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Optimization of Ultraviolet Irradiate Conditions for Vitamin D_2 Synthesis in Shitake Mushrooms (*Lentinula edodes*) by Using Response Surface Methodology

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Abstract The determination of the optimum conditions for the conversion of ergosterol to vitamin D_2 in shitake mushrooms (*Lentinula edodes*) was studied using response surface methodology (RSM). The effects of the three main variables ambient temperature (20–40°C), exposure time (60-180 min), and irradiation intensity (0.6–1.8 W/m^2) were investigated. According to the RSM ridge analysis, the optimum conditions were as follows: ambient temperature of 34.2°C, exposure time of 175.6 min, and irradiation intensity of 1.41 W/m^2. Under these optimum conditions, the maximum vitamin D_2 content of 117.93 μg/g in shitake mushrooms was obtained, which agreed fairly well with the predicted value of 122.60 μg/g.

Keywords ergosterol · response surface methodology · shitake mushroom · ultraviolet irradiate · vitamin D_2

Vitamin D is important in human nutrition as a regulator of the metabolism of calcium and phosphate (Ko et al., 2008); it promotes absorption of calcium and influences the process of bone mineralization. Deficiency and insufficiency of Vitamin D can cause many health problems (Roberts et al., 2008), such as rickets in children and osteoporosis in adults. Humans obtain vitamin D mainly from sunlight exposure, although the actual intake is affected by many factors, such as ethnicity, latitude, season shift, and age (Koyyalamudi et al., 2009). When the vitamin D requirement cannot be met by endogenous production, dietary intake becomes essential. Most foods contribute a relatively low amount of vitamin D, unless they are fortified; while unfortified, only eggs, milk, and certain fish species contribute useful amounts of vitamin D (Shrapnel and Truswell, 2006).

There are two distinct forms of vitamin D: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2 is the synthetic form of vitamin D that is formed via UV irradiation of the plant steroid ergosterol (Jasinghe and Perera, 2005) and is assumed to have the same biological activity as vitamin D_3 (Holick et al., 2008), which is only found in oily fish such as salmon, sardine, and tuna, as well as in eggs and red meat (Shrapnel and Truswell, 2006).

Vitamin D_2 is the form that is generally used in food and pharmaceutical supplementation (Jasinghe and Perera, 2005). Though shitake mushrooms contain a small amount of vitamin D_2, researchers have found them to be rich in ergosterol (Mattila et al., 2002). In addition, shitake is the most popular mushroom in the eastern Asian countries (Ogra et al., 2004), widely accepted by vegetarians and non-vegetarians. Therefore, shitake mushrooms are chosen as a source of supplemental vitamin D in the present study.

Several studies have reported that vitamin D_2 forms in mushrooms that have been exposed to UV irradiation through conversion of ergosterol, pro-vitamin D_2 (Ko et al., 2008; Roberts et al., 2008). Though irradiate factors such as irradiation dose (or intensity), exposure time, ambient temperature and moisture content have been investigated (Jasinghe and Perera, 2005; Jasinghe and Perera, 2006; Ko et al., 2008), the optimum conditions of vitamin D_2 conversion in shitake mushrooms have not been reported. Response surface methodology (RSM) which is a collection of experimental strategies, mathematical methods and statistical inference, is used

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to explain the combined effects of all the factors in an experiment process (Tanyildizi et al., 2005; Deepak et al., 2008). Thus, the objective of this study, achieved using response surface methodology, is to investigate the optimum conversion conditions of ergosterol to vitamin D_2 in shitake mushrooms.

