Phytochemistry, Vol. 23, No. 5, pp. 999–1001, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

BIOTRANSFORMATION OF CODEINONE TO CODEINE BY IMMOBILIZED CELLS OF *PAPAVER SOMNIFERUM*

TSUTOMU FURUYA, TAKAFUMI YOSHIKAWA and MEGUMI TAIRA

School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

(*Received* 21 *October* 1983)

Key Word Index—*Papaver somniferum*; Papaveraceae; immobilized cell; biotransformation; bioreactor; codeinone; codeine; GC/MS; SIM.

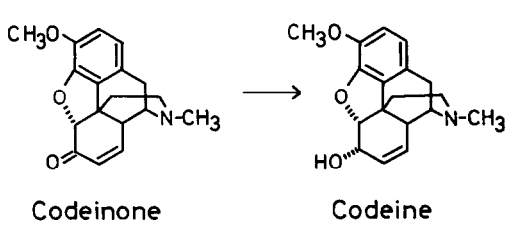
Abstract—*Papaver somniferum* (opium poppy) cells were immobilized in calcium alginate, where they continued to live with their biological activity for 6 months. The immobilized living cells performed the biotransformation of (−)-codeinone to (−)-codeine in both a shake flask and a column bioreactor. The biotransformation ratio in the shake flask (70.4%) was higher than that in the cell suspension (60.8%). Furthermore, 88% of the codeine converted was excreted in the medium. The column bioreactor was functional for 30 days under optimal conditions (20°, 3.75 vvm in aeration), whereas the ratio was 41.9%.

INTRODUCTION

The immobilized microbial cell systems have rapidly developed during recent years. Practically, these systems have been applied to the industrial production of useful compounds such as foodstuff additives and drugs [1–4]. At first, the immobilized cells were used as a non-viable catalyst for single enzyme reactions. Recently, the application of immobilized living cells to the production of useful compounds utilizing multi-enzyme reactions has been the subject of many papers [5–9].

A variety of matrices have been employed [10, 11], but calcium alginate or carrageenans appear to be favoured because of several operational advantages, i.e. mild conditions and high retention of cell viability, as the entrapment. Of these two matrices, alginate has been especially used in the immobilization of plant cells [12–15].

In our studies on immobilized plant cells, various reactions concerning steroids and alkaloids, described in our earlier papers, have been attempted for the production and biotransformation of secondary metabolites. In this paper we concentrate on the reduction of codeinone to codeine (Scheme 1) by immobilized living *Papaver somniferum* cells entrapped in calcium alginate beads. In a previous paper, we described in detail the biotransformation of codeinone to codeine by the cell suspension culture and the cell-free system [16]. This reduction required NADH as a cofactor in the enzyme system. In the immobilized cell system, however, this reaction proceeded without NADH. Using a column bioreactor packed with the immobilized living cells, we investigated, with the effect of various conditions such as temperature and aeration, the conversion of codeinone to codeine, and the cell viability.



Codeinone Codeine

Scheme 1. Biotransformation of codeinone to codeine.

RESULTS AND DISCUSSION

Immobilization of Papaver somniferum *cells*

Intact cells of *Papaver somniferum* were entrapped in calcium alginate beads under sterile conditions. The immobilized cells slightly increased in the beads when they were cultured in a flask supplemented with growing medium in a rotary shaker. However, the *P. somniferum* cells never leaked from the beads into medium, although the other plant cells (*Digitalis purpurea, Nicotiana tabacum, Glycyrrhiza echinata*) were excreted into medium after 1–2 weeks. It can be presumed by microscopic observation that the phenomenon (non-leak from the beads) is due to the very large size of the *P. somniferum* cultured cells. Moreover, we confirmed from respiratory activity that the cells continued to live over 6 months on subculturing at intervals of 1 month. In this experiment we used the immobilized living cells after 1 week culture in a shake flask and investigated them under various reaction conditions.

Biotransformation of codeinone to codeine in a shake flask by the immobilized cells

(−)-Codeinone (5 mg) was administered to the immobilized cells after 1 week culture. After shaking for 3 days in a rotary shaker, the cultures were harvested. The immobilized cells and medium were separately extracted with chloroform–isopropanol (3:1) at pH 8.5, and codeine (3.10 mg in the immobilized cells and 0.42 mg in the medium) was identified by TLC and determined by a SIM (selected ion monitoring) method (*m/z* 299 M^+) in GC/MS. The total conversion ratio was 70.4% to the

codeinone administered. This ratio was higher than that of the cell suspension culture (60.8% on 3 days). Furthermore, 88% of the codeine converted by the immobilized cells was excreted into the medium. This excretion (or release) is of considerable importance for the utilization of immobilized plant cells for the production of secondary metabolites. Therefore this reaction is described as the focus for an investigation of the application for the production by a bioreactor.

