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Production of hydrocortisone by *Absidia coerulea* in moderate pressure bioconversion system

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Abstract−The effects of moderate pressure (0.1-2.5 MPa) on viability, cell membrane permeability and catalyzing activity of *Absidia coerulea* for RSA were investigated. A new method for improving the production of Hydrocortisone (HC) from 17α-hydroxypregn-4-en-3, 20-dione-21-acetate by *Absidia coerulea* in moderate pressure was developed. The results showed that the morphology of *Abasidia coerulea* mycelium was changed in moderate pressure, *Absidia coerulea* mycelium seemed to be loosed, and cell membrane permeability of *Abasidia coerulea* mycelium was improved. However, the viability of *Abasidia coerulea* mycelium could keep high level. Moreover, the yield of HC was improved over 1.25-fold as compared with that of the control (untreated cells), to give the yield of HC as 350 mg/*l*, when the *Abasidia coerulea* mycelium was treated with 0.5 Mpa the atmosphere as the pressure media. Especially, the production of HC with atmosphere as the pressure media (0.5 MPa) could be increased by the addition of H_2O_2 (60 mmol/*l*); the relative yield of HC in moderate pressure was enriched by over 4.5% in comparison with the control. The major composition of bioconverted mixture was reduced. It was indicated that the new approach (moderate pressure) obtained in this work possessed a high potential for the industrial production of HC.

Key words: *Abasidia coerulea*, Hydrocortisone, Permeability, Viability, Moderate Pressure, Biotransformation

INTRODUCTION

Hydrocortisone (HC) belongs to a class of drugs called "corticosteroids". It is an effective anti-inflammatory drug and one of the important precursors of other steroid drugs. HC, which is also used to treat certain types of cancer, can be produced by several chemical processes and microbial transformation [1]. In recent years, different features of microbial transformation of HC have been investigated [2,3]. The incorporation of a hydroxyl group in the 11β-position of 17α-hydroxypregn-4-en-3, 20-dione-21-acetate (RSA) can be performed by a great number of microorganisms in fermentation culture and/or in immobilized cells. However, the production of HC from RSA was of limited success, partly because of the relatively low solubility in water of RSA and O_2 during the bioconversion. There is a growing interest in improving solubility in water of RSA and O_2 by different methods. The solubility in water of RSA could be increased by the addition of water-miscible organic solvents (cosolvents) [4-6]. The solubility in water of O_2 could be improved by enhancing aeration or agitation speed [7]. The solubility in water of RSA and O_2 were, however, disappointingly low. Thus, in terms of large-scale production of HC for industrial and medical uses, the solubility in water of RSA and O_2 needs to be improved substantially. Pressure is one of the most important factors for the fermentation process. There are some investigations about the effects of high pressure (more than 10 MPa) on microorganism cells. High pressure brings about significant changes on a microorganism's morphology [8,9], metabolic flux [10,11], gene expression [12,13], cell membrane damage [14,15] and enhancing the oxygen transfer rate [16,17]. However, very few works have been reported about the effects of moderate pressure (0.1-2.5 MPa) on viability, metabolism and catalyzing activity of microorganism cell. Especially, no literature report has been found on HC bioconversion by the use of moderate pressure. In this study, the effects of moderate pressure on viability, cell membrane permeability and catalyzing activity of *Absidia coerulea* (*A. coerulea*) mycelium for RSA were investigated. We attempted to improve the production of HC from RSA by *A. coerulea* in moderate pressure.

EXPERIMENTAL

1. Chemicals

RSA and HC were purchased from Tianjin Pharmaceutical Co., Tianjin, P. R. China. All other chemicals were of analytical grade and commercially available.

