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Review

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Pigment and Saikosaponin Production Through Bioreactor Culture of *Carthamus tinctorius* and *Bupleurum falcatum*

Wenyuan Gao[1], Lei Fan[1], Eun-Joo Hahn[2], Kee-Yoeup Paek[2*]

[1]Hong Kong Institute of Biotechnology LTD., 2 Biotechnology Avenue, 12 Miles, Tai Po Road, Shatin, New Territories, Hong Kong; [2]Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, 361-763, Korea.

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Abstract

Traditional culture technology of medicinal plants mainly depends on the field culture, which has many problems. With progress of modern culture technology, it has become possible to produce valuable secondary metabolites from medicinal plants. In this paper, we discuss about the pigment and saikosaponin production from too medicinal plants, *Carthamus tinctorius* and *Bupleurum falcatum*, through bioreactor culture system. A two-stage bioreactor culture system was established for the production of yellow and red pigments and saikosaponins by cell suspension cultures of *Carthamus tinctorius* and *Bupleurum falcatum*. In *Carthamus tinctorius*, balloon type airlift bioreactors and column type airlift bioreactors were employed for the cell culture and for the pigment production, respectively. The greatest pigment production was obtained on White medium supplemented with 4 mg/L kinetin, high levels of sucrose concentration and photosynthetic photon flux. In *Bupleurum falcatum*, adventitious roots were cultured in balloon type airlift bioreactors and the root growth was greatest on SH medium containing 5 mg/L IBA and 0.2 mg/L kinetin. HPLC analysis showed that the contents of main active saikosaponins a, c, and d in adventitious roots were almost the same as those in field cultured root.

Introduction

Chinese medicine is being used traditionally since thousands of year. On the basis of general survey on Chinese medicine, animals, plants and minerals that are medicinally available has totally amounted to 12,807 kinds: 11,146 kinds of them are medicinal plants, 1,581 kinds are medicinal animals, and the other 80 kinds are medicinal minerals, which means that medicinal plants are the main group of the traditional Chinese medicine (Xiao, 1998).

Medicinal plants have provided a wide variety of natural products with diverse structures and biological activities, many of which have been applied in the health sciences. For years, synthetic chemicals have been afforded the challenge of developing syntheses of such components but often due to structural complexity the resulting multi-step synthesis rarely find application in large scale production as required in the pharmaceutical drug industry (Kutney, 1997). As a result, starting materials for such drug production or indeed the final clinical drug are frequently obtained from tedious and often costly extraction form the living plant. But this solution is often fraught with many problems: a) The active agent is present in minute amounts in the plant extract; b) Varying concentrations of the target compound depending on seasons during which plant collection is performed; c) Desired plant species growing in geographically or politically inaccessible regions; e) Use large field which is limited for cultivation of crop. Appropriate solutions to at least some of the above difficulties are possible by the use of plant cell culture

* Corresponding author, E-mail; paekky@chungbuk.ac.kr
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methodology, particularly when such studies are coupled with chemical methods. The advantages of plant cell, tissue and organ cultures, in terms of secondary metabolite production, are clear: a) growth conditions are laboratory controlled, therefore, reproducible yields of end product are achieved; b) growth parameters such as pH, changes in nutrient media, temperature, etc. can be optimized to achieve metabolite production in yields significantly higher than in the living plant; c) separation of target compounds is much easier due to lower complexity of extract; d) cloning of cell lines provides further optimization for end product yields; plant cell cultures are an excellent source of enzyme availability, which result in more opportunities to perform bio-synthetic and/or biotransfromation experiments related to metabolites production (Kutney, 1998). Interest in applying the methods of plant cell, tissue and organ cultures to controlled production of secondary metabolites on a commercial scale has grown considerably since late 1950s when West and Mika (1957) found that atropine was synthesized by and accumulated in roots of *Atropa belladonna*. Since then, the number of publications on the production of secondary metabolites from plant cultures has increased dramatically, and two commercial processes, the production of shikonin and berberine, have emerged (Yeoman, 1996). Along with the new drug development from plants, such as taxol, taxane, vinblastine, ajmalicine, and artemisinin for anti-cancer and anti-malaria, more and more large scale plant cell cultures have been reported in *Taxus* (Hezari et al., 1997), *Artemisia annua* (Liu et al., 1998), *Cartharanthus tinctorius* (Gao et al., 2000) and *Panax ginseng* (Yu et al., 2000). Up to date, large-scale bioreactor culture system is suggested as an attractive approach to supply plant-derived sources (Son et al., 1999). In this paper, we report pigment and saikosaponin production by bioreactor culture system in too medicinal plants *Carthamus tinctorius* and *Bupleurum falcatum*.

