MOLECULAR CHARACTERISATION OF LIGNOCELLULOSE DEGRADATION IN *ROSEITHERMUS SACCHARIPHILUS* STRAIN RA

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ABSTRACT

An enormous amout of available scientific publications are experimental findings and knowledge of well-known prokaryotic strains. Very little attention has been given to uncommon prokaryotes. The current work focused on the molecular characteristic of strain RA, a minority bacterium of Ayer Hangat hot spring, to understand its strategies to survive the harsh environment. The work also aimed to investigate the ability of strain RA to hydrolyse lignocellulosic compounds and its role in microbial consortium of the hot spring. This work used methods of microbiology, genomic, transcriptomic, molecular biology, enzymology, and bioinformatics. Strain RA is the closest in similarity with *Roseithermus sacchariphilus* strain MEBiC09517^T with slight differences in its genome rearrangement and sets of unique genes. Strain RA is a halo-thermophile which uses thermophilic lipids, has high genome GC, stable enzyme, and 'salt-in' strategy by different transporters to thrive in the salted and heated ecosystem. When strain RA was cultured in MB media added with xylan, the bacterium exhibited higher growth rate, better enzymatic activities, and greater degree of differentially expressed genes which was significantly higher than when other carbon sources (EFB, CMC, glucose, and xylose) were used. Genes encoding auxiliary activity enzymes (AA), proteins that act on lignin, were non-responsive in all of the experimental setups. Strain RA harbours 54 glycosyl hydrolase (GH) genes that are affiliated with 30 families. Majority of these genes, especially cellulose and hemicellulose-acting GHs, were upregulated when strain RA was grown in MB+xylan. Xylanase XynRA1 was one of the upregulated GHs. Recombinant protein of the enzyme was purified and biochemically characterised. XynRA1 achieved maximum activity at pH 8 and 60°C, and exhibited an activity half-life ($t_{1/2}$) of at least one hour at 50−60°C. It can thus be concluded that strain RA is a subspecies of *R. sacchariphilus*. Findings accumulated in this work showed that strain RA adapts itself to a warm and salty environment by multiple strategies, instead of using a single approach. Even though strain RA is a minority *in-situ*, it plays a role in plant litter decomposition using, in particular the hemicellulose fraction. Strain RA most probably collaborates with other bacteria to form a multi-species biofilm that attach to the plant litter for complete degradation. The bacterium is a rare species in the hot spring because of its slow growth rate, and is less competitive compared to other prokaryotes due to its low preference for glucose. To better survive against ecological changes, strain RA generates non-homologous halo-thermostable enzymes with unique biochemical characteristics. The current work successfully elucidated the molecular features of *R. sacchariphilus* strain RA. It is proposed that strain RA can be an excellent candidate to be included in bacteria consortia for lignocellulosic biomass degradation or used in enzyme cocktail formulation.

