Modeling the Effect of Bed Height and Particle Size for Vitamin K2 Production in a Static Bed Fermenter

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Abstract— **Soy granules were used as a substrate for microbial production of Vitamin K_2 using *Bacillus subtilis*. Static bed "tray type" fermentation was carried out and the operation of this fermentation was optimized using full factorial statistical method of analysis. The optimum parameters were obtained as: bed thickness; particle size, incubation time. The results indicated that the MK7 yield reductions caused by elevated bed heights were reduced considerably with the corresponding elevations in particle sizes by improved substrate porosity. The polynomial model fitted the experimental data well with R^2 = 0.85 and R^2 (adj) = 0.78. MK7 production was increased from 104.1 mg/kg to 106.4±2.1 mg/kg when the strain was cultivated at optimum conditions predicted by statistical approach (50 mm bed thickness, 1-1.4 mm particle size, 8 days incubation time). The results of this investigation via statistical modeling of solid state fermentations assist to build a framework for quantitative and mechanistic fermentation design. They present a challenge for scale up.**

Index Terms— **Solid state fermentation (SSF), Vitamin K_2, Tray fermenters, mathematical modeling, Menaquinone 7, *Bacillus subtilis***

I. Introduction

Solid-state fermentation (SSF), in which microbial activity takes place on a moist solid surface having no free-flowing water, offers many advantages over submerged fermentation. SSF can be of special interest in those processes where the crude fermented product may be used directly as food supplement like Japanese fermented food natto rich in menaquinone 7 (MK7) (15.4 - 23.1 mg/kg)[1]. Recently research has demonstrated that MK7 may reduce the risk of bone fractures [2-5] and cardiovascular disorders [6-8]. Menaquinone 7 is the predominant and nutritionally interesting long chain menaquinone [9] belongs to the Vitamin K_2 sub group of Vitamin K family. This family of fat soluble vitamers which was discovered more than 75 years ago as an antihemorrhagic factor capable of correcting dietarily induced bleeding disorders in chickens [10].

Solid state fermentation of Vitamin K_2 in static bed reactors is limited researched area [11-16], where the relevant phenomenon are yet to be fully understood. For that statistical models have an important role to play in fermenter design, optimization and scale up. This work addresses the design and operation of solid phase fermentation under conditions where intermittent forced aeration is used but the substrate bed is not mixed. This mode of operation is appropriate for MK7 production via SSF processes in which mixing of the substrate bed is not desirable during the fermentation due to deleterious effects on "shear intolerant" *Bacillus subtilis* growth [17]. The aim of the present study is to evaluate the suitability and utility of soy protein granules as best substrate for optimization of key fermentation factors including incubation time, particle size and substrate bed thickness.

Particle size of the substrate is an important factor affecting the SSF. Small particles might lead to poor heat and mass transfer due to substrate agglomeration while coarser particles might result in poor accessibility of substrate to microorganism due to the reduction of the surface area per unit volume of the particles [18]. Similarly, the thickness of the substrate layer under any natural fermentation conditions plays a key role in the desired end product formation due to varied temperature gradients and O_2 transfer implications [19]. Further, it also affects the growth and MK7 production of the organism involved during the fermentation.

Hence the research herein has been carried out to cater the need for industrial production of supplementary MK7 by mimicking the solid sate fermentation conditions of natto to produce superior MK7 concentrations as most of the published research has been carried in Liquid state fermentation (LSF).

II. Materials and Methods

Microorganism

Strain *Bacillus subtilis var. natto* isolated from commercially available *natto* after screening different types for highest MK7 producing strain as described in Berenjian *et al.* 2011 [20]

Inoculum

Spores of *Bacillus subtilis* incubated on a liquid culture were suspended in 0.9 % NaCl solution to obtain the standard spore solution (10.8±0.04 $\log_{10}$CFU/mL).

Medium

Soy granules were of commercial origin was used, containing 9% w/w moisture. Substrate was autoclaved with 50% of moisture at 121 ^{o}C for 20 minutes before inoculation. The water content after inoculation was adjusted to 70 %.

Sieve analysis

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The dry substrate was sieved through standard mesh sieves to determine the particle size distribution in the substrate. Particles from five individual mesh sizes were used.

Fermentation

Fermentation was carried out in specimen jars with a spore loading of (8.4 ± 0.04) $\log_{10}$CFU/g. inside a fixed-climate incubator (37 ^{o}C, 95% RH) where humidified air was introduced at the top at a defined rate to increase the O_2 concentration.

Vitamin K_2 extraction and determination

MK7 was extracted from the fermentation media using 12 mL *2-propanol: n-hexane* (v:v 1:2) and determined using High performance liquid chromatography HP 1050 (Hewlett-Packard, USA) using the method mentioned in Berenjian *et al.* 2011 [20]. The LC-MS system (LCMS-2010EV, Shimadzu, Kyoto) were used to confirm the structure of MK7.

