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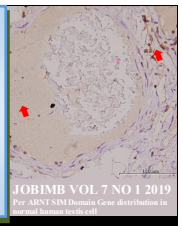

Encapsulation of *Lactobacillus plantarum* with Mannan and Sodium Alginate Improves its Cell Production

Muhamad Amin bin Jahari[1]*, Shuhaimi Mustafa[1,2], Muhamad Akhmal Hakim Roslan[3], Dhilia udie Lamasudin[2] and Yazid Abd Manap[1]

[1]Halal Products Research Institute, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia.
[2]Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia.
[3]Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author:

Muhamad Amin bin Jahari
Halal Products Research Institute,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia
Tel: +03-97691830
Fax: +03-89439745
Email: muhamadamin@upm.edu.my

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ABSTRACT

Probiotic bacteria are utilized in aquaculture as they exert a crucial function in promoting and maintaining the fish health. Probiotic strains should be present in a viable form during consumption and throughout the gastrointestinal tract for maximum health benefits. Many reports stated that there is poor survival of probiotic in products containing free probiotic cells. Providing probiotic living cells with physical barrier to resist adverse environmental conditions is therefore an approach currently receiving considerable interest. In this study, *Lactobacillus plantarum* was encapsulated with mannan and sodium alginate to increase probiotic viability. Response surface methodology (RSM) based on Box-Behnken design was used to optimize the encapsulation process with mannan concentration (5 to 30 % w/v) and sodium alginate concentration (1 to 5 % w/v) as the independent variables evaluated. According to the regression coefficients and significance of the polynomial model, the optimum encapsulation parameters were as follows: 24.73 % w/v mannan; 1.6 % w/v sodium alginate. Under these conditions of encapsulation, the total cell production of the *Lactobacillus plantarum* was increased to 5.3 (10^8 CFU/g) as compared to the free cell culture, 3.2 (10^8 CFU/g).

INTRODUCTION

Probiotics can be defined as small living organisms that serve as living feed supplement for the host by promoting the health of host intestinal [1]. Recently, the use of probiotic has been continually polished with health-promoting properties of aquatic animals [2-5]. A distinguish meaning of probiotic for aquatic life has been suggested as 'relation between bacteria and host is not only restricted to the intestinal part, rather also involving nature of fish farming that deals with the waters harbours microbial communities' [6]. According to author in [7], 'probiotic for aquaculture is a live, dead or constituent of a bacterial cell that, when embodied via the feed or to the rearing water, profited the host by building up either disease safeguarding, health status, growth performance, feed utilisation, stress response or general vigour, which is achieved at least partly either by improving the hosts or the environmental microbial balance." There are a lot of probiotic strains that were isolated from the aquatic animal including *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Streptococcus lactis [8]*. According to authors in [9], probiotic that was added into feed can give several health benefits such as enhance immune system and improve the function of gastrointestinal. The application of probiotics has revealed a fresh beginning era in commercial fish farming and its solicitation are attaining development of commercial and scientific concern in which shows a relatively increase in health-promoting foods to growth supplements, prophylactic and therapeutic [10-13].

Lactobacillus is a Gram-positive bacterium, catalase negative and non-sporulating with a rod-like shape [14]. It is also common to find this species of bacteria as an inhabitant of the gastrointestinal tract (GI) of various vertebrates, including freshwater fish [14], as it can ferment various carbohydrates mainly to lactate and acetate. Some ecological role played by *Lactobacillus* spp. are contributing to the production of antimicrobial substances thus enhancing the immune response and improving resistance level of a fish against bacterial pathogens which in turn will increase the availability of nutrients and use of some non-digestible carbohydrates [15].

