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Bioconversion of yolk cholesterol by extracellular cholesterol oxidase from *Brevibacterium* sp.

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Abstract

An efficient process for reducing yolk cholesterol by enzymatic treatment was developed in this paper. Extracellular cholesterol oxidase (COD) from a mutant *Brevibacterium* sp. ODG-007, showed a strong capacity in bioconversion of yolk cholesterol to cholest-4-en-3-one, especially supplemented with NaCl and lipase C, as a yolk granule solubilizer. The bioconversion process was investigated first, to obtain basic information of the process and was further optimized by analysis of parameters, including COD concentration, dilution ratio and incubation time on the cholesterol conversion, employing Response Surface Methodology (RSM) and Central Composite Design (CCD). Under the optimum operational conditions: COD concentration of 5.39 U/g yolk powder, water: solid ratio of 3.54 and incubation time of 13.75 h, up to 85.6% yolk cholesterol was reduced, and the remaining cholestenone, an effective anti obesity medicine in the product, may raise its commercial value.

Keywords: Bioconversion; Yolk cholesterol; Cholesterol oxidase; Optimization; RSM; Cholestenone

1. Introduction

Yolk cholesterol is believed to play an important role in yolk formation (Griffin, 1992). However, high dietary cholesterol is known to promote the formation of atherosclerotic plaques in arteries, leading to coronary heart disease. High cholesterol content in egg is a major defect contributing to a decline of egg consumption (Sheather, 1992). Hence, various approaches have been made to reduce egg yolk cholesterol.

It is reported that lovastatin, a 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-COA) reductase inhibitor, shows a cholesterol-reducing capacity of 19–38% when fed to laying hens (Elkin et al., 1999; Mori, Mendonca, & Santos,1999). Copper sulphate or acetate, supplemented in the diet of chickens, was also beneficial in reducing the cholesterol content of the yolk (Ankari, Najib, & Hozab, 1998). Some other attempts to modify yolk cholesterol, by feeding laying hens on unsaturated fatty acids including garlic oil (Reddy, Lightsey, & Maurice, 1991), failed to change yolk cholesterol content. In short, these manipulations of feedstuff were not very effective in reducing yolk cholesterol. Besides, they are more complicated than direct treatment of yolk cholesterol by solvent extraction, supercritical fluid extraction, β-cyclodextrin and other methods.

Solvent extraction (Martucci & Barges, 1997) or oil extraction (Jackeschky & Martin, 2001) seems simple and easy to practise; however, it might cause protein denaturation and residual solvent in the product. Supercritical fluid extraction provides another means to reduce yolk cholesterol in an efficient way (Froning, Wehling, & Cuppett, 1990), but with high investment. Besides, lipid soluble components might be extracted during the process, which would incur some undesirable effects on the functional properties of the egg yolk (Arntfield, Bulley, & Crerar, 1992). Adsorption of yolk cholesterol by β-cyclodextrin (β-CD) has been reported (Cully & Volbrecht, 1994; Manohar, Basappa, Rao, & Divakar, 1998; Smith, Awad, & Bennink, 1995) and the process already has some commercial applications in the food industry. However, the deficiency of this process is obvious; β-CD inevitably remains in the product; it is not an approved ingredient in some countries, and the cost is rather high.

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Microbial and enzymatic treatment of yolk cholesterol provides an alternative way to reduce yolk cholesterol economically and efficiently, but there are only a few reports in this field. Aihara, Watanabe, and Nakamura (1988) degraded over 60% yolk cholesterol by *Rodococcus equi No. 23* without steroid intermediate accumulation. A 40% bioconversion was reached in a 60 min period, also by *Rodococcus equi* (Terrance, Johnson, & George, 1990). A bioconversion of 93%, over a 3-day incubation with cholesterol oxidase (COD) from *Pseudomonas fluorescens* and *Nocardia erythropolis*, was reported (Speroulla, Tran, Maurie, & Robert, 1994). However, no information is available on further optimization of the process.

