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Study on the effects of Carbohydrate-Protein-Coconut Oil on the Viability of *Lactobacillus bulgaricus* during Spray Drying, Simulated Gastrointestinal Conditions and Unrefrigerated Storage by Simplex-Lattice Mixture Design

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Ideally, probiotics bacteria should be metabolically stable during processing, storage under unrefrigerated condition, survive passage through the upper digestive tract in large numbers for colonisation and proliferation in the large intestine, and have beneficial effects when in the intestine host. This study helps to understand the effect of feed composition towards viability of *L. bulgaricus* by using carbohydrate-protein-coconut oil mixtures. The encapsulation work was focused on the performance of carbohydrate-protein-coconut oil as the protective barrier. A simplex-lattice design was employed to study the effect of different feed formulation of the carbohydrate-protein-coconut oil mixtures on three response variables; the viability of *L. bulgaricus* after spray drying, under simulated gastrointestinal condition, and after 6 weeks storage in unrefrigerated environment. Analysis of variance and mixture design techniques were applied to identify the optimal feed composition. The optimal feed composition that results in the viability beyond the therapeutic minimum dose consisted of 30 % gum Arabic, 16.95 % gelatine and 53.05 % coconut oil. The percent of viability after spray drying, under simulated gastrointestinal condition, and after 6 weeks of storage were 4.24 %, 11.43 % and 77.36 % respectively. In conclusion, the mixture of gum Arabic - gelatine - coconut oil shows more protecting to protect against damaging of oxidative stress during storage under unrefrigerated condition.

1. Introduction

There is a growing trend in health awareness all over the world where consumers and health professionals are adopting health promotion and disease preventive strategy. Probiotics, on the other hand, promote a desirable gastro-intestinal microflora which is known to enhance the overall wellness. Given that probiotic microorganisms play an important role in promoting and maintaining health (Amara and Shibi, 2015). It has stimulated considerable interesting incorporating them into functional foods and healthcare products (Meybodi and Mortazavian, 2017). It is recommended that probiotic products should contain at least 10^6 to 10^9 live microorganisms per g or per mL in order to obtain the desired therapeutic effects (Spigno et al., 2015). Lactobacilli sp. has big potential use in the probiotic industries based on its high stability during processing and resistant to acidic and bile condition of gastrointestinal route (Malek et al., 2010). Several studies have shown several encapsulation agents may protect the probiotic bacteria during processing. Most of the known encapsulation agent though, intrinsically lack in the ability to withstand the harsh conditions of the gastrointestinal track and maintained viability of the probiotic cell during storage (Yonekura et al., 2014). Various strategies have been developed to increase the viability upon reaching the colon. The environmental condition in gastrointestinal tract such as pH, microflora, enzymes, reducing medium and transit time should be taken into consideration (Dianawati et al., 2016). It is known that certain type of polysaccharide such as inulin, sodium alginate, carrageenan gum, gum Arabic, fructo-oligosaccharide are used as encapsulating agents (Iravani et al., 2015). Proteins are also the favourable encapsulation agents because the cells are likely

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to be buffered by the protein and protected to the extreme pH in the stomach (El-Salam and El-Shibiny, 2015). The presence of polysaccharide alone though, will result in poor survival characteristic during spray-drying and under gastrointestinal condition and product storage (Rodrigues et al., 2017). It is noted that the mixtures of encapsulation materials using carbohydrate-protein-lipid mixtures are more stable microcapsules than protein-protein mixtures or carbohydrate – carbohydrate mixtures alone because of a phase separation within the formulation matrix during drying (Trojanowska et al., 2017). The aim of this study is to investigate effects of the encapsulating mixtures of carbohydrate-protein-coconut oil that can protect probiotic cells during spray drying, storage at unrefrigerated condition and they can reach the small intestine and the colon in the viable state. Mixture design methodology has been proposed to study the influence of individual factors and their interactive effects of the mixtures, as well as obtaining an optimal formulation with desirable viability during spray-drying and under gastrointestinal condition and storage (Zen et al., 2015).

