Agric. Chem. Biotechnol. **43**(2), 109-111(2000)

Improved Optimization of Indirubin Production from Bioreactor Culture of *Polygonum tinctorium*

Choong Sik Chung, Kyung Il Kim, Geun Won Bae, Youn Hyung Lee, Hyong Joo Lee[1], Young Am Chae[1] and In Sik Chung*

Institute of Genetic Engineering, Kyung Hee University, Suwon 449-701

[1]Research Center for New Bio-Materials in Agriculture, Seoul National University, Suwon 441-744, Korea

Received May 8, 2000

Effect of the two-stage operation and cell concentration on indirubin production was investigated using bioreactor culture of *Polygonum tinctorium*. Two-stage culture was operated successfully for 110 days without any adverse effects on continuous indirubin production. Maximum indirubin concentration was found to be at 80 mg/bioreactor. Initial cell concentration significantly affected indirubin production. The indirubin production at 29.2% PCV was improved by 845%, compared to that at 5% PCV. For high-density bioreactor culture of *P. tinctorium*, a maximum production rate of 10.2 mg indirubin/L day was obtained. Indirubin recovery for bioreactor operation was also examined using XAD-2, XAD-4, XAD-7, and solid silicon. XAD-4 was 1.6-fold more effective than that for solid silicon in indirubin recovery.

Key words: *indirubin, bioreactor, silicon, two-stage operation,* Polygonum tinctorium.

Indirubin and indigo related compounds are of significant interest both as natural colorants and treatment for chronic granulocytic leukemia.[2,6,12] *In vitro* production of indirubin and indigo-related compounds by plant cell culture is receiving more attention because native plants such as *Indigofera spp., Isatis tinctoria, Polygonum tinctorium,* and *Lonchocarpus cyanescens* produce only small amounts of the compounds over one to two years of growing period.[7] Maier *et al.*[8] and Kim *et al.*[5] studied biotransformation pathway for indirubin and indigo production from precursors such as indole and tryptophan using plant cell culture of *P. tinctorium* cells. Other investigators reported on the effects of adding precursor, potassium nitrate,[1] fungal elicitation,[9] and chitosan elicitation[4] on indirubin production using plant cell culture of *P. tinctorium* cells. However, indirubin production could be further improved through optimization on bioreactor culture of *P. tinctorium*. Therefore, in this study, we investigated the effects of the two-stage operation, cell concentration, and adsorbent types on indirubin production using bioreactor culture of *P. tinctorium* cells.

Materials and Methods

Cell line, culture medium and precursor preparation. The calli and the suspension cells of a Korean cultivar of *P. tinctorium* were induced and established as described previously.[10] For cell maintenance and suspension culture, modified B5 medium[3] supplemented with 0.2 mg 2,4-D, 1 g yeast extract, and 30 g sucrose per liter was used. The initial pH of the medium was adjusted to 5.7 before autoclaving. Indole dissolved in ethanol was kept as a stock solution. Unless otherwise specified, 20 mM indole was added to the suspension culture medium.

Shake flask and bioreactor operation. Shake flask experiments were performed in duplicates at 100 rpm and 27°C using 200 ml baffled shake flasks with a working volume of 50 ml. Reactors used for this study were internal-loop air-lift bioreactor (Eyela, Japan, 2 l), external-loop air-lift bioreactor and split-flow air-lift bioreactor, the working volumes being, 1250, 500, and 200 ml, respectively. Aeration for all experiments was 0.1 vvm. The medium used in all experiments was identical to that specified above. For the analysis of two-stage operation effects, a continuous system using internal-loop air-lift bioreactors was established. The first-stage bioreactor was designed for the cultivation of *P. tinctorium* cells and the second-stage bioreactor was for indirubin production. The working volume of both the first and the second stage bioreactors was maintained at 1250 ml. Two-stage bioreactor culture was operated at 27°C. For the analysis of cell concentration effects, a cell-recycled bioreactor was used. Cell recycle was done through natural sedimentation of *P. tinctorium* cells within a cell settler placed vertically inside the bioreactor. The dimensions of the glass settler for air-lift bioreactor were 2.5 cm×15.0 cm (diameter×height). Other conditions