Previously, quantitative work has been done on the properties of both rough and purified COD from a mutant, ODG-007, of *Brevibacterium* sp., the only steroid-like bioconversion product of cholesterol was cholest-4-en-3-one (Han, 1997; Ji, 2000). So it can be deduced that the bioconversion product of yolk cholesterol, by cholesterol oxidase from the same enzyme source, might be cholestenone alone. The converted choelstenone was characterized as a major component of oviductus ranae, a traditional Chinese medicine, and is reported to have an anti-obesity effect (Kashima, 1993; Suzuki, 1993) without any detectable clinical or necropsy anomaly.

The process of yolk cholesterol conversion, catslyzed by COD from a mutant ODG-007 of *Brevibacterium* sp., was thoroughly studied profoundly to obtain detailed information, and also optimum conditions, for the bioconversion of yolk cholesterol in a cost-efficient way, which might be applicable in the food industry.

2. Materials and methods

2.1. Material

Yolk powder was supplied by Changxing egg products Co. Ltd. (Changxing, China); all the other chemicals were commercial products of analytical purity.

2.2. Preparation of rough COD (cell-free extracts)

Brevibacterium sp. ODG-007, preserved in the lab, was used throughout this study. The culture medium contains, in g/l: cholesterol 2, glucose 20, yeast extract 7.5, NaCl 1, CH_3COONH_4 2, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.05, $FeSO_4{\cdot}7H_2O$ 0.01, $CaCl_2$ 0.1, and pH was adjusted to 7.5 by 0.1M NaOH. The medium was subjected to autoclaving for 20 min. The cultivation was carried out in a 500 ml Hinton's flask containing 50 ml culture medium at 30 °C and mixed at 200 rpm for 36 h in an orbital shaker. Cell free extract, used as enzyme source, was harvested by centrifuging the fermentation broth at 7000 rpm and 4 °C.

2.3. Enzymatic treatment of yolk cholesterol

Yolk powder was suspended in a 50 mM pH7.5 phosphate buffer, supplemented with cell free extract in a final concentration of 0.1 g/ml and a COD concentration of 3 U/g yolk powder, with vigorous mixing. The mixture was subject to incubation in a 500 ml Hinton's flask with a total volume of 100 ml at 220 rpm in an orbital shaker. Experimental conditions (temperature, pH) depended on the experiment and were varied in the range 20–70 °C, 4–11, respectively. pH 7.5 and 39 °C were fixed as the pH and treatment temperature, respectively, by results obtained earlier. Several metal ions, including Mg^2, Co^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, Ag^{+}, and EDTA, were added to the mixture in a final concentration of 5 μM to investigate their effects on the yolk cholesterol degradation. Effects of several factors, including 0.58M NaCl, lipase C, pepsin and 0.58M $(NH_4)_2CO_3$, which might improve yolk granule solubility (Causeret, Matringe, & Lorient, 1991; Chang, Powrie, & Fennema, 1977) on the yolk cholesterol degradation, were investigated, based on the results obtained above.

In the further optimization of the process by RSM, results obtained earlier were used. That is, the mixture contained yolk powder, cell-free extract and 0.58M NaCl as granule solubilizer and incubation in a 50 mM pH7.5 phosphate buffer system. The incubation was carried out at 39 °C, pH 7.5 and 220 rpm in an orbital shaker. Response surface methodology (RSM) was applied to optimize the process, using a 3×3 factorial central composite design (CCD), according to Box and Behnken's (1960) method. Based on preliminary experiments, three independent variables were: COD activity (IU):yolk powder weight (g) ratio (X_1), water volume (ml):yolk powder weight (g) ratio (X_2) and incubation time (h) (X_3) (see Table 1) and the dependent variable was cholesterol conversion rate (%).

