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Research Article

PREDICTION OF YIELDS IN GLUCOSYLATION OF RGOCALCIFEROL THROUGH RESPONSE SURFACE METHODOLOGY AND ARTIFICIAL NEURAL NETWORK ANALYSIS

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ABSTRACT
β-Glucosidase from sweet almond catalyzed synthesis of 20-*O*-(D-glucopyranosyl) ergocalciferol was analyzed using response surface methodology (RSM) and artificial neural network (ANN) analysis. In RSM a central composite rotatable design involving 30 experiments with four variables at five levels was employed to develop a predictive equation. The variables employed were immobilized β-glucosidase 20 - 100% w/w of D-glucose, pH 4.0 - 8.0, buffer concentration 0.04 - 0.2 mM (0.4 - 2 ml) and incubation period 10 - 50 h. Surface plots clearly brought out the behaviour of immobilized β-glucosidase in the glucosylation of ergocalciferol in exhibiting a crossover point at 60% (w/w D-glucose) immobilized β-glucosidase, 0.1 mM buffer concentration and pH 6.0. A maximum yield predicted by RSM of 26% under 100% (w/w D-glucose) immobilized β-glucosidase, pH 6.0, 0.12 mM (1.2 ml) buffer concentration and 30 h incubation period corresponded with an experimental yield of 26% under the same conditions. ANN analysis carried out with a set of training data also showed good correspondence to the testing data.

***Key Words*:** *Ann, Ccrd, Enzyme Concentration, Ergocalciferol, β-Glucosidase, Incubation Period, 20-O-(D-Glucopyranosyl) Ergocalciferol, RSM*

INTRODUCTION
Ergocalciferol (Vitamin D_2) is a very essential micronutrient for physiological functions of bone formation (skeletogenesis) and hormonal regulations. Vitamin D is available in two distinct forms, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). These are officially regarded as equivalent and interchangeable (Lisa and Vieth 2006). Although sunshine exposure and fish consumption provide vitamin D in the form of D_3, a different bioactive form is derived from plants as vitamin D_2. Vitamin-D deficiency develops osteoporosis or gradual loss of bone resulting in thinner and porous bone, leading to reduced bone density and sudden bone fracture. Most of the elderly population has vitamin-D deficiency (Lanske and Razzaque). Supplementation of vitamin D results in either prevention or treatment for a number of immune mediated diseases which include tuberculosis (Liu *et al.,* 2006), cancers of colon, breast and prostate (Bouillon *et al.,* 2006), cardiovascular diseases, some forms of arthritis, transplant rejection, autoimmune diseases such as multiple sclerosis (Munger *et al.,* 2006) and Type 1diabetes (Hypponen *et al.,* 2001).
Vitamin D_2 is a fat-soluble vitamin sensitive to light and heat (Lehninger 1977), which makes supplementation of vitamin D_2 a very challenging task. To such drawbacks can be overcome by synthesizing glycosides with enhanced solubility and stability. Enzymatic glycosylation is better then chemical glycosylation as it involves milder reaction conditions, easy work-up, easy recovery, less pollution and a cost effective process (Vijayakumar and Divakar 2007). Enzymatic glycosylation can be effected by glucosidases (Sivakumar *et al.,* 2006; Vijayakumar *et al.,* 2005; Vijayakumar *et al.,* .2006). Glycosidases are hydrolytic in nature, but abnormal conditions involving organic solvents with small amount of water, direct the enzymes towards glycosylation.

Response surface methodology (RSM) is a statistical method for the prediction of many chemical and biological processes (Jeong and Park 2006). In recent years, this methodology has been applied to some glycosylation reactions as well. The objective of these studies is to evaluate the optimum conditions within the parameters employed to achieve a maximum conversion yield. RSM involving a Central Composite Rotatable Design (CCRD) have been carried out on amyloglucosidase catalyzed synthesis of vanillin-maltoside (Sivakumar *et al.,* 2006), n-octyl-D-glucoside (Vijayakumar *et al.,* 2005) and curcumin-bis-α-D-glucoside (Vijayakumar *et al.,* 2006) in our laboratory. Artificial neural network (ANN) is an analytical learning tool applied in the field of biotechnology in terms of functional analysis, expression profile, genomics and proteomic sequences (Almedia 2002; Ozbay *et al.,* 2007). ANN can also be applied to study complex biological systems like enzymatic reactions.