*Corresponding author
Phone: 82-331-201-2436; Fax: 82-331-202-9885
E-mail: ischung@nms.kyunghee.ac.kr

Abbreviations: PCV, packed cell volume; vvm, volume per volume minute.

and medium composition were the same as those described above. For the analysis of adsorbent effects, XAD-2 (Sigma, U.S.A.), XAD-4 (Sigma, U.S.A.), XAD-7 (Sigma, U.S.A.), and solid silicon (Korea Ace, Korea) were used.

Analytical methods. The suspension cells were centrifuged at 1100 g for 20 min in a 15 ml graduated tube. The percentage of cell volume after centrifugation was reported as the packed cell volume (% PCV). Indole content was measured using a gas chromatograph (Carlo Erba, Italy) equipped with a flame ionized detector. Indirubin content was determined as follows: for the cellular phase, cells were separated from the culture broth by filtration, washed with distilled water, dried, and immersed in $CHCl_3$ solution for 48 hr in dark condition. For the medium phase, the culture broth was extracted for 24 hr with $CHCl_3$ in dark condition. For the adsorbent phase, the adsorbent was separated from the culture broth through centrifugation, extracted with ethanol for 24 hr, vacuum-evaporated, and immersed in $CHCl_3$ solution. The absorbance was measured at 534 nm (UV-160A spectrophotometer, Shimadzu, Japan) and converted into indirubin concentration.

Results and Discussion

Effects of two-stage operation. To determine the optimum condition for bioreactor operation, several runs were made by varing the dilution rate from 0.077 to 0.2 day^{-1}. Cell concentration was the highest at the dilution rate of 0.1 day^{-1} (Table 1). As such two-stage bioreactor culture was operated at this dilution rate, which corresponded to a residence time of 10 days. In principle, cell suspension of the continuously operated cell growth bioreactor (Stage 1) was pumped into the second stage bioreactor (Stage 2), and a second feed stream of indole was added intermittently to the second-stage bioreactor to keep indole level above 10 mM. Indirubin production was found to be 80 mg/bioreactor (Fig. 1), which corresponds to a volumetric production rate of 6.4 mg indirubin/l day. Two-stage culture was operated successfully for 110 days without any adverse effects (Fig. 1). Our results indicate that continuous indirubin production with *P. tinctorium* cell culture is possible using the two-stage operation.

Table 1. Effect of dilution rate on steady-state cell density.

Dilution Rate (Day^{-1})	Cell Growth (%PCV)
0.077	11.32
0.1	21.12
0.2	4.1

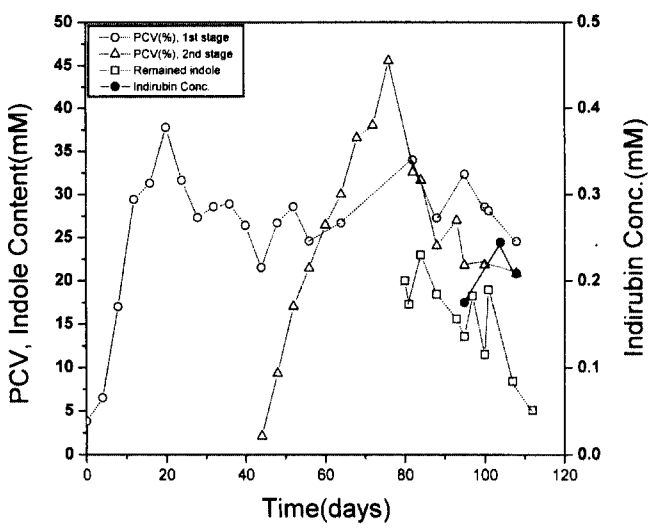

Fig. 1. Cell growth and indirubin production in continuous two-stage culture.