Biotransformation of codeinone to codeine by a column bioreactor

Column bioreactor. The bioreactor used in this experiment was designed by revising a column. An air supply to the entrapped cells was achieved by direct upward aeration, i.e. airlift mode. After reacting for an appropriate time, samples were taken at the base of the column by stopping the air pump. The fresh medium previously reserved in the tank was supplied from the top of the column by a pump. The substrate (codeinone) was injected from the upper inlet through a milipore filter. The continuous utilization of the immobilized *P. somniferum* cells was investigated by following the biotransformation of codeinone to codeine using the bioreactor under three different temperatures and three rates of aeration.

Effect of temperature. After 3 days' preincubation in the bioreactor, 5 mg codeinone was administered and incubated at three different temperatures (20°, 25°, 30°). After 3 days, these media were collected and extracted according to the Experimental and subsequently the content of codeine was determined by GLC. The bioreactor was again filled with the same fresh medium, and at the same time 5 mg codeinone was injected. These operations were carried out every 3 days. The results are shown in Table 1.

At 30°, the immobilized cells immediately lost their biotransformation potential. At the same time, the cells changed to brown and lost their viability (measured by respiration rate). At 25°, the cells showed highest activity at 6 days (41.9% in biotransformation ratio), but the activity was rapidly lost from the third time (9 days) and almost reached zero by the seventh time (21 days). However, at 20°, the biotransformation potential was maintained constantly for 27 days, although the rate was lower, 20–30%, than that at 25° (41.9%). In all cases, the biotransformation activity (41.9%) in the bioreactor was lower than that in the shake flask experiment (70.4%). This may be because of the difference in the contact of the substrate with the immobilized cells. Therefore the effect of aeration or agitation on the biotransformation was studied using the bioreactor.

Table 1. Effect of temperature on the biotransformation of codeinone to codeine in a bioreactor using immobilized living cells of *P. somniferum* at 3.75 vvm

Age of immobilized cells (days)	Biotransformation ratio (%) Temperature of bioreactor 20°	25°	30°
3	26.9	32.6	4.8
6	20.7	41.9	1.6
9	29.1	18.9	1.6
12	17.0	7.6	0.6
15	23.6	3.6	—
18	25.0	2.1	—
21	24.2	1.3	—
24	29.4	—	—
27	12.3	—	—
30	6.3	—	—

Effect of aeration. The effects of aeration and agitation were investigated under three different conditions of aeration: 0.94, 1.88 and 3.75 vvm, at 20°, the most stable temperature. As shown in Fig. 1, at both 0.94 and 1.88 vvm the biotransformation activity rapidly fell after 6 days and almost reached zero after 15 days.

At the same time the immobilized cells changed to brown and lost their viability. It was shown that the biotransformation rate could be correlated with the viability of the immobilized cells. It was considered that both the conditions (0.94–1.88 vvm) suffice for the supply of oxygen for respiration of the cells, since the aeration in the shake flask or jar fermentor culture is usually 0.5–1.0 vvm [17]. However, the immobilized cells entrapped in alginate beads required 5 times aeration, 3.75 vvm. At 3.75 vvm aeration, the immobilized cells maintained activity for 30 days at 20°, while at 25° and 30° the cells rapidly lost their activity. This fact suggests that the cells entrapped in the beads require more vigorous aeration or agitation at 25° and 30°, since the metabolism in the cell becomes active at the higher temperature.

In this experiment, a search was made first for the intensity of aeration, although a study of aeration mode has already been reported by Veliky and Jones [18].