2. Microorganism

A. coerulea AS365 was provided by microorganism lab, Tianjin University of science and technology, Tianjin, P.R. China.

3. Media and Culture Conditions

Potato dextrose agar (PDA) slants cultivation medium contained the following (g/*l* distilled water): white potato 200, agar 20, and glucose 20. The fermentation medium contained the following (g/*l* distilled water): glucose 10.5, yeast extract 27, $(NH_4)_2SO_4$ 6, corn steep liquor 10, pH was adjusted to 6.5 with NaOH. *A. coerulea* AS365 mycelium was inoculated on PDA slants at 28 °C for 6 days.

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The spores were washed with sterile water and the spore suspension was prepared as the inoculum. The spore concentration was 1×10^6/ml. 3 ml inoculum culture was added to 100 ml fermentation medium in a 500 ml Erlenmeyer flask. The culture was incubated in a rotary shaker at 28 °C and 220 r/m for 24 h.

4. Preparation of the Cell Suspension

The fermentation broth of the scale-up culture of *A. coerulea* was centrifuged at 4,000 r/m for 10 min, and then the cells were washed twice with double distilled water. The harvested cells were suspended in the double distilled water (2%, w/w) as cells suspension [18].

5. Bioconversion of RSA

The transformation was performed as follows: RSA (300 mg) dissolved in 100 ml of 80% ethanol solution was added slowly to the above *A. coerulea* cells suspension in the pressurizable bioreactor (see Fig. 1, designed by our laboratory) and dispersed by shaking at the same time. The pH was maintained at 6.5. The conversion was carried out at 28 °C in the pressurizable bioreactor at 170 r/m, the whole transformation cycle was 48 h, and the bioreactor was pressurized with atmosphere or N_2 in certain transformation time (the details were referred to in the results section). The control sample was performed under atmosphere pressure (0.1 MPa).

6. Analytical Methods

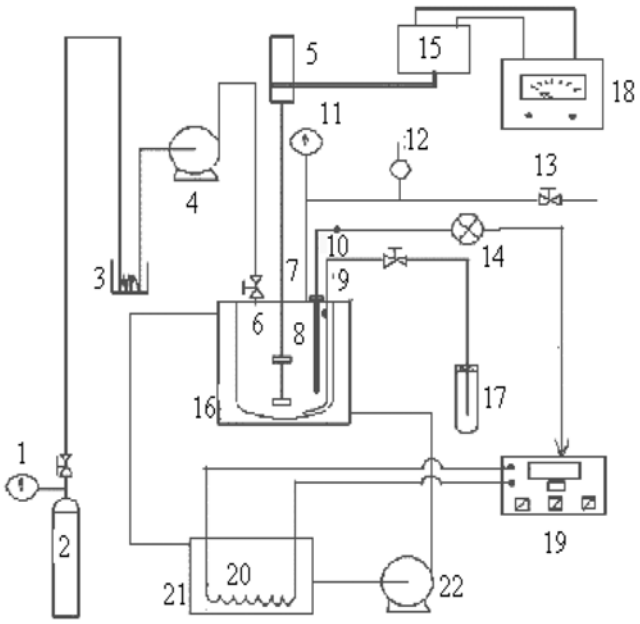

Fig. 1. The flow chart of the pressure bioreactor.

1. Pressure gauge
2. Carbon dioxide steel bottle
3. Cooling apparatus
4. Liquid booster pump
5. Stirrer
6. Air intake
7. Manometric measurement hole control
8. Temperature measurement hole
9. Sampling hole
10. Thermocouple
11. Precision pressure gauge
12. Safety valve
13. Air release valve
14. Transmitter
15. Electric machinery
16. Aqueous thermostat
17. Gather device
18. Speed regulator
19. Artifical intellingence temperature apparatus
20. Heater
21. Water tank
22. Centrifugal water pump

