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Physical factors and reuse affect the production of cholesterol oxidase in reactors containing calcium alginate-immobilized *Rhodococcus equi* No. 23

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Summary

Some physical factors including initial pH of medium, cultivation temperature and shaking speed as well as reuse affecting the production of cholesterol oxidase (CholOx) in reactors containing calcium alginate-immobilized cells of *Rhodococcus equi* No. 23 were investigated. Results revealed that the free cells showed the maximum CholOx in the culture with an initial pH of 5.0, while culture inoculated with immobilized cells exhibited a broad pH range, 6.0–9.0, for maximum CholOx production. The immobilized and free cells produced the maximum CholOx in the culture incubated at 30 and 25°C, respectively. The CholOx production decreased upon increasing the cultivation temperature. Increasing CholOx activity was also noted for both immobilized and free cells of *R. equi* No. 23 in the culture with increasing shaking speed. Under the optimal culture conditions, that were established, a higher maximum CholOx production of 0.94 unit/ml was found for immobilized *R. equi* No. 23 compared to that of 0.84 unit/ml for free cells after 48 h of cultivation. Furthermore, no gel leakage was noted after re-use of the calcium alginate-immobilized *R. equi* No. 23 for seven consecutive 48 h batch culture. The CholOx production in the seventh cycle was about 60.4% of that obtained in the first cycle.

Introduction

Cholesterol oxidase (CholOx, EC 1.1.3.6) catalyses the conversion of cholesterol to 4-cholesten-3-one. It has been used for the determination of cholesterol in blood serum and in the production of the precursor for chemical synthesis of steroid hormones (Watanabe *et al.* 1986; Lolekha *et al.* 2004). Furthermore, various investigators have demonstrated the degradation of cholesterol and low density lipoprotein by CholOx and indicated that the use of CholOx may provide an economic and practical method for reducing the amount of dietary cholesterol (Aihara *et al.* 1988; Christodoulou *et al.* 1994; Wu *et al.* 1995; Watanabe *et al.* 1989a; Kim *et al.* 2002), which is partially related to cardiovascular disease, and hence serves to improve human health (Kaunitz 1978).

Rhodococcus equi No. 23 was reported to produce CholOx and was regarded as a potential industrial strain for CholOx production (Aihara *et al.* 1988; Watanabe *et al.* 1989b; Chou *et al.* 1999). In an attempt to improve fermentation processes and to increase CholOx productivity, we have previously immobilized cells of *R. equi* No. 23 cells with calcium alginate and found that the immobilized cells produced a significantly higher amount of CholOx than the free cells (Chang & Chou 2002).

In the present study, effects of initial pH of the medium, cultivation temperature and shaking speed on he production of CholOx in reactors containing immobilized cells of *R. equi* No. 23 were further investigated. In addition, the performance and stability of the immobilized cells were also examined.

Materials and methods

Microorganism

Rhodococcus equi No. 23 CCRC 13634 obtained from the Culture Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan) was used in this study and was maintained on nutrient agar (NA, Difco, Detroit, MI, USA) at 4 °C.

Culture conditions

Growth and production of CholOx were performed in a 250 ml screw-capped Erlenmeyer flask containing

100 ml culture medium consisting of (g/l) cholesterol 2.0; yeast extract 8.0; NaCl 1.0; NH_4Cl 1.0; KH_2PO_4 0.5; Na_2HPO_4 0.25; L-valine 0.10; L-tyrosine 0.15; $MgSO_4 \cdot H_2O$ 0.15; $ZnSO_4 \cdot 7H_2O$ 0.01; $FeSO_4 \cdot 7H_2O$ 0.10; and Tween 80 4.0 ml; pH 6.2. For fermentation, the culture medium was inoculated with 14.0 g gel beads (immobilized cells) or 1.0 ml of the active culture of free cells and cultivated for a period of 48 h unless otherwise specified.

When the effect of medium pH was studied, the initial pH of the medium was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 by titration with sterilized 1 M HCl or 1 M NaOH. The culture was maintained at 25–45 °C and 10–250 rev/min for studying the effects of culture temperature and shaking speed, respectively. Other operating conditions were as specified in the Results and Discussion section.

