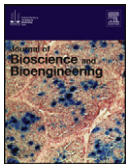

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Large-scale production of saikosaponins through root culturing of *Bupleurum falcatum* L. using modified airlift reactors

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Modification of internal configuration of a bubble column, airlift and stirred tank reactor (10–200 L) was made for root cultures of *Bupleurum falcatum* L. Agitation with an impeller covered with partition mesh was ineffective for a 10-L modified reactor, because it caused intensive foaming and subsequent overflow of the culture medium even at a low rotation speed of 50 rpm and a low aeration rate of 0.1 vvm (volume per volume of medium). In contrast, efficient aeration through a ceramic sparger placed at the bottom of a 20-L bubble column reactor yielded approximately 25 g/L of dry roots and 500 mg/L of saikosaponin-a and saikosaponin-d over 42 days. On a 200-L scale, however, the roots became flocculated under the upper perforated plate initially positioned near the middle of the reactor, forming a firm disk of roots and a large empty space between the disk and the medium. Thus, the roots had poor contact with the medium, which severely suppressed their growth. To avoid this flocculation, a bottom perforated plate and draft tube were installed as a partitioning device separating the culturing area (outside the draft tube) from the aeration area (inside the draft tube). The draft tube was made of a stainless steel mesh rather than a solid material, and the tube greatly increased the root yield in the 20-L reactor. This configuration was successfully applied at the 200-L scale, yielding 500–600 mg/L of saikosaponin-a and saikosaponin-d over 56 days.

[**Key words:** Saikosaponin; *Bupleurum falcatum* L.; Root culture; Large-scale culture; Airlift reactor]

Bupleurum falcatum L. is a perennial herb that has been widely used in a variety of traditional Chinese medical formulae since ancient times for treating fever in the common cold, alternating chills and fever, distension and pain of the chest and costae, malaria, prolapse of the uterus and rectum, and irregular menstruation (1). The root, known as Radix Bupleuri or Chai-Hu, contains several different forms of saikosaponins, which have a common steroid-like structure and are considered to be the pharmacologically active components of the plant (2,3). In particular, saikosaponin-a and saikosaponin-d have been reported to have various physiological effects, including antiallergic, analgesic, antiinflammatory, immunoregulatory, and hepatoprotective activities (4–8). The effects of derivative compounds such as saikosaponin-b1, saikosaponin-b2, and their metabolized products (prosaikogenins and saikogenins) have also been studied (9,10).

The production of secondary metabolites in field-grown plants is greatly affected by climate, soil conditions, and various other factors. In addition, considerable variations in growth, morphological characteristics, and saikosaponin content occur in *B. falcatum* L. due to genetic variations among individual plants (11). Therefore, as for the other economically important plants, *in vitro* production of saikosaponins using cultured cells or roots of *B. falcatum* L. has been studied (12–14). Although it has been reported that saikosaponins could not be found in the callus of *B. falcatum* L., they have been found in adventitious roots differentiated from the callus, suggesting that differentiation of these cells into root tissues is important for the biosynthesis of saikosaponins (15,16). Saikosaponin production using the root culture of *B. falcatum* L. has been improved through the optimization of nitrogen sources and their concentrations (17). Further enhancement of saikosaponin production has also been achieved through a 2-step culture method (18) and by adding methyl jasmonate to the root fragments (19). Untransformed roots have primarily been employed in saikosaponin production studies, with the exception of a recent report using a hairy root culture of *B. falcatum* L. (20). All of these studies, however, have been conducted on a small scale; little information is available on large-scale root culturing of *B. falcatum* L.

In contrast to suspension cultures, in root cultures, the roots are easily entangled to form large clumps and are vulnerable to shear stress caused by agitation or aeration (21). These morphological properties of the roots often create an uneven environment in reactors and present challenges to scaling up cultures. To overcome these problems, various reactor designs, including modified stirred tank (22), turbine-blade (23,24), rotating drum (25,26), submerged

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convective flow (27), and nutrient mist (28,29) reactors, have been proposed for root and organ cultures. Most of these studies have used small-scale bioreactors (<20 L). One exception is *Panax ginseng*, which has been reported to have been cultured in a 500-L balloon-type bubble bioreactor (BTBB) (30). The BTBB, a type of aeration reactor with a spherical shape, has also been used for mass propagation of Siberian ginseng (*Eleutherococcus senticosus*) somatic embryos (31), which have an aggregation behavior similar to that of roots. However, more detailed information is still needed for scaling up root cultures of other plant species.