ABSTRAK

Sejumlah besar hasil kajian dan pengetahuan yang telah diterbitkan dalam pelbagai penerbitan saintifik adalah berkaitan strain prokariot terkenal. Sangat kurang perhatian diberikan kepada prokariot nadir. Kajian ini memfokus kepada pencirian molekul strain RA yang wujud sebagai prokariot minoriti di dalam kolam air panas Ayer Hangat dan bertujuan untuk memahami strategi kemandirian bakteria tersebut dalam persekitaran melampau. Kajian ini turut menyelidik keupayaan strain RA bagi hidrolisis sebatian lignoselulosa dan peranannya dalam konsortium mikrob kolam air panas Ayer Hangat. Perlaksanaan kajian ini melibatkan penggunaan kaedah mikrobiologi, genomik, transkriptomik, biologi molekul, enzimologi, dan bioinformatik. Strain RA paling menyerupai *Roseithermus sacchariphilus* strain MEBiC09517^T dengan sedikit perbezaan dari segi penyusunan genom dan set gen-gen unik. Strain RA adalah bakteria halo-termofil yang menggunakan lipid termofil, mempunyai genom dengan kandungan GC yang tinggi, enzim yang stabil, dan strategi *'salt-in'* menggunakan pelbagai gen pengangkut untuk kelangsungan hidup dalam ekosistem yang bergaram dan bersuhu tinggi. Strain RA menunjukkan kadar pertumbuhan, aktiviti enzim, dan ekspresi gen yang lebih baik dalam kultur media MB yang telah ditambah dengan xilan berbanding sumber karbon lain (EFB, CMC, glukosa, dan xilosa). Enzim beraktiviti auksiliari (AA) adalah protein yang boleh mengurai lignin dan kebanyakan gen ini tidak bertindakbalas dalam semua persediaan eksperimen dalam kajian ini. Strain RA mempunyai 54 gen glikosida hidrolase (GH) daripada 30 keluarga yang berbeza. Majoriti gen berkenaan, terutamanya GH yang mengurai selulosa dan hemiselulosa dihasilkan dalam kadar yang tinggi apabila strain RA dikultur menggunakan media MB+xilan. Enzim xilanase XynRA1 merupakan salah satu GH yang dihasilkan pada kadar yang tinggi. Protein rekombinan XynRA1 telah ditulenkan dan dicirikan secara biokimia. XynRA1 menunjukkan aktiviti maksimum pada pH 8.0 dan suhu 60°C, serta mempunyai separuh hayat aktiviti sekurang-kurangnya satu jam pada suhu 50–60°C. Dapatan utama daripada kajian ini menyimpulkan bahawa strain RA merupakan sub-spesis kepada *Roseithermus sacchariphilus*. Kesemua dapatan kajian ini turut menunjukkan strain RA mampu hidup dalam keadaan bergaram dan bersuhu tinggi melalui penggunaan pelbagai strategi, dan bukannya melalui pendekatan tunggal. Walaupun strain RA adalah bakteria minoriti dalam kolam air panas Ayer Hangat, namun ia terlibat dalam penguraian sisa tumbuhan, terutamanya penguraian fraksi hemiselulosa. Strain RA mungkin bekerjasama dengan bakteria lain bagi membentuk saput biologi pelbagai spesis dan melekat pada sisa tumbuhan bagi memastikan penguraian lengkap sumber berkenaan. Bakteria ini adalah spesis nadir di dalam kolam air panas Ayer Hangat kerana mempunyai kadar pertumbuhan yang perlahan dan kurang berdaya saing berbanding prokariot lain kerana kurang memilih glukosa sebagai sumber tenaga. Strain RA turut menghasilkan enzim termostabil dengan ciri biokimia yang unik bagi memastikan kemandirian apabila berhadapan dengan perubahan ekologi. Kajian ini berjaya mengungkai ciri-ciri molekul *Roseithermus sacchariphilus* strain RA. Dicadangkan, strain RA berkemungkinan adalah calon yang amat baik bagi konsortium bakteria untuk penguraian biojisim lignoselulosa atau sumber bagi formulasi koktail enzim.

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LIST OF ABBREVIATIONS

AA	-	Auxiliary activity enzyme
ABC	-	ATP-binding cassette
AFTR	-	AraC family transcriptional regulator
ANI	-	Average nucleotide identity
antiSMASH	-	Antibiotics and Secondary Metabolite Analysis Shell
ASM	-	American Society for Microbiology
ATP	-	Adenosine triphosphate
BacDive	-	The bacterial diversity metadatabase
BCA	-	Bicinchoninic acid
BGL	-	β-glucosidase
BSA	-	Bovine serum albumin
BxlRA	-	β-xylosidase from strain RA
CAZy	-	Carbohydrates-Active Enzyme
CBH	-	Cellobiohydrolase
CBM	-	Carbohydrate-binding module
cDNA	-	complementary DNA
CDS	-	Protein-coding sequence
CE	-	Carbohydrate esterase
CFU	-	Colony-forming unit
CMC	-	Carboxymethyl cellulose
COG	-	Cluster of Orthologous genes
CoL	-	Catalogue of Life
dbCAN	-	A database of CAZyme sequence and annotation
dDDH	-	Digital DNA-DNA hybridization
DDH	-	DNA-DNA hybridization
DEGs	-	Differentially expressed genes
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DNS	-	Dinitrosalicyclic acid
DP	-	Degree of polymerization

