Biotechnology Letters Vol 12 No 8 603-608 (1990)
Received as revised 3rd July

ENHANCEMENT OF PRODUCING CATHARANTHINE BY SUSPENSION GROWTH OF CATHARANTHUS ROSEUS

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SUMMARY

Increased production of catharanthine (about 200 μg/mL) was obtained at low light intensity under chemostat cultivation as compared with batch and fed-batch processes. Photo-inhibition was observed at high light intensity. Cells after being frozen with 5 % DMSO produced more catharanthine (up to 350 μg/L) in batch culture than those from conventional culture techniques.

INTRODUCTION

Recently many efforts have been made to produce pharmaceutically active secondary metabolites through plant cell cultivation processes (Pu, et al., 1989 ; Schmidt, et al., 1989; Frazie, 1989). However, most of the metabolites are retained inside the plant cells, not secreted into culture broth (Deus and Zenk, 1982; Parr et al., 1986); the cells grow very slowly compared to bacteria and animal cells, and only produce their products using special induction media and/or processes for selected cell lines (Curtin, 1983; Bisson et al., 1983; Zenk et al., 1975). Therefore, it is absolutely necessary to develop culture process for high production of secondary metabolites. In this report, kinetics of cell growth and indole alkaloids production is investigated by suspension culture of Catharanthus roseus for three different cultivation systems, namely as batch, fed-batch and chemostat operations. A culture technique is also introduced to improve the production yield of catharanthine.

MATERIALS AND METHODS

A subclone of Catharanthus roseus G.Don was generously supplied by Dr. J. Berlin (Institut for Biochemie, GDF) and maintained using defined media and culture conditions (Konobloch and Berlin, 1980; Merillon at al., 1984). For suspension cultivation, an air-lift type

fermentor (1.5 L of working volume) was prefilled with Zenk production media (Zenk et al., 1975) enriched with 2 μM of alpha-naphthylacetic acid (NAA) and 2.0 g of fresh cells per liter was inoculated at 80 rpm of agitation by a paddle type impeller. Fresh production media were added into and cells were removed out of the reactor by a peristaltic pump for fed-batch and chemostat cultivations, based upon cell density in the system. Light intensity was measured by a Lux meter (DM-28, Japan) and adjusted by changing the distance between the reactor and light sources,white cool fluorescent lamps. To increase the production of catharanthine from C. roseus, those cells were cultivated with 1 M of sorbitol for 20 hours and frozen by adding 5 % dimethylsulfoxide (DMSO) as described elsewhere (Tony et al., 1984), and thawed after maintaining cells at - 40 ^{o} C for 48 hours. Then, thawed cells were grown under batch cultivation to measure cell growth and catharanthine accumulation.

Cells were collected from the reactor every one or two days and dried at 105 ^{o}C for 12 hours to measure dry cell density (Schmidt et al., 1989) and analyze catharanthine concentration by HPLC (Kutney et al., 1980). Size of cell aggregates was measured by a graduated microbial colony counter after spreading 10 mL of liquid sample from the culture flask every ten days in batch cultivation. Relative size distridution of size of cell aggregates was calculated by the ratio of the number of cells within a range of cell size to that of total cell aggregates in a colony counter. Cell size represents average radius of cell aggregates, assuming that cell aggregates are spheres.

RESULTS AND DISCUSSION

Cell growth of C. roseus was remarkably improved by addding 2 or 5 μM of NAA in batch cultivation, compared to growth in the absence of NAA; however, growth rate after adding 5 μM of NAA was decreased after only 15 day operation (Figure 1). Maximum cell density was obtained (11.5 g-dry wt./L) by adding 2 μM of NAA with relatively shorter lag period than in the case without NAA. In the first day of cultivation, average diameter of cell aggregates in inocula was about 3 mm increasing up to 10 mm in 20 days of batch cultivation (Figure 2). However, after 23 day cultivation,cell size was suddenly divided into two major sections ; very small size, about 1 mm in diameter and large particle, about 10-12 mm. Possibly large aggregates could no longer stand the shear stress at 80-100rpm in the stationary phase and were broken into small pieces even though total cell density was increased up to 10 g-cell/L.

