Increased Production of Digoxin from Digitoxin by Biotransformation Using Plant Cell Culture

Hee-Jeon Hong, Jong-Eun Lee and Dong-Il Kim

Department of Biological Engineering, Inha University,
Incheon, 402-751, Korea

Abstract

Production of a cardiac glycoside, digoxin, by 12β-hydroxylation from digitoxin was studied in plant cell suspension cultures of *Digitalis lanata*. In order to increase the conversion yield, various culture conditions including immobilization were investigated and optimized. Since digoxin was released in the medium temporarily and converted further into a glucosylated product, deacetyllanatoside C, *in situ* adsorption of digoxin was employed to recover the product continuously. Amberlite resin XAD-8 showed the best adsorption characteristics for digoxin among the examined resins, and an integrated process was developed to increase the productivity. In addition, it was found that the utilization of β-cyclodextrin to entrap digoxin during the culture enhanced the biotransformation yield significantly.

Introduction

Biotransformation by plant cell cultures implies the utilization of enzymes in plant cells for the conversion of any organic chemicals into more valuable structures. Although microorganisms are well-known for their biotransformation capabilities including steroid bioconversion, utilization of plant cell cultures in biotransformation has been limited (1). However, it is very important to use plant

cells in culture for the production of useful compounds originated from plant via biotransformation (2). Especially for the case of phytochemicals with stereospecificity and complex structure which prevents chemical synthesis, biotransformation using plant cell cultures can play an important role in commercial production (3). Reactions which are restricted only to plant cells and which produces chemicals of high economic value can be of commercial interest and biotechnological relevance. One of the most distinguished examples is the conversion of digitoxin into digoxin by *Digitalis lanata* cultures (4, 5). Cardiac glycosides(cardenolides) such as digitoxin and digoxin are important pharmaceuticals in the treatment of certain cardiac disease and are widely used in medicine (6). They can only be extracted from *Digitalis* plants, but digoxin is mostly used in cardiac therapy. Therefore, the need for digitoxin is decreasing and many researches have been performed to find ways to make better use of this compound. Biotransformation of card-enolides by *Digitalis* cell cultures has been studied extensively (7-10). Cardenolides consist of a steroid nucleus and a sugar side-chain of variable length. When digitoxin is added as a substrate, two major different reactions occur including 16′-O-glucosylation and

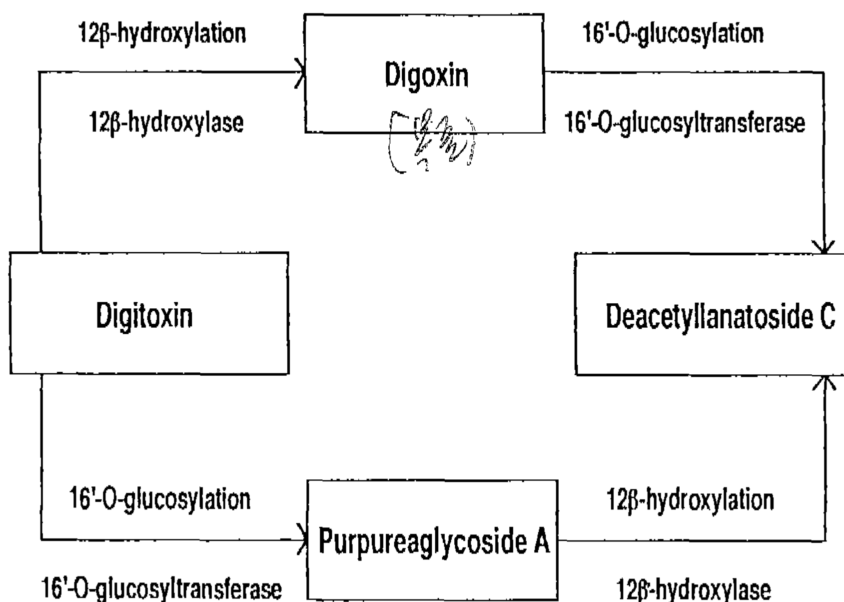


Fig. 1. Biotransformation of digitoxin by *Digitalis lanata* cell suspension cultures.

