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Optimization of ergosterol to vitamin D_2 synthesis in *Agaricus bisporus* powder using ultraviolet-B radiation

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Abstract Response surface methodology (RSM) was applied to determine the optimum circumstances for conversion of ergosterol to vitamin D_2 via ultraviolet-B (UV-B) in white button mushroom (*Agaricus bisporus*) powder. Three independent variables, namely, exposure time, ambient temperature, and irradiation intensity were investigated using a central composite rotatable design. The RSM ridge analysis determined the following ideal states: exposure time of 10.4 min, ambient temperature of 26.33°C, and UV-B irradiation intensity of 1.36 W/m^2. A vitamin D_2 content of 741.50±23.75 μg/g (the predicted value was 780.4 μg/g) was obtained under these ideal conditions.

Keywords: *Agaricus bisporus*, mushroom, vitamin D_2, synthesis, ultraviolet irradiation

Introduction

Vitamin D, which regulates the way calcium and phosphate are metabolized, is essential for human health (1). Vitamin D deficiency or insufficiency has been associated with disorders such as rickets in children and osteoporosis in adults (2) and has also been proven to be linked with other disorders such as certain types of cancers, heart diseases, diabetes, and obesity (3). Humans mainly obtain vitamin D through cutaneous synthesis by sunlight exposure. However, many people are unable to get enough sunlight exposure for production of adequate levels of vitamin D owing to their ethnicity, living conditions, age, and use of sunscreen (4,5). Under these circumstances, a dietary intake of vitamin D is essential. However, only few types of natural foods, such as oil-rich fish, fish liver oils, and egg yolks, provide a relatively useful amount of vitamin D (6). This suggests that people who eat non-vegetarian food can easily consume vitamin D through food sources, whereas people who refrain from eating animal products are more vulnerable to vitamin D deficiency disorders.

There are two primary types of vitamin D: vitamin D_2 and vitamin D_3. The former can be synthesized from a plant sterol called ergosterol via ultraviolet irradiation (7); whereas the latter is mainly obtained from the transformation of cholesterol in the skin under sunlight exposure (8). Vitamin D_2 is considered to behave in a similar biological manner as vitamin D_3 (9). Both of them can be transformed into a metabolite of 25-hydroxyvitamin D (25(OH) D) in the liver, which is considered as the functional indicator of dietary reference for vitamin D intake (10).

Vitamin D_2 hardly exists in a majority of cultivated mushrooms; however, researchers have ascertained that they are abundant in ergosterol (11), the provitamin D_2. The transformation of ergosterol in mushrooms to vitamin D_2 under UV irradiation has been studied for years. It has also been noted that ultraviolet-B (UV-B) radiation is more efficient compared with other light sources for the synthesis of vitamin D_2 (5,12). In addition, irradiation intensity, exposure time, ambient temperature, and moisture content are considered as the important factors for the conversion of ergosterol to vitamin D_2 under irradiation (5,12-14).

According to Jasinghe and Perera (7), mushrooms require a long period of UV irradiation to yield a relatively high amount of vitamin D_2. However, high amounts of ultraviolet irradiation can negatively affect the surface coloration in mushrooms and cause the moisture to evaporate and create texture changes, leading to an undesirable decrease in their market value (4). Furthermore, the porous epidermal structure of mushrooms makes them highly perishable; hence, they can only be stored for up to three days under usual shipping and marketing conditions (15). The use of dried mushroom powder instead of fresh mushrooms can be a solution to these problems as mushroom powder has a longer shelf life and absorbs UV irradiation more easily. The aim of this study is to establish the optimal UV-B irradiation conditions for vitamin D_2 synthesis in white button mushroom powders that are abundant in ergosterol using response surface methodology (RSM).

Materials and Methods

Materials Ergosterol and vitamin D_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium hydroxide (AR grade), sodium hydroxide (AR grade), sodium *L*-ascorbate (AR grade), *n*-pentane (EP grade), and 95% ethanol (EP grade) were purchased from Samchun Pure Chemical (Pyeongtaek, Korea). Methanol (HPLC grade), ethanol (HPLC grade), and acetonitrile (HPLC grade) were purchased from J.T. Baker (Avantor Performance Materials, Center Valley, PA, USA). UV-B lamps (G15T8E; Sankyo Denki, Tokyo, Japan) emitting ultraviolet rays in the range of 280–360 nm (with a peak at 306 nm) were used as the irradiation source. Samples of fresh mushrooms including white button, pine, beech, shiitake, oyster, enoki, and king trumpet mushrooms were purchased from local farmers (Jeonju, Korea) and were immediately freeze-dried.