Fed-batch process was operated by adding fresh production media when maximum cell density was maintained after 10 days of batch cultivation (Figure 3). Residual sucrose concentrations remained relatively constant after 16 days of operations corresponding to the point of reaching steady state of cell growth after passing transient periods. This fed-batch experiment was maintained for 20 days by step-wise

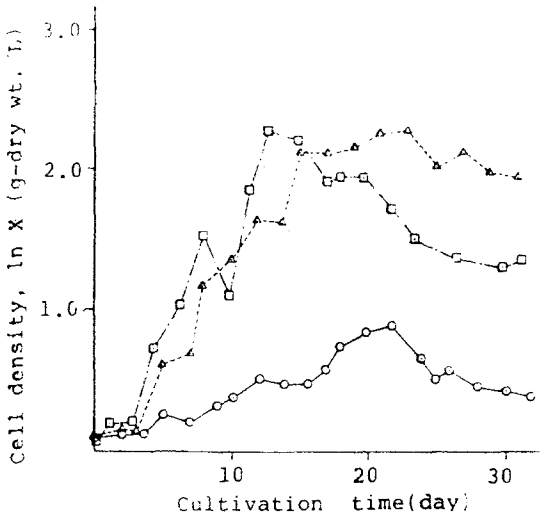

Fig.1 Effect of an auxin, α-naphthylacetic acid on growth of C. roseus in batch cultivation: ⊙ and ———, none; △ and - - - - - - - , 2μM of NAA; ⊡ and —·— , 5μM of NAA.

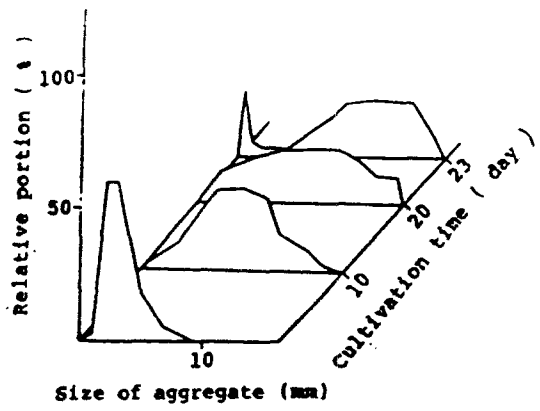

Fig.2 Distribution of cell aggregates according to cultivation time in batch suspension cultures at constant illumination of 5 klux and 80 r.p.m..

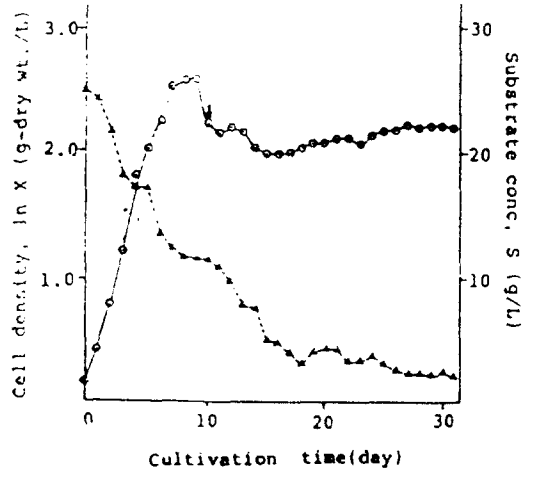

Fig.3 Growth of Catharanthus roseus switched from batch to fed-batch cultivation on sucrose as a growth-limiting substrate with 10 klux of continuous illumination: ⊙ , ———, cell density (g-dry wt./L); △ , - - - - - - - - , sucrose conc. (g/L). An arrow indicates the point of starting feeding fresh medium.

