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공기부양 생물반응기에서의 쪽 (*Polygonum tinctorium*) 세포배양의 생육조건 및 생육특성

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Cultural conditions and growth characteristics of indigo (*Polygonum tinctorium*) cells in an air-lift bioreactor

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ABSTRACT

To find out the optimum conditions for indigo cell culture in air-lift bioreactor, effects of media composition including nutrients and precursors of the indigo colorants on the cell growth, and characteristics of the cell growth under various cultural conditions were analyzed. Optimum cultural conditions were tested and the growth characteristics were analyzed in external and internal loop type air-lift bioreactors during 14-day culture. Better cell growth was obtained when the inoculum size was higher in the range of 0.5~2.5% packed cell volume tested. In the sucrose concentration of 2 to 4%, the cell growth was better when the sucrose concentration was 4% (w/w) in both types of reactors. Sucrose was used up in the early stage of exponential phase of growth. At the optimum concentration of a precursor tryptophan at 1 mM, DCW was 3.8 g/ℓ in internal loop bioreactor, and 3.5 g/ℓ in external one after 14 days of cultivation. Addition of indole showed negative effect on cell growth of suspension culture in air-lift biorector culture, and cell mass of 2.5 g/ℓ and 2.2 g/ℓ were obtained in external and internal loop bioreactor, respectively. Selected inorganic nitrogen source, potassium nitrate showed about 110% increase in cell growth than that of control. DCW was 16.34 g/ℓ under optimum conditions during 14-day cultivation in internal loop bioreactor.

INTRODUCTION

Attractive and acceptable colors and flavors are important in foods. Currently most of the food colorants are synthesized chemically. However, many consumers prefer the natural colorants to the synthetic ones because of safety. Green No. 3, Red No. 2, Blue No. 1 and 2, and Yellow No. 5 and 6 have been used as acceptable synthetic colorants in food processing in Korea(1). Although the preference for natural colorants is worldwide, the supply is limited because the productivity is very low,

and extracting the colorants from natural sources requires complex processing which resulted in high cost. Plant cell culture has been proposed as an effective way of colorant production that can substitute the traditional extracting methods.

Numerous studies of the pigment production using plant cell culture have been reported, such as anthocyanins from *Vitis* cells (2, 3) or carrot cells (4, 5), shikonin from *Lithospermum erythrorhizon* (6–8), purpurin from *Rubia cordifolia* (9, 10), betalain from red beet and *Phytolacca americana* (11–15). Among them, the shikonin is produced in commercial scale.

Indigo is a blue colorant that is widely used as a natural dye and food colorant. Several species of *Indigofera*, *Polygonum tinctorium*, and *Isatis tinctoria* have been used as sources of the blue dye indigo. Natural indigo was obtained by direct extraction from whole plant tissue so far. These plants contain the colorless glucoside indican, which can be enzymatically hydrolyzed into glucose and indoxyl after extraction. Indoxyl is transformed to indigo by oxidation in the air. The structure of indigo (16), kinetics of oxidation of indigo carmine (17), and biosynthesis of indoxyl derivatives in *Polygonum*, *Indigofera*, and *Isatis* species (18–20) have been studied. However, the characteristics and condition of indigo cell culture have not been reported.

Air-lift bioreactor (ALB) has been regarded as one of the possible reactor types for the production of secondary metabolites in plant cell culture (21–23). The advantages of ALB are sufficient air supply with low shear, simple structure, easy scale-up, and uniform flow pattern. Internal loop bioreactor is easy to scale-up, and external one has an advantage of more uniform flow pattern (24–27).

In the present study, the cultural conditions for indigo cells were analyzed in internal loop and external loop ALB.

MATERIALS AND METHODS

Maintenance of callus

Callus of indigo (*Polygonum tinctorium* Lour.) obtained from Prof. Chae Yong Am in Seoul National University was subcultured in a test tube every four weeks. B5 medium was a basal one containing 2ppm of 2, 4-dichlorophenoxyacetic acid (2, 4-D, Sigma Chemical Co., St. Louis, Mo., U.S.A.) as a growth regulator, 1 g/ℓ of yeast extract (Difco, Detroit, U. S.A.), and 1% of agar. The pH of the medium was adjusted to 5.7 with 0.5 N NaOH. Callus was cultivated in an incubator at 27℃ with cool white fluorescent light for 16 hr per day.