The biotransformation rate in the bioreactor was lower than that in the shake flask (70.4%) and in the free cells (60.8%). Evidently this is caused by the inhibitory effect of the permeability in the alginate beads, because the immobilized cells entrapped in the beads require five times the amount of oxygen compared to that in the free cells. The cells gradually lost their viability by the lack of

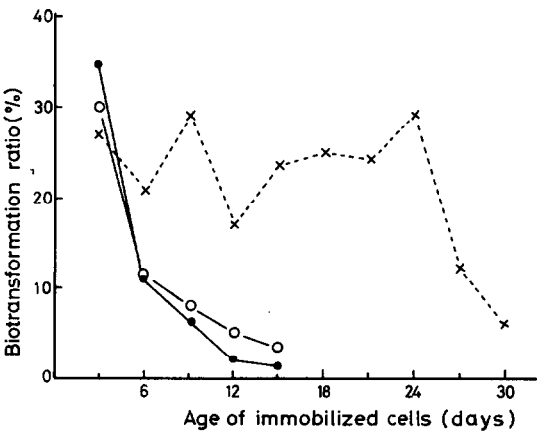


Fig. 1. Effect of aeration on the biotransformation of codeinone to codeine in a bioreactor using immobilized living cells of *Papaver somniferum*. The immobilized cells were cultured in a flask for 1 week and then transferred to a column bioreactor. After preincubation for 3 days, the bioreactor was operated under three aeration rates as follows; ●——●, 0.94 vvm; ○——○, 1.88 vvm; ×----×, 3.75 vvm. The medium was removed after 3 days and again fresh medium was supplied with the substrate. This operation was carried out repeatedly at intervals of 3 days.

oxygen and at the same time lost their biotransformation potential. Therefore in order to utilize the immobilized cells for long periods, a modification of the column bioreactor or the other matrices must be devised.

Brodelius and Mosbach [19] have pointed out two biotransformations as the most valuable application for the production of plant metabolites; one is the stereospecific hydroxylation of digitoxin to digoxin, and the other is the conversion of thebaine via codeinone to codeine. In this experiment, the biotransformation of codeinone to codeine was proved to be possible with the immobilized cells. The biotransformation of thebaine via codeinone and codeine to morphine is now being investigated with the other strain of *P. somniferum* cell cultures.

EXPERIMENTAL

Tissue culture. The tissue culture used in this experiment was derived from the seedling of *Papaver somniferum* L. var. Ikkanshu (opium poppy) in 1976, and subcultured for *ca* 6 years on modified Murashige and Skoog's tobacco medium containing 1 ppm 2,4-D, 0.1 ppm kinetin, 7% coconut milk and 3% sucrose (CM medium). After 3 weeks' static culture at 26°, the suspension cultures were grown on the same medium for 1 week in a rotary shaker. The medium (250 ml) was dispensed in a 500 ml flask.

Preparation of the immobilized cells. After leaving the suspension cultures for 1 week, the small cell aggregates were collected with nylon cloth (20–34 mesh). The immobilized cells were prepared using the alginate method, as follows. Packed cells (5.0 cm^3) were suspended in 4% Na alginate (50 ml). The suspension was added dropwise to 0.1 M $CaCl_2$ soln. The Ca alginate beads that formed (mean diameter 3.0 mm) were collected with nylon cloth (20 mesh) and then cultured in the same growth medium in a rotary shaker (140 rpm) at 25°. The living immobilized cells obtained as above were used for this experiment. All these procedures were carried out under sterile conditions. The viability of the immobilized cells was determined by respiratory activity, measured with an oxygen electrode and the manometric method.

Biotransformation in the shake flask and column bioreactor. In a shake flask, (−)-codeinone (5 mg) was administered after 1 week and cultured for 3 days under the same conditions (25°, 140 rpm in a rotary shaker). In the case of the column bioreactor, the immobilized cells cultured in a flask for 1 week were transferred to a column bioreactor (*d*: 30 mm, *h*: 200 mm) and preincubated in the same medium for 3 days. (−)-Codeinone (5 mg) dissolved in MeOH (2 ml) was injected from the upper sterile filter (Dualex, Millipore Co.) after 3 days and incubated with upward aeration by an air-pump for 3 days. The medium, after 3 days' reaction, was removed with the substrate and/or product from the lower sampling outlet, and then extracted according to the method described below. Subsequently, the same fresh medium was added from the medium reservoir, and (−)-codeinone (5 mg) was injected again. The bioreactor was operated under various conditions as follows: aeration, 0.94, 1.88, 3.75 vvm; temp., 20°, 25°, 30°.