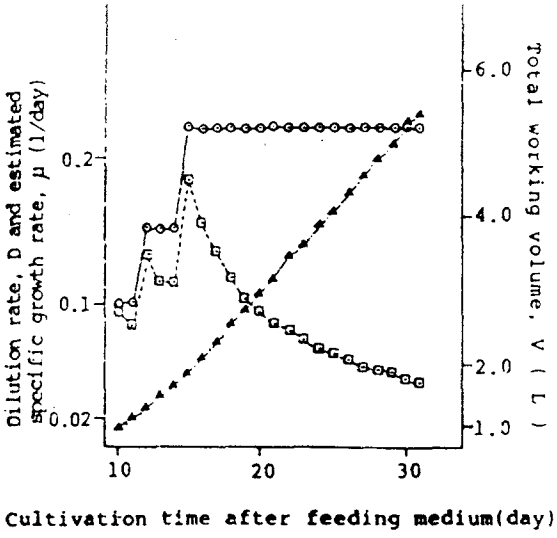

Fig.4 Comparison between rate and specific growth rate along with the increase of working volume after starting fed-batch cultivation: ⊙ , ———— , dilution rate (1/day); ⊡ , - - - - - - - , estimated specific growth rate (1/day); △ , —·—·— , total working volume (L).

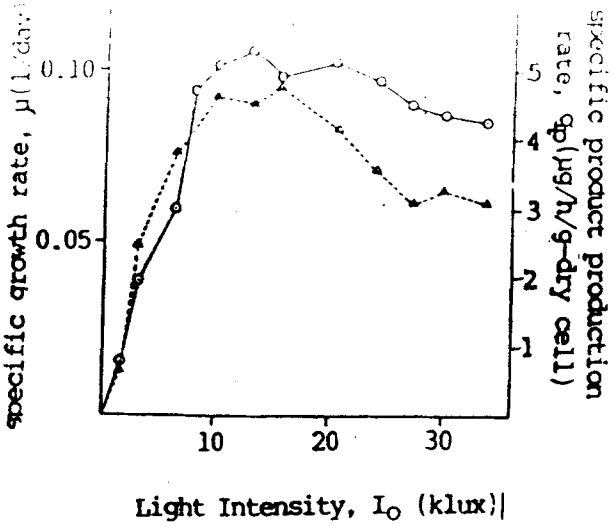

Fig.5 Specific growth rate and specific alkaloid production rate as a fuction of incident light intensity under chemostat cultivation: ⊙ and ——— , specific growth rate(1/h); ▲ and ----- , specific catharanthine production rate(μg/h/g-cell).

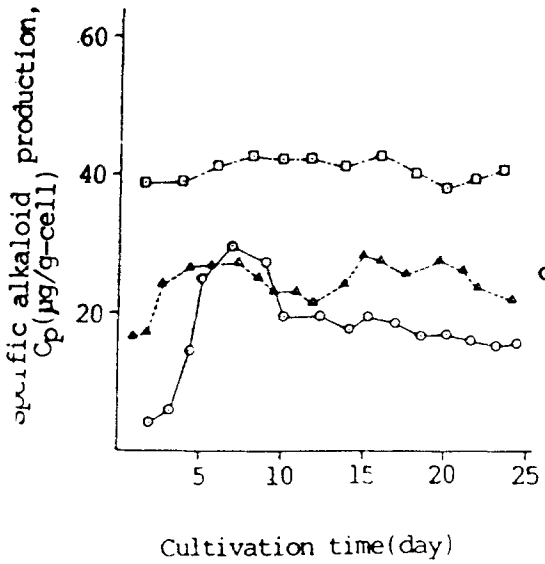

Fig.6 Comparision of kinetics of producing catharanthine from C. roseus among batch, fed-batch and continuos cultivation illumination of 10 klux: ⊙ and ——— , batch data; ▲ and ----- , fed-batch data; ⊡ and —·— , chemostat data.

