Production of cholesterol oxidase by *Rhodococcus equi* No. 23 in a jar fermenter

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***Rhodococcus equi* No. 23 was grown in a batch fermenter. The effects of cultivation temperature, pH of the culture medium, aeration rate and agitation speed on the production of cholesterol oxidase (CholOx) by the test organism were examined. Results revealed that the cultivation temperature, the pH of the medium, the aeration rate and the agitation speed all affected the production of CholOx by *R. equi* No. 23. Adjusting the operation variables during the cultivation period increased the production of CholOx effectively and prevented the occurrence of overflow of foam during the fermentation period. A maximum CholOx activity of 0.34 unit/ml with a volumetric production rate of 0.011 unit/h per ml could be achieved in 30 h of cultivation at an aeration rate of 5.0 l/min, if the pH of the culture medium, the cultivation temperature and the agitation speed were controlled at 6.5, 39 °C and 200 rev./min respectively during the first 24 h of cultivation, then shifted to 7.5, 37 °C and 300 rev./min respectively.**

Introduction

Rhodococcus equi No. 23 is one of the micro-organisms capable of producing cholesterol oxidase (CholOx) which catalyses the conversion of cholesterol to 4-cholesten-3-one [1–3]. CholOx has received much attention owing to its application in the determination of cholesterol in blood serum and food and in the production of a starting material for the chemical synthesis of pharmaceutical steroid [1]. In addition, CholOx can be employed to improve human health by degrading dietary cholesterol, which has been implicated in cardiovascular disease [4].

R. equi No. 23 was first isolated by Watanabe and Adachi [5] from butter and is regarded as a potential industrial strain for CholOx production because of its high yield [1,6,7]. Purifying and characterizing CholOx from *R. equi* No. 23, Watanabe et al. [7] showed that some properties of this CholOx are different from the enzyme from *Brevibacterium sterolicum* [8] and *Streptomyces violascens* [9].

We have previously investigated the nutritional factors that affect CholOx production by *R. equi* No. 23 [2]. In addition, a study with the use of response surface methodology was further conducted to improve the culture medium for CholOx production by this organism [3]. Here we report the influence of culture temperature, medium pH, aeration rate and agitation speed on growth behaviour and CholOx production by *R. equi* No. 23 in a jar fermenter. Furthermore, a profile of operation variables that improved the production of CholOx by the test organism is also described.

Materials and Methods

Micro-organism

R. equi No. 23 (CCRC) obtained from the Culture Collection and Research Center (CCRC) (Food Industry Research and Development Institute, Hsinchu, Taiwan) was used in this study and was maintained in nutrient agar (Gibco Laboratories, Madison, WI, U.S.A.) at 4 °C.

Culture conditions

Fermentation for the production of CholOx was performed in a 5-litre fermenter (Model 205; Hotech, Taipei, Taiwan) consisting of a standard cylindrical culture vessel with a working volume of 3 litres. The tank diameter was 17 cm and the ratio of height to tank diameter was approx. 1.5. The culture medium consisted of 2.0 g/l cholesterol, 8.0 g/l yeast extract, 1.0 g/l NH_4Cl, 1.0 g/l NaCl, 0.50 g/l KH_2PO_4, 0.25 g/l Na_2HPO_4, 0.10 g/l L-valine, 0.15 g/l L-tyrosine, 0.15 g/l $MgSO_4,7H_2O$, 0.01 g/l $ZnSO_4,7H_2O$, 0.10 g/l $FeSO_4,7H_2O$ and 4.0 ml/l Tween 80. For fermentation, the culture medium was inoculated with active preculture at a level of 10% (v/v) and cultivated for a period of 96 h.

When the effect of pH control was studied, the pH of the medium was maintained at various values (6.0, 7.0 and 8.0) by titration with sterile 0.1 M NaOH or 0.1 M HCl.

Abbreviation used: CholOx, cholesterol oxidase.

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When the effects of culture temperature, aeration and agitation were examined, the culture was maintained at 35–40 °C, subjected to an aeration rate of 4.0–6.0 l/min maintained at an agitation speed of 100–300 rev./min. Other operation conditions were as specified in the Results and discussion section. All experiments were performed in duplicate; the average values are reported.

