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Noor Azwani Zainol, A Rafidah A Mohd Yunos

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AFFORDABLE IN VITRO PERMEABILITY MODEL USING VERO CELL LINE

Sugania Malar Chinapayan[1], Saleha Shahar[1]

[1]*Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia 81310 UTM Johor Malaysia*
cmsuganiamalar@gmail.com, saleha@fbb.utm.my

ABSTRACT
Primary vascular endothelial cells are commonly used to study paracellular permeability of vascular leakage, a phenomenon underlying various pathological conditions from chronic to acute diseases. However, primary cells are technically difficult to use due to its inherent biological character and too cost-rigid to sustain high-throughput protocols such drug screening. We explored the potential of Vero epithelial cell-line as alternative cells for paracellular permeability model. Vero cells were grown to confluent on transwell membrane inserts then exposed to tumour necrosis factor-alpha at 0.2 ng/ml – 2 µg/ml. The integrity of Vero cell paracellular barrier was assessed by permeation of trypan blue-stained bovine serum albumin across transwell membrane. Tumour necrosis factor-alpha effectively reduced Vero cells barrier with the lowest concentration (0.2 ng/ml) causing increased of trypan blue-stained bovine serum albumin permeation by 51.6% ± 4.7% (t-test; $p<0.001$) compared to untreated control. We tested the paracellular barrier model in pathological condition using dengue virus infection. Low amount of dengue virus (multiplicity of infection = 0.01) significantly increased trypan blue-stained bovine serum albumin permeation across Vero cells barrier caused by tumour necrosis factor-alpha, similar to that reported in primary endothelial cells. This showed Vero epithelial cell-line behaved similarly as endothelial cells and can be used in placed of primary endothelial cells to model paracellular permeability. We estimated the cost of operating Vero-based *in vitro* permeability model would be half of the conventional method and would be more cost effective for high throughput protocols of drug screening.

Keywords: Vero; dengue virus; vascular permeability; endothelial; drug screening

1. INTRODUCTION

Dysfunctional endothelial barrier is key to vascular leakages underlying many human diseases such as cancers, diabetes mellitus, atherosclerosis, and viral infections [1]. Cytokines pivotal in inflammatory cascade including tumour necrosis factor-α (TNF-α), Interleukin-6 and Interleukin-1β are important regulators of paracellular permeability in vascular leakages [2]. Endothelium is a layer of endothelial cells (ECs) that lines the interior surface of blood and lymphatic vessels. The layer regulates, among others, vascular wall tonicity, vascular homeostasis and a battlefield for inflammatory responses [1]. Often, endothelial dysfunction is crucial in human diseases pathogenesis and is critical target for control and treatment [3]. Human umbilical vein (HUVEC), bovine brain microvascular and human coronary artery are common sources of primary ECs for *in vitro* model to study endothelial barrier dysfunction. However, primary ECs have very limited life span at an average of 10-serial passages and culture-life to only 5 months [4]. Thereafter, the cells enter senescence when they stop proliferating, form giant multinucleated cells, and die [5]. Primary ECs also lose their primary characteristics and responsiveness to stimuli after the sixth passages [5]. Thus, long-term *in vitro* experiments using primary ECs would be too cost demanding. Epithelium is a layer of epithelial cells (EpC) that lines the inner surfaces of internal organs such as the lumen of digestive tract. EpC regulates trafficking of molecule across epithelium layer, similar to ECs of endothelial layer [6]. The regulation is done by cell-cell junctional proteins that form the tight junction (TJ), adherens junction (AJ) and gap junction (GJ), all are present in endothelial and epithelial layers. The similarities of cell-cell junctional proteins in epithelial layer with endothelial layer suggest the possibility of using EpCs to study the physiology of vascular permeability in place of primary ECs. In addition, unlike primary ECs, many EpCs are established cell lines with longer culture-life and stable response to stimuli. Therefore, this study explores the feasibility of using Vero epithelial cell line as cheaper alternative to primary ECs for *in vitro* model of paracellular permeability. It uses dengue infection as pathological model of dysfunctional paracellular permeability.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Trypsin, Foetal calf serum (FCS) and Eagle's minimum essential medium (EMEM) were purchased from Life Technologies. Penicillin-streptomycin solution was obtained from Life Science. Leibovitz's-15 medium, crystal violet, trypan blue, bovine serum albumin (BSA), and Tryptose Phosphate Broth (TPB) were obtained from Sigma-Aldrich.

2.2. Cell lines and virus

Vero cell-line and dengue virus serotype-2 (DENV-2) strain New Guinea C (NGC) were available from Virology Laboratory, Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia. Vero cells were maintained in EMEM supplemented with 10% FCS, and 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in humidified air containing 5% CO_2. Cells were passaged when 70-80 % confluent, two times a week. DENV-2 was propagated in 90-100% confluent C6/36 cells at RT for 6-8 days until cytophatic effect (CPE) reached 100%. Virus titre was determined using 50% tissue culture infectivity dose (TCID50) by Reed-Muench method.

2.3. Vero paracellular permeability model

Vero cells were grown on 12 mm transwell inserts with 3 µm-pore membrane (Milipore, Ireland Ltd) at 37°C with 5% CO_2 for 24 hours. After incubation, spent medium from upper and lower chamber was removed and replaced with Trypan blue-BSA (T-BSA) medium in the upper chamber and blank medium in the lower chamber. T-BSA medium was prepared as described [7]. The transwell inserts were then returned to 37°C with 5% CO_2 for 60 minutes incubation. After that, medium in the lower chamber was collected into 96-well plate and the OD was measured at 595 nm by microplate reader to determine the amount of T-B that permeated through Vero monolayer. Vero cells monolayer in the transwell inserts was also stained with Trypan blue to determine viability or with crystal violet to determine integrity of monolayer after incubation or treatment. To mimic paracellular permeability in pathological condition, Vero monolayer in transwell inserts were infected with M.O.I. 1 of DENV-2 for 90 minutes, followed by incubation with the presence or absence of TNF-α for three days. Subsequently, permeation of T-BSA across infected Vero cells was tested as described above.

2.4. Statistical analysis

All results were expressed as means ± standard deviation (SD) from three independent experiments. Statistical comparisons between groups were assessed using unpaired Welch's t-test when variance between groups was unequal or Student's t-test when variance between groups was equal. A significant difference was established at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Vero cells responded to inflammatory cytokine with increased paracellular permeation

Increased paracellular permeability of the endothelial layer is one of the hallmarks of inflammatory response. The physiological changes are mediated, among others by TNF-α, a potent cytokine which activates ECs and enhances capillary permeability [8]. In this study, we showed that Vero cells also responded to TNF-α with increased paracellular permeation of BSA. Low concentration of TNF-α (0.2 ng/ml) increased permeability of Vero monolayer by 51.6% ± 4.7% compared to untreated monolayer. The effect becomes more profound with higher concentration of TNF-α (Figure 1A). Crystal violet staining showed no differences between Vero monolayer treated with 0.2 ng/ml TNF-α compared to Vero without treatment (Figure 1B). Similarly, differences in trypan-blue staining of the two were not significant (Figure 1C). This proved permeability increased by TNF-α was not due to structural loss of monolayer such as cell death due to cytotoxic effect.

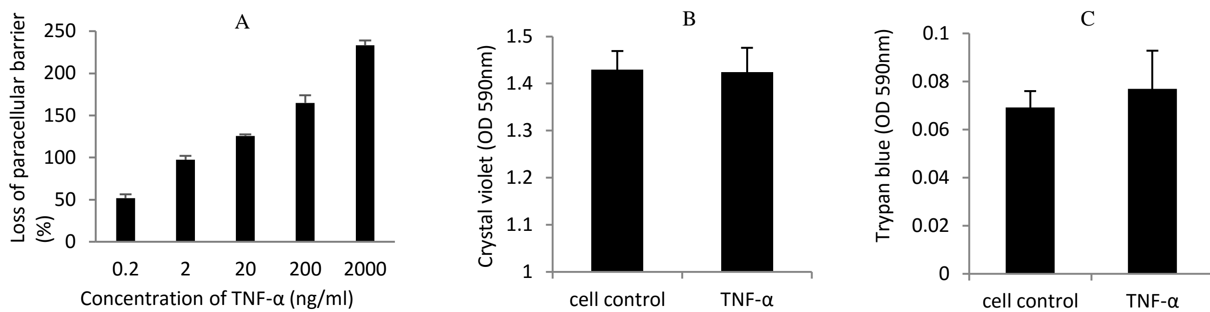

Figure 1 (A) Effect of tumor necrosis factor (TNF-α) on Vero cell monolayer permeability. (B) Determination of cell number of Vero cells treated with 0.2 ng/ml TNF-α by crystal violet. (C) Determination of cell viability of Vero cells treated with 0.2 ng/ml TNF-α by trypan blue (t-test, p>0.05).

3.2 Vero cells mimics endothelial response in pathological condition

One of the common uses of ECs is to model vascular permeability of severe dengue infection. Studies showed that although dengue could replicate in ECs, this by itself was not be enough to weaken endothelial barrier to explain vascular leakage of severe dengue [9]. However, permeability increased when infection is accompanied with TNF-α [10]. This is a corner stone of cytokine storm underlying increased vascular permeability in severe dengue infection. In this study, we showed DENV2 infection alone did not caused Vero monolayer to lose its paracellular control (Figure 2). Instead, permeability

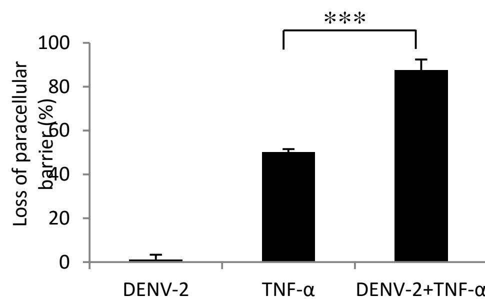

Figure 2 Effect of dengue virus (DENV)-2 and TNF-α (0.2 ng/ml) on Vero cell monolayer permeability. Results are the mean of three independent experiments. Statistical analysis was determined by Student's t-test analysis.

increased when TNF-α was present, 1.7-fold higher with dengue infection than without infection. Altogether, our results showed Vero epithelial cells behaved similarly as ECs in their control of paracellular permeability in response to inflammatory cytokine and to dengue infection. Our meta-data analysis (details in full paper) showed the degree of paracellular permeation in Vero monolayer was not much different from permeation of ECs in respond to immune modulators: TNF-α, IL-1, IL-1β and IFN-γ [11-13]. We also compared variable costs for culturing HUVEC (primary cells) and Vero cell-line. In general, the cost for HUVEC cells, from initial frozen stock was estimated to be between RM 2,103 to RM 2, 230 (estimates based on Cell Application Inc.). Thereafter, the cost may drop to RM 757 for a maximum 13-round of passages (taking into account three passages were used to establish in-house frozen stocks). On the other hand, the cost for Vero cell-line from initial frozen cells is approximately RM 1,974 (estimates based on SIGMA-Aldrich). Thereafter, the recurrent cost could drop to RM411. However, unlike primary cells the recurrent cost is for indefinite number of passages. Thus, taking the advantage of established cell-line, Vero cell-line has better cost-output elasticity than primary HUVEC cells when used to model *in vitro* paracellular permeability.

4. CONCLUSION

This study showed Vero epithelial cells can be physiologically relevant to model *in vitro* paracellular permeability in placed of primary endothelial cells. Work is ongoing to determine signalling pathway and morphological structure involved in paracellular control shared between Vero EpCs and ECs as well as its limit to represent endothelial barrier.

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IMPLEMENTATION OF HALAL ASSURANCE SYSTEM IN BROILER CHICKEN FARMING

Mohd Hasli Ramli[1], Arieff Salleh Rosman[1], Muhammad Talhah Ajmain @ Jima'ain[1]

[1]*Faculty of Social Science and Humanities, Universiti Teknologi Malaysia*
[1]*hasli8685@gmail.com,* [2]*aswar@utm.my,* [3]*mtalhah.uda@gmail.com*

ABSTRACT
Demand for halal product food was a significant in Malaysia and to the world's market due to the integrity of the product and the strong integrity to the product itself which was based on the halal concept. Hence, Department of Islamic Development Malaysia (JAKIM) had made it compulsory for the application of Malaysia halal certificate at industry level to implement Halal Assurance System (HAS). The main purpose and focus to develop this halal system was to upgrade the integrity of halal product that involved from the raw material production up to the end user in the chain. In the application of HAS, the focus was only at the level of receiving of raw material until the end user, instead started from the highest chain of food supply. Therefore, qualitative methodology through document analysis was used in order to obtain the source of information and to explain the element of HAS such as Halal Critical Point in broiler chicken farming activities. This systematic system and halal certification policy will benefit on farming sector and make it able to be the main reference to halal player's integrity because it will give benefit the consumer from the top of the halal food chain.

Keywords: halal concept, halal assurance system, broiler chicken farm, halal critical point

1. INTRODUCTION

In the development of halal Malaysia concept by JAKIM couple of initiative had been released to ensure the integrity of halal in order for food industry to produce food which full fill the halal handling system and concept. Hence, since 2013 Department of Islamic Development Malaysia (JAKIM) had enrolled an initiative to make it compulsory for any application of Halal Certificate related with multinational industry to implement Halal Assurance System in the production of halal product. Apart from that, food industry player also recommended to create this system where it functioned as additional value to the halal management, thus will increase consumer's trust toward halal food product [1].

The main guideline of Halal Assurance System or known as HAS was established by Department of Islamic Development Malaysia (JAKIM) in order to meet the requirement of Halal Standard Certification System and for the system itself to be effective in managing comprehensive halal system starting from the 'From Farm to Fork' concept. Therefore, industrial party had to provide HAS procedure as a guideline to meet the requirement of Halal Certificate and validation of halal integrity. In particular, the main element that has to be developed was the establishment of control center in which each process and steps will be meticulously checked according to HAS concept in halal toyyiban food (HT) production concept [2]; [3]. In order to study how the implementation of HAS and development plan for Halal Critical Control Point (HCCP), this research need to explore on activities of chicken farming to identify critical control point that involved in chicken broiler industry.

Therefore, this paper will discuss and analyze closely on the implementation of principle in food security system along with HAS that had become an important element within food chain and animal breeding and farming industry. This paper adheres to qualitative methodology through document analysis that involved literature material resources such as guidelines and manual which related to management of HAS. Study on this resources gave a clear view and input about the issue and requirement of HAS specifically in broiler chicken industry.

2. LITERATURE REVIEW

2.1. Halal concept and food safety in food industry

From the terminologies of halal (حلال) is an arabic word which means permissible or unforbidden by syara' law [4]. From the language view, halal means permissible and from terminology view, the halal word means those which are not forbidden [5]. From Islamic perspective, it involved the harmony and prosperity concept among community regardless of nation, religion and culture. For muslim, halal food concept is a command from Allah to ensure a healthy body, mind and spirit. On the other hand, for non-muslim halal was understood as safe, pure and healthy product and give advantage to the mankind in food resources [6].

Other than producing food according to halal concept, cleanliness and safety aspect of food also should be emphasized so that is free from any sources that may harm community such as contamination form bacteria, pathogen-carrier disease. In conclusion halal food concept is not only free from any forbidden entity, it will also give good impact to the consumer. Other than that, aspect of cleanliness assurance system and food safety that was implemented today, was based on some guideline application like *Good Hygiene Practice* (GHP), *Hazard Analysis Control Point* (HACCP) and *ISO series* - International Standard Organization. All the system mentioned above are the common quality system used which are complement to each other in halal system of Malaysia [7].

2.2. The need of element in halal assurance system

According to Malaysia Halal Certification System, which is established by JAKIM authorities, had gave good opportunity to those in halal food industry to upgrade or revise current halal certification system so that food product produced become more competent and assured. The guidelines of Halal Certification System (HCS), introduced by JAKIM are still new in Malaysia compared to other countries. Most of those in halal industry gave good commitment in the development of Halal Certification System to assure the halal integrity and reputation in marketing of halal food product [8]. In HCS, it is a documentation mechanism system to control, supervise, improve and prevent any misconduct in halal system or contamination of haram product during processing of food. This system become a catalyse and established by those in food industry in order to supervise product produced was surely halal and safe.

According to [9]; [10] and [11], it is stated that HCS is a managing system and halal documentation that should be implemented by producer for assurance of halal status to the food product produced. The principle of system exists based on this 3 concept which are including zero limit (no haram substance used in production), zero defect (no haram product produced) and zero risk (no risk should be taken by producer or industry involved in halal product aspect). In the context of this research, HCS referred to halal managing system which involved procedure development, documentation and practicality in an organization to ensure the halal farm animal product meet the principal of halal and toyyiban concept in farming sector industry.