12β-hydroxylation. The main reaction is the 12β-hydroxylation to convert digitoxin to digoxin. Once the side reaction, i.e. 16′-O-glucosylation, proceeds, unwanted products such as purpureaglycoside A and deacetyl-lanatoside C are synthesized from digitoxin and digoxin, respectively. This relationship is summarized in Figure 1.

In order to increase the production of digoxin, 16′-O-glucosylation should be suppressed while maximizing 12β-hydroxylation. In this study, production of a cardiac glycoside, digoxin, by 12β-hydroxy-lation from digitoxin was studied in plant cell suspension cultures as well as in immobilized cultures of *Digitalis lanata*. In order to increase the conversion yield, various culture conditions were investigated and optimized. In addition, *in situ* adsorption was applied to enhance the productivity further.

Materials and Methods

Plan cell culture and culture medium

The *Digitalis lanata* cell line K 3 OHD was kindly provided by Dr. W. Kreis (Pharmazeutisches Institut, Eberhard-Karls-Universitat Tübingen, Germany) and has been maintained in modified Murashige and Skoog medium without growth regulators (8). The suspension cultures were grown in 100 ml Erlenmeyer flasks with 50 ml of medium on a gyratory shaker at 150 rpm under dark condition. The temperature was kept at 25℃ and subcultures have been performed in every 10 days.

Biotransformation

For the production of digoxin from digitoxin, pure 8% (w/v) glucose solution without any other nutrients at pH 5.5 was used as a production medium (9). Cells in the late exponential growth phase in growth medium were used for inoculation into production medium. In order to avoid heterogeneity of the inoculum, all the

cells from different flasks were collected in an autoclaved large flask and mixed well by shaking. The cells were filtered through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum and washed with fresh production medium. As fresh weight, 8 g of cells were inoculated into a 100 ml Erlenmeyer flask containing 50 ml of production medium. In all experiments, digitoxin was supplied as needed in the form of a concentrated solution of 30 g digitoxin per liter of dimethyl sulfoxide (DMSO).

Analysis of cardenolides

The total methanolic extract of the culture was obtained by adding the same amount of methanol as that of the culture broth and sonicating for 20 min. Supernatant after the centrifugation was used for the determination of cardenolides. A filtered sample was injected into the HPLC system (Model 910, Young-In Scientific Co., Korea) with UV detector. Curosil G column (4.6 x 250 mm, 6 μm, Phenomenex Inc., USA) was used for the analysis. The mobile phase was a mixture of acetonitrile and water (35:65, v/v). The flow rate was 1 ml/min and the measuring UV wavelength was 220 nm. Standard cardenolides for HPLC analysis was purchased from Roth (Germany) and the solvent was obtained from Fisher Scientific (USA).

Results and Discussion

Optimization of culture conditions

For the enhanced production of digoxin by using biotransformation in suspension cultures of *Digitalis lanata* cells, a two-stage culture process was optimized. Typical time course profiles of digitoxin biotransformation are shown in Fig. 2. Digitoxin was added 3 days after the transfer of cells from growth medium to production medium. Digitoxin added in the production medium disappeared rapidly. The concentration of target product, digoxin, increased until

the 2nd day after the addition of substrate and began to decrease afterwards. On the other hand, the level of deacetyllanatoside C increased continuously until the end of the culture. Therefore, it was found that the optimization of culture process is essential to maximize digoxin production (11).