Immobilization of R. equi *No. 23*

Alginate-immobilized cells of *R. equi* No. 23 were prepared according to the procedure described by Chang & Chou (2002). Briefly, a 3% (w/v) sodium alginate solution was autoclaved and cooled to room temperature before adding the precultured cells. This mixture was extruded dropwise through a silicon glass tube that had a tip into a gently stirred 3% $CaCl_2$ solution. After 1 h, the calcium alginate beads with a diameter of ca. 2.2 mm were washed three times with sterile water.

Performance and stability of immobilized cells

The performance and stability of the alginate-immobilized cells were assessed using the same beads in 7 consecutive 2-day batch fermentations. At the end of each 2-day cycle, gel beads were collected by decanting the spent medium. After washing twice with sterile saline solution and soaking in 3% CaCl solution for 30 min, the gel beads were placed in fresh medium for the next cycle. The viable populations of *R. equi* No. 23 and CholOx activity at the end of each cycle were determined.

Analytical methods

The cell population of *R. equi* No. 23 in the media was measured by counting colonies developed from viable cells on NA and incubating at 37 °C for 3 days. Immobilized cells inside gel beads were determined according to that described by Ogbonna *et al.* (1989). The alginate beads were first collected by filtrating the culture through sterile filter paper. After washing three times with sterile distilled water, the gel beads were dissolved in 0.2 M citrate buffer (pH 5.0) and then viable cells were counted. On the other hand, viable cells in the filtrate of the immobilized cell culture (cells outside the gel beads) and the culture with free cells only were also determined. These were centrifuged at $5600 \times g$ (5 °C) for 15 min and the supernatant was used as the enzyme source. Assay of CholOx activity was based on the conversion of cholesterol to 4-cholesten-3-one. Briefly, substrate solution was prepared by adding 5.0 ml of cholesterol solution in isopropanol (30 mg/10 ml) to 300 ml of 0.1 M phosphate buffer (pH 7.5) containing 0.05% (v/v) Triton X-100. For reaction, 50 μl of enzyme solution was added to 3.0 ml of substrate solution and incubated at 30 °C for 5 min. Formation of 4-cholesten-3-one was determined by measuring the absorbance at 240 nm. One unit of CholOx is defined as the amount of enzyme required to form 1 μmol 4-cholesten-3-one per min under the test condition. Dry weight of gel beads was determined according to the AOAC method (AOAC 1984) . Details of these analytical procedures were described previously (Chang & Chou 2002).

Statistical analysis

The mean values and standard deviation were calculated from data obtained from three separate experiments. These data were then compared by Duncan's multiple range method (SAS 1989).

Results and discussion

Effect of initial pH

Initial pH of the medium is known to affect the enzymes or metabolites produced by microorganisms (Bajpai & Margaritis 1987; Buzás *et al.* 1989; Lee *et al.* 1997b). Table 1 shows the growth and production of CholOx by the free and immobilized cells of *R. equi* No. 23, respectively, in the medium with various initial pH values.

Immediately after the inoculation of the free cells, samples of culture were taken to determine the viable population of test organism. No significant difference ($P > 0.05$) in the number of viable *R. equi* No. 23 was noted in the media with various pH values (data not shown), indicating that the initial pH of the medium tested did not cause any immediate detrimental effect on the test organism. As shown in Table 1, the final population of test organism ranged between 8.31 and 8.62 log c.f.u./ml in the media after 48 h of incubation. The highest CholOx activity of 0.20 unit/ml was noted in the culture with an initial pH 5.0, while the media with an initial pH 7.0–9.0 had the lowest CholOx activity determined after 48 h of cultivation.

In the culture containing immobilized cells of *R. equi* No. 23 with various initial pHs, the gel bead was found to contain a viable population of ca. 8.07–8.77 log c.f.u./g after 48 h of cultivation (Table 1). The final viable population found outside the gel beads in culture with different initial pHs did not show significant difference ($P > 0.05$).

At the end of cultivation, the maximum CholOx activity of 0.19 unit/ml in the medium having an initial pH of 7.0 and was inoculated with the immobilized cells of *R. equi* No. 23 (Table 1). However,

Table 1. Effect of initial pH on the growth and cholesterol oxidase production by *R. equi* No. 23[a].