Experimental design

The characterization of different factors for Vitamin K_2 production was optimized by applying the RSM (MODDE v7.0, Umetrics). The lower and upper limits for process conditions were determined based on the preliminary data. The statistical model was obtained using the Full factorial design with three independent variables Bed thickness (mm), Particle size (mm) and Incubation Time (days).

III. Results and Discussion

SSF modeling is challenging due to the heterogeneous nature of fermentation. The fractional–factorial experimental design was adopted to optimize the packed-bed reactor system with respect to time, bed thickness and particle size of the substrate. The range of values for each parameter was chosen from data obtained in previous work [13]. These values are presented in Table 1, incubation time ranged from 4-8 days, bed thickness from 5-15 mm and particle size from 0.15 mm-1.4 mm. Inoculation density has been tested initially as a single variable, and found that in our case increasing the inoculum loading from 8.4±0.04 logCFU/g to 10.8±0.01 logCFU/g reduced the MK7 concentration [11]. The loading of logCFU/g = 8.4±0.04 was used for the rest of experiments to achieve high yield [11].

Table I: Experimental range and levels of the three independent variables

Variables	Levels		
	-1	0	+1
Bed thickness (mm)	5	10	15
Particle size (mm)	<0.150	0.5-0.75	1-1.4
Incubation Time (days)	4	6	8

The full experimental plan with respect to their values, in coded form, is shown in Table 2. Upon completion of the experiments, the average of MK7 production (mg/kg of dry weight) was taken as the response.

Table II: Experimental data for the Three-Factor, Three-Level Full factorial design and responses

Exp No	Bed thickness	particle size	Time	MK7 (mg/kg)
1	-1	-1	-1	47.3
2	0	-1	-1	32.1
3	1	-1	-1	28.7
4	-1	0	-1	39.8
5	0	0	-1	37.2
6	1	0	-1	25.3
7	-1	1	-1	60.1
8	0	1	-1	50.8
9	1	1	-1	22.8
10	-1	-1	0	76.3
11	0	-1	0	45
12	1	-1	0	40.3
13	-1	0	0	64.6
14	0	0	0	73.8
15	1	0	0	35.5
16	-1	1	0	75.1
17	0	1	0	70.6
18	1	1	0	45.7
19	-1	-1	1	83.5
20	0	-1	1	41.8
21	1	-1	1	26.7
22	-1	0	1	108.1
23	0	0	1	70.2
24	1	0	1	56.9
25	-1	1	1	100.6
26	0	1	1	72.3
27	1	1	1	71.3
28	0	0	0	74.1
29	0	0	0	53.9
30	0	0	0	67.2

It was found that bed thickness, particle size and incubation time were key factors on MK7 production (p<0.05, Table 3). At the 8th day of fermentation MK7 concentration was 100.6 mg/kg, 72.3 and 71.3 mg/kg (Table 2) and at bed thicknesses of 0.5, 10 and 15 mm, respectively at highest particle size, corroborating that bed thickness was a significant factor within the range examined (p<0.05). In addition it was found that particle size and incubation time were key factors on MK7 production (p<0.05, Table 3). The average MK7 concentration of day 4, 6 and 8 were 38.2, 58.5 and 70.2 mg/kg, respectively confirming the significance of fermentation time (p<0.05).

Table III: Statistical analysis: Full factorial design

Term	Coeff.	Std. Err.	P value	Conf. int(±)
Const.	61.59	3.91	9.56E-13	8.15
X_1	-13.23	1.90	9.18E-07	3.96
X_2	6.46	1.90	0.002819	3.96
X_3	12.58	1.90	1.88E-06	3.96
X_1^2	-0.08	2.42	0.97428	5.06
X_2^2	-1.79	2.42	0.468468	5.06
X_3^2	-3.30	2.42	0.188608	5.06
X_1*X_2	0.80	1.83	0.668287	3.82
X_2*X_3	-3.46	1.83	0.073438	3.82
X_1*X_3	3.45	1.83	0.074637	3.82

N = 30	Q2 = 0.55
DF = 20	R2 = 0.85
Conf. lev. = 0.95	R2 Adj. = 0.78

X_1 = Bed thickness X_2 = Particle size X_3 = time

Adequacy of the model was examined by comparing experimental data in Table 2 with the predicted value and then by performing extra independent experiments at the optimal conditions. The *p*-values were used to assess the significance of each coefficient where $p < 0.05$ indicate the significant of that specific factor. The calculated coefficients of regression model, the standard error, and the corresponding *p*-value are given in Table 3. The significance of each coefficient was determined using the *p*-values. The smaller the magnitude of *p*-value, the more significant is the corresponding coefficient.