dsDNA	-	Double-stranded DNA
DyP	-	Dye-decolorizing peroxidase
EC	-	Enzyme Commission
EDTA	-	Ethylenediaminetetraacetic acid
EFB	-	Empty fruit bunch
EG	-	Endoglucanase
ELSD	-	Evaporative light scattering detector
et al.	-	And others
FC	-	Fold change
FCB	-	*Fibrobacter-Chlorobi-Bacteroidetes*
FDR	-	False discovery rate
FPKM	-	Fragment Per Kilobase of transcript sequence per Millions base-pair sequenced
GC	-	Guanine-cytosine
gDNA	-	genomic DNA
GH	-	Glycosyl hydrolase
GO	-	Gene ontology
GT	-	Glycosyltransferase
HGAP	-	Hierarchical Genome Assembly Process
HPLC	-	High performance liquid chromatography
HTH	-	Helix-turn-helix
IJSEM	-	International Journal of Systematic and Evolutionary Microbiology
IL	-	Ionic liquid
IMAC	-	Immobilized metal affinity chromatography
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
IUBMB	-	International Union of Biochemistry and Molecular Biology
JGI	-	Joint Genome Institute
KEGG	-	Kyoto Encyclopedia of Genes and Genomes
LCB	-	Locally collinear block
LDA	-	Lignin-degrading auxiliary
Lhalo	-	*Longimonas halophila*
LiP	-	Lignin peroxidase

LME	-	Lignin-modifying enzymes
LMPO	-	Lytic polysaccharide mono-oxygenase
LPSN	-	List of prokaryotic names with standing in nomenclature
Lsali	-	*Longibacter salinarum*
LTTR	-	LysR-type transcriptional regulator
MA	-	Marine Agar
MB	-	Marine Broth
MB+CMC	-	Marine broth supplemented with carboxymethyl cellulose
MB+EFB	-	Marine broth supplemented with empty fruit bunch
MB+xylan	-	Marine broth supplemented with beechwood xylan
MCP	-	Methyl-accepting chemotaxis protein
MEGA	-	Molecular Evolutionary Genetic Analysis
MLSA	-	Multilocus Sequence Analysis
MnP	-	Manganese peroxidase
MP	-	Maximum-Parsimony
mRNA	-	messenger RNA
MW	-	Molecular weight
NCBI	-	National Centre for Biotechnology Information
ncRNA	-	Non-coding RNA
ND	-	Not determined
NGS	-	Next-generation sequencing
NJ	-	Neighbor-joining
Npro	-	*Natronotalea proteinilytica*
NRPS	-	Nonribosomal peptide synthetase
OD	-	Optical density
OGT	-	Optimum growth temperature
PacBio	-	Pacific Biosciences
padj	-	Adjusted p-value
PBS	-	Phosphate-buffered saline
PCR	-	Polymerase chain reaction
PE	-	Paired-end
PFOR	-	Pyruvate-ferredoxin/flavodoxin oxidoreductase
PGAP	-	Prokaryotic Genome Annotation Pipeline

pH	-	Potential hydrogen
PHAST	-	Phage Search Tool
pI	-	Isoelectric point
PL	-	Polysaccharide lyase
*p*NP	-	*para*-nitrophenol
*p*NPG	-	*para*-nitrophenyl-β-D-glucopyranoside
*p*NPX	-	*para*-nitrophenyl-β-D-xylopyranoside
POCP	-	Percentage of conserved protein
PPP	-	Pentose phosphate pathway
Q20	-	Quality Score of 20
QC	-	Quality check
qPCR	-	Quantitative polymerase chain reaction
RA	-	Strain RA
Rcmar	-	*Rubricoccus marinus*
RHH	-	Ribbon-helix-helix
RIN	-	RNA integrity number
RLE	-	Relative log expression
Rmar	-	*Rhodothermus marinus* DSM 4252
RNA	-	Ribonucleic acid
RNA-seq	-	RNA sequencing
rRNA	-	Ribosomal RNA
Rssac	-	*Roseithermus sacchariphilus* MEBiC09517^{T}
Rvmar	-	*Rubrivirga marina*
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	-	Scanning electron microscope
Sira	-	*Salinivenus iranica* CB7
Slon	-	*Salisaeta longa* DSM 21114
SMRT	-	Single molecule, real-time
SOPs	-	Standard operating procedures
sp.	-	Species
spp.	-	Several species
Srub	-	*Salinibacter ruber* DSM 13855

