

Cell suspension culture of *Orostachys cartilaginosa* in bioreactor systems for bioactive compound production and evaluation of their antioxidant properties

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Received: 16 August 2016/Revised: 10 October 2016/Accepted: 5 February 2017/Published online: 8 February 2017
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Abstract For selecting a suitable culture period during bioreactor culture of *Orostachys cartilaginosa* cells, the kinetics of cell biomass and bioactive compound accumulation were investigated. Cell biomass accumulation had a typical lag phase from 0 to 5 days, an exponential phase from 10 to 25 days, and an obvious declining phase from 25 to 30 days. Total polysaccharide and phenolic accumulation was synchronized with cell growth; their maximum contents were observed at 25 days. Among the flavonoids, quercetin (Qc) and kaempferide (Ke) reached maximum levels at 20 days, whereas the epicatechin gallate (Ecg), quercetin 3-*O*-glucoside (Qc-3-glc), and kaempferol-3-*O*-rutinoside (Kp-3-rut) content peaked at 25 days. The suitable culture period of 25 days was confirmed by majority of the observed bioactive compound accumulation. The effect of the air volume was also studied. The cell biomass accumulation was optimal at an air volume of 150 ml min⁻¹. However, the highest content for different bioactive compounds was determined at the varied air volume, i.e., total polysaccharides, total phenolics, and total flavonoids at an air volume of 150 ml min⁻¹, flavonoid monomers of Kp-3-rut at 100 ml min⁻¹, Ke and Ecg at 150 ml min⁻¹, Qc and Qc-3-glc at 200 ml min⁻¹. However, the maximum productivity for all bioactive compounds was at the air volume of 150 ml min⁻¹. The *O. cartilaginosa* cell

cultures had higher antioxidant properties. The cell extract concentration providing 50% of 1,1-diphenyl-2-picrylhydrazyl and alkyl radical-scavenging activity were 0.0487 and 0.11 mg ml⁻¹, respectively. Therefore, the bioreactor-cultured cells of *O. cartilaginosa* can be used as potential material sources for production of desirable bioactive compounds and products of *O. cartilaginosa*.

Keywords Air volume · Culture period · Electron spin resonance · Flavonoid monomer · Kinetic study

Abbreviations

MS	Murashige and Skoog
BA	Benzylaminopurine
NAA	α -Naphthalene acetic acid
FW	Fresh weight
DW	Dry weight
μ	Specific growth rate
T_d	Doubling time
Qc	Quercetin
Ke	Kaempferide
Ecg	Epicatechin gallated
Qc-3-glc	Quercetin 3- <i>O</i> -glucoside
Ke-3-rut	Kaempferol-3-rutinoside
HPLC	High-performance liquid chromatography
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ESR	Electron spin resonance
SE	Standard error

Communicated by H. Peng.

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Introduction

Plants are the major source of secondary metabolites, which are used as pharmaceuticals, flavors, fragrances, coloring agents, food additives, and agrochemicals. Plant

materials can be obtained in two ways, i.e., collection of the wild resources and cultivation in the field. However, wild resource are lacking in terms of the amount of plant species. The cultivable species have various disadvantages, including low yield and unstable metabolite content caused by geographical, seasonal, and environmental variations (Murthy et al. 2014). Therefore, plant cell, tissue, and organ cultures have emerged as alternatives to whole plants for the production of valuable metabolites (Ramachandra and Ravishankar 2002). Bioreactor systems have recently been developed and successfully applied in large-scale cultures of plant cells, tissues, and organs.

During bioreactor culture, mixing, air supply, shear stress, and other such factors are critical parameters of bioprocess engineering. Air supply is especially important for plant cell cultures in air-lift bioreactor systems. The air added into the air-lift bioreactors provides the dissolved oxygen for metabolic activities and agitates the cells (Ahmad et al. 2008). A high aeration volume is generally beneficial for speeding up oxygen transfer into bioreactors, as well as improving cell growth and metabolite accumulation (Zhong et al. 1993). However, at a high air volume, physiological damage can be caused by excessive agitation and shear stress (Meijer and Libbenga 1993). Therefore, the aeration volume should be controlled in bioreactor cultures for the optimization of biomass growth and secondary metabolite production. Acquiring the maximum biomass and metabolites at the shortest time is one of the aims of plant cell culture; the confirmation of a stable culture period shows a crucial event for maximum biomass and metabolite production. The kinetic study of cell growth and metabolite accumulation has been frequently conducted in a bioreactor culture to determine the exact stage at which the maximum biomass and metabolite production occurs (Thanh et al. 2014; Wang et al. 2013). Furthermore, the evaluation of the bioactivity in tissue cultures is essential before marketing the raw material for commercial use in the pharmaceutical and food industries. Therefore, the antioxidant (Ali et al. 2005), anti-inflammatory (Li et al. 2013), and anti-cancer (Jiang et al. 2015) activity of extracts from some plant cultures have been determined. Among studies regarding the determination of bioactivity in plant cultures, the antioxidant activity has been more actively evaluated to date.