2.3. Halal assurance system management in Malaysia

Growth of halal food industry had take place in Malaysia since 1970's and currently advancing along with current food technology. With the rapid growth of food industry along with the development of Halal Food Certification System by Jakim as early as 1974 till now to cover the need in industry and muslim's demand on the halal product. Generally, the competent authorities JAKIM had implement standard guidelines to be used as main and formal reference in Malaysia's Halal Certificate which is MS 1500 be used (Production, preparation, handling and storage - General guidelines)

Halal standard had been used for the first time since 2004 and had been improvised and revised for third time at early 2019 which is MS1500: 2019 [12]. Establishment of halal standard by Department of Standard Malaysia (DSM) are for the main reference to handle and provide halal fodd starting from

receivement of raw material to the ready-product. Since then, authorities had took proactive steps and initiative to develop and improvise current halal certification system by developing halal assurance system as a main condition in applying halal certificate in Malaysia.

It become a turning point to the development of the system in which there are additional of new elements and it become a main requirement for industry player's especially those in multinational stages, small to medium industries to develope this HAS in their company's organisation accordingly. This is one of the positive progress from JAKIM and industry enterpreneur to intensify more about the integrity and quality of halal food. Therefore, to empower and increase exposure to the industry, a lot of other iniciative done such as customer education about the halal system, HAS was implemented in order to increase understanding and application according to halal system [10]; [13].

However in Malaysia, JAKIM and DSM had co-operate together to come out with iniciative and improvement toward Manual Procedure Malaysia Halal Certification (MPHM), Manual System Halal Management (MSHM) which involved with preparation of documentation, the need of HAS and Malaysia halal standard (MS1500:2019). All of the guidelines and manual was reviewed with much details according to industry requirement and they have to meet all the Islamic law based on the understanding of Islamic scholar in Malaysia. If viewed from the development of HAS since it was established in 2011, there are changes occured in the its requirement and implementation. The changes was on the scope of guidelines usage which involved multinational, small to medium industry [14]. This issues can be referred at the schedule below:

Table 1.0: Requirement component suggested SPHM (2019). Quoted from Manual of Malaysia's Halal Handling System 2019 [14]

Category of Small and Micro Industry	Category of Big and Medium Industry
Internal Halal Comittee(IHC)	**Halal Assurance System (HAS)**
1. Halal policy 2. Control of raw material 3. Traceability	1. Halal Policy 2. Appointment of Halal Executive 3. Internal Halal Comitte 4. Halal Risk Control 5. Raw Material Control 6. Internal Halal Audit 7. Training program 8. Traceability 9. Implementation of control measure 10. Laboratory lab analysis 1. Development of product recall procedure 2. Documentation system and management of records

Therefore was that HAS development was not only for application of Malaysia Halal certificate but it also increase compliance and trust among Muslims. Its even important in emphasizing halal integrity and in preventing any potential harm that may accur along the production process of halal product. In the context of this research, eventhough HAS only focus on production of halal food industry, it may give significant impact to the farming sector of halal animal in which it should be monitored from the first food chain. In conclusion, this research shown that a complete HAS should be started from the early stage of halal food supply.

3. CONCLUSION

Halal and haram issues are always a hot topics, even meat issues. Malaysia halal food industry had been fighting for HAS in which it is a necessity and policy in production of halal certificate. From the context of halal food chain, food resources, medication issues and animal welfare also one of the important aspect that should be looked up. Therefore, application of effective HAS may help industry players

especially in farming industry and at the same time it maintain the credibility as it benefits all in the community starting from early stages in halal food chain. Furthermore, it increase awareness among community especially consumer to aware that the source of halal food starting right from the farm. This reserach also recommend that HAS to be applied to chicken broiler breeding sector including other halal animal such as ruminant animals.

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SYNERGISTIC EFFECT OF GRAPHENE OXIDE AND ZINC OXIDE NANOCOMPOSITE ON ANTIMICROBIAL ACTIVITY

Wen Ching Liew[1], Ida Idayu Muhamad*[1,2]

[1] *Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.*
wenching94@hotmail.com

[2] *IJN-UTM Cardiovascular Engineering Centre, V01 Universiti Teknologi Malaysia,81310 Skudai, Johor, Malaysia.*
idaidayu@utm.my

ABSTRACT

Engineering of multiple nanoparticles as single nanomaterial is currently in demand for electrical, medical, energy and environmental applications due to their promising synergistic effects. One of the approaches is the combination of graphene oxide (GO) nanosheet and zinc oxide (ZnO) nanoparticle as nanocomposite for their fascinating nanostructures. Previous studies have addressed their remarkable synergistic effects on mechanical, thermal and antimicrobial properties of composites, however, the relationship between GO and ZnO concentration on antimicrobial activity is in the lack of knowledge. Therefore, this study aims to investigate the effect of different GO and ZnO concentrations on the synergistic antimicrobial activity of GO/ZnO nanocomposite. The GO nanosheets are firstly synthesized from graphite using modified Hummer's method, then added into ZnO nanoparticle suspension to form GO/ZnO nanocomposite via ultrasonication. The concentrations of GO/ZnO are varied at 1:4, 2:3, 3:2, 4:1 wt% while neat GO nanosheet as control. The chemical properties of synthesized GO/ZnO nanocomposites are examined with FTIR. Results revealed that no remarkable change of peaks in GO/ZnO nanocomposite, implying the addition of ZnO does not affect the functionally of GO. Meanwhile, their synergistic antimicrobial activities are demonstrated on common spoilage microorganisms i.e. *Bacillus subtilis* and *Escherichia coli*. All ratios inhibit the microbial activity effectively and the optimum ratio for GO/ZnO concentration is found at 2:3 wt%. In conclusion, the potential GO/ZnO nanocomposite as superior antimicrobial agent could be designed and utilized in food packaging, pharmaceutical and biomedical applications.

Keywords: Graphene oxide nanosheet; zinc oxide nanoparticle; synergistic antimicrobial activity; inhibition zone; optimum ratio

1. INTRODUCTION

Nanomaterials as reinforcement particles have gained great interest and continue to develop because of their attractive intrinsic properties: as nanoscale dimensions, unique morphology, high surface area, mechanical strength and low density. Its market had been valued at $14,741.6 million in 2015 and expected to reach $55,016 million by 2022 with a CAGR of 20.7% [1]. Recently, graphene oxide (GO) nanosheet has been emerged as ideal nanofillers for its tremendous potential in improving mechanical, thermo-mechanical, thermal conductivity and gas barrier properties in order to produce high-performance polymer composites. GO is a single monomolecular layer of graphite with a large number of oxygen-containing functional groups include carbonyl, carboxyl, hydroxyl and epoxy. These functional groups make GO attractive to interact with many polar organic molecules and polymers, enduring it a chemical tunable fascinating platform [2]. It is also a strongly hydrophilic material that can be dispersed well in water to form stable and homogeneous GO suspension, allowing various graphene macrostructures to assemble easily thereby providing other tunable entrancing properties [3]. Instead of using single nanoparticle for desirable purposes, it is found that combination of two different nanoparticles could simultaneously improve the performance of nanocomposites due to their synergistic effects. One of the approaches is the combination of GO and zinc oxide (ZnO) [4]. Zinc oxide is a non-toxic inorganic material that has great antimicrobial activity. It is recognized as one of the five zinc compounds that listed as GRAS in food industry by United States FDA [5]. Its high biocompatibility of ZnO nanoparticle also makes it applicable in various productions [6]. GO/ZnO nanocomposite is reported has remarkable synergistic antimicrobial effect towards *Escherichia coli* and *Staphylococcus*

aureus [4], however, the contribution of individual nanomaterial towards the synergistic antimicrobial effect is in the lack of knowledge. Therefore, this study aims to investigate the effect of different GO and ZnO nanocomposite concentrations on the synergistic antimicrobial effect.

2. MATERIALS AND METHODS

2.1 Materials

All the materials were purchased as follow: Graphite (HmbG Chemicals, Germany), Hydrochloric acid (Merck, US), Sulphuric acid (J.T. Baker, Thailand), Potassium permanganate (Fisher Scientific, US), Hydrogen peroxide (Systerm Chemicals, Malaysia), Zinc oxide suspension (Sigma-Aldrich, US).

2.2 Fabrication of GO/ZnO nanocomposite

Graphite powder was used as precursor to synthesize graphene oxide according to modified Hummer's method [7]. GO powder obtained was then used to fabricate GO/ZnO nanocomposite with modification method [8]. Firstly, prepared GO (0.1g) was added into ZnO nanosuspension (0.4g of ZnO) and ultrasonicated for 1.5 hour to get uniform suspension. The dissolved GO/ZnO solution was centrifuged and washed with distilled water repeatedly to obtain pH 7. Finally, the GO/ZnO nanocomposite was recovered by drying in oven for future use. Sample with GO/ZnO concentration ratio of 1:4wt% was name as GO1/ZnO4. Ratios were then varied at 2:3, 3:2, 4:1wt% and name accordingly to examine their synergistic relationship. Neat GO nanosheet was used as control.

2.3 Characterization of GO/ZnO nanocomposite using FTIR

Structural changes of GO/ZnO nanocomposites were observed through Fourier-Transform Infrared (FTIR) spectra. FTIR analysis will be performed with Perkin-Elmer Spectrum One FT-IR Spectrometer [9]. The resolution of 4/cm in the range of 4000 to 400/cm will be used and averaged over 16 scans. Only three ratios of GO/ZnO nanocomposite concentration were selected, which were neat GO, GO1/ZnO4 and GO4/ZnO1. This was for the reason that large ratio difference between GO and ZnO concentration would have more remarkable change in structural compared to comparable ratios (GO2/ZnO3 and GO3/ZnO2)

2.4 Antimicrobial activity assay

Antimicrobial activity of GO/ZnO nanocomposite at different ratios were studied on Gram negative (*Escherichia. coli*) and Gram positive (*Bacillus subtilis*) bacteria using disc diffusion method [10]. Spread plates were prepared by sampling 1mL of E. *coli* and *Bacillus subtilis* culture separately. Whatman filter paper was cut into approximately 1.4cm in diameter to be used as disc. Discs were placed in petri dish and autoclaved at 121°C for 15mins to sterilize them before use. Different ratios of GO/ZnO nanocomposites were dissolving in distilled water to get final concentrations of 50mg/mL. These suspensions were then ultrasonicated for 1.5 hour to homogenize them. Subsequently, single disc was taken and dipped into the ultrasonicated solution then placed at the centre of agar plate. All plates were incubated at 37°C for 24 hours to examine their inhibition zones which represent the antibacterial activity of GO/ZnO nanocomposites.

3. RESULTS AND DISCUSSION

3.1 Characterization of GO/ZnO nanocomposite

FTIR spectra of all three samples as in
Figure ***1*** presented the main absorbance regions in the range of 1000- 3600cm^{-1}. Overall, their absorbance peaks are similar indicating they are exhibiting the same chemical composition. Peak near 1210cm^{-1} represented the C-O vibrations of graphitic domains whereas 1060cm^{-1} indicated the existence of C-OH groups. Furthermore, the C=C groups and C=O groups peaked at 1568cm^{-1} and around 1750cm^{-1} respectively. GO nanosheet also peaked around 2667cm^{-1} and 3200cm^{-1} corresponded to the O-H

stretching that comes from carboxylic acid. In overall, the presence of C-O, C=O and O-H groups have verified the oxidation of graphene into GO [10]. No significant changes of peaks in GO/ZnO nanocomposite spectra implies that addition of ZnO nanoparticle would not affect the chemical structural of GO nanosheet. Therefore, GO/ZnO nanocomposite could work well as a promising nanocomposite.

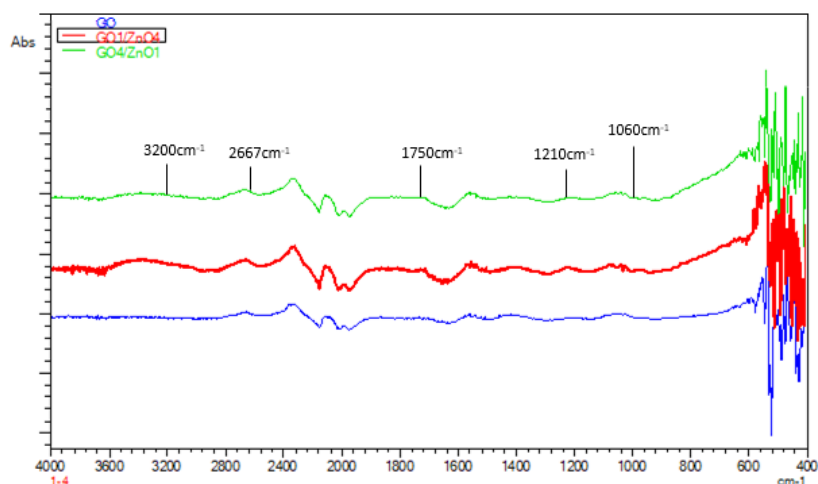

Figure 1 FTIR spectra of GO, GO1/ZnO4 and GO4/ZnO1 samples

3.2 Synergistic antimicrobial activity of GO/ZnO nanocomposite

The antimicrobial activity of GO/ZnO nanocomposite was demonstrated by the zone of inhibition as shown in Table 1. Results revealed that it inhibited *Bacillus subtilis* more effectively than *Escherichia coli*. By comparing the across different GO/ZnO nanocomposite concentration against *Bacillus subtilis*, it was found that GO2/ZnO3 had the largest inhibitory zone. Meanwhile, antimicrobial testing against *Escherichia coli* showed that GO1/ZnO4 exhibited largest inhibitory zone, followed by GO2/ZnO3. The synergistic antimicrobial activity of GO/ZnO nanocomposite was again proven in this study. Results also showed that the antimicrobial property was more significant when the concentration of ZnO increases, ie. decrease of GO concentration. This implied that ZnO nanoparticles have greater antimicrobial property than GO nanosheet and dominated the synergistic antimicrobial effect of GO/ZnO nanocomposite. The optimum ratio of GO/ZnO nanocomposite concentration for great synergistic antimicrobial activity was 2:3 wt%. Although the antimicrobial mechanism of metal oxide nanomaterials has not been fully explored, the most likely mechanism proposed is the attack of cellular membranes then enter to disrupt microbial mechanism. In detailed, special two-dimension of GO nanosheet interacts with the bacterial lipid bilayer thus attracts those lipid molecules to attach on it instead of binding with bacterial membrane. Bacterial membrane is then destroyed without the support from lipid molecules. For ZnO nanoparticles, zinc ions (Zn^{2+}) released binds with the negative charges on bacterial membrane owing to the Coulomb forces of attraction [11]. This phenomenon solidifies the protein on membrane as well as destroys the synthetase. As a result, bacteria cannot proliferate as usual. Synergistic antimicrobial activity is also possibly performed when electrons transferring rapidly between GO and ZnO nanocomposite, thereby absorbing surface oxygen to form various reactive oxygen species. This leads to the formation of lipid peroxide and destroys the bacterial membrane ultimately [8].

Table 1 The mean diameter of inhibitory zone of GO/ZnO nanocomposite at different concentration against *Bacillus subtilis* and *Escherichia coli*

Composition	**Zone of inhibition (cm)**	
	Bacillus subtilis	***Escherichia coli***
GO	1.638 ± 0.096	1.477 ± 0.021

Composition	Zone of inhibition (cm)	
	Bacillus subtilis	***Escherichia coli***
GO4/ZnO1	1.700 ± 0.150	1.773 ± 0.079
GO3/ZnO2	1.845 ± 0.050	1.635 ± 0.015
GO2/ZnO3	1.853 ± 0.052	1.833 ± 0.047
GO1/ZnO4	1.800 ± 0.082	1.850 ± 0.050

4. CONCLUSION

GO/ZnO nanocomposite could be fabricated successfully and function well as addition of ZnO nanocomposite did not affect the composition of GO nanosheet. It could inhibit both *Escherichia coli* and *Bacillus subtilis* effectively but more significant synergistic antimicrobial activity observed when the concentration of ZnO increased. This novel GO/ZnO nanocomposite is potentially designed as superior antimicrobial agent by manipulating the GO/ZnO concentration at its optimum ratio of 2:3 wt%. Further study on its toxicology is highly encouraged to provide a comprehensive understanding on its safety use before exploitation.

ACKNOWLEDGMENTS

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ENUMERATION OF VIBRIO SPECIES FROM THREE COMMERCIAL AQUACULTURE SHRIMP FARMS IN SARAWAK

Diyana, Z.[1], Nillian, E.[1*], Grace, B.[1], Amirah, Z.[1]

[1]*Faculty of Resource Science and Technology, Universiti Malaysia Sarawak,*
94300, Kota Samarahan, Sarawak, Malaysia
18020052@siswa.unimas.my

ABSTRACT

Bacteria are among the groups of microorganisms giving the negative impacts on global shrimp industries. Vibrio genus, including *V. cholarae, V. paraheamolyticus, V. mimicus, V. harveyi, V. alginolyticus*, and *V. vulnificus* has been described as pathogenic species in shrimp production environment. These pathogens cause serious infections and decreased the amount of production. This study was conducted to enumerate the *Vibrio* spp in three different commercial shrimp farm in Sarawak which practiced different pond management systems. Method for *Vibrio* spp isolation and quantification were standardized for all three locations in order to compare the distribution of the *Vibrio* spp in the farm for one cycle of production. Two ponds were selected from each farm. Water and sediment samples were collected from the inlet and outlet of the ponds for every two weeks within one cycle of production starting from stocking until the harvesting period. Samples dilution were performed up until 10^{-5} using APW. Based on 32 water samples and 32 sediment samples collected from each location, total plate count per cfu/ml of water and total plate count per cfu/g of sediments on TCBS agar were quantified. Throughout the sampling period, the pH of water ranged from 5.95 to 8.13, salinity ranged from 14.0 to 34.5 psu, and temperature ranged from 28.00°C to 32.85°C. This study provides the data on quantification of *Vibrio* spp from three different commercial shrimp farm with different pond management systems, hence highlighting the importance of preliminary steps to find effective and environmentally friendly approaches to overcome infections caused by *Vibrio* spp hence improving shrimp health and yields.

