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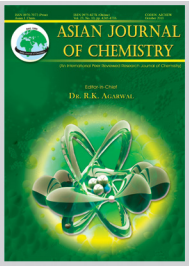

Evaluating the Effects of Process Variables on Protease-loaded Nano-liposome Production by Plackett-Burman Design for Utilizing in Cheese Ripening Acceleration

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Placket-Burman design was applied to evaluate the impacts of physicochemical parameters on multicomponent nano-liposomal formulations of protease. Liposomes (lecithin: cholesterol) were prepared by heating method without employing any toxic solvent or detergent. The aim of the present study was screening the effects of eleven factors including the pH, the cholesterol/lecithin ratio (w/w), the powder form food grade lecithin proportion (Ultralec®P) (% w/w), the enzyme/lecithin ratio (w/w), the stirring time (min), the process temperature (ºC), the speed of stirrer (rpm), the rate of stirrer diameter/tank diameter, the homogenization, the way of adding enzyme and the centrifugation conditions on the encapsulation efficiency (EE %) of nano-liposome. This nano-liposome was prepared according to 11-factor 12-run statistical model. The results showed that the lecithin (Ultralec®P) proportion, the rate of stirrer/tank diameter, the enzyme/lecithin ratio, the cholesterol/lecithin ratio (w/w), the speed of stirrer (rpm), the stirring time, the process temperature had significantly effect on encapsulation efficiency (EE %) ($p < 0.05$). The mean diameter and polydispersity index of successful formulation of flavourzyme-loaded liposome was 178 nm and 0.2 respectively.

Key Words: Plackett-Burman design, Nano-liposome, Heating method, Flavourzyme.

INTRODUCTION

Microencapsulation is a rapidly expanding technology with a great potential in different areas such as food industry[1]. It is a process by which core materials (solids, liquids or gaseous) are packaged within a wall to form microcapsules that can release their contents at controlled rates[2]. Liposome technology is among the most common techniques to produce encapsulated products[3]. The attractiveness of liposomes is based on many factors including the possibility of producing them from natural ingredients and in different sizes both in micrometer and nanometer scales[3-5].

Presence of protease and lipase in cheese may adversely affect the acceleration of cheese ripening without control[6]. Flavourzyme® (EC: 3.4.11.1) is a peptidases and proteases used for acceleration of cheese proteolysis[6-8]. Commercially, flavourzyme (an endo and amino-peptidase) is in a powder form and taken from *Aspergillus oryzae*[8]. However, addition of uncapsulated enzymes to cheese milk results in the premature attack of casein and the release of the majority of enzymes into the whey[9]. Currently, enzyme-loaded liposomes are used for acceleration of cheese ripening[10]. Recent papers showed nutrease[11], trypsine[12], α-chymotripsine[13,14], chymosine[15], neutral protease[16], cyprosine[17], fungal protease, flavourzyme, platase and microbial lipase[18,19] were encapsulated in liposome for acceleration of cheese ripening by a different methods. Different methods like reverse-phase evaporation, dehydration-rehydration, microfludization technique and proliposome usage have been used for liposome manufacture and their encapsulation efficiency varied from 10 to 40.3 %. However, most of them (except microfluidization technique and proliposome usage) require organic solvent, which caused enzyme inactivation[20] and are unacceptable in food products[13,21]. The main disadvantage of the microfluidization technique is the employment of very high shearing forces that can potentially damage the structure of compound to be encapsulated[22,23]. Other disadvantages of this method are material loss, its contamination and being relatively difficult to scale-

up. Proliposomes also are not cost-effective and their manufacture process is a multi-step, lengthy procedure[24]. On the other hand, heating method is a novel, scalable and robust method, which does not require employment of high mechanical stress, any harmful chemicals or extreme values of pH during their preparation[3].

The Plackett-Burman design (PBD) has been frequently used for screening of process variables. The use of a Plackett-Burman design can reduce considerably the number of preparations to 12 (N+1) for 11(N) factors at two levels each[25]. Consequently, Plackett-Burman design is useful for liposome studies due to the fact that each liposomal preparation is time consuming and requires costly materials. The use of Plackett-Burman design in the pharmaceutical applications of liposomes has been reported, recently. The study investigated compositional and procedure variation on the stability of multicomponent dehydration-rehydration liposomes[26].

The present study aimed to prepare flavourzyme-loaded nano-liposomes by a novel method (heating method) without employing toxic solvent and high shear forces or applying any other harmful procedure for the first time. PBD was used to assess the effect of chemical components (the lecithin (Ultralec®) (% w/w), the cholesterol/lecithin ratio (w/w), the enzyme/lecithin ratio (w/w) and pH) and process variables including the stirring time, the temperature, the rate of stirrer diameter/tank diameter, the centrifuge condition, the speed of stirrer, the way of adding enzyme and the homogenization on the encapsulation efficiency (EE %). The effective factors and their levels were selected based on the review of literature and also according to our previous experiences (unpublished data).