Orostachys (DC.) Fisch. belongs to the Crassulaceae family, which has important and valuable medicinal value. *Orostachys* is traditionally used as an anti-inflammatory, anti-febrile, hemostatic, antidote, and anti-cancer agent (Je et al. 2009). Whole plants of *Orostachys* contain polysaccharides, phenolics, flavonoids, steroids, and triterpenoids (Park et al. 1991). Extracts of *Orostachys* plants can efficiently decrease and scavenge radicals, delay aging, and

prevent and treat human diseases (Kim et al. 2004). *Orostachys cartilaginosa* A. Bor. is one of the *Orostachys* species, which mainly distributes in the Changbai Mountain area of China and has high bioactive activity (Li et al. 2015). In China, the large market demand exists for *Orostachys* products; the raw material for their production is entirely derived from wild plants. However, wild resources are scarce, and some species may even be endangered because of excessive collection; meanwhile, an artificial cultivation system for the large-scale production of *Orostachys* plants has not yet been established (Li et al. 2014). Fortunately, plant cell cultures recently can be used as alternative materials for product production; this approach may be cost-effective to meet the market demand (Ramachandra and Ravishankar 2002). To the best of our knowledge, studies on the production of bioactive compounds by cell culture of *Orostachys* species are lacking. Therefore, the establishment of an *Orostachys* cell culture system is likely to address the short supply of material resources.

To obtain a large amount of cell cultures with high bioactive compounds, we used air-lift balloon-type bioreactors to study the kinetics of *O. cartilaginosa* cell growth and bioactive compound (polysaccharides, phenolics, and five flavonoid compounds) accumulation and the effect of air volume on bioactive compound accumulation. Furthermore, the antioxidant properties of the cell culture extracts were evaluated by determining the activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and alkyl radical scavenging with an electron spin resonance (ESR) spectrometer.

Materials and methods

Cell induction and maintenance

The leaves of tissue-cultured plantlets of *O. cartilaginosa* were cut into pieces of approximately 0.5 cm × 0.5 cm and inoculated in Petri dishes ($\Phi \times h = 9 \text{ cm} \times 1.5 \text{ cm}$) containing 20 ml half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 4 mg l⁻¹ benzylaminopurine (BA), 0.5 mg l⁻¹ α -naphthalene acetic acid (NAA), 30 g l⁻¹ sucrose, and 7 g l⁻¹ agar. Medium pH was adjusted to 5.8 before autoclaving. Petri dishes were maintained at 25 ± 2 °C under 30 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity, with a 16 h photoperiod. The induced cells were collected and transferred into a 3 l air-lift balloon-type bioreactors containing 2 l MS medium with 3.5 mg l⁻¹ BA, 0.1 mg l⁻¹ NAA, and 30 g l⁻¹ sucrose (pH 5.8). The bioreactor was maintained at 25 °C under 30 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity, with a 16 h photoperiod, aerated at 100 ml min⁻¹ air.

Kinetics of cell growth and bioactive compound accumulation

Three liter air-lift balloon-type bioreactors were used in the experiments of the present study. Each bioreactor was supplied 2 l half-strength MS medium containing 3.5 mg l⁻¹ BA, 0.1 mg l⁻¹ NAA, and 30 g l⁻¹ sucrose (pH 5.8) and inoculated 20-day-old bioreactor-cultured cells of 15 g l⁻¹ FW. Bioreactors were aerated with 100 ml min⁻¹ air and maintained at 25 °C under 30 μmol m⁻² s⁻¹ light intensity, with a 16 h photoperiod. Cell samples were obtained from the bioreactors at 5 days intervals over a 30 days culture period. Cell biomass (fresh weight, FW; and dry weight, DW), specific growth rate (μ), and doubling time (T_d) were recorded. Contents of total polysaccharides and phenolics, and five flavonoid compounds [quercetin (Qc), kaempferide (Ke), epicatechin gallate (Ecg), quercetin 3-*O*-glucoside (Qc-3-glc), and kaempferol-3-*O*-rutinoside (Kp-3-rut)] were also determined.

Effect of air volume on cell growth and bioactive compound accumulation

A 3 l bioreactor was added 2 l half-strength MS medium supplemented with 3.5 mg l⁻¹ BA, 0.1 mg l⁻¹ NAA, and 30 g l⁻¹ sucrose. Medium pH was adjusted to 5.8. Each bioreactor was inoculated with 15 g l⁻¹ FW of 20-day-old bioreactor-cultured cells and aerated at various air volumes of 50, 100, 150, and 200 ml min⁻¹. Bioreactors were maintained at 25 °C under 30 μmol m⁻² s⁻¹ light intensity with 16 h photoperiod. After 25 days, the cell biomass and bioactive compound content were determined.

Effect of the cell extract on antioxidant property

The dry sample (15 g) of 25-day-old bioreactor-cultured cells was ground, soaked in 80% (v/v) methanol (200 ml) for 1 h, and filtered. Three replicates were obtained for each sample under the same conditions with a new solvent. The methanol extracts were filtered and evaporated under reduced pressure with a vacuum rotary evaporator (R 206, Shanghai Shensheng Science Technology Co., Ltd., China). The extracts were stored in the dark at 4 °C and further used for determining antioxidant activity.

Determination of cell biomass, specific growth rate (μ), and doubling time (T_d)

Cells were separated from the culture medium, passed through a stainless steel sieve, and washed twice or thrice with tap water to remove the medium. FW was measured after blotting away the surface water. The collected fresh cells were dried at 50 °C in a drying oven (GZX-

9070MBE, Boxun, Shanghai, China) for 48 h to a constant the weight before the DW was recorded.

The μ value of the cells was defined as

$$\mu = 1/X \cdot dX/dt$$

where X is the cell FW (g l⁻¹), t is the time (day), and μ is the specific growth rate (per day). The doubling time (T_d) of the cells was defined as $T_d = \ln 2/\mu = 0.693/\mu$.