6-1. The Enzyme Assay

A. coerulea dehydrogenase activity was determined by triphenyltetrazolium chloride (TTC) method [19]. First, *A. coerulea* mycelium were recovered from the culture by centrifugation, and the cells were washed twice with double distilled water. The harvested *A. coerulea* mycelium was treated in a certain moderate pressure (treatment details are referred to in the results section). TTC was dissolved in pH 8.5 potassium phosphate buffer (50 mmol/*l*) to give a concentration of 0.5% (w/v). Then, 3 ml of TTC solution was added to *A. coerulea* mycelium (about 0.1 g) in assay tubes (11×90 mm) and incubated without shaking for 18-20 h at 30 °C. After incubation, *A. coerulea* mycelium were rinsed briefly with distilled water and transferred to another set of tubes (35×60 mm). To extract the red formazan, 30 ml 95% ethanol was added to each of the tubes, and incubated for 2 h at 60 °C. After filtration, absorbance of the extract was measured at 485 nm with a 752 spectrophotometer (Shanghai precision and scientific instrument Co., China).

6-2. The Morphological Change Determination and the HPLC Assay of the HC Contents

Morphologic changes of *A. coerulea* mycelium by treating with moderate pressure were examined by scan electron Microscopy (XL-28 SEM, Nikon Ltd., Japan). HC concentration in bioconverted mixture was quantified by HPLC. The highly purified HC (>99%) was dissolved in dichloromethane : aether : methanol : water (385 : 60 : 28 : 2, V/V). A standard curve was established for HC using 0.5, 1.0, 2.0, 3.0, 4.0 g/*l* HC versus the corresponding area number of spectra. Sample preparation for HPLC was performed by extracting 2 ml of bioconverted mixture with 2 ml of chloroform under reflux for 5 min. After clear separation of the organic phase from the aqueous phase, 0.1 ml of organic fraction was mixed with 2.9 ml of dichloromethane : aether : methanol : water (385 : 60 : 28 : 2, V/V) and filtered. The filtrate was analyzed by HPLC. HPLC analysis was performed on a reverse phase C18 column (4.6×250 nm, ODS-100S, Shimadzu, Japan) using dichloromethane : aether : methanol : water (385 : 60 : 28 : 2, V/V) as the eluent. Detection was at 242 nm (using a LabAlliance Model 500 UV detector, USA). The sample size injected was 20 ml. The flow rate was 0.8 ml/min. The relative yield of each product expressed as percentage of the sum of the total transformed products.

RESULTS AND DISCUSSION

1. Effect of Moderate Pressure on Morphology of *A. coerulea* Mycelium

Fig. 2 shows the effect of moderate pressure on the morphology of *A. coerulea* mycelium. *A. coerulea* mycelium under atmosphere pressure at 0.1 MPa had distinct and dense outlines (Fig. 2B, 2D, 2F); however, in the moderate atmosphere pressure at 0.5 MPa, the mycelium seemed to be loosed (Fig. 2A, 2C, 2E). The interstices between *A. coerulea* mycelia were augmented. These changes increased the contact between the substrate (RSA) and *A. coerulea* mycelium, enhanced mass transfer of substrate, and as a result, improved RSA conversion rates.

2. The Effects of Different Moderate Pressures on the Viability of *A. coerulea* Mycelium

As shown in Fig. 3, the moderate pressure almost failed to effect significantly on the viability of *A. coerulea* mycelium. In this exper-