Initial pH	Free cells		Immobilized cells		
	Viable count (log c.f.u./ml)	CholOx activity (unit/ml)	Viable count (log c.f.u./ml or g)		CholOx activity (unit/ml)
			Inside gel beads	Outside gel beads	
control	8.62 A[b]	0.18 B	8.51 B	8.35 A	0.16 B
5.0	8.56 A,B	0.20 A	8.77 A	8.32 A	0.16 B
6.0	8.49 A,B,C	0.17 B	8.31 C	8.34 A	0.18 A,B
7.0	8.41 B,C	0.12 C	8.31 C	8.31 A	0.19 A
8.0	8.48 A,B,C	0.13 C	8.71 A	8.38 A	0.17 A,B
9.0	8.31 C	0.12 C	8.07 D	8.48 A	0.19 A

[a] Culture medium with various initial pHs was incubated at 37 °C in a rotary shaker (150 rev/min) for 48 h.[b]Means in the same column with different capital letters differ significantly ($P < 0.05$) according to Duncan's multiple range test.

CholOx activity in the medium with an initial pH between 6.0 and 9.0 did not show any significant difference ($P > 0.05$). Compared to culture inoculated with the free cells, which showed the optimum CholOx production at an initial pH of 5.0, the immobilized *R. equi* No. 23 exhibited a wide pH range, 6.0–9.0, for maximum CholOx production. Change in the optimum pH for CholOx production with the immobilized *R. equi* No. 23 as observed in the present study was also reported by various investigators (Buzás *et al.* 1989; Tan & Day 1998). Tan & Day (1998) observed that bioconversion of limonene to α-terpineol by alginate-immobilized *Penicillium digitatum* took place across a wider pH range, from 3.5 to 8.0, than free cells. Buzás *et al.* (1989) indicated that the fermentation capacity of a *Saccharomyces cerevisiae* free cell system had a pH dependence with an optimum around pH 4.0, while the fermentation capability of immobilized yeast cells was practically independent of the hydrogen ion concentration between pH 2.5 and 6.2. Protection of microbial cells entrapped in the Ca-alginate gel beads from environmental conditions may contribute to these observed phenomena. The greater insensitivity of the immobilized cells to pH as observed in the present study may allow a less precise pH control in the bioreactor operating with an immobilized *R. equi* No. 23, thereby reducing operation cost.

Effect of cultivation temperature

Table 2 shows the growth and CholOx production in culture inoculated with free cells and immobilized cells after 48 h of incubation at various temperatures. In general, growth of the inoculated free cells of *R. equi* No. 23 was best in the culture incubated at 25 °C. As the incubation temperature increased, the final population of *R. equi* No. 23 decreased. After 48 h of incubation at 45 °C, the culture originally inoculated with free cells contained the least viable population of only 2.26 log c.f.u./ml compared to 9.26 log c.f.u./ml, which was the highest, in the culture incubated at 25 °C. Meanwhile, viable cells inside the gel beads or outside the gel beads detected in the culture originally inoculated with immobilized cells were also found to be the highest when the culture was incubated at 25 °C among the various temperatures tested.

The medium inoculated with free cells exhibited the maximum CholOx activity of 0.26 unit/ml at 25 °C, while medium inoculated with immobilized *R. equi* No.23 showed the highest CholOx activity of 0.27 unit/ml when incubated at 30 °C. This showed that the immobilization of *R. equi* No. 23 with alginate enables the test organism to exhibit a higher optimum temperature for maximum CholOx production than free cells did. Similar phenomena were also observed by Bajpai & Margaritis (1987) and Tan & Day (1998). The former reported that the specific ethanol production rate was maximum at 35 °C with free

Table 2. Effect of cultivation temperature on the growth and cholesterol oxidase production by *R. equi* No. 23[a].

