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Bio-based anti-coagulant agent from microorganisms for natural rubber latex preservation

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BIO-BASED ANTI-COAGULANT AGENT FROM MICROORGANISMS FOR NATURAL RUBBER LATEX PRESERVATION

By

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Thesis Submitted to the School of Graduates Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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BIO-BASED ANTI-COAGULANT AGENT FROM MICROORGANISMS FOR NATURAL RUBBER LATEX PRESERVATION

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Chairman : Wan Zuhainis Saad, PhD

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Natural rubber (NR) latex is whitish fluid derived from latex ducts which are in a layer outside the cambium of rubber trees (*Hevea Brasiliensis*). Latex is a stable dispersion of cis-1,4-polyisoprene rubber in an aqueous phase that contain non-rubber substances such as carbohydrates, proteins and amino acids and a range of enzymes. Non-rubber constitute in NR latex encourage the proliferation of bacteria that causes latex destabilization and coagulation due to their metabolism activities. Preserved NR latex is referred to latex that will not undergo coagulation process to form a solid natural rubber. Ammonia and others chemical-based preservative agent such as tetramethylthiuramdisulphite/ zinc oxide (TMTD/ZNO) have negative drawbacks to the environment and human health. The approach on searching for biological based format for NR latex preservation was due to the current focus on generating biologically processing system. Therefore, the objective of the present study is to search for biological based anti-coagulant agent for NR latex short-term preservation with antimicrobial and surfactant properties from microorganisms present in environment related with NR. A total of 28 isolates comprises 20 bacterial isolates and eight fungal isolates were successfully obtained from field NR latex, coagulated NR latex and soil from rubber plantation area. Screening for antimicrobial activity was performed using disc diffusion method. The surfactant activity was evaluated by measuring the surface tension of the extracts. The emulsification capability was determined by measuring the droplets size and distribution of oil in water emulsions. The field NR latex stability was characterized by means of bacterial population, volatile fatty acid numbers (VFA), and NR latex viscosity. Out of 28 isolates, only four isolates exhibited antimicrobial activity namely, *Aspergillus fumigatus* S14, *A. flavus* S16, *Phaeomoniella chlamydospora* EM19 and *Bacillus amyloliquefaciens* S10b. Meanwhile, in surfactant activity screening assay, only five isolates out of 28 isolates exhibited surfactant activity in which comprises of one fungi *Lambdasporium* sp. FS31 and four bacteria (*Enterococcus feacalis* F11, *Myroides odoratus* F5, *Bacillus pumilus* S1b and EM23). Microbial extracts from *B. amyloliquefaciens* S10b performed better than the other isolates that exhibited

antimicrobial activity in acting as antimicrobial agent in field NR latex. Surfactant activities from *Lambdasporium* sp. FS31 showed greater potential to enhance the colloidal stability of the rubber particles. Out of the nine isolates, four isolates namely, *P. chlamydospora* EM19, *B. amyloliquefaciens* S10b, *Lambdasporium* sp. FS31 and *M. odoratus* F5 were prepared in a mixture. Field NR latex in the presence of microbial extracts from *B. amyloliquefaciens* S10b with *Lambdasporium* sp. FS31 showed less bacterial activity and slow increment of VFA number resulting in minimal changes of the NR latex viscosity indicated a good NR latex stability. In conclusion, this study showed that these microorganisms have potential to be used as an anti-coagulant agent for NR latex preservation.