Stock suspension culture

About 2–3 g of callus was suspended with 100 ml of liquid B5 medium in 250 ml baffled flask. Stock culture was incubated at 27℃ and 100 rpm with 16 hr of light condition per day. Suspended cells were broken into fine cells or smaller aggregates after 15 days. To stabilize the cells in liquid medium, cells were subcultured every week for several times. Then the cells were transferred into 120 ml of liquid B5 medium in 500 ml round flask and subcultured every four weeks.

Preparation of reactors

Detailed aspects of the internal loop bioreactor and the external loop bioreactor are shown in Fig. 1A and Fig. 1B, respectively. The internal loop bioreactor vessel consisting of a glass cylinder of 7.5 ㎝ diameter, inner draft tube of 3.5 ㎝ diameter, and height of 25 ㎝ had a working volume of 0.8 l. With these dimensions, the riser to downcomer cross sectional area ratio was 0.28, and the bottom clearance area was 2.2×10^{-3} ㎡.

The external loop bioreactor consisted of two cylinders. One cylinder was a riser part with 4 ㎝ diameter, and the other was a downcomer with 3.3 ㎝ diameter. The height was 60 ㎝ with 0.8 l working volume. The cross sectional area ratio was 1.47 and the clearance area was 3.8×10^{-3} ㎡. A teflon stopper was equipped with a condensor at the top of the reactor.

Analysis of cell growth

The sample of 5.0ml was put into a graduated conical centrifuge tube and centrifuged for 5 min at 200 × g. The packed cell volume (PCV) was the

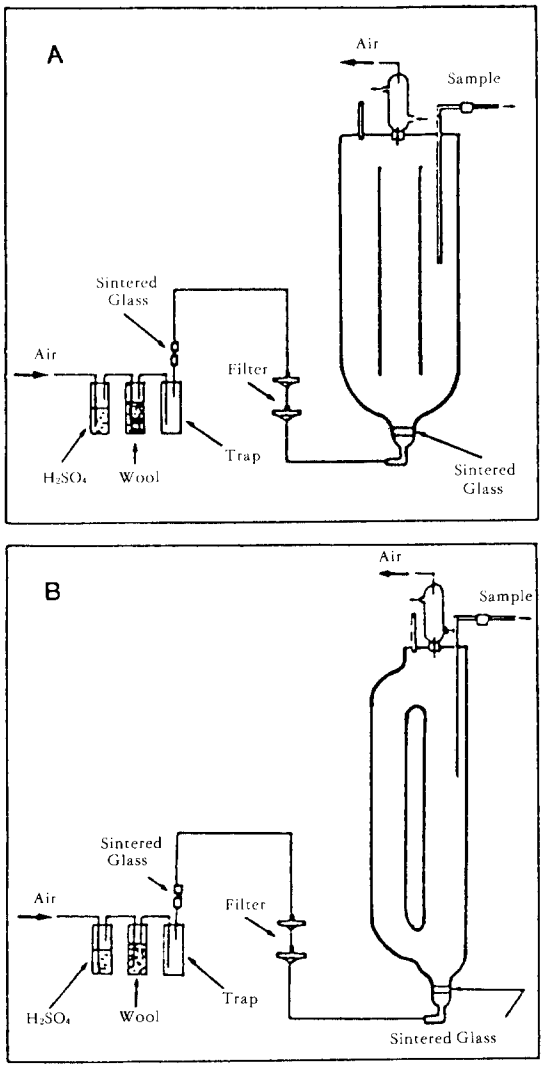

Fig. 1. Schematic diagram of the internal loop bioreactor (A) and external loop bioreactor (B) used for batch culture of indigo cells.

volume of the pellet over the volume of culture, and expressed as percentage (28).

