UNIVERSITI PUTRA MALAYSIA

ANALYSIS OF Lactobacillus plantarum PA21 WITH SURFACE ANCHORED HETEROLOGOUS PROTEIN MICROENCAPSULATED WITH POLYSACCHARIDE

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ANALYSIS OF *Lactobacillus plantarum* PA21 WITH SURFACE ANCHORED HETEROLOGOUS PROTEIN MICROENCAPSULATED WITH POLYSACCHARIDE

By

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Microencapsulation technology is retention of cells or active compounds within the microencapsulation matrix as a protection from harsh environments. The oral administration of microencapsulated probiotics carrying heterologous proteins through the Gastro intestinal tract (GIT) can be a novel approach for vaccine delivery. The peptidoglycan cell walls of Gram positive bacteria functions to transport and assemble of protein that capable to interact with a surrounding. These properties will enable non-genetically modified organism of *Lactobacillus plantarum* Pa21 to be manipulated as a carrier by anchoring externally added heterologous protein such as the AR antigens of *Mycobacterium tuberculosis*. To date, the protective effect of microencapsulation of *Lb. plantarum* Pa21 surface anchored with heterologous proteins is still unknown. The microencapsulation is believed to enhance the viability of cells in the intestine, this mechanism would also be capable to up-regulate the protection toward AR antigens anchored on *Lb. plantarum* Pa21 surface area. The purpose of this study is to investigate the protective effect of microencapsulation of *Lb. plantarum* Pa21 surface anchored with AR antigen againts simulated gastrointestinal fluid. Firstly, *Lb. plantarum* Pa21 without cell wall-anchored heterologous proteins were microencapsulated with different concentration of alginate and chitosan. The effect of 2% (w/v), 3% (w/v)and 4% (w/v) sodium alginate concentration coated with 0.2% w/v and 0.4% w/v chitosan concentration on the cell survival, hardness and cells released was studied. The beads produced were tested under stress conditions designed by incubation in Simulated Gastric Juice (SGF) solution at pH 1.8 followed by incubation in Simulated Intestinal Juice (SIF) solution at pH 7.45. Three percent alginate coated with 0.4 % (w/v) chitosan beads provided the best protection for *Lb. plantarum* in all treatments. Unencapsulated *Lb. plantarum* Pa21 survived the first 60 minute of the SGF solution treatment with $2.99x10^2$ CFU/ml viable cells compared to 3% (w/v) alginate coated with0.4 % (w/v) chitosan microencapsulated cells with $6.25x10^8$ CFU/ml viable cells after 120 minutes incubation. Secondly, a total amount of 50 µg/ml of AR protein was purified and attached to $2.19 x10^9$ CFU/ml of *Lb. plantarum* Pa21. The confirmation of protein surface display was detected by SDS-PAGE and western blot before subsequently microencapsulated in 3% (w/v) alginate coated with 0.4 % (w/v) chitosan matrix. After SGF solution test, whole cell ELISA detection of

microencapulated AR protein anchored to *Lb. plantarum* Pa21 in alginate coated chitosan has the higher value excitation of horseradish peroxide conjugate on secondary antibody compared to unencapsulated AR protein anchored to *Lb plantarum* Pa21. Subsequent microencapsulated AR protein anchoring to the cell wall of *Lb. plantarum* Pa21 also showed a microscopic excitation of FITC conjugate on secondary antibody which signified protection of microencapsulated AR protein binding on the surface of *Lb. plantarum* Pa21 along the GIT passage.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISA *Lactobacillus plantarum* PA21 DENGAN PROTEIN ASING YANG DILEKAT PADA PERMUKAAN SEL DAN DI KAPSUL MENGGUNAKAN POLISAKARIDA

