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RESEARCH

Isolation and Characterization of Antioxidation Enzymes from Cells of Zedoary (*Curcuma zedoaria* Roscoe) Cultured in a 5-l Bioreactor

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Abstract In this study, a cell suspension culture system for zedoary (*Curcuma zedoaria* Roscoe) was developed, using 50 g/l of fresh weight inoculum in a batch culture. The highest cell biomass obtained from a 5-l bioreactor equipped with three impellers after 14 days of culture was utilized to extract secondary metabolites (essential oil and curcumin) and determine the activities of antioxidant enzymes (peroxidase, superoxide dismutase, and catalase). For essential oil and curcumin, zedoary extracts were recovered via a variety of methods: steam distillation, volatile solvents, and Soxhlet. After 14 days of culture using volatile solvents, the optimal yield of essential oil (1.78%) was obtained when using petroleum ether at 40°C in 6 h of extraction, and the best curcumin yield (9.69%) was obtained at 60°C in 6 h via extraction with 90% ethanol. The activities of antioxidant enzymes from zedoary cells were also assessed. The specific activities of peroxidase, superoxide-dismutase, and catalase reached maximum values of 0.63 U/mg of protein, 16.60 U/mg of protein, and 19.59 U/mg of protein after 14 days of culture, respectively.

Keywords Antioxidant enzymes · Catalase · Cell suspension · *Curcuma zedoaria* · Curcumin · Essential oil · Peroxidase · Superoxide-dismutase

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Abbreviations

BAP	Benzylamino purine
CAT	Catalase
2,4-D	2,4-Dichlorophenoxyacetic acid
EDTA	Ethylenediaminotetraacetic acid
MS	Murashige and Skoog
POD	Peroxidase
SOD	Superoxide dimutase

Introduction

Zedoary (*Curcuma zedoaria* Roscoe), a member of the Zingiberaceae family, is a species, which grows wild in the eastern Himalayas and is cultivated in India, Sri Lanka, China, Japan, Thailand, and Vietnam. The essential oil, curcumin, and terpenoids, the primary secondary metabolites of zedoary, have been determined to be principally responsible for the pharmacological activities associated with the plant. Zedoary essential oil evidences antimicrobial and antimutagenic activities. The constituents of zedoary rhizome oil have been investigated extensively, and zedoary has been recognized as a rich source of terpenoids [1, 2]. Curcumin is renowned for its antitumor, antioxidant, antiamyloid, and antiinflammatory properties. Curcumin functions as a free radical scavenger and antioxidant, and inhibits lipid peroxidation and oxidative DNA damage [3, 4].

Plant cell cultures are currently being considered as a method for the production of a number of plant-derived pharmaceutical compounds. Free cell suspension is generally regarded to be the most suitable for large-scale applications in the biotechnology industry. Most applications of plant-cell-suspension cultures in biotechnology are

targeted toward the production of naturally occurring secondary metabolites. These metabolites include berberine [5], shikonin [6], ginsenosides [7], ajmalicine [8], anthocyanin [9], and, recently, important antitumor agents such as taxol [10], vinblastine, and vincristine [11].

The primary objective of the present study was to determine the possible use of zedoary cells in the production of essential oil and curcumin, and to evaluate the activities of antioxidant enzymes (POD, SOD, and CAT) from zedoary cell extracts. We have established a model batch culture system in a 5-l bioreactor equipped with three impellers. After the stabilization of growth, the quantities of essential oil and curcumin were determined, and the activities of antioxidant enzymes were evaluated. These primary results may prove useful in the development of a large-scale production protocol.

Materials and Methods

Establishment of Tissue Cultures

0.5 × 0.5 cm leaf-base explants were excised from zedoary plantlets [12] and cultured on MS [13] solid medium supplemented with 3 mg/l of BAP, 3 mg/l of 2,4-D, and 2% (w/v) sucrose, at a pH of 5.8 for the induction of callus formation. The calli, which are light yellow, hard, and friable, formed after 4 weeks of culture, and were subcultured and maintained in the same fresh medium for the entirety of the 4 week experimental period. The cultures were conducted at 25 ± 2°C with a photoperiod of 10-h daylight at an intensity of 2,000-3,000 lux.