The present study deals with prediction of yields in enzymatic glucosylation of ergocalciferol (vitamin D_2) in terms of enzyme concentration, pH, buffer concentration and incubation period using immobilized β-glucosidase through RSM and ANN analysis.

MATERIALS AND METHODS

Enzymes and chemicals

β-Glucosidase isolated from swe*et al.,* mond and immobilized on to calcium alginate beads was employed for the glucosylation reactions. Immobilized β-glucosidase activity determined by Colowick and Kaplan (Colowick and Kaplan 1976) method was found to be 0.078 mmol /(mg. Immobilized enzyme. min). Protein content by lowry's method was found to be 4.2%. D-Glucose purchased from SD Fine Chemicals (Ind.) Ltd. and ergocalciferol and sodium alginate from Sigma-Aldrich Chemical Co. USA were used as such. Solvents – di-isopropyl ether, DMF and HPLC grade acetonitrile from SD fine Chemicals (Ind.) Ltd. were employed after distilling once.

Glycosylation procedure

Glucosylation of ergocalciferol (0.5 mmol) involved refluxing with D-glucose (1 mmol) in a brown coloured 150 ml two necked flat bottom flask fitted with a brown coloured condenser containing 100 ml di-isopropyl ether in presence of 20 - 100 % (w/w D-glucose) immobilized β-glucosidase, pH 4.0 – 8.0, 0.04 - 0.2 mM (0.4 - 2 ml of 10 mM) buffer and 10 - 50 h incubation. Acetate buffer for pH 4.0 and 5.0, phosphate buffer for pH 6.0 and pH 7.0 and borate buffer for pH 8.0 were employed. The reactions were carried out under a nitrogen atmosphere. After incubation, the solvent was distilled off and the enzyme was denatured by holding the reaction mixture in a boiling water bath for 5 - 10 min. Unreacted D-glucose and products were dissolved in 15 - 20 ml of water, extracted with hexane to remove ergocalciferol and the aqueous portion was evaporated to dryness to get unreacted carbohydrate and the product glycosides. Work-up and isolation of the compound was carried out in dark, as ergocalciferol is a light sensitive compound. The glucoside was also stored in dark. The dried residue was subjected to HPLC analysis by injecting 20 μl of Phenomenex guard pretreated sample into a 250 mm × 4.6 mm aminopropyl column using acetonitrile : water in 70:30 ratio (v/v) as the mobile phase at a flow rate of 1ml/min and detecting with refractive index detector. Conversion yields were determined from HPLC peak areas of the glycoside and free carbohydrate with respect to the free carbohydrate employed. Error in HPLC measurements will be ± 10%. The glycosides formed were separated through size exclusion chromatography using Sephadex G-10 eluting with water. Eventhough the glycosides were separated from unreacted aglycon and carbohydrates, the individual glycosides could not be separated from their reaction mixtures due to similar polarity of the glycosides formed.

The isolated glycosides were characterized by recording UV, IR, MS and 2D NMR (HSQCT) spectra, which confirmed the product formation. ^{1}H and ^{13}C NMR spectra were recorded on a Brüker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz ^{13}C). About 40 mg of the sample dissolved in

DMSO-d_6 was used for recording the spectra at 35 ^{o}C. Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard to within ± 0.01 ppm. Two-dimensional Heteronuclear Single Quantum Coherence Transfer spectra (2D HSQCT) were recorded for the glucosides. In the NMR data, only resolvable signals are shown. Some assignments are interchangeable. Ergocalciferol and glucose signals are unprimed. Since, the compounds are surfactant molecules, they aggregate in the solvent to result in broad signals, thus, making it difficult to resolve the coupling constant values accurately.

