PRODUCTION AND DEVELOPMENT OF NUTRACEUTICALS USING *BACILLUS SUBTILIS* NCIM 2708 UNDER SOLID STATE FERMENTATION BY RESPONSE SURFACE METHODOLOGY

Md Faruque Ahmad
Department of Clinical Nutrition, Faculty of Applied Medical Sciences,
Jazan University, Jazan, Kingdom of Saudi Arabia (KSA)
Syed Aamir Ashraf
ZR Azaz Ahmad Azad
Department of Food Technology, Faculty of Engineering and Interdisciplinary Sciences,
Hamdard University, New Delhi, India
Bibhu Prasad Panda
Department of Pharmaceutical Biotechnology, Faculty of Pharmacy,
Hamdard University, New Delhi, India

Abstract:

Nutraceuticals is a broad umbrella term used to describe any product derived from food sources that provides further health benefits in addition to the essential nutritional value found in foods intense interest the public towards worth full effect not only for medicinal but also as a nutraceutical products. This research aimed to maximize production of Menaquinone-7(MK-7) by solid state fermentation process. Menaquinone-7 (MK-7) was produced by solid state fermentation process of *Glycine max* and *Phaseolus vulgaris* in presence of a co-cultured by *Bacillus subtilis*. Three separate nutritional parameters were screened by each experiment for the production of MK-7 using Plackett–Burman experimental design. Parameters were optimized by Box–Behnken design of response surface methodology for the production of menaquinone-7 (MK-7). In present study, 16.39 µg g^{-1} MK-7 produced of *Glycine max* in optimised formula in the medium contains sorbitol 51.22g kg^{-1}, urea- 3.40g kg^{-1} and $MnCl_2$-0.20g kg^{-1}. With *Phaseolus vulgaris* 31.35 µg g^{-1} of MK-7 produced per gram of the substrate in a optimised media containing glycerol-54.05g kg^{-1}, urea-3.40g kg^{-1} and soybean extract-15.00g kg^{-1}.

Keywords: Menaquinone-7, *Bacillus subtilis*, Response surface methodology, Placket Burman Design

Introduction

When we talk of vitamin K, naturally first thing which comes on our mind is coagulant vitamin and its role in blood clotting processes. Menaquinone-7 (MK-7) has laid us a new prospective of thinking, being a highly valuable member of the vitamin–K family. Vitamin K occurs in two forms: Phylloquinone (vitamin K1), which is present in green plants; and menaquinone (vitamin K_2), which is produced by some intestinal bacteria (Briggs and Calloway 1979; Conly and Stein 1992; Bentley and Meganathan, 1982). MK-7 is a highly bioactive homologue of Vit-K (Sato et al. 2001) MK-7 having seven isoprene units, one of the analogue of Vit-K2 is abundant in fermented soybean (natto) (Tsukamoto, 2004) and has significant effect on preventing osteoporosis and cardiovascular diseases besides its positive effects on blood coagulation. (Gast et al. 2009; Schurgers et al. 2007; Yamaguchi et al. 1999). MK-7 is a part of the family known as Vit-K2, and is necessary for the synthesis of blood coagulation factor, the activation of proteins involved in the building of bones and inhibition of vascular calcification (Brug et al. 2011; Rheaume et al. 2012). MK-7 has an anabolic effect on bone calcification in rat femoral tissue. Zinc has been shown to enhance the effect of MK-7 in increasing bone calcium content in vitro (Ehara et al. 1996). The combined administration of zinc and MK-7 was found to have a synergistic or additive enhancing effect on bone components in the femoral tissues of

female elderly rats. (Ma et al. 2001) MK-7 may be siginificant in preventing osteoporosis with increasing age. More recently, in an in vitro study, it has been demonstrated that MK-7 can directly stimulate calcification in femoral metaphyseal tissue obtained from normal rats. (Sato et al. 1996; Yamaguchi et al. 1999).

Materials and Methods

Microorganisms

The cultures of *Bacillus subtilis* NCIM 2708 were obtained from National Collection of Industrial microorganism (NCIM), National Chemical Laboratory (NCL), Pune, Maharashtra, India, was maintained on slants of starch agar peptone medium at 4ºC, and sub-cultured in 30-day intervals.

Preparation of seed culture and fermentation

Spore suspension of *Bacillus subtilis* NCIM 2708 were prepared from actively growing slants. The suspension was inoculated to conical flask containing nutrient broth liquid media (0.5% peptone, 1.5% beef extract, 0.5% sodium chloride, quantity sufficient in 100ml of distilled water adjusted pH to 7.2 ± .2). These cultures were incubated at 37ºC for 24 hrs in shaker incubator at 125 RPM using solid state fermentation.

