(a)

Figure 4.11: Subsystem for nitrogen metabolism. Enzymes for (a) allantoin, glyoxylate and urea utilization and (b) ammonia assimilation. The abundance of the genes in the samples was indicated by the size of the circle



(b)

Figure 4.11, continued.

4.10 Genes Functions using eggNOG Database

Annotated genes sequences were classified into clusters of orthologous group(s) (COG) for information storage and processing, cellular processes and signaling and metabolism using eggnog database (Figure 4.12). Within COG for information storage and processing, genes were further classified into 5 COGs (Figure 4.12). In COG for cellular processes and signaling, annotated gene sequences were further classified into 9 COGs (Figure 4.12). In COG for metabolism, annotated gene sequences were classified into COGs for energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, carbohydrate transport and metabolism, coenzyme transport and metabolism, lipid transport and metabolism, inorganic ion transport and metabolism, and secondary metabolites biosynthesis, transport and catabolism (Figure 4.12). In COG for carbohydrate transport and metabolism, genes coded for enzymes in chitin, starch, cellulose and xylose utilization were further studied. Besides, in COG for amino acid transport and metabolism, genes coded for peptidases and enzymes involved in ammonia and urea utilization were further studied.

Genes coded for chitinases, which catalyzed the degradation of chitin into chitobiose, chito-oligosaccharides and *N*-acetylglucosamine, were found in all samples (Figure 4.13). Genes coded for enzyme *N*-acetylglucosamine 6-phosphate deacetylase was found in samples BKT6.2 and BKT19, and Genes coded for enzyme glucose-6-phosphate deaminase was found in all samples except sample BKT9 (Figure 4.13). In *N*-acetylglucosamine utilization pathways, *N*-acetylglucosamine 6-phosphate deacetylase converts *N*-acetylglucosamine-6-phosphate to glucosamine-6-phosphate. Subsequently, glucosamine-6-phosphate can be converted to fructose-6-phosphate by glucose-6-phosphate deaminase. Besides, gene coded for cellulase, the key enzyme for cellulose utilization was found in sample BKT19 (Figure 4.13). In addition, gene coded

for beta-glucosidase for cellulose utilization was found in all samples (Figure 4.13). Gene coded for enzyme glycosidase, which catalyzed the hydrolysis of glycosidic bonds in chitin and cellulose degradation was found in sample BKT6.2 (Figure 4.13).

On the other hand, genes coded for xylose isomerase and carbohydrate kinase, which were the key enzymes in xylose utilization by isomerase pathway, were found in this study (Figure 4.13). In xylose utilization by isomerase pathway, xylose was converted into xylulose by xylose isomerase. Subsequently, xylulose was converted into xylulose-5-phosphate by kinase. The gene coding for alpha-amylase, that converts starch into glucose, was found in all samples (Figure 4.13).

In urea utilization pathway, urea and urea-1-carboxylate are degraded into ammonia and carbon dioxide by urea amidohyrolase and allophanate hydrolase, respectively. Genes coded for urea amidohyrolase alpha, beta and gamma subunits were mainly found in *N. gracilis* samples (Figure 4.14). Allophanate hydrolase subunit 1-coding gene was found in all samples and allophanate hydrolase subunit 2-coding gene was found in samples BKT5.2, BKT19 and N5 (Figure 4.14). Besides, gene coded for ureidoglycolate hydrolase, which converts ureidoglycolate into glyoxylate and urea was found in samples BKT5.2, FR5, N1, N3, N4 and N5 (Figure 4.14).

Genes coded for glutamate synthase, glutamine synthetase, and transaminase for ammonia assimilation was found in all samples (Figure 4.14). In ammonia assimilation pathway, glutamine synthetase catalyzes the conversion of ammonia and glutamate into glutamine. The glutamine was converted into glutamate by glutamate synthase and glutaminase and supplied into ammonia assimilation pathway. Besides, 2-oxoglutarate, which is also a product of glutamine synthetase activity, is also converted into

glutamate and succinate semialdehyde by transaminase. In addition, glutaminase-coding gene was found in samples BKT5.2, BKT19, FR5, N1 and N5 (Figure 4.14).

In COG for amino acid transport and metabolism, gene coded for peptidases and protease catalyzed the protein and peptides catabolism (Figure 4.15a). Gene coded for peptidase M24 was found in all samples, meanwhile gene coded for peptidases was only found in sample N1 (Figure 4.15a). Besides, genes coded for oligopeptidase, aminopeptidase, carboxypeptidase and proline iminopeptidase were found in all samples (Figure 4.15a). Dipeptidase-coding gene was found in all samples except sample BKT5.2 (Figure 4.15a). On top of COG for amino acid transport and metabolism, peptidases in COGs for cell wall control, cell division and chromosome partitioning, cell wall or membrane or cell envelope biogenesis, posttranslational modification, protein turnover and chaperones were found in all samples (Figure 4.15b).

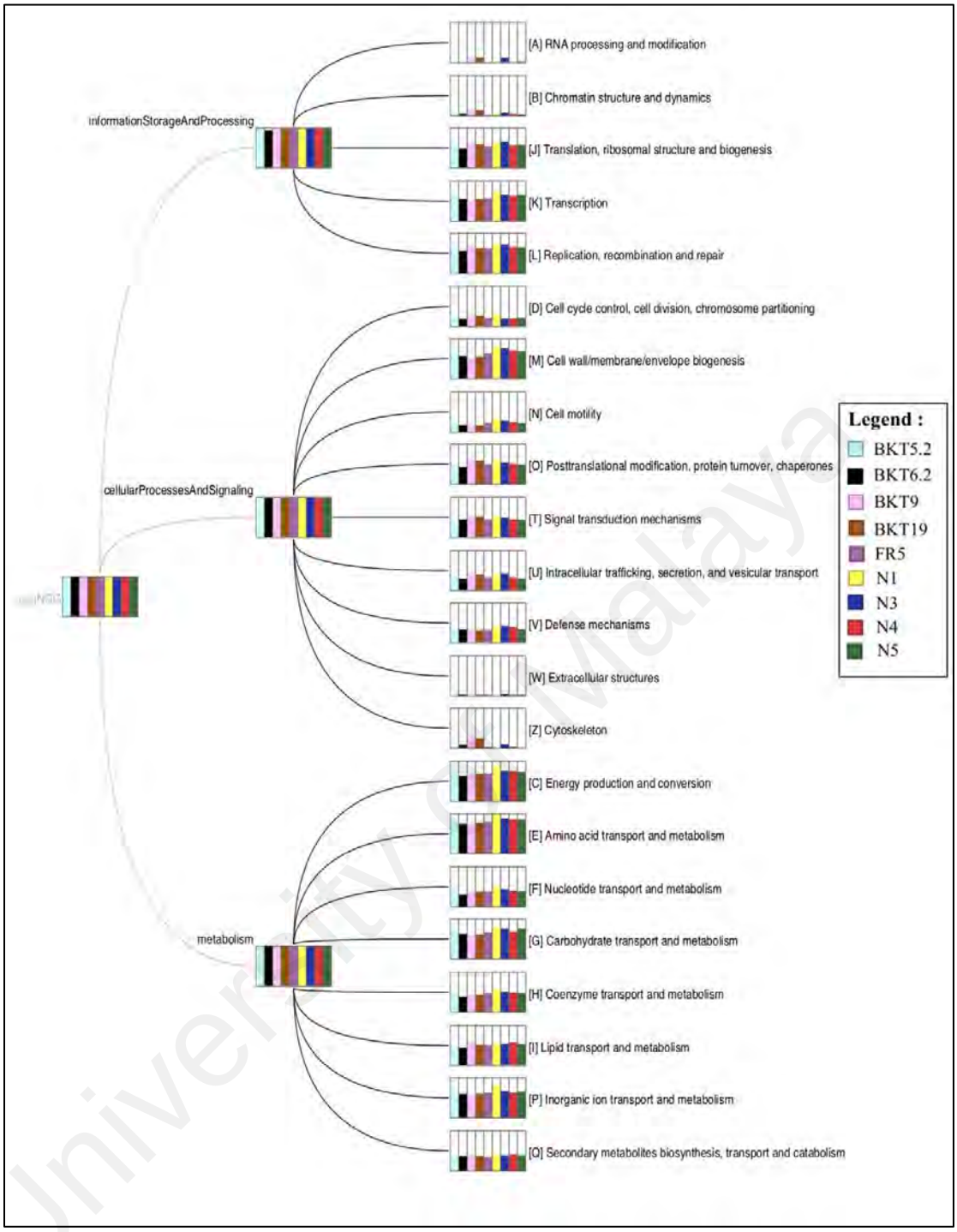


Figure 4.12: Gene functions by eggNOG classification. Annotated gene sequences were classified into COGs for information storage and processing, cellular processes and signaling and metabolism. The abundance of the genes in each COG was indicated by the height of the bar graph

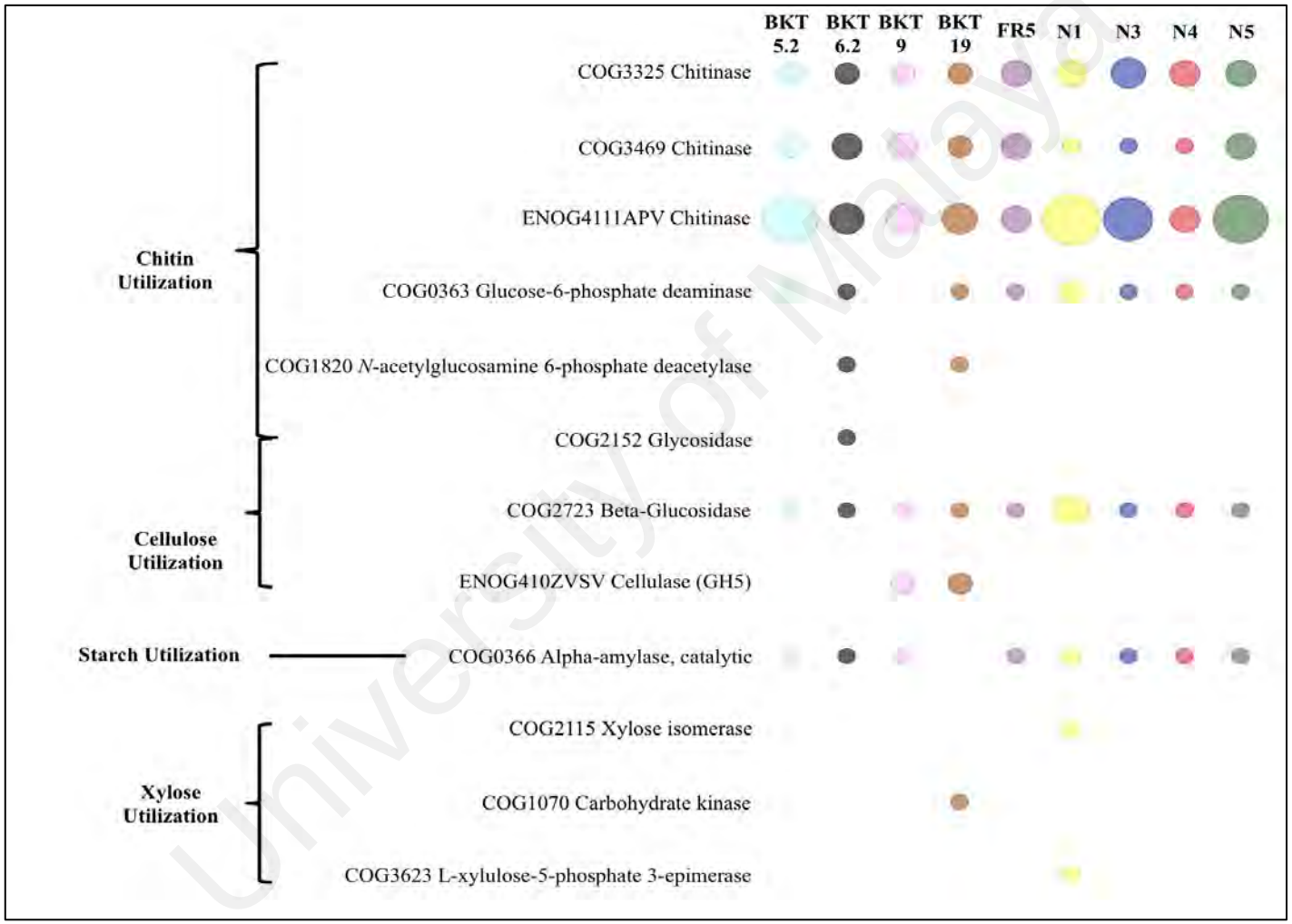


Figure 4.13: Enzymes for chitin, cellulose, starch and xylose utilization in COG for carbohydrate metabolism. The abundance of the genes in the samples was indicated by the size of the circle

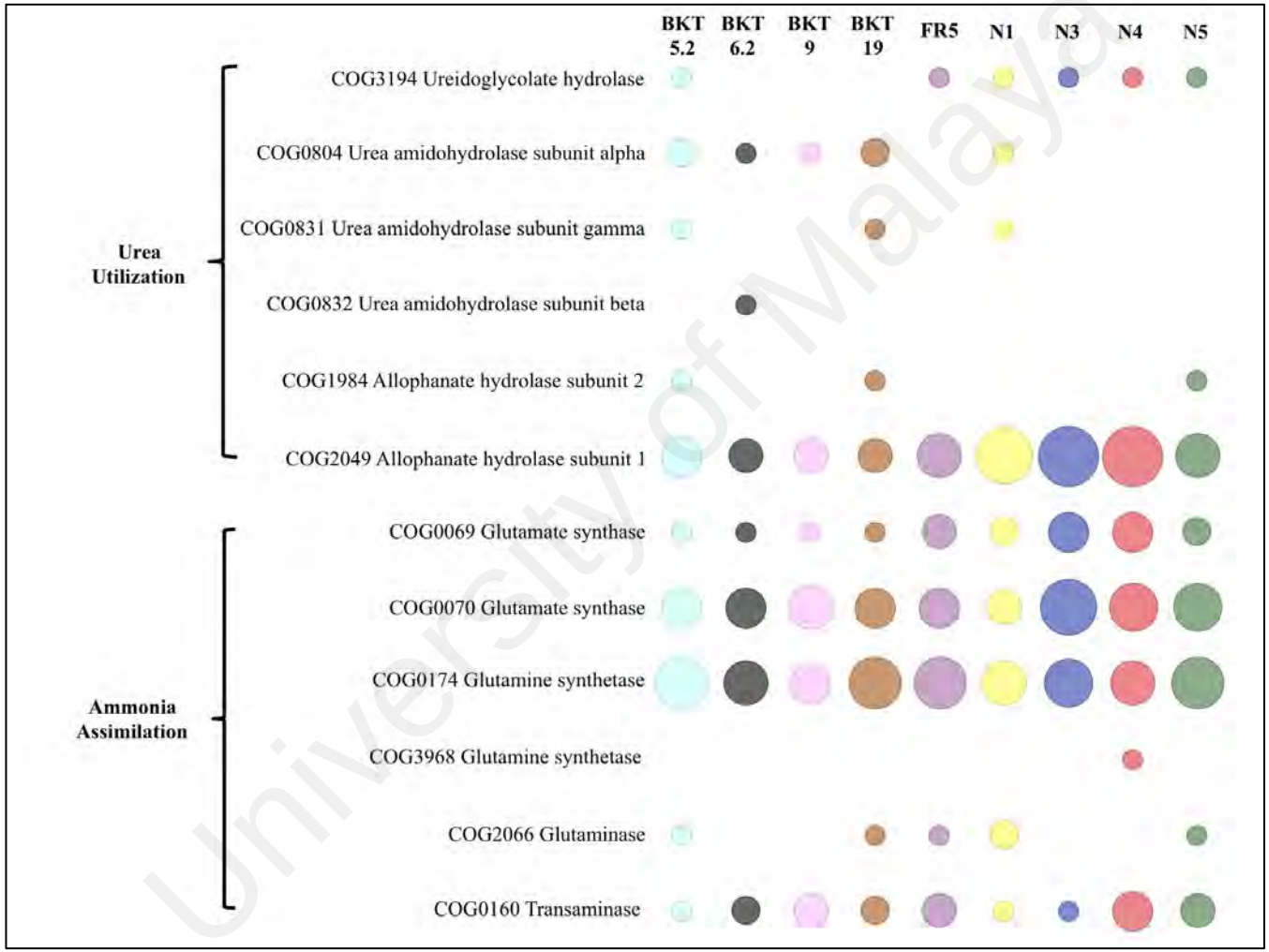
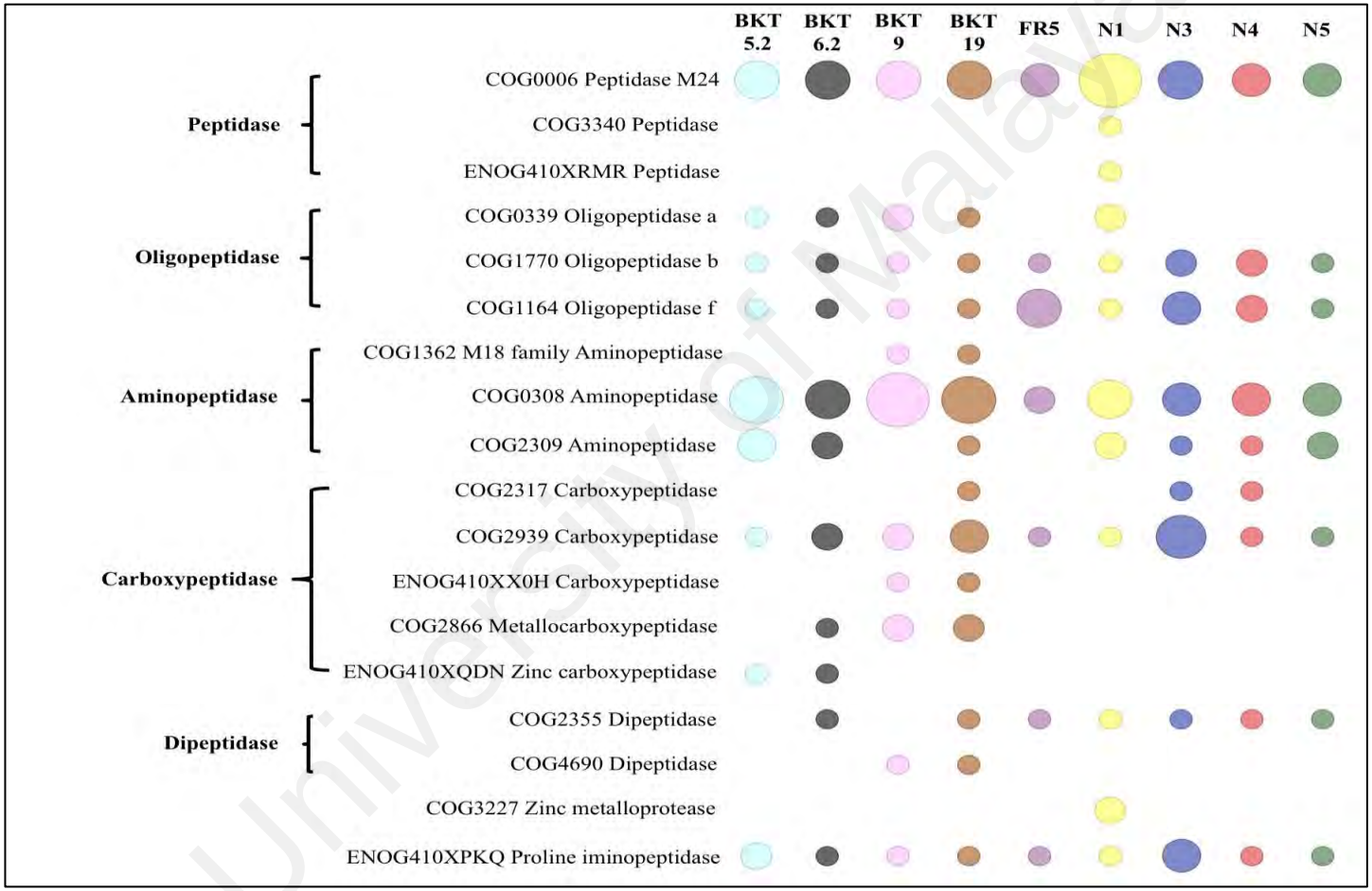
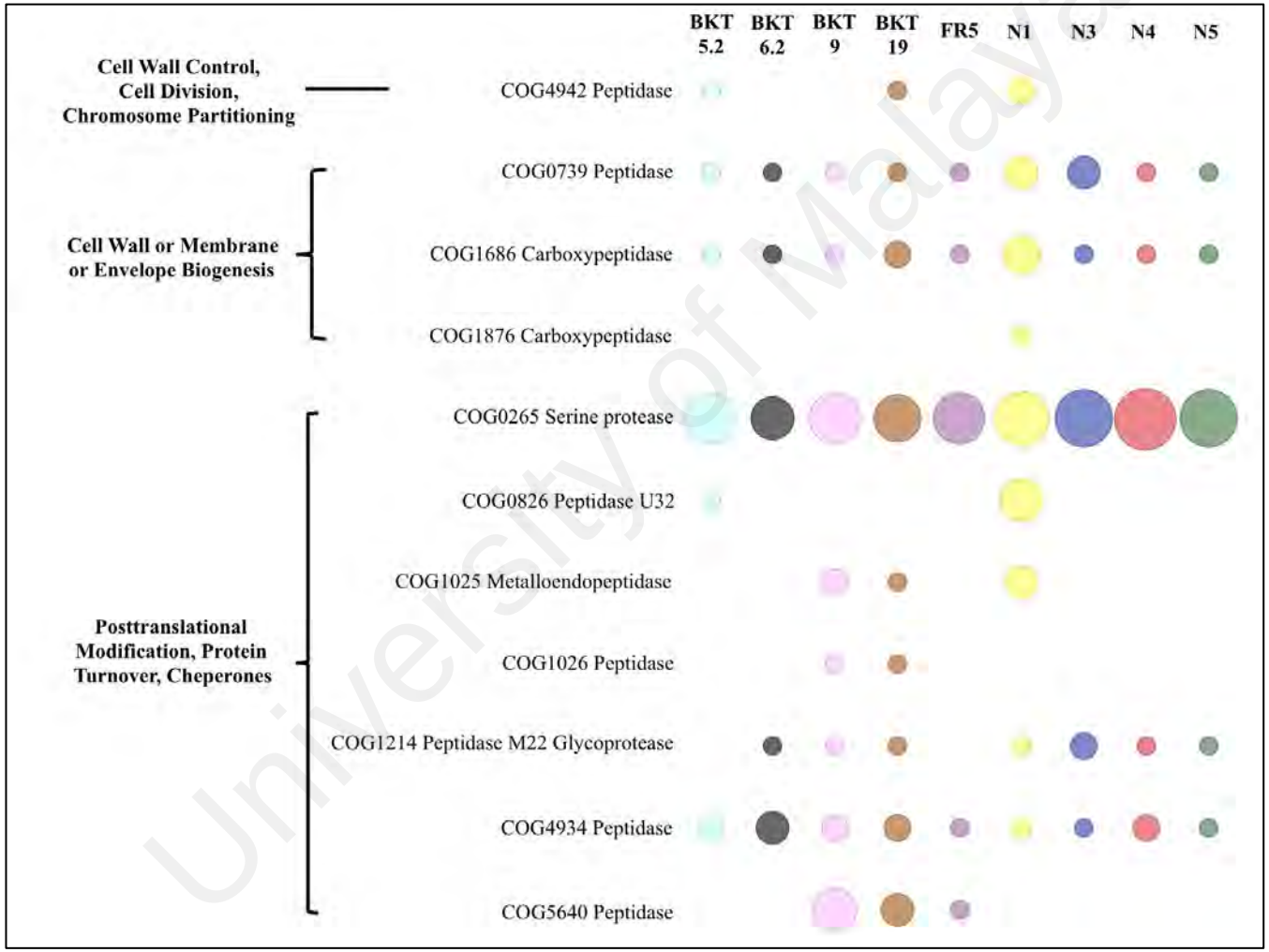


Figure 4.14: Enzymes for urea and ammonia utilization in COG for amino acid transport and metabolism. The abundance of the genes in the samples was indicated by the size of the circle



(a)

Figure 4.15: Protease and peptidases in a) COG for amino acid transport and metabolism and b) COGs for cell wall control, cell division and chromosome partitioning, cell wall, membrane or envelope biogenesis and posttranslational modification, protein turnover and cheperones. The abundance of the genes in the samples was indicated by the size of the circle



(b)

Figure 4.15, continued.