Ergocalcifero

Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 3524 M^{-1}), 293.0 nm ($n\rightarrow\pi^*$, $\varepsilon_{293.0}$ – 360 M^{-1}), 326 nm ($n\rightarrow\pi^*$, $\varepsilon_{326.0}$ 260 M^{-1}); IR (KBr stretching frequency cm^{-1}): 3280 (OH), 1371 (C=C), 2958 (CH); 2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13): 2.81 (H-2), 1.36 (H-4), 6.52 (H-5), 5.95 (H-8), 4.67 (H-9), 4.79 (H-10), 1.84 (H-11a), 1.83 (H-11b), 2.30 (H-12a), 2.33 (H-12b), 1.95 (H-13a), 1.90 (H-13b), 1.47 (H-14), 2.44 (H-15), 1.63 (H-17), 1.44 (H-18a), 1.43 (H-18b), 0.49 (H-19), 3.65 (H-20), 1.45 (H-21), 1.43 (H-21b), 1.60 (H-22), 2.01 (H-23), 1.31 (H-24a), 1.29 (H-24b), 0.88 (H-25), 1.01 (H-26), 0.79 (H-27), 0.81 (H-28); ^{13}C NMR δ_{ppm} (125 MHz): 45.3 (C1), 55.8 (C2), 136.6 (C3), 55.9 (C4), 121.1 (C5), 145.6 (C6), 140.6 (C7), 117.6 (C8), 135.4 (C9), 131.5 (C10), 23.1 (C11), 32.2 (C12), 46.0 (C13), 40.1 (C14), 32.6 (C15), 111.8 (C16), 42.2 (C17), 39.1 (C18), 12.1 (C19), 68.0 (C20), 35.5 (C21), 35.9 (C22), 28.4 (C23), 27.2 (C24), 19.8 (C25), 19.5 (C26), 21.9 (C27), 21.0 (C28).

20-O-(D-Glucopyranosyl)ergocalciferol

Solid; UV (H_2O, λ_{max}): 191.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.0}$ – 4894 M^{-1}), 221.5 nm ($\sigma\rightarrow\pi^*$, $\varepsilon_{221.5}$ 1016 M^{-1}), 261.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{261.0}$ – 566 M^{-1}), 293.5 nm ($n\rightarrow\pi^*$, $\varepsilon_{293.5}$ – 566 M^{-1}), 327 nm ($n\rightarrow\pi^*$, $\varepsilon_{327.0}$ 2888 M^{-1}). IR (KBr stretching frequency cm^{-1}): 1080 (C-O-C aryl alkyl symmetrical), 1364 (C-O-C aryl alkyl asymmetrical), 3494 (OH), 1458 (aromatic C=C), 2956 (CH). MS (*m/z*). 2D-HSQCT (DMSO-d_6) C1α-glucoside ^{1}H NMR δ_{ppm} (500.13 MHz) Glu: 4.88 (H-1α, d, J = 2.8 Hz), 3.49 (H-3α), 3.11 (H-4α); **Erg:** 1.55 (H-14), 1.67 (H-17), 3.66 (H-20); ^{13}C NMR δ_{ppm} (125 MHz) Glu: 97.2 (C-1α), 74.1 (C-2α), 73.2 (C-4α); Erg: 24.5 (C-11), 70.3 (C-20), 35.8 (C-21), 35.9 (C-22), 30.3 (C-23), 28.1 (C-24), 19.6 (C-26); C1β-glucoside ^{1}H NMR δ_{ppm} Glu: 4.46 (H-1β, d, J = 6.5 Hz), 3.08 (H-3β), 2.93 (H-2β); ^{13}C NMR δ_{ppm} Glu: 103.2 (C-1β), 76.1 (C-2β), 79.7 (C-3β); 6-O-arylated ^{13}C NMR δ_{ppm} Glu: 68.1 (C-6).

NMR data clearly confirmed the formation of three glucosides: 20-*O*-(β-D-Glucopyranosyl)ergocalciferol, 20-*O*-(α-D-glucopyranosyl)ergocalciferol and 20-*O*-(6-D-glucopyranosyl)ergocalciferol (Scheme 1).