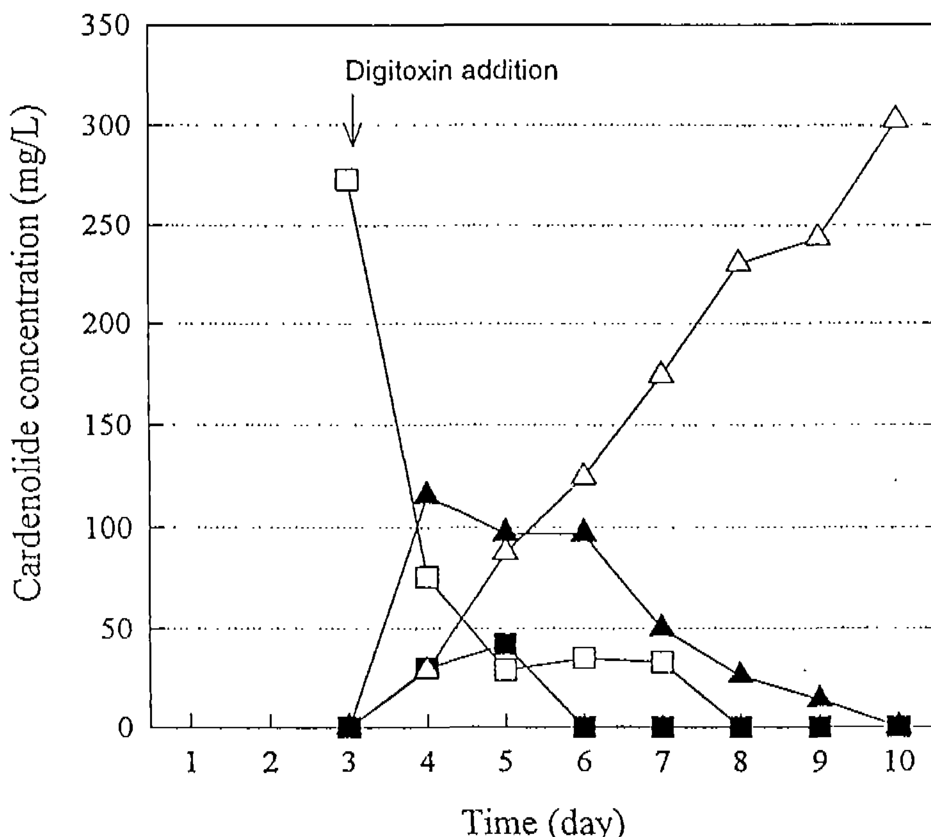


Fig. 2. Time course profiles of digitoxin biotransformation by suspension cultures of *Digitalis lanata* in 8% glucose solution: (□) digitoxin, (■) digoxin, (▲) purpureagly-coside A, (△) deacetyllanatoside C.

Most of the components in growth medium except phosphate reduced the efficiency of digoxin formation. Besides, peptone and beef extracts inhibited 12β-hydroxylation, while promoting glucosy-lation. It was apparent that light enhanced the formation of digoxin significantly.

When the cells were cultivated for 10 days in the growth period, 12β-hydroxylation capacity was the best. It was also found that the most suitable amount of digitoxin as substrate was 400 mg/L with initial cell density of 21%. In this case, maximum productivity was achieved 5 days after transfer of cells to production medium. Sucrose and fructose provided similar digoxin yield as that in glucose.

Biotransformation by immobilized cells

Since the hydroxylated product digoxin is released into the medium and the glucosylated byproducts are stored inside the cells, advantages of immobilization technique can be considered(12,13).

A comparative study on the cultivation of *D. lanata* suspension cells in polyurethane foam matrix and Ca-alginate gel was performed to investigate the effects of immobilization on the production of digoxin from digitoxin by biotransformation. Compared to polyurethane foam immobilization which did not affect cell viability, the growth of Ca-alginate entrapped cells was considerably inhibited.

As shown in Fig. 3, when 200 mg/1 of digitoxin was added, maximum digoxin production of 51.12 mg/1 was achieved at the first day of biotransformation in free cell suspensions. On the other hand, maximum digoxin production was 52.37 mg/1 in polyurethane foam entrapped cell cultures after 2 days of biotransformation (Fig 4). In contrast, as can be seen in Fig 5, very small amount of digoxin(25.43 mg/1) was obtained in Ca-alginate immobilized cell cultures. This may be due to the suppressed cells growth and low viability.

It was also found that the cells immobilized in polyurethane foam matrix produced much less digoxin compared to free cell suspensions with addition of 400 mg/1 of digitoxin, even though the digoxin productivities of both cultures were almost the same in the case when digitoxin addition was below 200 mg/1. It can be thus concluded that the addition of high concentration of substrate should be prevented for immobilized cells.