2. Materials and Methods

2.1 Inoculum preparation

The probiotic strain used in this research, *Lactobacillus bulgaricus* obtained from Universiti Teknologi Malaysia, Johor Bahru, Johor, Malaysia. Cells were obtained in frozen glycerol culture and were activated on MRS agar medium, DeMan, Rogosa, Sharpe Agar. MRS medium was composed of (g/L): Glucose, 20.0; Yeast extract, 5.0; Peptone, 10.0; Beef extract, 10.00; Polysorbate 80, 1.0; Ammonium citrate, 2.0; Sodium acetate, 5.0; Magnesium sulphate, 0.1 and Dipotassium phosphate, 2.0. The pH was adjusted to 6.5 before autoclaving and incubated 37 °C. After 24 h of incubation, the cells on the solid medium were dissolved with glycerol. Stock cultures were preserved in glycerol (50 %, v/v) at -80 °C as working cell bank which was used as starting culture for inoculum preparation for each experiment.

2.2 Preparation of cells before spray drying

L. bulgaricus was cultivated in the MRS broth. When growth of *L. bulgaricus* reached the stationary phase (12 h), the cells were harvested by centrifugation at 6000 x g for 15 min and were then washed twice with PBS. The cell paste was kept at 4 °C and was used at the same day.

2.3 Statistical experiments design

The simplex-lattice design of mixture design was used to evaluate the effect of gum Arabic, gelatine and coconut oil on the viability of *L. bulgaricus* during spray drying, under simulated gastrointestinal tract and during storage for 6 weeks at 25 °C. Component proportions were expressed as fractions of the mixture with a sum (gum Arabic + gelatine + coconut oil) of 100 %. The value of the maximum and minimum of the independent variables were obtained from the preliminary experiments when the percentage of gum Arabic between 30 - 40 %, gelatine at 20 - 25 % and coconut oil 40 - 55 %. In the simplex lattice design, 14 experiments were carried out. The following polynomial Eq(1) of function β_i was fitted for each factor assessed at each experimental point. This polynomial model differs from full polynomial models because it does not contain a constant term (intercept equal to zero). The polynomial model Eq(1) was used where Y is the estimated response; β_1, β_2, β_3, β_{12}, β_{13} and β_{23} are constant coefficients for each linear and non-linear (interaction) term produced for the prediction models of processing components. The analysis was analysed by Design Expert (V.6.0.8).

$$Y = \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} AA + \beta_{22} BB + \beta_{33} CC + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC \quad (1)$$

2.4 Preparation of the encapsulating agents

The composition of the mixtures was formulated as mentioned in Table 1. Gum Arabic and gelatine were dispersed in distilled water at 50 °C to fully dissolve of biopolymer solutions. The obtained solutions were heat treated at 80 °C for 15 min to destroy contaminants. The coconut oil was separately heated at 80 °C for 15 min. The heated coconut oil was cooled at 40 °C before mixed with probiotic cells-paste. The mixtures were mixed well together before spray drying processes.

2.5 Spray drying process

The feed suspensions were spray dried in a laboratory spray dryer (LU 228 Labultima, India) to produce the encapsulation powder. Inlet air temperature was filtered and electrically heated to a constant inlet air temperature of 150 °C for moisture content of powder below 4 %. The outlet temperature was stabilised at 80-85 °C by controlling the flow rate at 4 mL/min. The mixtures were sprayed into drying chamber by using atomiser nozzle, and the product dried almost instantaneously. The powders were then transferred to sterile bottles with screw-on lids and stored as required for viability assessment.

2.6 Enumeration of strain viability using spread plate technique

The viability of the *L. bulgaricus* was assessed using the spread plate method on MRS agar. The plates were incubated at 37 °C for 24 h. The cell counts were performed in duplicate and means are reported. The viability of *L. bulgaricus* was evaluated based on % viability before and after subsequent treatments.