ssDNA	-	Single-stranded DNA
T3PKS	-	Type III polyketide synthase
TCA	-	Tricarboxylic acid
TF	-	Transcription factors
TMM	-	Trimmed Mean of M-values
tRNA	-	Transfer RNA
USP	-	Universal stress protein
UV	-	Ultraviolet
v	-	Variable
VP	-	Versatile peroxidase
X1	-	Xylose
X2	-	Xylobiose
X3	-	Xylotriose
X4	-	Xylotetraose
X5	-	Xylopentaose
X6	-	Xylohexaose
XOS	-	Xylooligosaccharides
XynRA1	-	Xylanase 1 from strain RA
XynRA2	-	Xylanase 2 from strain RA

LIST OF SYMBOLS

-	-	negative result
–	-	From … to …
%	-	Percentage
~	-	Unregulated/non-DEG
+	-	positive result
<	-	Less than
=	-	equivalent to
>	-	More than
×	-	Times
×g	-	Times gravity
≈	-	Approximately
°C	-	Degree Celsius
Å	-	Angstrom unit
bp	-	Base pair
CFU/mL	-	Colony-forming unit per milliliter
CV	-	Coefficient of variation
g	-	Gram
g/L	-	Gram per liter
h^{-1}	-	per hour
hr	-	Hour
kb	-	Kilobase pair
K_{cat}	-	Turnover number
kDa	-	Kilo Dalton
km	-	Kilometers
K_m	-	Michaelis-Menten constant
kPa	-	Kilopascal
M	-	Molar
Mb	-	Megabase pair
mg/L	-	Milligram per liter
mg/mL	-	Milligram per milliliter

min	-	Minute
mL	-	Milliliter
mM	-	Millimolar
Na^+	-	Sodium ion
ng/µL	-	Nanogram per microliter
nm	-	Nanometer
p	-	*para*
R^2	-	Pearson correlation coefficient
rpm	-	Revolutions per minute
s	-	Second
SLM	-	Standard liter per minute
$t_{1/2}$	-	Half-life
T_d	-	Doubling time
U	-	Unit of enzyme activity
U/mg	-	Unit of enzyme activity per milligram
U/mL	-	Unit of enzyme activity per milliliter
V	-	Volt
v/v	-	Volume per volume
V_{max}	-	Maximum velocity
w/v	-	Weight per volume
α	-	Alpha
β	-	Beta
µ	-	Micro
µg	-	Microgram
µg/mL	-	Microgram per milliliter
µL	-	Microliter
µmol	-	Micromole
σ	-	Sigma
Φ	-	Phi
↗	-	Upregulated
↘	-	Downregulated

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The microbial world comprises tiny organisms from different taxonomy memberships, for instance, fungi, archaea, bacteria, yeasts, protozoa, and viruses. The total available bacteria species is far more diverse than we have thought. Approximately 9.2×10^{29}–3.2×10^{30} bacteria cells exist worldwide (Kallmeyer *et al.*, 2012). However, not every bacterium can be cultivated in the lab due to the 'Great Plate Count Anomaly'. The 'unculturable bacteria', or other scientists referred it as 'microbial dark matter', 'rare biosphere' or 'undomesticated', is believed to be necessary for the study in ecology and evolutionary (Stewart, 2012). The percentage of culturable bacteria that formed on plates is less than 1% of the total available microorganisms (Pande *et al.*, 2017). For example, 0.001−0.1% of total ocean bacteria could be grown in the lab; while for soil, it was around 0.1% to 5.2% (VanInsberghe *et al.*, 2013).