EXPERIMENTAL

Preparation of liposome: The Ultralec®P lecithin (ADM, Netherlands) is powder food grade soy bean lecithin (consist primarily phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) which utilized in liposome production. The water used for solution was obtained from a Milli Q water purification unit (Millipore, MA, USA). Preparation of liposome was based on the heating method[3,27]. All variables were introduced in Table-1. Cholesterol was first dissolved in glycerol (3 % w/v in final concentration) at 120 ºC. Flavourzyme (gifted from Novozyme, Tehran, Iran), Ultralec® lecithin (powder form) and cholesterol solution (in water) were added to a preheated (50 or 60 ºC) mixture of tris buffer (0.01 M) (pH: 6 or 8) as indicated in Table-2. Enzyme addition was conducted in two ways: A) addition of enzyme, lecithin and cholesterol in buffer solution simultaneously and B) addition of cholesterol and lecithin suspension in buffer after the complete solution of the enzyme. The mixture was stirred at 500 or 1000 rpm using hotplate stirrer (HCR_2, Gerhardt, Germany) with different stirrer diameter/tank diameters (0.45 or 0.9), while heating (at 50 or 60 ºC) for 5 or 15 min. After preparation of the liposomal samples, some of them were homogenized (10000 rpm, 1 min) according to Table-1. All the experiments were conducted in duplicate and the samples were kept overnight in 4 ºC under nitrogen atmosphere.

Determination of enzyme activity: Activity of flavourzyme was measured by the procedure of Kailasapathy *et al.*[28] and Anjani *et al.*[29] using L-leucine-*p*-nitroanilide (leu-*p*-Na) as a substrate based on the reaction rate and introduced as leucine aminopeptidaes units per milliliter (LAPU mL^{-1}).

TABLE-1
HIGHER AND LOWER LEVELS ASSAYED FOR THE 11 VARIABLES IN PLACKETT-BURMAN DESIGN

	Variable	Low level (-1)	High level (+1)
X_1	pH	6.00	8.00
X_2	Cholesterol/ lecithin (w/w)	0.05	0.10
X_3	Lecithin (% w/w) (Ultralec®P)	2.00	4.00
X_4	Enzyme/Lecithin ratio (w/w)	0.10	0.20
X_5	Stirring time (min)	5.00	15.00
X_6	Temperature (ºC)	50.0	60.0
X_7	Speed of stirrer (rpm)	500	1000
X_8	Homogenization (rpm)	0.00	10000
X_9	Way of enzyme addition	A	B
X_{10}	Centrifugation condition	40000 (60 min)	45000 (75 min)
X_{11}	Rate of stirrer diameter/tank diameter	0.45	0.9

[A]:Addition of enzyme, lecithin and cholesterol solution into the buffer solution; [B]:Addition of cholesterol and lecithin in buffer after complete solution of enzyme in buffer solution; [C]: Homogenized for 1 min

TABLE-2
EXPERIMENTAL PLACKETT-BURMAN DESIGN MATRIX AND ENCAPSULATION EFFICIENCY OF FLAVOURZYME-LOADED LIPOSOME PREPARED BY HEATING METHOD

Run no	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	EE (%)
1	1	-1	1	1	-1	-1	1	1	1	-1	1	13.86
2	1	1	-1	-1	-1	-1	-1	1	1	1	-1	9.125
3	-1	1	1	1	1	-1	-1	0	1	1	1	15.210
4	1	-1	1	-1	-1	1	-1	0	-1	1	1	16.195
5	1	1	-1	-1	1	-1	1	0	-1	-1	1	11.900
6	1	1	1	1	1	1	-1	1	-1	-1	-1	10.685
7	-1	1	1	-1	-1	1	1	0	1	-1	-1	10.445
8	-1	-1	1	-1	1	-1	1	1	-1	1	-1	14.270
9	-1	-1	-1	-1	1	1	-1	1	1	-1	1	14.103
10	1	-1	-1	1	1	1	1	0	1	1	-1	7.450
11	-1	1	-1	1	-1	1	1	1	-1	1	1	8.340
12	-1	-1	-1	1	-1	-1	-1	0	-1	-1	-1	8.365