Vitamin D_2 extraction and analysis Vitamin D_2 was extracted using a previously reported method (4) with slight modifications. 1 g of freeze-dried white button mushroom powder was added in 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 M NaOH), 10 mL of 50% KOH, and 50 mL of ethanol (95%). The mixture was saponified under reflux at 80°C for 1 h and then cooled to room temperature before transferring to a separating funnel. The mixture was first extracted with 15 mL deionized water, followed by 15 mL ethanol and then with a three-stage *n*-pentane extraction of volume 50 mL (three times). The pooled organic layers were washed twice with 50 mL of 3% KOH in 5% ethanol, and finally washed with deionized water until they were neutralized. The organic layers were transferred to a rotary evaporator and the residue was dissolved with 10 mL HPLC-grade ethanol. The solution was then filtered through a 0.45-μm polytetrafluoroethylene membrane syringe filter (Chromdisc, Daegu, Korea) and the filtrate (1 mL) was used for HPLC analysis. A volume of 20 μL of the filtrate was injected into a Waters Millennium system using a Waters 600 Controller gradient pump equipped with Waters UV-486 detector with the detection wavelength set to 264 nm (Waters Corp., Milford, MA, USA) and eluted through a reverse phase SunFire C18 analytical column (2.6x250 mm, 5 μm particle size, Waters Corp.) 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 as per a standard calibration curve.

Selection of mushroom source and irradiation conditions Seven types of mushroom powder were irradiated under UV-B to identify and select the one that yielded the maximum amount of vitamin D_2. The irradiation conditions were as follows: ambient temperature was 25°C, exposure time was 10 min, and irradiation intensity was 0.9 W/m^2.

Each effect of ambient temperature, exposure time, and irradiation intensity on vitamin D_2 synthesis was investigated through preliminary experiments. The first step was to identify an appropriate temperature for vitamin D_2 synthesis. This was done through experiments performed in an ambient temperature range of 15–45°C, a UV-B irradiation intensity of 0.9 W/m^2, and an exposure time of 10 min. The second step was to choose an appropriate UV-B irradiation intensity. On the basis of the optimum ambient temperature determined in the first step, white button mushroom powder was exposed to UV-B radiation for 10 min in the intensity range of 0.3–1.8 W/m^2. The last step was to determine the appropriate exposure time. Mushroom powder was exposed to UV-B radiation with the irradiation time ranging from 0.5 to 40 min. The exposure was performed at the optimum temperature determined in the first step and optimum irradiation intensity determined in the second step. On the basis of the results of the preliminary experiments (data not shown), the range and levels of the three independent variables as inputs for RSM were determined.

RSM experimental design and statistical analysis The optimization of vitamin D_2 synthesis in *A. bisporus* powder was performed with RSM. The preliminary study indicated that ambient temperature, irradiation intensity, and exposure time were significant variables for vitamin D_2 synthesis. The analysis a factorial central composite rotator design for three factors with replicates at the center point. The lower (1) and higher (+1) values of the three variables were as follows: ambient temperature, 15 and 35°C; exposure time, 5 and 15 min; and irradiation intensity, 0.9 and 1.5 W/m^2 (data not shown).

A mathematical second-order equation was established to identify the relations among the process index (the yield of vitamin D_2) and the three exposure factors. The yield of vitamin D_2 was regressed multiple times with respect to exposure parameters using the least squares method as follows:

$$y=b_0+\Sigma_{i=1}^{j}b_i x_i+\Sigma_{i=1}^{j}\Sigma_{j=1}^{j}b_{ij}x_i x_j+\varepsilon \quad (1)$$

where, y is the predicted response, x_i and x_j are the coded independent variables (ambient temperature denoted by A, exposure time denoted by B, and irradiation intensity denoted by C), b_0 is a constant, b_i is the linear effect coefficient, and b_{ij} is the interaction effect coefficient. The accuracy and general performance of the above polynomial model were evaluated using R^2.