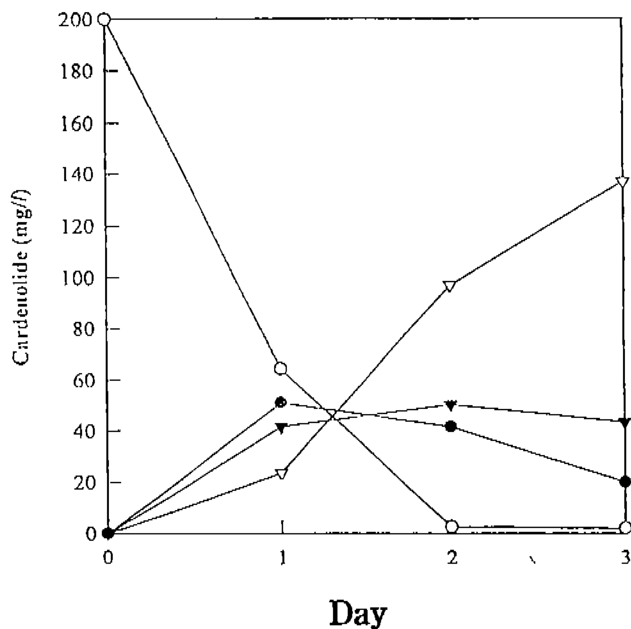


Fig. 3. Digitoxin biotransformation by freely suspended cells: (○) digitoxin, (●) digoxin, (▼) purpureaglycoside A, (▽) deacetyllanatoside C.

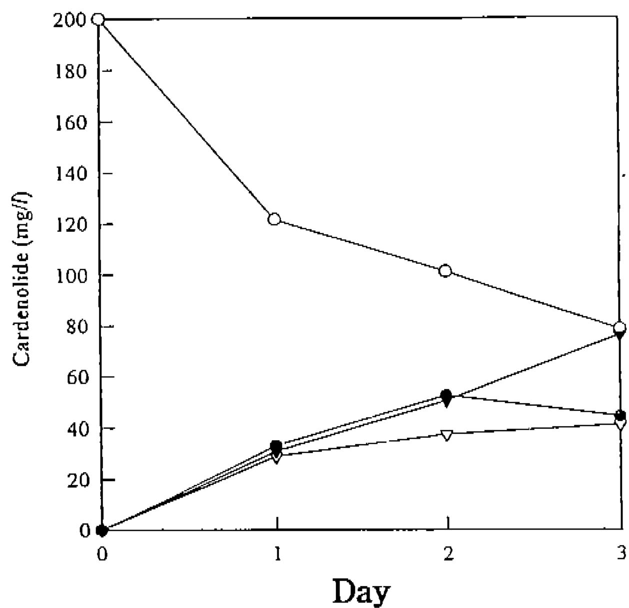
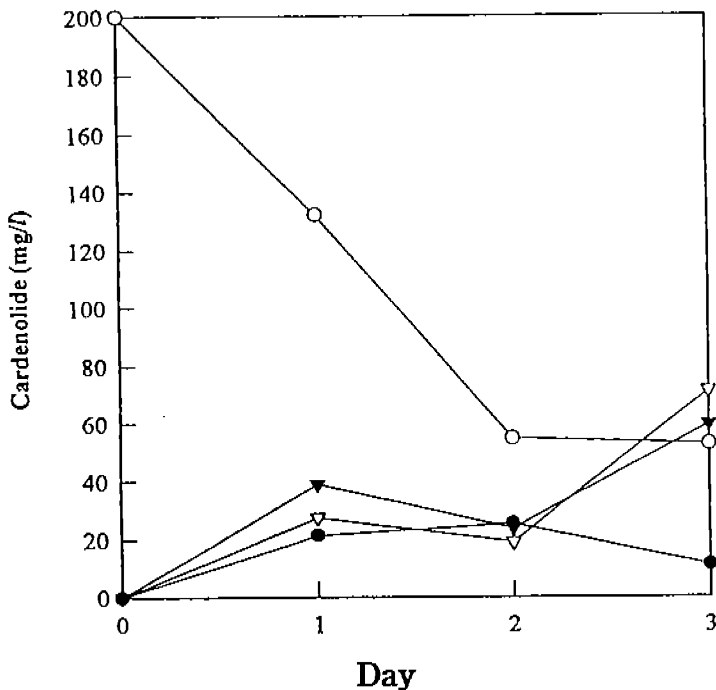


Fig. 4. Digitoxin biotransformation by the cells immobilized in polyurethane foam: (○) digitoxin (●) digoxin (▼) purpureaglycoside A, (▽) deacetyllanatoside C.



fFig. 5 Digitoxin biotransformation by the cells entrapped in Ca-alginate: (○) digitoxin, (●) digoxin, (▼) purpureagly-coside A, (▽) deacetyllanatoside C.