Determination of total polysaccharide content

Total polysaccharides were extracted according to Li et al. (1990). In brief, dried cells were powdered and 0.1 g of the powder was soaked in 90% (v/v) ethanol for 6 h at 25 °C. The precipitate was collected and mixed with 90% (v/v) ethanol to discard the interfering compounds (monosaccharides, oligosaccharides, and glycosides). Subsequently, 20 ml of distilled water was added to the extract. After 30 min of ultrasonic treatment, the extract was filtered, and the filtrate was collected. The remaining residue was soaked in water and ultrasonically treated thrice at 125 W for 30 min. All filtrates from a single sample were pooled. The polysaccharides were quantitatively determined by spectrophotometry (UV-2600, Shimadzu Corporation, Kyoto, Japan) at an absorbance of 490 nm according to the phenol–sulfuric acid assay (Dubois et al. 1956), with glucose (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China) as the reference standard.

Determination of total phenolic content

Powdered cells (0.1 g) were incubated at 80 °C for 2 h with 10 ml of 80% (v/v) methanol in a thermostatic water bath (HH-S, Jintan Hengfeng Instrument Manufacturing Co., Ltd., Jintan, China). After filtration and centrifugation (IMac CR22G, Hitachi Instruments Co., Ltd., Shanghai, China) at 5000 rpm for 10 min, the supernatant was collected and adjusted to 25 ml with 80% (v/v) methanol. The total phenolic content in the extract was determined by the Folin–Ciocalteu method (Singleton et al. 1999). Subsequently, 2 ml of each extract was added to test tubes containing 0.2 ml of 10% (v/v) Folin–Ciocalteu reagent and 1 ml of 2% (w/v) sodium carbonate. The tubes were thoroughly shaken and allowed to stand in the dark for 1 h. The total phenolic content was spectrophotometrically measured (UV-2600, Shimadzu Corporation) at an absorbance of 760 nm. Gallic acid (Tianjin Kemiou Chemical Reagent Co., Ltd.) was used as standard to obtain a calibration curve.

Determination of flavonoid compounds

Flavonoids were extracted and determined according to the method described by Lee et al. (2011) with slight

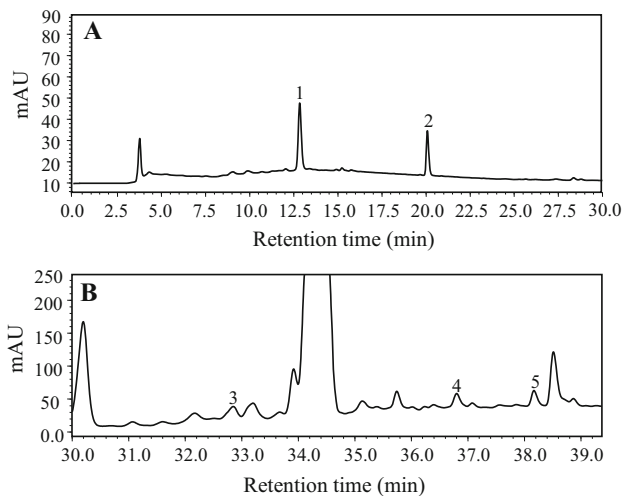


Fig. 1 HPLC chromatograms of extracts in *O. cartilaginosa* cells under UV wavelength 366 nm (a) and 280 nm (b). 1, Quercetin (Qc) 2, Kaempferide (Ke) 3, Epicatechin gallated (Ecg) 4, Quercetin 3-*O*-glucoside (Qc-3-glc) 5, Kaempferol-3-rutinoside (Kp-3-rut)

modification. In brief, the cell powder (1 g) was soaked in 80% (v/v) methanol (containing 5% hydrochloric acid) at a reflux temperature of 80 °C for 1 h and filtered. The filtrate was evaporated under reduced pressure with a vacuum rotary evaporator (R 206, Shanghai Shensheng Science Technology Co., Ltd.). The concentrated solution was washed thrice with ethyl acetate. The supernatant was collected and concentrated under reduced pressure. The extract was dissolved in chromatographic methanol and filtered with a 0.45 µm filter membrane to measure the flavonoid compound content (Qc, Ke, Ecg, Qc-3-glc, and Kp-3-rut) by high-performance liquid chromatography (HPLC) (Fig. 1). HPLC analysis used an ODS-C₁₈ reverse phase column (4.6 mm × 250 mm, 5 µm; Thermo Scientific, Waltham, MA, USA). The Qc and Ke contents were determined with a UV detector (SPD-15C, Shimadzu Co. Kyoto, Japan) at 366 nm (Fig. 1a). The mobile phase was 0.1% (v/v) phosphoric acid (a) and methanol (b). The gradient elution profile was: 0–15 min, 45% B; 16–30 min, 80% B. The flow rate of the mobile phase was 0.8 ml min⁻¹. The Ecg, Qc-3-glc, and Kp-3-rut contents were determined with a UV detector (SPD-15C, Shimadzu Co.) at 280 nm (Fig. 1b). The mobile phase was 0.05 M ammonium formate (a) and methanol (b), with a linear gradient of 10–90% B over 40 min. The flow rate was 0.8 ml min⁻¹.

Determination of antioxidant activity

The antioxidant activity was evaluated by determining the activities of DPPH and alkyl radical scavenging in the present study. DPPH radical scavenging activity was measured with the method described by Nanjo et al.