Vitamin D_2 was extracted using the method described in Koyyalamudi et al. (2009), with slight modifications. Briefly, 1 g of freeze-dried shitake mushroom powder was placed into a 250 mL round-bottom flask and mixed with 4 mL of sodium ascorbate solution (17.5 g of sodium ascorbate in 100 mL of 1 mol/L NaOH), 10 mL of 50% potassium hydroxide, and 50 mL of ethanol (95%). The mixture was saponified under reflux at 80°C for 1 h, then cooled to room temperature before transferring into a separating funnel. The mixture was extracted first with 15 mL deionized water, followed by 15 mL ethanol, then three times with 50 mL of n-pentane. The organic layers were pooled, washed twice with 50 mL of 3% KOH in 5% ethanol, and finally with deionized water until neutralized. The organic layer was transferred to a rotary evaporator, and the residue was dissolved with 10 mL of high-performance liquid chromatography (HPLC)-grade ethanol. Then, the solution was filtered through a 0.45-mm PTFE membrane syringe filter (Chromdisc, Korea), and 1 mL was used for HPLC analysis. A 20-μL filtered sample was injected into a Waters Millenium system with a Waters 600 Controller gradient pump equipped with a degasser, a Waters 717 Plus autosampler, and a Waters UV-486 detector with the detection wavelength set to 264 nm (Waters, USA). Each sample was separated on a SunFire C18 analytical column (2.6×250 mm, 5 μm, Waters, Ireland) at a fixed temperature of 30°C. The mobile phase was methanol/acetonitrile (25:75, v/v) at a flow rate of 1.0 mL/min. Vitamin D_2 concentration was measured according to a standard calibration curve.

Different UV irradiation sources have different influences on vitamin D_2 synthesis in mushrooms (Jasinghe and Perera, 2006; Jasinghe et al., 2007; Teichmann et al., 2007; Wu and Ahn, 2014). These studies indicated that UV-B is more efficient than other UV sources for vitamin D_2 synthesis. In addition, exposure to UV-B irradiation produces no other nutritionally or toxicologically significant changes in mushroom composition (Simon et al., 2011). As a result, UV-B was chosen as the source for determining the optimum conditions for vitamin D_2 synthesis in shitake mushrooms. In addition to the effect of the UV irradiation source, the effects of ambient temperature, exposure time, and irradiation intensity were investigated in a preliminary experiment. The first step of the preliminary experiment was to choose an appropriate temperature for vitamin D_2 synthesis, performed in the ambient temperature range of 20 to 50°C at a UV-B irradiation intensity of 1.2 W/m^2 and an exposure time of 90 min per side. The second step was to choose an appropriate UV-B irradiation intensity. Based on the optimum ambient temperature determined in the first step, each side of the shitake mushrooms was exposed to UV-B for 90 min in the intensity range of 0.3 to 1.5 W/m^2. The last step of the preliminary experiment was to determine the appropriate exposure time. Mushrooms were exposed to UV-B with irradiation time ranging from 30 to 150 min. The exposure was performed at the optimum temperature determined in the first step and the optimum irradiation intensity determined in the second step. The results of the preliminary experiments are shown in Table 1. Based on the results, the range and levels of the three independent variables were determined as inputs for RSM.

The preliminary study indicated that ambient temperature, irradiation intensity, and exposure time were significant variables for vitamin D_2 synthesis. A factorial central composite rotator design using RSM was conducted in the optimization of vitamin D_2 synthesis. The lower (−1) and higher (+1) levels of the variables were: ambient temperature, 20 and 40°C; exposure time, 60 and 180 min; and irradiation intensity, 0.6 and 1.8 W/m^2. The range and levels of the variables investigated in this study are given in Table 2.

The relationships between the process index (the yield of vitamin D_2) and the three irradiate factors were analyzed and processed using the "Design Expert" (Version 8.0.6, Stat-Ease Inc., USA) statistical package. The combinations of the three independent variables together with the response are shown in Table 3. The response measured was vitamin D_2 content.

Crude protein and crude fat were determined in freeze-dried material using AOAC (1990) methods: crude protein, by the

Table 1 Effects of independent variables on vitamin D_2 synthesis in shitake mushrooms under UV-B irradiation

Independent variables		Vitamin D_2 (μg/g)
Temperature (°C)	Control	N.D[1)]
	20	86.46±2.17[b2)]
	30	98.19±9.50[a]
	40	89.04±8.92[ab]
	50	62.02±3.14[c]
Time (min)	30	78.82±1.73[b]
	60	87.8±7.54[ab]
	90	103.55±5.23[a]
	120	113.71±19.10[a]
	150	115.48±14.87[a]
Intensity (W/m^2)	blank	0±0[e]
	0.3	44.95±0.99[d]
	0.6	68.71±10.94[c]
	0.9	83.07±12.44[b]
	1.2	100.25±7.28[a]
	1.5	103.78±4.33[a]

[1)]Not detectable;
[2)]Values are expressed as mean±SD; Means with the different letters within the same column are significantly different at p <0.01 by Duncan's multiple range tests.