Keywords: Aquaculture practice, Commercial shrimp farming, Microbial, *Vibrio* spp

1. INTRODUCTION

In the early 1970s, modern shrimp farming, marine shrimp cultivation in impoundments, ponds, raceways, and tanks, have been started, and today there are over fifty countries involved in shrimp farming industries. Thailand, Vietnam, Indonesia, India, and China are the rulers in the Eastern Hemisphere, and there are big sectors in Malaysia, Taiwan, Bangladesh, Sri Lanka, the Philippines, Australia, and Myanmar (Burma). Commercial shrimp aquaculture in many countries including Malaysia has been prompted due to increasing demand of international market and decreasing volume of shrimp catch [1].

However, the shrimp farming industry is constantly under threat due to the outbreak of infectious diseases and environmental problems. Bacteria are among the groups of microorganisms giving the negative impacts on global shrimp industries. Vibrio genus, including *V. cholarae, V. paraheamolyticus, V. mimicus, V. harveyi, V. alginolyticus,* and *V. vulnificus* has been described as pathogenic species in shrimp production environment [2]. These pathogens cause serious infections and decreased the amount of production.

Disease transmission via water, sediments, or as a result of ingestion of infective material, although there is some evidence that wounds can also provide a means of entry and occur rapidly. The infections caused by *Vibrio* species is referred as vibriosis. Vibriosis can be detected due to several symptoms such as black shell disease, tail rot, septic hepatopancreatic necrosis, brown gill disease, swollen hind gut syndrome, and luminous bacterial disease. Those infections have several number of clinical signs such as lethargy, loss of appetite, luminescence, yellowing of the gill tissue, and red discoloration of the body [3].

Since the environmental condition such as poor water quality and pond bottom management system are major factors associated with *Vibrio* diversity of pond culture systems, therefore monitoring the bacterial count for *Vibrio* species is important in order to prevent any vibriosis infections to occur [4]. The aim of this this study is to enumerate the *Vibrio* spp in water and sediment samples from three different commercial shrimp farm in Sarawak starting from 1st Day of culture (DOC) until 100th DOC.

2. MATERIALS AND METHODS

2.1 Collection of water and sediment samples

Water and sediment samples were collected from the inlet and outlet of two selected ponds from three commercial shrimp farm (Location 1: Telaga Air, Location 2: Santubong, Location 3: Tanjung Manis) for every two weeks starting from 1st DOC until 100th DOC.

2.2 Water quality measurement (pH, Temperature, and Salinity)

Water quality measurement for pH, Temperature (°C), and Salinity (psu) were recorded immediately at the sampling site for every water samples collection.

2.3 Enumeration of *Vibrio* spp.

Vibrio spp was enumerated by using the spread plate method. Serial dilutions of the samples were performed using Alkaline Peptone Water (APW). The samples were diluted from 10^{-1} to 10^{-5}. 0.1 ml of the diluted sample was spread on a Thiosulfate-citrate-bile salts-sucrose agar (TCBS). The plates after inoculation were incubated in an inverted position at 37°C for 20 - 24 hours. After incubation, colonies on TCBS were counted and recorded [5].

2.4 Data Analysis

Based on the total plate count on the TCBS agar, colony formation unit per ml in water and per gram in sediment samples were calculated using the following formula:

Cfu/ml or Cfu/g = (Number of colonies X Dilution factor) / Volume of culture

3. RESULTS AND DISCUSSION

A total of 32 samples of water and 32 samples of sediment collected for each location throughout the sampling duration for every two weeks starting from 1st DOC until 100th DOC. Figure 1-6 provide the information on the enumeration of *Vibrio* spp. by the total plate count on TCBS agar from dilution 10^{-2}.

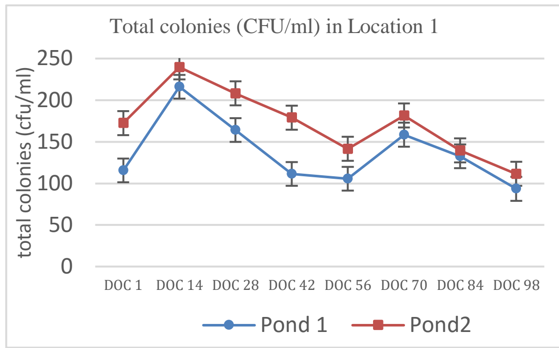

Figure 1 : Graph showing total colonies in water samples (CFU/ml) of Pond 1 and Pond 2 in Location 1

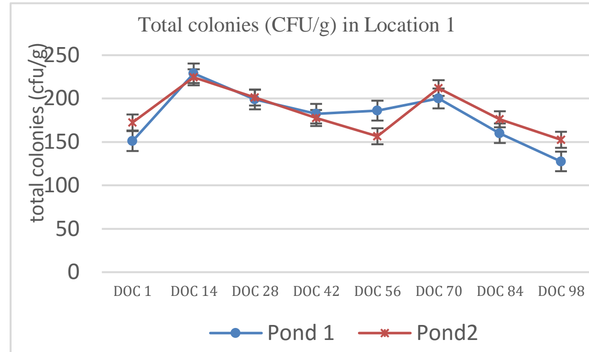

Figure 2: Graph showing total colonies in sediment samples (CFU/g) of Pond 1 and Pond 2 in Location 1

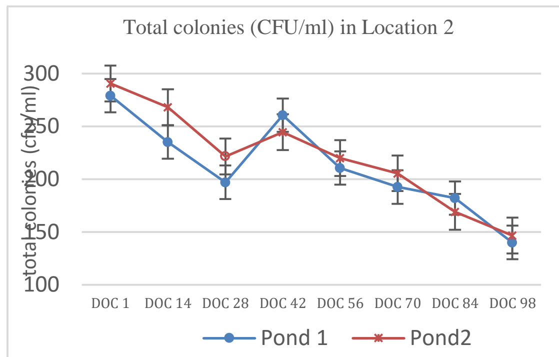

Figure 3: Graph showing total colonies in water samples (CFU/ml) of Pond 1 and Pond 2 in Location 2

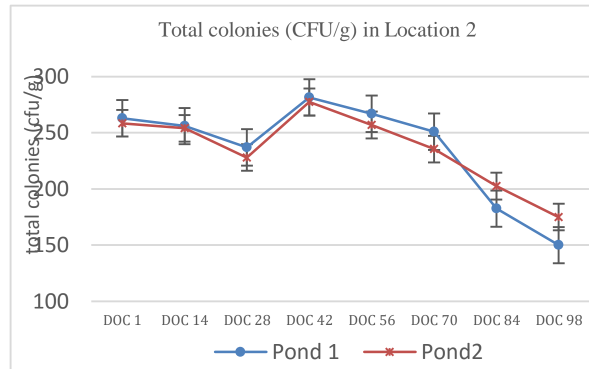

Figure 4 : Graph showing total colonies in sedimen samples (CFU/g) of Pond 1 and Pond 2 in Location 2

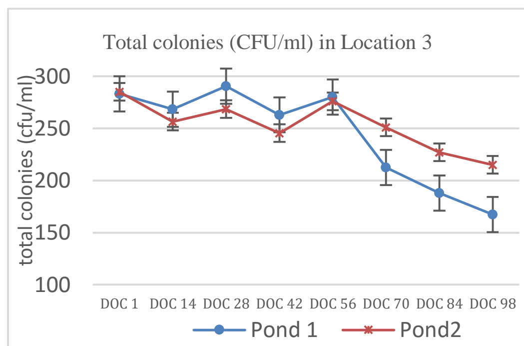

Figure 5 : Graph showing total colonies in water samples (CFU/ml) of Pond 1 and Pond 2 in Location 3

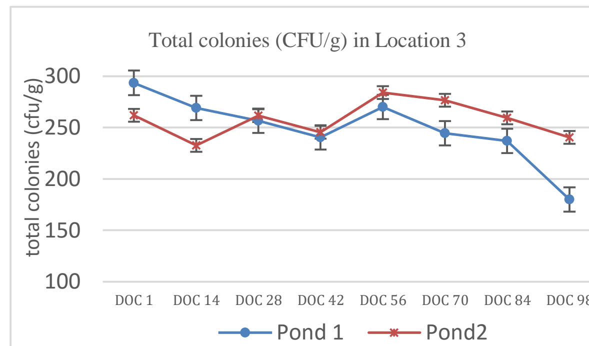

Figure 6 : Graph showing total colonies in sedimen samples (CFU/g) of Pond 1 and Pond 2 in Location 3

The enumeration was done using spread plate method on thiosulfate-citrate-bile salts-sucrose (TCBS) agar, a highly selective agar that commonly used to isolate *Vibrio* spp. that meets the nutritional requirements of the selected species. After 24h incubation at 37°C, sucrose fermenters bacteria will produce yellow colonies on the media, whereas other *Vibrio* spp which are non-sucrose fermenters will produce green colonies on the media [6].

Throughout the observation, the highest population of *Vibrio* spp in water and sediment samples at Location 1 was obtained on DOC 14. Based on water quality parameters recorded, pond 2 have greater value of temperature and salinity compared to pond 1, hence reflecting the population of *Vibrio* species on DOC 14.

However, the highest population of *Vibrio* spp in water samples for Location 2 and Location 3 shown on DOC 1. Based on the water quality parameters recorded, the range for temperature and salinity on DOC 1 for both Location 1 and Location 2 are high. For sediment samples, Location 2 have highest distribution of *Vibrio* spp on DOC 42 whereas Location 3 have the highest distribution of *Vibrio* spp on DOC 56.

4. CONCLUSION

Bacteria count in water and sediments are several factors that contributes to the disease transmission. Therefore, monitoring on pathogenic bacteria in aquaculture industries are important to increase the production thus eliminating negative impact on the overall financial efficiency of the business. With the reference to the findings of this project, long term monitoring and screening programmes are required to control the distribution of pathogenic *Vibrio* spp.

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DETECTION OF CHOLERA TOXIN (CTXA AND CTXAB) GENES IN VIBRIO CHOLERAE ISOLATED FROM CLINICAL AND ENVIRONMENTAL SAMPLES IN LIMBANG SARAWAK BY MULTIPLEX POLYMERASE CHAIN REACTION (PCR)

Amirah, Z. J. [1], Elexson, N. [1]*, Grace, B. [1], Diyana, Z. [1]

[1] *Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia.*
nelexson@frst.unimas.my

ABSTRACT

Cholera epidemics have been occurred in Malaysia since 1991 till 2003 which can be proved from the records by the Infectious Diseases Division of the Ministry of Health. Moreover, there were also course of cholera epidemics from the year 1994 to 2003 had been described in Sarawak. Cholera outbreaks in Malaysia mostly occur caused by the El Tor O1 *Vibrio cholerae* serogroup. Previous research has been focusing on the presence of virulence-associated factors in *V. cholerae* populations include cholera toxin (*ctx*A).The aims of this study were to detect the presence of *Vibrio cholerae* in clinical and environmental samples (n=28) from Limbang Sarawak and to detect the toxin genes from the isolates. Two types of PCR were used in this study, 16s rRNA and multiplex PCR. The results obtained from the study showed 16 out of 28 (57.14%) samples were confirmed to be *Vibrio cholerae* species. Four primers specific for *Vibrio cholerae* were used in multiplex PCR (O1 type, O139 type, *ctx*A and *ctx*AB) to confirm the species type and the toxin genes. All samples shown positive for *Vibrio cholerae* O1 type. For the detection of *ctx*A and *ctx*AB genes, all 16 isolates were positive to carry both genes. From this study, it showed that PCR can be used for research purposes in molecular genetics field.

Keywords: *Vibrio cholerae*, O1 serogroup, *ctx*AB genes, polymerase chain reaction (PCR)

1. INTRODUCTION

Vibrio cholerae is a Gram-negative bacteria with non-spore rods that caused cholera disease. Vibrio species which found in marine environment most likely to prefer conditions such as salty, warm and alkaline. There are more than 200 serogroups of *Vibrio cholerae* that successfully identified, but two biotypes of *Vibrio cholerae* serogroup O1 are mostly pathogenic which are the “classical” and the El Tor [1]. Each O1 biotype has three serotypes which are Ogawa, Inaba and Hikojima. The most prevalent causative agents of cholera epidemics are serogroups O1 and O139 [2]. According to [3], cholera outbreaks in Malaysia mostly occur caused by the El Tor 01 *V. cholerae* serogroup. Cholera epidemics have been occurred in Malaysia since 1991 till 2003 (Infectious Diseases Division of the Ministry of Health) and had been described in Sarawak from the year 1994 to 2003. Lack of proper treated water supply and poor sanitation could lead and facilitated the spread of the diseases in Sarawak especially in rural area which confronted by the toxigenic *Vibrio cholerae* [4]. Moreover, [5] in their research has been focusing on the presence of virulence-associated factors in *V. cholerae* include cholera toxin (*ctx*A). The polymerase chain reaction (PCR) is experimental method that can be used to detect the *V. cholerae* regulator and toxin genes [3]. PCR is a proper method for detecting small amounts of microbial DNA especially the direct PCR amplification and sequencing of bacterial genes that encode the small subunit rRNA. The rRNA (rrn) operon's genetic information offers useful taxonomic data [6] and essential in high copy numbers [7]. Multiplex PCR detecting multiple pathogens simultaneously in a single tube reaction has the potential to save time and effort, reducing the laboratory costs associated with testing [8]. PCR is an extremely particular, fast detection and delicate technique for detecting *Vibrio cholerae* toxigenic strains [9]. Therefore, the aims of this study are to identify bacterial species through molecular approach by 16s rRNA PCR and to detect the toxin genes of the bacteria (*V. cholerae*) using multiplex PCR.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

A total of twenty-eight (n = 28) bacterial strains from Limbang, Sarawak in collaboration with Sarawak Government Hospital (SGH) were used in this study. There were four different samples which consist of rectal swab, Moore swab, water sample, and stool. All strains were isolated from all of the samples [10].

2.2 DNA Extraction for PCR Amplification

The boiling-cell method was used to extract DNA in this study according to the method by [11] with modifications.

2.3 Amplification of 16s rRNA PCR

The PCR amplification of 16s rRNA PCR was carried out using two universal primers as represented in Table 1. According to the method by [11], the cycling conditions used to amplify DNA were as followed: 95 °C for 10 mins; 26 cycles of 94 °C for 30 secs, 55 °C for 1 min, 72 °C for 1 min 30 secs; and 72 °C for 10 mins. Each PCR reaction was optimized and performed in 30 µl reaction mixture consists of exTEN 2x PCR Mastermix (Base Asia), 10 µM of each primer, sterile ddH_2O, and DNA template.

2.4 Amplification of Multiplex PCR

Four primers were used to perform multiplex PCR amplification (Table 1). According to the protocol by [12], amplification was proceeded with cycling conditions as followed: 94 °C for 5 mins; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; and 72 °C for 7 mins. A total of 30 µl reaction mixture consists of exTEN 2x PCR Mastermix (Base Asia), 10 µM of each primer, sterile ddH_2O, and DNA template was performed in PCR reaction for multiplex PCR.

Table 1. Primers used in the molecular study of *Vibrio cholerae* strains isolated in Limbang, Sarawak.

Techniques	Primers	Primer Sequences (5'-3')	Amplicons (bp)	References
16s rRNA PCR	27F 1492R	F: AGAGTTTGATCMTGGCTCAG R: TACGGYTACCTTGTTACGACTT	1500	[13]
Multiplex PCR	O139F2 O139R2	F: AGCCTCTTTATTACGGGTGG R: GTCAAACCCGATCGTAAAGG	449	[12]
	O1F2-1 O1R2-2	F: GTTTCACTGAACAGATGGG R: GGTCATCTGTAAGTACAAC	192	
	VCT1 VCT2	F: ACAGAGTGAGTACTTTGACC R: ATACCATCCATATATTTGGGAG	308	
	CTXAB-F CTXAB-R	F: GCCGGGTTGTGGGAATGCTCCAAG R: GCCATACTAATTGCGGCAATCGCATG	536	[14]

3. RESULTS AND DISCUSSION

In this study, a total of 28 clinical and environmental samples of *Vibrio cholerae* from the outbreak in Limbang, Sarawak were isolated as represented by Sarawak Government Hospital (SGH). However, there were only 16 out of 28 samples survived (57.14%) and obtained positive results for identification and detection of *Vibrio cholerae* (Table 2).