2.7 Survival of bacteria strain in simulated gastric juice and bile solutions

To evaluate the survival of encapsulated probiotics under conditions that mimic in vivo human upper gastrointestinal transit, an in vitro methodology was modified and prepared based on the method of Crittenden et al. (2006) and Sathyabama et al. (2014). The transit tolerance of the encapsulated of *L. bulgaricus* was determined by exposing 1 g of particles in simulated gastric juice (pH 2) and was vortexed for 20 s for complete mixing the dispersion. Initial sample (0 h) was taken immediately after mixing to determine the viability of cell. The mixtures were then incubated at 37 °C for 2 h. Following incubation in simulated gastric juice (SGJ), the pH of the samples was adjusted to 6.8 with 1N NaOH, and the bacteria were diluted 10-fold in simulated small intestinal juice (SIJ) 2.0 % at pH 6.8 and incubated for a further 4 h at 37 °C. The reaction was stopped by placing the samples in ice for 5 min. Viable counts of the bacteria were compared before and after passage through both stages in duplicate. The 1.0 mL samples taken immediately after mixing the particles or free cells with SGI and SIJ were mixed with 9 mL PBS and centrifuged for 5 min and washed twice with saline solution. Then serially diluted with PBS and pour plated on MRS agar. Colonies were counted after incubation at 37 °C for 48 h.

2.8 Survival of spray dried *L. bulgaricus* during storage

The survival of encapsulated *L. bulgaricus* cells was assessed during unrefrigerated storage over a 6-week period (Crittenden et al., 2006). The bacterial samples were stored in closed containers and kept at 25 °C.

3. Results and discussion

3.1 Interpretation of experimental data

Table 1 shows the results of the experimental work based on Simplex-Lattice Design with 2 replication of run.

Table 1: The Simplex-Lattice Design with 14 experiments of % viability encapsulated of L. bulgaricus after spray drying, under simulated gastrointestinal condition and after 6 weeks storage at 25 °C

Run	A	B	C	Response (% of viability)		
	Gum Arabic (%)	Gelatine (%)	Coconut oil (%)	After spray drying	Under simulated gastrointestinal	After 6 Weeks of Storage
1	40.00	15.00	45.0	5.00	8.89	32.9
2	30.00	25.00	45.00	2.65	8.71	33.30
3	30.00	25.00	45.00	2.57	9.57	30.30
4	31.67	16.67	51.67	5.65	8.17	70.30
5	33.33	18.33	48.33	9.43	7.64	38.47
6	36.67	16.67	46.67	11.26	6.60	35.75
7	30.00	20.00	50.00	3.38	13.07	60.59
8	35.00	20.00	45.00	15.7	10.56	60.40
9	40.00	15.00	45.00	4.87	9.76	39.00
10	35.00	20.00	45.00	15.3	9.87	52.54
11	30.00	15.00	55.00	5.86	8.00	90.00
12	35.00	15.00	50.00	5.58	4.19	36.8
13	31.67	21.67	46.67	7.87	10.00	36.20
14	30.00	15.00	55.00	4.86	9.15	89.2

The highest percent of viability after spray drying was observed when gum Arabic and gelatine at the maximum limit, while the coconut oil at the minimum limit in the feed composition. It was shown in run 8 at the % viability of 15.7, the amount of gum Arabic, gelatine and coconut oil were 35 %, 20 % and 45 %. The percent of viability under simulated gastrointestinal condition was at the highest when the mixture of gelatine and coconut oil at the maximum and the gum Arabic is at the minimum in the feed composition. It was observed in run 7, at 13.07 % viability under simulated gastrointestinal condition, the mixture of the gum Arabic, gelatine and coconut oil are 30 %, 20 % and 50 %. The percent of viability after storage is highest when the mixture gelatine and coconut oil at the maximum limit, while the gum Arabic at the minimum limit of

the feed composition. It was shown in run 11 at the % viability after storage is 90 %, the amount of gum Arabic, gelatine and coconut oil are 30 %, 15 % and 50 %. The coconut oil increased the % viability after storage while the increase of gum Arabic and gelatine concentration make the % viability after storage decreased.