Cultivation temperature (°C)	Free cells		Immobilized cells		
	Viable count (log c.f.u./ml)	CholOx activity (unit/ml)	Viable count (log c.f.u./ml or g)		CholOx activity (unit/ml)
			Inside gel beads	Outside gel beads	
25	9.26 A[b]	0.26 A	8.81 A	8.97 A	0.24 B
30	8.87 B	0.23 B	8.44 B	8.94 A	0.27 A
37	8.45 C	0.13 C	8.50 B	8.77 B	0.18 C
40	7.94 D	0.07 D	7.53 C	0.95 C	0.04 D
45	2.26 E	0.00 E	0.00 D	0.00 D	0.00 E

[a] Culture medium with an initial pH 7.0 was incubated at various temperatures in a rotary shaker (150 rev/min) for 48 h. [b]Means in the same column with different capital letters differ significantly ($P < 0.05$) according to Duncan's multiple range test.

Table 3. Effect of shaking speed on the growth and cholesterol oxidase production by *R. equi* No. 23[a].

Rotary speed (rev/min)	Free cells		Immobilized cells		
	Viable count (log c.f.u./ml)	CholOx activity (unit/ml)	Viable count (log c.f.u./ml or g)		CholOx activity (unit/ml)
			Inside gel beads	Outside gel beads	
0	7.55 D[b]	0.02 E	7.13 C	7.32 E	0.00 D
100	8.86 C	0.18 D	8.42 B	8.85 D	0.18 C
150	8.86 C	0.28 C	8.90 A	9.02 C	0.30 B
200	9.53 A	0.39 B	9.02 A	9.14 B	0.28 B
250	9.40 B	0.84 A	9.03 A	9.82 A	0.94 A

[a] Culture medium with an initial pH 7.0 was incubated at 30 °C in a rotary shaker with various shaking speeds. [b] Means in the same column with different capital letters differ significantly ($P < 0.05$) according to Duncan's multiple range test.

cells of *Kluyveromyces maxianus*, while the alginate-immobilized cells exhibited its maximum specific ethanol production rate at 40 °C. The latter investigated the production of α-terpineol by *Penicillium digitatum* and indicated that the immobilized cells exhibited a higher optimum temperature than the free cells.

Growth and CholOx production by the test organism, regardless of immobilization, reduced significantly ($P < 0.05$) at an incubation temperature of 40 °C or higher. This phenomenon was especially pronounced with the immobilized cells. The reduced dissolved oxygen in the medium at higher cultivation temperatures may result in the reduced CholOx production observed. Furthermore, limitation of oxygen transfer through the outer cell matrix layer of the gel (O'Reilly & Crawford 1989; Chapatwala *et al*. 1998) and the high local cell density may also lead to the reduced growth and CholOx production by the immobilized *R. equi* No. 23 at 40 °C or higher temperatures.

Effect of shaking speed

Shaking speed showed a significant effect on the growth and production of CholOx in the cultures inoculated with the free or immobilized cells of *R. equi* No. 23 (Table 3). Inoculated with free cells, the static culture (0 rev/min) among the various shaking speeds tested showed the lowest final population of 7.55 log c.f.u./ml and CholOx activity of 0.02 unit/ml after 48 h of cultivation. Consistent with the observation of Wu *et al*. (1995), in general, the final population and CholOx production increased upon increasing the shaking speed.

Similar trend concerning the effect of shaking speed on the growth and CholOx production was also observed in the culture with the immobilized *R. equi* No 23. Although the final population inside the gel beads noted in the cultures shaking at a rotary speed of 150–250 rev/min showed no significant difference ($P > 0.05$), viable population outside the gel beads was found to be the highest in the culture shaken at 250 rev/min. It is interesting to note that CholOx production by immobilized cells in the culture with a shaking speed between 0 and 200 rev/min was either less or equivalent to that produced by the free cells under similar shaking conditions. In the present study, cholesterol serves as the main carbon source in the medium. Utilization of cholesterol as the carbon source involves a series of oxidation and degradation reactions and was considered to be an oxygen-consuming process (Stadtman *et al*. 1954). Therefore, the limited amount of dissolved oxygen present in the medium and the oxygen diffusion barrier of the immobilization matrices under the shaking speeds of 0–200 rev/min (O'Reilly & Crawford 1989; Chapatwala *et al*. 1998) may reduce the performance of the immobilized *R. equi* No. 23 and thus lead to the lower CholOx production observed in the present study. On the other hand, the amount of dissolved oxygen in the medium apparently increased and the oxygen diffusion problem was overcome as the shaking speed was increased to 250 rev/min. It thus enabled the immobilized cells of *R. equi* No. 23 to increase their CholOx production to a maximum of 0.94 unit/ml (Table 3) which was higher than the 0.84 unit/ml produced in the culture with the free cells.