4.11 Genes Functions using InterPro2GO

InterPro2GO classified 86.86% of the annotated genes sequences in the normalized data into the 3 domains of Gene Ontology (GO), the biological process, cellular component and molecular function. The annotated gene sequences were assigned to 38 nodes in GO "biological process" domain, 19 nodes in GO "cellular component" domain and 19 nodes in GO "molecular function" domain (Figure 4.16 and Table 4.6). In GO "biological process" "cellular component" and "molecular function" domains, highest gene counts was observed in GO-terms transport, membrane and transferase activity, respectively (Table 4.6). InterPro2GO result shows that 13.14% of the annotated genes in the normalized data were assigned to InterPro families with no GO assignment.

GO-term "peptidase activity", a node of GO-term "hydrolase activity" in the GO "molecular function" domain was further studied. Genes coded for peptidases, proteases and proteins related to peptidase activities were classified into 67 InterPro families in GO-term "peptidase activity" (Figure 4.17). Proteases and metalloprotease for protein and peptide catabolism were found in all samples (Figure 4.17). Besides, wide range of peptidases, includes carboxypeptidases, aminopeptidases, metallopeptidases, dipeptidases, oligopeptidases, endopeptidases and peptidase were found in the samples (Figure 4.17). This includes gene coded for aspartic peptidase, which was found in samples N3, BKT9 and BKT19 (Figure 4.17).

Gene coded for bacteria chito-oligosaccharide deacetylase, ChbG, which catalyzed the removal of an acetyl group from acetylated chito-oligosaccharides, was found in sample N1 (Figure 4.18). Besides, gene coded for exo-beta-D-glucosaminidase, which hydrolysed chitosan or chito-oligosaccharides in chitin degradation was found in all

samples (Figure 4.18). Genes coded for *N*-acetyl-D-glucosamine kinase, *N*-acetylglucosamine-6-phosphate deacetylase and *N*-acetylglucosamine-6-phosphate deacetylase, which are the key enzymes in N-acetylglucosamine utilization, were found in this study (Figure 4.18). On the other hand, gene coded for 1,4-beta cellobiodyrolase, which degrades cellulose, was found in samples N4 and N5 (Figure 4.18). Gene coded for xylulokinase and bacterial xylose isomerase, which were they key enzymes in xylose degradation through isomerase pathway were also detected in all samples (except sample N4, where gene coded for xylulokinase was not found) (Figure 4.18).

Gene coded for allantoinase, which converts allantoin into allantoate was found in samples BKT6.2, FR5, N1 and N5, and gene coded for allantoicase, which converts allantoate into ureidoglycine were found in sample BKT19 (Figure 4.19a). Besides, gene coded for allantoate amidohydrolase which converts allantoate to ureidoglycolate was found in samples FR5, N1 and N5 (Figure 4.19a). Gene coded for bacterial ureidoglycolase lyase, which degrades ureidoglycolate into glyoxylate and urea, was found in all samples (Figure 4.19a). Genes coded for glyoxylate carboligase and glycerate kinase, which involved in the degradation of glyoxylate into phosphoglycerate were found in all samples (except glyoxylate carboligase was not found samples BKT9 and N4 and glycerate kinase was not found in sample N4) (Figure 4.19a). In addition, urea produces from allantoin degradation was converted into ammonia and carbon dioxide by urease. Urease and urease accessory protein coding genes were found in all samples except samples FR5 and N4 (Figure 4.19b). The ammonia was utilized by glutamine synthetase during the conversion of glutamate into glutamine in ammonia assimilation pathway. Gene coded for glutamine synthethase type I was found in all samples except sample N3, while glutamine synthethase type III was found in samples BKT5.2, BKT6.2, BKT19 and FR5 (Figure 4.19c). In addition glutamate synthase,

which converts glutamine into glutamate and supplied glutamate to ammonia assimilation pathway were detected in all samples (Figure 4.19c).

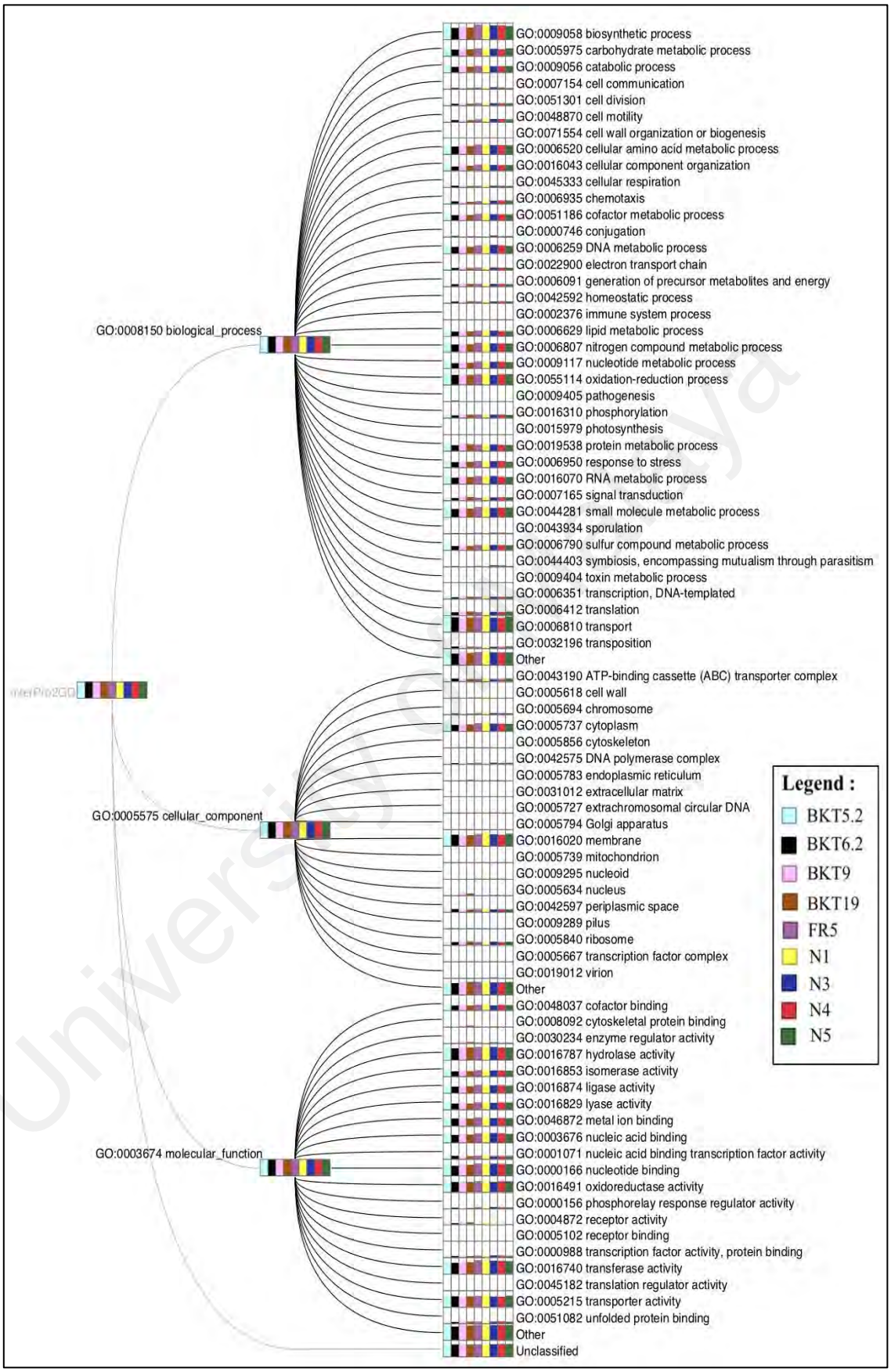


Figure 4.16: Classification of gene functions using InterPro database. GO second-tier nodes in GO "biological process", "cellular component" and "molecular function" domains

Table 4.6: Gene counts in each GO-term of GO "biological process", " cellular component" and "molecular function" domains.

GO Domain	GO-term	Number of Genes
Biological Process	GO:0006810 Transport	11,991
	GO:0009058 Biosynthetic process	9,873
	GO:0055114 Oxidation-reduction process	6,225
	GO:0044281 Small molecule metabolic process	4,821
	GO:0006807 Nitrogen compound metabolic process	4,681
	GO:0006520 Cellular amino acid metabolic process	4,560
	GO:0006935 DNA metabolic process	3,367
	GO:0016070 RNA metabolic process	3,166
	GO:0005975 Carbohydrate metabolic process	2,875
	GO:0019538 Protein metabolic process	2,355
	GO:0009056 Catabolic process	2,259
	GO:0009117 Nucleotide metabolic process	2,205
	GO:0006950 Response to stress	2,129
	GO:0051186 Cofactor metabolic process	2,077
	GO:0016043 Cellular component organization	1,834
	GO:0006629 Lipid metabolic process	1,530
	GO:0006790 Sulfur compound metabolic process	1,398
	GO:0006412 Translation	757
	GO:0007165 Signal transduction	672
	GO:0016310 Phosphorylation	628
	GO:0006935 Chemotaxis	519
	GO:0048870 Cell motility	449
	GO:0006091 Generation of precursor metabolites and energy	447
	GO:0022900 Electron transport chain	336
	GO:0051301 Cell division	314
	GO:0006351 Transcription, DNA-templated	286
	GO:0032196 Transposition	216
	GO:0042592 Homeostatic process	206
	GO:0045333 Cellular respiration	185
	GO:0007154 Cell communication	138
	GO:0000746 Conjugation	77
	GO:0044403 Symbiosis, encompassing mutualism through parasitism	59
	GO:0043934 Sporulation	55
	GO:0009404 Toxin metabolic process	35
	GO:0009405 Pathogenesis	17
	GO:0071554 Cell wall organization or biogenesis	15
	GO: 0002376 Immune system process	1
	GO:0015979 Photosynthesis	1
	Other	8,687

Table 4.6, continued.

GO Domain	GO-term	Number of Genes
Cellular component	GO:0016020 Membrane	7,478
	GO:0005737 Cytoplasm	3,063
	GO:0005840 Ribosome	690
	GO:0042597 Periplasmic space	502
	GO:0043139 ATP-Binding cassette(ABC) transport complex	456
	GO:0005694 Chromosome	139
	GO:0042575 DNA Polymerase complex	123
	GO:0005634 Nucleus	101
	GO:0005618 Cell wall	33
	GO:0009295 Nucleoid	20
	GO:0005783 Endoplasmic reticulum	18
	GO:0031012 Extracellular matrix	13
	GO:0019012 Virion	9
	GO:0009289 Pilus	8
	GO:0005667 Transcription factor complex	7
	GO:0005794 Mitochondrion	5
	GO:0005856 Cytoskeleton	4
	GO:0005727 Extrachromosomal circular DNA	4
	GO:0005794 Golgi Apparatus	3
	Other	8,913
Molecular Function	GO:0016740 Transferase activity	9,111
	GO:0016787 Hydrolase activity	9,098
	GO:0000166 Nucleotide binding	7,571
	GO:0005215 Transporter activity	6,772
	GO:0016491 Oxidoreductase activity	6,574
	GO:0003676 Nucleic acid binding	4,452
	GO:0046872 Metal ion binding	3,692
	GO:0016874 Ligase activity	3,192
	GO:0016829 Lyase activity	2,629
	GO:0048037 Cofactor binding	2,256
	GO:0016853 Isomerase activity	2,019
	GO:0001071 Nucleic acid binding transcription factor activity	643
	GO:0051082 Unfold protein binding	276
	GO:0000988 Transcription factor activity, protein binding	215
	GO:0000156 Phosphorelay response regulator activity	130
	GO:0030234 Enzyme regulator activity	110
	GO:0008092 Cytoskeletal protein binding	32
	GO:0045182 Translation regulator activity	4
	GO:0004872 Receptor binding	2
	Other	12,363

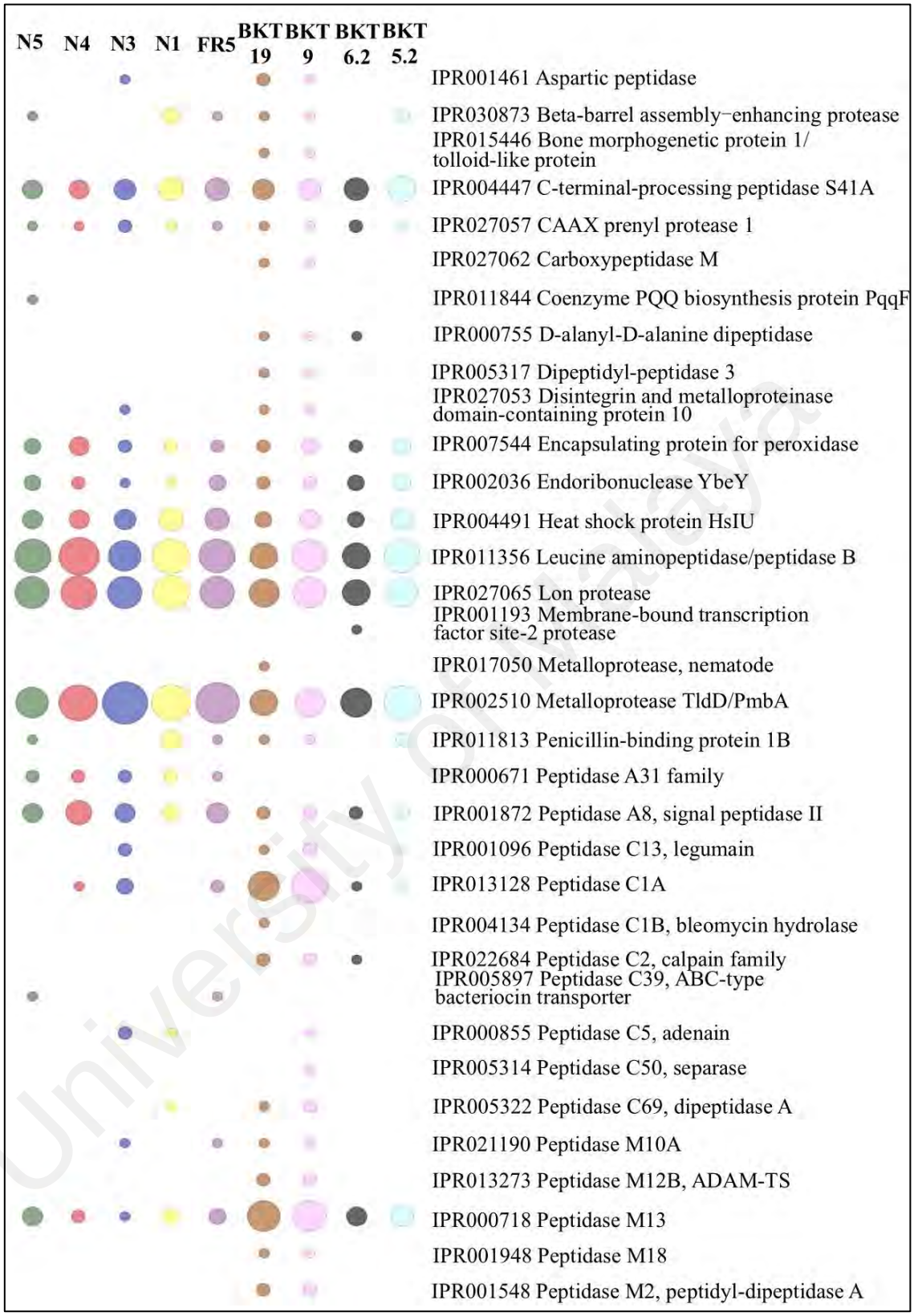


Figure 4.17: InterPro families in GO-term "peptidase activity" (third node in GO "molecular function" domain)

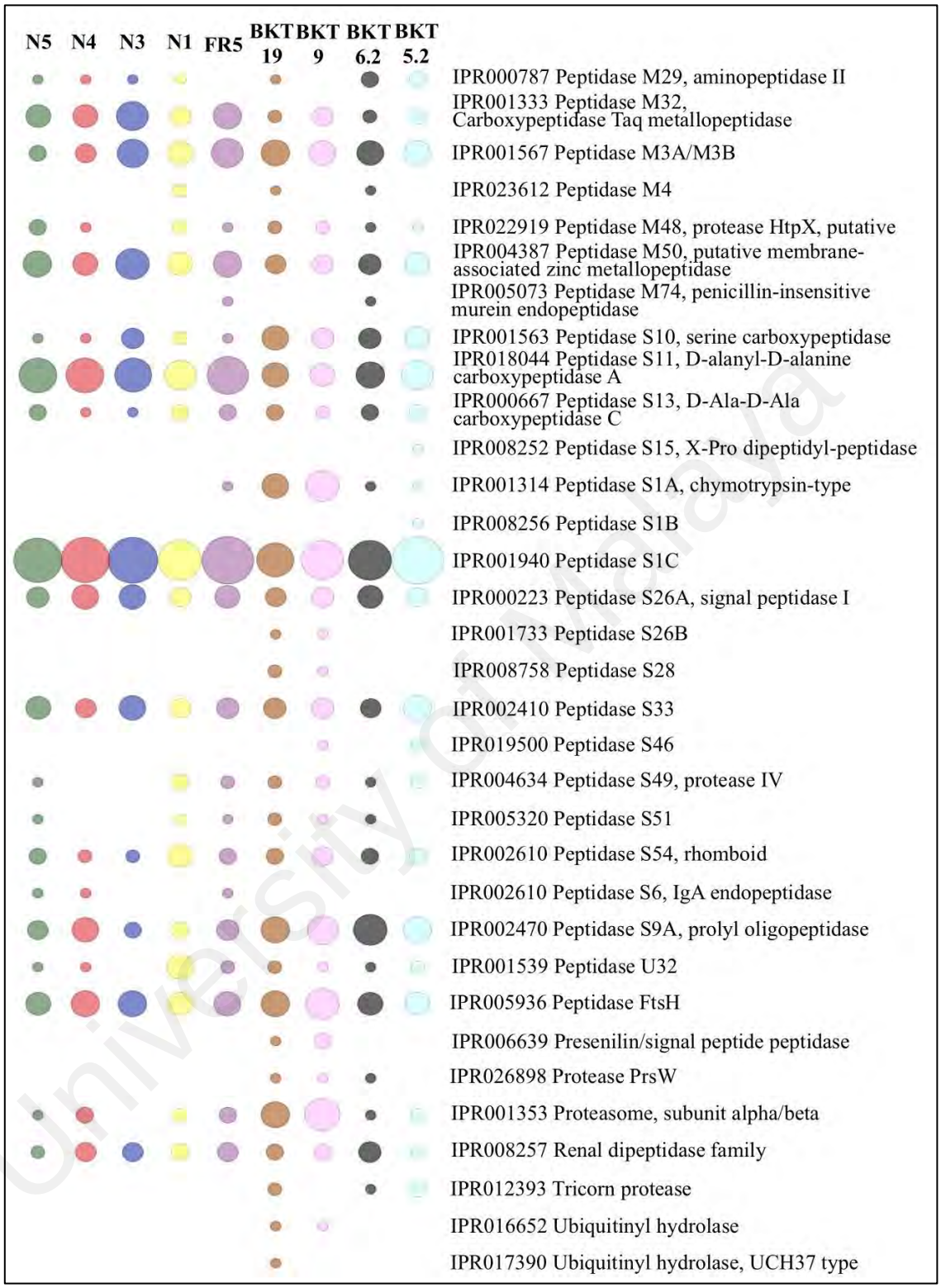


Figure 4.17, continued.