Analysis

For the determination of CholOx, the cultured medium was centrifuged at 5600 ***g*** and 5 °C for 15 min; the supernatant was used as the enzyme source. The assay of CholOx activity was based on the method described by Richmond [10]. One unit of CholOx is defined as the amount of enzyme required to form 1 mmol of 4-cholesten-3-one/min under the assay conditions.

Cell growth was determined by measuring D_{600} with a spectrophotometer or with a plate count on YM medium.

The percentage volume reduction was calculated by the method of Cheryan [11] and is expressed as $100(V_i - V_t)/V_i$, where V_i is the initial volume of the medium and V_t is the volume of medium at time t of cultivation.

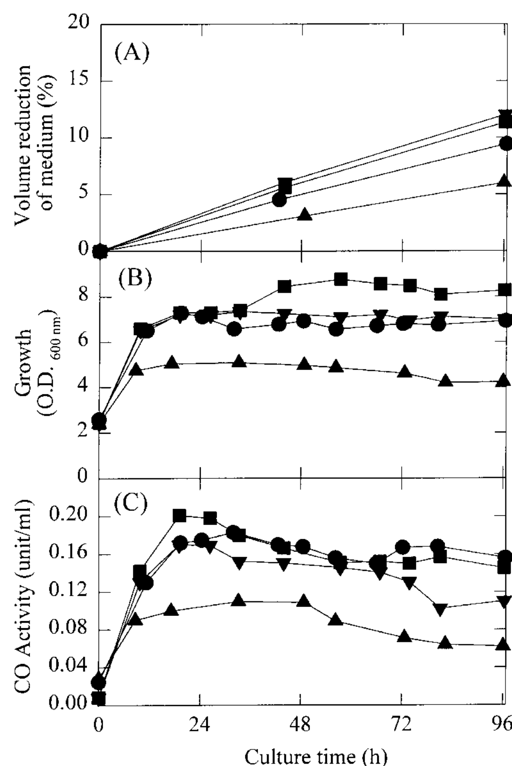

Figure 1 Effect of cultivation temperature on volume reduction of medium (A), growth (B) and production of CholOx (CO) (C) by *R. equi* No. 23

Symbols: ▲, 35 °C; ●, 37 °C; ■, 39 °C; ▼, 40 °C. Operating conditions: agitation speed 100 rev./min; aeration rate 5.0 l/min; pH 7.0. Abbreviation: $O.D._{600\ nm}$, absorbance at 600 nm.

Results and discussion

Effect of cultivation temperature

Perry and Green [12] indicated that increasing the cultivation temperature increases the vapour pressure of the cultivation medium, thus increasing the evaporation rate of the medium. As shown in Figure 1(A), the percentage volume reduction of the medium increased with cultivation time and cultivation temperature. At the end of the 96 h cultivation period, the volume reduction was 6.0%, 9.0%, 11.5% and 12.0% at cultivation temperatures of 35, 37, 39 and 40 °C respectively.

With flask cultures, Wu [13] indicated that the growth of *R. equi* and its CholOx production were optimal at 37 °C. The effect of cultivation temperature on the growth of *R. equi* in a jar fermenter is shown in Figure 1(B): the growth of *R. equi* was least at 35 °C and greatest at 39 °C after 96 h of cultivation.

Regardless of the cultivation temperature, the CholOx activity in all media increased rapidly during the initial period of cultivation (Figure 1C). The highest maximum CholOx activity of 0.20 unit/ml was noted in the medium after 20 h of cultivation at 39 °C. At the end of fermentation, no significant difference in CholOx activity was noted between the cultures incubated at 37 °C and at 39 °C. On the basis of the maximum CholOx production, the volumetric production rates were 0.003, 0.006, 0.010 and 0.009 unit/h per ml respectively at 35, 37, 39 and 40 °C.

Effect of pH of medium

Under the experimental conditions, the volume reduction in the medium was approx. 7.0–8.5% after 96 h of cultivation (Figure 2A). The maximum growth in the culture at pH 6.0, 7.0 and 8.0 occurred after approx. 26, 20 and 30 h of cultivation respectively. In general, the growth of the test organism was better in medium at pH 6.0 and 7.0 than at pH 8.0 (Figure 2B). Regardless of medium pH, the CholOx activity in all the media increased with time during the first 24 h of fermentation.