The second-order polynomial coefficients were analyzed and processed using the "Design Expert" (Version 8.0.6; Stat-Ease Inc, Minneapolis, MN, USA) statistical package, and the model was validated for the experimental conditions used in this study. The combinations of the three independent variables along with the responses for the measured vitamin D_2 content are shown in Table 1.

Results and Discussion

Selection of mushroom source Seven types of mushroom powder were irradiated under UV-B radiation to identify the mushroom

Table 1. Experimental design and central composite design results

Run	Irradiation conditions			Vitamin D_2 (μg/g)	Ergosterol (μg/g)
	Time (min)	Temperature (°C)	Intensity (W/m^2)		
1	-1(5)	-1(15)	-1(0.9)	525.13±24.64	4032.76±86.29
2	-1(5)	+1(35)	-1(0.9)	761.61±16.02	3536.13±51.57
3	+1(15)	-1(15)	-1(0.9)	682.87±11.31	3831.50±154.61
4	+1(15)	+1(35)	-1(0.9)	656.85±46.43	2969.67±107.14
5	-1(5)	-1(15)	+1(1.5)	676.66±28.63	3813.46±167.27
6	-1(5)	+1(35)	+1(1.5)	700.91±19.11	2916.54±85.37
7	+1(15)	-1(15)	+1(1.5)	749.95±3.69	3326.20±112.83
8	+1(15)	+1(35)	+1(1.5)	675.39±54.27	2557.01±113.30
9	0(10)	-α(8.2)	0(1.2)	406.74±10.17	4321.50±51.81
10	0(10)	+α(41.8)	0(1.2)	630.98±39.10	2542.85±31.92
11	-α(1.6)	0(25)	0(1.2)	658.87±18.53	3721.75±108.70
12	+α(18.4)	0(25)	0(1.2)	684.28±38.51	3052.09±62.59
13	0(10)	0(25)	-α(0.7)	710.86±31.22	3731.74±77.84
14	0(10)	0(25)	+α(1.7)	704.83±39.35	2809.78±50.65
15	0(10)	0(25)	0(1.2)	797.15±20.84	3176.27±110.37
16	0(10)	0(25)	0(1.2)	756.03±16.88	3217.79±43.50
17	0(10)	0(25)	0(1.2)	784.52±30.45	3198.34±50.38
18	0(10)	0(25)	0(1.2)	750.76±10.57	3185.97±42.47

species that yielded the maximum vitamin D_2 content (data not shown). The B ring of ergosterol is broken by photochemical reaction under UV light, leading to the production of ergocalciferol (vitamin D_2) (13). Thus, the ergosterol content in mushrooms exposed to UV light reduces and vitamin D_2 content increases. The amount of ergosterol in white button mushrooms was found to be considerably greater than that in other mushroom species, which explains the remarkably high concentration of vitamin D_2 in the button mushroom powder after UV-B irradiation. Thus, white button mushrooms were selected as the only source in this study for investigating the best irradiation states for vitamin D_2 synthesis.

Optimization of the key factors for vitamin D_2 synthesis in white button mushroom powder A central composite rotatable design was adopted to examine the ideal status of the key elements, including exposure time, ambient temperature, and irradiation intensity, along with their cooperative effects on vitamin D_2 synthesis in a detailed manner. After conducting a multiple regression analysis (Table 1), the content of vitamin D_2 was estimated using the following second-order polynomial equation:

$$Y=770.63+38.00A+16.59B+21.11C-42.88AB-30.31AC-2.93BC-81.00A^2-20.19B^2-9.08C^2 \quad (2)$$