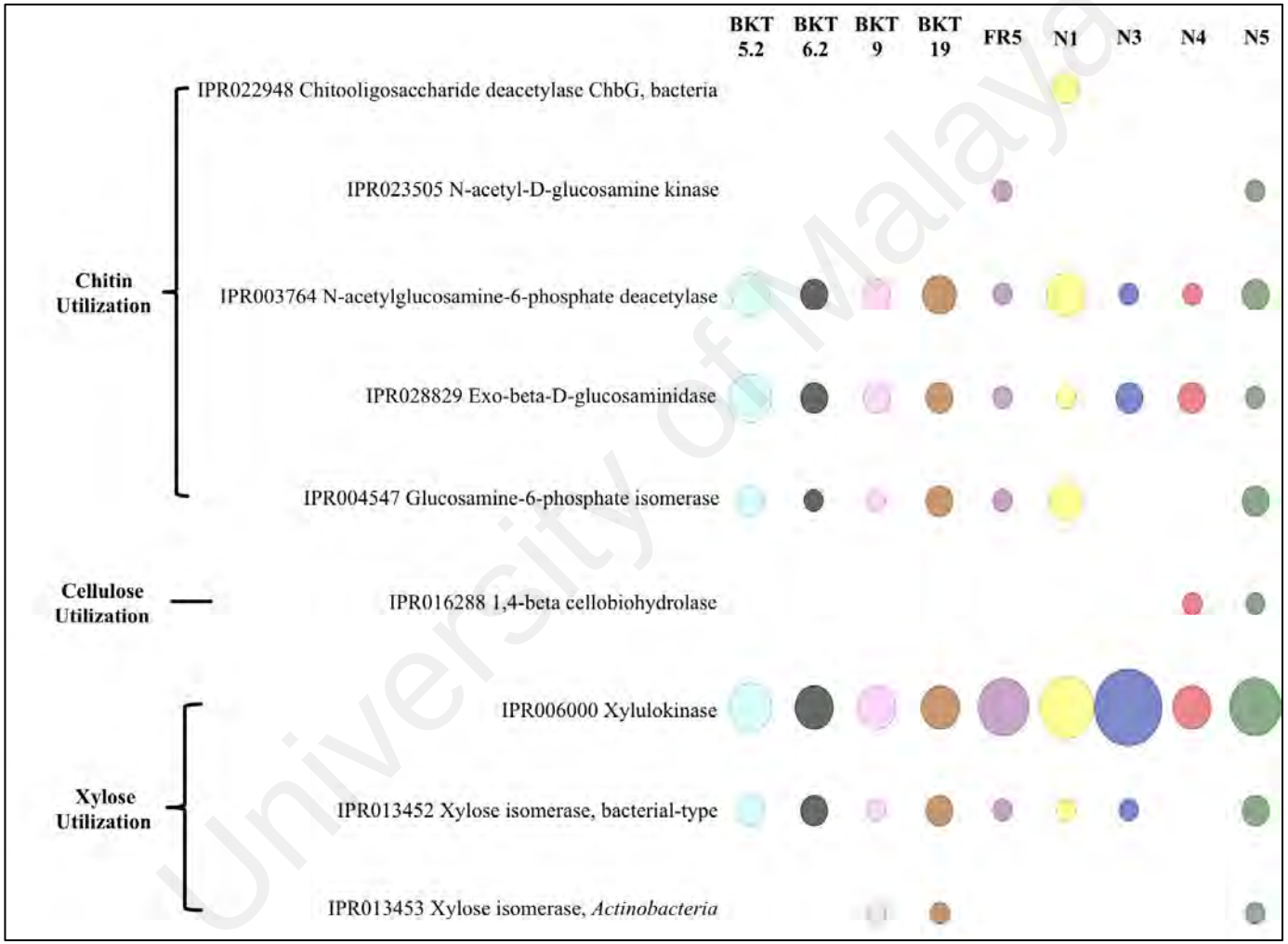
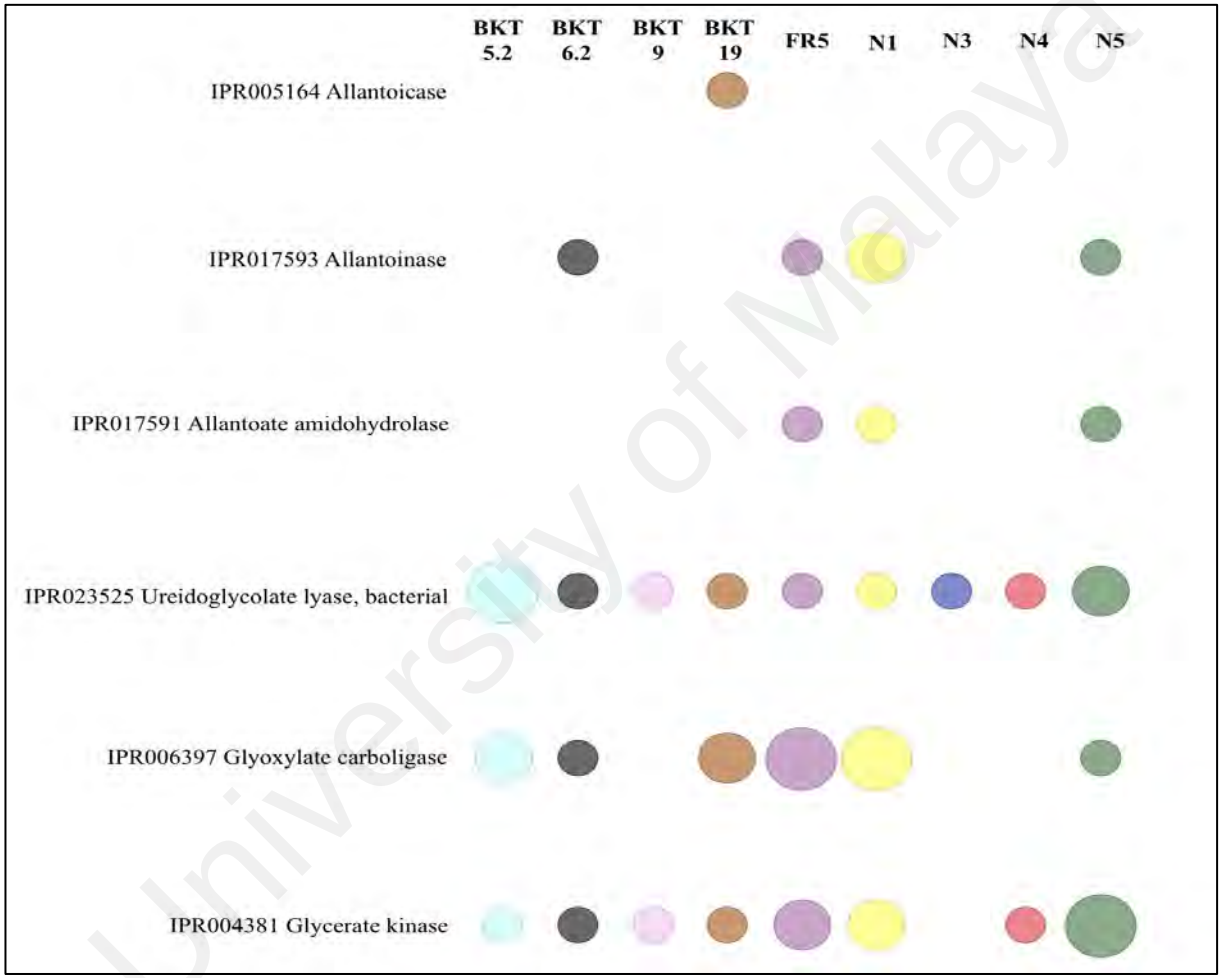
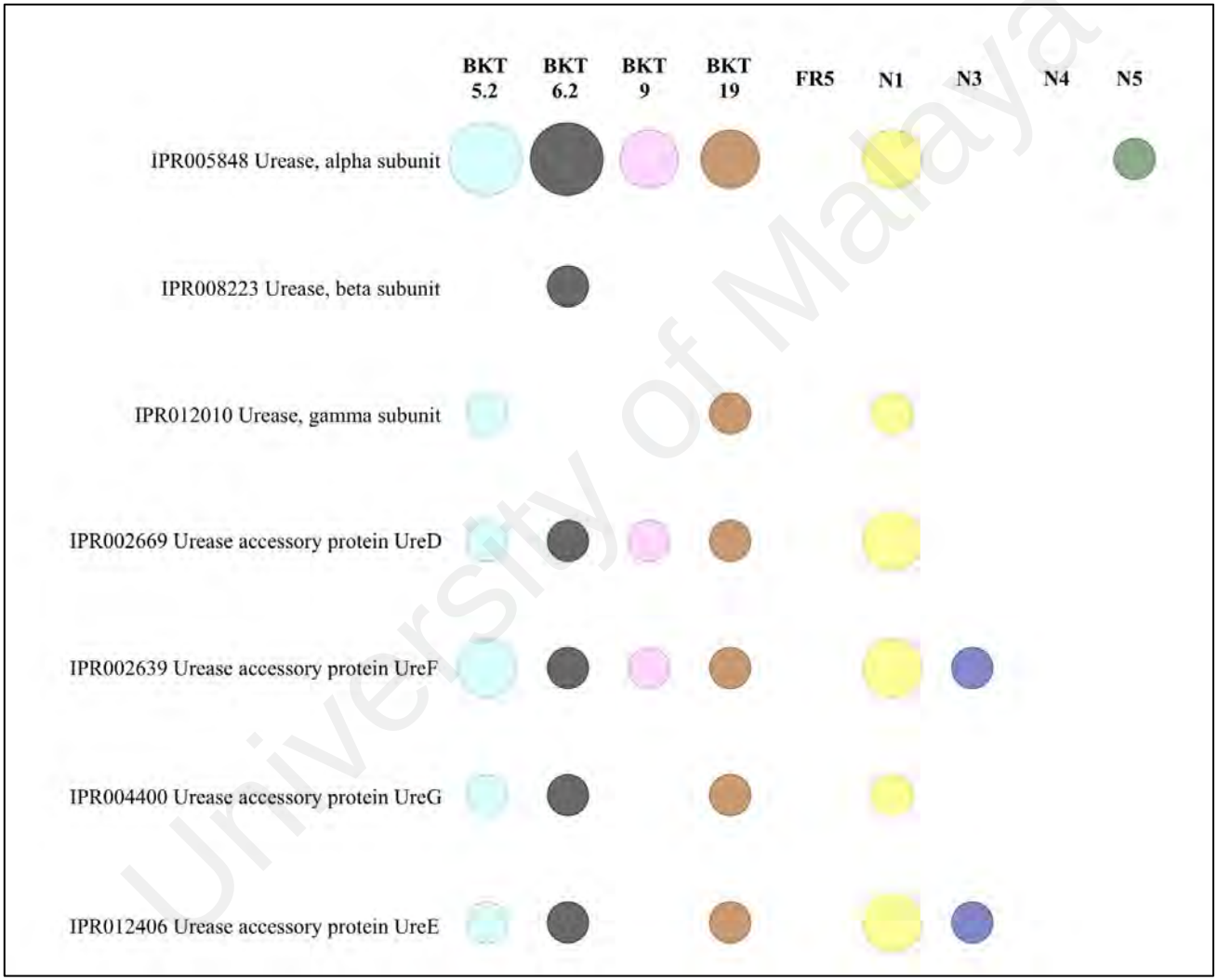


Figure 4.18: InterPro families on enzymes for chitin, cellulose and xylose utilization. The abundance of the genes in each sample was indicated by the size of the circle



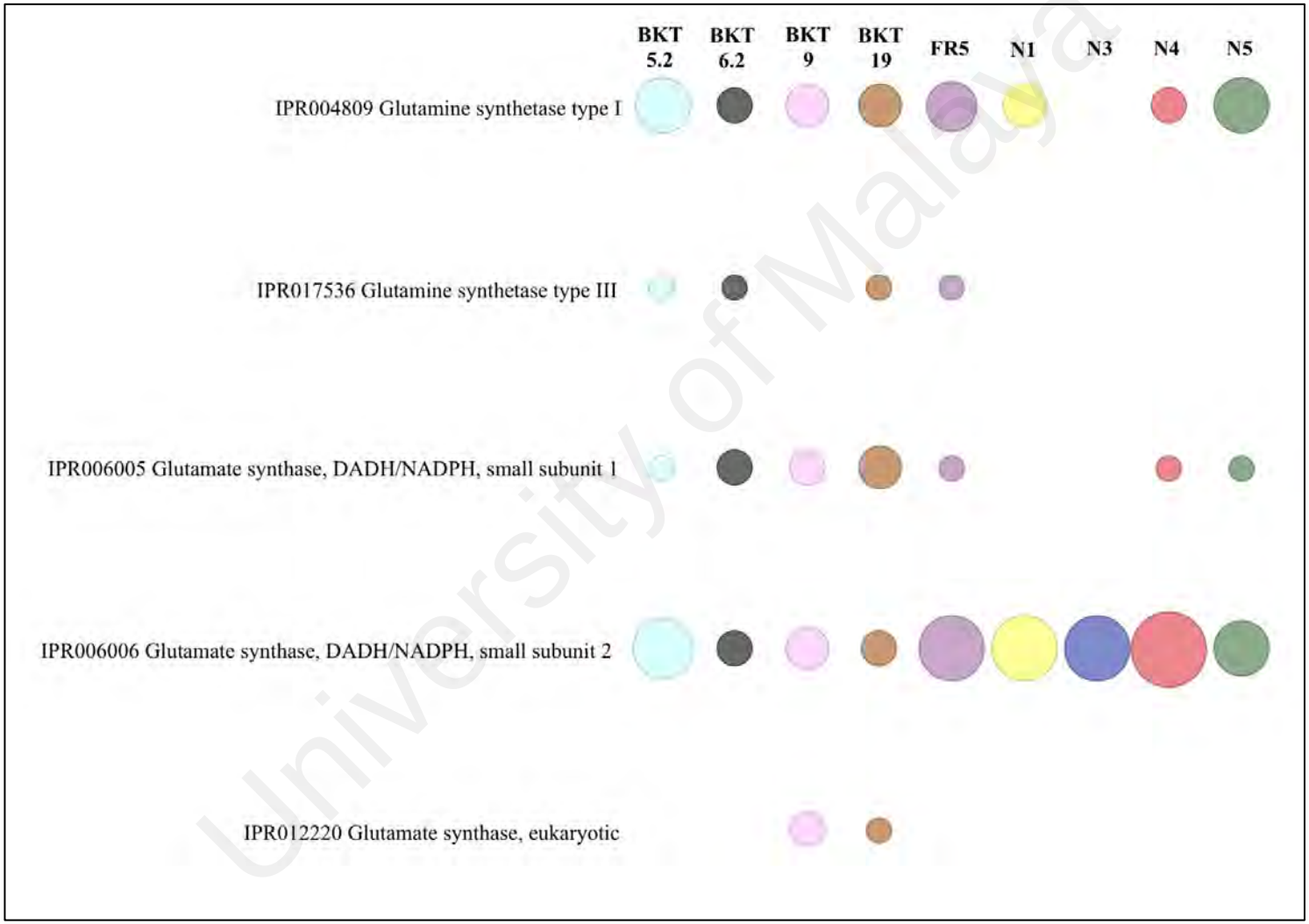
(a)

Figure 4.19: InterPro families on enzymes for (a) allantoin degradation, (b) urea degradation, (c) ammonia assimilation, glutamine and glutamate utilization. Predicted gene sequences were annotated using InterPro database. The abundance of the genes in each sample was indicated by the size of the circle



(b)

Figure 4.19, continued.



(c)

Figure 4.19, continued.

4.12 Bacterial Morphology, Size and Gram Staining Results

Six Gram-positive and 12 Gram-negative bacteria (Table 4.7 and Appendix I) were isolated from sample H1 using LB agar. The size of the bacterial cells was measured from the SEM images (Appendix J). Among the bacteria isolates, largest cell size was observed from the SEM image of strain H1a (5.04 μm) and smallest cell size were observed from the SEM image of strain DH1b and PH1c (0.83 μm) (Table 4.7 and appendix J). The morphologies (colour, form, surface and elevation) of bacteria isolated were observed from the bacterial colonies cultured on LB medium at 28 °C and tabulated in Table 4.7. Besides, pleasant scent was emanated by the culture of strain H1bi.

Table 4.7: Gram staining results, bacterial cells size, and the morphologies of bacterial colony.

Strain	Gram	Size, μm	Morphology			
			Colour	Form	Surface	Elevation
H1a	+	5.04	White	Filamentous	Rough	Flat
H1g	-	1.05	White	Circular	Smooth	Convex
H1h	-	1.87	White	Irregular	Smooth	Raised
H1k	+	2.31	White	Irregular	Smooth	Raised
H1l	-	1.39	Green	Irregular	Smooth	Raised
H1m	+	4.51	White	Irregular	Rough	Flat
H1n	-	1.16	White	Circular	Smooth	Convex
H1q	-	1.00	Red	Circular	Smooth	Convex
H1r	-	1.32	White	Irregular	Smooth	Convex
H1w	-	1.12	White	Circular	Smooth	Convex
H1ai	-	1.33	Orange	Punctiform	Smooth	Convex
H1aii	+	1.27	Yellow	Circular	Smooth	Convex
H1bi	-	1.31	White	Irregular	Smooth, Glistening	Raised
DH1b	+	0.83	Yellow	Punctiform	Smooth	Convex
DH1f	-	1.21	White	Circular	Smooth	Convex
PH1a	-	1.51	White	Circular	Smooth	Convex
PH1b	-	1.27	White	Circular	Smooth	Convex
PH1c	+	0.83	White	Circular	Smooth	Convex

4.13 Bacterial Identity

Bacterial identity was determined using MALDI TOF MS Biotyper and phylogenetic analysis on the bacterial 16S rRNA gene sequences (Appendix K). The identity of each strain was tabulated in Table 4.8. The bacteria identities obtained using MALDI TOF MS Biotyper were collaborated with the results from phylogenetic analysis on the bacterial 16S rRNA gene sequences (Table 4.8). MALDI TOF MS Biotyper failed to identify strains H1ai, H1aii and PH1c (Table 4.8). The bacterial 16S rRNA genes sequences were deposited in DDBJ/EMBL/GenBank with accession number as listed in Table 4.8.

Table 4.8: Bacterial Identity by MALDI TOF MS Biotyper and phylogenetic analysis on bacterial 16S rRNA gene sequence.

Strain	MALDI TOF MS	16S rRNA	16S rRNA Gene Accession Number
H1a	*Bacillus mycoides*	*Bacillus* sp.	KF557587
H1g	*Klebsiella oxytoca*	*Klebsiella oxytoca*	KF557591
H1h	*Pseudomonas koreensis*	*Pseudomonas* sp.	KF557592
H1k	*Lysinibacillus fusiformis*	*Lysinibacillus fusiformis*	KF557593
H1l	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	KF557594
H1m	*Bacillus thuringiensis*	*Bacillus* sp.	KF557595
H1n	*Serratia fonticola*	*Serratia* sp.	KF557596
H1q	*Serratia marcescens*	*Serratia marcescens*	KF557597
H1r	*Morganella morganii*	*Morganella morganii*	KF742682
H1w	*Serratia fonticola*	*Serratia* sp.	KF557599
H1ai	Not Identified	*Sphingobacterium* sp.	KF742683
H1aii	Not Identified	*Leifsonia aquatic*	KF742684
H1bi	*Myroides odoratimimus*	*Myroides odoratimimus*	KF742685
DH1b	*Microbacterium* sp.	*Microbacterium paraoxydans*	KF557585
DH1f	*Achromobacter* spanius	*Achromobacter* sp.	KF557586
PH1a	*Serratia marcescens*	*Serratia marcescens*	KF557600
PH1b	*Pseudomonas corrugate*	*Pseudomonas* sp.	KF557601
PH1c	Not Identified	*Leucobacter* sp.	KF557602

4.14 Screening of Biocatalytic Activity

Positive result in amylolytic activity screening test was indicated by the formation of yellow halo zone around the bacterial colony after the addition of iodine onto 0.5% (w/v) starch agar. Positive result in proteolytic activity screening test was indicated by the presence of a halo zone around the bacterial colony on opaque 5% (w/v) skim milk agar. Positive result in cellulolytic activity screening test was indicated by the discolouration of congo red around the bacterial colony on 0.15% (w/v) microcrystalline cellulose agar.

In this study, amylolytic activtity was detected in *Bacillus* sp. strain H1a, *Bacillus* sp. strain H1m and *Sphingobacterium* sp. strain H1ai (Table 4.9 and Appendix L). Proteolytic activity was detected in *Bacillus* sp. strain H1a, *P. aeruginosa* strain H1l, *Bacillus* sp. strain H1m, *S. marcescens* strain H1q, *Sphingobacterium* sp. strain H1ai, *M. odoratimimus* strain H1bi, *Microbacterium* sp. strain DH1b, *S. marcescens* strain PH1a and *Pseudomonas* sp. strain PH1b (Table 4.9 and Appendix M). Cellulolytic activity was detected in *Bacillus* sp. strain H1a, *Pseudomonas* sp. strain H1h, *Bacillus* sp. strain H1m, *Sphingobacterium* sp. strain H1ai, *M. odoratimimus* strain H1bi, *Microbacterium* sp. strain DH1b, *S. marcescens* strain PH1a and *Pseudomonas* sp. strain PH1b (Table 4.9 and Appendix N). Chitinolytic activity was detected in *K. oxytoca* strain H1g, *S. marcescens* strains H1q and PH1a and *Pseudomonas* sp. strain PH1b (Table 4.9). Xylanolytic activity was detected in *Pseudomonas* sp. strain H1h, *P. aeruginosa* strain H1l, *Sphingobacterium* sp. strain H1ai and *Pseudomonas* sp. strain PH1b (Table 4.9). The results of chitinolytic assays and xylanolytic activity screening test were described in the subsequent sections (4.15 and 4.16).