Table 2. *Vibrio cholerae* isolates from Limbang, Sarawak positive for 16s and Multiplex PCR.

Samples	16s rRNA PCR	Multiplex PCR			
		O139-*rfb*	O1-*rfb*	*ctx*A	*ctx*AB
Hiryana (Rectal swab)	-	-	-	-	-
Zainia (Stool)	+	-	+	+	+
Abdul (Rectal swab)	+	-	+	+	+
Masdinah (Rectal swab)	-	-	-	-	-
Mohd Shahiwan (Stool)	-	-	-	-	-
Ismail (Rectal swab)	-	-	-	-	-
Nur Suhadah (Rectal swab)	+	-	+	+	+
Fadillah (Stool)	+	-	+	+	+
SA001 (Water sample 1)	+	-	+	+	+
SA002 (Water sample 2)	+	-	+	+	+
SA004 (Water sample 4)	+	-	+	+	+
MS001 (Moore swab 1)	-	-	-	-	-
MS002 (Moore swab 2)	+	-	+	+	+
MS003 (Moore swab 3)	+	-	+	+	+
MS004 (Moore swab 4)	-	-	-	-	-
MS005 (Moore swab 5)	-	-	-	-	-
MS006 (Moore swab 6)	-	-	-	-	-
MS007 (Moore swab 7)	-	-	-	-	-
NA1 (Rectal swab)	+	-	+	+	+
NA3 (Rectal swab)	+	-	+	+	+
NA4 (Rectal swab)	+	-	+	+	+
NA6 (Rectal swab)	+	-	+	+	+
NA7 (Rectal swab)	+	-	+	+	+
NA24 (Rectal swab)	+	-	+	+	+
NA25 (Rectal swab)	-	-	-	-	-
NA26 (Rectal swab)	+	-	+	+	+
NA28 (Rectal swab)	-	-	-	-	-
NA39 (Rectal swab)	-	-	-	-	-

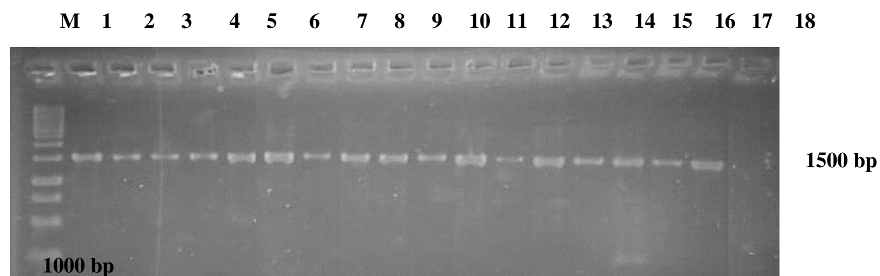

Figure 1. 16s rRNA PCR profile of 16 strains of *Vibrio cholerae* isolates obtained using universal primers 27F and 1492R. Lane M = 1 kB DNA ladder; Lane 1: Control; Lane 2 – 17 = Nur Suhadah, Fadillah, SA001, SA002, NA1, NA3, NA4, NA6, NA7, NA24, NA26, Abdul, Zainia, SA004, MS002, MS003; Lane 18 = Negative control

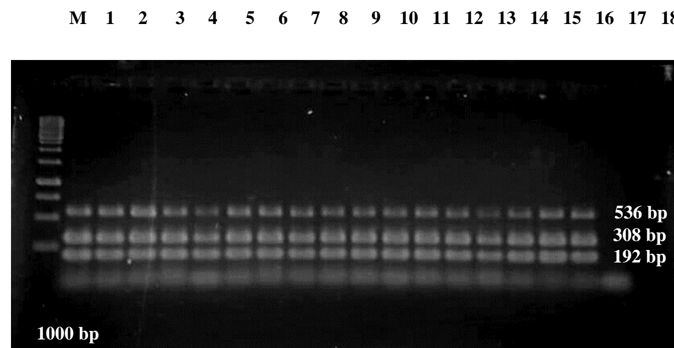

Figure 2. Multiplex PCR profile of 16 strains of *Vibrio cholerae* isolates obtained using *Vibrio cholerae* specific primers, O1-*rfb* (192 bp), *ctx*A (308 bp) and *ctx*AB (536 bp). Lane M = 1 kB DNA ladder; Lane 1 = Positive control; Lane 2 -17 = Nur Suhadah, Fadillah, SA001, SA002, NA1, NA3, NA4, NA6, NA7, NA24, NA26, Abdul, Zainia, SA004, MS002, MS003; Lane 18 = Negative control

4. CONCLUSION

From this research, the bacterial isolates were successfully identified and the toxin genes of *Vibrio cholerae* have also been detected. Molecular characterization of bacterial strains provides useful information about the ecology of the specific bacteria. Therefore, phenotyping or fingerprinting techniques can be used to further characterize the *Vibrio cholerae* strains such as RAPD-PCR and ERIC-PCR.

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THE EFFECTS OF ENZYMATIC TREATMENT ON THE PHYTOCHEMICAL CONTENT AND ANTIOXIDANT PROPERTIES OF MESOCARP OF *Borassus flabellifer*

Rodiah M. H.[1,2], Jamilah B.[1*], Kharidah. M.[1], Russly A. R.[1]

[1]*Universiti Putra Malaysia, Faculty of Food Science and Technology, 43400 Serdang, Selangor, Malaysia*
jamilah@upm.edu.my

[2]*Universiti Selangor, Faculty of Engineering and Life Sciences, Department of Science and Biotechnology, Bestari Jaya Campus, Jalan Timur Tambahan, 45600 Bestari Jaya, Selangor, Malaysia.*
rodiah@unisel.edu.my

ABSTRACT
The mesocarp of *Borassus flabellifer* has been proven to have good potential as a food ingredient; however, the presence of bitterness in it is a deterrent to its acceptable application. Enzymatic treatment using naringinase was carried out to remove the strong bitter taste and the effects of naringinase on the phytochemical content and antioxidant properties of the mesocarp were investigated. The enzymatic treatment using naringinase was found to preserve the bioactive compounds such as saponin, phenol and tannin. Naringinase treatment also led to the marked increases in total phenolic content and free radical scavenging activity. Besides, the ESI-MS base peak chromatogram confirmed that naringinase enables to remove the bitter compounds such as Flabelliferin C, Borassoside F and part of Flabelliferin II. This investigation proved that enzymatic treatment using naringinase has improved the bioactive compounds and antioxidant activities of debittered mesocarp.

Keywords: DPPH; FRAP; Naringinase; palmyrah palm

1. INTRODUCTION

The Palm tree of palmyrah palm (*Borassus flabellifer*) is widely grown in India, Thailand, Bangladesh, Sri Lanka and Malaysia [1]. Beverage and food products can be prepared commercially from the palmyrah fruit pulp and the demand for the food products is remarkably high but some consumers despise the bitterness of the fruit pulp. Hence, the more significant part of the annual production of palmyrah fruits is either to be utilized as animal feed or discarded into the environment [2]. In recent years, naringinase enzyme is extensively utilized in debittering of palmyrah jams, cordial and citrus juice industries [3]. A study has reported that the decrease in bitterness is due to the enzymatic process which improves the quality and commercial value of lemon fruit and other juices. Consequently, enhance the maintenance of healthy properties and the consumer's acceptance [4].

Skin from immature *Borassus flabellifer* (*B. flabellifer*) mainly the mesocarp contained a considerable number of bioactive compounds (tannin, saponin, and phenol), dietary fibre, and antioxidant properties [5] which give promises for the utilization of this by-product as a food ingredient. However, the presence of bitterness in it is also a deterrent to its acceptable application. The previous study proved that the bitterness associated with the mesocarp could be significantly reduced using naringinase (unpublish). Therefore, in this approach, the effect of naringinase treatment on the phytochemical and antioxidant activities of mesocarp of *Borassus flabellifer* was evaluated.

2. MATERIALS AND METHODS

2.1 Plant materials and enzymatic treatment

The mesocarp powder was prepared as the previous study [5]. Mesocarp was treated using naringinase enzyme according to the optimum condition (enzyme concentration: 2.0 g/L, time: 5 h, pH: 5.0 and temperature: 55 °C) of the previous study (unpublished data). The treated mesocarp was recognized as the debittered mesocarp (DM) and mesocarp without any treatment as control.

2.3 Preparation of crude extract and HPLC-ESI-MS/MS

Extraction of bitterness compounds from the mesocarp was adopted from Ariyasena et al. [1] as well as Wickramasekara and Jansz [6]. The identification of bitterness compounds was performed using the high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS/MS) [7].

2.4 Determination of phytochemical

The bioactive compounds were analysed for alkaloids, saponins, flavonoids, steroid, cardiac glycosides, tannins, terpenoids, and anthraquinone according to the standard procedures [8].

2.5 Determination of radical scavenging assay (DPPH) and ferric reducing antioxidant power (FRAP)

The antioxidant capacity of the samples was estimated by the free radical scavenging capacity [9] and the Ferric reducing antioxidant power [10].

3. RESULTS AND DISCUSSION

3.1 Peak identification and assignment

The ESI-MS base peak chromatogram (data not shown) from the control sample shows a complex mixture mainly peaks of steroidal saponins (Table 1). Four types of steroidal saponins; flabelliferin I, flabelliferin II, flabelliferin B and Borassoside F were contained in the control sample. DM showed only two steroidal saponins (Table 2) after the enzymatic treatment which was characterized as Flabelliferin, FII isomer and hydroxylated Flabelliferin B. The data obtained confirmed that naringinase enables to remove the bitter compound.

Table 1. Mass spectrometric data and identification of bitterness compounds in the mesocarp (Control) of *B. flabellifer*

Peak No	t_R	[M]+ (*m/z*)	MS/MS (*m/z*)	Compound
1	3.49	869.04	-	Flabelliferin C (F_C)
2	3.87	1031.04	869.31, 725.28	Flabelliferin II (F II)
3	4.48	1030.97	869.82, 725.54	Flabelliferin II (F II)
4	4.87	870.13	726.24	Flabelliferin B (F_B)
5	5.57	1032.03	870.21, 726.27	Flabelliferin II (F II)
6	6.74	869.13	725.08	Flabelliferin B (F_B)
7	11.15	1015.22	-	Borassoside F

Note: tR = Retention time

Table 2. Mass Spectrometric Data and Identification of Bitterness Compounds in The Debittered Mesocarp (DM) of *B. flabellifer*.

Peak No	t_R	[M]+ (*m/z*)	MS/MS (*m/z*)	Compound
1	6.00	1033.36	-	Flabelliferin FII isomer
2	6.90	886.32	868.38	Flabelliferin B-hydroxylated

Note: t_R= Retention time

2.2 Phytochemical Composition and antioxidant activities

Only phenols, saponin and tannins were present in all samples (Table 3). Similar compounds were also found in the aqueous and methanolic extracts of raw palmyra palm fruit pulp (RPFP) and thermally processed palmyra palm fruit pulp (PPFP) of *B. flabellifer* at the mature stage [11]. The total tannin content in control and DM were also lower if compared to the ethanolic extract of husk from *C. nucifera* [12]. Total phenolic compound (TPC) of control and DM was lower than the *B. flabellifer* fresh fruit and ethanol extract of the mesocarp husks of *C. nucifera* [13:12]. The TPC in DM was significantly ($p<0.05$) increased almost 2-fold compared to control when treated with naringinase. Enzymatic hydrolysis was reported as a possible method to remove the bitterness and could acquire compounds with enhanced biological activities [14].

The DM had significantly ($p<0.05$) lower reducing power (126.38 mM Fe^{2+}/g) as compared to the control (157.05 mM Fe^{2+}/g, Table 3). These values were also lower than those of the methanolic extract from *C. nucifera* mesocarp [15] and alkaline extract of *C. nucifera* mesocarp [5]. Meanwhile, DPPH levels of the DM has increased by 16.8% than the control and proved that naringinase resulted in a significant ($p<0.05$) increase towards DPPH value.

Table 3. Phytochemical composition and antioxidant activities of mesocarp (Control) and debittered mesocarp (DM) of *Borassus flabellifer*

Compounds and antioxidant activities	Control		Debittered mesocarp	
	Availability	Amount	Availability	Amount
Compounds				
Alkaloids (%)	ND	Negligible	ND	Negligible
Saponins (%)	D	$16.76^{a}\pm1.44$	D	$41.06^{a}\pm5.21$
Flavonoids (QE/g)	ND	Negligible	ND	Negligible
Steroid (ng/ ml)	ND	Negligible	ND	Negligible
Cardiac glycosides (%)	ND	Negligible	ND	Negligible
Phenol (GAE/g)	D	$3.23^{b}\pm0.00$	D	$6.08^{a}\pm0.00$
Tannins (TE/g)	D	$0.01^{b}\pm0.01$	D	$3.26^{a}\pm0.02$
Terpenoids (LE/g)	ND	Negligible	ND	Negligible
Anthraquinone (%)	ND	Negligible	ND	Negligible
Antioxidant activity				
FRAP (mM Fe2+/g)	D	$157.05^{a}\pm0.08$	D	$126.38^{b}\pm0.03$
DPPH (mM TE/g)	D	$29.90^{b}\pm0.06$	D	$35.93^{a}\pm0.02$

Values are the mean of triplicate experiments. $^{a-b}$ Different superscript between control and debittered mesocarp denote significant differences ($p< 0.05$). ND: Not detected; D: Detected

4. CONCLUSION

These finding showed that the naringinase can act directly on the mesocarp sample in debittering process with less bitterness but with improving bioactive compounds and antioxidant activities.

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THE GROWTH KINETICS AND FUNCTIONAL CHARACTERISTIC OF EXOPOLYSACCHARIDE FROM *LACTOBACILLUS PLANTARUM* ATCC 8014

***Nor Zalina Othman**, Zaheda Mohamad Azam, Mohamad Azzuan Rosli, Siti Nor Azlina Abd Rashid, Nur Amalina Mohd Ropi, Muhammad Helmi Nadri, Mohamad Roji Sarmidi

Innovation Centre in Agrictechnology, Universiti Teknologi Malaysia, 84600 Pagoh, Muar, Johor, Malaysia
norzalina@utm.my

ABSTRACT
Lactobacillus plantarum ATCC 8014 produces water soluble exopolysaccharide. Exopolysaccharide(EPS) which is well known compound application in the biotechnological applications mainly by their prebiotic properties. For industrial application, a low cost medium should be design to compensate the total production cost which is high. The batch cultivations in 16-L stirred tank bioreactors under controlled pH yielded cell mass and EPS concentrations of 5.14 g L^{-1} and 6.74 g L^{-1} respectively. At this stage study, the optimized medium as the new formulation of production medium has the potential to be a cheap carbon source for the cultivation of *L. plantarum* ATCC 8014 concurrently for high cell mass and EPS production. The functional properties of EPS were further evaluated for *in* vitro antioxidant assay using ABTS radical scavenging capacity test. The results showed significant antioxidant activity assay expressed as IC50 values at 150 ug ml^{-1}. Cell proliferative activity of the EPS was evidenced by significant inhibit growth of human dermal fibroblast (HDF) proliferation compared to control. Furthermore, the EPS also showed antiproliferative activity against colorectal cells with an IC_{50} of 234.04 µg mL^{-1}. These functional properties of the EPS have a big prospect in the food and pharmaceutical industry.

Keywords: *L. plantarum* ATCC 8014, cell mass, exopolysaccharide, HDF cell proliferation, antioxidant

1. INTRODUCTION

The probiotic bacteria can provide health benefits to us [1]. These benefits are thought to result from the ability of probiotics to restore the natural balance of gut bacteria with varied types of metabolism in the intestine, thereby influencing the host's health, including nutrition, physiological function, immunological responses and resistance to infection and other stress [2]. Each of the intestinal micro floras possesses diverse enzymes, capable of converting substances into beneficial and detrimental compounds. Exopysaccharides (EPS) are well known biopolymer secreted into culture medium producing by several lactic acid bacteria (LAB). Different strains of LAB have been reported to be good producer, especially some of lactobacilli which divided to be the probiotic bacteria produce EPS [3,4]. Extracellular polymeric substances (EPSs) are natural polymers of high molecular weight secreted by microorganisms into their environment. EPS are important ingredients in many food, pharmaceutical and cosmetic products, since they play an important role in the production and stabilisation of emulsions [5]. Most EPS are water soluble as they readily dissolve in aqueous solutions. In this study, partially important functional properties of EPS are investigated. All these functional properties exhibited by EPS are dependent on the structure, molecular weight and concentration of the polysaccharides present [6,7,8]. Therefore, in this study the functional characteristics and cell growth kinetics under uncontrolled pH cultivation were investigated for the groath of *L. plantarum* ATCC 8014.