Table 2. Anova for response surface quadratic model for vitamin D_2 synthesis

Source	Statistics				
	Sum of squares	Df†	Mean square	*F*-value	*p*-value
Model	1.358E+005	9	15088.03	7.06	0.0057**[2)]
A	19723.90	1	19723.90	9.23	0.0161*
B	3759.98	1	3759.98	1.76	0.2213
C	6085.88	1	6085.88	2.85	0.1300
AB	14709.56	1	14709.56	6.88	0.0305*
AC	7350.78	1	7350.78	3.44	0.1008
BC	68.91	1	68.91	0.032	0.8620
A^2	82984.87	1	82984.87	38.83	0.0003***
B^2	5154.98	1	5154.98	2.41	0.1590
C^2	1043.83	1	1043.83	0.49	0.5044
Residual	17098.09	8	2137.26		
Lack of fit	15990.81	5	3198.16	8.66	0.0527
Pure error	1107.29	3	369.10		
Corrected Total	1.529E+005	17			
R^2	0.8882			Adeq Precision 8.788	

†Degree of freedom.
*Significance at a level of 0.05; **significance at a level of 0.01; ***significance at a level of 0.001.

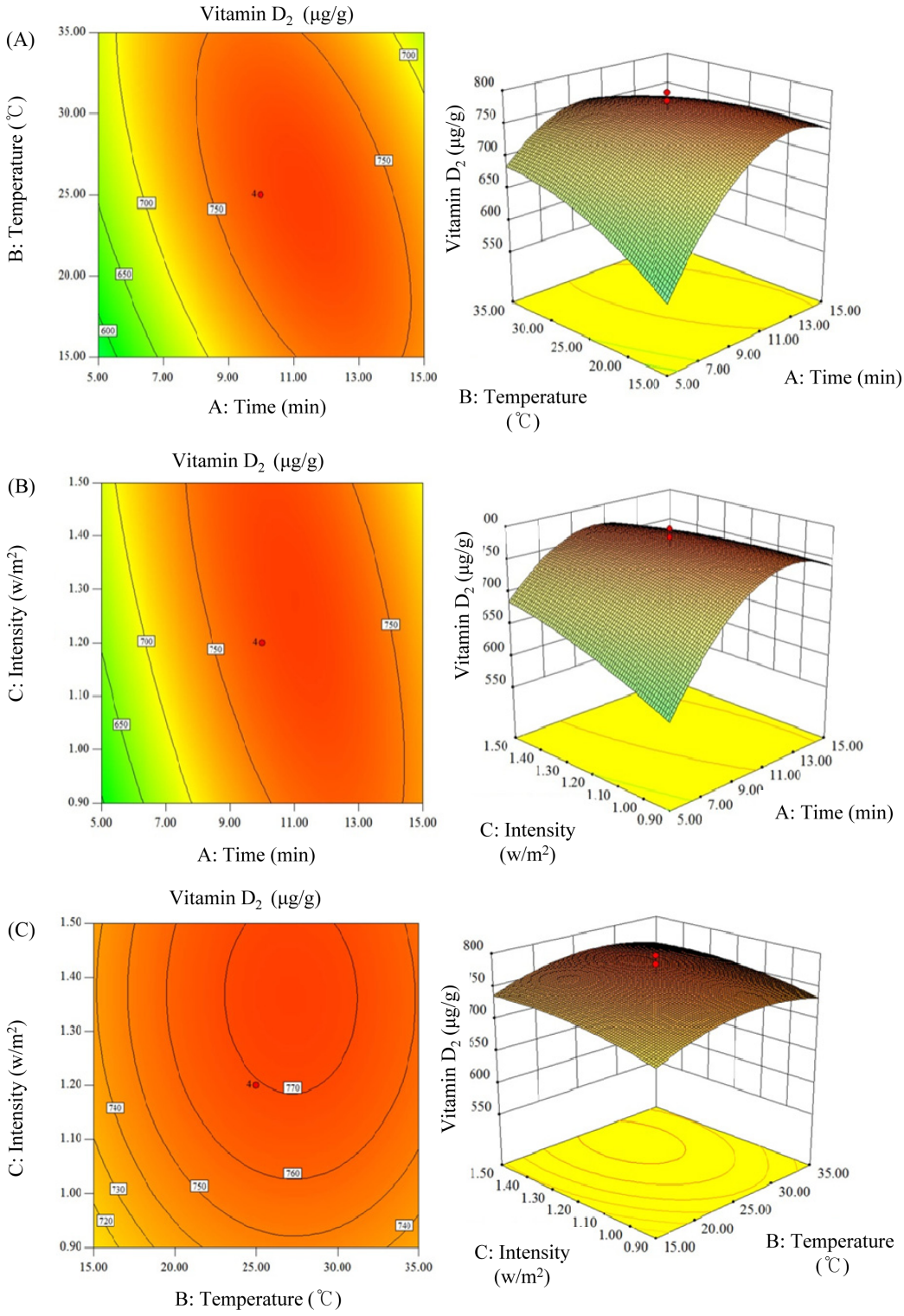

Fig. 1. Response surface and contour plots for vitamin D_2 production in white button mushroom powder. (A) Effects of ambient temperature and exposure time on vitamin D_2 synthesis in white button mushroom powder, with irradiation intensity of 1.2 W/m^2; (B) Effects of exposure time and irradiation intensity on vitamin D_2 synthesis in white button mushroom powder, with ambient temperature of 25°C; (C) Effects of ambient temperature and irradiation intensity on vitamin D_2 synthesis in white button mushroom powder, with exposure time of 10 min.

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

The significance of the fit of the second-order polynomial equation for the experimental data was assessed by the analysis of variance (ANOVA) test (Table 2). The model *F*-value of 7.06 indicates

that the model is significant. In addition, there is only a small chance (0.57%) that a "Model *F*-value of this size could occur due to noise. *P*-values less than 0.05 are considered significant model terms. In this case, model terms A, AB, and A^2 are significant (Table 2). The R^2 value of 88.82%, obtained in this study, can also explain the 88.82% variability in the response. The value of R^2 should be 0.75 or greater, and values above 0.90 represent a very high correlation (16).

To investigate the correlation among the three variables and determine the optimum level of each factor for production of vitamin D_2 in white button mushroom powder, response surface plots and contour plots were derived from the proposed regression model (Fig. 1A-1C). Figure 1A describes the effects of cooperation of ambient temperature (15–35°C) and exposure time (5–15 min) at a fixed irradiation intensity of 1.2 W/m^2, indicating quadratic effects of the two