Lactobacillus had been administered as a dietary supplement in recent years in order to enhanced growth, disease resistance and innate immune responses of *Epinephelus coioides* [16], Nile tilapia, *Oreochromis niloticus* [11,17] and *Epinephelus bruneus* [18] and protect fish from various infectious diseases [19]. Among numerous strains of *Lactobacillus*, *L. plantarum* probably is best strain to be used in aquaculture field as many previous studies had demonstrated positive effects of this bacterium on various fish species [11, 20, 21]. For instance, authors in [20] reported *O. niloticus* that had been given *L. plantarum* NIOFSD018 showed significantly better growth, feed utilization and improved enzyme activities of lipase, protease and amylase in the gastrointestinal tract (GI). Similarly, authors in [21] also claimed that the fish showed reduced viable culturable heterotrophic bacteria counts thus increase the lactic acid bacteria number in the fish gut which increase the number of leucocytes and thrombocytes, that in turn, increases the final weight and feed efficiency when they fed on *L. plantarum* supplemented diet for 12 weeks.

The probiotic advantageous is strongly influenced by their survival and multiplication in the host [22]. Numerous investigations have revealed low viability of probiotics within dairy based products like yogurt and fermented milk [23-25]. Thus, to improve viability of probiotic in both intestinal tract and food products, and for easy handling during mass production, dairy fermentations with encapsulation in hydrocolloidal beads has been proposed to protect the probiotics [26-28].

Encapsulation is a mechanical and chemical procedure whereby active substance within particle are concealed by a film of another element, that act as shield and controller of releasing the main ingredients [22]. This technique plays a major role to protect the probiotic, and subsequently increase its viability within the host. According to author in [29], functional properties of the capsules influenced by the composition of the coating materials. Therefore, sodium alginate is preferred among all other coating materials due to its simplicity, inexpensive, biocompatibility, and non-toxicity [30,31]. Authors in [32] defined alginic acid as a type of polyuronic acid (which also known as natural polymer) that is composed of various proportions of 1-4 linked β-D-mannuronic (M) and α-L-guluronic (G) acids, extracted from seaweeds. Presence of these residues are in various proportions, depending on the source of alginic acid [32].

Alginic acid and its salts are block copolymers that consist of mixed blocks containing M and G unit of irregular sequences and both homopolymer blocks of MM and GG [32]. The constitution and arrangement of the block residues affect the binding divalent cations and the subsequent gel formation [33]. The GG blocks contains a favourable binding site for divalent counter-ions, such as Ca^{2+}, and the bound ions interact with other GG blocks to form linkages that lead to gel formation [32]. On addition of sodium alginate solution to calcium chloride solution, instantaneous interfacial polymerization with precipitation of calcium alginate is rapidly occurred [32]. The distance between outlet and the coagulation solution, the viscosity of the polymer solution, and the diameter of the orifice will influence the beads size [34,35]. The encapsulation technique showed a better coating capability when use mixture of prebiotics, sodium alginate and calcium chloride. This was proven by authors in [22] when they utilize prebiotics (fructooligosaccharides), a growth promoter (peptide) and sodium alginate as coating substance to encapsulate various probiotics include *L. acidophilus, L. casei, B. bifidum and B. longum*.

A combination of sodium alginate (1 % w/v) mixed with peptide (1 % w/v) and fructooligosaccharides (3 %) as coating substances generated the best survival in terms of probiotic count [22]. The appropriateness of combining probiotics and prebiotics demonstrating greater effectiveness compared with probiotics alone [36]. According to authors in [22], incorporation of prebiotics and sodium alginate acts as coating material may deliver better protection for probiotics in food and yet the intestinal tract due to the potential for synergy between probiotics and prebiotics. So far, however, there has been little discussion about the utilization of mannan as prebiotic in encapsulation process, which lead to this research on optimizing the encapsulation process using mannan and alginate as encapsulation matrices to increase the cell production.

MATERIALS AND METHODS

Sample Preparation

Lactobacillus plantarum ISO14 was purchased from Halways Sdn Bhd (Serdang, Selangor, Malaysia). The bacterium was cultivated and propagated using MRS broth media. Cultures were incubated anaerobically at 37ºC in an anaerobic bottle on a rotary shaker set at the speed of 100 rpm. Cultures were harvested after 3 days by centrifugation (3000 × g, 10 min at 4 °C), washed and resuspended twice in saline solution [22]. Mannan and sodium alginate were obtained from Triovator Industries (Kulim, Kedah, Malaysia) and Sigma-Aldrich Co. (Oakville, ON, Canada) respectively. The hardening agent was 0.1 M calcium chloride solution.