Pigment production in *Carthamus tinctorius* L.

Active compounds of *C. tinctorius* are red and yellow pigments (Akihisa et al., 1994). The functions are to invigorate blood, to release stagnation and to promote menstruation. It is used to stimulate blood flow and relieve pain by removing stagnant blood for the treatment of amenorrhea, chest and abdominal plain, painful swelling due to pain from traumatic wounds (Liu and Xiao, 1993). Moreover, interest in food colorants has been directed to the use of naturally occurring pigments because of restrictions on using synthetic pigments. Yellow and red pigments obtained from the petals of safflower are safely applicable to processed foods and soft drinks as naturally harmless color additives. Use of petals of *C. tinctorius* for pharmaceuticals and food pigments has increased but the yield of petals is not enough for industrial utilization.

Plant cell culture was considered as an alternative method to solve this problem. There are reports of safflower red pigment production by cell culture (Hanagata et al., 1993; Hanagata and Karube, 1994; Wakayama et al., 1994; Wakayama, 1995). On the other hand, there are few reports in the yellow pigment production though it is also important in pharmaceuticals and color additives for food (Danisova and Subinova, 1995; Yu and Xu, 1997). Furthermore, the results were mostly obtained from the cultures grown in 100 to 300 ml Erlenmeyer flasks and only one paper was related to small-size bioreactor cultures (Hanagata et al., 1992). We applied large-scale cell cultures to pigment production in *Carthamus tinctorius* using bioreactors for commercial use.

Establishment of tissue culture

Seeds of *Carthamus tinctorius* were germinated on MS solid media in the dark at 25℃ followed by callus induction from the cotyledons on MS medium supplemented with 1-2 mg/L naphthalene acetic acid (NAA) and 1-2 mg/L kinetin in the dark at 25℃. After three weeks of callus culture, the cultured cells were selected for cell suspension cultures, which were maintained on the same medium containing 30 g/L sucrose. During the cell subculture, the actively dividing cell lines were selected according to their growth rate and maintained under the same conditions described above.

Cell growth and pigment induction

A two-stage culture system was applied to the cell growth and pigment production. For cell growth, the selected cell line was maintained on 1/2 MS medium supplemented with 1 mg/L NAA and 0.1 or 0.2 mg/L kinetin for 20 days in the balloon-type bioreactor. Cultured cells were transferred to the column-type bioreactor containing modified White medium supplemented with 1 or 2 mg/L NAA and 0.1 or 0.2 mg/L kinetin. Pigments were produced after 7 to 10 days of culture and the amount of yellow and red pigment were measured as described by Hanagata et al. (1992) and expressed as the total pigments from cell and medium.

Production of yellow and red pigment

Only a small amount of pigment was produced during the first two days. The amount of pigment increased exponentially during days 2-4, and reached the highest value after 4-7 days. Therefore, the cells can be harvested after culturing in pigment production medium for 7 days. In previous pigment production, 10 times higher concentration of kinetin (2 mg/L) was required as compared to NAA (0.2 mg/L) (Hanagata et al., 1992). On this basis, we increased kinetin concentration in the medium up to 10 mg/L and the results showed that 4 mg/L kinetin was optimal for yellow and red pigment production. Higher concentrations such as 6, 8, 10 mg/L lowered the pigment production.

The pigment accumulation was also related to sucrose concentration and light condition. In our experiment, high concentration of sucrose (8-12 %) increased the pigment production but the concentrations more than 14% did not affect the pigment production. In field culture, pigments of the petals of *C. tinctorius* are synthesized under light. On the other hand, previous experiments with *C. tinctorius* cell cultures were performed under dark conditions (Hanagata et al., 1992, 1993; Wakayama et al., 1994; Wakayama, 1995). We compared the pigment production under light and dark conditions and the result indicated that light condition (70 $\mu mol \cdot m^{-2}s^{-1}$ PPF under 16 h photoperiod) resulted in significantly higher pigment production compared to dark condition. In addition, the yellow pigment content was much higher than the red pigment content and the higher the yellow pigment content, the higher the red pigment content. This result indicated that yellow and red pigments might have originated from the same precursors, and they can be converted to each other under optimal conditions. Onodera et al. (1995) reported that yellow pigment (Safflower yellow B) was transformed to red pigment (carthamin and safflomin-A) in cultured *C. tinctorius* cells. This result suggests that red pigment maybe biogenetically derived from yellow pigment. In addition, Kumazawa et al. (1995) confirmed that carthamin, a red pigment in the flower petals of *C. tinctorius*, maybe formed via an unstable yellow precursor. The model compounds of the precursor were synthesized, and these yellow model compounds could be converted into red carthamin-type compounds.