Time course of growth and CholOx production

The time course of growth and production of CholOx by immobilized cells of *R. equi* No. 23 in medium is shown in Figure 1. The viable population of cells inside or outside the gel beads in the culture all increased from the start of cultivation. No increase in the viable population of cells inside gel beads was noted after 24 h of cultivation, while population of free cells further increased until 48 h of cultivation. Generation time of the newly released cells from gel beads was estimated to be about 1.21 h compared to 2.72 h noted with cells inside the gel beads. The higher growth rate of the cells released from the alginate gel beads, similar to that reported by Mori *et al*. (1989) who studied the growth behaviour of immobilized *Acetobacter* sp. K-1024, might contribute in part to the higher CholOx production by the immobilized cells of *R. equi* No. 23.

Stability of immobilized cells

Being able to be used repeatedly to improve productivity is one of the important advantages of immobilized cells (Paik & Glatz 1994; Saswathi *et al*. 1995). To test the stability and performance of the immobilized *R. equi*

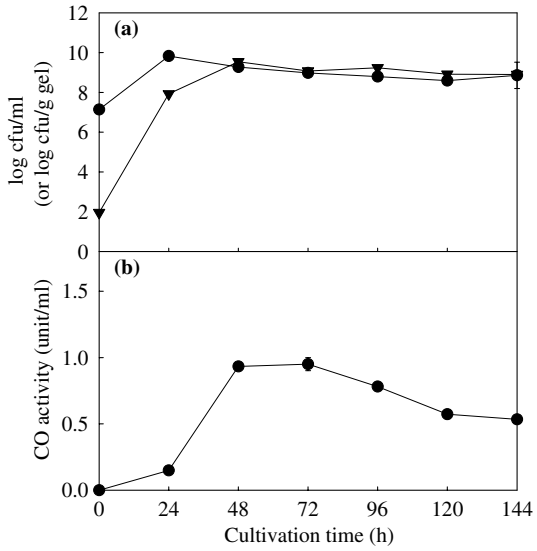

Figure 1. Time course of the growth and cholesterol oxidase production by immobilized cells of *R. equi* No. 23. Culture medium having an initial pH 7.0 was incubated at 30 °C with a shaking speed of 250 rev/min. Viable cells inside gel beads, ●; viable cells outside alginate beads, ▼ (a) and total cholesterol oxidase activity (b).

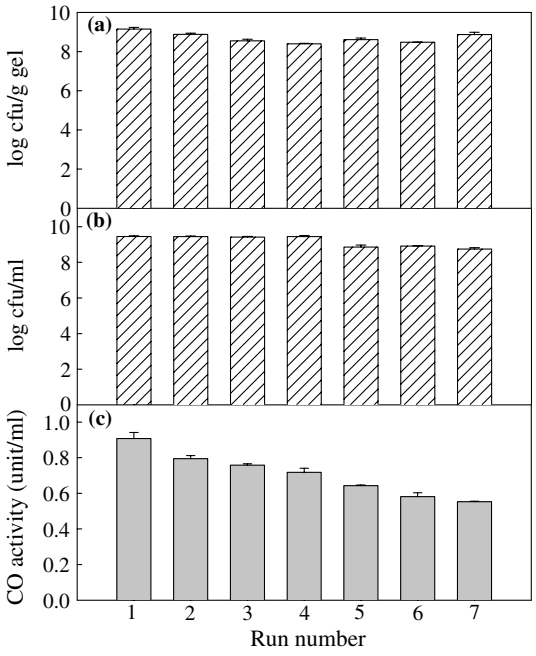

Figure 2. Growth and cholesterol oxidase production by immobilized cells of *R. equi* No. 23 after 48 h of cultivation in 7 consecutive batch cultivation with the same immobilized cells reused for the fermentation. Viable cells inside gel beads (a), viable cells outside gel beads (b) and total cholesterol oxidase activity (c).