In summary, 3 bacterial strains isolated from *Nepenthes* pitcher fluid showed positive result in amylolytic activity, 9 bacterial strains showed positive result in proteolytic activity, 8 bacterial strains showed positive result in cellulolytic activity, 4 bacterial strains showed positive result in chitinolytic assays and 4 bacterial strains showed positive result in xylanolytic activity (Table 4.9). Positive result in the tested biocatalytic activities was not observed in *L. fusiformis* strain H1k, *Serratia* sp. strains H1n and H1w, *M. morganii* H1r, *L. aquatic* strain H1aii, *Achromobacter* sp. DH1f and *Leucobacter* sp. strain PH1c (Table 4.9). The biocatalytic activities by the bacterial strains isolated from *Nepenthes* pitcher fluid were summarized and tabulated in Table 4.9.

Table 4.9: Amylolytic, proteolytic, cellulolytic and xylanolytic activities of bacteria Isolated from *Nepenthes* pitcher fluid.

Strain	Identity	Amylolytic	Proteolytic	Cellulolytic	Chitinolytic	Xylanolytic
H1a	*Bacillus* sp.	+	+	+	-	-
H1g	*Klebsiella oxytoca*	-	-	-	+	-
H1h	*Pseudomonas* sp.	-	-	+	-	+
H1k	*Lysinibacillus fusiformis*	-	-	-	-	-
H1l	*Pseudomonas aeruginosa*	-	+	-	-	+
H1m	*Bacillus* sp.	+	+	+	-	-
H1n	*Serratia* sp.	-	-	-	-	-
H1q	*Serratia marcescens*	-	+	-	+	-
H1r	*Morganella morganii*	-	-	-	-	-
H1w	*Serratia* sp.	-	-	-	-	-
H1ai	*Sphingobacterium* sp.	+	+	+	-	+
H1aii	*Leifsonia aquatica*	-	-	-	-	-
H1bi	*Myroides odoratimimus*	-	+	+	-	-
DH1b	*Microbacterium paraoxydans*	-	+	+	-	-
DH1f	*Achromobacter* sp.	-	-	-	-	-
PH1a	*Serratia marcescens*	-	+	+	+	-
PH1b	*Pseudomonas* sp.	-	+	+	+	+
PH1c	*Leucobacter* sp.	-	-	-	-	-

- "+": Positive result
- "-" : Negative result

4.15 Chitinolytic Activities

Bacterial chitinases (in specific β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase) activities were assessed using chitinase assay kit (Sigma, USA). β-*N*-acetylglucosaminidase, chitobiosidase and endochitinase activities were observed from *S. marcescens* strains H1q and PH1a (Table 4.10). Meanwhile, *Pseudomonas* strain PH1b performed chitobiosidase and endochitinase activities (Table 4.10). Only chitobiosidase activity was observed from *K. oxytoca* strain H1g (Table 4.10).

Table 4.10: β-*N*-glucosaminidase, chitobiosidase and endochitinase activities from bacterial isolated from *Nepenthes* pitcher fluid.

Sample	Bacteria Identity	β-*N*-acetylglucosaminidase[a] (nmole min^{-1} mL^{-1})	Chitobiosidase[b] (nmole min^{-1} mL^{-1})	Endochitinase[c] (nmole min^{-1} mL^{-1})
Blank	Not available	0.00	0.00	0.00
Chitinase	Not available	73.70	71.93	58.90
H1a	*Bacillus* sp.	0.07	0.21	0.03
H1g	*Klebsiella oxytoca*	0.26	**2.38**	0.00
H1h	*Pseudomonas* sp.	0.12	0.14	0.05
H1k	*Lysinibacillus fusiformis*	0.07	0.23	0.02
H1l	*Pseudomonas aeruginosa*	0.08	0.18	0.06
H1m	*Bacillus* sp.	0.08	0.19	0.03
H1n	*Serratia* sp.	0.17	0.30	0.05
H1q	*Serratia marcescens*	**2.15**	**3.75**	**2.54**
H1r	*Morganella morganii*	0.08	0.16	0.05
H1w	*Serratia* sp.	0.20	0.24	0.16
H1ai	*Sphingobacterium* sp.	0.08	0.38	0.04
H1aii	*Leifsonia aquatica*	0.05	0.16	0.07
H1bi	*Myroides odoratimimus*	0.08	0.21	0.03
DH1b	*Microbacterium paraoxydans*	0.03	0.22	0.04
DH1f	*Achromobacter* sp.	0.03	0.17	0.09
PH1a	*Serratia marcescens*	**2.15**	**3.86**	**2.06**
PH1b	*Pseudomonas* sp.	0.02	**3.79**	**1.41**
PH1c	*Leucobacter* sp.	0.10	0.14	0.00

a. Substrate: 4-Nitrophenyl *N*-acetyl-β-$_D$-glucosaminide
b. Substrate: 4-nitrophenyl *N*,*N*'-diacetyl-β-$_D$-chitobioside
c. Substrate: 4-nitrophenyl β-$_D$-*N*,*N*',*N*''-triacetylchitotriose
* Positive results were in bold.

4.16 Xylanolytic Activity

Bacterial xylanase activity was screened using EnzChek® Ultra Xylanse Assay kit (Thermo Fisher Scientific, USA). The rate of product formation by bacterial xylanase was not quantified in this assay. Samples with fluorescence reading higher or similar to than standard 4 (S4, 25 mU/mL xylanase from *Trichoderma viride*) were recorded as positive results.

Xylanolytic activity was observed from *Pseudomonas* sp. strains H1h and PH1b, *P. aeruginosa* strain H1l, and *Sphingobacterium* sp. strain H1ai (Table 4.9 and Figure 4.20). In this study, the fluorescence signal detected in *Sphingobacterium* sp. strain H1ai was strongest among the bacterial isolated from *Nepenthes* pitcher fluid (Figure 4.20). The xylanase produced by *Sphingobacterium* sp. strain H1ai was active for at least 4 hours at 25 °C (Figure 4.20). The fluorescence signal detected in *Pseudomonas* sp. strain and PH1b and *P. aeruginosa* strain H1l were slightly higher than S4 (Figure 4.20). The fluorescence signal detected in *Pseudomonas* sp. strain H1h was slightly lower than S4 (Figure 4.20). The xylanase activity of *Pseudomonas* sp. strain H1h was weakest among the xylanase positive strains.

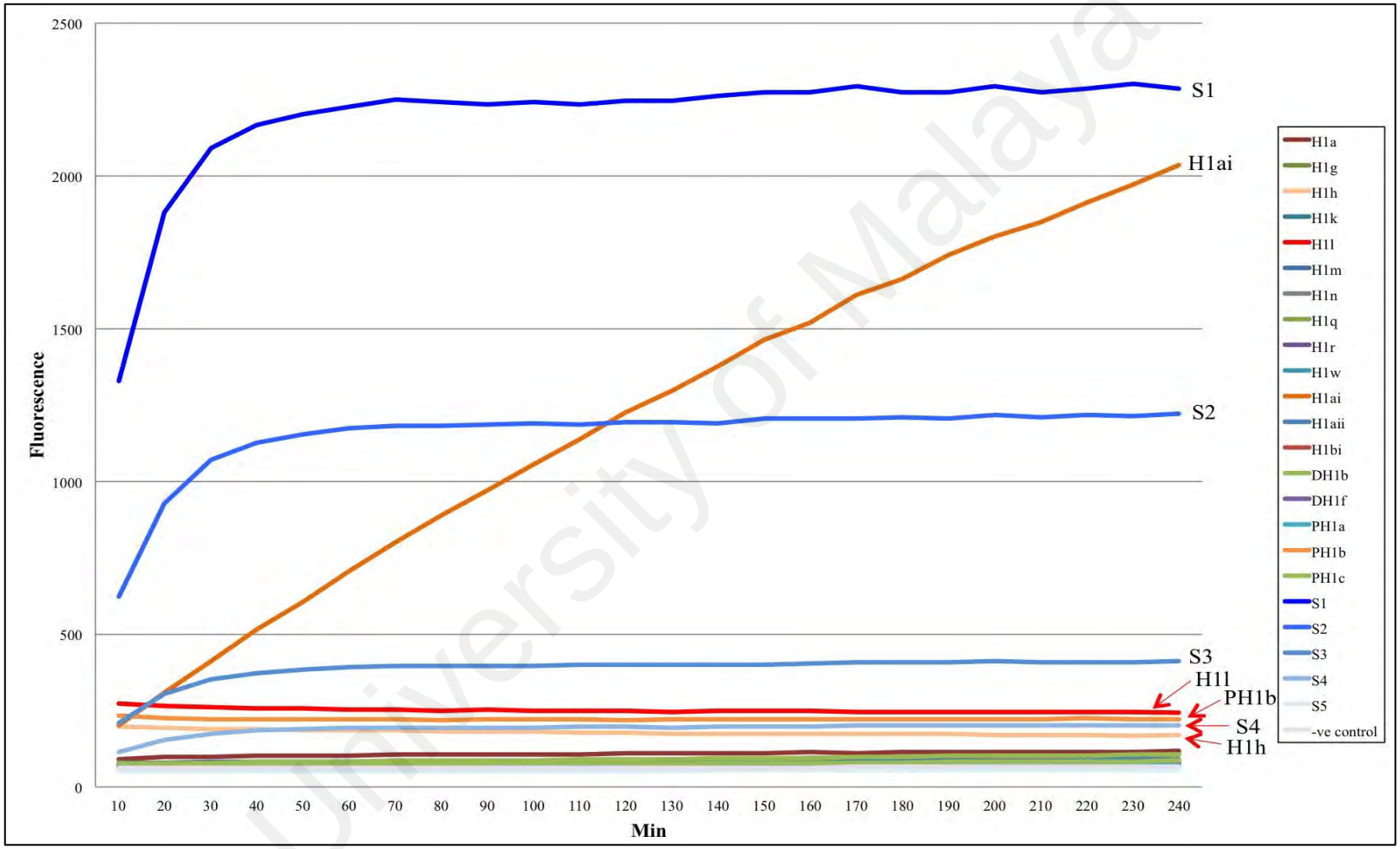


Figure 4.21: Screening of bacterial xylanase activity. Xylanase from *Trichoderma viride* was used as standard (S1: 200 mU/mL, S2: 100 mU/mL, S3: 50 mU/mL, S4: 25 mU/mL and S5: 0 mU/ml\L). Substrate (50 µg/mL) was used as negative control in this assay. Bacterial identity in the graph was cross-referred to the Table 4.9

4.17 Bacterial Genome

Among the isolates, smallest genome size was observed in *Leucobacter* sp. strain PH1c (3.1 Mbps) and largest genome was observed in *Pseudomonas* sp. strain PH1b (7.4 Mbps) (Table 4.11). The bacterial genomes were sequenced to at least 30-fold average coverage and assembled into less than 250 contigs (Table 4.11). The G+C content in the bacterial genome ranges from 34% to 71% (Table 4.11). The size, average coverage, G+C content, number of contigs and accession numbers of each bacterial genomes were listed in Table 4.11.

Whole genome shotgun projects were deposited at DDBJ/EMBL/GenBank with accession numbers from AYME00000000 to AYMV00000000. The version described in this thesis is version AYME01000000 to AYMV01000000.

Table 4.11: The sizes, average coverage, G+C %, number of contig and accession numbers of the bacterial genomes.

Strain	Bacteria Identity	Genome Size (Mbps)	Average Coverage	G+C %	Number of Contig	Accession Number
H1a	*Bacillus* sp.	5.8	120	35.0	167	AYMH00000000
H1g	*Klebsiella oxytoca*	5.8	142	56.0	68	AYMI00000000
H1h	*Pseudomonas* sp.	6.4	74	60.3	78	AYMJ00000000
H1k	*Lysinibacillus fusiformis*	4.8	87	37.3	60	AYMK00000000
H1l	*Pseudomonas aeruginosa*	6.4	72	66.4	40	AYML00000000
H1m	*Bacillus* sp.	5.7	34	35.0	178	AYMM00000000
H1n	*Serratia* sp.	6.1	58	53.8	247	AYMN00000000
H1q	*Serratia marcescens*	5.2	132	59.2	60	AYMO00000000
H1r	*Morganella morganii*	4.5	85	50.1	118	AYMP00000000
H1w	*Serratia* sp.	6.1	88	53.8	240	AYMQ00000000
H1ai	*Sphingobacterium* sp.	6.7	88	39.0	66	AYMG00000000
H1aii	*Leifsonia aquatica*	4.6	108	70.4	73	AYMR00000000
H1bi	*Myroides odoratimimus*	3.9	72	34.0	183	AYMS00000000
DH1b	*Microbacterium paraoxydans*	3.6	153	70.2	47	AYME00000000
DH1f	*Achromobacter* sp.	7.1	105	66.4	148	AYMF00000000
PH1a	*Serratia marcescens*	5.2	86	59.2	56	AYMT00000000
PH1b	*Pseudomonas* sp.	7.4	94	62.9	89	AYMU00000000
PH1c	*Leucobacter* sp.	3.1	108	71.3	73	AYMV00000000

4.18 Chitinase Genes

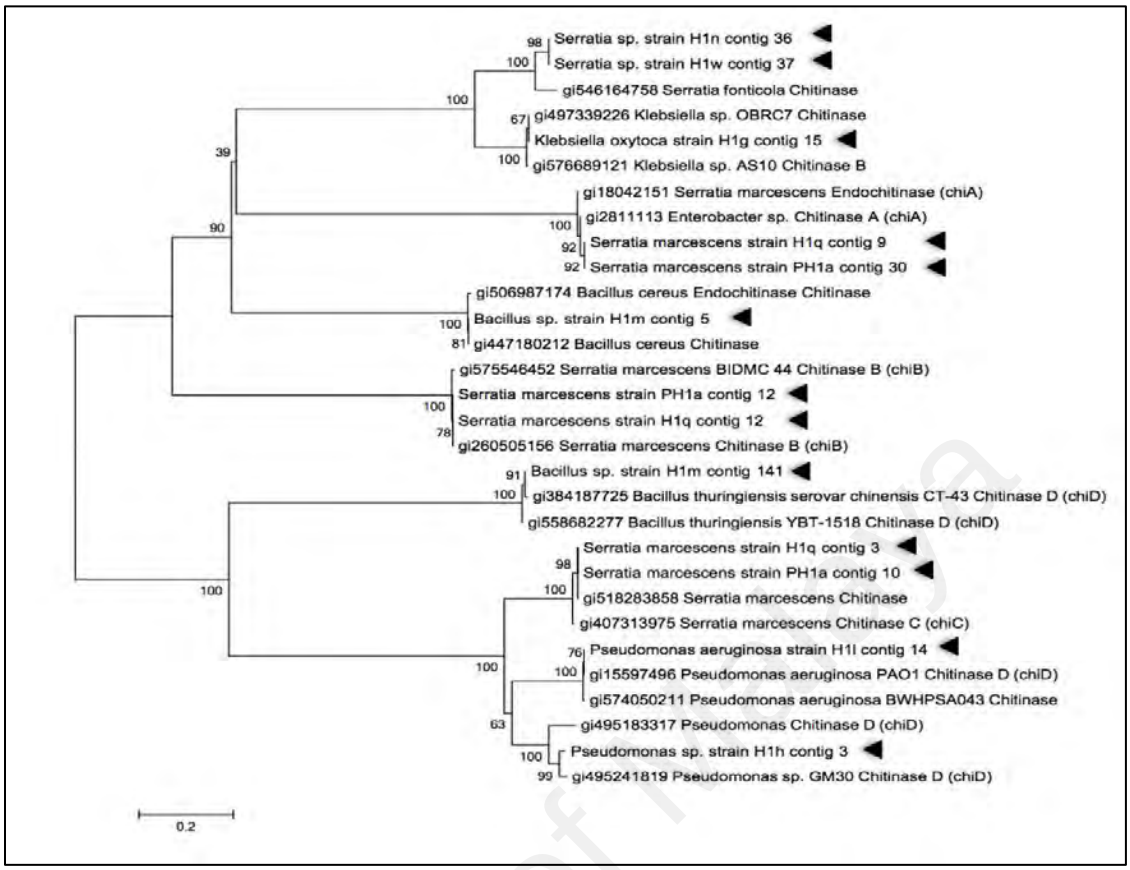
Fifteen putative chitinase-coding genes were identified from the annotated bacterial genomes. In brief, 2 putative chitinase-coding genes were found in the genomes of *K. oxytoca* strain H1g, *Pseudomonas* sp. strain H1h and *Bacillus* sp. strain H1m; 1 putative chitinase-coding gene was found in the genomes of *P. aeruginosa* strain H1l and *Serratia* sp. strains H1n and H1w; 3 putative chitinase-coding genes were found in the genomes of *S. marcescens* strains H1q and PH1a. Chitobiosidase and endochitinase activities were observed from *Pseudomonas* sp. strain PH1b in chitinase assay (Table 4.10) but the chitinase-coding gene was not identified from the bacterial genome.

Based on the phylogenetic analysis, 13 putative chitinase-coding genes identified in this study were clustered into glycoside hydrolase 18 (GH18) family chitinase (Figure 4.21a). Putative chitinase genes of *Serratia* sp. strains H1n and H1w were closely related to GH18 chitinase of *S. fonticola* (Figure 4.21a). Putative chitinase-coding genes in contig 15 of *K. oxytoca* strain H1g was closely related to chitinase B (ChiB) of *Klebsiella* sp. strain AS10 (Figure 4.21a). Putative chitinase-coding genes in contig 9 of *S. marcescens* strain H1q and contig 30 of strain PH1a were closely related to chitinase A (ChiA) of *Enterobacter* sp. (Figure 4.21a). The putative chitinase-coding genes in contig 12 of *S. marcescens* strains H1q and PH1a were closely related to ChiB of *S. marcescens* (Figure 4.21a). Besides, putative chitinase-coding genes in contig 3 of *S. marcescens* strain H1q and contig 10 of strain PH1a were closely related to chitinase C (ChiC) of *S. marcescens* (Figure 4.21a). Putative chitinase-coding genes in contig 5 and contig 141 of *Bacillus* sp. strain H1m were closely related to chitinase of *Bacillus cereus* and chitinase D (ChiD) of *B. thuringiensis*, respectively (Figure 4.21a). The putative chitinase-coding genes in contig 14 of *P. aeruginosa* strain H1l and contig 3 of

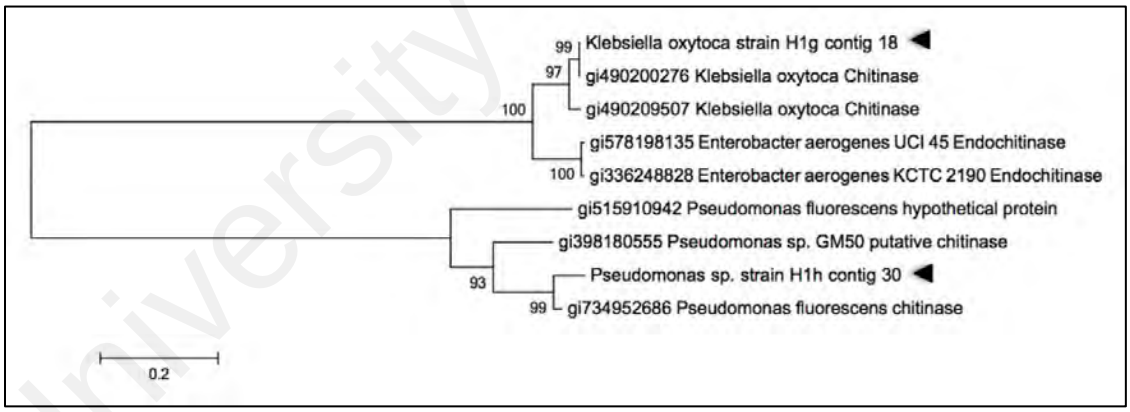
Pseudomonas sp. strain H1h were closely related to ChiD of *P. aeruginosa* strain PAO1 and ChiD of *Pseudomonas* sp. strain GM30, respectively (Figure 4.21a).

Based on the phylogenetic analysis, 2 putative chitinase-coding genes identified in this study were clustered into GH19 family chitinase (Figure 4.21b). Putative chitinase-coding genes in contig 18 of *K. oxytoca* strain H1g was closely related *K. oxytoca* chitinase (Figure 4.21b). The putative chitinase-gene of *Pseudomonas* sp. strain H1h was closely related to GH19 chitinase of *Pseudomonas fluorescens* (Figure 4.21b).

Putative chitinase gene sequences were deposited at DDBJ/EMBL/GenBank with accession numbers KT921876 to KT921890 (Appendix O).



(a)



(b)

Figure 4.21: Putative chitinase genes from bacterial genome were clustered into (a) GH18 chitinase and (b) GH19 chitinase. The phylogenetic trees were constructed using amino acid sequences of the bacterial putative chitinase genes. The putative chitinase genes from the genomes of the bacterial isolated in this study were indicated by filled triangles

4.19 Chitinase Activities of Chitinase Transformants

Putative chitinase gene sequences in contig 9, contig 12, and contig 3 of *S. marcescens* strain H1q were identical to the putative chitinase gene sequences in contig 30, contig 12, and contig 10 of *S. marcescens* strain PH1a, respectively. Similarly, putative chitinase gene sequence of *Serratia* sp. strain H1n was identical to putative chitinase gene sequence of *Serratia* sp. strain H1w. Thus, only chitinase genes of *S. marcescens* strain H1q and *Serratia* sp. strain H1n were synthesized, cloned and preceded to chitinase assay.

β-*N*-glucosaminidase activity was detected in the transformants with chitinase genes from contig 15 of *K. oxytoca* strain H1g and contig 36 of *Serratia* sp. strain H1n (Table 4.12). Chitobiosidase and endochitinase activities were detected in the transformants with chitinase genes from contig 3 of *Pseudomonas* sp. strain H1h, contig 14 of *P. aeruginosa* strain H1l, contig 141 of *Bacillus* sp. strain H1m, and contig 3 of *S. marcenscens* strain H1q (Table 4.12). The transformants of these chitinase genes showed stronger chitobiosidase activity compared to endochitinase activity (Table 4.12).