The objective of this study was to develop a simple large-scale root culturing system for saikosaponin production based on a 2-step culture method reported previously, which had high biomass and saikosaponin yields (25–30 g/L of dried roots containing 2.5% saikosaponin-a and saikosaponin-d) at a flask scale (18). Cylindrical reactors (stirred tank and bubble column reactors) with aspect ratios between 1.0 and 2.2 were used as a base system for simple modifications of the internal structure. The technological aspects of the development of the 200-L culture system are described in detail below.

MATERIALS AND METHODS

Plant materials Seeds of *B. falcatum* L. (kindly provided by Dr. Motoyoshi Satake of Tsukuba Medicinal Plant Research Station, Tsukuba, Japan) were sterilized with 70% alcohol for 30 s and subsequently with 10% sodium hypochlorite solution for 30 min. The seeds were washed with sterilized water and germinated on Linsmaier–Skoog (LS) solid medium (32). The roots of the seedlings were then collected and cultured on LS solid medium containing 0.1 mg/L indolebutyric acid (IBA) at 23 ± 2°C under a 12/12 h light-dark cycle. Adventitious roots formed in the culture were alternately subcultured in Gamborg B5 liquid medium (33) supplemented with 5 mg/L IBA for 21 days and without IBA for 42 days at 23 ± 2°C on a gyratory shaker (105 rpm). Roots cultured for 42 days without IBA were used in the experiments. IBA was sterilized by filtration though Millex-GS filters (Nihon Millipore, Tokyo, Japan) upon use.

Large-scale preparation of root inoculums The subcultured roots with the medium were transferred to a 5-L sterile beaker for disentangling the root clumps. The roots were dispersed into the medium with a rod-shaped impeller consisting of 4 rods (210 × 3 mm) arranged in parallel with a rotating shaft at a distance of 25 mm (2 rods) and 56 mm (2 rods) from the axis. The impeller was driven by a 40-W reversible motor (Oriental Motor, Tokyo, Japan) with periodic inversions of 0.5 s for 1 min. A set of reduction gears (1:12.5) was installed between the motor and the rotating shaft. The motor was regulated by a control unit (SS21M-SSSD; Oriental Motor) at maximum acceleration and brake settings. At this setting, the impeller rotated approximately 180° per 0.5-s interval. This processing device has been previously described in detail (Kusakari, K., Inomata, S., Yokoyama, M., Ichikawa, T., and Kuroiwa, I., Japanese patent application, Publication number 11–009263, 1999).

Culture conditions for saikosaponin production After the disentanglement process, the roots (0.5% fresh weight of medium volume) were transferred into a reactor containing B5 medium supplemented with 4 mg/L IBA and 1% sucrose in a clean room (class 100). The roots were cultured at 23°C with aeration for up to 56 days. Sucrose solution sterilized by autoclaving (121°C for 20 min) was added to the medium at a final concentration of 6% at 14 days, when the lateral roots emerged from the inoculated roots.

Reactors The configurations of the reactors used are summarized in Fig. 1A–F and Table 1 and included a modified stirred tank reactor (10-L ST) (Fig. 1A), bubble column reactors (20-L and 200-L BC) (Fig. 1B, E), a modified airlift reactor (20-L AL) (Fig. 1C), and modified airlift reactors equipped with a mesh draft tube (20-L AM and 200-L AM) (Fig. 1D, F). The components of each reactor are listed in Table 1. To prevent the roots from extending out of the medium, a perforated plate was installed on the top of culture medium in all reactors. An additional perforated plate was installed at the bottom of the bubble column and airlift reactors to reserve the bottom clearance (clearance height: 15 mm for 20-L reactors and 30 mm for 200-L reactors) for the circulation of culture medium. The central areas of the perforated plates in the modified airlift reactors (Fig. 1C, D, F, and G) were excised out in a circle. The impeller in the 10-L ST was covered with a partition mesh (40 mesh) to prevent the roots from being damaged by stirring. Porous stainless steel tubes (10 mm diameter × 20 mm length, nominal pore size, 20 μm) or ceramic tubes (20 mm diameter × 100 or 150 mm length) (Aquarium air stones; Kainuma Sangyo, Nagoya, Japan) were used as spargers. The ratio of draft tube diameter to the reactor diameter (Di/Do) was approximately 0.14. The aeration rate was monitored either with a rotameter (RK 1600R; Kofloc, Tokyo, Japan) or a mass flow meter (SEF-52; STEC, Kyoto, Japan). In experiments using the 200-L AM, pH, dissolved oxygen (DO), and exhaust CO_2 were monitored with a sealed pH sensor (pH Fermprobe; Broadley-James Corp., Irvine, CA, USA), a galvanic cell-type oxygen sensor (SP-5, closed-type; ABLE, Tokyo, Japan), and a nondispersive infrared CO_2 detector (CD-602A; Flow System, Kyoto, Japan), respectively.