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AGEN ANTI-PENGGUMPALAN BERASASKAN BIOLOGI DARIPADA MIKROORGANISMA UNTUK PENGAWETAN LATEKS GETAH ASLI

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Lateks getah asli (GA) merupakan cecair putih yang dihasilkan daripada saluran lateks yang merupakan lapisan luaran kambium pokok getah (*Hevea Brasiliensis*). Lateks adalah penyebaran stabil cis-1,4-polyisoprene getah di dalam fasa bendalir yang mengandungi komponen selain getah seperti campuran karbohidrat, protein dan asid amino dan pelbagai enzim. Komponen ini menggalakkan pembiakan bakteria yang menyebabkan ketidakstabilan lateks kesan daripada aktiviti metabolisma. Lateks GA yang diawet merujuk kepada lateks yang tidak melalui proses pembekuaan bagi menghasilkan lateks yang stabil. Ammonia dan agen pengawetan berasaskan kimia yang lain seperti tetramethylthiuramdisulfida/ zink oksida (TMTD/ZnO) memberi kesan negatif kepada alam sekitar dan kesihatan manusia. Oleh itu, pencarian bahan pengawet yang berasaskan biologi merupakan langkah yang diambil berdasarkan matlamat untuk menghasilkan sistem pemprosesan hijau. Oleh itu, objektif kajian ini adalah untuk mencari sebatian anti-penggumpalan untuk pengawetan lateks GA dalam jangka masa pendek yang berasaskan biologi dengan sifat-sifat antimikrob dan surfaktasi daripada hasil ekstrak mikroorganisma yang diperolehi daripada kawasan sekitar yang melibatkan GA. Sejumlah 28 mikroorganisma terdiri daripada 20 jenis bakteria dan 8 jenis fungi telah berjaya diperolehi daripada lateks GA, lateks beku dan tanah dari kawasan tanaman getah. Saringan terhadap aktiviti antimikrob daripada ekstrak mikroorganisma telah dijalankan dengan menggunakan kaedah resapan agar. Manakala saringan aktiviti surfaktasi dijalankan dengan mengukur kadar ketegangan permukaan air dengan kehadiran ekstrak mikroorganisma tersebut. Keupayaan emulsifikasi ekstrak mikroorganisma tersebut pula ditentukan dengan mengkaji pengedaran dan saiz titik kecil yang terbentuk daripada proses emulsifikasi minyak di dalam air. Kestabilan lateks GA dicirikan melalui kandungan bakteria didalam lateks GA, pembentukan asid lemak meruap dan kelikatan lateks GA. Daripada sejumlah 28 jenis mikroorganisma, hanya empat sahaja yang menunjukkan sifat antimikrob iaitu *Aspergillus fumigatus* S14, *A. flavus* S16, *Phaeomoniella chlamydospora* EM19 dan *Bacillus amyloliquefaciens* S10b. Manakala,

daripada saringan aktiviti surfaktasi hanya lima mikroorganisma yang terdiri daripada satu fungi *Lambdasporium* sp. FS31 dan empat bakteria (*Enterococcus feacalis* F11, *Myroides odoratus* F5, *Bacillus pumilus* S1b dan EM23) yang menunjukkan sifat surfaktasi. Ekstrak mikroorganisma daripada bakteria *B. amyloliquefaciens* S10b menunjukkan aktiviti antimikrob yang lebih baik berbanding mikroorganisma yang lain yang menunjukkan aktiviti yang sama. Aktiviti surfaktasi daripada ekstrak *Lambdasporium* sp. FS31 pula, menunjukkan potensi untuk meningkatkan kestabilan koloid antara zarah-zarah getah didalam lateks GA. Daripada sembilan jenis mikroorganisma yang terdiri daripada empat mikroorganisma dengan ciri-ciri antimikrob dan lima mikroorganisma dengan ciri-ciri surfaktasi, hanya empat mikroorganisma iaitu *P. chlamydospora* EM19, *B. amyloliquefaciens* S10b, *Lambdasporium* sp. FS31 dan *M. odoratus* F5 dipilih dan disediakan di dalam bentuk campuran. Lateks GA yang dicampurkan dengan campuran daripada ekstrak *B. amyloliquefaciens* S10b bersama *Lambdasporium* sp. FS31 menunjukkan kadar aktiviti bakteria yang kurang dan peningkatan kadar asid lemak meruap yang perlahan. Seterusnya menyebabkan perubahan yang tidak ketara dalam kadar kelikatan lateks GA yang merupakan petunjuk kepada kestabilan lateks GA yang baik. Kesimpulannya, kajian ini menunjukkan hasil daripada ekstrak mikroorganisma mempunyai potensi untuk digunakan sebagai bahan anti-penggumpalan untuk pengawetan lateks GA.