Optimization of nutrient

Glycerol, Mannitol, Sorbitol, Maltose, Yeast, soybean extract, Urea, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$ and $MnCl_2$ constituents were selected for the study. The Plackett-Burman experimental design for eleven variables i.e. nine nutritional components (independent variables) and two dummy variables, were used to evaluate the relative importance of various nutrients for a higher production of menaquinone-7. For each nutrient variable, two different concentrations i.e. high (+) and a low (–), tested. Data analysis was carried out by the standard procedure of Plackett-Burman experimental design (Plackett & Burman 1946).

Three important nutrient parameters (Sorbitol, Urea and MnCl2) as per Plackett-Burman experimental design were selected, when *Glycine max* was the solid substrate. In case of *Phaseolus vulgaris*, three important parameters (Glycerol, Urea and Soybean extract) as per Plackett-Burman experimental design were selected for the determination of optimal value for menaquinone-7 production. An experimental design of 17 runs containing 5 central points was made according to Box-Behnken's response surface design for selected three parameters using DESIGN EXPERT 7.1.3 software (Statease Inc., USA). The relative effects of two variables on MK-7 were identified from the contour and response surface plot. Optimum values of the parameter for maximum production of MK-7 were determined by the point prediction tool of the software.

Extraction and analysis of MK-7

Extraction process of MK-7 was carried by using acetonitrile. 10gm fermented beans were extracted with 25ml acetonitrile. The mixture is shacked for 10 minutes. Transferred the solution into centrifuge tube and did the centrifugation for 5 min at 6000 rpm to get the decant solvent. Now concentrate the sample over nitrogen chamber. Transfer the sample into vials for further analysis and till that keep in refrigerator.

MK-7 were estimated by HPLC (Shimadzu, Japan) using 250 mm x 4.6 mm ID Lichrosper® 100 C_{18} column of 5 μm particle size and 20 μl loop injector in presence of mobile phase acetonitrile and methanol 1:1 v/v with the flow rate of 1ml min^{-1} and detection was carried out by UV detector at 254 nm. The chromatogram was analyzed by HPLC software class-VP.

Results and discussion

To identify the concentration of key nutrients influencing production of MK-7 Plakette Burman Design was used. Medium components for substrate *Glycine max* (Sorbitol, Urea and $MnCl_2$), While in case of *Phaseolus vulgaris* (Glycerol, Urea and Soybean extract) screened by Plakette Burman Design. An experimental design of 17 runs containing 5 central points was made according to Box–Behnken response surface design for 3 selected media parameters for each MK-7. The individual and interactive effects of nutrients variables were studied by conducting the fermentation run (Tables 1 and 2). The response was measured in terms of MK-7 production. The results of experimental data and simulated values are listed (Tables 1 and 2). Data collected for MK-7 concentration in each run were analyzed using the software DESIGN EXPERT 7.1.6 and fitted into a multiple nonlinear regression model proposes following equation (in the coded factor) for MK-7.

(Substrate *Glycine max*) Menaquinone-7 (µg/100gm) =

$1426 + 76.87 \times A + 144.32 \times B + 87.41 \times C – 20.57 \times A \times B – 1.4725 \times A \times C – 23.15 \times B \times C – 369.786 \times A^2 - 100.04 \times B^2 + 141.21C^2$

Where A, B and C represent sorbitol, urea and $MnCl_2$ respectively in g kg^{-1}.

(Substrate *Phaseolus vulgaris*) Menaquinone-7 (µg/100gm) =

$2481.15 + 89.08 \times A + 739.6525 \times B + 14.51 \times C + 46.9225 \times A \times B - 8.4425 \times A \times C + 120.6675 \times B \times C - 415.044 \times A^2 - 128.999 \times B^2 + 11.77125C^2$

Where A, B and C represent glycerol, urea and soyabean extract respectively in g kg^{-1}.

The effects of all nutrient parameters on MK-7 production can be compared with the help of perturbation plots Fig. 1 (a, b). The lines in the graph represent influence and sensitivity of respective factor for MK-7 production. This model resulted in response surface graphs. The response surface plots of calculated model for MK-7 production are shown in Fig. 2 (a-f). The relative effect of medium components on MK-7 production was depicted in response surface graphs. The analysis of variance of regression for MK-7 production was summarized in (Table 3).