3.2 Analysis of variants (ANOVA) of the response, encapsulated *L. bulgaricus* in carbohydrate-protein-coconut oil mixtures

The polynomial model obtained from the experiment was verified with a hypothesis test. The tabulated F-value ($F_{0.05,\ 5,8}$) for % viability and after storage was 4.82 and the tabulated F-value for under simulated gastrointestinal condition ($F_{0.05,\ 6,7}$) was 4.21 which is greater than the F value of the model means that the model is accepted. The analysis of variance is tabulated in Table 2 indicate that F value for % viability after spray drying, under simulated gastrointestinal condition and after storage are 151.95, 30.23 and 21.32. In these experiments, all responses gave R^2 above 90 % indicated that this study signifies a good correlation between experimental data and the predicted value which overall only 10 % was not explained by the model. The acceptances of the model were supported by the p-value < 0.05 which were significant for all responses (Karaman et al., 2015).

Table 2: ANOVA analysis of the responses

Response	F calculated	F Tabulated	R^2	Adjusted R^2	p-Value
Viability after spray drying	151.95	4.82	0.9896	0.9831	<0.05
Simulated gastrointestinal condition	30.23	4.21	0.9628	0.9310	<0.05
After 6 weeks storage at 25 °C	21.32	4.21	0.9032	0.8866	<0.05

3.3 Interpretation of contour plots

The interactions amongst the three components of feed formulation on the responses were studied in the 14 assays. The model equation was adequate for predicting the responses as shown in Figure 1. The residuals that is the difference between experimental and the predicted value from the regression are falling on the straits line which implies that the errors are distributed normally which also reflected the accuracy and applicability of the feed composition to the responses. Figure 1a shows the interaction between all three factors (gum Arabic, gelatine and coconut oil) and the % viability after spray drying. The sharpest contour on the response surface curve plotted presents the highest % viability. The area of the highest % viability is located on the left edge of the plots. The contour plot shows that the highest % viability after spray drying when gum Arabic and gelatine at the maximum limit, while the coconut oil at the minimum limit in the feed composition. As shown in mathematical equation of Eq(2), AB (gum Arabic : gelatine) presented a stronger effect on the % viability of encapsulated *L. bulgaricus* cell after spray drying process. As shown in mathematical equation of Eq(3), ABC (gum Arabic : gelatine : coconut oil) presented a stronger effect on the % viability of *L. bulgaricus* cell under simulated gastrointestinal condition. The area of the highest % viability under simulated gastrointestinal condition was located on the base of the plots as shown in Figure 1b. The contour plot shows that the % viability under simulated gastrointestinal condition was at the highest when the mixture of gelatine and coconut oil is at the maximum and the gum Arabic is at the minimum in the feed composition. The contour plot in Figure 1c shows the % viability after storage is highest when the coconut oil at the maximum limit, while the gum Arabic and gelatine at the minimum limit of the feed composition. The coconut oil increased the % viability after storage while the increase of gum Arabic and gelatine concentration result in the % viability after storage to decrease. The second order terms in mixture models of Eq(4). AC (gum Arabic x gelatine) presented stronger effects on preference as demonstrated by the higher coefficients.

$$\textit{Viability index} = +5.11A + 2.52B + 5.29C + 47.1AB - 3.22BC + 2.55AC \quad (2)$$

$$\textit{Gastrointestinal index} = +9.25A + 9.14B + 8.63C + 3.79AB - 19.14AC + 17.18BC - 45.14ABC \quad (3)$$

$$\textit{Storage index} = +35.90A + 30.88B + 91.93C + 75.94AB - 115.22AC - 16.98BC \quad (4)$$

3.4 Validation experiments

To verify the region predicted by contour plot as a region of maximum for the responses, validation experiments were made using the formulation composed of gum Arabic, gelatine and coconut oil at 30.00 %, 16.95 %, 53.05 % respectively. In this study, 4.09 % viability after spray drying was obtained at the value is 0.15 % lower than predicted. For the viability of *L. bulgaricus* under simulated gastrointestinal condition, 11.43 % was observed higher than predicted value. For the % viability after storage is lowered by 1.8 % than optimisation experiment, which is 75.54 %. It was observed that experimental values are closed to the

predicted value of the model. The spray-dried microcapsules produced were smaller from 5 to 15 µm in diameter as in Figure 2 with moisture contents lower than 4 %.