Determination of encapsulation efficiency: According to Kheadr *et al.*[19] the liposomes were separated from uncapsulated enzymes by centrifugation at 40000 × g for 1 h or 45000 × g for 1.5 h at 4 ºC (centrifuge power was another variable) (Table-1) as shown in Tables 1 and 2. The activity of the encapsulated and free enzyme (as LAPU mL^{-1}) was used to estimate the EE (%) according to the following equation:

$$EE\ (\%) = \frac{\text{Encapsulated (LAPU mL}^{-1})}{\text{Uncapsulated (LAPU mL}^{-1}) + \text{encapsulated (LAPU mL}^{-1})}$$

Experimental design: A well defined statistical experimental design is considered to be necessary for optimization of a liposome preparation, since it would be possible to get more information through conducting fewer measurements during the process. The first optimization step was to identify the variables, which have significant effects on the encapsulation efficiency. Plackett-Burman design is one of the highly fractional designs, which allows for the study of k = (N-1)/(L-1) factors, each with L levels in N experimental trials.

Choice of these factors was based on previous experience (unpublished data) for preparation flavourzyme-loaded liposome with powder form food grade Ultralec by heating method and selection of settings reflects a wide but reasonable numerical range. As it is known, only 2 levels can be considered in the Plackett-Burman design for each factor[25]. Table-1 shows the selected variables, which include chemical components (the lecithin (Ultralec®P) (% w/w), the cholesterol/lecithin (w/w), the enzyme/lecithin (w/w) and pH) and process variables (the stirring time, the process temperature, the rate of stirrer/ tank diameter, the centrifugation condition, the speed of stirrer (rpm), the way of adding enzyme and the homogenization). The corresponding levels in this table were chosen on the basis of the preliminary tests. Table-2 shows selected experimental factors and a Plackett-Burman design for conducting twelve experimental trials. All the trials were done in duplicate. The elements, + (high level) and - (low level) represent the two different levels of the independent factors examined.

RESULTS AND DISCUSSION

The effects of 11 physicochemical factors including the pH, the cholesterol/lecithin ratio (w/w), the lecithin (% w/w), the enzyme/lecithin ratio(w/w), the stirring time (min), the temperature (ºC), the speed of stirrer (rpm), the rate of stirrer/ tank diameter, the homogenization, the way of adding enzyme and the centrifugation condition on the encapsulation efficiency (%) of flavourzyme-loaded nano-liposome (which was produced for the first time in the present work by heating method) were evaluated. Table-2 provides comparison of the experimentally determined the encapsulation efficiency (%) with those predicted. An R-squared value of the experimentally determined the encapsulation efficiency (%) to those predicted was 94.81 %.

The Minitab 14 software was used to reconfirm the experimental matrix (24 runs); two replicates were used and the experiments were randomized. The response was encapsulation efficiency (%). The coefficients for the eleven variables were determined by:

$$A_i = (1/N)\sum_{0}^{n} X_i \cdot K_i$$

where A_i is coefficient value of the variable, X_i is experimental yield, Ki is the coded value of each variable corresponding to the respective experimental yield (X_i) and N is the number of experiments.

Predicted responses are given by: $Y_i = \sum_{i=0}^{N} A_i \cdot K_i$

For i = 0, a dummy level of +1 was used and the coefficient obtained was called A_0. The standard error (S_e) was determined as the sum of the squares of the difference between the experimental and predicted yields for each run. The estimated error (S_b) is given by:

$$S_b = \sqrt{S_e^2/N}$$

The student's t-test was used to determine the significance of each variable employed (t-value = coefficient/S_b)[30].

Encapsulation efficiency (%) of flavourzyme-loaded heating method liposome varied from 7.45 % (formulation No.10) to 16.95 % (formulation No. 4) (Table-2). Statistical calculations for the variance analysis of the encapsulation efficiency (%) of flavourzyme-loaded heating method liposome are summarized in Table-3. The confidence levels above 95 % (p value < 0.05) were accepted as significant variables. The results show that the lecithin (Ultralec®P), the rate of stirrer/ tank diameter, the enzyme/lecithin ratio, the cholesterol/ lecithin ratio, the speed of stirrer, the stirring time and the process temperature are significant, as their p-value were less than 0.05. All of significant variables had positive effect on the encapsulation efficiency (%), except the cholesterol/ lecithin ratio and the process temperature which had negative effect. These results are in line with practitioners of thi s field that it is generally agreed that lecithin is the major component in liposome formulation, while cholesterol, used as a membrane stabilizer in smaller quantity than lecithin and decreased encapsulation efficiency (%)[2,3,5].

