CHARACTERIZATION OF BIOFILM FORMATION IN *BACILLUS SUBTILIS* ISOLATED FROM RADIATION AND NON RADIATION-EMITTING HOT SPRINGS

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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May 2019

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Biofilms can be defined as a bacterial communication form, in which the bacteria adhere to surfaces and to each others using slimy, glue-like substances called the extra-cellular matrixes. Production of biofilms is a critical key for the bacteria to express multiple kinds of biological processes, including resistance against sanitizers and environmental stresses. Ramsar hot spring, is an active, radon-containing hot spring situated in north of Iran, has the highest background of natural radiation in the world (260 mGy/year). Investigation of the microbial biofilm formation in these environments may contribute to understand their significance in food industries, in terms of cellular resistance to environmental stresses and to ionizing sterilization methods commonly used for food materials and equipment. In this work, *B. subtilis* (RAM-04) was isolated from water and mud samples obtained from Ramsar hot spring, and was found to survive following exposure to 20 kGy gamma radiation with a viable growth of 20 CFU/mL. *B. subtilis* (GAD-28) was isolated from water and biofilm samples obtained from Gadek hot spring, Malaysia, where no dose of natural radiation was reported, and was therefore used as a control. Biofilm Formation assay was performed using Microtitre Plate technique and Scanning Electron Microscopy (SEM). Gene expression analysis to quantify the critical genes involved in *B. subtilis* biofilm formation was performed using direct sequencing method and quantitative PCR (q-PCR). Biofilm formation under food-related stresses, including incubation time, temperature, pH, nutrient concentration, and subsequent chlorine treatment, was also estimated using the Microtitre Plate technique for both isolates. Results of the Microtitre Plate technique and the Scanning Electron Microscopy showed that RAM-04 formed stronger biofilms compared with GAD-28. In addition, the results of gene expression analysis of RAM-04 revealed that of 13 genes in total, seven genes showed genetic variations at multiple places in the genome sequences. The q-PCR revealed an up regulation of *yqxM-sipW-tasA* operon genes in RAM-04 with the folding change of 13, 11, and 8 respectively, and a down regulation in both *ccpA* and *slrR* genes with the folding change of 0.2 and 0.3 respectively. There was a marked variability in the food-related stress profiling, in which temperature, pH and media concentration showed a significant

effect on biofilm formation by both isolates, whereas, chlorine and incubation time had a non-significant effect. As a conclusion, RAM-04 was able to produce a biofilm three times stronger compared to that of GAD-28. In addition, assuming that RAM-04 utilizes the operon *yqxM-sipW-tasA* to produce the extracellular matrix that is necessary to build such a strong architecture of cell associations or biofilms. This in turn will be potential strategies to survive and resist extreme ionizing environmental stress. However, the biofilm formation under food-related-stress offers possible practical applications, like usage of physical applications (sub-thermal temperatures and nutrient deficiency) to control the development of biofilms in both isolates. On the other hand, the research highlighted the possible disadvantage of chlorine usage as an active sanitizer in food industries.

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PENCIRIAN PEMBENTUKAN BIOFILM DALAM *BACILLUS SUBTILIS* YANG DIPENCILKAN DARIPADA KOLAM AIR PANAS YANG MEMANCARKAN DAN YANG TIDAK MEMANCARKAN RADIASI