Encapsulation of probiotics

Cells of *L. plantarum* ISO14 were encapsulated by emulsification–internal gelation technique in mannan-alginate beads [30]. The gel matrix was prepared by dissolving the mannan (5 to 30 % w/v) and sodium alginate (1 to 5 % w/v) in boiling water with continuous stirring and autoclaved at 121ºC for 15 minutes. The gel matrix (temperature 30ºC) and cell suspension were mixed and stirred continuously until uniform mixture was obtained.

The cell suspension mixture was hardened by adding it into cold sterilized 0.1 M $CaCl_2$ solution by using syringe and was left for 10 minutes to form beads. The beads were gently washed 4 times with sterile distilled water. All procedures were executed aseptically. The encapsulated cell was reproduced to form high cell density capsules by incubation in nutrients broth at 30ºC for 3 days with shaking at 100 rpm. The viable cells were counted via plating on nutrient agar, using the pour plate method, followed by incubation for 48 h at 37 ºC in anaerobic conditions.

Optimization of encapsulation matrix using response surface methodology (RSM)

Response surface methodology (RSM) based on the Box-Behnken was used as the experimental design for the encapsulation matrices with two independent variables [37]. **Table 1** shows the coded and actual variables of the two independent variables consisting of mannan (X_1, %) and sodium alginate (X_2, %). The percentage mannan and sodium alginate were the amount of materials to be used as encapsulated-cell production experiment. The biomass yield or cell production was taken as the dependent variable of the encapsulation. The experimental design, which included 13 runs, was developed using Design Expert 7.0.0 software (Stat-Ease, Tnc., USA). Regression analysis was performed to analyse the results of the dependent variables.

Statistical Analysis

All experiments were carried out in three replicates. Design Expert 7.0.0 software (2005) was used for the analysis of variance (ANOVA) of the experimental results. Minitab software version 16.2.4 (2013, Minitab Pty Ltd, Sydney, Australia) were used for t-test and ANOVA analysis. All significant differences were considered at significant level of $p < 0.05$.

RESULTS AND DISCUSSIONS

Response surface modelling (RSM) for optimization of encapsulation matrices

The experimental and predicted models for cell production in the encapsulation matrices were generated and presented in **Table 1**. This study revealed that combination of two independent variables (mannan and sodium alginate) in 13 separation runs showed the significant change of biomass yield range of 2.3 (CFU/g) to 6.6 (CFU/g), under different experimental conditions.

Table 1. Experimental design for the cell production of encapsulated-cell cultures under different combination of encapsulation matrices with respective coded factors, actual variable levels and response function for biomass yield.

Run	Coded variables		Uncoded variables		Response	
	X_1	X_2	Mannan (X_1 % w/v)	Sodium alginate (X_2 % w/v)	Biomass yield (10^8 CFU/g sugar consumed)	
					Experimental	Predicted
1	-1.00	-1.00	10.00	1.00	3.10	2.73
2	1.00	-1.00	30.00	1.00	2.60	2.68
3	-1.00	1.00	10.00	5.00	2.80	2.78
4	1.00	1.00	30.00	5.00	3.50	3.92
5	-1.41	0.00	5.86	3.00	2.40	2.69
6	1.41	0.00	34.14	3.00	3.80	3.46
7	0.00	-1.41	20.00	0.17	2.30	2.52
8	0.00	1.41	20.00	5.83	3.70	3.43
9	0.00	0.00	20.00	3.00	6.50	6.38
10	0.00	0.00	20.00	3.00	6.60	6.38
11	0.00	0.00	20.00	3.00	6.10	6.38
12	0.00	0.00	20.00	3.00	6.30	6.38
13	0.00	0.00	20.00	3.00	6.40	6.38

Table 2 shows the ANOVA for the optimisation of encapsulation matrices condition for cell production. The models showed an insignificant lack of fit ($P > 0.05$), which indicated the models could be used to predict values for the cell production during encapsulation of probiotics. The model is not a good indicator for the response and should not be used for prediction if it has a significant lack of fit [38].