(1996). The AR extract of 30 µl was added to 30 µl of DPPH (60 µM) in a methanol solution. After mixing vigorously for 10 s, the solution was transferred into a 100 µl Teflon capillary tube. The scavenging activity of the extract on DPPH radical was measured with a JES-FA ESR spectrometer (Jeol Ltd., Tokyo, Japan). A spin adduct was measured on an ESR spectrometer after exactly 2 min. Alkyl radicals were generated by 2,2-azo-bis (2-amidino-propane) hydrochloride (AAPH). The phosphate-buffered saline (pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM of (4-pyridyl-1-oxide)-*N*-*tert*-butylnitron, and specific concentrations of the extract samples were incubated at 37 °C in a water bath for 30 min and transferred to a 100 µl Teflon capillary tube. The spin adduct was recorded on an ESR spectrometer (Jeol Ltd.). Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; gain, 6.3×10^5 ; temperature, 25 °C; microwave power, 5 mW for DPPH radicals (Li et al. 2013) and 1 mW for alkyl radicals (Lee et al. 2011). The extract concentration providing 50% of radical scavenging activity (IC₅₀) was obtained by interpolation from linear regression analysis.

Experimental design and data analysis

All data were expressed as the mean ± standard error (SE) of triple experiments. The mean values were subjected to Duncan's multiple range test with the SAS system (Version 6.12, SAS Institute Inc., Cary, NC, USA). Values with $P < 0.05$ were considered significant.

Results and discussion

Kinetics of cell growth and bioactive compound accumulation

To confirm the suitable culture period for the efficient production of bioactive compounds, a kinetic study of cell growth and bioactive compound accumulation during bioreactor culture was investigated. A similar change in the cell FW and DW was observed. The pattern of cell biomass accumulation in *O. cartilaginosa* exhibited a typical lag phase from 0 to 5 days, an exponential phase from 10 to 25 days, and an obvious declining phase from 25 to 30 days (Fig. 2). The maximum cell biomass was found at 25 days, where the FW and DW were 8.8- and 7.5-fold, respectively, greater than that of the initial culture level. Figure 3 shows that the cells gradually became a deeper green color with increasing culture days, but lost their green color after 25 days of culture. The decreased cell biomass in the later period of culture can be attributed to the increased nutrient consumption in the medium during cell culture, as well as

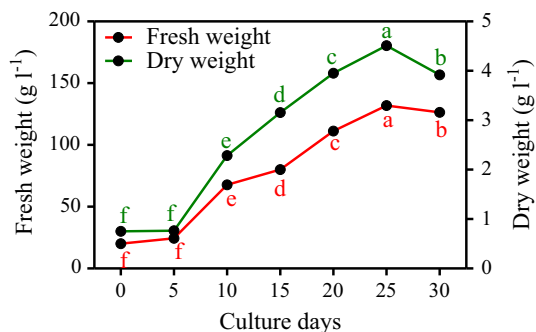


Fig. 2 Changes of cell biomass with the elongation of culture days during bioreactor culture of *O. cartilaginosa*. The different letters within the same curve mean significantly different by Duncan's multiple range test at $P < 0.05$

the disruption and death of the cultured cells (Zhong and Zhu 1995). The specific growth rate (μ) of cells changed during the culture, with the peak rate of 0.355 and the doubling time (T_d) of 0.227 d at 10 days; after which, μ rapidly declined (Table 1). A similar pattern was found during cell culture of various plant species. For *Rhodiola sachalinensis*, the FW and DW of calluses cultured in 3 l air-lift balloon-type bioreactors had gradually increased and peaked at 25 days, followed by a decrease of both parameters (Li et al. 2016). In *Taxus media*, cell growth exhibited an initial lag phase from 1 to 4 days, an exponential growth phase between 4 and 17 days, and a deceleration phase from 27 to 32 days during cell suspension culture in a stirred 15 l bioreactor (Zhang et al. 2013). By contrast, cell growth in *Glycyrrhiza inflata* showed a stationary phase from 24 to 27 days without a declining phase in a stir-tank bioreactor (Wang et al. 2010a). These results suggest that the cell growth pattern relates to bioreactor size or types and is typically species-dependent.

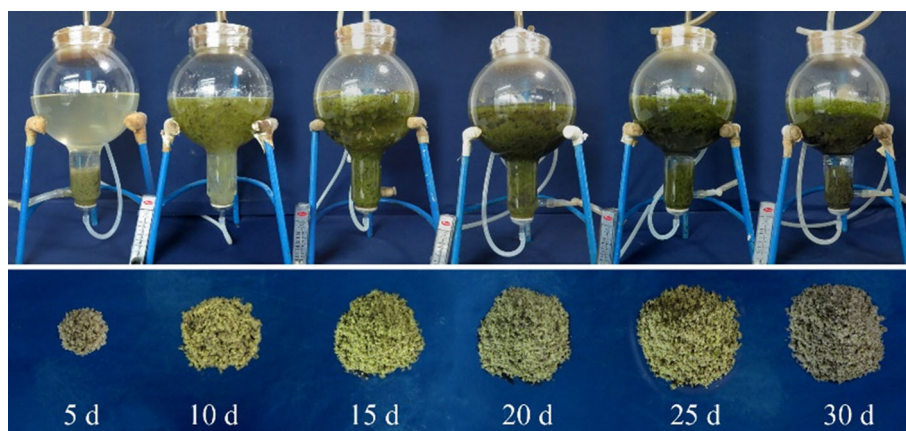
The contents of total polysaccharides and phenolics decreased from 0 to 5 days before it gradually increased with increasing culture days within 20 or 25 days before