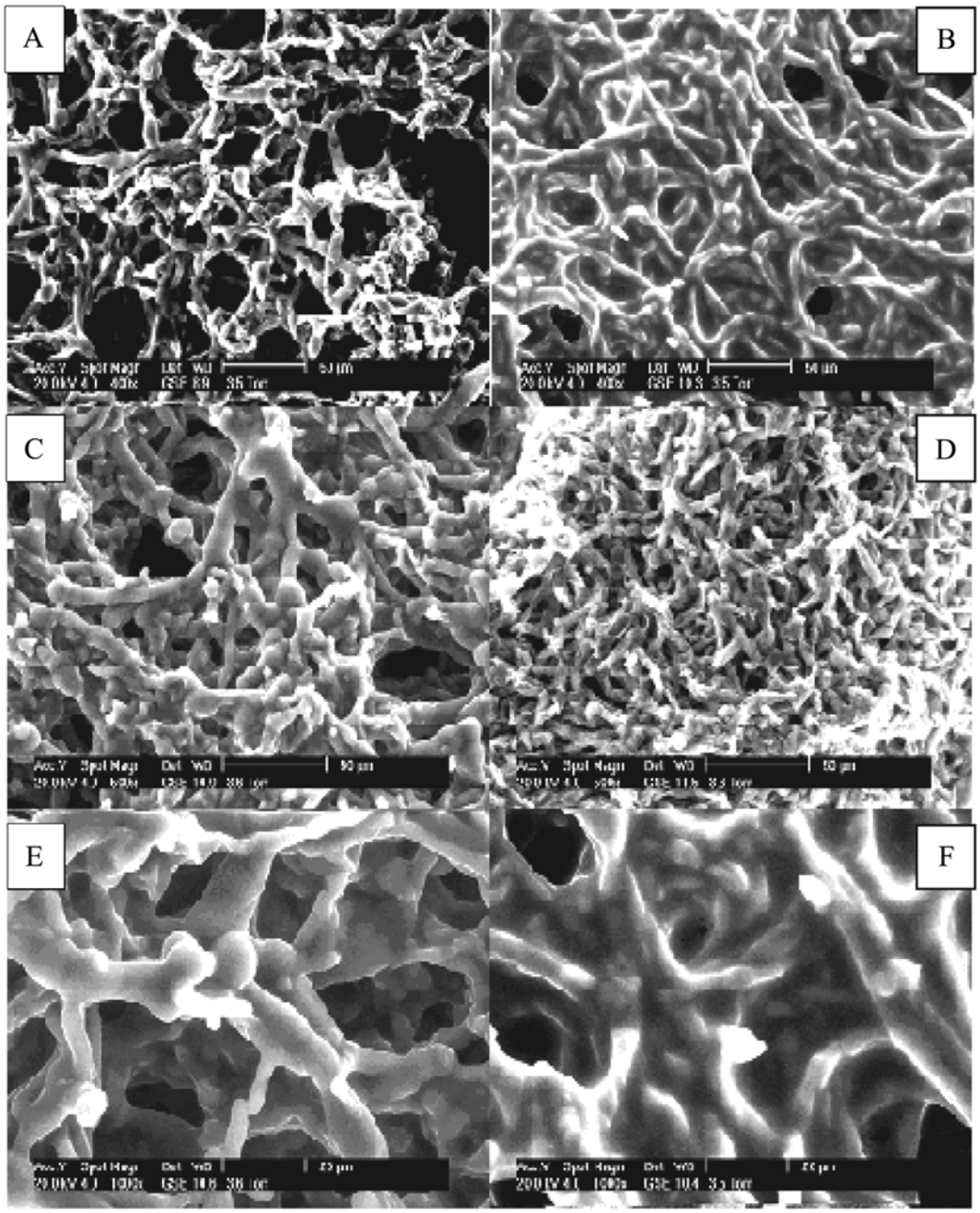

Fig. 2. The effect of moderate pressure on the morphology of *A. coerulea* pellet. A: 0.5 MPa, 400×; B: 0.1 MPa, 400×; C: 0.5 MPa, 500×; D: 0.1 MPa, 500×; E: 0.5 MPa, 1,000×; F: 0.1 MPa, 1,000× 50 ml cell suspension diluted with double distilled water was put in the preheated 300 ml pressurizable bioreactor at 28 °C, and then the reactor was pressurized with 0.5 MPa atmosphere for 6 h.

iment, the dehydrogenase activity of *A. coerulea* in atmosphere pressure at 0.1 MPa (control) was assumed as 100%, the specific viability rate kept high level (above 80%) in moderate pressure. It is possible that the oxygen transfer rate and dissolved oxygen in the solution was increased; as a result, the viability of A. *coerulea* mycelium in moderate pressure could still keep high level.