2.4. Statistical analysis

A software package (SAS Institute, 1985) was used for analysis of variance and to fit the second order models to the dependent variables using the following equation:

$$Y = \mathrm{Ao} + \Sigma\ \mathrm{AiXi} + \Sigma\ \mathrm{AiiXi}^2 + \Sigma\ \mathrm{AijXiXj}$$

Table 1
Variables and levels for central composite design used to optimize enzymatic modification of yolk cholesterol

Variables	Symbol	Coded levels		
		−1	0	1
COD:solid ratio	X_1	1	3	5
Water:solid ratio	X_2	3	5	7
Time	X_3	9	12	15

where Ao, Ai, Aii, Aij are constant and regression coefficients of the model, and Xi the independent variables in coded values. These graphs were drawn by imposing a constant value equal to zero (central point) on one of the three independent variables.

2.5. COD activity in cell free extract

COD activity was assayed by quantifying the H_2O_2 produced with the enzyme-coupled assay with the use of horseradish peroxidase (0.01 mg/ml) and 4-aminophenazone, as previously described (Ji, 2000; Richmond, 1973).

2.6. Residual cholesterol in the product

Referring to Zhang's, Li, Liu, Chen, and Rao (1999) HPLC method, 1 ml sample was saponified and dried in a nitrogen bath, then dissolved in 1 ml isopropanol and subjected to HPLC. HPLC was carried out on a reverse phase column (ZORBAX C^{18}, *Hp*., America, 250×4.6 mm, 5 μm) with an eluent (acetonitrile:isopropanol ratio of 3:1) at a flow rate of 1 ml/min and 25 °C with a detector wavelength of 208 nm.

2.7. Cholesterol oxide in the product

Cholesterol oxide, present in the ethylacetate extracts, was analyzed by TLC according to the method of Aihara et al. (1988).

3. Results and discussion

Granule, an important microparticle, contains about 70% lipovitellin, 12% low LDL and 16% phosvitin (Burley & Cook, 1961). These subunits are connected by a ionic bridge (Causeret et al., 1991). Yolk cholesterol is mainly located in low-density lipoprotein (LDL) particles (Gornall & Kuksis, 1971).

3.1. Effect of temperature and pH on cholesterol conversion

Cholesterol oxidase (COD) from *Brevibacterium* sp., which dehydrogenated cholesterol to form the only steroid-like product, cholest-4-en-one (Han, 1997), had a broad temperature and pH optimum (Uwajima, Yagi, & Terada, 1974; Ji, 2000). Nevertheless, optimum temperature and pH for yolk cholesterol bioconversion was even broader (see Fig. 1). COD activity remained relatively high at 20 °C (Ji, 2000), but cholesterol conversion became low at 20 °C (also see Fig. 1). About 37% of the maximum conversion rate at 39 °C, can be ascribed to the poor solubility of yolk granule at low temperature, thus affecting contact between yolk cholesterol and COD. A 40% COD activity loss occurred at 50 °C (Ji, 2000); cholesterol conversion remained high, about 96% of the maximum conversion rate, maybe due to the better solubility of granule at a higher temperature.

As shown in Fig. 2, yolk cholesterol can be effectively degraded in a wide pH range of 6–10, despite COD activity loss at basic pH (Ji, 2000). Likewise, this might be ascribed to better solubility of the granule at extreme pH (Causeret et al., 1991).

3.2. Effect of metal ions on cholesterol conversion

Ca^{2+} and EDTA had a stabilization effect on COD activity, while Fe^{3+}, Co^{2+}, Hg^{2+}, Zn^{2+} and Ag^{+} had a negative effect on COD activity (Han, 1997). Bivalent or trivalent cations, especially Fe^{3+}, at low concentration

Table 2
Effect of metal ions and EDTA on cholesterol conversion

Metal ions	Cholesterol conversion rate (%)	Residual COD activity[a]
Control	64.4±2.56	100
Mg^{2+}	64.1±2.16	91.2
Co^{2+}	53.3±3.61	51.4
Mn^{2+}	38.8±2.22	97.3
Fe^{3+}	35.9±1.43	38.1
Zn^{2+}	43.4±3.72	51.8
Ca^{2+}	42.6±2.16	105
Hg^{2+}	12.5±0.74	0
Ag^{+}	45.2±1.44	38
EDTA	56.2±2.55	101

[a] Data obtained from Han's (1997) thesis.