To increase red and yellow pigments production, further studies are necessary on biosynthesis of red and yellow pigment formation, and key enzymes related with synthesis and bioconversion of yellow and red pigments in cultured *Carthamus tinctorius* cells. Saito (1992) found glucose oxidase from *Aspergillus niger* could change bright-orange florets to reddish-orange when incubated in floret paste even without the addition of glucose. After a series of experiments, it was postulated that glucose oxidase might have participated indirectly in the carthamin formation via the process of generating its by-product, hydrogen peroxide, which could react directly with precarthamin (Saito, 1993). However, there is still no evidence that glucose oxidase and hydrogen peroxide are key enzymes related with the yellow and red pigment formation. Further work is required to investigate the biosynthesis routes of yellow and red pigments in *C. tinctorius*.

Saikosaponin production in *Bupleurum falcatum*

Bupleurum falcatum is a perennial herb and needs at least two years to harvest the roots. Dry roots have been used in traditional Asian medicine over 2000 years and the main active compounds in the roots are saikosaponins that have a number of biological actions (Jung et al., 1998). *Bupleurum falcatum* has been used for treatments of disorders that are accompanied by inflammation, liver stasis, hepatomegaly, splenomegaly, indigestion, adrenal fatigue, and for common cold with chills and fever (Huang, 1993). It has also been used in combination with other herbs for treatment of hepatitis (Izumi et al., 1997; Ohtsu et al., 1997). Along with the development of pharmaceuticals, yield of *Bupleurum falcatum* is not enough for the increasing market demand.

Production of saikosaponins in *Bupleurum falcatum* can be enhanced by application of adventitious root culture. There are papers regarding the production of saikosaponins through adventitious root cultures of *Bupleurum falcatum* (Yamamoto and Kamura, 1997; Ahn et al., 1993). However, it seems necessary to determine optimal culture conditions and other factors to increase biomass and saikosaponin content and especially to enlarge the culture scale for commercial purpose. In this paper, we report results of adventitious root culture and saikosaponins production in *Bupleurum falcatum*.

Establishment of tissue culture

Seeds of *Bupleurum falcatum* were germinated aseptically on MS solid medium without hormones in the dark and the seedlings obtained were grown under light condition (70 $\mu mol \cdot m^{-2}s^{-1}$ PPF under 16 h photoperiod) at 25℃. All media used for induction and growth of callus and adventitious roots were determined after the

experiments. Calli were induced from the leaves and mesocotyls of the seedlings on MS solid medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L kinetin. The calli were subcultured on 1/2 MS medium supplemented containing 4 mg/L NAA and 0.2 mg/L kinetin and transferred to 1/2 MS medium supplemented with 2 mg/L IBA and 0.2 mg/L kinetin for four weeks to induce multiple adventitious roots. Root growth was greater in SH medium rather than in MS medium and two times macronutrients of the normal concentration in SH medium significantly increased the biomass of adventitious roots. In the hormone experiment, NAA leaded to higher biomass increase, while IBA was better for root enlargement. In addition, root growth was greatest under the combination of auxin and cytokinin in the culture medium: 5 mg/L IBA combined with 0.5 mg/L kinetin. Sucrose concentration also affected the root growth: low sucrose concentra tions (10 to 30 g/L) increased fresh weight and root length, while high sucrose concentrations (50 to 70 g/L) increased dry weight and root thickness. Root growth was also enhanced by raising contents of Fe^{2+} and vitamins.

Adventitious root culture and saikosaponin production in bioreactors

The adventitious roots were grown in different types and volumes of culture vessel: 300 mL flasks, 5000 mL balloon-type airlift bioreactors, and 10,000 mL balloon-type airlift bioreactors. The results confirmed that the 10,000 mL balloon-type airlift bioreactor resulted in the greatest growth rate (40 times of the inoculated weight), followed by the 5000 mL balloon-type airlift bioreactor (30 times of the inoculated weight). The growth rate in the 300 mL Erlenmeyer flask was less than 10 times of the inoculated weight.

The airflow in the airlift bioreactor has two functions, one is supplying O_2, and the other is agitating the root cultures, which are very important for root growth. We determined optimal air speed as 100 cc/min/1000 mL medium for fresh and dry weight increase. It was also better for dry weight increase to lower the air speed at the beginning of culture followed by raising it at the end of culture. The multiplication of adventitious roots in bioreactors was greatest under the culture of 3 to 5 g fresh weight per 1000 mL medium. Finally, saikosaponin contents of adventitious roots were compared with those of field-grown roots by HPLC, which resulted in the similarities of the main saikosaponin contents, a, c, and d between the two kinds of roots. This result indicated that the adventitious root culture of *Bupleurum falcatum* was suitable for mass production of saikosaponins.

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