Table 2 Experimental range and levels of the independent variables

Independent variables	Unit	Symbol	Code levels				
			−α	−1	0	+1	+α
Temperature	°C	A	13.2	20	30	40	46.8
Time	min	B	19.1	60	120	180	220.9
Intensity	W/m^2	C	0.19	0.6	1.2	1.8	2.21

Table 3 Effects of ambient temperature, exposure time and irradiation intensity on vitamin D_2 content

Run	Irradiation conditions			Vitamin D_2 (μg/g)
	Temperature (°C)	Time (min)	Intensity (W/m^2)	
1	−1(20)	−1(60)	−1(0.6)	53.83±10.88
2	+1(40)	−1(60)	−1(0.6)	59.19±14.33
3	−1(20)	+1(180)	−1(0.6)	73.74±8.85
4	+1(40)	+1(180)	−1(0.6)	108.48±6.03
5	−1(20)	−1(60)	+1(1.8)	83.01±9.06
6	+1(40)	−1(60)	+1(1.8)	81.12±10.44
7	−1(20)	+1(180)	+1(1.8)	115.09±8.77
8	+1(40)	+1(180)	+1(1.8)	104.66±12.43
9	−α(13.2)	0(120)	0(1.2)	85.11±10.33
10	+α(46.8)	0(120)	0(1.2)	108.59±8.44
11	0(30)	−α(19.1)	0(1.2)	59.24±6.69
12	0(30)	+α(220.9)	0(1.2)	119.79±13.87
13	0(30)	0(120)	−α(0.19)	46.14±1.68
14	0(30)	0(120)	+α(2.21)	109.98±10.67
15	0(30)	0(120)	0(1.2)	109.06±7.97
16	0(30)	0(120)	0(1.2)	105.03±3.14
17	0(30)	0(120)	0(1.2)	103.92±4.32
18	0(30)	0(120)	0(1.2)	118.24±10.75

Table 4 ANOVA for response surface quadratic model for vitamin D_2 synthesis

Source	Statistics				
	Sum of squares	Df[1)]	Mean square	*F*-value	*P*-value
Model	9323.58	9	1035.95	14.15	0.0005***[2)]
A	331.34	1	331.34	4.52	0.0661
B	3761.58	1	3761.58	51.36	<0.0001***
C	2813.11	1	2813.11	38.41	0.0003***
AB	54.29	1	54.29	0.74	0.4143
AC	343.48	1	343.48	4.69	0.0622
BC	23.05	1	23.05	0.31	0.5901
A^2	285.04	1	285.04	3.89	0.0840
B^2	681.55	1	681.55	9.31	0.0158*
C^2	1641.09	1	1641.09	22.41	0.0015**
Residual	585.87	8	73.23		
Lack of fit	458.93	5	91.79	2.17	0.2783
Pure error	126.93	3	42.31		
Cor Total	9909.44	17			
R^2	0.9409		Adeq Precision		11.311

[1)]Degree of freedom.
[2)]*Significance at a level of 0.05; **significance at a level of 0.01; ***significance at a level of 0.001.

macro-Kjeldahl method and a conversion factor of 4.38 was used to quantify the nitrogen percentage of the crude protein; crude fat, by Soxhlet extraction with petroleum ether. Total carbohydrate, was analyzed by a phenol-sulfuric acid method (Albalasmeh et al., 2013).

A central composite rotatable design was conducted to further explore the optimal levels of the key factors (ambient temperature, exposure time, and irradiation intensity) and the effects of their interactions on vitamin D_2 synthesis. After applying multiple regression analysis on the experimental data shown in Table 3, the following second-order polynomial equation was obtained to explain vitamin D_2 synthesis:

$$Y = 109.16 + 4.93*A\ 16.60*B + 14.35*C + 3.61*A*B - 6.55*A*C - 1.70*B*C - 4.75*A2 - 7.34*B^2 - 11.39*C^2$$

where Y is the predicted vitamin D_2 content (μg/g, dry weight); A, B, and C are the coded values of ambient temperature, exposure time and irradiation intensity, respectively.