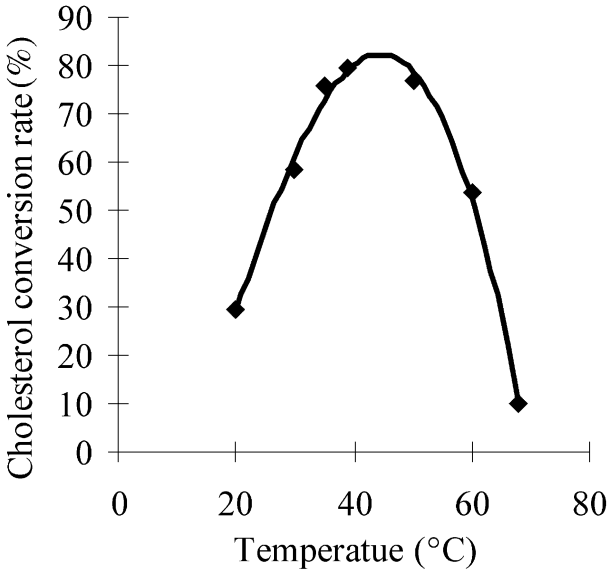

Fig. 1. Effect of temperature on cholesterol conversion.

can form complexes by co-ordinate bonds, thus strengthening the granule structure (Causeret, Matringe, & Lorient, 1992). Integrity of the structure was known to cause insolubility of granule and consequently affected contact between yolk cholesterol and COD. Most of the bivalent and trivalent metals tested had an adverse effect on the cholesterol conversion (see Table 2), which can be explained by reasons mentioned above. EDTA, by chelating all the cations, including Fe^{3+} and Ca^{2+}, can break the ionic bridge easily (Causeret et al., 1992) and had a positive effect on COD activity. Interestingly, the cholesterol conversion rate is still low with EDTA addition. A further study was needed to explain this result.

3.3. Effect of granule disrupter on cholesterol conversion

Hydrolysis of LDL by pepsin can consequently lead to granule structure disassociation (Chang et al., 1977), and application of lipase C, in attacking plasma LDL, can also stimulate cholesterol oxidation by COD (Michael, Chrissy, & Nelson, 1991). Substitution of bivalent cations by monovalent (e.g NaCl) can bring about a break-up of the phosphocalcic bridges (Causeret et al., 1991), too, and $(NH_4)_2CO_3$ was reported to have a similar ability (Cully & Volbrecht, 1994).These factors, which can disassociate granule particles and thus improve contact between COD and yolk cholesterol, were investigated for their effect on yolk cholesterol conversion (Fig. 3). Lipase C and NaCl greatly enhance cholesterol conversion, while $(NH_4)_2CO_3$ has a markedly adverse effect on conversion rate, probably due to its alkalinity. Conversion rate in the protease group was also low, maybe due to the proteolysis of COD.

Analysis of cholesterol oxide by TLC showed that the major cholesterol oxide was cholestenone, which was in agreement with previous studies (Han, 1997; Ji, 2000). But only about 40% of the yolk cholesterol was converted to cholestenone (data not shown), the rest probably being degraded to non-steroid compounds as previously reported (Aihara et al., 1988).

In short, addition of NaCl proved to be a practical and effective way of improving the conversion rate. Also, a temperature of about 39 °C was beneficial to both COD activity and granule solubility, and a pH of 7.5 was also suitable for the conversion. From results above in the first step, it was demonstrated that contact between COD and yolk cholesterol might be very significant in cholesterol bioconversion, and might be a rational approach to benefit cholesterol conversion. Other factors, including enzyme concentration, dilution rate and time, might also play important roles in cholesterol conversion. Therefore, effects of these three factors on cholesterol conversion need further study.