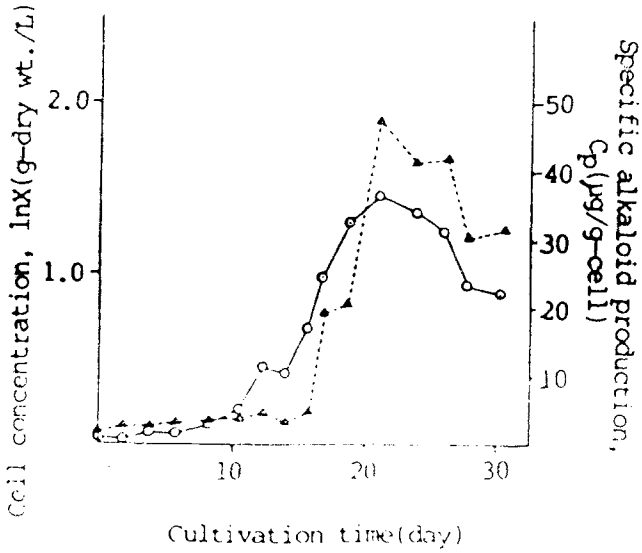

Fig.7 Cell growth and alkaloid production in batch cultivation from C. roseus after freezing by production medium with 10% DMSO for 48 hours at -70°C: ⊙ and ——— , cell density (g-dry wt./L); ▲ and ----- , specific catharanthine concentration(μg/g-dry cell).

increasing media flow rates up to 0.225 (1/day) (Figure 3), while the working volume was gradually increased up to 6 L. Specific growth rates were calculated, based upon dilution rates and working volume by employing quasi-steady state theory (Kutney et al., 1978) (Figure 4). The estimated specific growth rates were gradually decreased as dilution rates were increased due to the increase of working volume.

Specific growth rate and catharanthine production rate were observed as a function of light intensity in continuous cultivation (Figure 5). Maximum specific growth rate, μ_{max}, half-saturation constant, K_I and photo-inhibition constant, K_i were estimated as 0.127 (1/day), 1.86 (Klux) and 19.4 (Klux)$^{-1}$, respectively in using conventional photo-inhibition model (Lee et al.,1987), not considering simultaneous sucrose-limiting growth since both cell growth and product production rates were obviously decreased at high light intensity of 20 Klux (Figure 5). Specific alkaloid production rate was sharply decreased at high light intensity, compared to cell growth rate, because of less formation of catharanthine in photo - inhibition period. The Concentration of catharanthine was about 200 µg/L in 4.8 g-dry cell/L at maximum specific growth rate of 0.120 (1/h) , which is 0.0046 % of production yield (dry basis). It was also found that catharanthine production rate was faster at low light intensity than at medium and high light intensities.

Specific catharanthine production rates from three different culture processes were compared, and chemostat cultivation showed the highest production as 40 (µg of catharanthine/g-dry wt.) at 0.10 (1/day) of dilution rate (Figure 6). During overall culture period, fed-batch process had better productivity than batch cultivation, except that of exponential growth of C. roseus in batch system where maximum concentration of catharanthine was about 66 µg/L in 2.2 g-dry wt./L. Catharanthine production was improved by subsequently thawing cells after freezing suspension cultures for 48 hours (Figure 7). Alkaloid production was higher after thawing cells than those from batch and continuous cultures even though cells did grow slowly and had long lag peroid while maintaining relatively low maximum cell density (Figure 1 and 6). Maximum concentration of catharanthine in the cell was about 185 µg/L; however, total production of catharanthine was larger than 200 µg/L produced by chemostat cultivation. This is possibly caused by releasing about 10 % of catharanthine from the vacuoles due to conformational changes of cell wall at low pH(5.5) after being thawed (Kartha et al., 1982).

CONCLUSIONS

Chemostat cultivation is proved to be most advantageous process to accumulate catharanthine in the cells among batch, fed-batch and

continuous processes since this system can produce larger amounts of alkaloids within the cell than batch cultivation and also continuously collect cells to separate catharanthine from them. In batch process NAA played an important role in controlling cell growth of C. roseus and size distribution of cell aggregates was investigated according to cultivation time. Light intensity is a key factor for cell growth and product production, with photo-inhibition growth pattern at high light intensity. A freezing-thawing culture technique proves to increase the productivity of catharanthine under batch operations.

ACKNOWLEDGEMENT

This work was supported in part by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 89-90.

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