The analysis of variance (ANOVA) test was conducted to test the significance of the fit of the second-order polynomial equation for the experimental data, as shown in Table 4. The model *F*-value of 14.15 implies that the model is significant. There is only a 0.05% chance that a "Model *F*-value could occur due to noise. The *p*-value is used to determine the significance of each variable. The smaller is the *p*-value, the greater is the significance of the corresponding variable (Guo et al., 2009). *P*-values less than 0.05 in this study indicate significant model terms. In this case, B, C, B^2, and C^2 are significant model terms. The R^2 value of 94.09% indicates good agreement between experimental and predicted values and signifies that the proposed model is reliable for vitamin D_2 synthesis.

Based on the proposed model, response surface plots and contour plots were obtained, as shown in Figs. 1, 2, and 3, in order to investigate the interactions among three variables and to determine the optimum levels of each factor in the synthesis of vitamin D_2 in shitake mushrooms. Tanyildizi et al. (2005) reported that the maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram. On the basis of analysis from the present study, the model predicted a maximum vitamin D_2 content of 122.60 μg/g, with a 95% confidence interval between 112.87 μg/g and 132.33 μg/g, under conditions where ambient temperature, exposure time, and irradiation intensity were 34.17°C, 175.61 min, 1.41 W/m^2, respectively. Under the above optimum conditions, the vitamin D_2 content in shitake mushrooms was 117.93±8.64 μg/g, within the 95% confidence of the predicted maximum value (122.60 μg/g). The excellent correlation of the predicted and measured values verifies the utility and practicability of the proposed model. A previous study has used a similar approach (RSM) to optimize the vitamin D_2 production in oyster mushroom (Wu and Ahn, 2014); however, for different species of mushrooms, due to their different texture and components, the optimum conditions for vitamin D_2 synthesis may be different from each other. Compared to oyster mushrooms (Wu and Ahn, 2014), shitake mushrooms need longer time (175.61 to 94.28 min), higher temperature (34.17 to 28.16°C) and stronger irradiate intensity (1.41 to 1.14 W/m^2) to maximize the production of vitamin D_2. The reasonable explanation for these differences could be that shitake mushrooms have harder texture, thicker surface and less gills compared with oyster mushrooms.

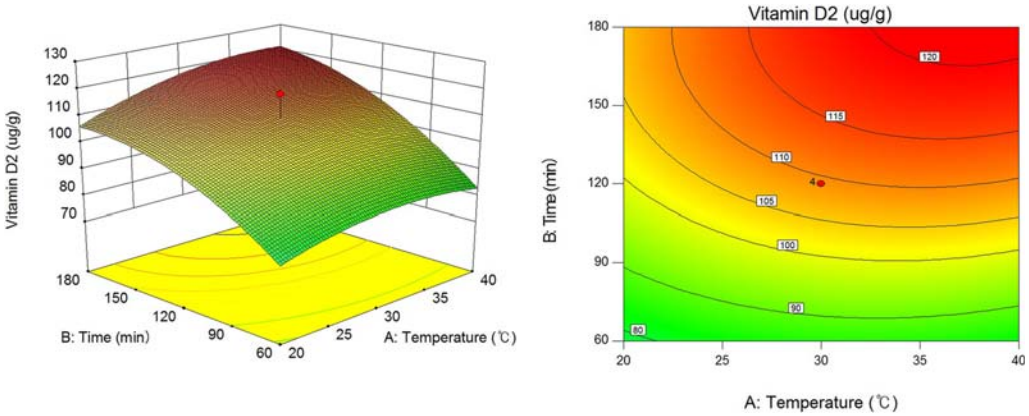

Fig. 1 The response surface plot and the corresponding contour plot showing the effects of ambient temperature and exposure time on vitamin D_2 synthesis in shitake mushrooms, with irradiation intensity of 1.2 W/m^2.