Table 1 Specific growth rate (μ) and doubling time (T_d) of *O. cartilaginosa* cells grown in bioreactors

Days	5	10	15	20	25
μ	0.126	0.355	0.036	0.078	0.037
$T_d = \ln 2 / \mu_{\max}$ (day)	0.227				

decreasing in the succeeding days (Fig. 4). The decrease in bioactive compound accumulation during the first 5 days was probably caused by the greater catabolism than anabolism of inoculated cells in the new culture medium. Catabolism provides the carbon skeleton and energy needed to promote cell growth as reflected in the biomass increase. After 5 days of culture, anabolism was enhanced, thereby dramatically increasing the content of the bioactive compounds. The number of days when the maximum content was observed had slightly differed among the bioactive compounds. The maximum polysaccharide content was determined at 20 days, which was earlier than that of the biomass. The primary and secondary metabolic events in plant cell cultures are governed by the active biomass (Schlatmann et al. 1995). The polysaccharide content reached a maximum point and subsequently decreased before cell growth entered the progressive deceleration phase. This result can be attributed to the decreasing carbon source concentration in the medium; in this case, the degradation rate of the polysaccharide was higher than its synthetic rate, thereby affecting the polysaccharide accumulation; the degraded polysaccharide was used as a carbon source by the cells (Wang et al. 2010b). The maximum phenolic content was determined at 25 days. The total content of flavonoid compounds also reached the maximum level at 25 days. However, the observed peak days differed among the flavonoid monomers. The maximum contents of Qc and Ke were determined at 20 days, whereas those of Ecg, Qc-3-glc, and Kp-3-rut were measured at 25 days (Fig. 5). During plant cell culture for bioactive compound production, the most

Fig. 3 Cell biomass at different culture days during bioreactor culture



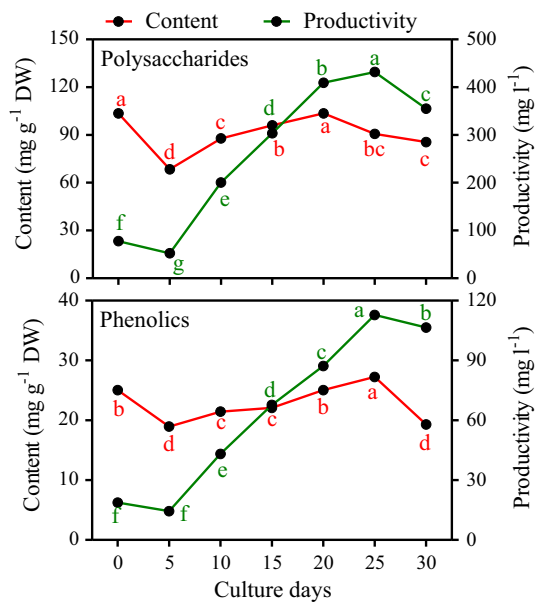
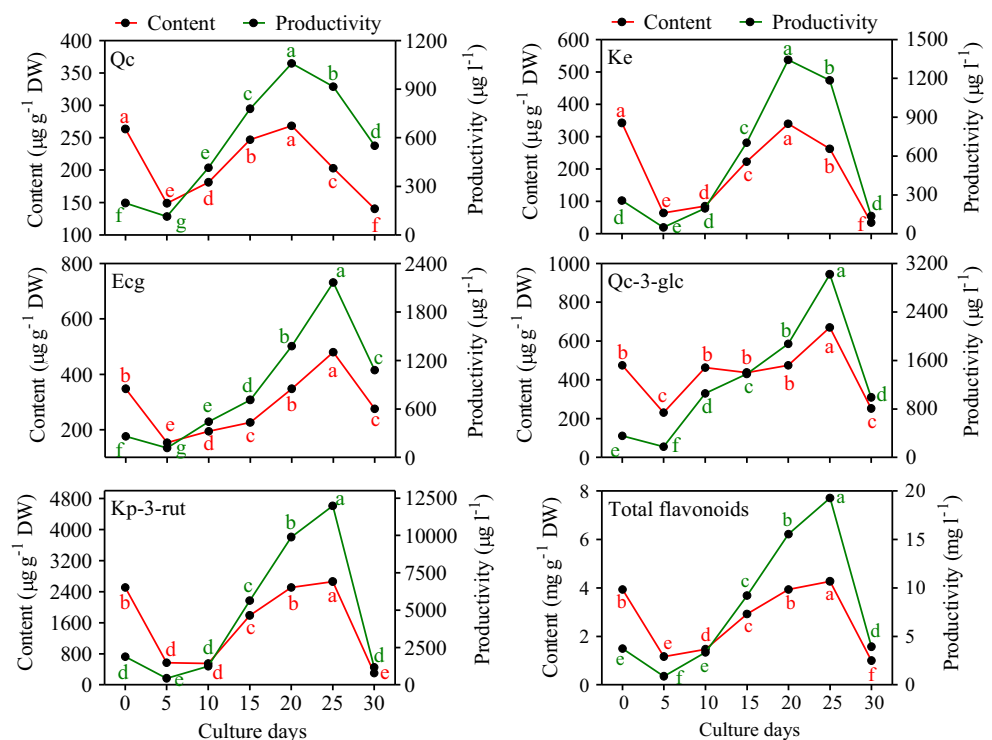