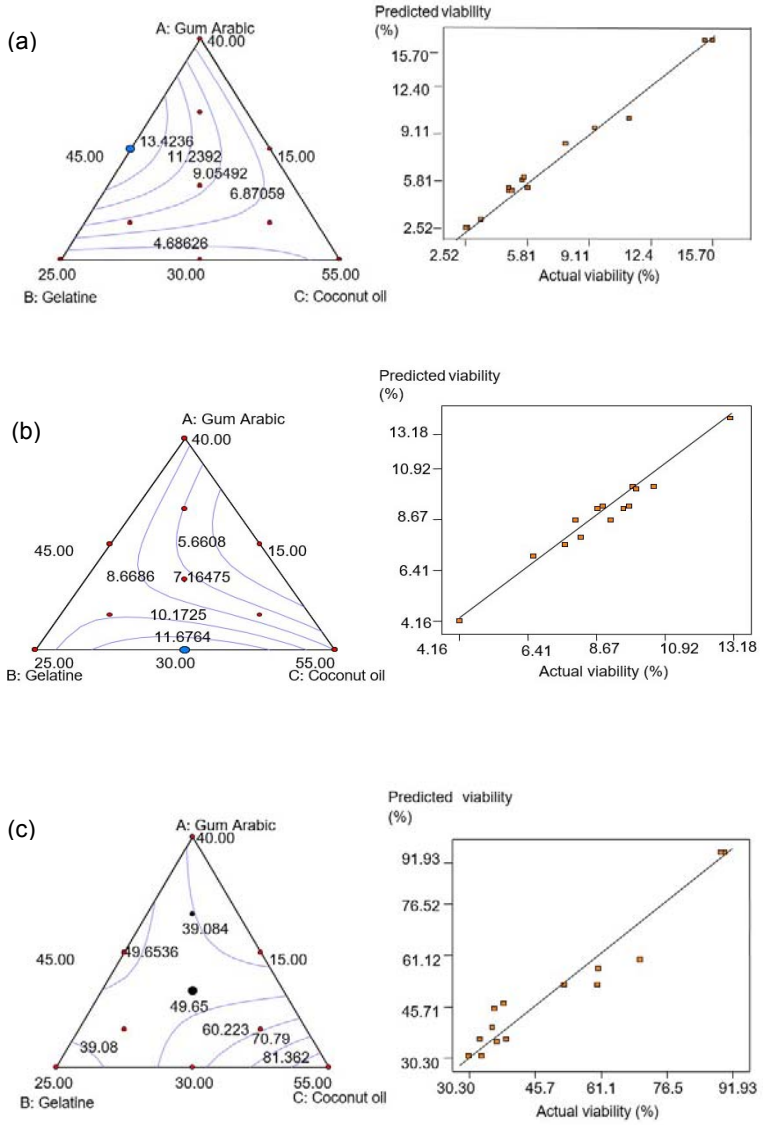

Figure 1: Mixture contour plots and fitted line plots of the experimental and predicted value of % viability (a) after spray drying, (b) under simulated gastrointestinal condition and (c) after 6 weeks storage at 25 °C

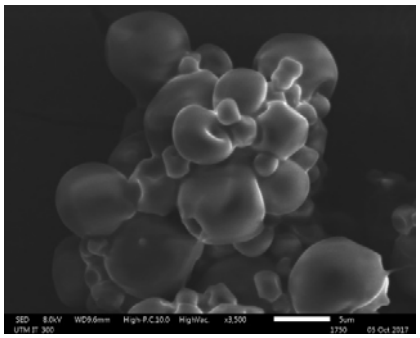

Figure 2: Scanning electron micrograph (magnification, x3,500) of spray-dried encapsulated L. bulgaricus

4. Conclusions

The concept of encapsulated allowed probiotic bacteria to be separated from its environment during food processing until reach human gastrointestinal tract for proliferation. This study demonstrated that, encapsulation of *L. bulgaricus* in the mixtures of carbohydrate-protein-coconut oil improved the viability of the probiotic cell during spray drying, under simulated gastrointestinal conditions and storage at 25 °C.

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