Extraction and determination of codeine. The immobilized cells and medium, incubated in a shake flask for 3 days, were separated by filtration with nylon cloth. The cells were homogenized with Ca alginate beads in cold MeOH, filtered, and the residue was refluxed with MeOH. The combined soln was evapd to dryness *in vacuo* and acidified with 1 M HCl. The acidic soln was extracted with *n*-hexane. The aq. soln was made basic to pH 8.5 with NH_4OH and extracted with $CHCl_3$–*iso*-PrOH (3:1). The medium separated from the immobilized cells was concd and extracted as above. A metabolite was detected from both the extracts on TLC (silica gel 60 F 254, R_f 0.22; $CHCl_3$–MeOH, 9:1). Codeine, 3.10 mg (biotransformation ratio 62%) in the immobilized cells and 0.42 mg (8.4%) in the medium, was identified and determined by the SIM (selected ion monitoring, *m/z* 299 M^+) method in GC/MS. GC/MS was carried out under the following operating conditions. A JMS-DX 300 GC/MS spectrometer was used; 0.3 × 200 cm glass column of 3% OV-17 on Chromosorb WAW; DMCS at 260°; ionizing energy 70 eV.

Reagents. (−)-Codeinone was synthesized from (−)-codeine according to ref. [20]. Codeine as phosphate was supplied by the Sankyo Co. Ltd. (Ginza, Tokyo). Sodium alginate (90–130 cps) was supplied by the Kimitsu Chemical Industry Co. Ltd. (Chiba, Japan).

Acknowledgements—We are grateful to Dr. I. Chibata and Dr. T. Tosa, Department of Biochemistry, Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co. Ltd., Osaka, Japan, for their helpful instructions.

REFERENCES

1. Tosa, T. Sato, T., Mori, T. and Chibata, I. (1974) *Appl. Microbiol.* **27**, 886.
2. Sato, T., Mori, T., Tosa, T., Chiba'a, I., Furui, M., Yamashita, K. and Sumi, A. (1975) *Biotechnol. Bioeng.* **17**, 1797.
3. Yamamoto, K., Tosa, T., Yamashita, K. and Chibata, I. (1976) *Eur. J. Appl. Microbiol.* **3**, 169.
4. Yamamoto, K., Tosa, T., Yamashita, K. and Chibata, I. (1977) *Biotechnol. Bioeng.* **19**, 1101.
5. Kennedy, J. F., Barker, S. A. and Humphrey, J. D. (1976) *Nature (London)* **261**, 242.
6. Kierstan, M. and Bucke, C. (1976) *Biotechnol. Bioeng.* **19**, 387.
7. Somerville, H. J., Masson, J. R. and Ruffel, R. N. (1977) *Eur. J. Appl. Microbiol.* **4**, 75.
8. Navarro, J. M. and Durand, G. (1977) *Eur. J. Appl. Microbiol.* **4**, 243.
9. Wada, M., Uchida, T., Kato, J. and Chibata, I. (1980) *Biotechnol. Bioeng.* **22**, 1175.
10. Takata, I., Tosa, T. and Chibata, I. (1977) *J. Solid-Phase Biochem.* **2**, 225.
11. Brodelius, P. and Nilsson, K. (1980) *FEBS Letters* **122**, 312.
12. Brodelius, P., Deus, B., Mosbach, K. and Zenk, M. H. (1979) *FEBS Letters* **103**, 93.
13. Alfermann, A. W., Schuller, I. and Reinhard, E. (1980) *Planta Med.* **40**, 218.
14. Jones, A. and Veliky, I. A. (1981) *Eur. J. Appl. Microbiol. Biotechnol.* **13**, 84.
15. Brodelius, P. and Mosbach, K. (1982) *Appl. Biochem. Biotechnol.* **7**, 31.
16. Furuya, T., Nakano, M. and Yoshikawa, T. (1978) *Phytochemistry* **17**, 891.
17. Noguchi, M., Matsumoto, T., Hirata, Y., Yamamoto, K., Katsuyama, A., Kato, A. Azechi, S. and Kato, K. (1977) *Plant Tissue Culture and its Biotechnological Application*, p. 85. Springer, Berlin.
18. Veliky, I. A. and Jones, A. (1981) *Biotechnol. Letters* **3**, 551.
19. Brodelius, P. and Mosbach, K. (1982) *Adv. Appl. Microbiol.* **28**, 1.
20. Rapoport, H., Naumann, R., Bissell, E. R. and Bonner, R. M. (1950) *J. Org. Chem.* **15**, 1103.