β-*N*-glucosaminidase, chitobiosidase and endochitinase activities were not detected in the transformants with chitinase genes from contig 18 of *K. oxytoca* strain H1g, contig 30 of *Pseudomonas* sp. strain H1h, contig 5 of *Bacillus* sp. strain H1m and contigs 9 and 12 of *S. marcenscens* strain H1q (Table 4.12).

Table 4.12: β-*N*-glucosaminidase, chitobiosidase and endochitinase activities of transformant inserted with a putative chitinase-coding gene.

Sample	β-*N*-acetylglucosaminidase[a] (nmole min^{-1} mL^{-1})	Chitobiosidase[b] (nmole min^{-1} mL^{-1})	Endochitinase[c] (nmole min^{-1} mL^{-1})
Blank	0.00	0.00	0.00
Chitinase	77.60	93.55	76.71
E. coli BL21	0.16	0.12	0.13
H1g contig 15	**1.58**	0.14	0.13
H1g contig 18	0.17	0.11	0.13
H1h contig 3	0.17	**3.70**	**0.99**
H1h contig 30	0.17	0.12	0.13
H1l contig 14	0.19	**2.47**	**1.81**
H1m contig 141	0.19	**4.56**	**0.83**
H1m contig 5	0.18	0.13	0.15
H1n contig 36	**1.45**	0.14	0.13
H1q contig 3	0.18	**1.97**	**0.93**
H1q contig 12	0.00	0.24	0.13
H1q contig 9	0.15	0.11	0.10

a. Substrate: 4-Nitrophenyl *N*-acetyl-β-$_D$-glucosaminide
b. Substrate: 4-Nitrophenyl *N*,*N*'-diacetyl-β-$_D$-chitobioside
c. Substrate: 4-Nitrophenyl β-$_D$- *N*,*N*',*N*''-triacetylchitotriose
* Positive results were in bold.

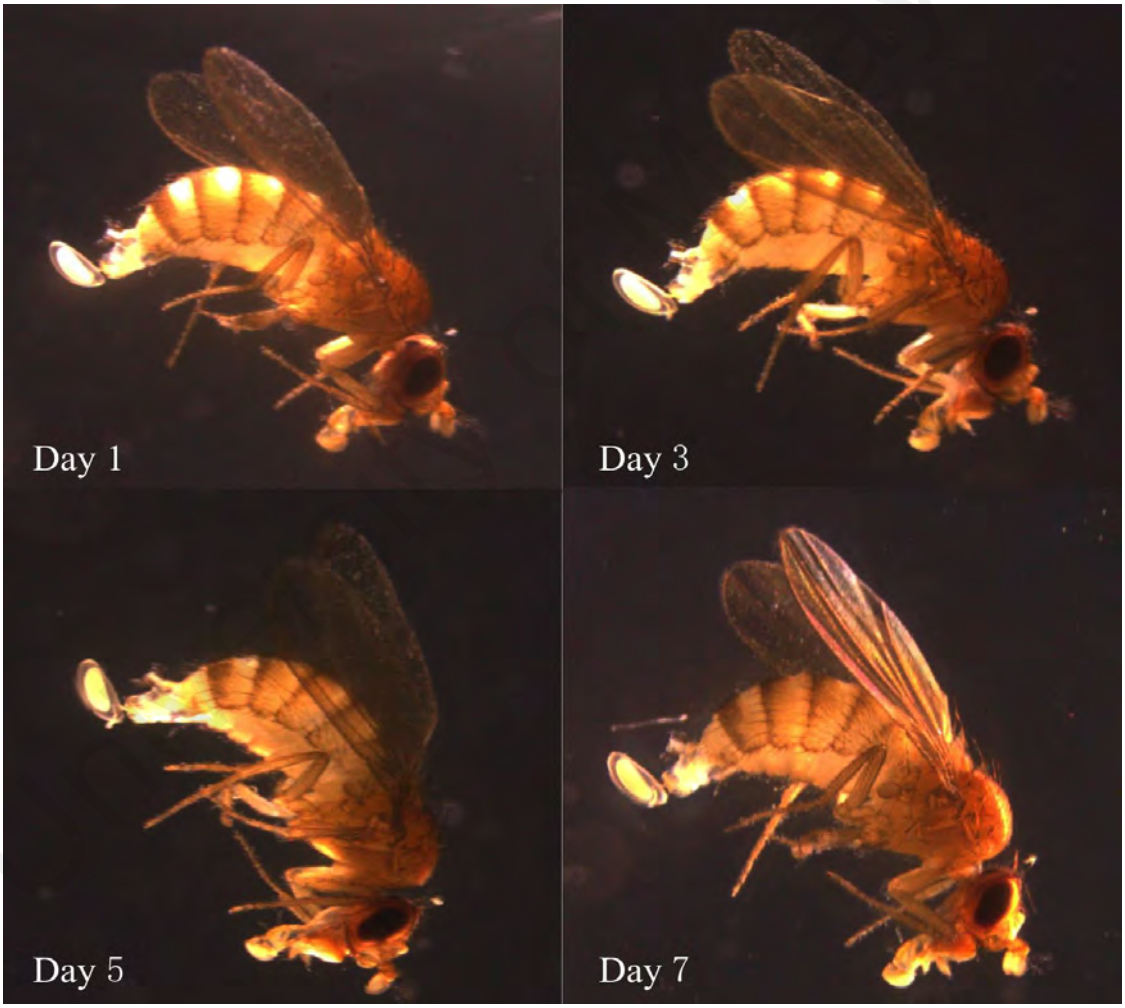
4.20 Insect Degradation by Bacteria Isolated from *Nepenthes* Pitcher Fluid

The insect degradation ability of the bacterial isolated from *Nepenthes* pitcher fluid was tested on *D. melanogaster*. *Bacillus* sp. strain H1a (amylolytic, proteolytic and cellulolytic activities), *P. aeruginosa* strain H1l (proteolytic and xylanolytic activities) and *S. marcescens* strain H1q (proteolytic and chitinolytic activities) were selected for insect degradation test based on their positive results in biocatalytic tests (Table 4.9). Besides, *Bacillus* sp. strain H1a with filamentous cell form and *S. marcescens* strain H1q that produced red pigment (at culture condition below 30 °C) were selected for insect degradation test due to the ease in observation.

Significant changes were not observed on the *D. melanogaster* immersed in autoclaved *Nepenthes* pitcher fluid (Figure 4.22a) and 1X PBS buffer (pH6.5) (Figure 4.22b) for 7 days. Bloated abdomen was observed on the *D. melanogaster* immersed in autoclaved *Nepenthes* pitcher fluid inoculated with *Bacillus* sp. strain H1a from day 3 to day 7 (Figure 4.22c). Physical destruction and bloated abdomen were not observed on the *D. melanogaster* immersed in autoclaved *Nepenthes* pitcher fluid inoculated with *P. aeruginosa* strain H1l for 7 days (Figure 4.22d). Bloated abdomen was observed on the *D. melanogaster* immersed in autoclaved *Nepenthes* pitcher fluid inoculated with *S. marcescens* strain H1q from day 3 to day 7 (Figure 4.22e).

Red pigment was formed on the head, thorax and abdomen of the *D. melanogaster* immersed in autoclaved *Nepenthes* pitcher fluid inoculated with *Bacillus* sp. strain H1a, *P. aeruginosa* strain H1l and *S. marcescens* strain H1q (hereafter refer as "mixed culture") on day 3 to day 8 (Figure 4.22f). The abdomen of *D. melanogaster* in mixed culture was slightly bloated on day 3 and day 5 (Figure 4.22f). The abdomen of *D. melanogaster* in mixed culture was significantly bloated on day 7 and day 8 (Figure

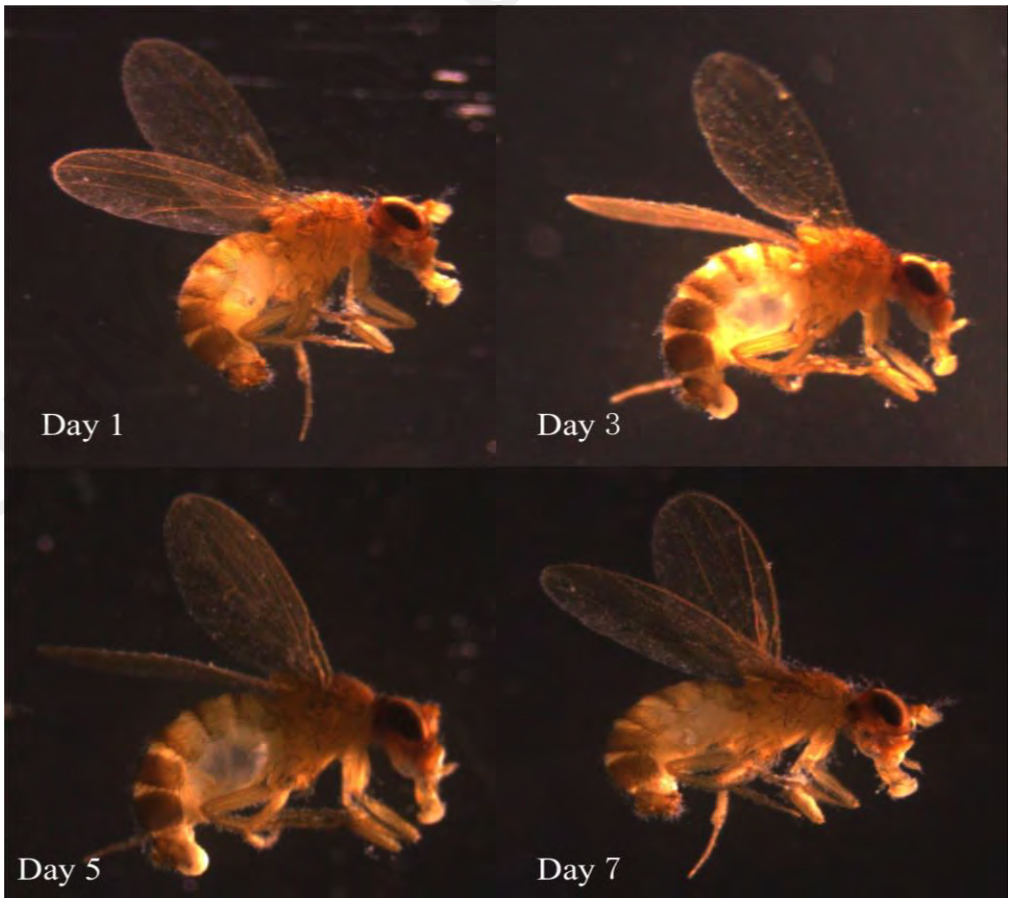
4.22f). Besides, filamentous-liked structure was observed on the head of the *D. melanogaster* in mixed culture on day 5 (Figure 4.22f). The filamentous-liked structure was formed around the head, thorax, wings and abdomen of the *D. melanogaster* in mixed culture on day 7 and day 8 (Figure 4.22f). In mixed sample, all three *D. melanogaster* in a well of the 6-well plate were bounded together at day 7 and day 8 of the insect degradation test (Figure 4.22f). Disintegration of *D. melanogaster* was not observed in any samples at the end of the insect degradation test.



(a)

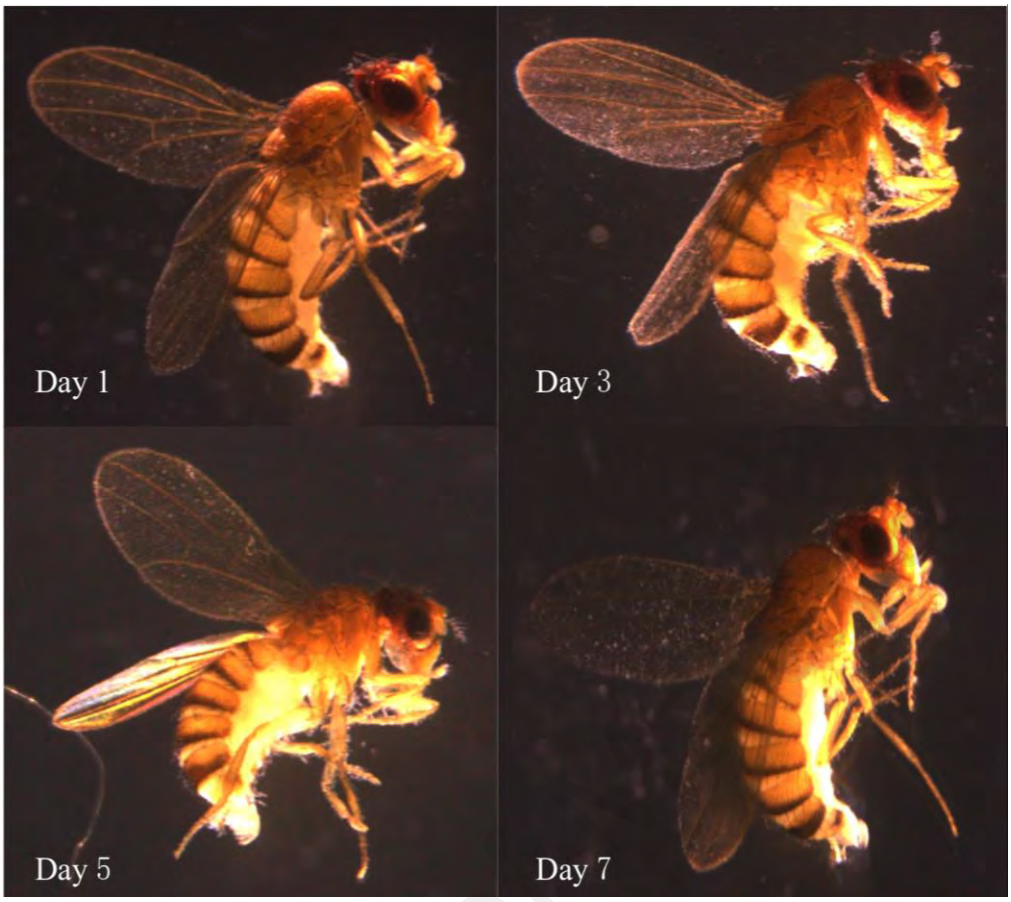
Figure 4.22: Degradation of *D. melanogaster* by bacteria isolated from *Nepenthes* pitcher fluid. *D. melanogaster* was incubated in (a) autoclaved *Nepenthes* pitcher fluid, (b) 1X PBS, (c) autoclaved *Nepenthes* pitcher fluid with *Bacillus* sp. strain H1a, (d) autoclaved *Nepenthes* pitcher fluid with *P. aeruginosa* strain H1l, (e) autoclaved *Nepenthes* pitcher fluid with *S. marcescens* strain H1q and (f) autoclaved *Nepenthes* pitcher fluid with *Bacillus* sp. strain H1a, *P. aeruginosa* strain H1l and *S. marcescens* strain H1q (mixed culture)

(b)

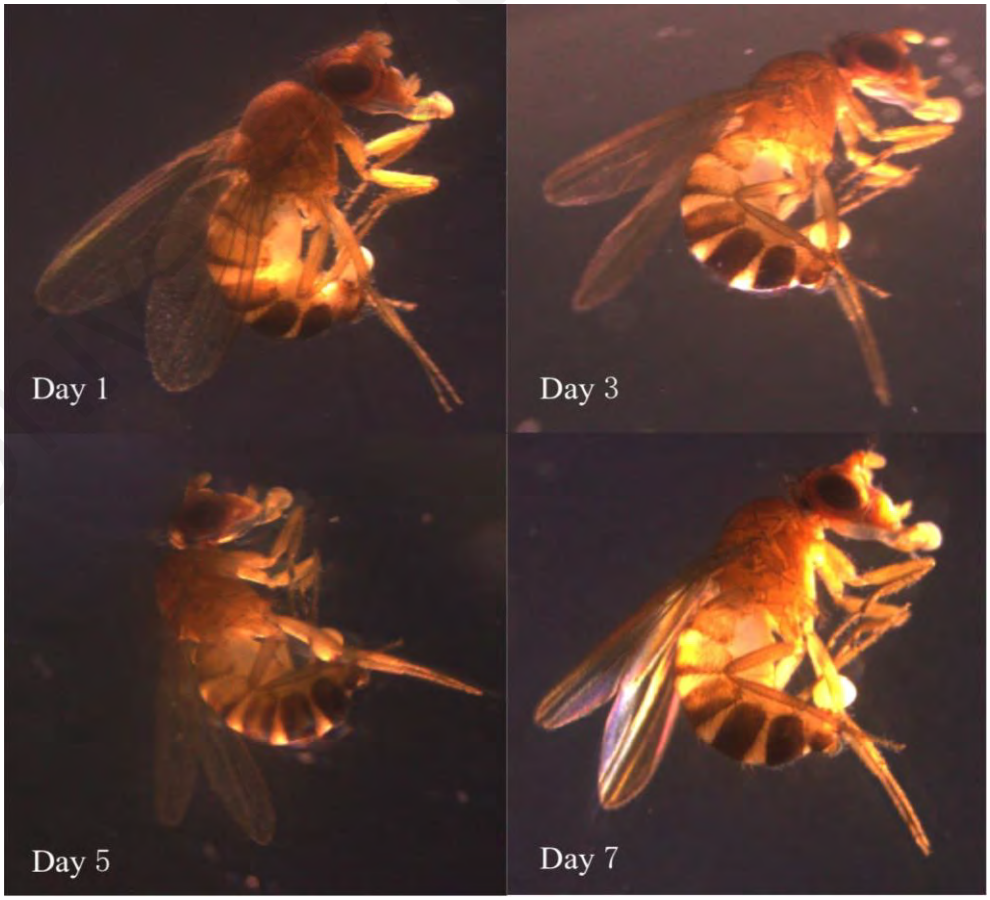


(c)

Figure 4.22, continued.

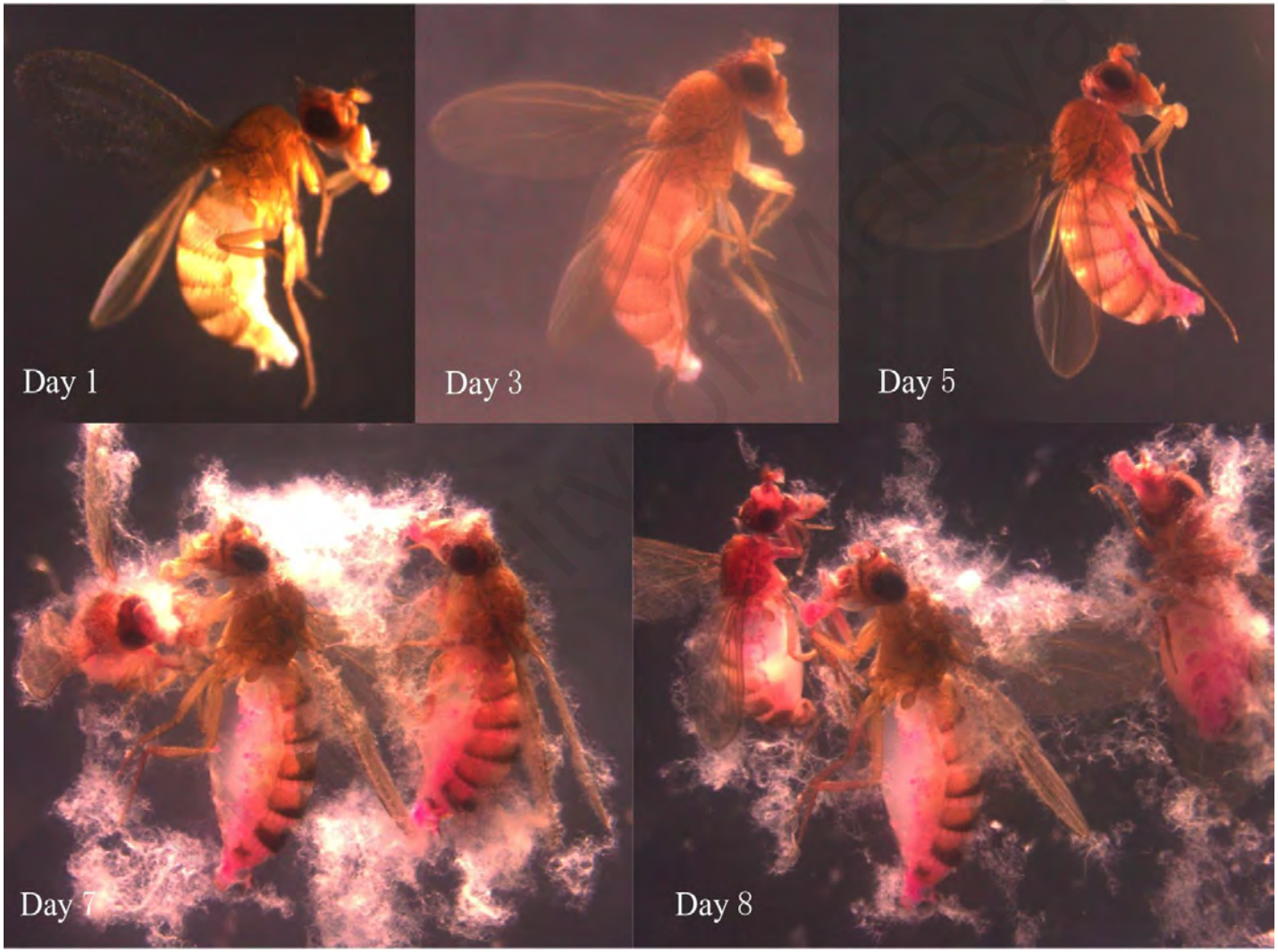


(d)



(e)

Figure 4.22, continued.



(f)

Figure 4.22, continued.