No. 23, consecutive batch fermentations each lasting 48 h with the same gel beads were conducted. At the beginning, the gel beads were rinsed twice with saline solution after each cycle before placing them in the medium for the next cycle. However, after 4 consecutive cycles, appearance of cracks and leakage of gel beads were noted. Apparently, the presence of phosphate, a calcium chelating agent, and certain cations such as Mg^{2+} or K^{+}, which are bound strongly to alginate and form soluble salts, in the medium disrupt alginate gels by solubilizing the bounds Ca^{2+} (Cheetham *et al.* 1979; Smidsrød & Skjåk-Bræk 1990). Therefore, the alginate gel beads were soaked in 3% $CaCl_2$ solution for 30 min to improve the alginate gel structure (Cheetham *et al.* 1979) in the present study. With this treatment, no crack or leakage of gel beads was noted throughout the entire experimental period.

Figure 2 shows the growth of the immobilized *R. equi* No. 23 and CholOx activity detected in the spent medium at the end of each cycle of the 7 consecutive batch fermentations. The number of cells found inside or outside the gel beads varied non-linearly throughout the consecutive cycles of growth (Figure 2A, B). The maximum CholOx activity of 0.91 unit/ml was detected in the spent medium after the first cycle. As the consecutive cycle is increased, CholOx activity detected in the spent medium is decreased (Figure 2C). In the seventh and last cycle tested, CholOx production (0.55 unit/ml) was ca. 60.4% of that obtained in the first cycle.

Conclusion

The results show that in the system inoculated with calcium alginate-immobilized cells of *R. equi* No. 23 exhibited a broader pH optimum for CholOx production than free cells. The cholesterol oxidase production in the culture with immobilized cells, estimated at 48 h culturing, under the fixed optimal conditions (pH 7.0, 30 °C, shaking at 250 rev/min), was higher than that with the free cells. In addition, the results also suggest that the calcium alginate cells of *R. equi* No. 23 soaked in 3% $CaCl_2$ solution for 30 min after each fermentation cycle could be used for several consecutive fermentation cycles with adequate and acceptable performance. Therefore, data gathered from the present study provide useful information when a calcium alginate-immobilized cell of *R. equi* No. 23 is employed for the practical production of cholesterol oxidase.

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References

Aihara, H., Watanabe, K. & Nakamura, R. 1988 Degradation of cholesterol in egg yolk by *Rhodococcus equi* No. 23. *Journal of Food Science* **53**, 659–660. ISBN 0935584242.

AOAC, 1984 *Official Methods of Analysis*, Washington, DC: 14th ed Association of Official Analytical Chemists.

Bajpai, P. & Margaritis, A. 1987 The effect of temperature and pH on ethanol production by free and immobilized cells of *Kluyveromyces marxianus* grown on Jerusalem artichoke extract. *Biotechnology and Bioengineering* **30**, 306–313.

Buzás, Zs., Dallmann, K. & Szajáni, B. 1989 Influnce of pH on the growth and ethanol production of free and immobilized *Saccharomyces cerevisiae* cells. *Biotechnology and Bioengineering* **34**, 882–884.

Chang, Y.C. & Chou, C.C. 2002 Growth and production of cholesterol oxidase by alginate-immobilized cells of *Rhodococcus equi* No. 23. *Biotechnology and Applied Biochemistry* **35**, 69–74.

Chapatwala, K.D., Babu, G.R.V., Vijaya, O.K., Kumar, K.P. & Wolfram, J.H. 1998 Biodegradation of cyanides, cyanates and thiocyanates to ammonia and carbon dioxide by immobilized cells of *Pseudomonas putida*. *Journal of International Microbiology Biotechnology* **20**, 28–33.

Cheetham, P.S.J., Blunk, K.W. & Bucke, C. 1979 Physical studies on cell immobilization using calcium alginate gels. *Biotechnology and bioengineering* **21**, 2155–2168.

Chou, C.C., Lee, M.T. & Chen, W.C. 1999 Production of cholesterol oxidase by *Rhodococcus equi* No. 23 in a jar fermenter. *Biotechnology and Applied Biochemistry* **29**, 217–221.