Buckland et al. [14] reported that controlling the medium pH at 6.7 favoured CholOx production by *Nocardia rhodocrous*. As shown in Figure 2(C), of the various pH values tested, *R. equi* produced the highest maximum CholOx activity of 0.20 unit/ml in medium at pH 6.0; this occurred after a shorter cultivation period (approx. 26 h) than in media at other pH values. For example, the maximum CholOx activity of 0.19 unit/ml was noted in medium at pH 8.0. After reaching its maximum, CholOx activity in the culture subsequently declined. The volumetric production rate of 0.008 unit/h per ml, calculated from the maximum CholOx production, was found in medium at pH 6.0. This

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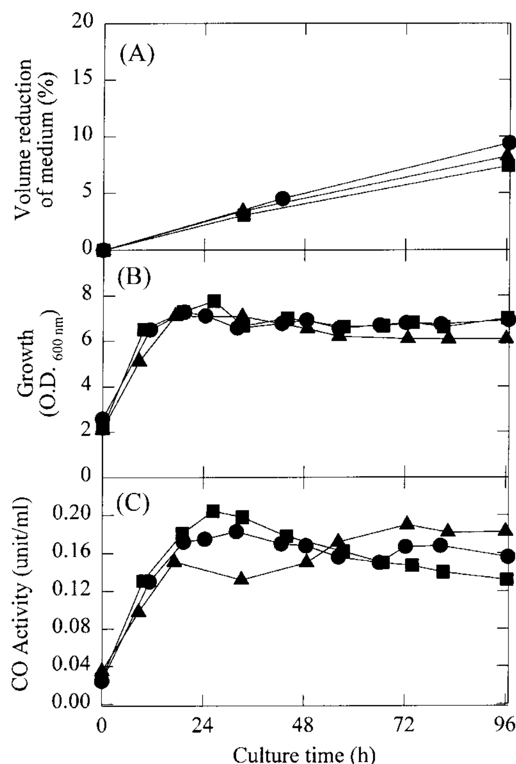

Figure 2 Effect of pH on volume reduction of medium (A), growth (B) and production of CholOx (CO) (C) by *R. equi* No. 23

Symbols: ■, pH 6.0; ●, pH 7.0; ▲, pH 8.0. Operating conditions: temperature 37 °C; agitation speed 100 rev./min; aeration rate, 5.0 l/min. Abbreviation: O. D.$_{600\ nm}$, absorbance at 600 nm.

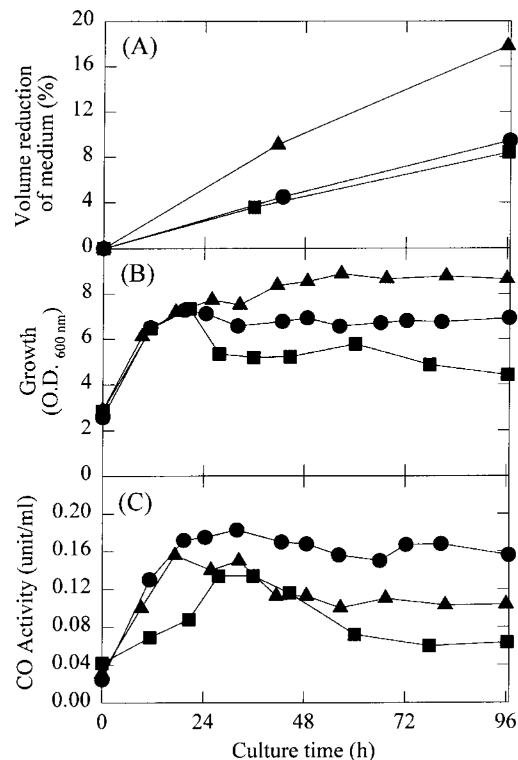

Figure 3 Effect of aeration rate on the volume reduction of medium (A), growth (B) and production of CholOx (CO) (C) by *R. equi* No. 23

Symbols: ■, 4.0 l/min; ●, 5.0 l/min; ▲, 6.0 l/min. Operating conditions: temperature 37 °C; agitation speed 100 rev./min; pH 7.0. Abbreviation: O.D.$_{600\ nm}$, absorbance at 600 nm.

value was higher than that in medium at with a pH of 7.0 or 8.0 (0.006 or 0.003 unit/h per ml respectively).