4.21 *N*-acetylglucosamine Detection from *Nepenthes* Pitcher Fluid

Pitcher fluid samples for *N*-acetylglucosamine detection were collected from 5 unopened pitchers and 19 opened pitchers of *N. gracilis* (Table 4.13). The pH values of the samples collected from unopened pitchers were pH5 (Table 4.13). The pH values of the samples collected from opened pitchers range from pH1 to pH5 (Table 4.13). The fluid conditions of samples collected from unopened pitchers were cleared. The fluid conditions of samples collected from opened pitchers ranges from clear through slightly brown to brown and turbid (Table 4.13).

The external standard, 0.01 g/mL *N*-acetylglucosamine was eluted at 0.8 min (Appendix P) using LCMS-qTOF as described in section 3.2.30. Based on the spectra extracted from the chromatogram, the molecular ion for *N*-acetylglucosamine standard was 222.0973 *m/z* and the product ion were 204.0869 *m/z* and 186.0771 *m/z* (Appendix Q).

In this study, the presence of molecular ion, 222.0973 *m/z* (±0.0004) in the extracted spectra of opened pitchers samples 1_OP, 2_OP, 3_OP, 4_OP, 6_OP, 10_OP, 11_OP, 12_OP, 14_OP, 15_OP, 16_OP and 19_OP indicated the presence of *N*-acetylglucosamine in the fluid of (Table 4.14 and Appendix Q). The product ion 204.0869 *m/z* (±0.0005) was detected in the spectra of samples 1_OP, 2_OP, 3_OP, 4_OP, 10_OP, 11_OP, 12_OP, 14_OP, 15_OP and 16_OP (Appendix Q). The product ion 186.0771 *m/z* (±0.0005) was detected in the spectra of samples 3_OP, 4_OP, 6_OP, 10_OP, 14_OP, 15_OP, and 16_OP (Appendix Q). On the other hand, *N*-acetylglucosamine was not detected in the fluid of unopened pitchers 5_OP, 7_OP, 8_OP, 9_OP, 13_OP, 17_OP and 19_OP (Table 4.13). *N*-acetylglucosamine was also not detected in the fluid of unopened pitchers (Table 4.13).

Table 4.13: Metadata and *N*-acetylglucosamine in the samples collected from *N. gracilis*.

Sample	Pitcher State	Fluid pH	Fluid Volume (mL)	Sampling Date	Fluid Condition	*N*-acetylglucosamine
1_CL	Unopened	5	1.0	19th Sept 2017	Clear	Not Detected
2_CL	Unopened	5	2.4	19th Sept 2017	Clear	Not Detected
3_CL	Unopened	5	1.8	19th Sept 2017	Clear	Not Detected
4_CL	Unopened	5	2.5	19th Sept 2017	Clear	Not Detected
5_CL	Unopened	4	2.0	9th Oct 2017	Clear	Not Detected
1_OP	Opened	4	2.7	19th Sept 2017	Brown and turbid	**Detected**
2_OP	Opened	2	0.5	19th Sept 2017	Brown and turbid	**Detected**
3_OP	Opened	2	3.6	19th Sept 2017	Brown and turbid	**Detected**
4_OP	Opened	2	4.5	19th Sept 2017	Slightly brown	**Detected**
5_OP	Opened	3	2.7	19th Sept 2017	Clear	Not Detected
6_OP	Opened	3	1.2	19th Sept 2017	Brown and turbid	**Detected**
7_OP	Opened	2	4.2	19th Sept 2017	Clear	Not Detected
8_OP	Opened	5	10.0	19th Sept 2017	Clear	Not Detected
9_OP	Opened	2	2.7	9th Oct 2017	Clear	Not Detected
10_OP	Opened	2	2.7	9th Oct 2017	Slightly brown	**Detected**
11_OP	Opened	1	0.8	9th Oct 2017	Slightly brown	**Detected**
12_OP	Opened	2	1.4	9th Oct 2017	Slightly brown	**Detected**
13_OP	Opened	2	1.2	9th Oct 2017	Clear	Not Detected
14_OP	Opened	2	6.8	9th Oct 2017	Brown and turbid	**Detected**
15_OP	Opened	3	5.5	9th Oct 2017	Clear	**Detected**
16_OP	Opened	3	5.2	9th Oct 2017	Slightly brown	**Detected**
17_OP	Opened	4	2.5	9th Oct 2017	Clear	Not Detected
18_OP	Opened	2	4.9	9th Oct 2017	Clear	Not Detected
19_OP	Opened	3	7.6	9th Oct 2017	Clear	**Detected**

CHAPTER 5: DISCUSSION

Nepenthes plants grow on nutrient depleted soil at moist and sunny environment (Clarke & Moran, 2016; Givnish et al., 1984; Pavlovič et al., 2007). In order to compensate for the nutrient depletion, *Nepenthes* develops pitchers through leaf modification for preys assimilation (Adamec, 2010; Owen & Lennon, 1999). The lid, peristome and upper part of the pitcher lured and slipped the preys into the pitcher fluid (Bauer et al., 2008; Benz et al., 2012; Bohn & Federle, 2004; Gorb et al., 2005). The preys are drown by the pitcher fluid and the carcasses are degraded by the hydrolytic enzymes in the pitcher fluid degrades the captive (Clarke, 1997; Phillipps & Lamb, 1996). Arthropod-based nutrients released into the pitcher fluids are absorbed by the plant through multicellular glands at the lower part of the pitcher (Thornhill et al., 2008).

Nepenthes plant conditioned its pitcher fluid into an acidic environment, which favours the activities of the plant hydrolytic enzymes (Athauda et al., 1998; Athauda et al., 2004; Takahashi et al., 2009). Some of the plant hydrolytic enzymes, for example chitinases and protease, are classified as PR proteins (Mithöfer, 2011). These dual functions hydrolytic enzymes degrades the prey in the pitcher fluid at the same time protect the plant from pathogens (Mithöfer, 2011). On top of hydrolytic enzymes, PR-1 protein, napthoquinones and its derivatives, which are antimicrobial, are secreted into the pitcher fluid upon induction or maturation of the pitcher (Buch et al., 2014; Buch et al., 2013; Eilenberg et al., 2009; Mithöfer, 2011; Raj et al., 2011). The acidic pitcher fluid supplied with antimicrobial compounds and PR proteins have led to the suggestion that *Nepenthes* pitcher fluids are harsh living environment for microorganisms (Buch et al., 2013; Buch et al., 2014; Eilenberg et al., 2009; Hatano & Hamada, 2008; Lee et al., 2016; Raj et al., 2011; Rottloff et al., 2016).

Despite of the harsh environment created by *Nepenthes* plants, the pitcher fluids are natural living habitats to bacteria, fungi, protist, insect's larvae, nematodes, annelids, mites and crustaceous (Adlassnig et al., 2010; Beaver, 1979; Beekmann, 2004; Bittleston et al., 2016; Chou et al., 2014; Clarke, 1998; Kanokratana et al., 2016; Mogi, 2004; Mogi & Chan, 1996; Mogi & Chan, 1997; Quisado, 2013; Sickel et al., 2016; Takeuchi et al., 2015). These inquilines can be symbiotic, commensalistic or parasitic to the *Nepenthes* plant.

5.1 Bacterial Community

Since the invention of NGS, number of publications on comprehensive bacterial community compositions in the pitcher fluid of different *Nepenthes* species has increased (Chou et al., 2014; Kanokratana et al., 2016; Sickel et al., 2016; Takeuchi et al., 2015). However, information on *Nepenthes*-associated bacteria remain sparse. In this study, the diversity and assemblage of bacterial communities in the pitcher fluids of *N. sanguinea*, *N. albomarginata* and *N. gracilis*, which are the native to Peninsula Malaysia, were assessed using NGS data. In addition, the bacterial community in the pitcher fluid of *N. hookeriana* was assessed in this study. To my knowledge, this is the first report of the bacterial community in the pitcher fluid of *N. sanguinea*.

In this study, bacterial diversity and species richness were higher in pitcher fluids of lower acidity. This result was supported by the bacterial taxa summary, where acidophilic *Acidocella* and *Acidisoma* dominated high acidity samples. Meanwhile, various bacteria taxa were found in the bacterial community of low acidity samples. An In acidic environment, inorganic acid and undissociate organic acid diffused into the bacterial cell leading to the decrement of intracellular pH level (Audia et al., 2001;

Bearson et al., 1997; Wesche et al., 2009). The lowered intracellular pH causes acid stress to bacterial cells and the effect is lethal if the bacteria failed to overcome the stress. Lower acid stress is exerted by low acidity pitcher fluid on its inhabitants compared to its high acidity counterpart. Thus, enable the survival and adaptation of broader range of bacteria in low acidity samples (Bearson et al., 1997). Significantly higher bacterial diversity and species richness were observed in samples H1 and LK compared to the other samples in this study. The combination of amplicons from several annealing temperature during sample preparation in order to capture as much bacteria species and NGS using 454-GS-FLX platform which generates longer read length were suggested as the cause of this observation.

In general, Proteobacteria is the predominant bacteria in the pitcher fluids of *N. albomarginata*, *N. gracilis*, *N. hookeriana* and *N. sanguinea*. Other dominating bacteria phyla were Bacteroidetes, Actinobacteria, Acidobacteria and Firmicutes. These results were corroborated to previous studies on the bacterial community compositions in the pitcher fluids of *N. rafflesiana*, *N. hemsleyana*, *N. ampullaria*, *N. gracilis*, *N. mirabilis*, *N. andamana*, *N. albomarginata*, *N. hirsute*, *N. smilesii* and *N. suratensis* (Chou et al., 2014; Kanokratana et al., 2016; Sickel et al., 2016; Takeuchi et al., 2015). Although different dietary preferences are observed in some of the mentioned *Nepenthes* species (for examples preference in detritivorous diet by *N. ampullaria*; preference in coprophagous diet by *N. hemsleyana* and termite specialization by *N. albomarginata*), the bacteria community compositions in the pitcher fluids of these *Nepenthes* species are similar to certain extent (Merbach et al., 2002; Moran et al., 2003; Moran et al., 2001; Schöner et al., 2017; Sickel et al., 2016). This suggested that *Nepenthes* plants exert influence on the bacterial community structure in its pitcher fluid.

Proteobacterial genus *Acidocella* dominated (up to 99.5%) the bacterial communities in high acidity (pH1 to pH4) samples. Similar result was found in previous study, where *Acidocella* accounted at least 30% of the bacterial communities in pitcher fluids with pH level lower than 4 (Kanokratana et al., 2016). Besides, high abundance of *Acidocella* was also found in the pitcher fluid of *N. rafflesiana* (unknown pH value) (Sickel et al., 2016). The highly acidic pitcher fluids (pH1 to pH4) have created favorable living environment that led to thrive of *Acidocella.* In addition to *Nepenthes* pitcher fluid, plant-related *Acidocella* are isolated from the roots of reedgrass grown on highly acidic sulfate soil and waterweed grown on highly acidic swamp (Kimoto et al., 2010; Oishi et al., 1999). Acidophilic *Acidocella* are also found in highly acidic environment such as acidic mine drainage, and natural acidic lake (Kishimoto et al., 1993; Kishimoto, et al., 1995; Servín-Garcidueñas et al., 2013; Wichlacz et al., 1986). The detection of lipolytic activity from the culture of *Acidocella* strain USBA-GBX-505 suggested the contribution of *Acidocella* in *Nepenthes* prey catabolism (Bernal et al., 2017).

Previous studies have detected *Acidisoma* in the fluid of both opened and unopened *Nepenthes* pitchers (Chou et al., 2014; Kanokratana et al., 2016; Sickel et al., 2016). In this study, strikingly high relative abundance (99.4%) of *Acidisoma* was detected in sample N7, which has pH value of 3. Proteobacterial genus *Acidisoma* is an acidophilic bacterium. It is found in various environments such as acidic *Sphagnum*-dominated tundra, acidic wetland, acidic lake, peatlands and decaying pinewood and lichen (Belova et al., 2009; Davis-Belmar et al., 2013; Grube et al., 2009; Kielak et al., 2016; Oloo et al., 2016; Printzen et al., 2012). Study on the type strains of *Acidisoma* show that they grow on medium MB with pH value ranging from pH3 to pH7 (Belova et al., 2009). The optimum pH for the growth of *Acidisoma* type strains range from pH3 to pH5.7 (Belova et al., 2009). The pH of sample N7, which falls within the pH optima for

the growth of *Acidisoma*, might be the reason of *Acidisoma* thrives in the sample. However, the contribution of *Acidisoma* in *Nepenthes* pitcher fluid remains unknown.

Reported soil and freshwater bacteria, such as candidate alphaproteobacterial order Ellin 329, Kaistia, Burkholderia, Sphingomonadaceae, Xanthomonadaceae, Microbacteriaceae, Curtobacterium, Mycobacterium, Sphingobacteriaceae, Sediminibacterium and Chitinophagaceae, were detected in low acidity samples (Baik et al., 2010; Brandt et al., 2002; Chen et al., 2013a; Chen et al., 2013b; Ducey et al., 2013; Huang et al., 2009a; Im et al., 2004; Kim & Jung, 2007; Kim et al., 2008a; Kim et al., 2008b; Kim et al., 2013; Lee et al., 2009; Lee et al., 2012; Madhaiyan et al., 2015; Nakai et al., 2015; Pinto et al., 2018; Primm et al., 2004; Sait et al., 2002; Sheu et al., 2015; Sun et al., 2007; Sun et al., 2006; Urai et al., 2008; Weon et al., 2008; Weon et al., 2009a; Weon et al., 2009b; Yi et al., 2010; Yoon et al., 2013). Besides, airborne bacteria such as *Xanthomonadaceae*, *Microbacteriaceae* and *Microbacterium* were also found in the samples (Jang et al., 2012; Kämpfer et al., 2010; Kim et al., 2012; Weon et al., 2013; Zlamala et al., 2002). The detection of soil, freshwater and airborne bacteria in the pitcher fluids suggested that part of the *Nepenthes* pitcher fluid bacteria are originated from the environment.

Apart from environmental bacteria, arthropods-associated bacteria such as *Acetobacteraceae*, Rhizobiales-related bacterium and *Microbacteriaceae* were detected in this study. Previous study has found *Microbacteriaceae* from the larvae of giant crane fly, *Tipula abdominalis* (Cook et al., 2008). Besides, *Acetobacteraceae* and Rhizobiales-related bacteria are found in the guts of ants species, *Camponotus chromaiodes* and *Paraponera clavata*, respectively (Brown & Wernegreen, 2016; Larson et al., 2014). Among the prey assemblages, ants are the commonest prey for

Nepenthes plants (Adam, 1997; Chin et al., 2014; Gaume et al., 2016; Hosoishi, 2012). Several ant species including *Camponotus* sp. are documented as the prey assemblage in *Nepenthes* pitcher fluid (Hosoishi, 2012). An exception is observed from ant species, *Camponotus schmitzi*, which practices mutualism with *N. bicalcarata* (Bonhomme et al., 2011a; Merbach et al., 2007; Scharmann et al., 2013; Thornham et al., 2012). The detection of arthropods-associated bacteria suggested that the bacteria are introduced to the pitcher fluid by preyed arthropods and arthropods that interacted with *Nepenthes* plants.

The inner surface of *Nepenthes* pitcher is not accessible externally before the pitcher opened, as the lid is tightly sealed to the pitcher (Owen & Lennon, 1999). Thus, the detection of putative endophytic bacteria in previous study suggested that endophytic bacteria is the early colonizer of *Nepenthes* pitcher fluid (Chou et al., 2014). In this study, reported endophytic bacteria of *Nepenthes* plant such as *Burkholderia*, Firmicutes, and *Xanthomonadaceae* are found in the samples (Bhore et al., 2013). In addition, other reported endophytic bacteria include Curtobacterium, Chitinophagaceae, and Sphingomonadaceae were also found in this study (Garrido et al., 2016; Khan et al., 2014; Diogo et al., 2014; Proença et al., 2017). This supported the proposed hypothesis, where endophytic bacteria accessed and colonized the pitcher fluid before the pitcher was exposed to the environment.

In order to survive on nutrient depleted soil, nutrient acquisition through arthropods assimilation is an important process for the *Nepenthes* plant (Adamec, 2010). The bacteria community is predicted to contribute in prey digestion and nutrient recycling. During arthropod degradation process, chitinase, chitosanase and *N*-acetylglucosaminase are the key enzymes for the degradation of the chitinous

exoskeleton and its derivatives (Beier & Bertilsson, 2013; Foster & Webber, 1961). The detection of putative chitinase and chitosanase producing bacteria such as *Chitinophagaceae* and *Microbacterium* from the samples, suggested the contribution of bacteria on insect degradation (Del Rio et al., 2010; Sun et al., 2007; Sun et al., 2006; Weon et al., 2009b; Zhang et al., 2013). On the other hand, the detection of putative mono, di- or polysaccharides degraders such as *Microbacterium*, candidate alphaproteobacterial order Ellin 329 and *Sphingobacteriaceae* suggested that contribution of bacteria in nutrient recycling from plant detritus in the pitcher fluid (Harbison et al., 2016; Kodama & Watanabe, 2011; Pankratov et al., 2007; Rivas et al., 2004; Sait et al., 2002; Takasaki et al., 1991).

The detection of considerable amount of putative nitrogen-fixing bacteria such as *Burkholderia*, *Bradyrhizobium* and *Methylosinus* from *Nepenthes* pitcher fluid was first highlighted in the study of bacterial community from *N. rafflesiana* and *N. hemsleyana* (Sickel et al., 2016). In corroboration to previous study, commonly known nitrogen-fixing bacteria, such as *Burkholderia*, *Cupriavidus*, Rhizobiales, *Bradyrhizobiaceae* and *Acetobacteraceae* were found in the pitcher fluids of N. sanguinea, N. albomarginata and N. gracilis collected for this study (Caballero-Mellado et al., 2004; Silva et al., 2012; Souza et al., 2014; Estrada-De et al., 2001; Gillis et al., 1989; Im et al., 2006; Itakura et al., 2009; Jones, 2015; Jourand et al., 2004; MacLean et al., 2007; Reis et al., 2004; Reis & Teixeira, 2015; Rivas et al., 2002; Samaddar et al., 2011; Vandamme et al., 2002). This suggested that the nitrogen-fixing bacteria are alternative nitrogen supplier to *Nepenthes* plant. Former study found that nitrogen-fixing bacteria on the leaf of northern carnivorous pitcher plant, *Sarracenia purpurea*, are capable to generate substantial amount of nitrogen for the plant's growth and development (Prankevicius & Cameron, 1991). Even though the utilization of bacterial fixed nitrogen by *S. purpurea*

plant is not experimentally confirmed, the nitrogen-fixing bacteria are suggested as important nitrogen suppliers of this carnivorous pitcher plant (Prankevicius & Cameron, 1991). In the contrary, the contribution of bacteria fixed nitrogen to *Nepenthes* plant was predicted to be of lower magnitude, especially in highly acidic pitcher fluids, as compared to *Sarracenia*. Two reasons were suggested for the low nitrogen fixation rate in highly acidic pitcher fluids. Firstly, low abundance or absent of putative nitrogen-fixing bacteria in highly acidic pitcher fluids as a result of intolerable acidic living condition. Second, bacterial nitrogen fixation is inhibited or suppressed by high concentration of ammonium, which induced the release of H^+ for pitcher fluid acidification (Higashi et al., 1993; Vintila & El-Shehawy, 2007). Comparable example is observed from the aquatic carnivorous plants, *Utricularia* in which the bacterial fixed nitrogen contributed less than 1% of the daily plant gain nitrogen, albeit significant relative abundance of nitrogen-fixing bacteria are detected in the traps of *Utricularia* (Sirová et al., 2014). The high concentration of ammonium in the *Utricularia* trap, which might inhibit the expression of microbial *nif* gene is the suggested cause for the low nitrogen fixation rate (Sirová et al., 2014).

5.2 Correlation of Bacterial Communities with Pitcher Fluid Acidity, Host Plant and Sampling Location

Variations of bacterial community compositions in the pitcher fluids of different *Nepenthes* pitchers are observed in both present and previous studies. However, report on the factors that shaped the bacterial community in *Nepenthes* pitcher fluid is very limited. Only 1 report on the correlation of bacterial communities dissimilarity and biotic factor is available, hitherto.

In corroboration with previous study, the bacterial community composition was strongly correlated to the pH value of *Nepenthes* pitcher fluid (Kanokratana et al., 2016). The acidity of *Nepenthes* pitcher fluids varies from pitcher to pitcher, albeit collected from the same *Nepenthes* plant on same season. *Nepenthes* plant alters the acidity of its pitcher fluid according to age of the pitcher, number of prey and types of prey captured (An et al., 2002a; Clarke, 1997; Clarke & Lee, 2004; Higashi et al., 1993; Higgins, 2001). The acidification of pitcher fluid is predicted to play important role in prey retention, prey killing, plant enzymes hydrolytic activities and nutrient uptake from pitcher fluid (An et al., 2001; Bazile et al., 2015; Eilenberg & Zilberstein, 2008; Michelet & Boutry, 1995; Moran et al., 2010; Shivas, 1984). As the opened pitcher has limited control on the bacteria introduced into its pitcher fluid, it was suggested that the acidity of the *Nepenthes* pitcher fluid also plays an important role in controlling the population of its bacterial inhabitants.

Strong influence of environmental pH on the bacterial community composition is consistently observed in soil bacterial community studies (Lauber et al., 2009; Rousk et al., 2010; Shen et al., 2013). Significant differences in bacterial community composition is observed from soil samples of different pH, albeit the sampling sites are 180m apart, emphasized the influence of pH on the bacterial community (Rousk et al., 2010). The narrow pH range for the growth of most bacteria taxa is proposed as an explanation for the strong influence of the soil pH on the bacterial community composition (Rosso et al., 1995; Rousk et al., 2010). However, pH in the bacterial living environment is not the only factor that influences the bacterial community composition. Other factors such as salinity, types of environment and temperature also affect the bacteria community composition (Herlemann et al., 2011; Lozupone & Knight, 2007; Tajima et al., 2007).