Response Surface Methodology (RSM) proved to be a powerful tool for optimizing menaquinone-7 production by *Bacillus subtilis* NCIM 2708. Three nutrients sorbitol, urea, $MnCl_2$ for *Glycine max* and glycerol, urea and soybean extract for *Phaseolus vulgaris* screened by Plackett-Burman Experimental design were optimized by Box-Behnken design of RSM with help of DESIGN EXPERT 7.1.6 software. From the studied nutrient variable different ingredients have different effects on MK-7 production. In case of *Phaseolus vulgaris* all the three parameters glycerol, urea and soybean extract were positively significant factors. The proposed model equation illustrate the interaction between two factors, from the equation it was found that urea is positively interacted with glycerol and soybean extract while glycerol negatively interacted with the soybean extract. In case of *Glycine max* all the three parameters sorbitol, urea and $MnCl_2$ were positively significant factors. The proposed model equation illustrates the interaction between two factors. From the equation it was found that sorbitol is negatively interacted with urea and $MnCl_2$ while urea is also negatively interacted with the $MnCl_2$. In present study, in case of *Glycine max* 16.39µg g^{-1} of menaquinone-7 production was obtained in optimized medium under solid state fermentation using *Bacillus subtilis* NCIM 2708. In case of *Phaseolus vulgaris* 31.35µg g^{-1} of menaquinone-7 production was obtained in optimized medium under solid state fermentation using *Bacillus subtilis* NCIM 2708. Menaquinone-7 production can further be increased by optimization of process parameters.

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Table 1 Box-Behnken design for production of MK-7 by *Glycine max* with result (actual and predicted)

Run	Sorbitol (g/l)	Urea (g/l)	$MnCl_2$ (g/l)	Menaquinone-7 (µg /100gm)	
				Actual	Predicted
1	35.00	2.00	0.10	674.55	714.395
2	65.00	2.00	0.10	932.5	909.295
3	35.00	3.14	0.10	1021	1044.205
4	65.00	3.14	0.10	1196.64	1156.795
5	35.00	2.71	0.00	1034.75	1031.673
6	65.00	2.71	0.00	1128.39	1188.393
7	35.00	2.71	0.20	1269.42	1209.448
8	65.00	2.71	0.20	1357.17	1360.248
9	50.00	2.00	0.00	1249.05	1212.283
10	50.00	3.14	0.00	1567.37	1547.243
11	50.00	2.00	0.20	1413.29	1433.418
12	50.00	3.14	0.20	1639	1675.768
13	50.00	2.71	0.10	1426	1426
14	50.00	2.71	0.10	1426	1426
15	50.00	2.71	0.10	1426	1426
16	50.00	2.71	0.10	1426	1426
17	50.00	2.71	0.10	1426	1426

Table 2 Box-Behnken design for production of MK-7 by *Phaseolus vulgaris* with result (actual and predicted)

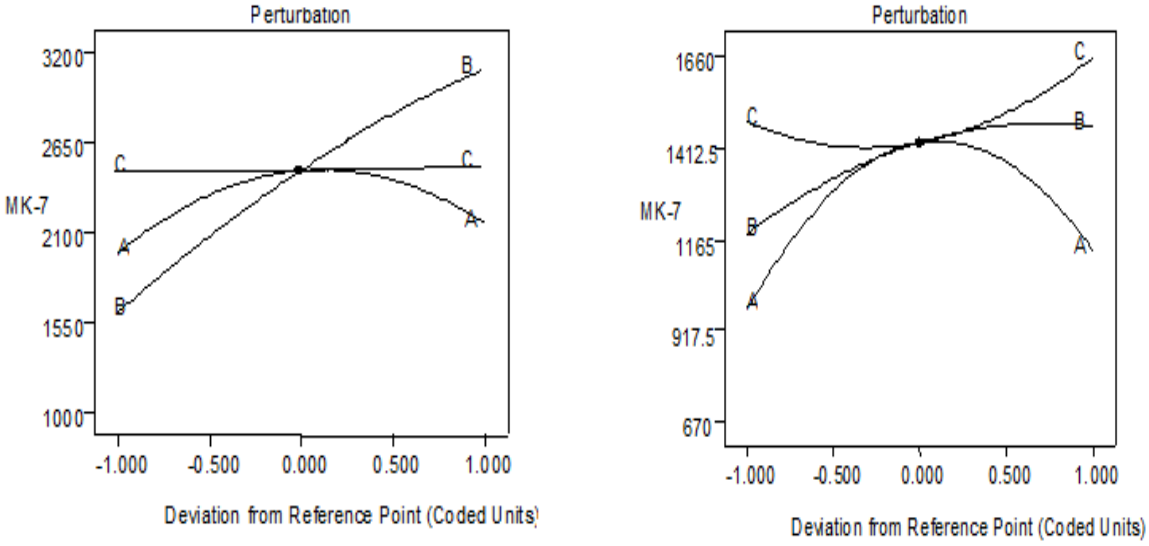
Run	Glycerol (g/l)	Urea (g/l)	Soybean Extract (g/l)	Menaquinone-7 (µg /100gm)	
				Actual	Predicted
1	35.00	2.00	10.00	1050.17	1155.298
2	65.00	2.00	10.00	1178.71	1239.613
3	35.00	3.40	10.00	2601.66	2540.758
4	65.00	3.40	10.00	2917.89	2812.763
5	35.00	2.70	5.00	1967.27	1965.845
6	65.00	2.70	5.00	2118.09	2160.89
7	35.00	2.70	15.00	2054.55	2011.75