Christodoulou, S., Hung, T.V., Trewhell, M.A. & Black, R.G. 1994 Enzymatic degradation of egg yolk cholesterol. *Journal of Food Protection* **57**, 908–912.

Kaunitz, H. 1978 Cholesterol and repair process in arteriosclerosis. *Lipids* **13**, 73–374.

Kim, K.P., Rhee, C.H. & Park, H.D. 2002 Degradation of cholesterol by *Bacillus subtilis* SFF34 isolated from Korean traditional fermented flatfish. *Letters in Applied Microbiology* **35**, 468–472.

Lee, M.T., Chen, W.C. & Chou, C.C. 1997a Medium improvement by orthogonal array designs for cholesterol oxidase production by *Rhodococcus equi* No. 23. *Process Biochemistry* **32**, 697–703 .

Lee, M.T., Chen, W.C. & Chou, C.C. 1997b Nutritional factors that affect the production of cholesterol oxidase by *Rhodococcus equi* No. 23. *Biotechnology and Applied Biochemistry* **26**, 159–162.

Lolekha, P.H., Srisawasdi, P., Jearanaikoon, P., Wetprasit, N., Sriwanthana, B. & Kroll, M.H. 2004 Performance of four sources of cholesterol oxidase for serum cholesterol determination by the enzymatic endpoint method. *Clinica Chimica Acta* **339**, 135–145.

Mori, A., Matsumoto, N. & Imai, C. 1989 Growth behavior of immobilized acetic acid bacteria. *Biotechnology Letters* **11**, 183–188.

O'Reilly, K.T. & Crawford, R.L. 1989 Kinetics of *p*-cradation by an immobilized *Pseudomonas* sp. *Applied and Environmental Microbiology* **130**, 866–870.

Ogbonna, J.C., Amano, Y. & Nakamura, K. 1989 Elucidation of optimum conditions for immobilization of viable cells by using calcium alginate. *Journal of Fermentation and Bioengineering* **67**, 92–96.

Paik, H.D. & Glatz, B.A 1994 Propionic acid production by immobilized cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. *Applied Microbiology and Biotechnology* **42**, 22–27.

SAS, 1989 In: *SAS user's Guide: Statistics* (Version 6 eds.). SAS Institute Inc., Cary, N.C., USA.

Saswathi, N., Sheela, R., Jamuna, R. & Ramakrishna, S.V. 1995 Synthesis of cyclodextrin glycosyl transferase by immobilized cells of *Bacillus circulans*. *Bioprocess Engineering* **12**, 283–286.

Smidsrød, O. & Skjåk-Bræk, G. 1990 Alginate as immobilization matrix for cells. *Trends in Biotechnology* **8**, 71–78.

Stadtman, T.C., Cherkes, A. & Anfinsen, C.B. 1954 Studies on the microbiological degradation of cholesterol. *Journal of Biological Chemistry* **206**, 511–523.

Tan, Q. & Day, D.F. 1998 Bioconversion of limonene to α-terpineol by immobilized *Penicillium digitatum*. *Applied Microbiology and Biotechnology* **49**, 96–101.

Watanabe, K., Aihara, H. & Nakamura, R. 1989a Degradation of cholesterol in lard by the extracellular and cell-bound enzymes from *Rhodococcus equi* No. 23. *Lebensmittel-Wissenschaft und -technologie* **22**, 98–99.

Watanabe, K., Aihara, H., Nakagawa, Y., Nakamura, R. & Sasaki, T. 1989b Properties of the purified extracellular cholesterol oxidase from *Rhodococcus equi* No. 23. *Journal of Agricultural and Food Chemistry* **37**, 1178–1182.

Watanabe, K., Shimizu, H., Aihara, H., Nakamura, R. & Suzuki, K.I. 1986 Isolation and identification of cholesterol-degrading *Rhodococcus* strains from food of animal origin and their cholesterol oxidase activity. *The Journal of General and Applied Microbiology* **32**, 137–147.

Wu, C.Y., Yu, R.C. & Chou, C.C. 1995 Decomposing cholesterol in egg yolk by enzymes of *Rhodococcus equi* No. 23. *Journal of Food Science* **22**, 69–76.