Cell suspension cultures were initiated via the agitation of 3 g of friable calli in 250-ml Erlenmeyer flasks containing 50 ml of MS liquid medium supplemented with 3 mg/l of BAP, 3 mg/l of 2,4-D, and 2% (w/v) sucrose until a suspension of free cells formed. The suspensions were then positioned on a rotary shaker running at 100 rpm for 12 days under the described physical conditions for the callus culture. 50 g of cell biomass were then collected from the shaking culture and transferred into a bioreactor (Biotron, Inc. Korea) with a 5-l working volume and three impellers, and then propagated at an agitation rate of 150 rpm and an aeration rate of 2.5 l/min for 14 days. Mixing and aeration were achieved using sterile gas from an air pump through a flow meter and an air filter. The online monitoring of the pH and temperature of the cultures (5.8 and 25°C, respectively) was conducted by connecting the pH electrode and temperature sensor to the bioreactor.

Samples were obtained under sterile conditions every 2 days in order to determine the cell mass in both fresh and dry weights, and to obtain the extracts. The fresh weights of the cells in the suspension cultures were determined via the filtration of the cell suspension through a filter paper under vacuum conditions, and the dry weight of cells was determined by drying the fresh cell biomass at 40°C until it reached constant weight. Zedoary rhizomes (control) were harvested and dried in sunlight. Prior to each extraction, 100 g of zedoary was triturated and mixed with an equal quantity of glass beads in order to prevent the compaction of the material inside the distiller [14].

Extraction of Essential Oil

Steam Distillation

The extract from steam distillation in the Clevenger apparatus was obtained using 4 g of dried samples (cell biomass and rhizome), and the process took 6 h. After being passed through the zedoary bed, the steam was condensed using cooling water at room temperature (RT), and then collected in a separation funnel together with the recovered essential oil. The mixture was then allowed to settle for 1 day; afterward, the essential oil was collected in vials closed with plastic lids and wrapped in Teflon.

Volatile Solvent

The extract with volatile solvent was obtained using 4 g of dried samples (cell biomass and rhizome) with an orbital shaker at 40°C and 100 rpm for 6 h. 50 ml of petroleum ether were used. Erlenmeyer flasks covered with rubber corks were weighed prior to and after the extraction in order to determine whether any loss of solvent had occurred during the extraction. After the extraction, the mixture was vacuum-filtered. The micelle (solvent plus oil) was then placed in a dark flask, covered, weighed, and placed into an oven at 40°C; the essential oil was thus recovered [14].

Curcumin Extraction

Volatile Solvent

After steam distillation for the recovery of essential oil, the residue of the dried samples (cell biomass and rhizome) was weighed and transferred to Erlenmeyer flasks, with the addition of 40 ml of 90% ethanol. The extractions were then conducted in batches at 60°C and 100 rpm for 6 h. The filtered materials were placed on Petri dishes and maintained at 40°C in order to evaporate the ethanol. After separation, the curcumin was recovered [14].

Soxhlet Method

The Soxhlet process was conducted using the residue of the dried samples (cell biomass and rhizome). After steam distillation and the addition of an ethyl acetate: acetone mixture (1:9), the system was maintained for 20 h under reflux conditions. The filtered materials were then placed on Petri dishes and maintained at 40°C in order to evaporate the solvent mixture. The curcumin was recovered after the separation [15].

Enzymatic Antioxidant Activity Assays

Catalase Activity

The extraction of soluble CAT was achieved via the homogenization of the samples (cell biomass and rhizome) in 0.1 M K_2HPO_4 at a ratio of 1:9 (w/v). The homogenate was shaken gently and maintained for 30 min at RT. It was then centrifuged for 5 min at 4°C and 7,000×*g*. The supernatant was then used for measurements of CAT activity.

Catalase activity was determined via a titrimetric method. The decomposition of hydrogen peroxide (H_2O_2) is followed by the measurement of H_2O_2 remaining in the mixture after a certain time via back-titration with potassium permanganate ($KMnO_4$). The sample was then incubated for 30 min with excess H_2O_2 at RT. The reaction was then halted via the addition of H_2SO_4. The quantity of H_2O_2 remaining in the reaction mixture (1 ml of sample + 20 ml of distilled water + 0.3 ml of 1% H_2O_2) after 30 min of CAT action was determined by titration with $KMnO_4$, a potent oxidizing reagent [16].