Fig. 4 Changes of polysaccharide and phenolic accumulation in *O. cartilaginous* cells with the elongation of culture days during bioreactor culture. The different letters within the same curve mean significantly different by Duncan's multiple range test at $P < 0.05$. Productivity (mg l^{-1}) = content (mg g^{-1} DW) \times dry weight (g l^{-1})

important index is the productivity of the bioactive compounds. The productivity is the product of the DW and compound content. The adjustment of a moderate culture condition is based on the productivity level. The maximum productivity of polysaccharides (432.0 mg l^{-1}), phenolics

Fig. 5 Changes of flavonoid accumulation in *O. cartilaginous* cells with the elongation of culture days during bioreactor culture. The different letters within the same curve mean significantly different by Duncan's multiple range test at $P < 0.05$. Total flavonoids = Qc + Ke + Ecg + Qc-3-glc + Kp-3-rut. Productivity (mg l^{-1}) = content (mg g^{-1} DW) \times dry weight (g l^{-1})



(112.8 mg l^{-1}), and total flavonoid compounds (19.3 mg l^{-1}) were measured at 25 days. However, the productivity among flavonoid monomers differed, with Qc and Ke reaching the maximum levels at 20 days. The maximum productivity for Ecg, Qc-3-glc, and Kp-3-rut were observed at 25 days.

The kinetic relationship between the rates of metabolite and biomass production can be divided into three patterns: coupling, partly coupling, and uncoupled. However, the kinetics of metabolite production is difficult to explain by uniform modeling because of the complicated pathways of intracellular biosynthesis (Qi and Wang 2007). The present result can serve as evidence for this hypothesis, wherein the maximum contents of different bioactive compounds were synchronous or ahead of the days when the maximum biomass occurred during the cell bioreactor culture of *O. cartilaginous*. The kinetic study is important to understand the regularity of bioactive compound biosynthesis; the study can also confirm the suitable culture period for the culture used. From the results of the present work, we selected 25 days as the suitable culture period for *O. cartilaginous* cell culture in bioreactors.

Effect of air volume on cell biomass and bioactive compound accumulation

Air volume ($50, 100, 150,$ and 200 ml min^{-1}) affected cell growth of *O. cartilaginous* (Fig. 6). Cell FW and DW increased with increasing air volume from 50 to

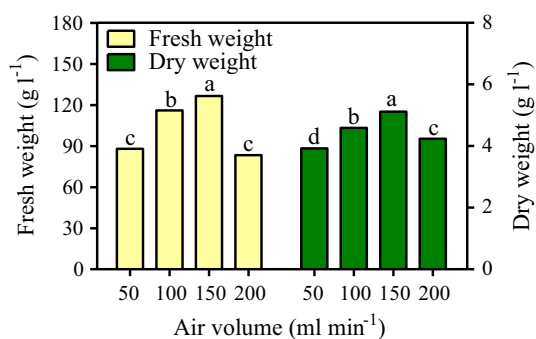


Fig. 6 Effect of air volume on cell biomass of *O. cartilaginosa* after 25 days of bioreactor culture. The different letters within the same column mean significantly different by Duncan's multiple range test at $P < 0.05$

150 ml min⁻¹ and peaked at an air volume of 150 ml min⁻¹. The cell biomass decreased when the air volume was greater than 150 ml min⁻¹. The effect of air volume on polysaccharide and phenolic accumulation was similar to its effect on biomass. The maximum levels were determined at 150 ml min⁻¹. The polysaccharide and phenolic content reached 123.9 DW and 25.2 mg g⁻¹ DW, respectively. The maximum productivity of polysaccharides and phenolics was also discovered at an air volume of 150 ml min⁻¹; these levels were 1.85- and 2.41-fold greater than that of the air volume measuring 50 ml min⁻¹, respectively (Table 2). In addition, flavonoids are the main bioactive compounds of *Orostachys* species (Lee et al. 2011). Air volume also influenced the accumulation of flavonoid compounds in *O. cartilaginosa* cells though the degree of the effect was different among the varied flavonoid compounds (Table 3). Qc and Oc-3-glc contents increased with increasing air volume and peaked at an air volume of 200 ml min⁻¹. However, the Ke, Ecg, and Kp-3-rut content increased with the increasing air volume ranged from 50 to 100 or 150 ml min⁻¹, followed by a drop beyond the range. The maximum content of Ke and Ecg were determined at 150 ml min⁻¹ and that of Kp-3-rut at 100 ml min⁻¹. The content of total flavonoid

compounds reached the maximum level (3.9 mg g⁻¹ DW) at 150 ml min⁻¹. The productivity considering the DW and content indicated that the maximum level was determined at 150 or 200 ml min⁻¹ for Qc and at 150 ml min⁻¹ for Ke, Ecg, Qc-3-glc, and Kp-3-rut. The maximum productivity (25.2 mg l⁻¹) of total flavonoid compounds was measured at 150 ml min⁻¹. These results confirmed that increased aeration improved cell growth and metabolite accumulation by oxygen transfer (Zhong et al. 1993). However, the high air volume can cause physiological damage on the cell because of excessive agitation and shear stress (Meijer and Libbenga 1993). The air volume requirement of cell cultures differs depending on the plant species and target compounds during bioreactor culture (Ahmad et al. 2008). Wang et al. (2013) supplied different volumes (20, 40, 60, 80, and 100 ml min⁻¹) to the culture medium; their group found the highest content of triterpenoid saponins and flavonoids in cell cultures of *Glycyrrhiza uralensis* at an aeration volume of 60 ml min⁻¹. Thanh et al. (2014) investigated the effects of aeration volume within 50, 100, 200, and 300 ml min⁻¹ on ginseng cell biomass and ginsenoside accumulation. Their group indicated that 100 ml min⁻¹ was the suitable air volume. In the present study, the accumulation of cell biomass and majority of the bioactive compounds exhibited the highest increase at an air volume of 150 ml min⁻¹, thus, the air for *O. cartilaginosa* cell culture should be applied with 150 ml min⁻¹ volume during the air-lift bioreactor culture to obtain the mass cells containing high contents of bioactive compounds.