To determine dry cell weight (DCW), the cells were washed with distilled water several times, filtered with a pre-weighed filter paper (Whatman No. 1), dried at 70℃ overnight in an oven to get a constant weight, and then the weight was measured (28).

Analysis of residual sugar in the media

A modified Anthron method (29) was used to determine the residual total sugar concentration in the medium. One hundred microliters of 30% potassium hydroxide was added to 100 μl medium filtrate, placed in a boiling water bath for 10 min, removed and left to cool. Three mililiters of anthrone reagent was added, placed in a 40℃ water bath for 20 min, and absorbance was measured at 620 nm.

Operation of air-lift bioreactor

Air supplied by an air pump was dried and sterilized by passing through H_2SO_4 trap and two air filters. Sintered glass sparger provided an aeration rate of 30 or 50 ml/min. Bioreactors were operated at 27℃ and illuminated for 16 hr per day. To avoid cell dispersion caused by bubble bursting forming a crust of cells on the surface of reactor and stopper, antifoam agent (silicon KM-72) and mineral oil were added.

RESULTS AND DISCUSSION

Effect of inoculum size

Growth curves with various inoculum sizes in ALB are shown in Fig. 2. In Fig. 2 A the cell growth was not good with inoculum size of 0.5% PCV in the internal loop bioreactor. Cell mass of 3.75 g/ℓ was obtained after 14 days of cultivaton using 2% PCV of inoculum. When inoculum size was 2.5% in PCV, DCW reached 3.27 g/ℓ after 8-day cultivation.

In case of external loop bioreactor, the result is presented in Fig. 2 B. DCW of 3.1 g/ℓ was obtained with 1.5% inoculum after 14 days of cultivation. Inoculum size of 1% and 2% resulted in 1.61 g/ℓ of cell mass after 14 days and 2.91 g/ℓ of cell mass after eight days, respectively. The external loop type showed a similar growth pattern as the internal one. It is clear that a larger amount of inoculum size will show a better results of growth.

The size of inoculum is important to initiate the cell growth. So, there is a critical minimum size of inoculum below which subcultures will not grow (34, 35). In this study, minimum inoculum size was 1% PCV(Fig. 2 A, B). Plant cells are grown in contact

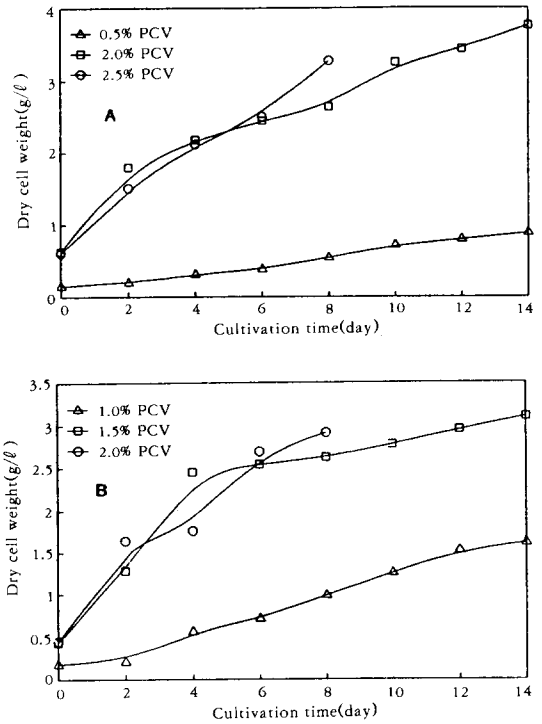

Fig. 2. Growth of indigo cells cultivated in B5 medium and cultured at 27℃ for 14 days in internal loop bioreactor (A) and external loop bioreactor (B). Inoculum size was 0.5, 2, and 2.5% in internal loop bioreactor and 1, 1.5, and 2% in external loop bioreactor.