3.4. Optimization of the process employing CCD and RSM strategies

CCD and RSM strategies were adopted as an efficient way to find the optimum conditions for the yolk cholesterol conversion, which included COD activity:yolk powder weight ratio (X_1), water volume: yolk powder weight ratio (X_2) and incubation time (X_3). Experiments and response values (Table 3) were then used to obtain a full second order polynomial model by a multiple regression (Box & Behnken, 1960). The regression models were tested for adequacy by analysis of variance (Table 4). The close R squared to 1, and a large F-value, implied that the model was strong and can well predict

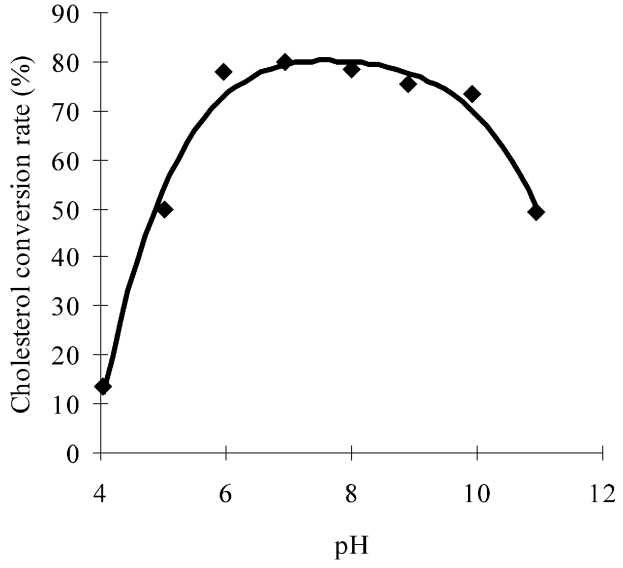

Fig. 2. Effect of pH on cholesterol conversion.

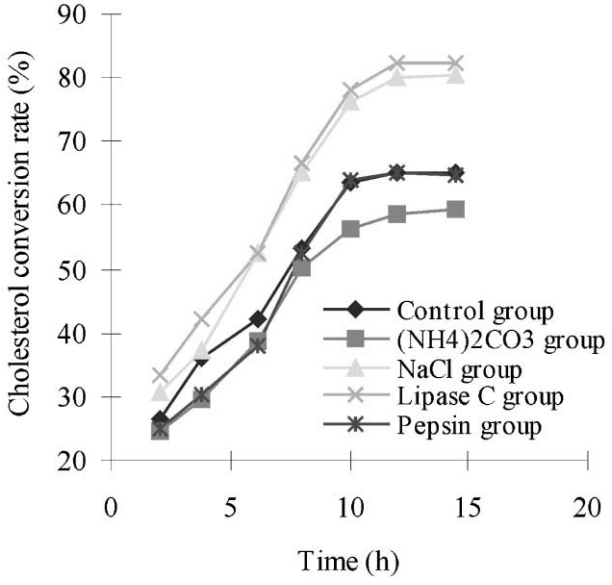

Fig. 3. Effect of granule solubilizer on cholesterol conversion.

responses, and 99% of the experimental variation can be explained by it. Moreover, the results also indicated that cholesterol conversion was complicated and characterized by linear, quadratic and interrelation terms.

Regression coefficients, as well as their Student's *t*-test values, for the models of cholesterol conversion are presented in Table 5. COD activity:yolk powder weight ratio was the most important factor affecting cholesterol conversion, due to its largest *t*-value among the three variables (Figs. 4 and 5). This result also suggested that contact between COD and yolk cholesterol was a key factor influencing the cholesterol conversion. As can also be seen in Table 5, all three variables showed significant linear and quadratic terms. Cholesterol conversion increased as COD:yolk powder ratio and incubation time increased. After 15 h incubation and in a COD:solid ratio of 5, a maximum cholesterol conversion of approximately 83% was reached, quite close to the maximum response predicted from the model (85.61%). Nevertheless, it is worth noting that large quantities of COD and long time courses are not recommended. Vast increments in COD could incur a much higher cost with just a minor increase in conversion rate. Likewise, a dramatic increase in time course can bring about more bacterial invasion with only a limited increase in conversion rate. Water:solid ratio exerted a slighter effect on the cholesterol conversion compared with the other two independent variables (Figs. 5 and 6). The maximum cholesterol conversion was located near the central point of water:solid ratio, indicating that yolk cholesterol can be reduced without high dilution rate, hence the energy consumption of the drying process would be practical for large-scale production.