Effects of cell concentration. The effect of initial cell concentration on indirubin production from *P. tinctorium* cell culture is shown in Table 2. Indirubin production increased with initial cell concentration. The indirubin production at 29.2% PCV was improved by 845%, compared to that at 5% PCV. For further analysis of cell concentration effects, cell-recycled bioreactor runs were made (Table 3). *P. tinctorium* cells were grown batchwise in air-lift bioreactors with working volume of 500 ml for 12 days. Thereafter, cell recycle operations were started by applying various dilution rates of 0.05, 0.1, and 0.2 day^{-1}. The cell growth was highest when the culture was operated at the dilution rate of 0.05 day^{-1}. Cell-recycled continuous culture attained a quasi-steady state within four residence times from the start-up of cell-recycle operations. The indirubin productivities at quasi-steady state were taken as representative values for each dilution rate (Data not shown). A maximum production rate of 10.2 mg indirubin/l day was obtained at the dilution rate of 0.05 day^{-1} (Fig. 2). Our results proved that a simple increase in cell concentration (high-density culture) enhanced the volumetric production rate of indirubin. Similar results on the effect of high cell concentration on secondary metabolite production were also reported for *Anchusa officinalis*.[11]

Table 2. Effect of initial cell density on indirubin production.

Initial Cell Density (%PCV)	Indirubin Concentration (mM)	Yield*
5.0	0.031	-
11.0	0.054	-
16.2	0.103	0.04
23.5	0.171	0.05
29.2	0.262	0.06

*Yield was calculated according to the following equation; production of indirubin(mg)/consumption of indole(mg)

Table 3. Effect of dilution rate on cell growth.

Dilution Rate(Day^{-1})	Cell Growth(%PCV)
0.2	55.2
0.1	52.3
0.05	57.1

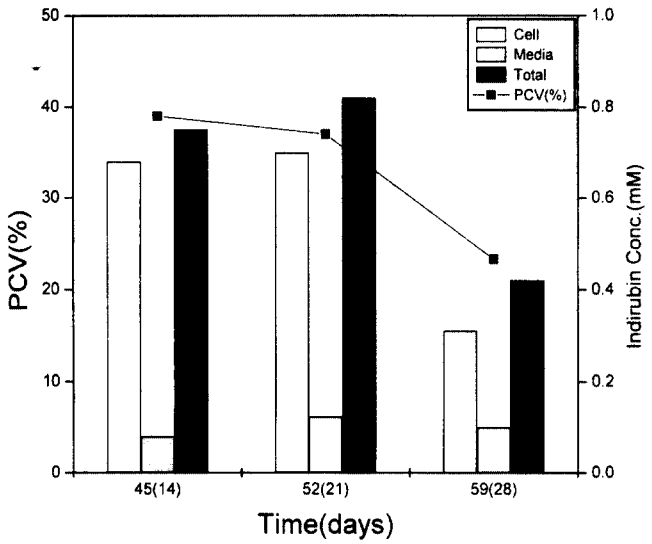

Fig. 2. Indirubin production in a cell-recycled bioreactor. The X-axis values in parenthesis indicate number of days after indole addition.

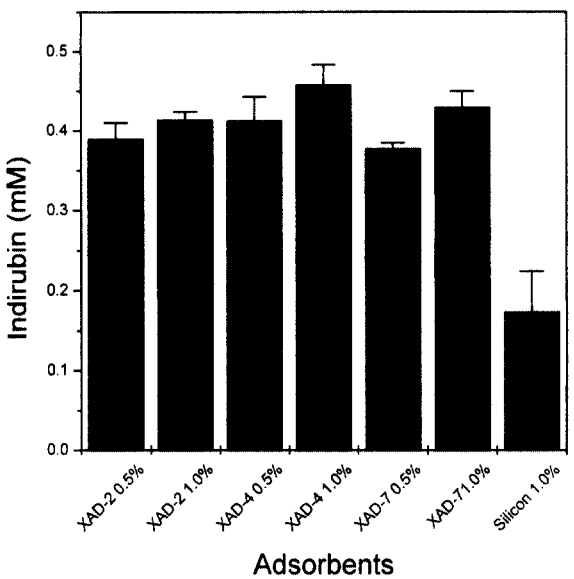

Fig. 3. Effect of adsorbent type on indirubin recovery.