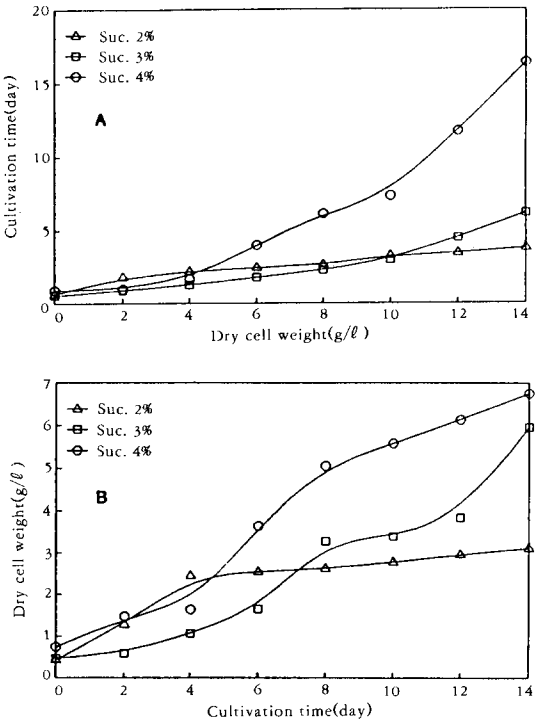

Fig. 3. Growth of indigo cells cultivated with sucrose concentrations of 2, 3, and 4% in B5 medium with 1 g/ℓ inoculum and cultured at 27℃ for 14 days in the internal loop bioreactor (A) and external loop bioreactor (B).

with each other, and there must be an unknown material in culture for growth at undifferentiated state. Song (32) investigated the effect of inoculum size on *Mentha* cell growth in an air-bubble bioreactor, and found the inoculum size of 2.0% in PCV was most suitable for the cultivation.

Effect of sucrose concentration

Effect of sucrose concentration on indigo cell growth in internal and external loop bioreactors is represented in Fig. 3 A and B. In the internal loop bioreactor (Fig. 3 A), the best growth was resulted with 4% of sucrose where the cell mass reached 16.3 g/ℓ after 14 days. The cell growth was also the best at the concentration of 4% where the cell mass was 6.75 g/ℓ in the external loop bioreactor(Fig. 3 B). It is not clear what makes these differences in cell growth in internal loop and external loop bioreactor.

The addition of sucrose as a carbon source is necessary to accelerate growth in callus and suspension cultures. Glucose and fructose may be substituted in some cases, glucose being as effective as sucrose and fructose being somewhat less effective. However, sucrose concentration also affects on the production of secondary metabolites as pointed by Dougall (30).

Choi (15) reported that the optimum sucrose concentration for red beet cell growth was 5% and betalain production was 3%. The optimum sucrose concentration was 3% for production of anthocyanin by *Vitis* cell (2). For betacyanin accumulation from *Phytolacca americana*, cell growth was best at 2% of sucrose (14). Maximum growth of *Lithospermum*

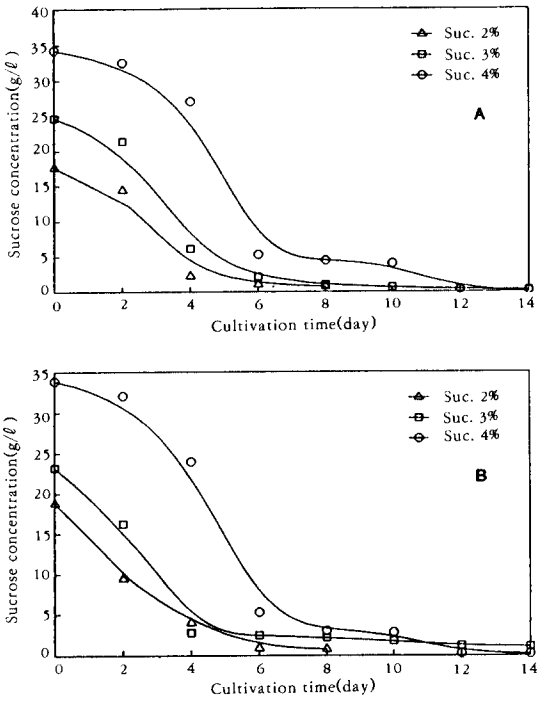

Fig. 4. Sucrose consumption during the batch culture of indigo cells in the medium containing 2, 3, and 4% sucrose in the internal loop bioreactor (A) and external loop bioreactor (B). This result is a same experiment to Fig 3.