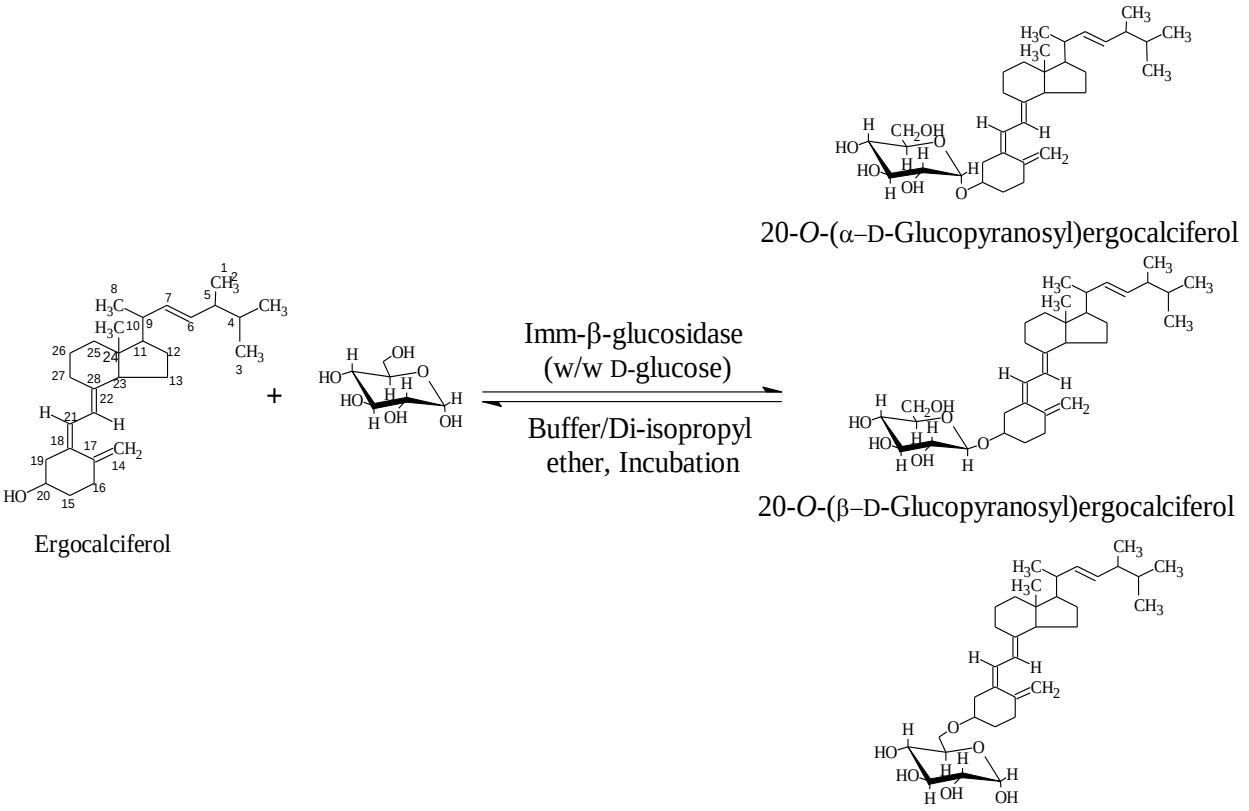
Response Surface Methodology

A four variable parametric study was employed for the CCRD analysis. Immobilized β-glucosidase concentration, pH, buffer concentration and incubation period was employed as variables. The experimental design consisted of 30 experiments of four variables at five levels (-2, -1, 0, +1, +2). Table 1 shows the coded levels and the equated actual values of the variables employed in the design matrix. Actual levels employed and the glucosylation yields obtained from the experiments are given in Table 2. A second order polynomial equation correlating the effect of variables in terms of linear, quadratic and cross product terms was employed to predict the glucosylation yield. The general equation is of the form,

$$Y = A_0 + \sum_{i=1}^{N} A_i X_i + \sum_{i=1}^{N} A_{ii} X_i^2 + \sum_{i=1}^{N-1} \sum_{j=i+1}^{N} A_{ij} X_i X_j \quad \text{.....(1)}$$

where Y is the glucosylation yield (%), A_o = constant term, X_i are the variables, A_i are the coefficients for the linear terms, A_{ii} are the coefficients for the quadratic terms, A_{ij} are the coefficients for the cross product terms and N is the number of variables.

Microsoft Excel software, version 5.0 was used for determining the coefficients of the equation and analysis of variance (ANOVA) for the final predictive equation. ANOVA is required to test the significance and adequacy of the model (Table 3). The response surface equation was optimized for maximum yield in the range of process variables using a KyPlot version 2.0 beta.