The earlier study proposed that microbial diversity could be represented by a low number of dominant species (Nemergut *et al.*, 2011). The dominant species remain stable over time, whereas the minority or the rare species fluctuate. Also, our current knowledge about the bacteria world is based on the dominant groups rather than the rare biosphere (Reid *et al.*, 2011; Lynch *et al.*, 2015). Thus, from the academic point of view, there is a need to explore and understand these rare biospheres. Some studies demonstrated that rare microorganisms are essential for the biogeochemical cycles on Earth, such as the carbon cycle and nitrogen cycle (Hua *et al.*, 2015; Mallon *et al.*, 2015). Besides, rare microorganisms may play some roles in human, animal and plant microbiome, which contribute to both positive and negative impacts on the health of the hosts (Hol *et al.*, 2015; Low-Décarie *et al.*, 2015). Rare microorganisms may be

useful for industrial applications. They contain a vast functional gene pool and can be a good reservoir for mining new enzymes or biocatalysts (Jousset *et al.*, 2017).

Hot springs are the natural habitat for thermophiles. Yellowstone hot spring located in the United States of America is among the earliest sites for microbial diversity studies. At that period, microbial diversity was carried out via culture-independent techniques involving amplification of complete 16S rRNA, cloned into a suitable vector and individually sequenced (Stahl *et al.*, 1985). Although low throughput, it was in this study that the hypothesis of 'unculturable bacteria' was introduced. Since then, this concept arose the interest of many worldwide scientists in exploring underexplored bacteria. Examples of other few milestone projects on the microbial diversity of hot springs include those located in Iceland, Romania, and Arctic (Roeselers *et al.*, 2007; Coman *et al.*, 2013). And recently, the 1000 New Zealand hot springs project produces extensive information on dominant and underexplored bacteria (Power *et al.*, 2018).

Regardless of which hot springs, the microbial population contains the dominant as well as rare or underexplored thermophiles. Similarly, a rare biosphere exists in any environment, for instance, in aquatic resources. Many rare halophiles were isolated from salty samples, for example, Antarctic ocean, coastal, mangrove swamp, deep sea, sub-seafloor and the saline lake (Ma *et al.*, 2010b; DasSarma *et al.*, 2017). Examples of rare halophiles are *Iamia majanohamensis*, *Salinispora arenicola* and *Marinactinospora thermotolerans* (Maldonado *et al.*, 2005; Kurahashi *et al.*, 2009; Tian *et al.*, 2009).

Several years ago, a microbial survey using 16S rRNA amplicon sequencing and culture collection was performed on several Malaysian hot springs. One of these sites, Ayer Hangat hot spring (Langkawi) is particularly interesting as this is the only known saline hot spring in Malaysia (Chan *et al.*, 2017). The hot spring contained a high concentration of salt ions such as Cl^- (13,832 mg/L), Na^+ (7,905 mg/L) and Mg^{2+} (394 mg/L) (Chan *et al.*, 2017). Ayer Hangat hot spring has a temperature of around 40−50°C and pH 7.1. Collectively, the majority of the bacterial members in the hot springs are halo-thermophiles. The major bacteria families in the hot spring are

Rhodobacteraceae, *Oscillatoriophycideae*, *Geobacteraceae*, and *Vibrionaceae*, which contributed 97.8% of the total population (Chan *et al.*, 2017). The rare microbial families present in this hot spring were *Dictyoglomaceae*, *Ignavibacteriaceae*, *Thermoanaerobacterales*, and others. Moreover, *Rhodothermaceae* occupied less than 0.001% of the total bacteria population in Ayer Hangat hot spring (Chan *et al.*, 2017).

The sampling site had a carbon to nitrogen ratio (C:N) of 5.0 (Chan *et al.*, 2017). The composition of carbon sources in AH hot spring is poorly understood. Yet, it is likely that the dissolved and particulate organic carbon in this site is contributed by lignocellulolytic materials from plant, humic substances from soil organic matters, amino acids, carbohydrates, biopolymers, and biomolecules. It is hypothesized that both the dominant and rare families as mentioned above functioned as a microbial consortium and work cooperatively in degrading and use these carbon sources.