8	65.00	2.70	15.00	2171.6	2173.025
9	50.00	2.00	5.00	1834.13	1730.428
10	50.00	3.40	5.00	2906.07	2968.398
11	50.00	2.00	15.00	1580.44	1518.113
12	50.00	3.40	15.00	3135.05	3238.753
13	50.00	2.70	10.00	2481.00	2481.45
14	50.00	2.70	10.00	2480.95	2482.15
15	50.00	2.70	10.00	2481.05	2482.15
16	50.00	2.70	10.00	2480.75	2478.15
17	50.00	2.70	10.00	2481.10	2481.19

Table 3 Analysis of variance of model of nutrient parameters for MK-7 production

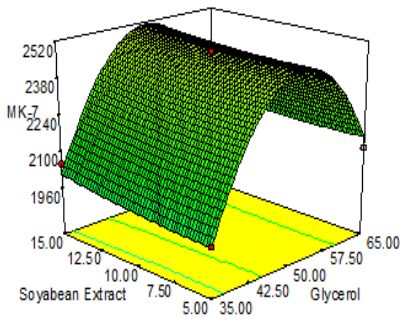
Parameters	*Glycine max* (MK-7)	*Phaseolus vulgaris* (MK-7)
Regression analysis of model		
Sum of squares	971075.4	5332011
Df	9	9
Mean squares	107897.3	592445.7
F-value	50.42	66.38854
*p-value	<0.0001	>0.0001
Regression analysis of Residual		
Sum of squares	14978.47	62467.4
Df	7	7
Mean squares	2139.78	8923.915
Correlation coefficient (R^2)	0.9848	0.96688

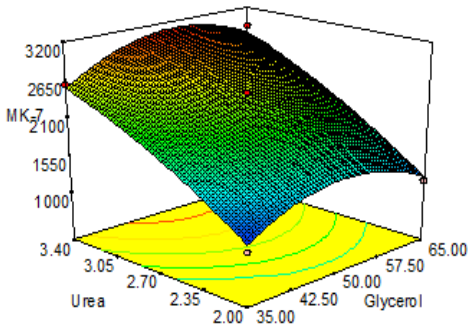
* Less than 0.0500 indicate model terms are significant.



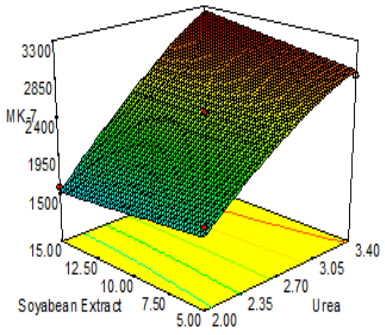
(a) (b)

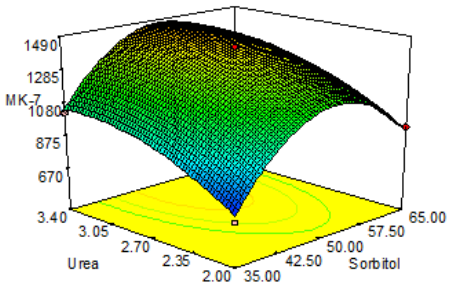
Figure 1 Perturbation plot showing the effect of all nutrients parameter on MK-7 production (µg g^{-1}).
Where A, B, and C represent glycerol, urea, and soyabean extract (Figure 1 a)
and sorbitol, urea and $MnCl_2$ (Figure 1 b)



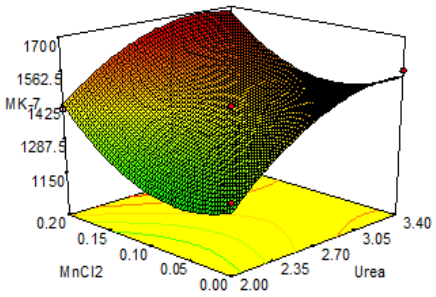


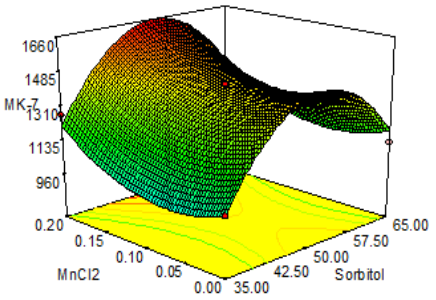
(a) (b)





(c) (d)





(f)

(e)

Figure 2 Response surface plots showing relative effect of two nutrient parameters for the production of MK-7 by *Phaseolus vulgaris* (a-c) and Glycine max (d-f) while keeping other nutrient at constant level.