2. MATERIALS AND METHODS

2.1 Microorganism

L. plantarum ATCC 8014, was first propagated in Man Rogosa Sharpe's (MRS) broth (Merck, Darmstadt, Germany) consisting of (g l^{-1}): peptone from casein 10.0; meat extract 10.0; yeast extract 4.0; D(+)-glucose 20.0; dipotassium hydrogen phosphate 2.0; Tween 80 1.0; diammonium hydrogen

citrate 2.0; sodium acetate 5.0; magnesium sulfate 0.2; manganese sulfate 0.04; agar-agar 14.0 and incubated at 30°C for 48 h. The arisen colonies were harvested by glycerol solution (50% w v^{-1}) and put in series of 2 ml cryogen vials. The vials were frozen at - 20°C for 24 h followed by further storage in the working cell bank at -80 °C for further use.

2.2 The growth kinetics of *L. plantarum* ATCC 8014 under uncontrolled pH condition in bioreactor 16 L

Batch cultivations in stirred tank bioreactor were carried out using 16-L pilot scale stirred tank bioreactor (BioEngineering, Wald, Switzerland) with a working volume of 8-L. The optimized medium was used and the growth in the bioreactor under uncontrolled pH at 37^{0}C for 72 hr cultivation.

2.3 Extraction of EPS

The cell-free clear supernatant was used for EPS determination. The crude EPS was then isolated by 95% ethanol precipitation at the ratio of 1:3. After centrifugation at 6339 ×g of 15 min, 4°C, the EPS pellet was dispersed in aqueous 95 % ethanol and centrifuged again. The final precipitate was dried to a constant weight at 55°C [9,10,11]. The EPS yield was measured.

2.4 Physical characterization of EPS by carbohydrate composition (HPLC), and the presence of functional groups by IR spectroscopy

2.4 The functional properties of EPS: Monomer compositions of EPS produced by *Lactobacillus plantarum* ATCC 8014 by HPLC.
2.4.1 *In* vitro antioxidant assay using ABTS radical scavenging capacity test.
2.4.2 Cell proliferative activity of the EPS in human dermal fibroblast (HDF) proliferation

3. RESULTS AND DISCUSSION

3.1 Growth kinetics of *L. plantarum* ATCC 8014 in new formulation of production medium in bioreactor 16L

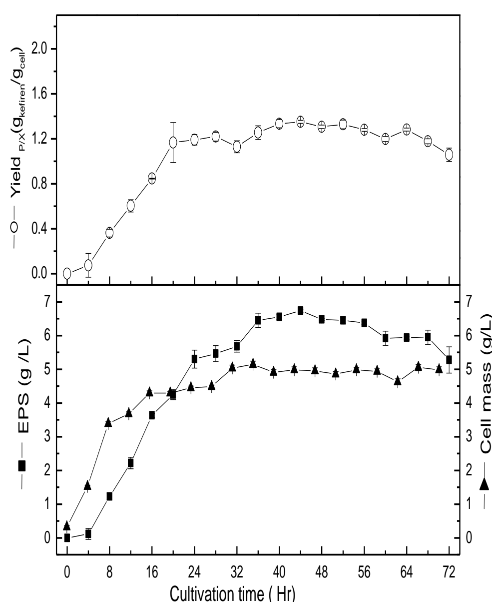

Figure 1. Kinetics of cell growth and EPS production by *L. plantarum* ATCC 8014 under uncontrolled pH when cultivated in bioreactor 16L

3.1 Physical characterization of EPS by carbohydrate composition

EPS produced by *L. plantarum* ATCC 8014 categorized as heteropolysaccharides consists of glucose, galactose, arabinose and ribose as monosaccharide building units (Table 1)

Table 1. Monomer compositions of EPS produced by *Lactobacillus plantarum* ATCC 8014

Sample	**Monomer composition (% w/w)**			
	Glucose	Galactose	Arabinose	Ribose
EPS	0.43	0.03	0.15	0.15

3.2 Functional characteristics of EPS produced by *L. plantarum* ATCC 8014

3.2.1 Free radical scavenging activity of EPS in different solvents

The present study utilized ABTS assay which based on the generation of the blue/green $ABTS^+$ that can be reduced by antioxidant molecules

Table 2. Free radical scavenging activity of EPS in different solvents

Sample	**Extract**	**Half minimal inhibitory [IC_{50} (µg/mL)]**
EPS from *L. plantarum* ATCC 8014	Ethanol	280
Ascorbic acid	-	2.5

3.2.2 Cell proliferative activity of the EPS in human dermal fibroblast (HDF) proliferation

Cell proliferative activity of the EPS was evidenced by significant inhibit growth of human dermal fibroblast (HDF) proliferation compared to control.

Table 3: Percentage of cells viability treated of EPS extract treated HT-29 (colorectal) cancer cell line

Sample	**Sample Concentrations (ug/mL)**	**% of Cells Viability**	**The half maximal inhibitory concentration IC_{50} (ug/mL)**
EPS	1	91.75±2.64	>625
	5	89.74±3.86	
	25	83.23±5.18	
	125	73.02±3.58	
	625	62.30±2.92	

As for comparison, cisplatin gave IC_{50}*value in the range of 0.71±0.026 (ug/mL) when tested on HT-29 (colorectal) cancer cell line*

4. CONCLUSION

EPS samples contained significant amounts of carbohydrate content and relatively low protein. Cell proliferative activity of the EPS was evidenced by significant inhibit growth of human dermal fibroblast (HDF) proliferation compared to control. All tested samples reduced ABTS+ molecule but their potency is lesser than ascorbic acid.

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COMPARISON OF CASEINOLYTIC ENZYMES PRODUCTION FROM *BACILLUS CEREUS* 13BN USING DIFFERENT TYPES OF MEDIA POTENTIALLY FOR CARDIOVASCULAR DISEASES

Mazlina Mohd Ariffin[1*], Zarita Zakaria[1], Huszalina Hussin[1], Nur Amira Syahirah Mazlan[1], Noor Aini Abdul Rashid[1]

[1]*Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor.*
mazlina.mohdariffin@gmail.com*, zarita@fbb.utm.my, huszalina@fbb.utm.my,
amirasyahirah_gmc@yahoo.com

ABSTRACT

Previously, a proteolytic enzyme producer from one of Malaysian fermented food - *belacan* was successfully isolated and named as *Bacillus cereus* 13BN. This caseinolytic enzyme is a systemic enzyme, and functionally involved in dissolving the excess fibrin of the blood clots, and belongs to serine protease family. Considering the potential of this enzyme as an alternative of commercially available anticoagulant, the best condition for *B. cereus* 13BN's enzyme production was optimized by slightly modifying the composition of nutrient broth, using five types of carbon source, which are lactose, galactose, maltose, glucose and glycerol. Five types of nitrogen source which are gelatine, casein, proteose peptone, ammonium sulphate and ammonium nitrate were also used in modifying the composition of nutrient broth, as well as the influence of different incubation period. It showed that maltose and casein had constantly increasing of caseinolytic activity during incubation. Both sources had the highest caseinolytic and activity at 36-hour incubation, which was 5.824±0.065 U/mL for maltose, and during 72-hour incubation, which was 3.948±0.772 U/mL for casein, respectively. As for control, the highest caseinolytic activity was at 36-hour incubation, which was 1.390±0.074 U/mL respectively. After combining these two sources, the analysis using statistical softwares showed that highest caseinolytic enzymes can be optimised during 72-hour incubation with the desirability of 0.540.

Keywords: *Bacillus cereus* 13BN; protease; caseinolytic activity; carbon and nitrogen source, incubation period

1. INTRODUCTION

Nowadays, various types of cardiovascular disease had already become one of the main reasons that lead to huge amount of death cases worldwide [6]. Symptoms that lead to cardiovascular diseases, or known as CVD such as hypertension, high blood pressure and unbalanced diet can be seen commonly occur to humankind worldwide. When a person has critical CVD, those symptoms can cause blood clotting in blood vessels, thus leading to thrombosis, and highly fatal to patients if not treated [6]. In order to reduce the deposition of blood clotting or thrombosis, a microbial enzyme known as nattokinase has been introduced to the pharmaceutical and medicinal world [4]. This is because, these enzymes are systemic enzyme, and come from serine protease family, are also critically involved in breaking down the excess fibrin of blood clots in the blood vessels [4]. Other commercialized natural thrombolytic enzymes such as urokinase, reptilase and also brinase have been utilized widely for clinical purposes [1]. Yet, these commercialized enzymes are still expensive [1]. Hence, *Bacillus cereus* 13BN [7] was used throughout this study. This is because the enzyme secreted by the bacteria was proved has an anticoagulant agent, known as t-PA. It is easily purified, can fully hydrolyse fibrin and fibrinogen without any help from other proteolytic enzymes, and gives high chance to be as affordable medicinal anticoagulant product [7]. Thus, this study is conducted to compare the production of caseinolytic enzymes from *B. cereus* 13BN by slightly modifying the carbon source and nitrogen source of the nutrient broth compositions, and the general nutrient broth, at different incubation periods.

2. MATERIALS AND METHODS

2.1 Bacterial strain

The *Bacillus cereus* 13BN strain that was used in this study was obtained from the previous research. It was isolated from shrimp paste, or known as *belacan* [7].

2.2 Preparation of Nutrient Broth (NB) medium

Throughout this research, *Bacillus cereus* 13BN strain was incubated in three types of nutrient broth (NB), which were modified carbon source NB (MCNB), modified nitrogen source NB (MNNB), modified carbon-nitrogen source NB and the general NB. These NB media were freshly prepared from the beginning. **Table 2.1 and 2.2** show the different compositions of NB used for culturing the bacteria;

Table 2.1 The modified composition of nutrient broth (NB) using different types of carbon source

Composition	Amount needed for 100mL (g)
Modified carbon source (lactose, galactose, maltose, glucose and glycerol)	0.2
Peptone	0.5
Sodium chloride (NaCl)	0.5
Distilled water	100 mL

Table 2.2 The modified composition of nutrient broth (NB) using different types of nitrogen source

Composition	Amount needed for 100mL (g)
Carbon source that has the highest proteolytic activity	0.2
Modified nitrogen source (gelatin, casein, proteose peptone, ammonium sulphate and ammonium nitrate)	0.5
Sodium chloride (NaCl)	0.5
Distilled water	100 mL

The media were calibrated to pH 7.0 and heated a little until the media were homogenised. This general NB also was used as control during this study.

2.3 Optimization of media and incubation period for the enzyme production

The influence of the incubation period using different sources on enzyme production was analysed within 0-72 hours, at 37ºC, at 200 rpm. To determine the optimum conditions for enzyme production, the caseinolytic activity assay was carried out using standard procedures as enzyme as described in subsection 2.4. The caseinolytic activity at the optimum time-frame was taken as 100%.

2.3.1 Effect of carbon and nitrogen sources and incubation period

The carbon source utilised in the general composition of NB, which is the yeast extract was substituted with five different types of carbon source at 1 % w/v, which were lactose, galactose, maltose, glucose and glycerol. For nitrogen source, peptone was replaced by five different types of nitrogen source at 1 % w/v (gelatin, casein, proteose peptone, ammonium sulphate and ammonium nitrate). The bacteria were incubated in these modified NB media at 37ºC for 0-72 hours at 200 rpm.

2.4 Enzyme assays

In general, *B. cereus* 13BN was cultured in three different types of nutrient broth (NB) before at 37ºC and 200 rpm within range of 0-72 hour incubation time. Next, the supernatant from each medium within

each incubation period was collected for estimation of protein concentration and caseinolytic activity assay. The supernatants were collected using centrifuge method at 25℃, at 4000 rpm for 15 minutes.

2.4.1 Determination of caseinolytic activity

The caseinolytic activity for *B. cereus* 13BN was measured using casein as substrate while tyrosine as standard with concentrations of tyrosine such as 0.055, 0.111, 0.221, 0.442 and 0.553 µmoles. This assay was carried out at 37°C for 10 minutes.

2.5 Statistical Analysis

The data analysis of samples obtained from both Lowry assay and enzymatic assay will be calculated and obtained using statistical computer software such as Microsoft Excel and Design Expert Software.

3. RESULTS AND DISCUSSION

3.1 The results for general composition of nutrient broth (control)

During this study, the common nutrient broth (NB) was used as control. Within 0-72 hours of incubation, every 12-hour period, 6 mL of culture was pipette and transferred into 15 mL Falcon tube. 2 mL of 6 mL culture was used for OD reading, using 600 nm of absorbance, while the other 4 mL was centrifuged for 15 minutes, at 25°C and at 4000 rpm. Then, the supernatant was collected for determining the protein concentration and also the specific caseinolytic activity using the assays stated before.

3.1.1 Determination of caseinolytic activity

After incubating the culture for 0-72 hours, the supernatants were collected and analysed to determine the caseinolytic activity. The results were recorded in **Figure 3.1** below;

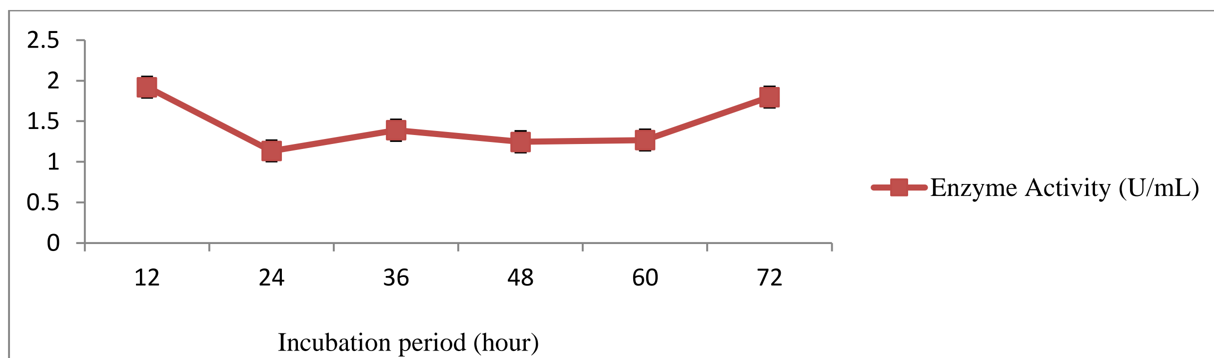

Figure 3.1 Readings of caseinolytic activity for each incubation period

Based on the results, it showed that at 12-hour incubation has the highest caseinolytic activity, which is 1.920±0.172 U/mL. Although the protein concentration recorded was 0.510±0.083 mg/mL, but still has the highest caseinolytic activity reading, and also specific activity, compared to other types of carbon used. At 24-hour incubation, it showed that the caseinolytic activity result was the lowest, which is 1.135±1.790 U/mL respectively. Both readings were shown in **Figure 3.1**.

3.2 Effects of modification of carbon and nitrogen source on nutrient broth composition

Throughout this study, the general composition of NB has been slightly altered, which is the carbon source in the NB, as stated in **Table 2.2** before. Hence, the processes in collecting the supernatant from each carbon source were exactly similar as stated in section **3.1**.

3.2.1 Determination of caseinolytic activity for both sources

The supernatant for each type of carbon source was collected at 12 hours, 24 hours, 36 hours, 48 hours, 60 hours, and 72 hours incubation time. After collecting the supernatants, they were tested to determine the caseinolytic activity and specific activity for each incubation period. The results were recorded in **Figure 3.3** for carbon source, while **Figure 3.4** for nitrogen source as below;

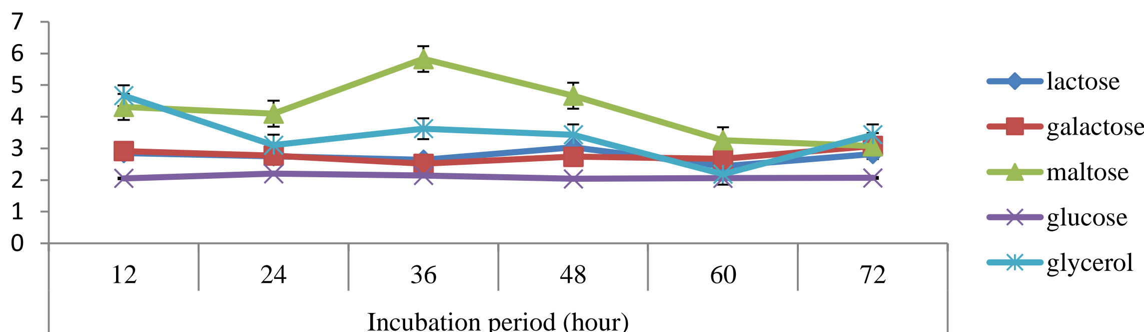

Figure 3.3 Caseinolytic activities of each carbon source during 12-72 hour

Based on the results shown in **Figure 3.3**, it showed that maltose has the highest caseinolytic activity among other types of carbon, which was 5.824±0.065 U/mL respectively. The reading was recorded the highest during 36-hour incubation. While glucose, has the lowest caseinolytic activity, which was 2.186±0.615 U/mL respectively. The reason why the caseinolytic activity of this bacteria when using maltose as the carbon source, compared to glucose is because of the composition of these carbons. Maltose is consisting of two molecules of glucose and bound together using glycosidic bond [2]. This compound can be found as an intermediate and also as the final product in starch and glycogen production [2][3]. Compared to other types of disaccharides, maltose can be digested easier due to its compounds and gives higher carbon source to the microbes, so that the cells can be more productive. On the other hand, glucose is actually does not give the maximum and the highest amount of nutrient for the microbes to obtain the energy. The metabolism of the microbes also is decreasing due to the lackness of carbon source (glucose) in the media. That is why the caseinolytic activities for *B. cereus* 13BN using glucose as its carbon source has the lowest reading compared to others.