Superoxide-dismutase Activity

The soluble SOD was extracted via the homogenization of the samples (cell biomass and rhizome) in the medium (0.1 M Tris–HCl buffer pH 7.8, containing 1 mM dithiothreitol and 1 mM EDTA) at a ratio of 1:5 (w/v). The homogenate was centrifuged for 10 min at 4°C and 12,000*g*. The supernatant was then used in the measurements of SOD activity.

Superoxide-dismutase activity was evaluated in accordance with the spectrophotometric method developed by Misra and Fridovich [17]. This method was predicated on the inhibition of the spontaneous degradation of adrenaline to adrenochrome by SOD at a pH of 10.2. Adrenaline stock solution was freshly prepared prior to measurement and contained 3×10^4 M adrenaline in 0.1 M HCl. The assay mixture contained 0.1 ml of supernatant, 2.8 ml of 0.05 M sodium carbonate buffer at a pH of 10.2, 0.6 μl of 1×10^4 M EDTA, and 0.1 ml of adrenaline stock solution. Adrenaline autooxidation was monitored with a BioMate-3 spectrophotometer (Thermo Spectronic, USA) at 480 nm, for 10 min at RT.

Peroxidase Activity

Soluble POD was extracted via the homogenization of the samples (cell biomass and rhizome) in 0.1 M acetate buffer at neutral pH at a ratio of 1:5 (w/v). The homogenate was centrifuged for 10 min at 4°C and 12,000×*g*. The supernatant was then used for measurements of POD activity.

Peroxidase activity was assessed in accordance with the method described by Zheng et al. [18]. For POD activity, guaiacol was used as an electron donor for oxidation. To 0.1 ml of supernatant, 0.09 ml of 30 mM H_2O_2, 50 μl of 1.5 mM of guaiacol, and 2.75 ml of 0.1 M phosphate buffer, pH 7 was added. The reaction mixture was shaken gently and absorbance readings were conducted after 15 and 30 s at 470 nm using a BioMate-3 spectrophotometer (Thermo Spectronic, USA).

Total Soluble Protein Concentration

The total soluble protein concentration in the supernatant was determined via the Bradford method [19] with bovine serum albumin as a standard. All enzyme-specific activities were assessed by dividing enzyme units by the total soluble proteins in the samples.

Statistics

The callus and cell suspension culture experiments were conducted with a minimum of three replicates. All experiments were repeated thrice. The data were analyzed in terms of means ± standard error followed by comparisons of the mean via Duncan's tests (one-way ANOVA). For all studies, the treatment groups were considered to be statistically significantly different from the controls at $P < 0.05$.

Results and Discussion

Plant Cell Suspension Culture

In order to evaluate the relationship between cell growth and the accumulation of natural compounds, we cultured 50 g of cells in a 5-l bioreactor at an agitation rate of 150 rpm, and an aeration rate of 2.5 l/min for 20 days. The

biomass concentration reached a maximum value of 67.73 g/l of fresh weight (approximately 6.77 g/l dry weight) after 14 days of culture. Determinations of the inoculum sizes, agitation rates, and aeration rates suitable for cell biomass production were presented in another article [20].

The batch culture of zedoary cells in the bioreactor evidenced a typical growth curve based on the fresh and dry cell weights. The zedoary cells exhibited a lag phase of approximately 2 days, while the cells were still adapting to the new medium, followed by a log phase (exponential growth period) from days 2 to 14, with intensive growth, and ending with a stabilization of growth (stationary phase). The stationary phase of these experiments is quite short, and difficult to predict. During the last days of the culture period (from days 16 to 20), the cell biomass evidenced a reduction in fresh and dry cell weights (Fig. 1). The zedoary suspension cell culture exhibited growth profiles characterized by a doubling time (T_d) of 5.5 days. This may be attributable to the exhaustion of nutrients or the accumulation of toxic substances in the medium [21]. The dynamic of total protein production is shown in Fig. 2. The intracellular total protein concentration increased continuously from days 2 to 12 of the culture, and reached a maximum value of 11.82 mg/g, then declined rapidly after day 14.

Essential Oil

Our results revealed an accumulation of essential oil in the zedoary cell biomass. Table 1 provides the total essential oil yield recovered from the zedoary cell biomass at various culture times, via steam distillation and the addition of volatile solvent.