Application of *in situ* adsorption

Because digoxin was released in the medium temporarily and converted further into a glucosylated product, deacetyllanatoside C, *in situ* adsorption of digoxin was employed to recover the product continuously. To adsorb digoxin selectively *in situ*, various kinds of ion-exchange resins were examined.

Among the tested adsorbents, nonionic resins such as Amberlite XAD showed good adsorption characteristics against both digoxin and digitoxin. Among them, adsorption capacity of XAD-8 was the best as shown in Fig. 6. It was also found that cell growth inhibition of XAD-8 was negligible. Because XAD-8 resin adsorbs not only the target product digoxin but also the substrate digitoxin, optimization of addition time is important to reduce the adsorption of digitoxin and to maximize the adsorption of digoxin. In order to determine the addition time, resins were added at different times and the results are shown in Fig. 7.

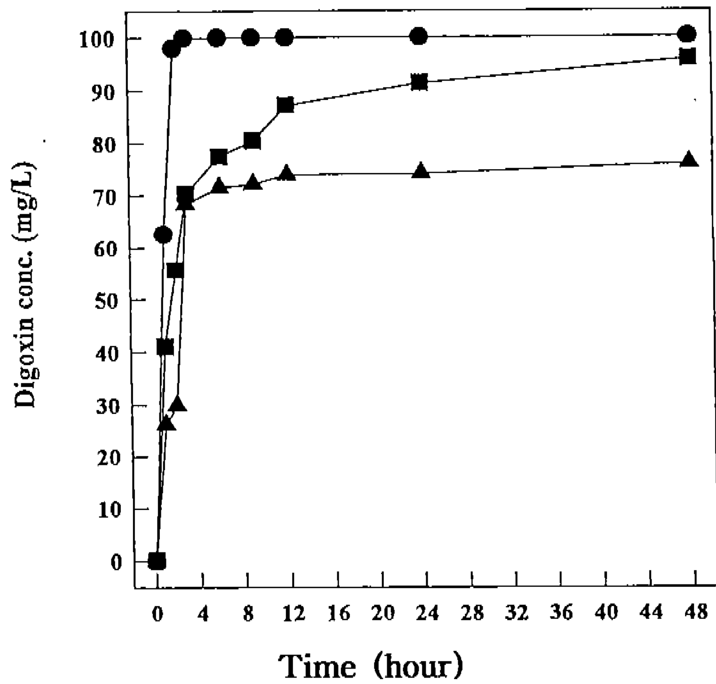


Fig. 6. Adsorption kinetics of digoxin: (■) XAD-4, (▲) XAD-7, (●) XAD-8

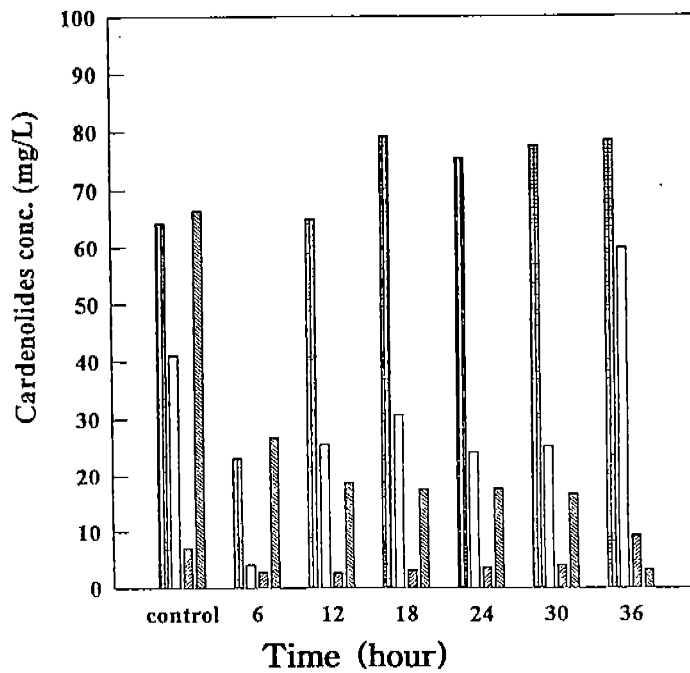


Fig. 7. Effect of resin addition time on the biotransformation of digitoxin. At every addition time, each bar represents deacetyllantoside C, digoxin, purpureaglycoside A, digoxin, respectively from the left.

When XAD-8 resin was added 36 hours after the addition of digitoxin as substrate, digoxin yield was the best. This may be due to the fact that digoxin concentration became the maximum around 36 hours after the beginning of bioconversion.