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Biofilms boleh ditakrifkan sebagai bentuk komunikasi bakteria, di mana bakteria melekat pada permukaan dan di antara satu sama lain dengan menggunakan bahan berlendir, seperti gam yang dipanggil matriks ekstra-selular. Pembentukan biofilm adalah kritikal bagi bakteria untuk menyatakan pelbagai jenis proses biologi, termasuk penentangan terhadap sanitizer dan tekanan alam sekitar. Kolam air panas Ramsar adalah aktif, merupakan kolam air panas yang mengandungi radon, terletak di utara Iran, dan mempunyai latar belakang radiasi semula jadi tertinggi di dunia (260 mGy / tahun). Penyelidikan berkenaan pembentukan biofilm mikrob dalam persekitaran ini boleh menyumbang kepada kefahaman terhadap kepentingan biofilm dalam industri makanan, dari segi rintangan selular terhadap tekanan persekitaran dan terhadap kaedah pensterilan pengionan yang biasa digunakan untuk bahan makanan dan peralatan. Dalam kajian ini, B. subtilis (RAM-04) telah diasingkan dari air dan sampel lumpur yang diperoleh dari kolam air panas Ramsar, dan didapati dapat terus hidup selepas pendedahan kepada radiasi gamma 20 kGy, dengan pertumbuhan sebanyak 20 CFU / mL. B. subtilis (GAD-28) telah diasingkan dari sampel air dan biofilm yang diperolehi dari kolam air panas Gadek, Malaysia, di mana tiada dos sinaran semula jadi dilaporkan, dan oleh itu, ianya digunakan sebagai kawalan. Ujian Formasi Biofilm dilakukan dengan menggunakan teknik Microtitre Plate dan Mikroskopi Pengimbasan Elektron (SEM). Analisis ekspresi gen untuk mengukur gen kritikal yang terlibat dalam pembentukan biofilm B. subtilis dijalankan dengan menggunakan kaedah penjujukan langsung dan kuantitatif PCR (q-PCR). Pembentukan biofilm di bawah tekanan berkaitan makanan, termasuk masa inkubasi, suhu, pH, kepekatan nutrien, dan seterusnya rawatan klorin, juga dianggarkan dengan menggunakan teknik Microtitre Plate untuk kedua-dua dipencilkan. Keputusan teknik Microtitre Plate dan Mikroskopi Pengimbasan Elektron menunjukkan bahawa RAM-04 membentuk biofilm yang kuat berbanding dengan GAD-28. Di samping itu, keputusan analisis ekspresi gen RAM-04 menunjukkan bahawa 13 gen keseluruhannya, tujuh gen menunjukkan variasi genetik di pelbagai tempat dalam urutan genom. Q-PCR menunjukkan regulasi menaik bagi yqxM-sipW-tasA operon gen dalam RAM-04

dengan perubahan lipat 13, 11, dan 8, masing-masing, dan regulasi menurun pada kedua-dua ccpA dan slrR gen dengan perubahan lipat sebanyak 0.2 dan 0.3, masing-masing. Terdapat variasi yang ketara dalam profil tekanan yang berkaitan dengan makanan, di mana suhu, pH dan kepekatan media menunjukkan kesan yang signifikan terhadap pembentukan biofilm oleh kedua-dua isolat, manakala, klorin dan masa inkubasi mempunyai kesan yang tidak signifikan. Sebagai kesimpulan, RAM-04 dapat menghasilkan biofilm tiga kali lebih kuat berbanding dengan GAD-28. Di samping itu, dengan mengandaikan bahawa RAM-04 menggunakan operon yqxM-sipW-tasA untuk menghasilkan matriks ekstra-selular yang diperlukan untuk membina struktur kesatuan sel atau biofilm. Ini seterusnya akan menjadi strategi yang berpotensi untuk terus bertahan dan menentang tekanan alam sekitar yang melampau. Walau bagaimanapun, pembentukan biofilm di bawah tekanan berkaitan makanan menawarkan aplikasi praktikal, seperti penggunaan aplikasi fizikal (suhu sub-haba dan kekurangan nutrien) untuk mengawal pembangunan biofilm di kedua-dua dipencilkan. Namun yang demikian, kajian ini menunjukkan kemungkinan kelemahan penggunaan klorin sebagai sanitizer dalam industri makanan.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CBD	Calgary biofilm device
CFU	Colony forming unit
CV	Crystal violet
CPDs	Cyclobutane pyrimidine dimers
EPS	Extracellular polymeric substances
HLNRA	High level natural radiation areas
kGY	Kilogray
LD	Lethal dose
MIC	Microbiologically influenced corrosion
MTP	Microtiter plate
mGy	Milligray
mSv	Millisievert
NA	Nutrient agar
NB	Nutrient broth
ppm	Part per million
PBS	Phosphate buffer solution
PPs	Photoproducts
PGA	Poly-acetylglucosamine
PCR	Polymerase chain reaction
PNAG	Poly-N-acetylglucosamine
RCF	Relative centrifugal force
SEM	Scanning electron microscopy
SRB	Sulfate reducing bacteria
TSB	Tryptic soy broth
VIS	Visible spectrophotometry

CHAPTER 1

INTRODUCTION

Bacteria are capable of switching between the planktonic and biofilm modes of growth. Unlike the free floating members, bacteria in a biofilm assemble and embed themselves in a self-generated glycocalyx containing extracellular polymeric substances (EPS) along with the surface associated structures (O'Toole, Kaplan, and Kolter, 2000).