In this study, bacterial community compositions in *Nepenthes* pitcher fluids were also influenced by host plant and sampling site. However, the influences of plant host and sampling site on the bacterial community compositions were not conclusive as the pitcher fluids were collected from different *N. sanguinea* plants at different sampling sites and seasons. Other possible factors that shaped the bacterial composition in *Nepenthes* pitcher fluid includes pitcher age, pitcher type (upper or lower pitchers), pitcher fluid salinity, level of oxygen in pitcher fluid, types of prey captured and plant secretion (Chou et al., 2014; Sickel et al., 2016; Takeuchi et al., 2015).

Besides, clustering of bacterial community profile (generated from shotgun metagenome data) by *Nepenthes* species was observed from the UPGMA tree suggested the influence of *Nepenthes* species on its pitcher fluid bacterial community composition. Since all shotgun metagenomic samples are highly acidic (pH1 to pH4), the influence of pitcher fluid pH on bacterial communities was minimized. This finding was contrary to previous study, in which the correlation of bacterial community structure and *Nepenthes* species is not significant (Kanokratana et al., 2016).

5.3 Eukaryotes in *Nepenthes* Pitcher Fluid

The insectivorous practice of *Nepenthes* plants have enable its thriving on nutrient-depleted environment (Ellison & Gotelli, 2001; Givnish et al., 1984). In addition to prey assimilation, studies on food webs, microbial community, symbionts and inhabitants of *Nepenthes* plants show that *Nepenthes* pitcher fluids are natural habitats to a range of microscopic and macroscopic eukaryotes (Adlassnig et al., 2010; Beaver, 1979; Bittleston et al., 2016; Clarke, 1998; Miyagi & Toma, 2007; Mogi & Chan, 1996; Mogi & Chan, 1997; Sota et al., 1998). In this study, insects, worms, arachnids, fungus and

unicellular eukaryotes were found in the pitcher fluids of *N. sanguinea* and *N. gracilis* plants.

Arthropods orders Diptera, Hymenoptera and Coleoptera are commonly reported as the main group of prey captured by *Nepenthes* (Chin et al., 2014; Di Giusto et al., 2009; Moran et al., 1999). In corroboration to previous reports, insects (arthropods order Dipteran, Hymenoptera and Coleoptera) formed the main eukaryotes composition in the *N. sanguinea* samples. On the other hand, low relative abundance of insect except Diptera was observed from the *N. gracilis* samples. The influence of the natural habitat was suggested as an explanation for the difference in the insect's composition between these two *Nepenthes* species that deployed similar pitcher characteristics (reduced peristome, waxy pitcher wall and absent of viscoelastic fluid) for prey trapping, but grow at environment of different altitude (Bauer et al., 2012b; Moran et al., 2013). Influence of natural habitat on the *Nepenthes*' prey spectrum is observed on the highland and lowland *Nepenthes* species. The highland *Nepenthes* species are found to capture more flying insects; meanwhile, the lowland *Nepenthes* species capture more crawling insects (Adam, 1997; Gaume et al., 2016; Moran et al., 1999; Peng & Clarke, 2015). This reported work supports my finding in which the relative abundance of flying insect (taxon order Diptera, Hymenoptera and Coleoptera) was higher in *N. sanguinea* samples (highland species) compared to *N. gracilis* samples (lowland species).

Diptera formed the largest group of insects in the samples collected in this study. Aside from the prey of *Nepenthes*, some Diptera species spent part of their life cycle in the *Nepenthes* pitcher fluid. To date, more than 20 genera of Dipterans are reported as the inhabitant in *Nepenthes* pitcher fluid (Adlassnig et al., 2010). Dipterans' larvae are

common inhabitant in *Nepenthes* pitcher fluid (Kato et al., 1993; Lam et al., 2017; Mogi & Chan, 1997; Mogi & Yong, 1992; Ratsirarson & Silander, 1996). It serves as the predators, detritus feeders and filter feeders in the food web in the pitcher fluids (Adlassnig et al., 2010; Beaver, 1979; Kato et al., 1993; Mogi & Chan, 1996; Mogi & Yong, 1992; Ratsirarson & Silander, 1996; Sota et al., 1998). The suggested contribution of Dipteran's larvae to the *Nepenthes* plant includes breaking up insect's carcass, removing detritus and controlling the population of eukaryotes and bacteria living in the pitcher fluid (Beaver, 1979; Lam et al., 2017; Mogi & Chan, 1996; Mogi & Yong, 1992; Sota et al., 1998). Besides, study by Lam et al. shows that the presence of Dipteran's larvae in the pitcher fluid of *N. gracilis* enhances the release of ammonium and soluble protein, which are the nitrogen source of *Nepenthes* plant, into the pitcher fluid (Lam et al., 2017).

Ants (taxon order Hymenoptera, family *Formicidae*) are consistently reported as the largest group of prey captured by *N. gracilis* (Adam, 1997; Chin et al., 2014; Gaume et al., 2016; Kato et al., 1993; Peng & Clarke, 2015). The waxy pitcher and flickable characteristic of the pitcher lid have largely increase the ant catching efficacy on *N. gracilis* during raining season (Bauer et al., 2012b; Bonhomme et al., 2011b; Gaume et al., 2016). In contrary to previous studies, the relative abundance of Hymenoptera was relatively low in the samples collected from *N. gracilis*. This result was in corroboration to Bittleston et al. (2016) study, which is also conducted using NGS data. In Bittleson et al. (2016) study, the authors suggested that DNA of the *Nepenthes* prey was largely degraded by the pitcher fluid's inhabitants. This hypothesis is supported by the detection of deoxyribonucleases and endonuclease from *Nepenthes* pitcher fluids (Chin et al., 2007; Ekanayaka, 2009; Lee et al., 2016). Besides, the removal of ant's carcasses

from the samples before DNA extraction also led to the low amount of ant's DNA in this study (Bittleston et al., 2016).

Arthropod class Arachnid is not the main prey target of Nepenthes, but it is occasionally found within the prey composition in Nepenthes pitcher (Adam, 1997; Chin et al., 2014; Di Giusto et al., 2008; Di Giusto et al., 2009; Gaume et al., 2016; Kato et al., 1993; Moran, 1996; Moran et al., 1999; Peng & Clarke, 2015; Ratsirarson & Silander, 1996; Rembold et al., 2010). In this study, Arachnids order Acari was detected in all samples, while Arachnids order *Araneae* and Opiliones were detected in *N. sanguinea* samples. These 3 orders of Arachnid have been reported as the prey of *Nepenthes* (Di Giusto et al., 2008; Kato et al., 1993; Marina et al., 2018; Peng & Clarke, 2015; Rembold et al., 2010). Although a few species of *Araneae* (*Synema obscuripes, Misumenops nepenthicola* and *Thomisus nepenthiphilus*) are reported as the inhabitant of *Nepenthes* pitcher, it was not reported as the inhabitant in *Nepenthes* pitcher fluid (Beaver, 1979; Chua & Lim, 2012; Pocock, 1898; Rembold et al., 2013). Thus, the *Araneae* and Opiliones detected in this study were speculated as the prey of *N. sanguinea*. Unlike *Araneae*, aquatic Acari are commonly found living in the *Nepenthes* pitcher fluid and might feed on the detritus in the pitcher fluid (Adlassnig et al., 2010; Beaver, 1979; Fashing, 2002; Fashing, 2005; Fashing, 2008). This suggested that the Acari's DNA was originated from both prey and inhabitant Acari in the samples.

Nematode orders Diplogasterida, Rhabditida, Plectida, Dorylaimida, Enoplida, Desmodorida and Chromadorida, are reported as the inhabitant of *Nepenthes* pitcher fluid (Ahmad et al., 2004; Bert et al., 2003; Menzel, 1922; Quisado, 2013; Van Oye, 1921). Former studies have suggested that nematodes play a role as the detritus and bacterial feeders in the pitcher fluid's food web (Devi & George, 2018; Sota et al.,

1998; Lieven & Sudhaus, 2000). In this study, nematode orders Diplogasterida and Rhabditida were the dominating nematode in the pitcher fluid of *N. gracilis* and *N. sanguinea*, respectively. The association of Diplogasterida with insects and the entomopathogenic characteristic of Rhabditida suggested that these nematodes were introduced to the Nepenthes pitcher fluid through the insect's larvae and insect preyed by *Nepenthes* plant (de Waal et al., 2018; Giblin & Kaya, 1984; Hazir et al., 2015; Herrmann et al., 2006; Knoff et al., 2017; Luong et al., 1999; Malan et al., 2016; Pascual et al., 2017). Besides, the predatory behavior of Diplogasterida, which kills other nematodes, was suggested as the cause of low abundance or absence of other nematode in the pitcher fluid of the *N. gracilis* samples, in the present study (Bajaj & Kanwar, 2015; Bilgrami & Jairajpuri, 1989; Chitambar & Noffsinger, 1989; Devi & George, 2018; Khan et al., 2016).

Fungi divisions Ascomycota, Basidiomycota, Myxomycota and Zygomycota are found living in *Nepenthes* pitcher fluid (Adlassnig et al., 2010; Mase et al., 2009; Okahara, 1933; Prabhugaonkar & Pratibha, 2017; Shivas & Brown, 1989; Van Oye, 1921). In addition, unidentified acid-resistant yeasts are also isolated from the pitcher fluid of *N. alata* and *N. madagascariensis* (An et al., 2001; An et al., 2002a; Ratsirarson & Silander, 1996). The fungi serve as the food source for filter feeder in the food web in pitcher fluid (Ratsirarson & Silander, 1996). Previous study shows that the abundance and diversity of yeast in pitcher fluid are influenced by the host species and the position of the pitcher (upper or lower pitchers) (Shivas & Brown, 1989). However, host specialization was not observed from the fungi and yeast community in this study.

In this study, fungus division Ascomycota (*Ophiostomataceae*, Sordariomycetes, Barbatosphaeria, Arthoniomycetes, Hypocreales, *Aliquandostipitaceae* and members of Saccharomycetales) was the main assemblage in the fungal community of the samples. Fungus division Zoopagomycota order Entomophthorales was also detected in the samples. The arthropods preyed and visited the *Nepenthes* pitchers were suggested as the agent that introduced the entomopathogenic Entomophthorales and Hypocreales, and the arthropods-associated *Ophiostomataceae* to the pitcher fluids (Feng et al., 1991; Furniss et al., 1995; Inglis et al., 2012; Jensen et al., 1998; Lacey et al., 2011; Papierok & Hajek, 1997; Posada & Vega, 2005; Romón et al., 2014; Watson et al., 1993). Besides, *Ophiostomataceae* (wood-staining fungi), Sordariomycetes (includes phytopathogen, endophyte and lichenized fungi), *Barbatosphaeria* (fungi that grows on decayed wood), Arthoniomycetes (lichenized fungi) and *Aliquandostipitaceae* (fungi that grows on decaying woods in tropical forest and freshwater) might be introduced to the pitcher fluid from the forest by wind, rain or organism that interact with the pitchers (Armstrong et al., 2018; Frisch & Ohmura, 2013; Furniss et al., 1995; Hodkinson, 2012; Hutchison & Reid, 1988; Inderbitzin et al., 2001; Lücking et al., 2016; Maharachchikumbura et al., 2016; Raja & Shearer, 2007; Reblova, 2007; Réblová et al., 2015; Solheim, 1986; Zhang et al., 2006).

In corroboration to Bittleston et al. (2016) study using NGS, members of yeast class Saccharomycetes were detected in this study. The *Saccharomycetales* detected in this study were from genera *Meyerozyma*, *Candida*, *Dekkera* and *Tetrapisispora*. In conjunction with the isolation of caseinase and invertase producing yeasts from the pitcher fluid, the detection of Saccharomycetes genera *Meyerozyma*, *Candida* and *Dekkera* which perform alcoholic fermentation using polysaccharides (includes cellobiose and xylose) and monosaccharides, suggested the involvement of yeasts in

prey and detritus digestion in pitcher fluid (An et al., 2001; An et al., 2002a; Aryuman et al., 2015; Blondin et al., 1983; Ciani & Ferraro, 1997; Delgenes et al., 1996; Du Preez et al., 1986; Martini et al., 2016; Mase et al., 2009; Suárez et al., 2007; Toivola et al., 1984). Besides, *Meyerozyma* and *Tetrapisispora* are reported as biocontrol agents on spoilage yeast in wine fermentation and phytopathogenic yeast of mango, respectively (Bautista-Rosales et al., 2013; Comitini & Ciani, 2010; Comitini et al., 2009; Oro et al., 2014). This suggested that *Meyerozyma* and *Tetrapisispora* may be protecting its' *Nepenthes* host by killing or suppressing the growth of phytopathogen.

Protist members of Amoebozoa, Alveolata, Ciliates, Excavata, Mastigophora, Rhizaria, Rhizopoda and Sarcodina are recorded as the inhabitant in the *Nepenthes* pitcher fluid (Adlassnig et al., 2010; Bittleston et al., 2016; Dover et al., 1928; Oye, 1921). In this study, Alveolates (*Cryptosporidium*, Ciliophora and Colpodida), Rhizaria (Cercozoa) and Excavata (Tetramitia) were detected in *N. gracilis* samples. Protist was not detected in the pitcher fluid of *N. sanguinea.* The cause for the absence of protist in *N. sanguinea* samples was not study in this project; thus, remains unknown. In the pitcher fluid, protist are important food source for filter feeder in pitcher fluid (Ratsirarson & Silander , 1996). In addition, the presence of bacterivorous Tetramitia, Ciliophora and Cercozoa, might play a role in controlling bacterial population in the pitcher fluid (Bass et al., 2009; Howe et al., 2011; Moon et al., 2017; Pánek & Čepička, 2012). Besides, protist may be helping the plant on prey and detritus degradation (Ratsirarson & Silander, 1996).

In brief, *Nepenthes* pitcher fluid provides food and shelter to wide range of macro and microscopic organisms. In this study, the preys (insects and arachnids) and inhabitants (dipteran's larvae, Acari, nematodes, fungi and protista) formed the eukaryotes assemblage in *Nepenthes* pitcher fluid. The preys of *Nepenthes* serve as food source for both *Nepenthes* plant and the inhabitants in *Nepenthes* pitcher. Meanwhile, the inhabitants may help in prey carcasses fermentation and degradation, kill and suppress the growth of phytopathogen and control the population of the trophic species in the food web of *Nepenthes* pitcher fluid (Adlassnig et al., 2010; Dover et al., 1928; Sota et al., 1998). In the pitcher fluid, the inhabitants are important predator, filter feeder, detritus feeder, and decomposer. Besides, the assessment of eukaryotic assemblage using NGS data from metagenome samples has allowed more comprehensive study on the inhabitants in *Nepenthes* pitcher fluid, especially the living microscopic organisms (fungi and protista). The identification of fungi and protista, which relies on their cultivability and observation through microscope, often causes the underestimation on the diversity. Host specialization was observed on certain groups of eukaryotes; for example, protists were only detected in *N. gracilis* samples in this study. The *Nepenthes* plant species, growth environment and pitcher fluid acidity were suggested as the factors that shapes the eukaryotic community in the *Nepenthes* pitcher fluid. However, experimental studies are needed in order to determine the factors that shape the eukaryotic community in the *Nepenthes* pitcher fluid.

5.4 Viral Community

Nepenthes plant-associated virus is one of the least study subjects among *Nepenthes* plant related studies. To my knowledge, *Beet western yellow virus* (polerovirus) is the only record on virus that naturally infecting *Nepenthes* plant (Miguel et al., 2016). In this study, *Beet western yellow virus* was not detected in the samples. Dasheen mosaic

virus (potyvirus), Vicia cryptic virus (partitivirus) and *Bombyx mori* nucleopolyhedrovirus (bactulovirus) were the major groups of viruses found in the samples.

Dasheen mosaic virus is commonly found infecting plants family Araceae (Babu et al., 2011a; Babu et al., 2011b; Pearson et al., 1998; Ram et al., 2003; Reyes et al., 2009; Zettler et al., 1970). Leaf mosaic symptoms and leaf distortion are observed from the araceous plants infected by Dasheen mosaic virus (Babu et al., 2011b; Pearson et al., 1998; Reyes et al., 2009; Simone & Zettler, 1991). In nature, arphids are the main vectors for Dasheen mosaic virus transmission (Babu et al., 2011b; Reyes et al., 2009; Simone & Zettler, 1991). The Dasheen mosaic virus detected in this study might be associated to the insects preyed by *Nepenthes* pitcher. Even though patches of red spots against the green background are often observed on *Nepenthes* pitchers, the infection of *Nepenthes* plant by Dasheen mosaic virus is at low possibility. This is because Dasheen mosaic virus infection is almost restricted to araceous plants (Babu et al., 2011a; Binoy Babu et al., 2011b; Pearson et al., 1998; Ram et al., 2003; Reyes et al., 2009; Zettler et al., 1970).

Vicia cryptic virus is discovered from several cultivars of *Vicia faba* (Abou-Elnasr et al., 1985; Blawid et al., 2007; Boccardo et al., 1987; Kenten et al., 1978). Unlike Dasheen mosaic virus, *Cryptic* virus infection resulted mild to no symptom on its host (Boccardo et al., 1987; Roossinck, 2015a). Vicia cryptic virus is strictly vertical transmitted virus and persistent in lineage of infected plant (Abou-Elnasr et al., 1985; Roossinck, 2012; Roossinck, 2015b). The pollens of infected plant that falls into the *Nepenthes* pitcher fluid were a possible source of Vicia cryptic virus found in this study.

The presence of *Dasheen mosaic virus* and *Vicia cryptic virus*, which are singled-stranded RNA (ssRNA) virus and double-stranded RNA (dsRNA) virus, respectively, in *Nepenthes* pitcher fluid were not conclusive. This is because RNAse A treatment, which is responsible for the removal of RNA from the metagenome samples, was included in the DNA extraction protocol. In addition, the NGS library preparation was not devoted to RNA sequencing. Similar issue was observed in studies by Dubinkina et al. (2017) and Philips et al. (2017) that deployed similar protocol (NGS on DNA libraries created from shotgun metagenome samples and virus components identification using MetaPhlAn2) (Dubinkina et al., 2017; Philips et al., 2017). Philips et al. (2017) has raised their concern on the issue and suggested 2 explanations for the incident. Firstly, the viral RNA was accidentally reverse transcript by the environmental reverse transcriptase or DNA polymerase used in NGS library preparation; secondly, misclassification due to mismapping of NGS reads to markers of RNA viruses (Philips et al., 2017). Besides, partitivirus-related sequences might be integrated to eukaryotic genomes (include plant, arthropods and protozoa) through horizontal gene transfer could be an explanation for the detection of *Vicia cryptic virus* (partitivirus) in the samples (Liu et al., 2010).

Bombyx mori nucleopolyhedrovirus (baculovirus) is common pathogen that infects and kills *Bombyx mori* (silkworm; arthropods order Lepidoptera) (Acharya et al., 2002; Cheng et al., 2014). It can be transmitted to silkworm through oral ingestion and vertical transmission (Jiang & Xia, 2014; Khurad et al., 2004). The chitinase and cathepsin of *Bombyx mori* nucleopolyhedrovirus promotes liquefaction on the carcass of infected silkworm (Daimon et al., 2007; Ohkawa et al., 1994; Wang et al., 2005). In this study, infected insects were the possible source of *Bombyx mori* nucleopolyhedrovirus found

in the pitcher fluid. Besides, the carcass liquefaction process by *Bombyx mori* nucleopolyhedrovirus might aids in *Nepenthes* prey catabolism.

Although most of the virus studies focus on the disease-causing virus, virus infection does not necessary harms the host (Dawe & Nuss, 2013; Nakatsukasa-Akune et al., 2005; Roossinck, 2011; Roossinck, 2012; Roossinck, 2015a; Roossinck, 2015b; Xu et al., 2008). For example, the tobacco mosaic virus increased the drought tolerance of infected *Nicotiana benthamiana* plant, enhancing the plant to survivability in prolong duration of water shortage (Roossinck, 2011; Xu et al., 2008). Thus, the ecological role of *Nepenthes*-associated virus might be linked to the survivability of *Nepenthes* plant on nutrient depleted soil.

The viral communities described in this study do not represent the total virus present in the *Nepenthes* pitcher fluid's sample. Most of the genetic material from RNA viruses was lost during sample preparation. The viruses' RNA was lost because (i) RNAse A treatment during DNA extraction, (ii) reverse transcription of viral RNA was not performed in this metagenomic study and (iii) NGS library preparation was devoted to DNA sequencing. Further studies are needed in order to determine the total composition, origin and functions of viral community associated to the *Nepenthes* pitcher fluid or pitcher plant.

5.5 Insect Degradation, Nitrogen and Carbohydrates Metabolism

In this study, gene coded for enzyme involved in chitin, *N*-acetylglucosamine, protein, ammonia, allantoin, cellulose and xylose utilization were detected from the metagenomic data. The metagenomic data were presumably composed of mainly the genetic material from the inhabitants in *Nepenthes* pitcher fluid. This is because first,

the *Nepenthes* plant genomic DNA was not extracted in this study and; secondly, the prey carcasses and debris were removed from the *Nepenthes* pitcher fluid prior to DNA extraction.

Nitrogen is an essential macronutrient for the survival of all living organisms (Bolin & Arrhenius, 1977; Rogers et al., 1998). Former study has reported the nitrogen scarcity (hardly detectable) in the fluid of unopened *Nepenthes* pitcher (Buch et al., 2013). Albeit the nitrogen deprived environment, present study shows that *Nepenthes* pitcher fluid hosted a range of bacteria, eukaryote and virus. Due to the insectivorous diet of *Nepenthes* plant, arthropod carcasses are presumably the primary nitrogenous substrates for the inhabitants in the pitcher fluid. Thus, nitrogen acquisition through arthropod carcass degradation is necessary for the *Nepenthes* pitcher fluid inhabitants, especially microbes; in order to survive in the nitrogen depletes pitcher fluid.