Scheme 1: Synthesis of 20-*O*-(D-Glucopyranosyl)ergocalciferol

Table 1: Coded and actual values of variables used in the experimental design

Variables	-2	-10	0	1	2
Imm-β-glucosidase (% w/w D-glucose)	20	40	60	80	100
pH	4.0	5.0	6.0	7.0	8.0
Buffer concentration (mM)	0.04	0.08	0.12	0.16	0.2
Incubation period (h)	10	20	30	40	50

Table 2: CCRD experimental design with predicted and experimental yields

Expt. no	Enzyme (% w/w D-glucose)	pH	Buffer concentration (mM)	Incubation Period (h)	Yield (%)	
					Experimental[a]	Predicted
1	40	5.0	0.08	20	7	17
2	40	5.0	0.08	40	6	10
3	40	5.0	0.16	20	6	7
4	40	5.0	0.16	40	6	5
5	40	7.0	0.08	20	5	7
6	40	7.0	0.08	40	10	12
7	40	7.0	0.16	20	3	3
8	40	7.0	0.16	40	7	13
9	80	5.0	0.08	20	18	18
10	80	5.0	0.08	40	10	13
11	80	5.0	0.16	20	11	11
12	80	5.0	0.16	40	7	10
13	80	7.0	0.08	20	7	9
14	80	7.0	0.08	40	10	15
15	80	7.0	0.16	20	6	8
16	80	7.0	0.16	40	27	19
17	20	6.0	0.12	30	28	19
18	100	6.0	0.12	30	26	26
19	60	4.0	0.12	30	14	7
20	60	8.0	0.12	30	8	6
21	60	6.0	0.04	30	25	14
22	60	6.0	0.2	30	6	8
23	60	6.0	0.12	10	8	3
24	60	6.0	0.12	50	11	7
25	60	6.0	0.12	30	9	10
26	60	6.0	0.12	30	9	10
27	60	6.0	0.12	30	9	10
28	60	6.0	0.12	30	10	10
29	60	6.0	0.12	30	10	10
30	60	6.0	0.12	30	10	10

[a]*Conversion yields calculated from the HPLC with respect to D-glucose. Error in the measurement will be ± 10%. Data are an average from two measurements.*

Table 3: Analysis of variance (ANOVA) of the response surface model along with coefficients of the response equation

Regression					
Multiple *R*	0.74				
Standard error	6.405				
ANOVA					
	Degrees of freedom	**Sum of squares**	**Mean sum of squares**	***F* ratio**	**Significance *F***
Regression	14	751.3	53.7	1.3	0.3
Residual	15	615.4	41.0		
Total	29	1366.8			
Coefficients	**Values of coefficients**	**Standard error**	***t*-Stat**	**p-value**	
A_1	1.721	1.307	1.3161	0.21	
A_2	-0.312	1.307	-0.2383	0.81	
A_3	-1.552	1.307	-1.1867	0.25	
A_4	0.995	1.307	0.7610	0.46	
A_{11}	3.250	1.223	2.6570	0.02	
A_{22}	-0.774	1.223	-0.6330	0.54	
A_{33}	0.426	1.223	0.3482	0.73	
A_{44}	-1.187	1.223	-0.9703	0.35	
A_{12}	0.189	1.601	0.1179	0.91	
A_{13}	0.724	1.601	0.4520	0.66	
A_{14}	0.256	1.601	0.1600	0.87	
A_{23}	1.444	1.601	0.9016	0.38	
A_{24}	2.849	1.601	1.7790	0.09	
A_{34}	1.241	1.601	0.7751	0.45	

Artificial Neural Network (ANN)

ANN can accommodate more then two variables to predict two are more parameters. ANN differs from conventional programs in their ability to learn about the system without a need of any prior knowledge on the relationships of the process variables. ANN has three layers - an input layer consisting of four nodes (variables), a hidden layer consisting of ten nodes and an output layer of one output node (Linko *et al.*, . 1993). The experimental data was trained using one of the various training algorithms such as Online Backpropagation procedure. A suitable activation function is selected to arrive at minimum average absolute relative deviation between experimental and predicted yields. ANN analysis was carried out by using Software QwikNet version 2.1.