Bacterial cells have been found to form biofilms in response to numerous environmental conditions such as unfavorable temperature, nutrient deficiency, pH changes, osmotic pressure and dehydration (Stoodley et al., 2002), the presence of chemical reagents (Mah and O'Toole, 2001) and ecological competition (Oliveira et al., 2015).

B. subtilis is a good test organism to characterize and study the biofilm formation processes since this bacterium possesses the ability to build a strong associated biofilm (Hamon and Lazazzera, 2001; Stanley et al., 2003; Hamon et al., 2004). Studies on *B. subtilis* biofilm have revealed the importance of regulatory mechanism in biofilm formation and development. Bacterial cells are arranged in long chains of cells that are connected together in bundles by an extracellular matrix of exopolysaccharide and the protein TasA. The exopolysaccharide is generated by enzymes modulated by the *epsA-O* operon. While, the gene regulator TasA is situated in the *yqxM-sipW-tasA* operon (Chai et al., 2008). In addition, the circuitry for extracellular matrix generation is controlled by a complex network involving the interplay of negatively and positively acting of four transcriptional regulatory proteins: (*spo0A, sinR, abrB* and *slr*) with two of them: (*sinR and slr*) being highly responsible for the controlling of the two operons implicated in the matrix production (Chu et al., 2008).

High Level Natural Radiation Areas (HLNRA) around the world provides interesting locations to investigate the different biological responses of high radiation exposure on humans and microorganisms. The level of natural background radiation in these areas is sometimes 10−100 times higher than normal areas (Ghiassi-nejad et al., 2002).

Food irradiation is achieved by exposing the food materials to ionizing radiations such as (a) gamma rays, the standard dose for food irradiation in the US is 1 kGy, which is believed to be adequate to eliminate most of the food-contaminating microorganisms since only a few strains of bacteria have the LD_{10} values higher than 1 kGy (Haire et al., 1997; Farkas, 2006). Therefore, radiation-resistant bacteria would significantly impact on the food industries (Collins et al., 2000). That being said, food irradiation at those doses (1 kGy) is not particularly recommended, and should be taken into consideration when gamma radiation is applied as a sterilization method of food and industrial supplies.

Biofilm formation in food-processing environments is considered an important issue as it represents a permanent source of microbial contamination that may result in food spoilage and transmission of diseases, as well as incurring economic losses (Whiteley, Pajkos, and Vickery, 2002). The raised resistance of biofilms to sanitization methods in the food industry results in huge problems which cost millions of dollars annually in terms of lost and quality poorness of the food products (Brooks and Flint, 2008).

Several attempts have been made to understand the bacterial biofilm formation under different environmental stresses, and the subsequent induction of their regulatory genes. Most studies in this field have been focused on biofilm expression in food-related environments. At present, there is a lack of documented reports on how biofilm is formed and regulated at high levels of natural radiation areas. Investigation on the ability of certain bacteria to form biofilm under these conditions is therefore important in developing a new control regime and technology to eliminate biofilms and to avoid the improper usage of sterilization procedure that may trigger further mechanisms of resistance. With regard to irradiation resistant biofilms, the reliable sterilization methods may not be sufficient to reduce or kill the bacteria, especially for those responsible in foodborne infections.

Therefore, the objectives of the present study were as follows:

1. To isolate, characterize, and molecularly identify ionizing and non-ionizing radiation-resistant bacteria
2. To assess the biofilm formation ability of the selected ionizing and non-ionizing radiation-resistant bacteria
3. To evaluate the expression of critical genes involved in biofilm formation of ionizing and non-ionizing radiation-resistant bacteria
4. To characterise the biofilm formation of ionizing non-ionizing radiation-resistant bacteria subjected to food-related stresses and chlorine treatment

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