A bacterium designated as strain RA was isolated from Ayer Hangat hot spring in 2015. The 16S rRNA sequence (KU517707) was submitted to NCBI, and the closest bacteria (87.5% 16S rRNA similarity) at that time was *Rhodothermus marinus*, a genus under *Rhodothermaceae* family. If a sequence shares low similarity to existing known species, NCBI would place the sequence in an 'unclassified' group under the most relevant order (Madden *et al.*, 2002; Yarza *et al.*, 2014). The automated NCBI taxonomy webpage placed strain RA in the unclassified group under the *Rhodothermaceae* family. The bacterium was tentatively named as *Rhodothermaceae* bacterium RA in 2015. Lately, in early 2019, *Roseithermus sacchariphilus* strain MEBiC09517^T was isolated from the coastal body in Korea (Park *et al.*, 2019). Strain MEBiC09517^T represents the first member of *Roseithermus*. Based on 16S rRNA similarity (99.3%), *Rhodothermaceae* bacterium RA is very closely related to strain MEBiC09517^T.

When the first member of order *Rhodothermales* was discovered three decades ago (Alfredsson *et al.*, 1988), the order consisted only family *Rhodothermaceae*. Lately, with more and more new taxa descriptions, members of *Rhodothermales* order have undergone several rounds of updating over the years (Munoz *et al.*, 2016; Park *et al.*, 2019). Now, *Rhodothermales* consisted of family *Rhodothermaceae* and newer

families such as *Salinibacteraceae*, *Salisaetaceae*, and *Rubricoccaceae* (Munoz *et al.*, 2016; Park *et al.*, 2019).

To date, *Rhodothermales* order consists of 10 genera. Family *Rhodothermaceae* consists of two genera− *Roseithermus* and *Rhodothermus* (Alfredsson *et al.*, 1988; Park *et al.*, 2019). Another family, *Salinibacteraceae*, consists of genera *Salinibacter* and *Salinivenus* (Antón *et al.*, 2002; Makhdoumi-Kakhki *et al.*, 2012). *Rubricoccaceae*, the third family in *Rhodothermales* order, consists of *Rubricoccus* and *Rubrivirga* genera (Park *et al.*, 2011; Park *et al.*, 2013). In relative, *Salisaetaceae* has more genera as it consists of *Salisaeta*, *Longimonas*, *Longibacter*, and *Natronotalea* (Vaisman *et al.*, 2009; Xia *et al.*, 2015; Xia *et al.*, 2016; Sorokin *et al.*, 2017). Genera *Roseithermus*, *Salisaeta*, *Rubricoccus*, *Rubrivirga*, and *Natronotalea* are represented by mono-species. Other genera stated earlier has no more than three species.

The lignocellulolytic ability of the bacteria in *Rhodothermales* is not widely discussed. So far, only *Rhodothermus* genus had been proven to be a good cellulose and hemicellulose degrader. This genus can produce relevant enzymes such as endoglucanase, β-glucosidase, xylanase, β-xylosidase, etc (Dahlberg *et al.*, 1995; Halldórsdóttir *et al.*, 1998). In this study, preliminary screening showed that strain RA had the ability in cellulose and hemicellulose degradation. The abilities of strain RA in enzyme production related to glycoside hydrolases (GHs) and auxiliary activities protein (AAs) would be discussed further based on the result from genomics, transcriptomics, and enzyme cloning.

1.2 Problem Statement

Rhodothermaceae bacterium RA is an underexplored bacterium isolated from a saline hot spring. Based on 16S rRNA similarity, strain RA is very closely related to *Roseithermus sacchariphilus* strain MEBiC09517^{T}. However, assigning taxonomy position purely based on 16S rRNA market is insufficient for distinguishing species-level differences. Therefore, more detail analyses shall be conducted to confirm the

taxonomy position of strain RA. Many fundamental questions regarding strain RA and *Roseithermus* genus remain unknown since there is only one publication on strain MEBiC09517^T (Park *et al.*, 2019). The strategies of how strain RA and *Roseithermus* genus survive at high temperatures or high salinity remained unsolved. It is hypothesized that their surviving strategies in the heated and saline environment should share some commonalities across the family and order members. Besides, comparative genomic among members of *Rhodothermales* have not been performed; thus, the understanding of diversity and evolution is poor.