Effects of adsorbent type. Several runs were made to determine appropriate adsorbent for indirubin recovery from *P. tinctorium* cell culture. XAD-2, XAD-4, XAD-7, and solid silicon were added to 50 ml of modified B5 medium containing indirubin in 200 ml flasks. The adsorption rate in the flask containing 5 or 10% XAD series was better than that in the flask containing 1% silicon (Fig. 3). Treatment with 1% XAD-4 gave the best results, being 1.6-fold more efficient than that for silicon in indirubin recovery, however the mechanism behind this phenomenon is not yet known. This result indicates that indirubin recovery depends on the adsorbent type and on the concentration employed. Our preliminary data suggest that XAD-4 may be an effective adsorbent for recovering indirubin in plant culture broth. Thus, if indirubin can be continuously adsorbed during the culture, it may provide a method to overcome product inhibition and to improve the overall economics of the process.

Acknowledgments. This work was supported by grants from Kyung Hee University and the Korea Science and Engineering Foundation through the Research Center for New Bio-Materials in Agriculture.

References

1. Chung, I. S., Kim, T., Bae, G. W., Lee, H. J. and Chae, Y. A. (1996) Stimulation of indirubin production by KNO_3 depletion in indole-supplemented suspension culture of *Polygonum tinctorium*. *Biotechnol. Lett.* **18**, 947-950.
2. Fox, M. R. and Pierce, J. H. (1990) Indigo past and present. *Textile Chemist and Colorist* **22**, 13-15.
3. Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50**, 148-151.
4. Kim, J. H., Shin, J. H., Lee, H. J., Chung, I. S. and Lee, H. J. (1997) Effect of chitosan on indirubin production from suspension culture of *Polygonum tinctorium*. *J. Ferment. Bioeng.* **83**, 206-208.
5. Kim, S. U., Song, K. S., Jung, D. S., Chae, Y. A. and Lee, H. J. (1996) Production of indoxyl derivatives in indole-supplemented tissue culture of *Polygonum tinctorium* Ait. *Planta Med.* **62**, 54-56.
6. Kohda., H., Niwa, A., Nakamoto, Y. and Takeda, O. (1990) Flavonoid glucosides from *Polygonum tinctorium*. *Chem. Pharm. Bull.* **38**, 523-524.
7. Lee, H. Y. and Kim, W. J. (1990) Production of natural colorants by plant cell biotechnology. In *Natural Spices and Pigments*, Hyangmoon Publishing Co., Seoul, Korea.
8. Maier, W., Schumann, B. and Groger, D. (1990) Biosynthesis of indoxyl in *Itatis tinctoria* and *Polygonum tinctorium*. *Phytochemistry* **29**, 817-819.
9. Marero, L. M., Jin, J. H., Shin, J. H., Lee, H. J., Chung, I. S. and Lee, H. J. (1997) Effect of fungal elicitation on indirubin production from suspension culture of *Polygonum tinctorium*. *Enzyme Microb. Technol.* **21**, 97-101.
10. Soh, E. H. and Chae, Y. A. (1993) Callus induction and culture for indigo production in *Polygonum tinctorium*. *Korean J. Breed.* **25**, 133-138.
11. Su, W. W. and Lei, F. (1993) Rosmarinic acid production in perfused *Anchusa officinalis* culture: Effect of inoculum size. *Biotechnol. Lett.* **15**, 1035-1038.
12. Xia, Z. Q. and Zenk, M. H. (1992) Biosynthesis of indigo precursors in higher plants. *Phytochemistry* **31**, 2695-2697.