To train an ANN model, a set of data containing input nodes and output nodes are fed into the software program. After training, the ANN is capable of predicting the output with any similar pattern that it has learned during the training. ANN is tested for the remaining set of experimental data. The learning rate and momentum value of the network is set to optimize with a targeted error value of 0.05. Table 4 shows the training and testing data considered for the analysis.

Table 4: Experimental variables values employed for training and testing of ANN

Enzyme (% w/w D-glucose)	pH	Buffer concentration (mM)	Incubation Period (h)	Yield (%)	
				Experimental[a]	Predicted
Training data					
40	5.0	0.08	20	7	12
40	5.0	0.16	20	6	12
40	5.0	0.16	40	6	11.3
40	7.0	0.08	40	10	10
40	7.0	0.16	20	4	12
40	7.0	0.16	40	7	5
80	5.0	0.08	40	10	11
80	5.0	0.16	40	7	7
80	7.0	0.08	20	7	7
80	7.0	0.16	20	6	7
80	7.0	0.16	40	27	12
100	6.0	0.12	30	28	12
60	8.0	0.12	30	8	9
60	6.0	0.04	30	25	24
60	6.0	0.12	10	8	12
60	6.0	0.12	50	11	11
60	6.0	0.12	30	10	5
Testing data					
100	5.0	0.1	25	18	12
100	4.5	0.08	30	8	13
90	5.5	0.11	25	13	5
80	6.0	0.125	35	10	19
100	8.0	0.2	50	11	12
100	8.0	0.14	50	16	13
60	8.0	0.125	50	10	12
60	7.0	0.04	20	6	4
20	4.0	0.1	10	10	18
20	5.0	0.08	25	8	12
40	5.0	0.08	40	6	11
40	7.0	0.08	20	5	12
80	5.0	0.08	20	18	8
80	5.0	0.16	20	11	7
80	7.0	0.08	40	10	12
20	6.0	0.12	30	28	15
60	4.0	0.12	30	14	10
60	6.0	0.12	30	9	5
60	6.0	0.12	30	10	19

Conversion yields calculated from the HPLC with respect to D-glucose. Error in the measurement will be ± 10%. Data are an average from two measurements

RESULTS AND DISCUSSION

The present work is attempted to study the effect of immobilized β-glucosidase concentration, pH, buffer concentration and incubation period on the glucosylation yield using the data shown in Table 2. For ANN training, some of the data were considered at random and the remaining data were considered for testing (Table 4).