TABLE-3
STATISTICAL DATA THE FOR THE VARIANCE ANALYSIS OF THE ENCAPSULATION EFFICIENCY OF FLAVOURZYME LOADED LIPOSOME PREPARED BY HEATING METHOD[a]

Variables	Effect	Coefficient	t-value	p-value
X_1: pH	-0.2705	-0.1352	-0.69	0.501
X_2: Cholesterol/lecithin (w/w)	-1.4055	-0.7028	-3.60	0.004
X_3: Lecithin (% w/w) (Ultralec®P)	3.5812	1.7906	9.18	0.000
X_4: Enzyme/lecithin ratio (w/w)	2.0038	1.0019	5.14	0.000
X_5: Stirring time (min)	1.2322	0.6161	3.16	0.008
X_6: Temperature (ºC)	-0.9362	-0.4681	-2.40	0.034
X_7: Speed of stirrer (rpm)	-1.2538	-0.6269	3.21	0.007
X_8: Homogenization	0.1188	0.0594	0.30	0.766
X_9: Way of enzyme addition	0.0905	0.0452	0.23	0.820
X_{10}: Centrifugation condition	0.2228	0.1114	0.57	0.578
X_{11}: Rate of stirrer diameter/tank diameter	3.2288	1.6144	8.28	0.000

[a]Each value is the average of two replications

A useful plot for identifying the factors that are important is the Pareto chart of effects (Fig. 1). This graph showed the factors main effect estimates plotted against the horizontal axis. The factors main effects were rank ordered according to their significance and, if there was an estimate of error variability available (standardized effects), this chart was included a vertical line to indicate the P = 0.05 threshold for statistical significance.

After completing the 12 runs, the Pareto chart showed that the most significant effect variable was the lecithin proportion (Ultralec®P) and the second most important factor was the rate of stirrer/tank diameter. The third important factor was the enzyme/lecithin ratio followed by the cholesterol/ lecithin ratio, the speed of mixing, the stirring time and the process temperature. The four most insignificant factors, as becoming evident from the Pareto chart, were the pH, the centrifuge condition, the homogenization and the way of enzyme addition.

The optimum parameters were obtained as lecithin (Ultralec®P) proportion (4 %), rate of stirrer diameter/tank diameter (0.9), enzyme/lecithin ratio (0.2), cholesterol/

lecithin ratio (0.05), speed of stirrer (1000 rpm), stirring time (15 min) and process temperature (50 ºC). The mean diameter and polydispersity index of successful formulation of flavourzyme-loaded nano-liposome using dynamic light scattering (DLS) technique employing a particle size analyzer 90 Plus (Brookhaven Instruments Corporation, USA) was 178 nm and 0.2 respectively.

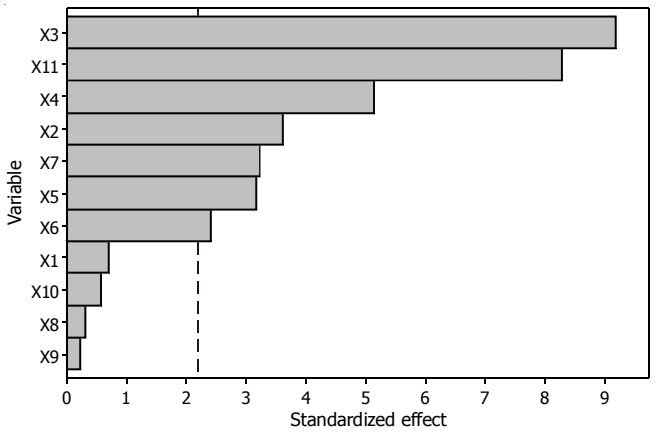

Fig. 1. Pareto charts for the factors main effect on encapsulation efficiency of Flavourzyme-loaded nanoliposome by powder lecithin Ultralec®; (X_1: pH, X_2: cholesterol/lecithin (w/w), X_3: Lecithin (% w/w) (Ultralec®P), X4: Enzyme/Lecithin ratio (w/w), X5: Stirring time (min), X6: Process temperature (°C), X7: Speed of stirrer (rpm), X8: Homogenization, X9: Way of enzyme addition, X10: Centrifugation condition, X11: Rate of stirrer diameter/tank diameter)

Conclusion

This study aimed to prepare flavourzyme-loaded nano-liposomes for the first time using a novel method (called heating method) by powder food grade lecithin (Ultralec®). The Placket-Burman design was used to assess the relative importance of chemical components (the lecithin, the cholesterol/lecithin ratio, the enzyme/lecithin ratio and the pH) and process variables (the stirring time, the temperature, the rate of stirrer/tank diameter, the centrifuge condition, the speed of stirring, the way of enzyme addition and homogenization) on the encapsulation efficiency. This study is the first use of Plackett-Burman design for the production of flavourzyme-loaded nano-liposome. The screening phase, which is the first optimization process, showed the significance of main variables included the lecithin, the rate of stirrer/tank diameter, the enzyme/lecithin ratio, the cholesterol/lecithin ratio, the speed of stirrer, the stirring time and the process temperature were affect on the encapsulation efficiency.