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

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LIST OF ABBREVIATIONS/SYMBOLS

<	Less than
=	Equal
>	More than
%	Percentage
µg	Microgram
µL	Microliter
µm	Micrometer
ABC	Assisted biological coagulation
ATCC	American Type Culture Collection
BA	Boric acid
C	Carbon
Cfu/mL	Cell forming unit per mililiter
Cp	Centripoise
CMC	Critical micelles concentrations
d	Day
DA	De-ammoniated latex
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRC	Dry rubber content
EUCAST	European Committee for Antimicrobial Susceptibility Testing
Fe	Ferum
g	Gram
g/L	Gram per liter
h	Hour
H	Hydrogen
HA	High ammoniated
K	Potassium
L	Liter
LA	Low ammonia
LA-TZ	Low ammonia - tetramethylthiuram disulphide, zinc oxide
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
Mg	Magnesium
MHA	Muller-Hinton Agar
MIC	Minimum inhibition concentration
min	Minutes
mL	Mililiter
mm	Milimeter
mN/m	Mili Newton per meter
mRNA	Messenger ribonucleic acid
MST	Mechanical stability time
MYEA	Molasses yeast extract agar
N	Nitrogen
NC	Negative control
NCCLS	National Committee for Clinical Laboratory Standards
nm	Nanometer
NH_3	Ammonia
NR	Natural Rubber

O	Oxygen
OD	Optical density
^{o}C	Celcius
P	Phosphorus
PC	Positive control
PDA	Potato dextrose agar
phr	Parts per hundred
PRBL	Atriazine/benzotriazole derivative
PRI	Plasticity Retention Index
rpm	Revolution per minit
S	Sulphur
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SLS	Sodium laurate sulphate
SPP	Sodium pentachlorphenate
sp.	Species
T_i	Surface excess concentration
TMTD	Tetramethylthiuram disulphide
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSC	Total solid content
VFA	Volatile fatty acid
Wt%	Weight percent
ZDC	Zinc diethyldithiocarbamate
ZnO	Zinc oxide
$ZnSO_4$	Zinc sulphate

CHAPTER 1

INTRODUCTION

Natural rubber (NR) latex is derived from latex ducts which are in a layer outside the cambium of rubber trees (*Hevea brasiliensis*) (John, 1982). When the tree bark is tapped, a milky fluid which comprising 30% - 40% of rubber hydrocarbon particles with a few percentage of non-rubber particles such as proteins, lipids, carbohydrates and sugars are immediately flow through the tapping panel on the tree bark and into the collecting cup (Angrove, 1964). The remaining major component of NR latex is water. In addition to rubber and water, latex contains small quantities of proteins, fatty acids, sterols, lipids, carbohydrates and other mineral matters (Hasma and Subramaniam, 1986). Due to mineral rich in latex serum, microorganisms were prone to propagate and used these substances as their energy sources (Taysum, 1960).

Natural rubber latex is contaminated with microorganisms after being tapped (Taysum, 1960). The main sources of contamination of field NR latex are the tree lace, tapping panel bark and the cup which receives the latex (Soeseno and Mansjoer, 1975). The bacterial population in field NR latex is always large in any routine collection and many early workers were familiar with microorganisms in latex and in rubber products. A wide variety, mostly strong acid producers are present in field NR latex. They represent species of *Bacillus, Bacterium, Corynebacterium, Escherichia, Micrococcus, Streptococcus, Serratia, Sarcina, Klebsiella, Listeria, Azotobacter, Proteus* and *Pseudomonas* (John, 1977; 1982). The destabilization of fresh and ammoniated NR latex occurs from a build-up of volatile fatty acids (VFA; primarily acetic, formic and propionic acids) due to microbial metabolic activities consuming the rich substrates that constitute the non-rubber phase (Juntarachat et al., 2013). During latex coagulation, the negatively charged protein membranes that surrounds many rubber molecules was neutralized by oxidation reaction due to presence of microorganisms cause the rubber particles repelling to each other and therefore flocculation of latex is occurred (Booten el al., 2011).