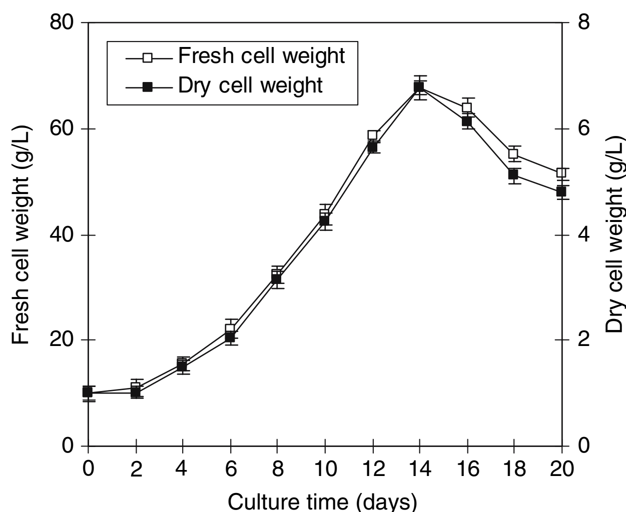

Fig. 1 Cell biomass production of zedoary in 5-l of medium in a bioreactor

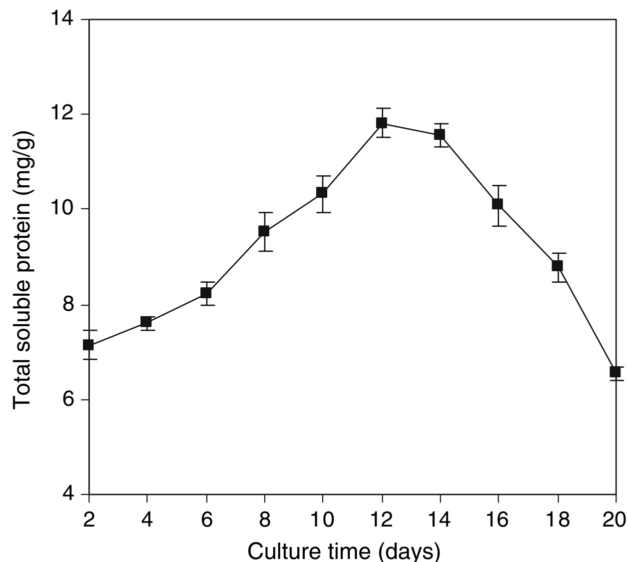

Fig. 2 Total soluble protein concentration during batch culture of zedoary cells

Table 1 Content of essential oil (% of dry cell weight) recovered from cell biomass

Culture time (days)	Content of essential oil	
	Petroleum ether	Steam distillation
2	0.38^{b}	1.15^{b}
4	0.40^{b}	1.29^{b}
6	0.47^{ab}	1.50^{ab}
8	0.51^{ab}	1.54^{ab}
10	0.55^{ab}	1.58^{ab}
12	0.60^{a}	1.73^{a}
14	0.69^{a}	1.78^{a}
16	0.61^{a}	1.49^{ab}
18	0.49^{ab}	0.83^{bc}
20	0.33^{b}	0.68^{c}
Control	0.61^{a}	1.51^{ab}

Different letters indicate significantly different means using Duncan's test ($P < 0.05$)

In general, the essential oil contents (percentage of dry cell weight) increased between days 2 and 14, and reached maximum values at the end of the log phase (14th day of culture) in both extractions via steam distillation (1.78%) and petroleum ether (0.69%). The total essential oil content of the control was 1.51% for steam distillation, and 0.61% for petroleum ether. We noted no significant differences in essential oil content recovered from the cell biomass on the 14th day and the control (for petroleum ether extraction). In other reports, the yield of essential oil recovered by

steam distillation (Clevenger apparatus) from fresh zedoary rhizomes was 0.22% (wt) [22], representing approximately 1.5% of dry weight [23].

Curcumin

Curcumin is one of four curcuminoids of zedoary. The other three curcuminoids are dihydrocurcumin, tetra hydrodemethoxycurcumin, and tetrahydrobisdemethoxycur cumin [24]. Curcumin is a water soluble powder with an orange-yellow color. It can be isolated via solvent extraction or the Soxhlet method from the dried rhizomes of *Curcuma* species.