The coefficients for the linear effect of bed thickness ($p<9.18\times10^{-7}$), particle size ($p < 2.8\times10^{-3}$) and fermentation time ($p < 1.88\times10^{-6}$) were found to be significant for the response. Among the higher order effects for MK7 yield, quadratic effects and mutual interactions were not found to be significant. Nevertheless, there are instances where researchers claim that it is probably better to accept higher *p* values (i.e. $p <0.1$) rather than taking the chance of missing an important factor [21]. This effect may apply to processes such as SSF, which are non-homogenous and usually show large variations. However, it would make negligible changes to the model optimization for MK7 production generated in this work. The fit of model was checked by the determination coefficient (R^2). In this case, the value of determination coefficient (R^2=0.85) indicates that only 10.5% of the total variations is not explained by the model. The value of adjusted determination coefficient (adjusted R^2=0.78) is also high indicating a good agreement between the experimental and predicted values for the MK7 production.

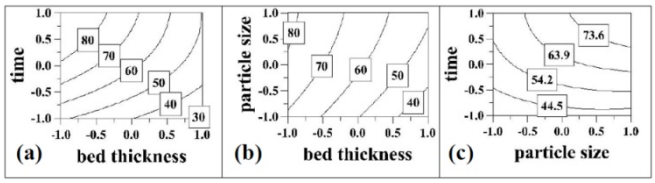

Fig 1: Response surface plots showing the effect of independent variables

As shown in Fig 1 (a), the MK7 yield increased with decrease in bed height (bed loading) and Fig 1 (b) & (c) suggests that with for larger particle sizes lower bed thicknesses are favoured and higher incubation periods. These results are in good agreement with Mahanama *et al.* 2011 where smaller beds increased the production of MK7 [11]. The layer of substrate in trays is limited due considerations of heat and O_2 transfer within the bed.

The findings may be affected by dissimilar moisture holding capacity of different particle sizes where initial moisture content used (~70%) in all different particle size categories are highly unlikely to match the moisture holding capacity. Excess moisture content present in the substrate may reduce the porosity of the bed hindering O_2 transfer to the bottom restricts microbial growth only to the surface of the bed. Further experiments should be carried out to analyze the effects of changing moisture and packing density of different particle sizes to understand the gas transfer phenomena associated with solid-state fermentation. These studies will elucidate the findings of Richard *et al.* 2004 where the reduction in air-filled porosity that accompanies increasing moisture content coincides with an increase in permeability which effects of moisture on particle and pore size distributions having clear physical impacts on permeability physiology phenomenon [22].

Equation 1: The linear equation in coded form for MK7 production

$$Y = 61.59 - 13.23X_1 + 6.46X_2 + 12.58X_3$$

X_1 = Bed thickness X_2 = Particle size X_3 = time

The regression equation obtained after the analysis of variance (ANOVA) represented the level of MK7 concentration (mg/kg) as a function of three independent variables. Apart from the linear effect of the variables on the fermentation process, quadratic and interaction effects did not influenced the equation.

Table IV: Design for validation of predictive model optimum values generated by the quadratic model

Bed thickness	Particle size	time	MK7 predicted	MK7 observed (mg/kg)
-1	1	1	104.1	106.4±2.1

The model was validated by replicate fermentation at the optimum conditions, shown in Table 4. The highest MK7 production of 104.1 mg/kg mg/kg was predicted by the model for conditions using lowest bed thickness (5 mm), highest particle size (1-1.4 mm) and highest fermentation period (8 days). The experimental value achieved under these conditions was 106.4±2.1 mg/kg, which was very close to the value predicted by the model with only ~2.1% error. SSF of *Bacillus subtilis* strain isolated from commercial *natto* demonstrated good potential to improve the production of MK7 on the economically viable substrates. The MK7 concentration was modelled and optimized using RSM. The prediction of empirical model

outside the range of the experiments is inaccurate and the deviation increased by moving away from the fitted region.

IV. Conclusion

The SSF process parameters were optimised for MK7 production. Unlike the classical method of optimizing process parameters, statistical techniques were performed, where the levels of variables were changed simultaneously to study their collective effect on MK7 production. The statistical optimization developed in this study established the basis of MK7 production by means of static cultivation. Bed thickness, particle size and incubation period demonstrated significant effect on MK7 yield. At the optimized conditions MK7 concentration was increased threefold compared with the commercially available fermented food, *natto.* SSF process technique might provide a better choice for MK7 production than Liquid state fermentation, considering the high productivity and lower production costs as the potential of using the vitamin enriched substrate as a food supplement bypassing extraction protocol.

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