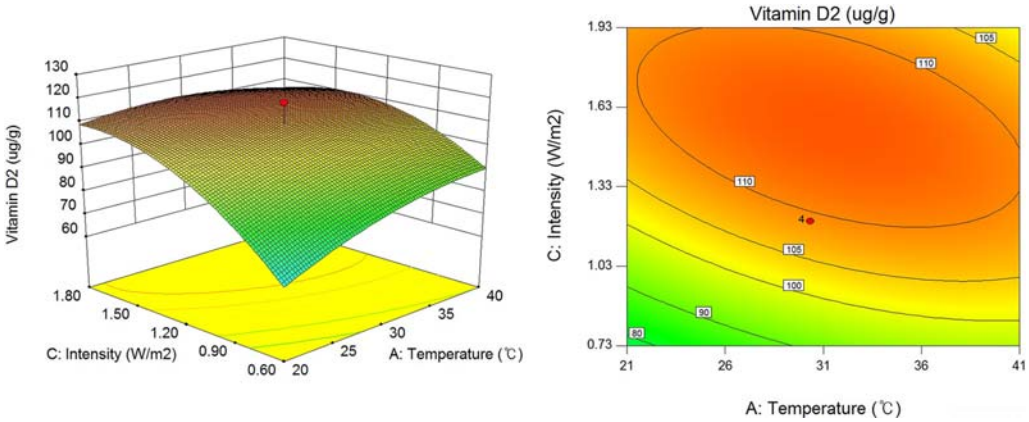

Fig. 2 The response surface plot and the corresponding contour plot showing the effects of ambient temperature and irradiation intensity on vitamin D_2 synthesis in shitake mushrooms, with exposure time of 120 min.

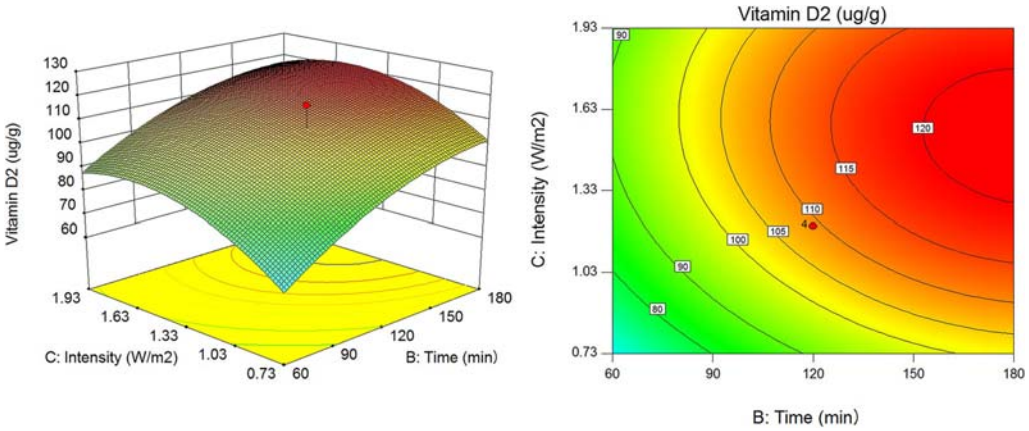

Fig. 3 The response surface plot and the corresponding contour plot showing the effects of exposure time and irradiation intensity on vitamin D_2 synthesis in shitake mushrooms, with ambient temperature of 30°C.

The major composites of the shitake mushrooms analyzed, including crude protein, crude fat and total carbohydrate, are shown in Table 5. Comparing mushrooms processed in the presence or absence of UV-B irradiation, there were only slight differences among the contents of those components from each group. These results are in accordance with a previous study

Table 5 Proximate composition (percent dry weight) of *Lentinula edodes* processed with or without UV-B irradiation

Parameter	Control (%)	UV-B (%)
Protein	14.71±0.01[1)]	14.46±0.06
Fat	1.89±0.19	2.05±0.00
Carbohydrate	13.80±0.75	14.47±0.37

[1)]Values are expressed as mean± SD.

(Simon et al., 2011), which reported that no other nutritionally significant changes in mushrooms were identified under UV irradiate condition. However, extensive research is still required to evaluate the safety and toxicity of the UV-B irradiated mushroom powder in vitro study. We will investigate that in our future studies.

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