Rhodothermus is the closest genus to *Roseithermus*. In the past, *Rhodothermus marinus* DSM 4252 and *Rhodothermus marinus* SG0.5JP17-172 are proven to produce important cellulosic and hemicellulosic hydrolases (Alfredsson *et al.*, 1988; Dahlberg *et al.*, 1995; Halldórsdóttir *et al.*, 1998). However, none of the other genera in the same order, including *Roseithermus*, had been studied for counterpart enzymes. Both *R. sacchariphilus* strain MEBiC09517^T and strain RA were isolated from the area with a high C:N ratio or lignocellulolytic-riched site. They have not been subjected to detail cellulolytic and hemicellulolytic analyses. It is hypothesized that strain RA and strain MEBiC09517^T are also lignocellulose degraders, similar to *Rhodothermus*. And most probably they are members of a microbial consortium in the environment that take part in lignocellulosic biomass decomposition. The total numbers of cellulolytic and hemicellulolytic enzymes in strain RA and strain MEBiC09517^T are unknown. It is also not known if strain RA can react to the lignin portion of plant biomass. Collectively, its actual role in the consortium, whether strain RA is good at targeting cellulose, hemicellulose or lignin portion, remains unsolved.

1.3 Research Objectives

The objectives of this research are:

(a) To identify the taxonomic position of *Rhodothermaceae* bacterium RA and its surviving strategies in high temperature and high salinity.

(b) To evaluate the lignocellulolytic abilities of strain RA and its major role in a microbial consortium of the hot spring.

(c) To purify and characterise an enzyme from strain RA, which is responsible for lignocellulose degradation.

1.4 Scope of Study

In this study, the rare microorganism *Rhodothermaceae* bacterium RA had been undergone the following analyses:

(a) To perform basic phenotypic characterisation on strain RA.

(b) To extract the bacterium genome, sequenced using PacBio RSII platform, assembly, and annotation.

(c) To verify the taxonomic position of strain RA via different analyses, including 16S rRNA similarity, percentage of conserved protein (POCP), average nucleotide identity (ANI), and digital DNA-DNA hybridization (dDDH).

(d) To compare the available genomes of the members in *Rhodothermales* order.

(e) To identify genes in this bacterium that are putatively responsible for lignocellulose degradation via genes and protein sequences analyses.

(f) To perform RNA extraction, sequencing using Illumina HiSeq 4000 and mapping of the transcriptome.

(g) To analyze differentially expressed genes (DEGs) of the bacterium via transcriptomics analysis.

(h) To amplify and clone a lignocellulolytic gene from strain RA.

(i) To purify and characterise recombinant xylanase from the aspects of optimum temperature, optimum pH, optimum salt concentration, thermostability, metal ion effects, enzyme kinetics, and substrate specificity.

1.5 Significance of Study

Rhodothermales order consists of underexplored genera from the aspects of genomic, transcriptomic and the ability to hydrolyze lignocellulolytic materials. Current knowledge about the bacteria world is based on the dominant groups rather than the rare biosphere. Many genera in *Rhodothermales* are represented by a mono-species, therefore this project will create new information for this order. For the first time, comparative genomic among members of *Rhodothermales* were performed. Thus, this will contribute valuable knowledge to the scientific public. Since *Roseithermus* is an underexplored bacterium, the analyses of transcriptomic will enhance the understanding of gene expression when growing the *R. sacchariphilus* strain RA in various experimental setups. *Rhodothermus*, the closest genera to *Roseithermus*, had been studied for several lignocellulolytic enzymes. It is hypothesized that enzymes produced by *R. sacchariphilus* strain RA are equally useful.

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1. Liew, K.J., Teo, S.C., Shamsir, M.S., Sani, R.K., Chong, C.S., Chan, K.-G. and Goh, K.M. (2018) 'Complete genome sequence of *Rhodothermaceae* strain RA with cellulolytic and xylanolytic activities', *3 Biotech*, 8, pp. 376. (Q3, IF: 1.786).

2. Liew, K.J., Ngooi, C.Y., Shamsir, M.S., Sani, R.K., Chong, C.S. and Goh, K.M. (2019) 'Heterologous expression, purification and biochemical characterisation of a new endo-1,4-β-xylanase from *Rhodothermaceae* bacterium RA', *Protein Expression and Purification*, 164, pp. 105464. (Q4, IF: 1.291)