To reuse the resins, direct contact of resins with cells should be avoided. Therefore, resins were immobilized in Ca-alginate gel beads, glass tubes with two openings, and paper bags made of tea-bag-paper(wood pulp) and were applied into the bioconversion. As expected, positive effect of *in situ* adsorption of digoxin was noticed and the use of wood pulp supported the highest biotransformation yield. The results are summarized in **Fig. 8.**

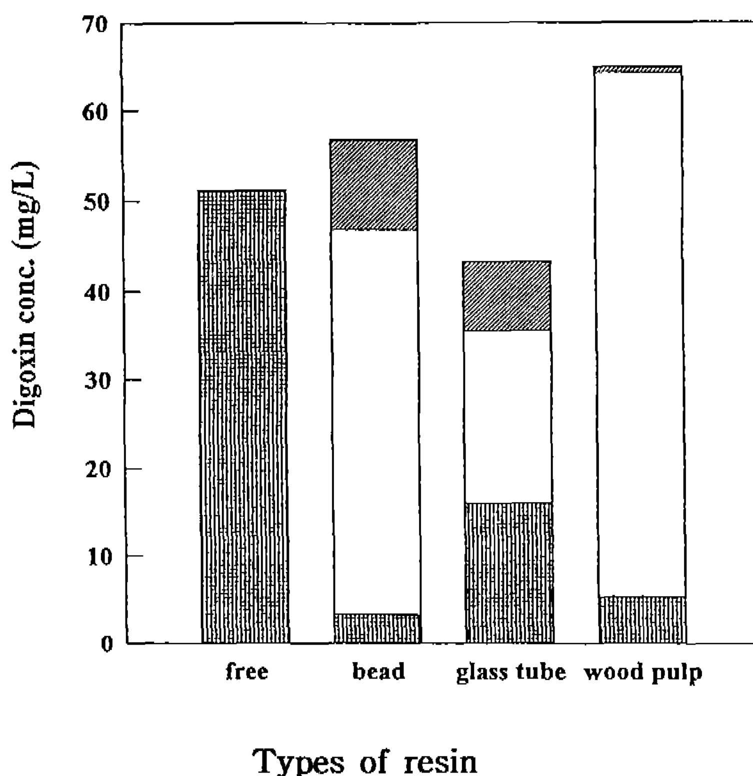


Fig. 8. Effects of various addition types of XAD-7 resin on digoxin yield. Distribution of produced digoxin is also shown at each bar. White bars represent digoxin adsorbed on resins. The bars with slanted lines represent digoxin in the cells, and the bars with crossed lines represent digoxin in the medium.

In addition, it was observed that the utilization of β-cyclodextrin to entrap digoxin during the culture enhanced the biotransformation yield significantly. As digoxin and digitoxin have very hydrophobic properties, they are almost insoluble in water. Therefore, the presence of β-cyclodextrin improves their solubility and bioconversion. After the formation of digoxin by 12β-hydroxylation, glucosylation into deacetyllanatoside C was reduced due to the molecular encapsulation of digoxin in β-cyclodextrin. In consequence, the level of digoxin was maintained to some extent.

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