Effect of aeration rate

Cholesterol can be used as the carbon source for the growth of *R. equi* and for its production of CholOx [2]. It was indicated that the utilization of cholesterol by micro-organisms involved a series of oxidation and degradation steps, which required an adequate supply of oxygen [15]. Therefore the growth of *R. equi* No. 23 and its production of CholOx were tested at different aeration rates (4.0, 5.0 and 6.0 l/min); the results are shown in Figure 3. The volume reduction of the medium increased with aeration rate (Figure 3A). After 96 h of cultivation, the highest volume reduction (17.8%) was found in the culture with an aeration rate of 6.0 l/min, whereas the culture with an aeration rate of 4.0 l/min had the smallest volume reduction (8.4%).

During the initial 20 h of cultivation, no significant difference in the growth of the test organism was observed in media at the various aeration rates tested. However, further cultivation showed that an aeration rate of 6 l/min was favourable for the growth of *R. equi* (Figure 3B).

The production of CholOx in the medium was greatly influenced by the aeration rate, as shown in Figure 3(C). The highest CholOx production was obtained at an aeration rate of 5 l/min. Increasing or decreasing the aeration rate resulted in the reduced production of CholOx. A similar phenomenon was observed by Buckland et al. [14], who examined the production of CholOx by *N. rhodocrous*. In addition, of the various aeration rates tested, we noted that the greatest volumetric CholOx production rate of 0.009 unit/h per ml occurred at an aeration rate of 6.0 l/min.

Effect of agitation speed

The effect of agitation speed on the production of CholOx by various organisms has been examined previously [14,16,17]. Data on *R. equi* growth and its production of CholOx as affected by agitation speeds are given in Figure 4.

The volume reduction of the medium increased with speed of agitation (Figure 4A). At the end of fermentation, the volume reduction was 9.5%, 10.0% and 14.0% at 100, 200 and 300 rev./min respectively. Although no significant difference in the growth of the test organism was noted between the cultures agitated at 100 and 200 rev./min,

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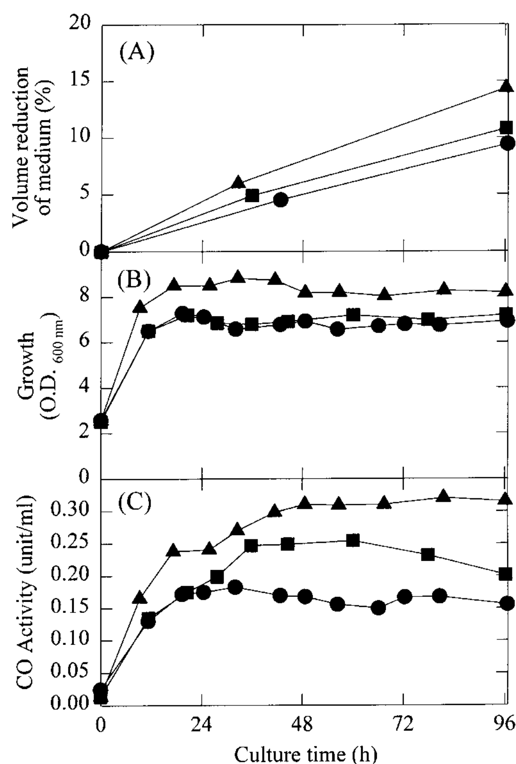

Figure 4 Effect of agitation speed on volume reduction of medium (A), growth (B) and production of CholOx (CO) (C) by *R. equi* No. 23

Symbols: ●, 100 rev./min; ■, 200 rev./min; ▲, 300 rev./min. Operating conditions: temperature 37 °C; aeration rate 5.0 l/min; pH 7.0. Abbreviation: O.D.,$_{600\ nm}$, absorbance at 600 nm.