Active antioxidant property

The antioxidant properties of *O. cartilaginosa* in cell cultures were evaluated by determining the DPPH and alkyl radical scavenging activities by ESR in the present study. The ESR signals of DPPH (Fig. 7a) and alkyl (Fig. 7b) radicals decreased with the dose increments of the extracts. DPPH radical-scavenging activities exceeded

Table 2 Effect of air volume on accumulation of polysaccharides and phenolics of *O. cartilaginosa* cells after 25 days of bioreactor culture

Air volume (ml min ⁻¹)	Polysaccharides		Phenolics	
	Content (mg g ⁻¹ DW)	Productivity (mg l ⁻¹)	Content (mg g ⁻¹ DW)	Productivity (mg l ⁻¹)
50	67.0 ± 1.7c	262.8 ± 4.2c	13.6 ± 0.2c	53.5 ± 0.9c
100	87.8 ± 1.3b	402.8 ± 5.9b	20.7 ± 0.7b	95.2 ± 2.2b
150	123.9 ± 2.9a	634.2 ± 3.3a	25.2 ± 1.3a	129.0 ± 1.7a
200	64.7 ± 3.1c	273.8 ± 3.6c	8.5 ± 0.3d	35.8 ± 2.4d

Data represent the mean ± standard error of three replicates

The different letters within the same column mean significantly different by Duncan's multiple range test at $P < 0.05$

Productivity (mg l⁻¹) = content (mg g⁻¹ DW) × dry weight (g l⁻¹)

Table 3 Effect of air volume on flavonoid accumulation of *O. cartilaginosa* cells after 25 days of bioreactor culture

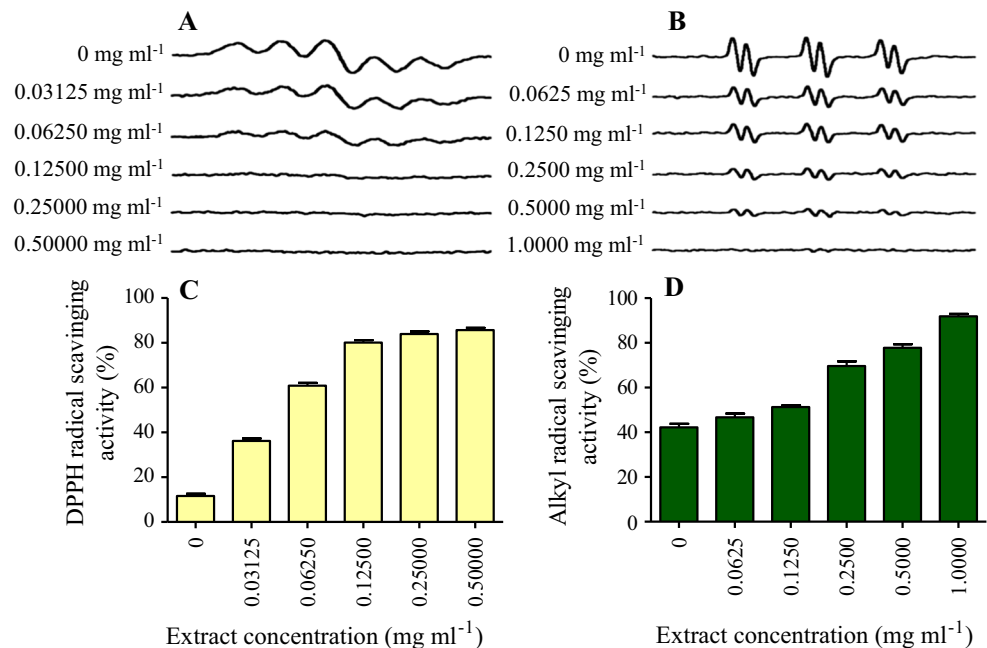
Air volume (ml min ⁻¹)	Qc Content (μg g ⁻¹ DW)	Ke Content (μg g ⁻¹ DW)	Ecg Content (μg g ⁻¹ DW)	Qc-3-glc Content (μg g ⁻¹ DW)	Kp-3-rut Content (μg g ⁻¹ DW)	Total content (mg g ⁻¹ DW)
50	72.8 ± 1.2d	120.2 ± 3.1d	375.0 ± 5.6d	368.3 ± 6.1d	2082.4 ± 13.5c	3.0 ± 0.03d
100	164.7 ± 1.8c	140.2 ± 3.5c	441.8 ± 6.1b	511.1 ± 8.3c	2399.4 ± 15.3a	3.7 ± 0.10b
150	204.3 ± 5.6b	252.1 ± 2.1a	553.7 ± 2.5a	608.8 ± 8.1b	2229.4 ± 16.5b	3.9 ± 0.03a
200	250.6 ± 4.2a	240.8 ± 2.6b	404.2 ± 5.4c	655.4 ± 9.3a	2018.7 ± 12.8c	3.5 ± 0.08c
Air volume (ml min ⁻¹)	Productivity (μg l ⁻¹)	Productivity (μg l ⁻¹)	Productivity (μg l ⁻¹)	Productivity (μg l ⁻¹)	Productivity (μg l ⁻¹)	Productivity (mg l ⁻¹)
50	285.5 ± 4.3c	471.4 ± 11.7d	1470.0 ± 21.8d	1443.8 ± 47.5d	8163.0 ± 53.1b	12.9 ± 0.09d
100	755.9 ± 8.3b	648.2 ± 24.1c	2027.8 ± 28.0b	2345.9 ± 33.4c	11,013.0 ± 70.3a	21.5 ± 0.30b
150	1046.0 ± 21.2a	1290.7 ± 13.5a	2834.7 ± 12.6a	3117.1 ± 93.9a	11,414.7 ± 33.0a	25.2 ± 1.04a
200	1058.8 ± 23.8a	1019.8 ± 16.8b	1711.6 ± 23.0c	2775.6 ± 68.0b	8549.2 ± 38.9b	15.0 ± 2.95c