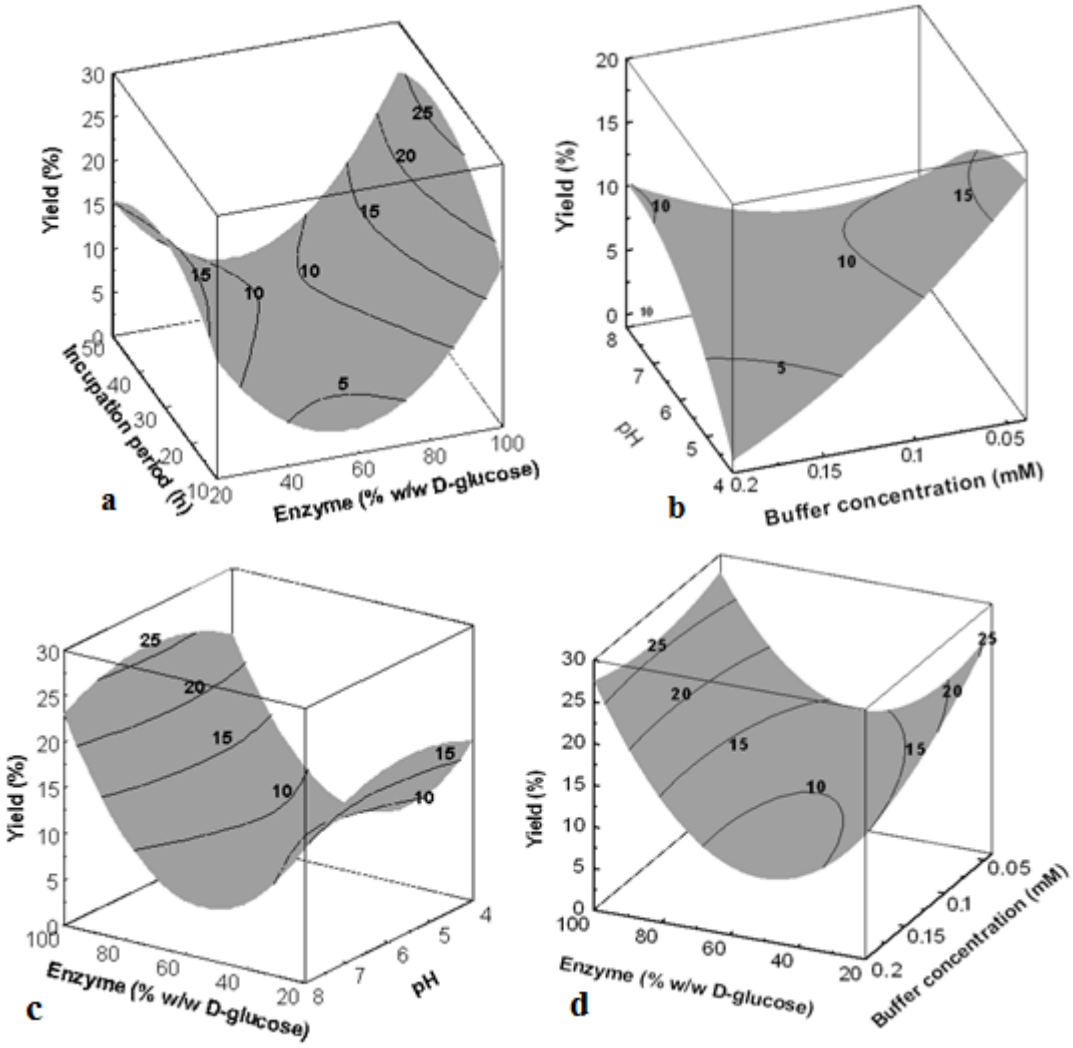


Figure caption

Figure 1a-d. Three-dimensional surface plots showing the effect of variables on the extent of glucosylation in the immobilized β-glucosidase catalyzed reaction

A. Effect of enzyme concentration and incubation period on the extent of glucosylation of ergocalciferol at 0.5 mmol ergocalciferol, 1 mmol d-glucose, ph 6.0 and 0.12 mm buffer concentration.

B. Effect of ph and buffer concentration on the extent of glucosylation at 0.5 mmol ergocalciferol, 1 mmol d-glucose, 60% (w/w d-glucose) immobilized β-glucosidase and 30 h incubation period.

C. Effect of enzyme and ph at 0.5 mmol ergocalciferol, 1 mmol d-glucose, 0.12 mm buffer concentration and 30 h incubation period.

D. Effect of buffer and enzyme concentration on the extent of glucosylation at 0.5 mmol ergocalciferol, 1 mmol d-glucose, ph 6.0 and 30 h incubation period.

Response surface methodology (RSM)

The data obtained using immobilized β-glucosidase were fitted to a second-order polynomial equation and the predictive equation obtained exhibited a R^2 value of 0.74 (Table 3a). Only few terms were found to be significant at 90% level.

Y=103.17167 - 1.092458 * E - 4.465417 * P - 46.66042 * B - 1.347 * T + 0.008124 * E * E -0.774167 * P * P + 2.6614583 * B * B + 0.011867 * T * T + 0.0094375 * E * P + 0.0904688 * E * B + 0.0012813 * E * T + 3.609375 * P * B + 0.284875 * P * T + 0.3103125 * B* T

Where E – Enzyme concentration, T – Incubation period, B – Buffer concentration, P – pH, Y - Yield. Table 2 shows the predictive yields obtained by RSM using this equation. Surface plots were generated using the predicted equation by varying any two variables at a time maintaining the other two variables at middle levels. All the reactions were carried out with a constant ergocalciferol concentration of 0.5 mmol and 1 mmol D-glucose.