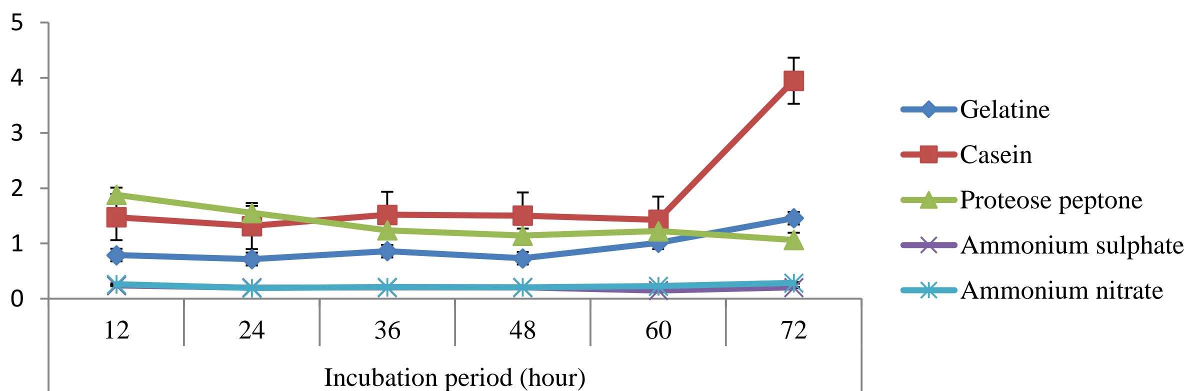

Figure 3.4 Caseinolytic activities of each nitrogen source during 12-72 hour

Based on **Figure 3.4**, it showed that casein has the highest caseinolytic activity, which was 3.948±0.772 U/mL, during 72-hour incubation. Ammonium sulphate has the lowest caseinolytic activity, which was 0.148±0.453 U/mL. Casein has two properties that make it differ than other milk proteins. The first one, it is easily dissolve in amorphous calcium phosphate solution quicker due to the presence of phosphoserine [5]. Secondly, caseins are easily bound and coagulate with each other, forming higher-order structures of morphology and different sizes. Hence, it is easier for them to deliver nutrients throughout the cells [5]. As for ammonium sulphate, it is less suitable due to its properties as inorganic

compound. Ammonium sulphate consists of 21% of nitrogen and 25% sulphur [8]. Since it is inorganic compound, *B. cereus* 13BN cannot get any nutrients from this because *B. cereus* 13BN is a heterotroph [7]. After analysing all results, overall it showed that maltose and casein has the highest caseinolytic activity among other types of sources. Hence, they were selected as the main carbon and nitrogen source for the modified carbon-nitrogen NB.

3.3 Statistical analysis for modified maltose-casein nutrient broth (MMCNB)

The analysis of caseinolytic activities of *B. cereus* 13BN for MMCNB has been calculated using Central Composition Design (CCD). Based on the analyses, it is shown that the results are in quadratic. Quadratic effect is used to determine whether the results can be used or not. The results are shown in **Figure 3.5**;

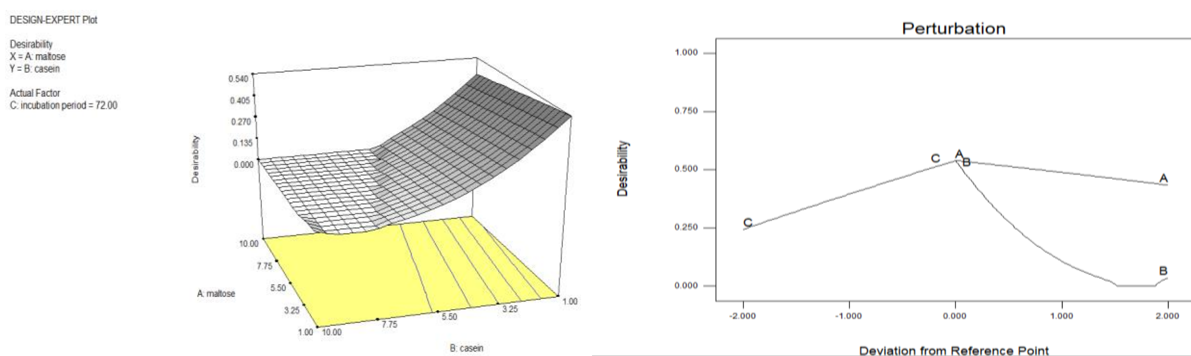

Figure 3.5 The perturbation and 3D-contour analysis for modified maltose-casein NB

Based on the analyses, it is shown that the caseinolytic activity has been produced the most during 72-hour incubation. The desirability shown from the analyses is 0.54, which is the higher than the general nutrient broth. Although the general nutrient broth has more nutrient than MMCNB, but it takes longer time for *B. cereus* 13BN to digest the nutrients, and maybe has less desirable nutrients demanded by this type of microbe. Hence, it is showed that MMCNB is more suitable for *B. cereus* 13BN to increase the optimisation of the caseinolytic enzymes production.

4. CONCLUSION

Based on the analyses, it showed that maltose and casein were the most ideal carbon and nitrogen source for optimising the production of caseinolytic enzymes from *B. cereus* 13BN. It is because both sources had shown a constant increasing of caseinolytic activities and specific activities during incubation time, with the desirability of 0.540 from the statistical analysis.

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DRYING PROCESS OF *Moringa oleifera* LEAVES - A REVIEW

A. S. Nabilah[1, *], A. Z. Dayang Norulfairuz[2], M. Ida Idayu[3], M. J. Yanti Maslina[4]

[1,3,4] *Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Skudai, Malaysia.*
nabilah.asahi@gmail,com, idaidayu@utm.my, yantimaslina@utm.my

[2] *Institute of Bioproduct Development, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Skudai, Malaysia*
dnorulfairuz@utm.my

ABSTRACT
Moringa oleifera is a well cultivated plant of genus *Moringa* belonging to family *Moringaceae* that can be widely found in West, East and South Africa, tropical Asia, and Latin America. It had been intensively use for medicinal and food purposes in most country due to plant contain a balanced level of amino acids as well as high amounts of essentials nutrients such as protein, calcium, carbohydrate, minerals, copper, iron, potassium, magnesium, manganese and zinc as well its ability to withstand in drought resistance. It also used extensively for treatment in malnutrition as it gives low-cost nutrition. Hence, with the high amounts of nutrients in *M. oleifera*, the best drying processing techniques can be applied to preserve the nutrients, phytochemical compound to be include in food application. Drying processing is a process to preserve appearance, nutritional characteristics and aroma of the raw herbs as maximum possible and store herbs so that a minimal loss of flavour, quality and avoidance of microbial growth.

Keywords: *Moringa oleifera* leaves; drying process

1. INTRODUCTION

Moringa oleifera Lam. is a fast-growing and a drought-resistant tree. It can be widely found in West, East and South Africa, tropical Asia, and Latin America countries. It is commonly known as *moringa*, drumstick tree due to the presence of the long, slender, triangular seed-pods, horseradish tree because due to the taste of the roots, which similar to horseradish, and as well as ben oil tree or benzoil tree as from the seeds it can produced oil.

Moringa Oleifera Lam. is among *Moringa* species which is the most frequent cited use for medicinal and food purposes in most country due to plant contain a balanced level of amino acids as well as high amounts of essentials nutrients such as protein, calcium, carbohydrate, minerals and vitamins and its ability to withstand in drought resistance. In addition, the plant is known to be a multipurpose tree and it is used extensively for treatment in malnutrition as it gives low-cost nutrition.

Previous study has stated that *M. oleifera* was also known as 'The Miracle Tree' due to its plenty uses in medicinal, food as well as in industries with lots of nutrients [1]. Besides, all parts of the plants starting from the roots, seed, woods, pods, oil, fruits, flower, and leaves have their own benefits. Hence, this plant had been widely cultivated in most country worldwide.

This review is highlighting the different drying processes of *M. oleifera* leaves and the responses towards different analysis such as chemical and biological properties.

2. DRYING PROCESS

The main purpose of drying of herbs is to preserve appearance, nutritional characteristics and aroma of the raw herbs as maximum possible and store herbs so that a minimal loss of flavor, quality and avoidance of microbial growth. Moreover, it is also important for packaging, transportation and distribution process. The principle of preservation is by dehydration process where removal of the moisture content of a material to a level where can prevent the growth of microorganisms and lead to spoilage [2]. Therefore, there are various methods that had been done for drying process such as

direct sunlight, shade sunlight, convection oven, microwave, freeze drying, dehydrator and blanching.

It has been reported that amount of vitamin A available is estimated about 20-40% when the leaves are dried under direct sunlight, in contrast, under shade dried there is around 50-70% of vitamin A retained [3]. Breakage of protein may occur when the leaves are dried under high temperature [4], longer drying duration, oxygen, and sunlight. It may also lead to nutrients loss [5]. The drawback of direct sunlight and shade drying is that it requires a longer time of drying process and has a high risk of contamination due to insects, rodents and birds [17].

Various studies have reported on the drying process for *M. oleifera* under convective oven as listed in Table 1. Different parameter conditions were used for the convective oven depending on the analysis of the research study. Ali et al. [5] has reported that drying of *M. oleifera* leaves using oven at 40°C was suitable for preserving maximum amount of protein content. At oven temperature 50°C it was able to preserve the highest content of vitamins especially water-soluble vitamins for instance vitamins C and B complex while there are no changes in preserving carbohydrates contents when the drying under oven at 40-60°C. Besides that, study by Premi et al. [6] has found that at temperature 60°C it gives an optimum of energy efficiency and product quality of the *M. oleifera* leaves powder. However, it is advisable to obtain an optimize drying conditions of every single herbs, [19] as different herbs have different tolerance toward temperature and time to obtain maximum nutrients contents, phytochemical compounds as well as good organoleptic characteristics such as color, texture and taste. The disadvantages that usually encounter when using oven drying methods are it cause excessive shrinkage, changes in color appearance and can cause product collapse [20].

The usage of microwave was becoming preferable method due to penetrate of foods and heat on both sides, the outer surface and inside the food. This makes the drying process more rapids, uniform penetration as compare to convection oven and ability to improve the quality of final products. The mechanisms under microwave which the energy dissipates depend on the characteristics of the medium and the frequency of the wave. Microwave drying process uses electromagnetic radiation and the uses electrical energy in the frequency range of 300 MHz to 300 GHz, and the most common frequency is at 2,450 MHz [18]. Moreover, freeze drying was a new and a promising method had recently become an interest among researchers. This is known to be a most accurate method to preserve nutrients and color quality of food product because in this process extriction of bioactive compounds increase as compared to other drying methods. This is due to dehydration by sublimation of frozen products [5]. However, this method requires high expenditure, energy consumption and maintenance as well as very time consuming [17]. Hence, an optimized drying process is required to preserve appearance, nutritional characteristics and aroma of the raw herbs as maximum possible and store herbs so that a minimal loss of flavour, quality and avoidance of microbial growth. Table 1 shows different drying process done by previous researchers on *M. oleifera*. leaves.

Table 1. Different Drying Process of *M. oleifera*

Drying Method	Parameter	Analysis	Reference
Oven	65°C for 48 h	Productivity and nutrient uptake efficiency of wheat plants	[7]
Oven	80°C until MC <5%	Effect of Extraction Process of Moringa Instant Tea on Its Sensory Quality	[8]
Oven	50°C for 12 h	Quality characteristics of wheat flour cookies	[9]
Sun dried	until constant MC	Optimization of drying process and grinding mechanism on the nutrients	[5]
Shade dried	30±7°C and relative humidity of 50–70% until constant MC		

Drying Method	Parameter	Analysis	Reference
Oven	40°C, 50°C, 60°C		
Microwave oven	660 W		
Freeze Dried	-106°C for 24 h		

Table 1. Different Drying Process of *M. oleifera*

Drying Method	Parameter	Analysis	Reference
Oven	48 ± 2°C for 3 h	Comparing the proximate composition of the blanched and un-blanched dehydrated leaves for MO biscuit	[1]
Shade dried and Oven	4 d, 50°C for 30 min	Processing of leaves	[3]
Oven	45°C for 72 h	Profiling of polyphenolics, nutrients and antioxidant	[10]
Oven	60°C for 8-12 h	For optimization of formulation and processing of *Moringa oleifera* and spirulina complex tablets	[11]
Air circulating oven	35°C for 24 h	For chemical characteristics and fractionation of proteins	[12]
Oven	65°C for 48 h	To determine protein and micronutrient contents at different localities in Sri Lanka	[13]
Oven	50°C for 8 h	Rheological, microstructural, nutritional, textural and organoleptic characteristics of cookies	[15]
Oven	40°C for 8 h	Drying kinetics and colour analysis	[14]
	50°C for 5.75 h		
	60°C for 2 h		
Oven	50°C for 10 h	Kinetics models during convective drying	[6]
	60°C for 8 h		
	70°C for 6.83 h		
Sun Drying	6 d	Effect of dehydration on the nutritive value	[16]
Shade dried	4 d		
Oven	60°C for 1 h		

3. CONCLUSION

In conclusion, before the production of a dietary supplement of *Moringa* leaves, it begins with drying process before any further processing to be done. Drying process is an important process in preserving the appearance, nutritional characteristics and aroma of the raw herbs as maximum possible and store herbs so that a minimal loss of flavor, quality and to prevent the microbial growth. Hence, different drying processes are required for different needs. An optimized condition of drying process needs to be investigated in order to obtain maximum amount nutritional value, phytochemical, antioxidants contents so that it can widely apply for medicinal and food purposes.

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EFFECT OF SOLVENTS ON POLYVINYLIDENE FLUORIDE NANOFIBER AND ITS POTENTIAL USE FOR *ESCHERICHIA COLI* IMMOBILIZATION

Norhamiza Mohamad Sukri[1], Rosli Md Illias[1]

[1]*School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310, Johor Bahru, Johor, Malaysia.*
nhamizasukri87@gmail.com, r-rosli@utm.my

ABSTRACT

In bioprocess industry, cell instability, bioconversion inefficiency and low product yield are among the common problems faced when using free cells as whole cell biocatalyst. Thus, cell immobilization can be a powerful tool to overcome the bottlenecks. This study used polyvinylidene fluoride (PVDF) nanofiber as the support for *Escherichia coli* immobilization. The PVDF nanofibers were prepared using dimethylformamide (DMF) and dimethylacetamide (DMAc) solvents. The solvents' effects on the morphology and physicochemical properties of PVDF nanofibers before and after immobilization were examined. The results revealed that both solvents produced smooth surface and regular morphology fibers with average diameters of 111±45 nm and 265±74 nm for DMF and DMAc, respectively. Upon immobilization, the higher number of cells ($8.6 \times 10^7 \pm 1.3$ ml^{-1}) adsorbed onto PVDF (DMF) nanofiber could be attributed to the electrostatic interaction between its positively charged surface (17.1 mV) and negatively charged *E. coli*. The increased intensity of OH and C=C peaks indicates the incorporation of *E. coli* on both nanofibers. The results showed that solvent affects the physicochemical properties of PVDF nanofiber and its preparation using DMF resulted in better support for *E. coli* immobilization.

Keywords: Whole-cell biocatalyst; immobilization; nanofiber; solvent; polyvinylidene fluoride

1. INTRODUCTION

The growing demand for value-added chemicals requires the scientific communities to use whole cell biocatalysis as an alternative to produce such chemicals with a much-reduced cost and environmental impact. The whole cell biocatalyst also provides higher selectivity and catalytic efficiency and milder operational conditions in comparison to the chemical catalyst [1]. A variety of microorganisms has been widely used as whole cell biocatalysts; either in the form of freely suspension or immobilized cells. Although mass transfer limitation barely happens when using free cell system, a decrease in cell's stability, substrate conversion efficiency and product yield are commonly observed when long reaction time or recyclability is employed in the system [2]. Such drawbacks could be overcome using cell immobilization. Immobilization allows easier separation from the fermentation medium, prolonged and repeated use of cells, simple downstream processing, and preservation of plasmid-bearing cells and reduces contamination risk [3]. Adsorption, encapsulation, entrapment and flocculation are among the generally used methods for immobilization [4]. The choice of support is also important as it can affect immobilization efficiency. This work used polyvinylidene fluoride (PVDF) nanofiber as the support for immobilization of recombinant *Escherichia coli*. Nanofibers could be an excellent immobilization support as it has large surface area-to-volume ratio, tailorable surface functionality and porosity, and great mechanical strength [5]. The fabrication of high-quality nanofibers depends on many factors such as solvent, polymer concentration and conductivity. Therefore, the effects of solvents on morphology and physicochemical properties of PVDF nanofiber as well as on *E. coli* immobilization were investigated.