Table 2. Analysis of variance (ANOVA) for optimization of the cell production of encapsulated-cell cultures.

Response	Total biomass yield (10^8 CFU/g)		
Source	Df	*F*-value	Prob>F
Model	5	64.80	<0.0001
X_1	1	5.28	0.0551
X_2	1	7.40	0.0298
X_1X_2	1	3.20	0.1167
X^2_1	1	168.96	<0.0001
X^2_2	1	179.34	<0.0001
Residual	7		
Lack of fit	3	5.76	0.0620
Pure error	4		
Cor total	12		
R^2	0.9789		
Adj R^2	0.9637		
C.V. %	7.77		

Based on the analysis, the predictive models developed for cell production via RSM was acceptable ($P < 0.0001$) with coefficient of determination (R^2) value of 0.9789, which shows only 2.11% of the total variation was not explainable by the model. The goodness of the model can be determined by coefficient of determination (R^2) value, as the better correlation between experimental results with the predicted indicates by the higher the R^2 value (more than 0.85) [39]. The value of adjusted coefficient of determination ($Adj\ R^2$) was 0.9637, which illustrated that the model was significant and adequate used as predictor model. Similarly, authors in [38] found that their RSM models to be adequate as predictor models, when the coefficient of determinations of regression equations ranged from 0.8561 to 0.9948 with significant probability values ($P < 0.0001$) and non-significant lack of fit.

For the predictive model of biomass yield, the linear coefficient (X_2) and quadratic coefficients (X_1, X^2_2) were found to be significant, indicated by the small *P*-value ($P < 0.05$). The other term coefficients (X_1, X_1X_2) were not significant with large *P*-values ($P > 0.05$). The analyses of both the independent and dependent variables resulted in the following polynomial regression model equation was fitted as follows (Equation 1):

$$\text{Biomass yield (CFU/g)} = -4.189 + 0.6436\,X_1 + 2.413\,X_2 - 0.01653\,X^2_1 - 0.4252\,X^2_2 + 0.01500\,X_1X_2$$

Optimizing combinations of coating materials

Fig. 1 shows the response surface plot simulated by the adjusted model. The 3D plot was acquired by plotting the biomass yield (CFU/g) on the Z-axis against two factors which are mannan (X-axis) and alginate (Y-axis). The cell production under different combination of alginate and mannan as encapsulation matrices, significantly enhanced ($P < 0.05$) biomass content of bacteria.

The response surface plots in **Fig. 1** illustrates the conditions for optimum biomass yield production. Interestingly, the highest value of biomass yield was obtained when the alginate content was high, with high mannan concentration. This outcome is similar to that of authors in [40] who found high levels of alginate provide better protection for encapsulated probiotics subsequently increases its yield.

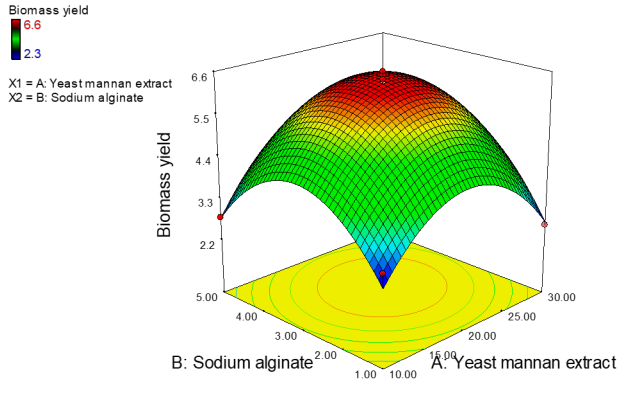

Fig. 1. The effect of sodium alginate and mannan extract on biomass yield.