3. The Effect of Moderate Pressure on Membrane Permeability of *A. coerulea*

As shown in Fig. 4, the conductivity, OD_{280} and OD_{260} of the cell suspension by treatment with the different pressure were higher than that of control (0.1 MPa); especially, the atmospheric pressure at 0.5 MPa had a more remarkable effect on membrane permeability of *A. coerulea* mycelium than the others. It indicated that the treatment strategy to *A. coerulea* mycelium with moderate pressure was effective on the improvement of membrane permeability of *A. coerulea* mycelium. Moreover, the specific viability rate of mycelium could still keep high level in different pressures (see Fig. 3). Thus, the improvement of membrane permeability allowed easier access of the substrate RSA to 11β-hydroxylase and excretion of the product, which increased the rates of transport of HC and RSA.

4. The Effects of Moderate Pressure on Yield of HC

Fig. 5 shows the effects of different pressure times and pressure

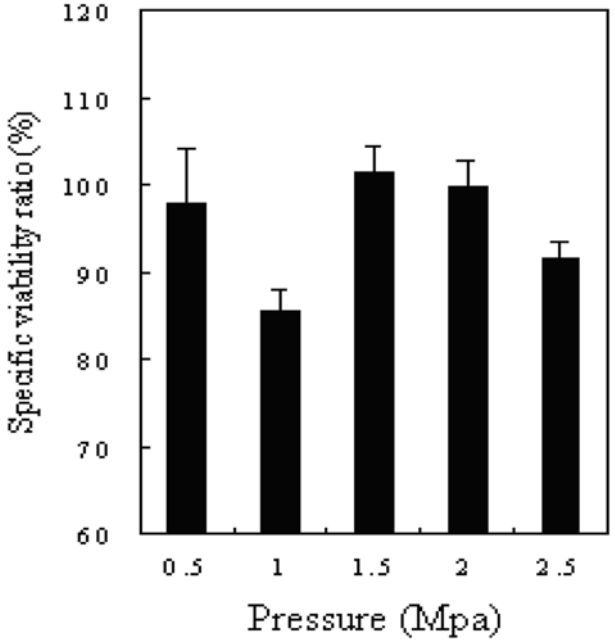

Fig. 3. The effect of mild pressure on the viability of *A. coerulea* mycelium pellet 50 ml cell suspension diluted with double distilled water was put in the preheated 300 ml pressurizable bioreactor at 28 °C, and then the reactor was pressurized with 0.5-2.5 MPa atmosphere pressure for 6 h, respectively. The specific viability rate indicates *A. coerulea* dehydrogenase activity by treatment with moderate pressure/ *A. coerulea* dehydrogenase activity in atmosphere pressure (0.1 MPa).

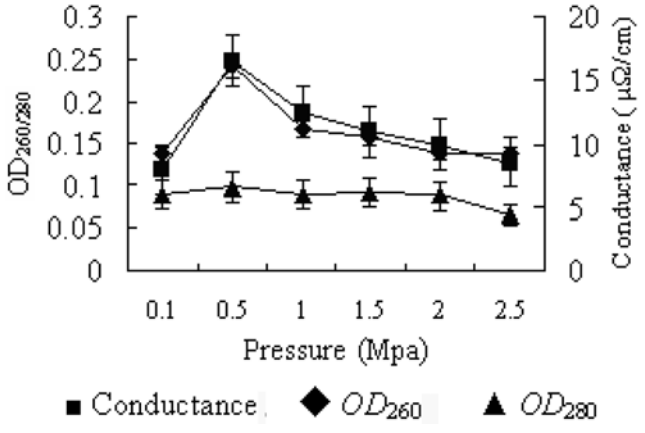

Fig. 4. The effect of moderate pressure on the membrane permeability of *A. coerulea* 50 ml cell suspension diluted with double distilled water was put in the preheated 300 ml pressurizable bioreactor at 28 °C, and then the reactor was pressurized with 0.1-2.5 MPa atmosphere pressure for 6 h, respectively.