Based on our findings, it seems that heating method is suitable for encapsulation of flavourzyme in liposome without employing any solvent or toxic material for application in food industry especially in cheese ripening acceleration.

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REFERENCES

1. F. Gibbs, S. Kermasha, I. Alli and C.N. Mulligan, *Int. J. Food Sci. Nut*., **50**, 213 (1999).
2. R. Mozafari, K. Khosravi-Darani, G.G. Borazan, J. Cui, A. Pardakhty and S. Yurdugul, *Int. J. Food Prop*., **11**, 833 (2008).
3. M.R. Mozafari and S.M. Mortazavi, Nanoliposomes From Fundamentals to Recent Developments, Trafford Publishing (2005).
4. M.R. Mozafari, *Cell. Mol. Biol. Let*., **10**, 711 (2005).
5. M.R. Mozafari, C. Johnson, S. Hatziantoniou and C. Demetzos, *J. Liposome Res*., **18**, 309 (2008).
6. P.F. Fox, Cheese: Chemistry, Physics and Microbiology, Chapman and Hall (1993).
7. P.F. Fox and M.B. Grufferty, in ed.: P.F. Fox, Food Enzymology, Elsevier Applied Science, pp. 219-269 (1991).
8. K.N. Kilcawley, M.G. Wilkinson and P.F. Fox, *Enzyme Microb. Tech*., **31**, 310 (2002).
9. M. El Soda, *Int. Dairy J*., **3**, 531 (1993).
10. M.G. Wilkinson and K.N. Kilcawley, *Int. Dairy J*., **15**, 817 (2005).
11. W. Alkhalaf, M.E. Soda, J.-C. Gripon and L. Vassal, *J. Dairy Sci*., **72**, 2233 (1989).
12. B. Larivière, M. El Soda, Y. Soucy, G. Trépanier, P. Paquin and J.C. Vuillemard, *Int. Dairy J.*, **1**, 111 (1991).
13. P. Dufour, J.C. Vuillemard, E. Laloy and R.E. Simard, *J. Microencapsul.*, **13**, 185 (1996).
14. E. Laloy, J.-C. Vuillemard, P. Dufour and R. Simard, *J. Control. Rel.*, **54**, 213 (1998).
15. A. Picon, P. Gaya, M. Medina and M. Nuaez, *J. Dairy Sci*., **77**, 16 (1994).
16. A. Picon, P. Gaya, M. Medina and M. Nunez, *J. Dairy Sci*., **78**, 1238 (1995).
17. A. Picon, C. Serrano, P. Gaya, M. Medina and M. Nunez, *J. Dairy Sci*., **79**, 1699 (1996).
18. E.E. Kheadr, L.C. Vuillemard and S.A. El-Deeb, *J. Food Sci*., **67**, 485 (2002).
19. E.E. Kheadr, J.C. Vuillemard and S.A. El-Deeb, *Food Res. Int.*, **36**, 241 (2003).
20. D.R. Rao, C.B. Chawan and R. Veeramachaneni, *J. Food Biochem*., **18**, 239 (1994).
21. S. Skeie, *Int. Dairy J*., **4**, 573 (1994).
22. Y.F. Maa and C.C. Hsu, *Pharm. Dev. Technol*., **4**, 233 (1999).
23. M.R. Kasaai, G. Charlet, P. Paquin and J. Arul, *Innov. Food Sci. Emerg. Tech.*, **4**, 403 (2003).
24. K.-H. Song, S.-J. Chung and C.-K. Shim, *J. Control. Rel.,* **84**, 27 (2002).
25. R.L. Plackett and J.P. Burman, *Biometrika*, **33**, 305 (1946).
26. Y.L. Loukas, *J. Pharm. Biomed. Anal*., **26**, 255 (2001).
27. J.C. Colas, W. Shi, V.S.N.M. Rao, A. Omri, M.R. Mozafari and H. Singh, *Micron*, **38**, 841 (2007).
28. K. Anjani, K. Kailasapathy and M. Phillips, *Int. Dairy J*., **17**, 79 (2007).
29. K. Kailasapathy, C. Perera and M. Phillips, *Int. J. Food Eng*., **2**, 1 (2006).
30. K. Khosravi-Darani and A. Zoghi, *Bioresour. Technol.*, **99**, 6986 (2008).