2. MATERIALS AND METHODS

2.1 Materials

Polyvinylidene fluoride (PVDF, Kynar® 740, $M_w = 1.56 \times 10^5$) pellet was used as received. *N, N*-dimethylformamide (DMF) and *N, N*- dimethylacetamide (DMAc) were obtained from J.T. Baker and

QreC, respectively. The recombinant *E. coli* expressing xylitol phosphate dehydrogenase (xpdh) gene with deletion of phosphoglucose isomerase (Δ*pgi*) was obtained from the Genetic Engineering Laboratory, Universiti Teknologi Malaysia.

2.2 Development of polyvinylidene fluoride nanofiber

Polyvinylidene fluoride (PVDF) solution was prepared by dissolving 15% (w/v) of PVDF pellet in *N, N*- dimethylformamide (DMF) or *N, N*- dimethylacetamide (DMAc) solvent at 60°C for at least 5 hours. The PVDF nanofibers were fabricated using electrospinning. The electrospinning was performed at the voltage of 15 kV, with the flow rate and tip-to-collector distance of 1 ml/h and 15 cm, respectively. A grounded conducting collector was used to collect the nanofibers. The collected nanofibers were dried overnight at 60° C to remove the residual solvent. The nanofibers were cut into 3 cm x 3 cm square sheets at the weight of 0.01 g and sterilized using ultraviolet (UV) light before using them for cell immobilization.

2.3 Cell immobilization

Cell immobilization was achieved by adding the sterile PVDF (DMF) or PVDF (DMAc) nanofibers into 50 ml of Luria-Bertani (LB) broth containing 34 µg/ml chloramphenicol and approximately 10^7-10^8 cfu/ml of *E. coli* Δ*pgi*/xpdh strain. The immobilization was carried out at 37°C and 100 rpm for 24 hours.

2.4 Characterization of PVDF nanofibers

Before immobilization, the morphology of PVDF (DMF) and PVDF (DMAc) nanofibers was analyzed using scanning electron microscopy and the average diameter of fibers was determined using ImageJ software. The physicochemical properties of the nanofibers were characterized using attenuated total reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and zeta potential. The immobilization performance was determined quantitatively using plate count assay, SEM and ATR-FTIR.

3. RESULTS AND DISCUSSION

As demonstrated in Figure 3.1, the fibers formed showed that both solvents have high degree of spinnability which is presumably due to their high dipole moment and low viscosity properties. According to Pattamaprom et al. (2006), dipole moment and viscosity of solvent play a significant role in determining the degree of spinnability and formation of bead-free fibers [6]. The SEM demonstrated that both solvents produced a smooth surface and regular morphology fibers with average diameters of 111±45 nm and 265±74 nm for DMF and DMAc, respectively. The higher dipole moment and lower viscosity of DMF compared to DMAc might explain this finding. As depicted in Figure 3.2, the viable cell count result exhibited that higher number of *E. coli Δpgi*/xpdh cells ($8.6 \times 10^7 \pm 1.3$ ml^{-1}) was attached on PVDF (DMF) compared to that on PVDF (DMAc) ($4.5 \times 10^7 \pm 1.5$ ml^{-1}). However, the SEM images could not precisely support the quantitative evaluation as the attachment performance of cells onto both nanofibers seemed comparable (Figure 3.1).

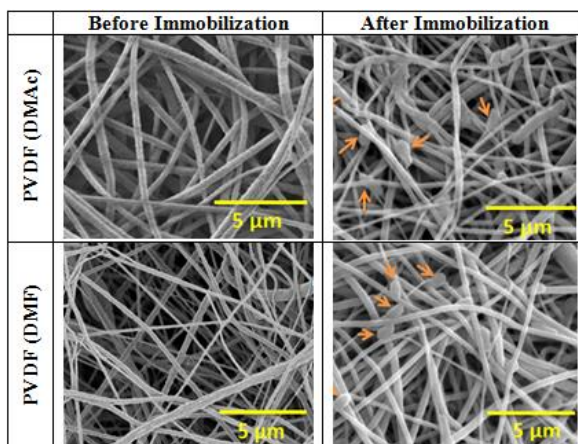

Figure 3.1. SEM Images of PVDF (DMAc) and PVDF (DMF) (Magnification: 5000; Arrows: *E. coli Δpgi*/xpdh cells).

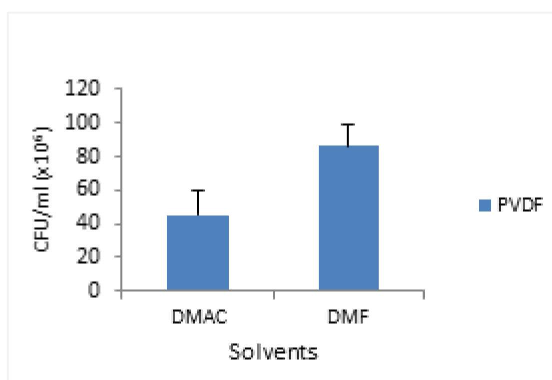

Figure 3.2. Plate Count Assay of PVDF (DMAc) and PVDF (DMF).

To understand the physicochemical properties of *E. coli* Δpgi/xpdh cells, PVDF (DMAc) and PVDF (DMF) nanofibers, were evaluated using ATR-FTIR and zeta potential measurement. Figure 3.3 shows the FTIR-ATR spectra for *E. coli* Δpgi/xpdh cells, immobilized PVDF nanofibers, and non-immobilized PVDF nanofiber. There are two characteristic peaks of PVDF; one at 1390 cm^{-1} was attributed to the CH_2 stretching vibration and the other at 1170 cm^{-1} was caused by the CF_2 stretching vibration. As depicted in the figure, both PVDF (DMAc) and PVDF (DMF) nanofiber showed an increase intensity of peaks at approximately 3340 cm^{-1} (OH stretching vibration) and at 1612 cm^{-1} (C=C symmetrical stretching) which indicates incorporation of foreign body i.e *E. coli* cells on the nanofibers.

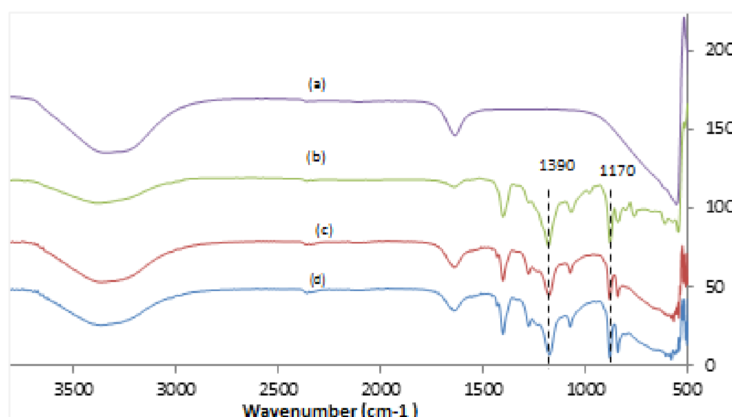

Figure 3.3. ATR-FTIR Spectra of (a) *E. coli Δpgi*/xpdh only, (b) Non-immobilized PVDF, (c) Immobilized PVDF (DMAc) and (d) Immobilized PVDF (DMF).

Zeta potential has been shown to correlate well with surface charge of any solid particle. One of the factors that influence the attachment of cells on material surfaces is electrostatic interaction. Therefore, zeta potential measurement of cells and materials could be indicative of cell attachment behavior. The surface charge of *E. coli* Δpgi/*XPDH* cells, PVDF (DMAc) and PVDF (DMF) nanofibers was measured and shown in Figure 3.4. The result shows that the surface charge of cells and PVDF (DMAc) are both negative (-16.5 mV and -34 mV, respectively) while PVDF (DMF) showed a slight positive charge (17.1 mV). The negative charged surfaces could cause repulsion force between cells and PVDF (DMAc) which might explain the low number of cells immobilized on the nanofiber described earlier.

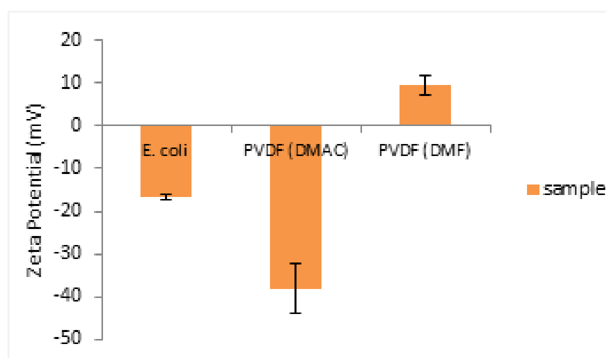

Figure 3.4. Zeta Potentials of *E. coli Δpgi*/xpdh, PVDF(DMAc) and PVDF(DMF) Nanofibers.

4. CONCLUSION

Although more analyses might be necessary to completely characterize the nanofibers, this early finding shows that solvent affects the physicochemical properties of PVDF nanofiber and its preparation using DMF resulted in better support for *E. coli* immobilization.

ACKNOWLEDGMENT

The authors thanked School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia (UTM). This research was supported by Q.J130000.3509.06G54 from Geran Universiti Penyelidikan (GUP), UTM. The authors also thanked UTM Zamalah Scholarship for the student financial aid.

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EFFECT OF RED LIGHT EMITTING DIOD (LED) ON *Nannochloropsis* SP. GROWTH, LIPID CONTENT AND NUTRIENTS REDUCTION EFFICIENCY USING PALM OIL MILL EFFLUENT (POME)

Rosnani Resdi[1] , Ani Idris[2,*], Lim Jeng Shiun[1]

[1]*Process Systems Engineering Centre (PROSPECT), Research Institute for Sustainable Environment, Chemical and Energy Engineering, 81310 UTM Johor Bahru, Johor, Malaysia*

[2]*Institute of Bioproduct Development (IBD), Faculty of Chemical and Energy Engineering, 81310 UTM Johor Bahru, Johor, Malaysia*
**Corresponding author: aniidris@utm.my*

ABSTRACT
Palm oil mill effluent (POME) is agriculture wastewaters with high organic content that causes algal bloom problem. Microalgae biomass thrives in wastewater and this is an advantage since it solves environmental problems and simultaneously produces lipids useful for various applications. POME is dark in colour which limits the light penetration for microalgae growth. However, there is a certain range of light wavelength produced from LED that can solve this problem. Thus, this study aims to evaluate the effect of continuous red LED on the culture growth at temperatures of 23º ± 10.5 ºC, pH 8.0 ± 0.2 under a light intensity of 150 µmol $m^{-2}s^{-1}$ for 14 days. Results revealed *Nannochloropsis* sp. grown in POME under red light LED has the highest amount of specific growth rate (0.256±0.0024d^{-1}), lipid content (73.14±0.336%), PUFA (58.86%), COD (75.95±11.817%), BOD (63.51±2.880%), TN (58.07±2.235%), and TP removal efficiency (85.56±6.482%).

Keywords: *Nannochloropsis* sp.; lipid content; red LED; Palm Oil Mill Effluent

1. INTRODUCTION

Palm oil production is a major industry in Malaysia. In POME treatment, ponding system is used and Malaysia has large available land as pond areas [1]. The large amount of POME consumes by microalgae promote the sustainable sound development in POME management. Microalgae can grow rapidly and some of them have high lipid content. They can produce oil content in the range 20 to 50% [2]. Microalgae cultivation requires light, CO_2, and nutrients for the growth of the microalgae [3]. However due to the dark colour of POME, light is limited and intermittent [4]. LED is considered as a good alternative compared to white fluorescent light because of its high energy efficiency and lower energy consumption [5]. Moreover, the supplied wavelength or spectrum of light can activate nutrients uptake in microalgae [6]. This study is a first attempt to enhance the biomass and lipid content in *Nannochloropsis* sp. under red LED in POME. The aim of the study is to investigate microalgae growth, lipid content and nutrients removal in POME under red LED in comparison to the control in Walne's medium under white florescent lamp. Fatty acid methyl ester (FAME) composition analysis of lipids in both treatments are studied too.

2. MATERIAL AND METHODS

All the materials and methods used in the work are described as below.

2.1 Experimental procedure

POME was collected from facultative ponds located at FELDA Bukit Besar palm oil mill, Kulai. POME concentration (60% POME + 40% Walne medium) selected from previous batch experiments (data not shown) was autoclaved. *Nannochloropsis* sp. used in this study were grown at 23º±0.5ºC, 24hrs photoperiod under red LED and 150µmol $m^{-2}s^{-1}$ light intensity for 14 days. This treatment was then compared with the control where *Nannochloropsis* sp. were cultivated in Walne's medium with same

conditions but under white florescent lamp and 100μmol $m^{-2}s^{-1}$ light intensity. All treatments were duplicated in 500mL operating volume in volumetric flask.

2.2 Wastewater and microalgae growth analysis

Samples were collected at 2 days interval. For wastewater analysis, the parameters were chemical oxygen demand (COD), biological oxygen demand (BOD), nitrogen (N), phosphorus (P) and potassium (K) were done by following APHA [7]. For microalgae growth, the parameters were optimal density (OD), cell concentration, biomass concentration, specific growth rate (μ) and division rate (D). The OD was determined using Shimadzu UV-vis spectrophotometer OD 620nm [8]. The cell was counted using a direct microscopic count with a 0.1 mm deep Neubauer haemocytometer under light microscope (Olympus CX21, Japan). Biomass was determined using Cell Dry Weight (CDW) method [9]. The specific growth rate (μ) was calculated by this equation (1) [10];

$$\mu, (day^{-1}) = \ (ln(X_2/X_1))/(t) \qquad (1)$$

where X is biomass at the time *t* during the exponential phase. *D* were evaluated by equation (2) [11]

$$D, (day^{-1}) = \mu/(\ln 2)\ . \qquad (2)$$

For lipid content, Folch method was used by homogenising the biomass in a mixture of 2:1 chloroform-methanol (v/v).

2.3 Transesterification and fatty acid methyl ester (FAME) analysis

Transesterification was done by BF3-MeOH method. The remaining FAME was diluted with 1.5 mL dichloromethane and filtered through 0.22 μm syringe filter. Then FAMEs were analysed using gas chromatography (GC) (Agilent Technologies, 7820A) and HP-88 capillary column (60m x 0.25 mm x 0.2 μm). The GC separation by the instrument was running under customized conditions. The samples were quantified by area percentage calculation using standard Supelco 37 Component FAME Mixture.

2.4 Statistical analysis

Most data will be analysed by using the Minitab 16 Statistical Software by one-way ANOVA. Tukey's test was used to compare the data when ANOVA was significant; the value of $P \leq 0.05$ was considered significant. All results were written as the mean ± standard deviation (S.D.).

3. RESULTS AND DISCUSSION

Figure 1 shows the higher optimum density reading trend achieved under red LED. The microalgae exponentially increased from day 0 to day 8 and start to achieve the stationary phase on day 10 to day 12. This higher optimum density is due to the higher cell concentration and biomass growth as depicted in Figure 2 and Figure 3 respectively when compared to the control. Table 1 illustrates the lipid content of *Nannochlopsis* sp. cultivated in red LED is significantly higher than the control due to higher biomass produced as shown in Figure 3. Previous study [12] on different LED (royal-blue, blue, orange-red, and red) at same intensity (150μmol m^{-2} s^{-1}) resulted in higher biomass and fatty acid yields when compared to the white LED. Red LED treatment showed the highest nutrients reduction efficiencies as shown in Figure 4. High N and P in POME supported *Nannochloropsis* sp. growth and their uptake was activated by the red LED spectrum. Furthermore, from Figure 5, the high COD and BOD concentration of POME can be reduced from 4000 mg/L and 1000 mg/L to 120 mg/L and 40 mg/L respectively. In Table 2, PUFA's, omega-3 and omega-6 content in red LED treatment are higher compared to the control where methyl linoleate is the main content.