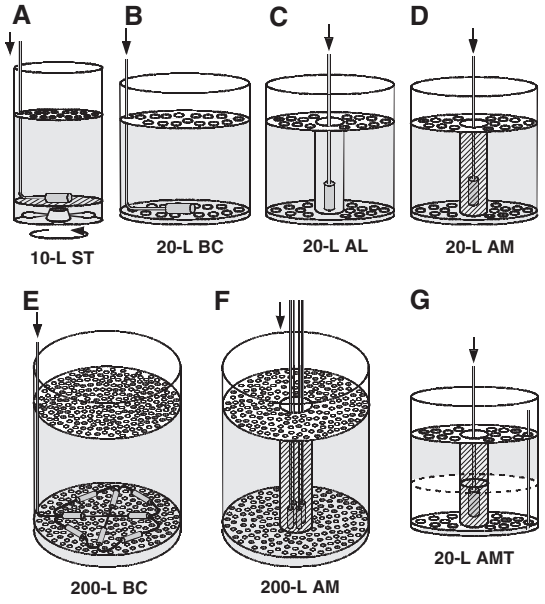

FIG. 1. Schematic illustrations of reactors used in the experiments. (A) 10-L modified stirred tank reactor (10-L ST), (B) 20-L bubble column reactor (20-L BC), (C) 20-L modified airlift reactor (20-L AL), (D) 20-L modified airlift reactor with a mesh draft tube (20-L AM), (E) 200-L bubble column reactor (200-L BC), (F) 200-L modified airlift reactor with a mesh draft tube (200-L AM), and (G) 20-L reactor with a tube manometer used in tracer experiments for evaluating liquid flow (20-L AMT). Arrows indicate the inlets for sterilized air sent to the spargers for aeration. Plates with small holes indicate perforated plates (aperture diameter, 5 mm and pitch, 8 mm) used for partitioning. The impeller in reactor A was covered with stainless steel mesh (40 mesh, 0.25-mm square openings). In the center of the modified airlift reactors (C, D, F, and G), a draft tube was placed to separate the culturing area (outside of the tube) from the aeration area (inside of the tube). Two types of draft tubes (solid stainless steel or stainless steel mesh) were used. Hatched areas indicate stainless steel mesh. Shaded areas indicate the culture medium level. A dotted line in reactor G indicates the top of the sand bed used for a substitute of roots.

Analysis of saikosaponins After being extracted from the dried roots with 90% methanol containing 0.5% KCl at 37°C for 24 h, saikosaponin-a and saikosaponin-d were analyzed using an HPLC system (LC100, Yokogawa Electric, Tokyo, Japan) with a Capcell

TABLE 1. Details of reactors.

Reactor type[a]	Working volume (L)	Diameter × height (mm)	Internal components	Reactor design (Fig. 1)
10-L ST	7	180 × 400	Impeller with a partition mesh (40 mesh), perforated plate (top), sparger	A
20-L BC	14	300 × 300	Perforated plates (top and bottom), sparger	B
20-L AL	14	300 × 300	Draft tube (40 × 180 mm), perforated plates (top and bottom), sparger	C
20-L AM	14	300 × 300	Mesh draft tube (40 × 180 mm, 20 or 40 mesh), perforated plates (top and bottom), sparger	D
200-L BC	140	550 × 900	Perforated plates (top and bottom), 8-piece sparger	E
200-L AM	140	550 × 900	Mesh draft tube (80 × 560 mm, 20 mesh), perforated plates (top and bottom), 4-piece sparger	F

[a] ST, BC, AL, and AM stand for stirred tank reactor, bubble column reactor, modified air-lift reactor, and modified air-lift reactor with a mesh draft tube, respectively.