media on the yield of HC. The moderate pressure had more remarkable effect on the yield of HC than that of the control (untreated cells). The yield of HC was improved over 1.25-fold as compared with that of the control, and the maximal yield of HC was obtained during conversion 36-42 h, to give the yield of HC as 350 mg/*l*, when the *Abasidia coerulea* mycelium was treated with the atmosphere

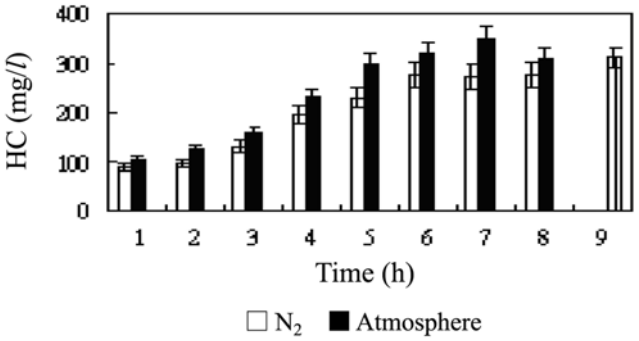

Fig. 5. The effects of different pressure times and pressure media on the yield of HC. The whole transformation cycle was 48 h, 0.5 MPa pressure was only held at certain time during transformation, other time kept 0.1 MPa atmosphere pressure.

1. 0-6 h 0.5 MPa,
2. 6-12 h 0.5 MPa
3. 12-18 h 0.5 MPa
4. 18-24 h 0.5 MPa
5. 24-30 h 0.5 MPa
6. 30-36 h 0.5 MPa
7. 36-42 h 0.5 MPa
8. 42-48 h 0.5 MPa
9. Control, 0-48 h 0.1 MPa

Table 1. The effect of different pressure on k_La during bioconversion reaction

Pressure medium	k_La (1/h)
Control (0.1 MPa atmosphere)	98
Atmosphere pressure at 0.5 MPa	134
Atmosphere pressure at 1.0 MPa	157

at 0.5 Mpa as the pressure media. It is the possible reason that the oxygen transfer rate and the dissolved oxygen in bioconverted mixture were enhanced with the atmosphere as the pressure media (0.5 MPa). To determine whether the oxygen transfer rate and the dissolved oxygen were improved, volumetric mass transfer coefficient of oxygen (k_La) in bioconverted mixture was measured according to Jung [20] (Table 1). With the atmosphere as pressure media, k_La in bioconverted mixture was higher than that of control (0.1 MPa). It was also indicated that the oxygen transfer rate and dissolved oxygen in bioconverted mixture were assuredly improved with the atmosphere as the pressure media (0.5 MPa). As a result, the yield of HC was significantly improved.

5. The Effects of Reaction Volume and H_2O_2 on Yield of HC

The yield of HC with the atmosphere as the pressure media (0.5 MPa) was higher than that with N_2 as the pressure media (0.5 MPa) and control (0.1 MPa); furthermore, the maximal yield of HC was obtained as the reaction volume was 50 mL (see Fig. 6A). With the atmosphere as the pressure media (0.5 MPa), the addition of H_2O_2 (60 mmol/*l*) helped in the conversion of RSA; the yield of HC was respectively improved about 1.22-fold and 1.08-fold as compared with the control (0.1 MPa) and no addition of H_2O_2, reached 380 mg/*l* (see Fig. 6B). This result maybe was explained by the fact that the addition of H_2O_2 enhanced the dissolved oxygen in the bioconverted mixture. It was indicated again that the dissolved oxygen was an important factor in the conversion of RSA.