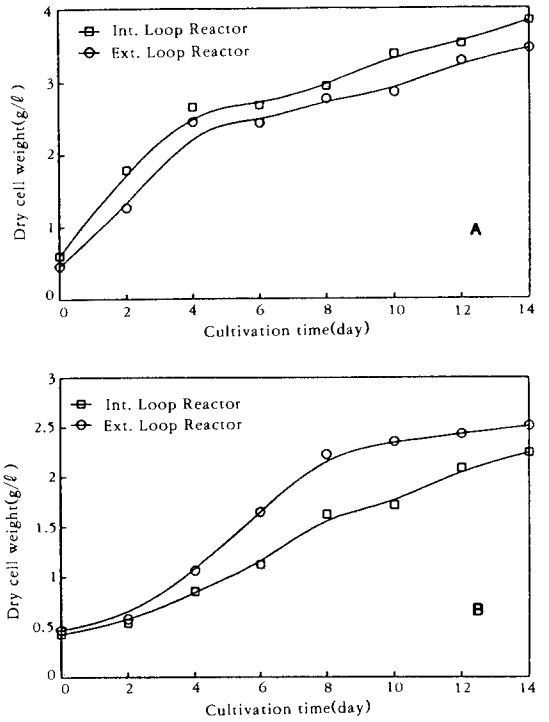

Fig. 5. Growth of indigo cells cultivated in B5 medium containing 1 mM of tryptophan at 27℃ for 14 days in internal and external loop bioreactors (A) and growth curves in B5 medium containing 1 mM of indole (B) in both bioreactors. Two percent of sucrose was added in the medium, and inoculum size was 1% PCV in both type reactors.

erythrorhizon was obtained at 5% of sucrose (6).

Time course changes of sucrose consumption

Fig. 4 shows the sucrose consumption during the cultivation of indigo cell in ALB's with the initial sucrose concentrations of 2, 3, and 4%, respectively. Most of sucrose was hydrolyzed during the first six days of cultivation. Sucrose was consumed in a similar pattern at various initial concentrations of sucrose in internal or external loop bioreactor as illustrated in Fig. 4 A and B. Sucrose was used up at the early stage of exponential phase of growth as compared to Fig. 3 A and B.

Effect of precursor

Fig. 5 A shows the effect of precursor on the cell growth. The cell growth was better at 1mM of tryptophan, where the PCV increased more than 1.5 times compared to that without precursor. DCW was 3.8 g/ℓ in internal loop bioreactor and 3.5 g/ℓ in external loop bioreactor after 14 days of cultivaton. Fig. 5 A (□) shows little difference in growth in internal loop bioreactor compared to control. Fig. 5 A (○) represents 13% increase of growth in external loop bioreactor compared to control.

Ensley et al. (31) reported the construction of a recombinant strain of *Escherichia coli* that excretes indigo, where precursors were tryptophan and indole. To investigate the effect of precursor on indigo cell growth, Maier et al. (18) administered L-[5-^{3}H] tryptophan to young plants of *Isatis tinctoria* and

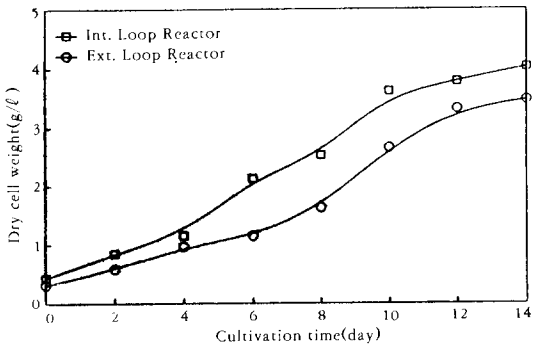

Fig. 6. Growth of indigo cells cultivated in B5 medium containing 2,500mg/ℓ of KNO_3 at 27℃ for 14 days in internal and external loop bioreactors. Two percent of sucrose was added in the medium, and inoculum size was 1% PCV in both type reactors.