factors on the response. Figure 1B shows that exposure time has a quadratic effect on the response, whereas the irradiation intensity factor linearly increases with short exposure time at an ambient temperature of 25°C and that the maximum yields of vitamin D_2 were achieved within 9–11 min. Figure 1C depicts the interactions of ambient temperature and irradiation intensity on the response, showing a similar quadratic trend of the two factors on the response. Tanyildizi *et al*. (17) reported that the surface confined in the smallest ellipse in the contour diagram indicates the maximum predicted value. On the basis of the analysis done in this study, the model estimated a maximum vitamin D_2 content of 780.4 μg/g (between 728.83 and 831.99 μg/g with a 95% confidence interval) under the following conditions: exposure time, 10.4 min; ambient temperature, 26.33°C; irradiation intensity 1.36 W/m^2.

Model verification Under the ideal circumstances mentioned above, the vitamin D_2 content in white button mushroom powder was 741.50±23.75 μg/g with 95% confidence for the predicted maximum value (780.40 μg/g). The outstanding correlation between the estimated and recorded values testifies the usefulness and feasibility of the model proposed in this paper. As per our understanding, this is the first study on vitamin D_2 optimization in white button mushroom powder. Furthermore, the vitamin D_2 content synthesized in white button mushroom powder is significantly greater than the other reported values (data not shown); In addition, under UV-B irradiation, nutritional or toxicological changes were not observed in white button mushroom composition (18), suggesting that these specifications can be employed for commercial purposes.

In summary, the vitamin D_2 level in white button mushroom powder was observed to be higher than that in other mushroom species. Ambient temperature, irradiation intensity, and UV-B exposure time were all proved to be effective factors that influence vitamin D_2 synthesis in white button mushroom powder, of which exposure time is the most crucial. The maximum vitamin D_2 content of 741.5 μg/g (dry weight) was derived under the optimum conditions of an ambient temperature of 26.33°C, a UV-B irradiation intensity of 1.36 W/m^2, and an exposure time of 10.4 min. The results indicated that the proposed experimental model can be important for vitamin D_2 synthesis at the industrial production scale.

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Disclosure The authors declare no conflict of interest.

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