6. Analysis of the Bioconverted Mixture

The HPLC profile of the bioconverted mixture is shown in Fig. 7. In this system, the retention time of HC was 10.12 min. Six major

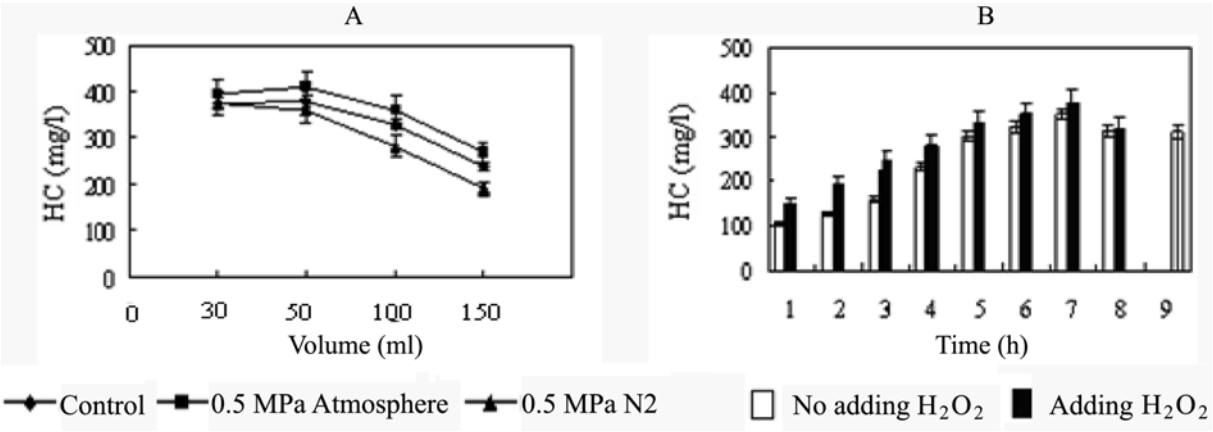

Fig. 6. The effects of reaction volume and H_2O_2 on the yield of HC. A. The effect of reaction volume on the yield of HC under 0.5 MPa atmosphere pressure transformation was carried out for 36 h under atmosphere pressure (0.1 MPa), subsequently, conversion reaction was continued for 6 h under 0.5 MPa atmosphere pressure, other time kept 0.1 MPa atmosphere pressure. B. The effects of H_2O_2 on the yield of HC under 0.5 MPa atmosphere pressure the whole transformation cycle was 48 h. 0.5 MPa atmosphere pressure was only hel at certain time during transformation, simultaneity, H_2O_2 (60 mmol/*l*) was added in bioconverted mixture, other time kept atmosphere pressure (0.1 MPa).

1. 0-6 h 0.5 MPa
2. 6-12 h 0.5 MPa
3. 12-18 h 0.5 MPa
4. 18-24 h 0.5 MPa
5. 24-30 h 0.5 MPa
6. 30-36 h 0.5 MPa
7. 36-42 h 0.5 MPa
8. 42-48 h 0.5 MPa
9. Control, 0-48 h 0.1 MPa

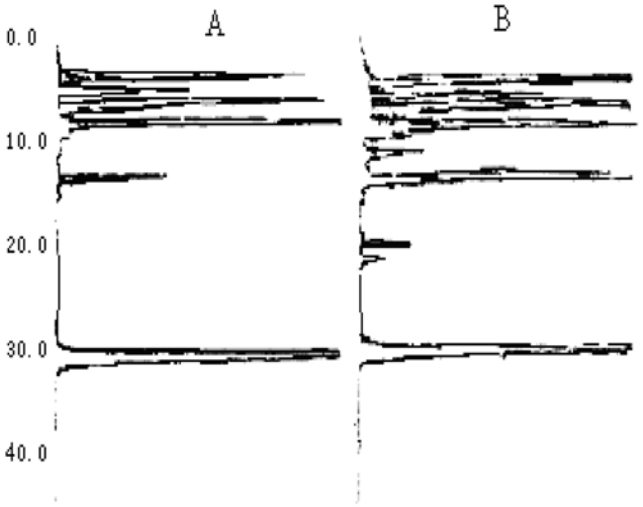

Fig. 7. HPLC profile of transformation metabolites of the bioconverted mixture. A. After 36 h under 0.1 MPa atmosphere pressure, transformation was carried out for 6 h under 0.5 MPa atmospheric pressure, then, transformation was continued for 6 h under 0.1 MPa atmospheric pressure. B. Control, transformation was held for 48 h under 0.1 MPa atmosphere pressure.