Polygonum tinctorium. Labeled indoxyl derivatives could be isolated from stems and roots of both plant species. Mizukami et al. (6) reported that phenylalanine stimulated *Lithospermum* cell growth up to 125% and shikonin formation with 367%. One milimole of phenylalanine was positively effective on cell grwoth as 1 mM of tryptophan on *Polygonum* cell growth in this study.

Indole was employed as an another precursor to produce indican. Effect of indole on indigo cell growth in ALB's is presented in Fig. 5 B. Cells were grown to 2.5 g/ℓ in external and to 2.2 g/ℓ in internal loop bioreactor after 14 days of cultivaton. In external loop bioreactor, indole decreased the cell growth to 80% compared to the control (○). Cell growth was also decreased to 41% with indole treatment in internal loop bioreactor(□). This result implied that reducing the concentration of indole as low as possible was important for growth of the indigo cells. Feeding experiments with labelled precursors were conducted by Zhi et al. (19) using C^{13} NMR and mass spectroscopy. Indole was the biosynthetic precursor for the indoxyl derivatives in plants as the precursor of indigo, and this result was contradictory to that of Maier et al (18).

Effect of inorganic nitrogen source

Fig. 6 shows the effect of media composition containing 2,500 mg/ℓ of potassium nitrate which was selected as the best nitrogen source in suspension culture on indigo cell growth in ALB's. Cell mass after 14-day cultivation was 4.02 g/ℓ and 3.46 g/ℓ in internal and external loop bioreactor, respectively. It was higher than that using the basal medium containing 2,500 mg/ℓ of potassium nitrate and 134 mg/ℓ of ammonium sulfate.

CONCLUSION

This study showed that indigo cells were successfully cultivated in ALB's. Cell mass as dry weight was 16.3 g/ℓ after 14 days of cultivation with 2% sucrose.

Other changes in cultural conditions showed little effect on cell growth. Precursor tryptophan and potassium nitrate were effective on slight increase of cell mass, but precursor indole exhibited a negative effect on cell growth.

Only cultural conditions were examined in this study. Further experiments to find out optimum conditions for indigo production, colorant isolation and recovery, and elicitations for high productivity should be followed.

요 약

공기부양 생물반응기에서 천연 청색 색소원인 쪽 (*Polygonum tinctorium*) 세포를 대량 배양하기 위한 배양조건을 찾기 위해 영양성분 및 색소 전구체를 포함한 배지 성분의 생육에 미치는 영향과 여러 배양조건에서 세포의 생육 특성에 대하여 실험하였다. 최적 생육조건과 생육 특성을 규명하기 위해 external loop와 internal loop 두 가지의 air-lift bioreactor 종류를 사용하여 14일간 배양하였다. 초기 접종량을 달리한 결과 각 reactor에서 공히 접종량이 0.5~2.5% packed cell volume보다 많을수록 생육에 좋은 결과를 나타내었다. 초기 당농도의 영향을 살펴보기 위하여 2~4%로 그 조건을 달리하여 14일간 배양한 결과 4% 초기 당농도가 생육에 가장 좋은 조건임을 알 수 있었다. Sucrose는 대수기 초기단계에서 모두 소비되었다. Tryptophan(1mM)을 첨가한 경우 14일 배양 후에 internal loop reactor에서 3.8g/ℓ, external loop reactor에서 3.5g/ℓ의 cell mass를 얻었

다. Indole을 첨가했을 때에는 세포 생육 저해효과를 나타내었는데 external과 internal loop reactor에서 각각 2.5g/ℓ와 2.2g/ℓ를 얻었다. 무기질소원으로는 potassium nitrate가 선발되었고, control에 비해 110%d의 생육도 증가가 있었다. 최적 조건하에서의 14일간 internal loop bioreactor 배양에서 세포 건조 중량 16.34 g/ℓ를 얻었다.

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