As presented in **Fig. 1**, the best sodium alginate and mannan concentration to produce highest biomass yield were 1.66 % (w/v) and 24.73 % (w/v), respectively. This finding is consistent with that of authors in [39] who also reported the optimal composition for alginate as encapsulation matrix, was 1.12 % (w/v), which is not far from our finding. Authors in [41] stated the concentrations of alginate used for gel formation fluctuate from 1.5 % to 2.5 % with 0.05 to 1.5 M $CaCl_2$. In this study, concentration of alginate in the range of 1 % to 5 % were tested as preliminary tests revealed that when blended with prebiotics it could enhance probiotic encapsulation.

The selected alginate concentration range was also in accordance to previous researches that have established optimal concentrations of the alginate used was in between 1 % to 2 % (w/v) [42, 43], because low concentrations (<1 % (w/v)) of alginate used as the supporting gel resulted in low viscosity, nonspherical and varied encapsulated bead shape; whereas, a high concentrations (>2 % (w/v)) of alginate gel along with mixture with other gels make it difficult for extrusion process due to high viscosity [44]. The current study found the optimal value (1.66 % (w/v) sodium alginate) and subsequently used it for the preparation of optimum capsules, which is consistent to that of authors in [41] finding.

The improvement of the encapsulation process was continuously done as an attempt to increase the viability of the encapsulated bacteria in the harsh condition through increment of alginate gel concentration from 0.75 % to 1.8 % (w/v) [45]. Our own findings were anticipated and proportional to most prior researches, suggesting a relatively high level of sodium alginate (1.66 % (w/v)) as coating materials could improve cell production. Contrarily, declination of alginate concentration 2.0 to 0.5 % persuasively reduce the cell survival rate which perhaps was due to the protecting ability of alginate [42].

Additionally, this study also suggests synergistic use of prebiotics with sodium alginate as coating materials for probiotic encapsulation did improve probiotics viability. This finding broadly supports the work of other studies in this area linking probiotics with prebiotics, and alginate as encapsulation matrix gave better protection for probiotics in food and eventually in the intestinal tract [36, 40, 46]. Although extensive research has been carried out on prebiotic use as encapsulation material, only a few studies exists which emphasizes on utilization of prebiotic mannan oligosaccharides (MOS) as encapsulation material to improve viability of probiotic [47-49]. On the other hand, several studies have investigated the use of mannan in feed formulation and its positive effects on the fish growth performance [50-52]. Therefore, this study provides new insights into use the of mannan as encapsulation materials for probiotic and its effect on fish growth rate when incorporated into aquafeed.

Verification experiment for predicted RSM models

Additional experiment was conducted in order to verify the optimum combination of encapsulation matrix on the effectiveness of encapsulation in retaining cell viability. The optimal combination of the encapsulating materials for the probiotic capsules is 24.73 % (w/v) mannan blended with 1.66 % (w/v) alginate. **Table 3** shows the response value derived from the verification experiment illustrates no significant different ($P < 0.05$) between experimental and predicted results, strengthen the previous statement stating this model adequate to be used as predictive model. Moreover, the experiment also proved encapsulation technique able to produce a higher yield of probiotic as compared to free cell culture ($P < 0.05$).

Table 3. Cell production of free and immobilized-cell culture.

	Free cell culture	Immobilized-cell culture (Experimental)*	Immobilized-cell culture (Predicted)
Biomass yield (10^8 CFU/g)	3.2[a]	5.3[b]	5.07[b]

Means ± standard deviation (n = 3) with different superscripts within rows are significantly different ($P < 0.05$).
*Cell immobilization parameters: yeast mannan extract, (24.73 % w/v); sodium alginate, (1.66 % w/v).

CONCLUSION

The present study demonstrated that encapsulation of *Lactobacillus plantarum* ISO14 with mannan (1.66 % w/v) and sodium alginate (24.73 % w/v) through response surface methodology (RSM) technique would produce the highest survival of the probiotic. The verification experiment yielded result close to the expected values, with no significant difference ($P > 0.05$) suggesting the model is adequate to be used as predictive model.

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