Chitin, a nitrogeneous polysaccharide composed of *N*-acetylglucosamine subunits, is the main component in insect exoskeleton (endo- and exocuticle) (Arakane et al., 2012; Chorvatovičová et al., 1998; Daraghmeh et al., 2011; Merzendorfer & Zimoch, 2003; Moussian et al., 2005; Prakash et al., 2010). Chitinase is the key enzyme in insect catabolism. It cleaves the β-1-4 glycosidic linkage within chitin chain (Fukamizo, 2000; Ishisaki et al., 2012; Kramer & Muthukrishnan, 1997). In nature, chitinases are produces by virus, bacteria, fungi, protista, insects, worms, higher plants and vertebrates (Bhattacharya et al., 2007; El Sayed & Aly, 2007; Prakash et al., 2010; Yan & Fong, 2015). Microbe mainly uses its chitinase for chitin catabolism meanwhile the role of plant and insect chitinases includes protection against pathogen and ecdysis, respectively (Beier & Bertilsson, 2013; Bhattacharya et al., 2007; Charnley & Leger, 1991; Merzendorfer & Zimoch, 2003; Metraux et al., 1988; Stintzi et al., 1993).

The detection of chitinase-coding genes in this study suggested the contribution of *Nepenthes* pitcher fluid inhabitant (especially microbial community) in insect degradation. The synergistic interaction of *Nepenthes* plant chitinases (class I, class III and class IV plant chitinases) and the chitinases from the pitcher fluid inhabitants might leads to the complete insect degradation, which supply insect-based nitrogen to the *Nepenthes* plant as well as inhabitant of the pitcher fluid (Eilenberg et al., 2006; Ishisaki et al., 2011; Ishisaki et al., 2012; Lee et al., 2016; Rottloff et al., 2011). Besides, studies on entomopathogenic fungi show that fungal chitinases, are directly involve in hydrolyzing insect exoskeleton (Charnley & Leger, 1991; Charnley, 2003). The synergistic effect of chitinases and mechanical pressure enables the entomopathogenic fungi to invade insect through exoskeleton penetration (Charnley & Leger, 1991; Ortiz-Urquiza & Keyhani, 2013; Pedrini et al., 2007). Unlike entomopathogenic fungi, almost all entomopathogenic bacteria initiate their invasion from insect's midgut (Charnley & Leger, 1991; Hajek & Leger, 1994; Ortiz-Urquiza & Keyhani, 2013). The degradation of insect carcasses by bacterial and fungal chitinases through different route might enhance the insect degradation efficiency.

The *N*-acetylglucosamine generate from chitin hydrolysis can be further degraded into fructose-6-phosphate (intermediate component in glycolysis), acetate and ammonia (Comb & Roseman, 1956; Comb & Roseman, 1958; Yan & Fong, 2015). The ammonia might be incorporated into glutamate by enzyme glutamine synthetase during glutamine biosynthesis (Hirel & Lea, 2001; Joy, 1988; Shapiro & Stadtman, 1970; Suzuki & Knaff, 2005). The glutamine generated in this ammonia assimilation served as the fundamental component for protein synthesis and the biosynthesis other compounds such as tryptophan, asparagine, purine and ureide (Hirel & Lea, 2001; Joy, 1988; Miflin & Lea, 1982; Shapiro & Stadtman, 1970; Yanofsky, 1971). In addition, the glutamine

can be reassimilated into glutamate by glutamate synthase, which catalyzes the conversion of L-glutamine and 2-oxoglutarate to L-glutamate (Hirel & Lea, 2001; Miflin & Lea, 1982; A. Suzuki & Knaff, 2005).

On top of chitin, insect exoskeleton is mainly composed of protein extracellular matrix (Arakane et al., 2012; Moussian et al., 2005; Samuels & Paterson, 1995). In fact, studies on the biochemistry of insect exoskeleton degradation by entomopathogenic fungi showed that cuticle-degrading proteolytic enzymes acts before chitinolytic enzymes on insect exoskeleton penetration (Fukamizo & Kramer, 1985; Leger et al., 1998; Smith et al., 1981; St Leger, 1993). To date, several cuticle-degrading proteases, such as subtilisin-like serine protease, Pr1, serine proteinase, Pr2, and cysteine proteinase, Pr4, are extracted and characterized from entomopathogenic fungi (Cole et al., 1993; Firouzbakht et al., 2015; Leger et al., 1987; Samuels & Paterson, 1995; St. Leger, 1995). Although insect degradation mechanism in *Nepenthes* pitcher fluid is not fully understood, it was postulated that the cuticle-degrading protease initiates the insect degradation. Subsequently, proteolytic enzymes synthesized by *Nepenthes* plant and the inhabitants in its pitcher fluid further hydrolyzed the proteins and peptides released from the cuticle digestion or deposited in insect hemolymph. Albeit wide range of proteases and peptidases were detected in this study, not all proteolytic enzymes are involved in prey catabolism. More work is needed in order to determine the proteolytic enzymes responsible for nitrogen assimilation from insect preyed by *Nepenthes* plant.

In this study, key enzymes involved in allantoin degradation pathway and urea degradation are detected from the metagenome samples. In nature, allantoin is an intermediate component generates in the purine degradation pathway of most organisms (Ma et al., 2016; Vogels & Van der Drift, 1976; Xi et al., 2000). When preferred nitrogen sources are not available, allantoin can be utilized as alternative nitrogen source by bacteria (for example *Bacillus subtilis* and *Klebsiella pneumoniae*) and yeast (Cooper & Sumrada, 1983; Ma et al., 2016; Magasanik & Kaiser, 2002; Vogels & Van der Drift, 1976). During allantoin degradation, ammonia, which is the preferred nitrogen source of microbe and carbon dioxide are released as end products (Bossinger et al., 1974; Riva et al., 2008; Ma et al., 2016; Nygaard et al., 2000; Trijbels & Vogels, 1966; Vogels & Drift, 1976). Meanwhile, glyoxylate is generated as intermediate components (Bossinger et al., 1974; Ma et al., 2016; Trijbels & Vogels, 1966; Vogels & Drift, 1976; Werner & Witte, 2011). The glyoxylate could be supply into physiological process such as glyoxylate cycle, or convert into metabolic intermediates such as 3-phosphoglycerate or 2-phosphoglycerate, which are utilized in glycolysis and Krebs cycle.

Xylose isomerase pathway is opted mainly by bacteria for xylose fermentation (Amore et al., 1989; Harhangi et al., 2003; Lawlis et al., 1984; Rygus et al., 1991; Scheler et al., 1991). In xylose isomerase pathway, xylose isomerase catalyses the conversion of D-xylose into D-xylulose, followed by phosphorylation of D-xylulose into D-xylulose-5-phosphate by xylulose kinase (Harhangi et al., 2003; Karhumaa et al., 2007). The D-xylulose-5-phosphate is an key intermediate in pentose phosphate pathway (Karhumaa et al., 2007). In this study, the detection of cellulase and key enzymes for xylose isomerase pathway (xylose isomerase and xylulose kinase xylose) from the metagenome samples suggested the fermentation of plant material (cellulose and xylose) by microbe (especially bacteria) inhabits in *Nepenthes* pitcher fluid (Gilbert &

Hazlewood, 1993; Karhumaa et al., 2007). The carbohydrates released from the cellulose and xylose catabolism could serve as energy sources for the growth of microbial inhabitants in the *Nepenthes* pitcher fluid. Nonetheless, the occurrence of cellulose and xylose catabolism events could also indicate the invasion of plant by phytopathogen.

5.6 Biocatalytic Activities of Bacteria Isolated from *Nepenthes* Pitcher Fluid

Previous studies have detected protease, lipase, putative β-D-glucosidase and putative β-D-glucosaminidase activities from bacteria inhabited in *Nepenthes* pitcher fluid (Hepburn, 1918; Higashi et al., 1993; Lüttge, 1964; Morohoshi et al., 2011; Takeuchi et al., 2011). However, most of the report does not reveal the bacterial identities. In this study, the isolation of 18 bacteria from *Nepenthes* pitcher fluid confirmed the presence of bacteria in the fluid of opened pitcher. Besides, the detection of several biocatalytic activities from the bacteria isolates supported the hypothesis that bacterial hydrolytic enzymes assist the organic material catabolism and nutrient recycling in *Nepenthes* pitcher fluid.

In soil, chitin degradation rate is positively correlated with the bacterial density suggested the importance of bacteria in nutrient recycling from chitin (Beier & Bertilsson, 2013; Kielak et al., 2013). Similar hypothesis was applied on bacteria inhabited in *Nepenthes* pitcher fluid. Although the mechanism for insect degradation in *Nepenthes* pitcher fluid is not fully understood, the assistance of bacterial community on the degradation of chitin rich insect exoskeleton (endo- and exocuticle) was postulated. In this study, β-*N*-glucosaminidase, endochitinase, and chitobiosidase producing bacteria were isolated from *Nepenthes* pitcher fluids. β-*N*-glucosaminidase and chitobiosidase catalyzed processive release of *N*-acetyl-β-D-glucosamine and

N-glucosaminidase, respectively, from the terminal non-reducing end of chitin and oligomeric substrates (Hamid et al., 2013; Parham & Deng, 2000). Meanwhile, endochitinase cleaves the internal points within chitin chain (Hamid et al., 2013). The β-*N*-glucosaminidase, endochitinase, and chitobiosidase-producing bacteria may aid in the degradation of chitin oligomeric substrates, which are generated during the chitin degradation by chitinases from *Nepenthes* plant and its eukaryotic inhabitant.

Several bacteria isolated in this study performed multiple chitinolytic activities or coded more than 1 chitinase gene in their genome. For example *S. marcescens* strains H1q and PH1a performed β-*N*-glucosaminidase, chitobiosidase and endochitinase activities. In addition, 3 chitinase-coding genes were found on their genomes. Extensive studies are performed on the chitinases (ChiA, ChiB and ChiC) of *S. marcescens* due to its high efficiency in chitin degradation (Bhattacharya et al., 2007; K. Suzuki et al., 2002). ChiA and ChiB processively hydrolyze chitin chain in opposite direction, while ChiC randomly hydrolyzes the internal point of chitin chain (Eva-Lena et al., 2005; Horn et al., 2006; Sikorski et al., 2006). The synergistic chitinases interaction is believed to be reason for efficient chitin utilization by *S. marcescens* (Arakane et al., 2012; Bhattacharya et al., 2007; Horn et al., 2006; K. Suzuki et al., 2002; Yan & Fong, 2015).

Chitinases are mainly classified into GH18 or GH19 families and subdivided into different classes based on the amino acid sequence and enzymatic mechanism (Fukamizo, 2000; Henrissat, 1991; Henrissat & Bairoch, 1993; Yan & Fong, 2015). The hydrolysis of β-1,4-glycosidic linkages by GH18 and GH19 chitinases produces β-anomer and α-anomer, respectively (Arakane et al., 2012). According to CAZy database, bacterial chitinases are mainly GH18 chitinases (Arakane et al., 2012;

Bhattacharya et al., 2007). The GH19 chitinases are assumed absent in bacteria before the documentation of GH19 chitinases from *Streptomyces grisues* strain HUT6037 (Ohno et al., 1996; Prakash et al., 2010). Evolutionary studies suggest that bacterial obtained GH19 chitinases from higher plant through horizontal gene transfer (Prakash et al., 2010; Watanabe et al., 1999). To date, more than 3000 bacterial GH19 chitinases are recorded in CAZy database. In this study, GH18 and GH19 chitinase-coding genes were detected from the bacterial genomes. The wide range of chitinases (including bacterial chitinases, chitinases synthesized by the inhabitants of *Nepenthes* pitcher and plant chitinases synthesized by *Nepenthes*) in the *Nepenthes* pitcher fluid might work synergistically for an efficient mechanism on nutrient recycling from arthropods carcass (Eilenberg et al., 2006; Ishisaki et al., 2012; Ishisaki et al., 2011; Lee et al., 2016; Rottloff et al., 2011).

Other than chitinase, proteases are key enzymes in releasing nitrogen from insect carcasses into *Nepenthes* pitcher fluid. The insect based nitrogen contributes at least 50% (up to 77%) of the total foliar nitrogen in *Nepenthes* plants (Moran et al., 2001; Scharmann et al., 2013; Schulze et al., 1997). In this study, protease activity was detected from *Bacillus* sp., *P. aeruginosa*, *Pseudomonas* sp., *S. marcescens*, *Sphingobacterium* sp., *M. odoratimimus*, and *M. paraoxydans* suggested that bacteria were involved in insect carcass catabolism. However, due to the slower protein degradation rate of bacterial protease compared to plant proteases, former study has hypothesized that the bacterial protease only play secondary role in insect digestion in *Nepenthes* pitcher fluid (Hepburn, 1918).

On top of preys, *Nepenthes* opened pitchers occasionally collect plant materials (example, seed, leaves and pollen) that fall from the surrounding environment. The present of cellulase and xylanase producing bacteria, such as *Sphingobacterium* sp. strain H1ai and *Pseudomonas* sp. strain PH1b in the *Nepenthes* pitcher fluid suggested their contribution in plant material degradation. During plant material catalysis, cellulase and xylanase cleaved the β-1,4-$_D$-glycosidic bond of cellulose and xylan, respectively, into monosaccharides and oligosaccharides (Gilbert & Hazlewood, 1993; Han et al., 1995). The carbohydrates released from the plant material may serve as energy source for the growth of bacteria and other microbial inhabitants in the *Nepenthes* pitcher fluid.

In brief, the presence of biocatalytic bacteria in *Nepenthes* pitcher fluid indicates the contribution of bacterial community on insect degradation and nutrient recycling. However, further study is needed in order to understand the importance and mechanisms of bacteria communities in *Nepenthes* nutrient assimilation.

5.7 *N*-acetylglucosamine Detection

In this study, the pitcher fluids in unopened pitchers were colorless and clear. Meanwhile the pitcher fluids in opened pitchers ranges from colorless and clear to browned and turbid condition. *Nepenthes* pitcher fluid changes from colorless to yellow color after opening its orifice (Hua & Li, 2005). Further study found that the color of the pitcher fluid only change in the presence of prey (Baby et al., 2017). Analysis on the yellowed pitcher fluid detected droserone and 5-*O*-methyl droserone, which are yellow colour compounds that dissolve in organic solvents (Baby et al., 2017). Furthermore, droserone and 5-*O*-methyl droserone are secreted into *Nepenthes* pitcher fluid after addition of chitin and insect prey (Eilenberg et al., 2009; Raj et al., 2011). Thus, it is

believed that the color changes in fluid of opened *Nepenthes* pitcher indicate prey digestion event. In this study, *N*-acetylglucosamine, the monomer of chitin was detected in browned fluid of opened pitchers, but not the colorless and clear fluid of and unopened pitchers suggested that *N*-acetylglucosamine was the generated in insect chitin degradation (Daraghmeh et al., 2011).

5.8 Insect Degradation by Bacteria in *Nepenthes* Pitcher Fluid

Findings of disintegrated insects in the pitcher fluid of wild *Nepenthes* plants and the observation of substrates dissolution by *Nepenthes* plant in laboratories confirmed the insect catabolism events in the pitcher fluids (Bauer et al., 2009; Kato et al., 1993; Schmid-Hollinger, 1997). Most hypothesizes state that the insect catabolism is primarily carried out by plant hydrolytic enzymes and assisted by the microbe and eukaryote inhabits in *Nepenthes* pitcher fluid (Adlassnig et al., 2010; Hepburn, 1918; Higashi et al., 1993; Lam et al., 2017; Scharmann et al., 2013). Even though hydrolytic enzymes producing bacteria are isolated and identified from *Nepenthes* pitcher fluid, insect degradation by bacteria inhabited in *Nepenthes* pitcher fluid is not experimentally tested.

In this study, insect degradation by bacteria isolated from *Nepenthes* pitcher fluid was tested on *D. melanogaster*. Disintegration of *D. melanogaster* was not observed in all experiments. However, possibility of *D. melanogaster* degradation by selected bacteria cannot be ruled out as colonization of bacteria on *D. melanogaster* was observed. In addition, observation of bloated abdomen on *D. melanogaster* in the medium inoculated with *Bacillus* sp. strain H1a and *S. marcescens* stain H1q indicated the presence of bacteria activities. The abdomen of *D. melanogaster* is bloated due the gases byproducts (such as hydrogen, carbon dioxide and methane) produced by bacteria

during putrefaction (Hyde et al., 2013; Vass, 2001). In aligned with former hypothesis, this results suggested that bacteria community in *Nepenthes* pitcher fluid contributed on the insect degradation.

Colonization of bacteria on *D. melanogaster* was more successful in samples with mixed culture compared to monoisolate indicates the cooperation of bacteria enhance the bacterial community survivability in nutrient limiting environment created in this insect degradation tests. However, despite of the varieties of hydrolytic enzymes produced by the selected bacteria strain, disintegration of *D. melanogaster* was not observed in this study. In nature, cadaver decomposition is completed by the synergistic actions of microbe, insect and scavengers in conjunction with abiotic factors (Carter et al., 2007; Hyde et al., 2013). The absent of hydrolytic enzymes, mainly chitinases and proteases, from *Nepenthes* plant and other pitcher fluid's inhabitants was suggested as the main reason for the inefficient insect degradation in this study.

In brief, the results in this study supported the hypothesis that bacterial community assisted in insect degradation in *Nepenthes* pitcher fluid. Besides, colonization of bacteria on *D. melanogaster* was more successful by mixed culture suggested that the synergistic interaction between pitcher fluid's inhabitants (microbes, virus and eukaryotes) and *Nepenthes* plants lead to an efficient nutrient recycling system. The efficient nutrient recycling system in the pitcher fluid enhances the survivability of *Nepenthes* plant and its pitcher fluid inhabitants in nutrient depleted environments.

5.9 Future Work

The profiles of microbial and eukaryotic communities in *Nepenthes* pitcher fluid were revealed in this study. However, more study is needed in order to determine the factors that shape the inhabitant communities. Besides, comprehensive study on the viral community, inclusive of the RNA viruses, is needed in order to understand the viral community profile in the *Nepenthes* pitcher fluid. In addition, further studies on the metagenomic and genomic data are needed, in order to understand the habitat-specific traits that might dominate in the system, the interaction within the inhabitants in the pitcher fluid and between the *Nepenthes* plant and the inhabitants. Moreover, the survival strategies of inhabitant community in the acidic and nutrient deplete *Nepenthes* pitcher fluid remain unknown. Thus, future work is needed in order to understand the ecosystem in the *Nepenthes* pitcher fluid.

CHAPTER 6: CONCLUSION

In conclusion, the pitcher fluid of *Nepenthes* harbours wide range of bacteria, eukaryotes (includes insects, nematodes, arachnids, fungi and protist) and viruses. The inhabitants of *Nepenthes* pitcher fluid might be introduced to the pitcher fluid from arthropods preyed by *Nepenthes* plant, the environment, the endophytic bacterial of *Nepenthes* plant phyllosphere and organisms that interacted with the pitcher fluid. Pitcher fluid acidity was significantly influencing its bacterial diversity assemblage; meanwhile, the eukaryote community assemblages in *Nepenthes* pitcher fluids were clustered by *Nepenthes* species. The factors that influence the virus community were unknown. Further study on the bacterial community in the *Nepenthes* pitcher fluids showed that the bacterial diversity and species richness were lower in the highly acidic pitcher fluids. These highly acidic pitcher fluids (pH1 to pH4) were predominated by the acidophilic protebacterial genus *Acidocella* and *Acidisoma*. Meanwhile, *Nepenthes* pitcher fluid with low acidity (pH5 to pH7) was not colonized by specific bacterial genera.

Hypothesis on the contributions of the inhabitants in *Nepenthes* pitcher fluid were always associated to prey digestion (physically and enzymatically), nutrient recycling from organic materials (for example insect carcasses and plant detritus) for the growth and development of the *Nepenthes* plant and the inhabitant community, controlling the microorganism population in the pitcher fluid, and supplying nitrogen source through nitrogen fixing process. Although disintegration of *D. melanogaster* was not observed in insect degradation test, better colonization of bacteria on *D. melanogaster* in samples with mixed culture compared to monoisolate indicates the cooperation of bacteria enhance the bacterial community survivability. Besides, the detection of genes coded for

enzymes involved in insect carcass and plant detritus catabolism in this metagenomic study, isolation of biocatalytic bacteria from the *Nepenthes* pitcher fluid and the insect degradation test supported the hypothesis that the microbial inhabitants of *Nepenthes* pitcher fluid aids in prey digestion. The complete degradation of insect carcass in *Nepenthes* pitcher fluid might be resulted from the interaction of enzymes synthesized by both the *Nepenthes* plant and its inhabitant.

N-acetylglucosamine was only detected in the fluid of opened *Nepenthes* pitcher suggested *N*-acetylglucosamine was generated by insect chitin degradation event. Besides, *N*-acetylglucosamine was not detected in the clear and colorless fluid of unopened *Nepenthes* pitchers suggested that the pigmentation of *Nepenthes* pitcher fluid was induced by prey digestion event. More work is needed in order to fully understand the host-inhabitant interaction and the ecosystem in *Nepenthes* pitcher fluid.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. Bittleston, L. S., Wolock, C. J., Yahya, B. E., **Chan, X. Y.,** Chan, K. G., Pierce, N. E., & Pringle, A. (2018). Convergence between the microcosms of Southeast Asian and North American pitcher plants. *eLife, 7*, 1-19.

2. **Chan, X. Y.,** Hong, K. W., Yin, W. F., & Chan, K. G. (2016). Microbiome and biocatalytic bacteria in monkey cup (*Nepenthes* pitcher) digestive fluid. *Scientific Reports, 6*, 1-10.

PAPER PRESENTED

1. **Chan, X. Y.** (2018). Microbiome in *Nepenthes* pitcher fluid. Paper presented at the Student Thesis Seminar, Kuala Lumpur, 16 March 2018, Malaysia.

2. **Chan, X. Y.** (2015). Insight to the microbiota of carnivorous plant, *Nepenthes*. Paper presented at the Postgraduate seminar, 27 November 2015, Kuala Lumpur, Malaysia.