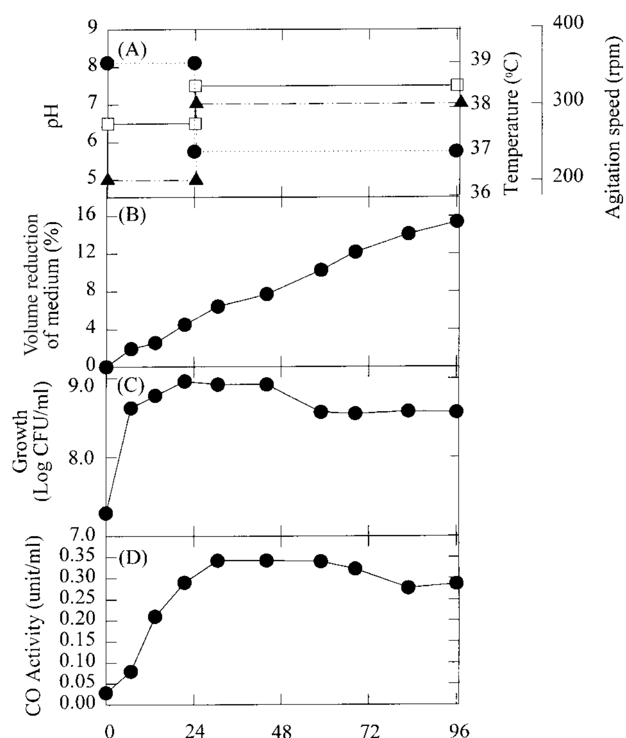

Figure 5 Operation profile (A) and its effect on the volume reduction of medium (B), growth (C) and production of CholOx (CO) (D) by *R. equi* No. 23

Symbols in (A): ●, agitation speed; □, pH; ▲, temperature. The abscissa is culture time (h). Abbreviation: CFU, colony-forming units.

growth was significantly greater at 300 rev./min (Figure 4B). A higher agitation speed increases the amount of dissolved oxygen and stimulates the dispersion of macromolecules in the medium [14]. It also decreases the viscosity of high-protein components that might hinder the growth of micro-organisms [18]. In addition, a high speed of agitation improves the utilization of poorly dispersed substrate by the organism [19]. These effects of high agitation speed might have contributed to the greater growth of the test organism found in the present study.

Along with the increased growth, CholOx production increased in culture with increasing agitation speed (Figure 4C). At an agitation speed of 300 rev./min, CholOx production was 0.31 unit/ml after approx. 48 h of fermentation, increasing to 0.32 unit/ml after approx. 80 h. The maximum CholOx activity in the culture agitated at 100 rev./min was 0.18 unit/ml at approx. 30 h of cultivation, whereas that at 200 rev./min was 0.25 unit/ml after approx. 60 h. The volumetric CholOx production rates were 0.006, 0.004 and 0.004 unit/h per ml at agitation rates of 100, 200 and 300 rev./min respectively.

Although a higher production of CholOx was achieved at 300 rev./min, this system was not stable. Overflow occasionally occurred during the first 24 h of fermentation owing to the foaming characteristics of yeast extract, one of the main components of the medium [20]. However, as the fermentation proceeded, the phenomenon of overflow disappeared as the yeast extract was degradaded into small peptides and amino acids. Therefore agitation at a speed of 300 rev./min at the beginning of fermentation is undesirable.

Growth and production of CholOx with an improved profile of operation variables

From the results of the previous experiments, a temperature–pH–agitation profile was determined so as to improve the production of CholOx in a batch culture. As shown in Figure 5(A), the 96 h fermentation period was divided into two phases. During the first phase, from 0 to 24 h of fermentation, the cultivation temperature, pH and agitation speed were controlled at 39 °C, 6.5 and 200 rev./min respectively. From the start of hour 25 (the second phase), these conditions were shifted to 37 °C, 7.5 and 300 rev./min respectively. In addition, during the whole fermentation period, the aeration rate was controlled at 5.0 l/min. Under these conditions, maximum growth occurred after 24 h of cultivation (Figure 5C). In contrast,

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the maximum production of CholOx of 0.34 unit/ml, with a volumetric CholOx production rate of 0.011 unit//h per ml, was obtained after 30 h of fermentation (Figure 5D). The amount of maximum CholOx obtained here was higher than the maximum CholOx (0.32 unit/ml) obtained in the culture at 300 rev./min after 80 h of fermentation as shown in Figure 4(C) and was approx. 1.7-fold that (0.24 unit/ml) reported previously [2]. Furthermore, the system was quite stable under these conditions. No overflow occurred during the entire fermentation period even though the agitation speed was increased from 200 to 300 rev./min after 24 h of cultivation. Thus it can be concluded that changing the operation variables can effectively increase the production of CholOx by *R. equi* under a stable fermentation system.

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Received 15 October 1998/25 November 1998; accepted 22 December 1998

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