Canonical analysis, a mathematical approach to examine the overall shape and locate the stationary

Table 3
The 3×3 factorial central composite designs and response values used to optimize enzymatic modification of yolk cholesterol

Trial	X_1	X_2	X_3	Cholesterol conversion (%)
1	−1	−1	0	52.7
2	−1	0	−1	49.7
3	−1	0	1	57.3
4	−1	1	0	58.3
5	0	−1	−1	64.9
6	0	−1	1	71.1
7	0	1	−1	64.4
8	0	1	1	73.5
9	1	−1	0	82.2
10	1	0	−1	74.4
11	1	0	1	83.9
12	1	1	0	77.1
13	0	0	0	75.5
14	0	0	0	74.4
15	0	0	0	73.9

Table 4
Analysis of variance for the regression model of cholesterol conversion obtained from the experimental results

Source	Degree of freedom	Sum of squares	*F*-value
Regression			
Linear	3	1369.5	692.9[a]
Quadratic	3	132.25	66.92[a]
Cross product	3	32.34	16.36[a]
Total regression	9	1534.1	258.7[a]
Residual			
Lack of fit	3	1.891	0.898
Pure error	2	1.403	
Total error	5	3.293	
R^2		0.9979	
Coefficient of variance		1.1783	

[a] Significant at 99% level.

Table 5
Regression coefficients of a full second-order polynomial model for cholesterol conversion

Term	Coefficients estimated	*T*
A0	74.61	−6.622[a]
A1	12.44	13.90[a]
A2	0.2938	5.422[a]
A3	4.059	8.438[a]
A11	−4.602	−10.89[a]
A22	−2.708	−6.672[a]
A33	−2.474	−5.857[a]
A12	0.4775	1.177
A13	0.7250	1.786
A23	−3.669	−8.686[a]

[a] Significant at 99% level.

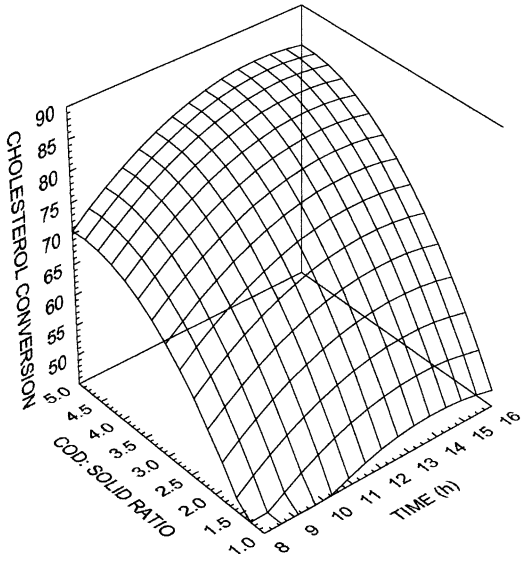

Fig. 4. Effect of COD:solid ratio and incubation time on cholesterol conversion at water:solid ratio of 5:1.

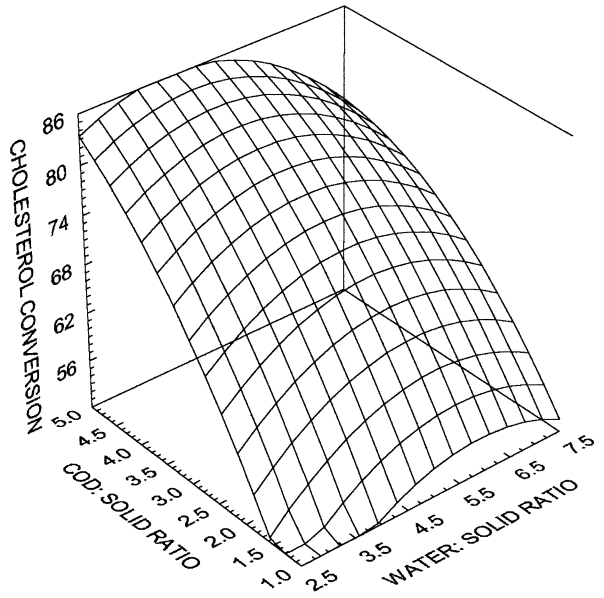

Fig. 5. Effect of COD:solid ratio and water:solid ratio on cholesterol conversion during incubation time of 12 h.