Oleh

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Teknologi penmikrokapsulan adalah pengekalan sel atau sebatian aktif di dalam matriks pemikrokapsulan sebagai perlindungan daripada persekitaran yang tidak memberansangkan. Pengambilan secara pemakanan probiotik mikrokapsul yang membawa protin asing melalui saluran usus mampu menjadi pendekatan baru dalam dunia penghantaran vaksin. Bakteria Gram positif mempunyai dinding sel peptidoglikan yang berfungsi sebagai pengangkut dan pemasangan protein di mana mampu untuk bertindak balas dengan persekitaran sel. Ciri-ciri ini membolehkan sel yang tidak perlu diubahsuai secara genetik seperti *Lactobacillus plantarum* Pa21 boleh dimanipulasi sebagai pembawa dengan melekatkan protein asing dari luar sel di mana protin yang dibawa adalah antigen AR untuk vaksin batuk kering. Setakat ini, kesan perlindungan pemikrokapsulan daripada *Lb. plantarum* Pa21 yang dilekatkan dengan protein asing di luar permukaan sel masih tidak diketahui lagi. Seiring dengan kepercayaan pemikrokapsulan mampu meningkatkan daya tahan sel di dalam usus, mekanisme ini juga mampu untuk memangkin perlindungan terhadap antigen AR yang melekat pada permukaan *Lb. plantarum* Pa21. Tujuan kajian adalah bagi menyiasat kesan perlindungan pemikrokapsulan *Lb. plantarum* Pa21 yang dilekat dengan antigen AR di permukaan sel daripada simulasi cecair gastrousus. Pertama sekali, *Lb. plantarum* Pa21 tanpa protin asing yang dilekat telah di mikrokapsulkan menggunakan kepekatan alginate dan kitosan yang berbeza. Kesan daripada 2% (w/v), 3% (w/v) dan 4% (w/v) kepekatan natrium alginate disalut dengan 0.2% (w/v) dan 0.4% (w/v) kepekatan chitosan pada sel hidup, ketahanan sel dan perlepasan sel telah dikaji. Mikrokapsul yang dihasilkan telah diuji di dalam persekitaran yang menyerupai persekitaran di dalam usus yang direka dengan menggunakan teknik pemeraman di dalam cecair simulasi gastrik (SGF) pada pH 1.8 diikuti oleh pengeraman di dalam cecair simulasi usus pada pH 7.45. Tiga peratus alginate bersalut 0.4% (w/v) kitosan memberikan perlindungan yang terbaik kepada *Lb. plantarum* Pa21 di dalam kesemua eksperimen. *Lb. plantarum* Pa21 yang tidak di mikrokapsulkan hanya mampu hidup selama 60 minit pertama semasa pengeraman SGF dengan $2.99x10^2$ CFU/ml sel-sel yang berjaya hidup berbanding dengan 3% (w/v) alginate bersalut 0.4% (w/v) sel mikrokapsul kitosan yang mengandungi $6.25x10^8$ CFU/ml sel yang hidup selepas tamat 120 minit pengeraman. Kemudian, sebanyak 50 µg / ml AR protin telah di tulenkan dan dilekatkan pada $2.19 x10^9$

CFU /ml *Lb. plantarum* Pa21. Pengesahan paparan protein di permukaan sel dikesan dengan menggunakan SDS-PAGE dan Western blot sebelum dimikrokapsulkan ke dalam 3% (w/v) alginate bersalut 0.4% (w/v) matriks kitosan. Selepas ujian pengerapan di dalam SGF, teknik pengesanan menggunakan keseluruhan sel ELISA menunjukan mikrokapsul *Lb. plantarum* Pa21 yang dilekat protin AR mempunyai nilai pengujaan yang lebih tinggi dengan menggunakan peroksida horseradish konjugat antibodi ke dua berbanding protin AR yang terlekat pada *Lb. plantarum* Pa21 tetapi tidak dikapsulkan. Selain itu, mikrokapsul AR protein yang dilekat pada dinding sel *Lb. plantarum* Pa21 juga menunjukkan pengujaan mikroskopik FITC konjugat antibodi ke dua yang menandakan perlindungan protein AR mikrokapsul mengikat permukaan *Lb. plantarum* Pa21 di sepanjang laluan GIT.