Figure 1a shows the effect of enzyme concentration and incubation period on the extent of glucosylation of ergocalciferol at pH 6.0 and 0.12 mM buffer concentration. At 20% immobilized β-glucosidase (w/w D-glucose), the glucoside yield is 15%. As the enzyme concentration increased, the conversion yield decreased to 5% at 60% enzyme concentration and later increased to 20% at 100% enzyme concentration. Increase in incubation period did not vary the conversion yield much. It is the enzyme concentration which dictated the extent of glucosylation.

Effect of pH and buffer concentration on the extent of glucosylation at 60% (w/w D-glucose) immobilized β-glucosidase and 30 h incubation period is shown in Figure 1b. There is a crossover point at pH 6.0 and 0.1 mM buffer concentration. At a lower buffer concentration of 0.05 mM the yield decreased from 15% to 0% as the pH increased from 4.0 to 8.0. Similarly, the yield increased to 10% at 0.2 mM buffer concentration as the pH was increased. The distinct crossover point clearly indicated that the behaviour of the enzyme changes at pH 6.0 and 0.1 mM buffer concentration to give a better yield at lower pH and lower buffer concentrations and higher pH and higher buffer concentrations.

A curved surface plot was again obtained (Figure 1c) showing the effect of enzyme and pH at 0.12 mM buffer concentration and 30 h incubation period. A trough region was observed at all pH values for 60% (w/w D-glucose) immobilized β-glucosidase concentration. This clearly indicated that irrespective of the pH, inhibition of the enzyme takes place at 60% (w/w D-glucose) immobilized β-glucosidase and with increasing enzyme concentration beyond 60% (w/w D-glucose) the yield increased. This behaviour is a clear indication of ergocalciferol binding to the enzyme from 20 - 60% (w/w D-glucose) enzyme concentration in preference to D-glucose. Once complete binding of ergocalciferol to the enzyme at 60% (w/w D-glucose) enzyme concentration had occurred, further increase in enzyme concentration facilitated the availability of both D-glucose and ergocalciferol to the other enzyme molecules, due to rapid exchange between bound and unbound forms of D-glucose in competition to ergocalciferol.

Similar behaviour has also been absorbed in Figure 1d which depicts the effect of buffer and enzyme concentration on the extent of glucosylation at pH 6.0 and 30 h incubation period.

The maximum yield predicted by RSM was 26% at 100% (w/w D-glucose) immobilized-β-glucosidase, pH 6.0, 0.12 mM buffer concentration and 30 h incubation period. Under these conditions an experimental yield of 26% was obtained. Validation experiments were also carried out at certain selected random reaction conditions and the results are shown in Table 5. A close correspondence between experimental and predicted yield were observed.

Artificial Neural Network (ANN)

The effect of variables on yield was analyzed based on ANN. Among the various algorithms tested, Online Backpropagation method gave the least error with gaussian activation function for hidden layer and logistic function for output layer. The ANN was carried out at 0.1 learning rate with 68203 learning cycles. About 17 training data were employed to facilitate the learning process and 19 data were employed for testing (Table 4). A maximum predicted yield of 24% was obtained from the ANN training data with 60% (w/w D-glucose) immobilized β-glucosidase, pH 6.0, 0.04 mM buffer and 30 h incubation

period. Under these conditions an experimental yield of 25% was obtained. Similar correspondence was also observed with testing data.

Table 5: Validation experimental data of ergocalciferol glucoside synthesis

Enzyme (% w/w D-Glucose)	pH	Buffer concentration (mM)	Incubation period (h)	Experimental yield (%)	Predicted yield (%) (RSM)
100	5.0	0.1	25	18	27
100	4.5	0.08	30	8	13
90	5.5	0.11	25	13	9
80	6.0	0.125	35	10	15
75	6.0	0.09	30	17	13
100	8.0	0.14	50	16	35
60	8.0	0.125	50	10	15
60	7.0	0.04	20	6	7
40	5.0	0.15	25	11	9
70	5.5	0.06	30	16	15

[a]*Conversion yields calculated from the HPLC with respect to D-glucose. Error in the measurement will be ± 10%. Data are an average from two measurements.*

Both the RSM and ANN more or less gave the same standard error for both the experimental and predicted yields. Also, both the experiments have clearly brought out the enzymatic behaviour of immobilized β-glucosidase in glucosylating ergocalciferol to produce a water soluble ergocalciferol glucoside

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