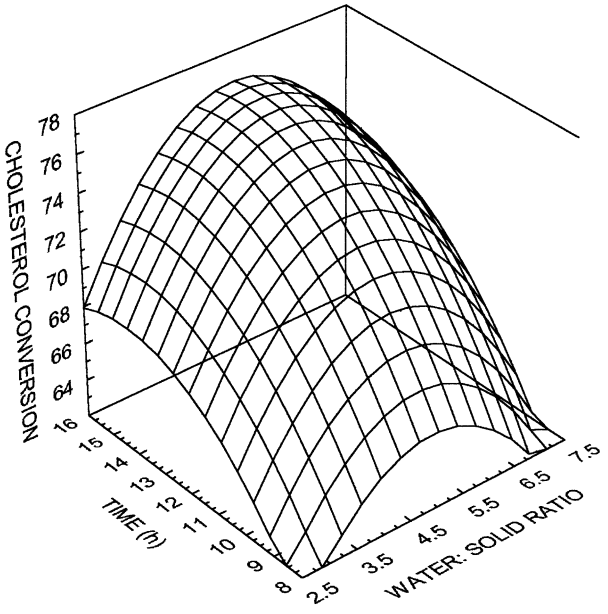

Fig. 6. Effect of water:solid ratio and incubation time (h) on cholesterol conversion at COD:solid ratio of 5:1.

point of the response surface, showed that the stationary point of the current response surface was a maximum response (Box & Behnken, 1960). Under the optimum operational conditions: COD concentration of 5.39 U/g yolk powder, water:solid ratio of 3.54 and incubation time of 13.75 h, up to 85.61% yolk cholesterol was degraded. A set of experiments was carried out to recheck the fitness of the model. After 13.75 h, a maximum conversion rate of 86.53% was reached (Fig. 7). As also shown in Fig. 7, the observed results and predicted response were in close agreement (about

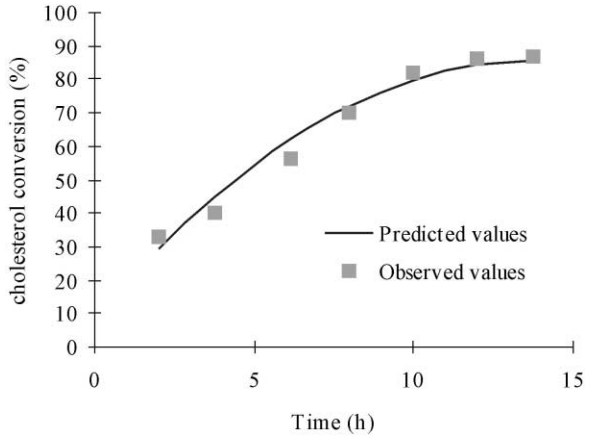

Fig. 7. Time course of yolk cholesterol conversion under optimum conditions.

90%). Thus the model developed might be considered to be very reliable for predicting conversion rate in this experiment.

4. Conclusions

Bioconversion of egg yolk cholesterol by COD from *Brevibacterium* sp. was thoroughly investigated. Some of the yolk granule solubilizers, such as NaCl and Lipase C, were found to enhance the cholesterol bioconversion dramatically. CCD and RSM were employed to further optimize the process. After the optimization, a cost-efficient process for reducing yolk cholesterol was developed. More than 86% of yolk cholesterol was reduced in the optimized process, and the cholestenone as a residual potential medicine, may add to its commercial value.

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