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I certify that a Thesis Examination Committee has met on 9 January 2017 to conduct the final examination of Sarah Safwah binti Md Sabidi on her thesis entitled "Analysis of *Lactobacillus plantarum* PA21 with Surface Anchored Heterologous Protein Microencapsulated with Polysaccharide" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This is to confirm that:

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LIST OF ABBREVIATIONS

~	approximately
°C	degree Celcius
µg	microgram
µl	microlitre
BSA	Bovine Serum Albumin
bp	base pairs
$CaCl_2$	calcium chloride
cDNA	complementary deoxynucleotide acid
Da	Dalton
dH_20	distilled water
DNA	deoxyribonucleotide acid
EDTA	ethylenediaminetetraacetic acid
eV	electron volt
g	gravity force
GRAS	Generally Regarded as Safe
h	hour
HRP	Horse Radish Peroxidase
kb	kilo base pairs
kDa	kilo Dalton
kV	kiloVolt
l	litre
LAB	Lactic Acid Bacteria
LB	Luria-Bertani
Lb. plantarum-AR	*Lb. plantarum* Pa21 anchored with AR protein
M	Molar
mA	milliampere
min	minute
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar

$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
OD	Optical Density
rpm	revolutions per minute
RT	retention time
sec	seconds
Ta	annealing temperature
Tm	melting temperature
V	volt
v/v	volume per volume
W	Watts
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Live probiotic microorganisms that are usually from Lactic Acid Bacteria (LAB) species are recognized as nutritious as they confer health benefits to the host. Unfortunately, some adverse conditions in the stomach can put the LAB into a vulnerable state, resulting in significant reduction of cells before they can reach the small intestine for uptake into the body. An alternative strategy to overcome this problem is to create a physical barrier between the probiotic and this harsh environment. Microencapsulation is an effective method which can overcome this problem as it protects viable microorganisms in the gastrointestinal tract after ingestion.

Many biomaterials have been studied by researchers for microencapsulation but alginate and chitosan are the most commonly used biopolymers This is due to their biocompability, non-toxicity, low cost and they can be prepared using mild gelation condition (Krasaekoopt *et al*., 2004). In addition, the pH dependent properties make these biopolymers suitable for targeted delivery application. When the entrapped probiotic is protected in an alginate matrix coated with chitosan reaches the stomach, the chitosan will prevent acidification and dissolution of the alginate core until it reaches the small intestine where dissolution happens at higher pH (Krasaekoopt *et al*., 2004).

Besides providing this added benefit as probiotic, the LAB species can also be utilized as a vaccine carrier by expressing heterologous antigens for oral administration. In many cases, antigens which are surface displayed on the carrier host pose better antigenicity compared to antigens expressed intracellularly. Basically, this application is achieved by the expression of antigen as fusions with anchoring domains that are capable of attaching to the LAB cell surface, allowing the antigen to have direct contact with the targeted environment.

There are two different modes of protein surface display; surface display from the inside of the cell (through covalent attachment) and surface display from the outside of the cell (through non-covalent attachment). In the first mode, protein display involves the expression of the gene of interest from the same carrier host (in this case, LAB). The protein of interest is targeted to the surface from inside the cell using a signal peptide. In the second mode, a different host is used for protein expression. The protein is then purified from the expression host and displayed on the LAB from the outside simply by mixing the carrier cells with the protein. In both modes, an anchor domain allows attachment of the protein to the surface of the cell. Surface display of proteins from the outside of the cell is advantageous as the carrier host surface displaying the protein need not be genetically modified offering an opportunity to use non-genetically modified bacteria as a vaccine carrier. Other strong reason to utilize LAB as a carrier is due to their Generally Regarded as Safe (GRAS) status and known capability to stimulate the innate immune system. Thus, triggering the adaptive immune system to generate strong and durable immune responses (Mbow *et al*. 2010).

Although microencapsulation has been proven to protect viability of probiotic cell, there is little study on how it affects the integrity of the protein which is bound to the surface of the cells from adverse conditions such as those found in the stomach. The *Lb plantarum* Pa21 anchored heterologous protein will face degradation and high acidity in the stomach. Thus, it is essential to protect it by microencapsulation., This study was carried out with the main objective to analyze the effect of microencapsulation on *Lb. plantarum* Pa21 displaying AR antigen against tuberculosis.

The specific objectives were:

1. To investigate the best microencapsulation material for *Lb. plantarum* Pa21 using different concentrations of alginate-chitosan.
2. To analyze the protective effect of microencapsulation of *Lb. plantarum* Pa21 surface anchored with AR antigen from simulated gastrointestinal fluid.

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