Natural rubber latex processing from raw materials until products manufacturing involved intensive chemical usage. At initial state, preservative agent is responsible to arrest microbial growth in field NR latex for certain of period and yet obtain rubber end products of acceptable properties (John, 1976). Preservation of latex is referred to latex that will not undergo coagulation process to form a solid rubber. Ammonia is a commonly used latex preservative agent is added into latex based on the weight of latex depending on the storage time and the condition of the latex (Ong, 1998). Ammonia became the most favourable anti-coagulant agent for NR latex due to its alkalinity and biocide characteristic (Cook, 1960). Although it seems to have many advantages, ammonia is not a suitable compound to be used in prolonging time (Loykulnant et al., 2009). Ammonia can cause detrimental effects to the environment, human health and quality of rubber products (Vivayganathan et al., 2008; Loykulnant et al., 2009). Ammonia could possibly pollute the environmental air by its pungent smell odour and it is easy to evaporate. Chemical residuals from NR latex based products also have been reported by Tinkler et al. (1998). In the study, dithiocarbamates which were being used as accelerators in the latex industry could possibly have carcinogenic

effect. Chemical residues in latex based medical devices have been reported to cause allergy reaction to the medical practitioners and patients (Yip and Cacioli, 2002). Study on latex allergenicity by De Jong et al. (2002) due to chemical sensitization activity revealed that chemicals that being used during NR latex glove manufacturing cause chemical sensitization prior to contact and through cross reaction. These chemical could produce substances that were also can trigger the allergen reactions. Extensive usage of chemicals increase the post operating management due to chemical spills or leakage has urged the industry to search for a more environmentally friendly processing that can reduce the dependancy on chemicals process.

Natural rubber industries continually seeks safer and simpler preservation systems and have to discover novel substance than can work without ammonia, be water soluble and be as cheap as tetramethylthiuramdisulphite (TMTD)/zinc oxide (ZnO). In this study the microbial preservative agent as biological alternative to the ammonia-based biocide chemical format was proposed. The simplest approach to this objective would be to search for potential microorganisms that have an advantage in producing antibiotics to suppress VFA producers and excreting surfactant-like substances to increase electrostatic repulsion on rubber particles. Therefore, this work is initiated to culture NR related environmental derived fungi or bacteria (e.g field NR latex, coagulated latex and soil in rubber plantation) and screen for antimicrobial and surfactant activities that might be produced by the isolated fungi and bacteria. This was followed by inoculating the isolates into NR latex and stability of the latex was characterized. The general objective of this study was to search for biological based anti-coagulant agents for NR latex preservation. The specific objectives of this study were:

i. To screening, isolate and characterize microorganisms from environment related with NR latex such as latex serum, coagulated NR latex and soil from rubber plantation area.
ii. To investigate the antimicrobial and surfactant activities from the isolated microbes.
iii. To characterize the properties of treated NR latex with selected microbial isolates.

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LIST OF PUBLICATIONS

Aziana, A.H., Amir-Hashim, M.Y. and Zuhainis S.W. (2015). Efficiency of Commercial Biological Compounds as Anti-Coagulant Agents in Natural Rubber Latex. *Journal of Rubber Research*. (Accepted).

Aziana, A.H., Manroshan, S., Zuhainis, S.W. and Rosfarizan, M. (2015). Microbial Surfactant for Preservation of Natural Rubber Latex. Monograph. Beneficial Microorganisms in Agriculture and Aquaculture. Microbiology Monographs. Springer. (Under Review)

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