peaks of transformation metabolites of the bioconverted mixture were obtained with the atmosphere as the pressure media (0.5 MPa, the addition of H_2O_2) (Fig. 7A), whereas, there were nine major peaks of transformation metabolites of the bioconverted mixture at atmospheric pressure (0.1 MPa) (Fig. 7B). It was indicated that the ingredients of the bioconverted mixture with the atmosphere as the pressure media (0.5 MPa, the addition of H_2O_2) were fewer than that of control (0.1 MPa atmospheric pressures). Moreover, the relative yield of HC in bioconverted mixture with the atmosphere as the pressure media (0.5 Mpa) was improved over 4.5% as compared with that of the control, reaching 50.06% (data not shown). These results show again that the moderate pressure (0.5 MPa atmospheric pressure) helped to enhance the yield of HC.

CONCLUSION

The morphology of *A. coerulea* mycelium seemed to be loosed but the viability could be kept at high level at moderate pressure. At the same time, cell membrane permeability of mycelium by treatment with moderate pressure was improved; the mass transfer of substrate and oxygen transfer rate also were enhanced during transformation. Thus, the production of HC from RSA was improved. It is indicated that this novel approach (moderate pressure) for improving the yield of HC has much applied value in industry.

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REFERENCES

1. A. F. Mohammad, T. Y. Mojtaba and S. A. Z. Gholamreza, *Steroids*, **67**, 869 (2002).
2. R. B. R. Porter, W. A. Gallimore and P. B. Reese, *Steroids*, **64**, 770 (1999).
3. S. Anmad, S. K. Garg and B. N. Johri, *Biotech. Adv.*, **10**, 1 (1992).
4. J. S. Dordick, *Enzyme. Microb. Technol*., **11**, 194 (1989).
5. S. Abramov, Y. Aharonowitz and M. Harnik, *Enzyme. Microb. Technol*., **12**, 982 (1990).
6. J. Wang, C. Chen and B. Li, *Enzyme. Microb. Technol*., **22**, 368

(1998).
7. S. R. Jia, B. Li and Y. S. Park, *J. Fermen. Biosci*., **2**, 191 (1996).
8. M. A. Z. Coelho, I. Belo and R. Pinheiro, *Appl. Microbiol. Biotechnol*., **66**, 318 (2004).
9. P. Maňas and B. M. Mackey, *Appl. Environ. Microbiol*., **70**, 1545 (2004).
10. F. Abe, C. Kato and K. Horikoshi, *Trends. Microbiol*., **11**, 447 (1999).
11. G. D. Bothun, B. L. Knutson and J. A. Berberich, *Appl. Microbiol. Biotechnol*., **65**, 149 (2004).
12. D. H. Bartlett, C. Kato and K. Horikoshi, *Res. Microbiol*., **146**, 697 (1995).
13. A. Sharma, J. H. Scott and G. D. Cody, *Science*, **295**, 1514 (2002).
14. M. Kato, R. Hayashi and T. Tsuda, *Eur. J. Biochem*., **269**, 110 (2002).
15. J. M. Perrier-Cornet, M. Hayert and P. Gervais, *J. Appl. Microbiol*., **87**, 1 (1999).
16. S. Sato, S. Mukataka and H. Kataoka, *J. Ferment. Technol*., **59**, 221 (1981).
17. T. Matsui, N. Shinzato and H. Ypkpta, *Process Biochem*., **41**, 920 (2006).
18. J. D. Cui and Y. Li, *Korean J. Chem. Eng*., (In print) (2009).
19. I. G. Byun, H. U. Nam, S. K. Song, I. S. Hwang, T. H. Lee and T. J. Park, *Korean J. Chem. Eng*., **22**, 917 (2005).
20. A. R. Jung and Y. H. Jeong, *Korean J. Chem. Eng*., **22**, 201 (2005).