Data represent the mean ± standard error of three replicates

The different letters within the same column mean significantly different by Duncan's multiple range test at $P < 0.05$

Total flavonoids = Qc + Ke + Ecg + Qc-3-glc + Kp-3-rut. Productivity (mg l⁻¹) = content (mg g⁻¹ DW) × dry weight (g l⁻¹)

Fig. 7 Free radical scavenging activity of the extracts in *O. cartilaginosa* cells. DPPH radical (a), DPPH radical spectra (b), alkyl radical (c), alkyl radical spectra (d). Data represent the mean ± standard error of three replicates



80% at extract concentrations greater than 0.125 mg ml⁻¹ (Fig. 7c); the IC₅₀ value was 0.0487 mg ml⁻¹. The alkyl radical scavenging activity reached 91.9% at 1 mg ml⁻¹ of the cell extract (Fig. 7d); the IC₅₀ value was 0.11 mg ml⁻¹. Free radicals influence the occurrence of numerous diseases and aging; these radicals also damage DNA, lipids, and proteins; therefore, the use of free radical scavengers may be important in the early prevention of these diseases (Lee et al. 2012). The antioxidant activity of *Orostachys* in field-grown plants has been reported by several researchers. Jin et al. (2016) extracted

Orostachys japonicas powder with four solvents and discovered that the higher antioxidant activity was determined with the ethanol extract. Lee et al. (2014) indicated that the dichloromethane and hexane fractions of *O. japonicas* displayed a dose-dependent DPPH radical scavenging activity; the IC₅₀ value was 32.05 μg ml⁻¹ for the dichloromethane fraction and 205.76 μg ml⁻¹ for the hexane fraction. Li et al. (2015) evaluated the antioxidant activity of *O. cartilaginosa* plants by determining the activity of DPPH, hydroxyl, and superoxide anion radicals. The radical scavenging activities were found to

reach 99.15% for DPPH, 94.55% for the hydroxyl radicals, and 61.33% for the superoxide anion. The lower metabolite content and bioactive activity are critical factors that affect the industrial application of cultured plants. The differences in cultured plant cells, tissues, and organs as compared with field-grown plants have been a concern among researchers. In the present study, the extract of *O. cartilaginosa* cell cultures exhibited higher antioxidant activity, which provides an important basic reference for the future use of the plant as an alternative material. In addition, most studies have used the colorimetric method to determine the antioxidant activities. The ESR spin trapping technique used in the present study for determining the activities of DPPH and alkyl radical scavenging is based on the measurement of transitions of unpaired electrons in a magnetic field, and the use of an ESR spectrometer with a proper radical trapper is a sensitive method for detecting free radicals (Peng et al. 2009). The ESR spin trapping technique was used for the first time in the present study for determining the antioxidant activity of *Orostachys* species, which is significant for accurately evaluating the antioxidant properties of the plant.

Conclusions

The maximum biomass, content and productivity of total polysaccharides, phenolics, and flavonoids, including the flavonoid monomers of Ecg, Qc-3-glc, and Kp-3-rut in *O. cartilaginosa* cells, were measured at 25 days of bioreactor culture. The flavonoid monomers of Qc and Ke reached maximum levels at 20 days. The recommended cell culture period was 25 days according to majority of the bioactive compound accumulations observed. The highest cell biomass and productivity of total polysaccharides, total phenolics, total flavonoids and five flavonoid monomers (Qc, Ke, Ecg, Qc-3-glc, and Kp-3-rut) were observed at an air volume of 150 ml min⁻¹. The extract of the *O. cartilaginosa* cell cultures showed higher antioxidant properties; the IC₅₀ levels of DPPH and alkyl radical scavenging were 0.0487 and 0.11 mg ml⁻¹, respectively. Therefore, cell cultures can be used as alternative materials for the commercial production of *O. cartilaginosa* products.

Author contribution statement All authors contributed extensively to the work presented in this paper (Cell suspension culture of *Orostachys cartilaginosa* in bioreactor systems for bioactive compound production and evaluation of their antioxidant properties). XCP and MLL designed the research, WBZ, JJ conducted bioreactor experiments, YHJ contributed plant materials, PJP, SEK conducted antioxidant experiment, and MLL wrote the paper.

Acknowledgements This research was supported by the National Natural Science Foundation of China (31660080 and 31260182).

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