Table 1 Results of maximum cell density, specific growth rate (μ) and division rate (k) of *Nannochloropsis* sp. grown in different treatments

Treatments	Specific growth rate, μ (d^{-1})	Division rate, *k* (d^{-1})	Max. Cell Density (x10E7 cell/mL)	Lipid content (%)
Control-White Lamp	0.228 ± 0.0003^{b}	0.330 ± 0.0004^{b}	3.823 ± 0.2858^{b}	55.82 ± 9.644^{b}
Red LED	0.256 ± 0.0024^{a}	0.369 ± 0.0035^{a}	7.708 ± 0.2121^{a}	73.14 ± 0.336^{a}

*Mean values with different superscript letters in the same column significantly different ($P \leq 0.05$)

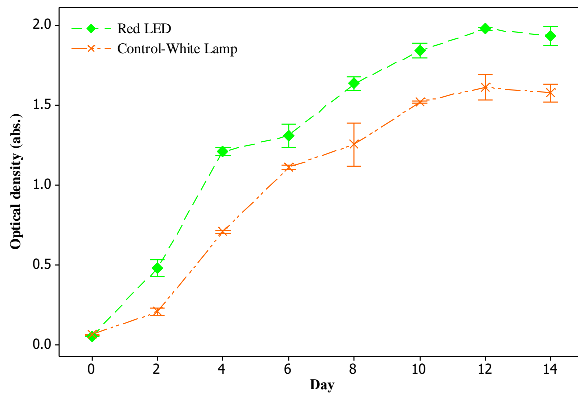

Figure 2. Optical density trend of Nannochloropsis sp. over time

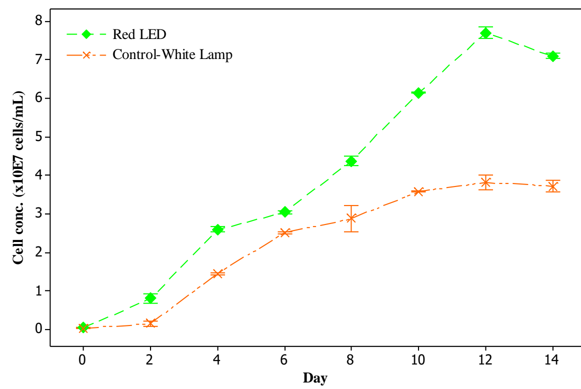

Figure 2. Cell concentration trend of *Nannochloropsis* sp. over time

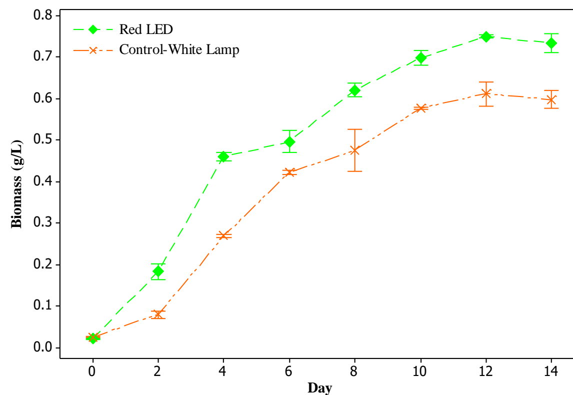

Figure 3. Biomass production trend of *Nannochloropsis* sp. over time

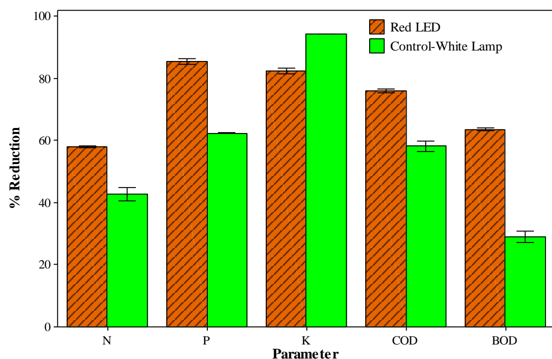

Figure 4. Efficiency of nutrients reduction used by *Nannochloropsis* sp.

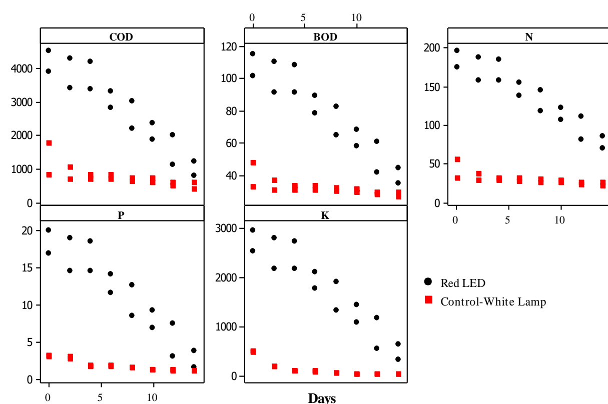

Figure 5. Nutrients (mg/L) reduction trends of POME with cultivation days

Table 2. Fatty acid methyl ester (FAME) composition analysis of lipids from microalgae

FAME species	Lipid number	Treatment	
		Control	Red LED
Methyl octanoate	C8:0	0.00	0.18
Methyl hexanoate	C10:0	0.00	0.21
Methyl laurate	C12:0	0.00	0.81
Methyl tridecanoate	C13:0	0.00	0.76
Methyl tetradecanoate	C14:0	0.39	0.31
Myristoleic acid methyl ester	C14:1	1.27	0.23
Methyl pentadecanoate	C15:0	0.00	0.52
cis-10-Pentadecenoic acid methyl ester	C15:1	0.00	0.18
Methyl palmitate	C16:0	24.00	22.12
Methyl Palmitoleate	C16:1n9c	1.52	0.24
Methyl heptadecanoate	C17:0	1.58	1.74
cis-10-Heptadecenoic acid methyl ester	C17:1	0.32	0.44
Methyl octadecanoate	C18:0	3.81	2.78
trans-9-Elaidic acid methyl ester	C18:1n9t	4.12	0.28
cis-9-Oleic acid methyl ester	C18:1n9c	13.84	4.72
Linolelaidic acid methyl ester	C18:2n6t	0.00	0.16
Methyl Linoleate	C18:2n6c	34.37	39.77
gamma-Linolenic acid methyl ester	C18:3n6	0.00	0.15
Methyl Arachidate	C20:0	4.17	3.65
Methyl Linolenate	C18:3n3	9.31	18.78
Methyl docosanoate	C22:0	0.88	1.33
Methyl Erucate	C22:1n9c	0.42	0.64
Saturated fatty acid (SFA)		34.83	34.41
Monosaturated fatty acid (MUFA)		21.49	6.73
Polysaturated fatty acid (PUFA)		43.68	58.86
Omega 6 (n-6)		34.37	40.08
Omega 3 (n-3)		9.31	18.78
Total FAME (% by weight)		100.00	100.00

4. CONCLUSION

Red LED treatment in POME medium was able to increase microalgae biomass, lipid content and nutrients reduction efficiency compared to the control. PUFA in red LED treatment are higher compared to the control.

ACKNOWLEDGMENT

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INITIAL STUDY OF PARAMETERS INFLUENCING THE PHYSICAL CHARACTERISTICS OF *Andrographis paniculata* LOADED CHITOSAN /HYALURONAN NANOPARTICLES

Hii, W.S.[1,2 *], Abdul Aziz, A [1,2], Abdul Hamid, M.[1,2,]

[1]*Institute of Bioproduct Development, University Technology Malaysia, Kuala Lumpur*
[2]*School of Chemical Engineering, University Technology Malaysia, Skudai Johor*
wangsinghii@yahoo.com, r-azila@utm.my, mariani@utm.my

ABSTRACT
Andrographolide, a diterpenoid lactone obtained from Andrographis paniculata, has numerous therapeutic benefits such as anti-inflammatory, antioxidant activities, antiviral and antibacterial. However, it is restricted with its low water solubility, short-life time and low permeability. Nanoencapsulation of the drug can increase the solubility and yet increase the release rate of the drug at the targeted site. The purpose of this study was to evaluate the potential of polyelectrolyte nanoparticles based on hyaluronan/chitosan (HA/CS) as carriers for Andrographis paniculata (AP) extract. Nanoencapsulation with ionic gelation method result the encapsulation efficiency of 83.99% at 1:1 of CS:HA ratio. The particle size and zeta potential is 135.73nm and - 4mV respectively. The size became larger with the increasing of HA amount. Influence of the pH on the physical characteristic of nanoparticles investigated. In conclusion, the HA/CS nanoparticles might be a promising carrier for Andrographis paniculata.

Keywords: Andrographolide, *Andrographis paniculata*, nanoencapsulation, ionic gelation, chitosan, hyaluronan

1. INTRODUCTION

Andrographis paniculata (AP) has used in Asian countries as a medicinal herb. The active compounds found in AP have more than 20 diterpenoids and 10 flavonoids [6]. The main diterpenoid is Andrographolide, which is the primary medicinal component of AP. Andrographolide, has the pharmacological activities include anti-inflammatory, antioxidant, antiviral and antibacterial [5]. However, its therapeutic benefits are limited with low water solubility, low permeability and short-life time [2]. Development of the delivery system for natural drugs can solve their limitations. The solubility of the drugs can enhance with nanonization technology via modification of particle size [2].

Polyelectrolyte nanoparticles (PENPs) are developed with two oppositely charged polyelectrolytes interact via the electrostatic attraction upon mixing in aqueous solution. PENPs can protect the encapsulated drugs against the chemical degradation via simple preparation steps [8]. Ionotropic gelation method involved the ionically cross-linked of the two oppositely charged polyelectrolytes. Addition of hyaluronan to chitosan-based nanoparticles enhance in vivo, the stability controlling the nanoparticles' aggregation phenomena [3].

The main purpose of this research was to study the formation of PENPs based on HA/CS-TPP and to evaluate the potential of PENPs as a carrier for AP extract. PENPs are characterized with the particle size, zeta potential and encapsulation efficiency.

2. MATERIALS AND METHOD

The *Andrographis paniculata* extract was purchased from IBD, UTM. Chemicals used in this study are chitosan (DD 90%), sodium hyaluronan, sodium tripolyphosphate, sodium hydroxide, and methanol.

2.1 Ionic gelation method

The preparation of AP loaded chitosan/hyaluronan nanoparticles was using ionic gelation method [7]. The concentration of chitosan and sodium tripolyphosphate (TPP) were constant at 0.625 mg/ml and 05 mg/ml respectively. 0.3125 mg/ml of hyaluronan was prepared and added dropwise into chitosan

solution under magnetic stirring. The step repeated with hyaluronan concentration of 0.625 mg/ml and 1.25 mg/ml. With three different concentrations of hyaluronan, the CS/HA solutions had three different weight ratio.

AP was initially added into TPP. The AP-TPP was added dropwise into CS/HA solution. Then the solution brought to ultrasonification for 3 minutes under 40% amplitude.

2.2 Characterization of the nanoparticles

Particle size, zeta potential and PDI of the three nanoparticles solutions were analysed using Zeta sizer Nano ZS (Malvern Instruments, UK). Encapsulation efficiency was determined by HPLC with the formula of:

$$\frac{Amount\ of\ total\ AP - Amount\ of\ free\ AP}{Amount\ of\ total\ AP} \times 100\%$$

2.3 Influence of pH on nanoparticles

The nanoparticle solution was adjusted to pH 5 using sodium hydroxide solution. The characterization of the solutions were determined using Zeta sizer in order to obtain particle size, zeta potential and PDI.

3. RESULTS AND DISCUSSION

The relationship between the weight ratio of CS to HA and the resulting characteristics are shown in Table 1. The particle size increase with the increasing of hyaluronan concentration but decrease for both zeta potential and encapsulation efficiency. The adsorption of polyanion on positively charged nanoparticles can result in agglomeration which lead to the high value in particle size [4]. The weight ratio of 1:1 appeared to have desired results with particle size of 135,73 nm and encapsulation efficiency of 83.99%. The electrostatic stabilization between the two oppositely charged polyelectrolytes lead to the desired results.

Table 1. Effect of the weight ratio of CS to HA on the PDI, zeta potential and particle size

CS:HA ratio	Particle size (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (%)
2:1	437.93	0.446	-19.67	72.18
1:1	135.73	0.338	-4.00	83.99
1:2	4273.33	0.037	-4.47	22.22

The pH of the solution determines the degree of ionization of carboxylic acids groups of the HA and amino groups of the CS. This will affect the particle size, seta potential, PDI value and encapsulation efficiency, which mean the formation of the nanoparticles [8]. The particle size increase for the pH 5 as shown in Figure 1. However, the particle size decreased for the weight ratio of 1:2. The electrostatic attraction among the oppositely charged polyelectrolytes affected by the pH [1]. Besides, the encapsulation efficiency is the highest for weight ratio 1:2 in pH5 as compared to other weight ratio, as shown in Figure 4. Tis phenomena is different with pH2.

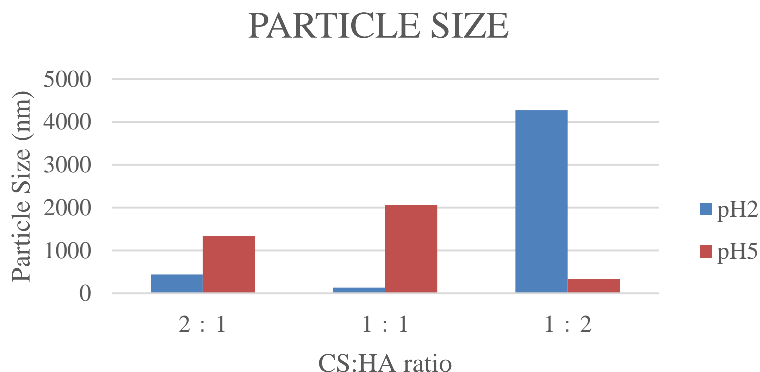

Figure 1. Particle size for nanoparticles of pH2 and pH5

All PDI values for the set of pH2 are under 0.5, reflecting homogenous preparations as shown in Figure 2. The highest pH lead to the increasing of PDI values which reflect the minor differences in the homogeneity of the polyplex suspensions [1].

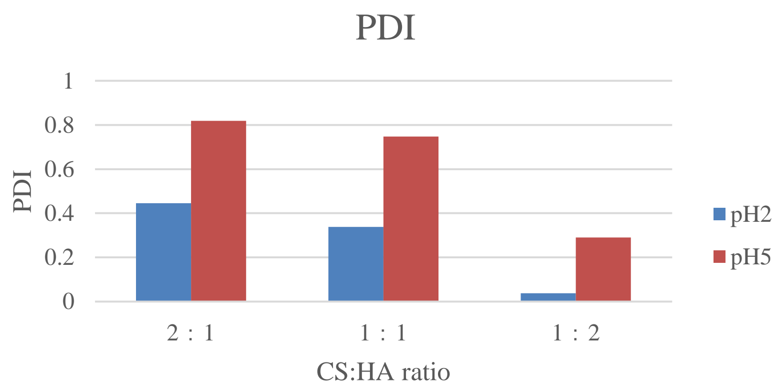

Figure 2. PDI for nanoparticles of pH2 and pH5

Zeta potential indicates the stability of the nanoparticles. The zeta potential for weight ratio of 1:2 for pH5 is higher than pH2, as shown in Figure3. This may due to the decreasing of the particle size of the nanoparticles.

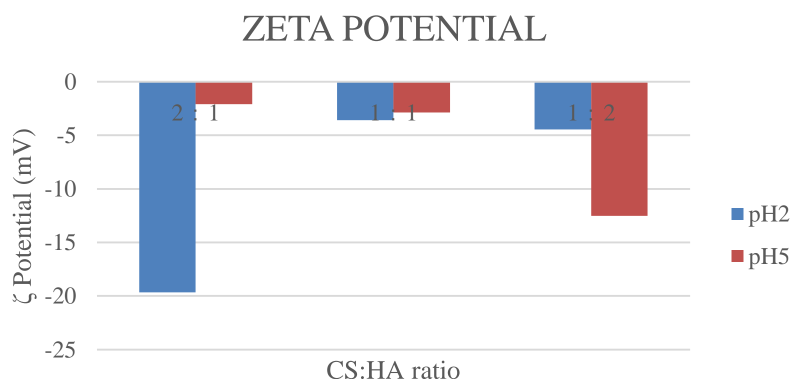

Figure 3. Zeta potential for nanoparticles of pH2 and pH5

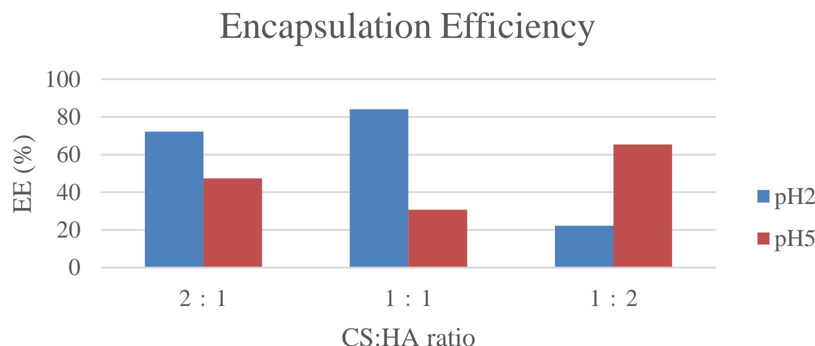

Figure 4. Encapsulation efficiency for nanoparticles of pH2 and pH5

4. CONCLUSION

CS:HA ratio of 1:1 showed the best result with the particle size less than 200nm and encapsulation efficiency 83.99%. However, the value of zeta potential quite low which will lead to the instability of the nanoparticles.

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