Table 2 shows the total curcumin yield recovered using volatile solvent and the Soxhlet method. Our experimental results also showed curcumin accumulation in the zedoary cell biomass. However, the increase in curcumin accumulation did not occur in a clear fashion from days 2 to 12 (highest value reached at the end of the log phase, 14th day of culture) via both ethanol (9.69%) and Soxhlet extraction (9.42%). However, the total curcumin content of the control was generally relatively lower (5.88% for ethanol and 5.99% for Soxhlet). The curcumin yield from dried zedoary rhizome is approximately 5% [15]. In general, no significant differences were observed in terms of curcumin content as a result of ethanol and Soxhlet extraction.

Since the productivity of any process, including plant cell culture, depends on growth rate, yield, and biomass concentration, the improvement of all three values should be considered worthy goals. A few studies have reported yields in excess of 10% of dry cell weight, which is considered to be a very high yield. Such yields have been achieved for other products as well, including berberine [24], shikonin [25], and anthraquinone [26]. Values of approximately 1.7% of essential oil and 9.6% of curcumin, as were obtained in this study, are considered promising.

The results of our experiments indicate that essential oil and curcumin production can be achieved by the culturing of zedoary cells in a bioreactor. Further work and study will, however, be required in order to determine the optimal culture conditions for the recovery of these secondary metabolites.

Table 2 Content of curcumin (% of dry cell weight) recovered from cell biomass

Culture time (days)	Content of curcumin	
	Ethanol	Soxhlet
2	8.20^{ab}	8.14^{ab}
4	8.31^{ab}	8.29^{ab}
6	8.34^{ab}	8.33^{ab}
8	8.52^{ab}	8.34^{ab}
10	8.55^{ab}	8.51^{ab}
12	8.63^{ab}	8.63^{ab}
14	9.69^{a}	9.42^{a}
16	8.63^{ab}	8.55^{ab}
18	8.31^{ab}	8.33^{ab}
20	8.22^{ab}	8.22^{ab}
Control	5.88^{b}	5.99^{b}

Different letters indicate significantly different means using Duncan's test ($P < 0.05$)

Antioxidant Enzymes

Changes in the activities of primary antioxidant enzymes (POD, SOD, and CAT) were assessed in zedoary cells cultured in a 5-l (working volume) bioreactor equipped with three impellers. In general, the activities of three antioxidant enzymes increased continuously over the first two weeks of the batch culture, and the highest levels of activity were observed after 14 days, followed by a decline over the final week. These results bolster the notion that a relationship exists between the accumulation of cell biomass and the induction of antioxidant enzymes over the culture period.

Peroxidase

The POD specific activity differed profoundly during the culture period. POD activity was shown to increase continuously from days 2 to 14 of the culture (0.01–0.63 U/mg protein) in the bioreactor, and the levels were consistently higher than those of the control (0.07 U/mg protein). After 14 days, POD activity dramatically declined, and dropped to levels lower than those of the controls (Table 3).

Table 3 Specific activities of POD, SOD and CAT in culture of zedoary cell

Culture time (days)	POD (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
2	0.01^{e}	13.12^{cd}	6.22^{de}
4	0.02^{e}	13.57^{cd}	7.73^{d}
6	0.13^{cd}	14.66^{c}	10.37^{c}
8	0.15^{cd}	14.76^{c}	16.11^{bc}
10	0.25^{c}	14.84^{c}	17.57^{b}
12	0.41^{b}	14.96^{c}	17.96^{b}
14	0.63^{a}	16.60^{b}	19.59^{a}
16	0.08^{d}	15.76^{bc}	12.84^{c}
18	0.04^{de}	14.77^{c}	10.41^{c}
20	0.01^{e}	12.17^{d}	7.77^{d}
Control	0.07^{d}	18.58^{a}	16.12^{bc}

Different letters indicate significantly different means using Duncan's test ($P < 0.05$)

Superoxide-dismutase

An increase in SOD specific activity was observed from days 2 to 14, and a decline was registered from days 16 to 20. SOD activity, which achieved a maximum value of 16.60 U/mg of protein, was lower after 14 days of culture in the bioreactor, in comparison with the controls (18.58 U/mg protein) (Table 3).

Catalase

An increase in specific CAT activity was observed in the zedoary cell culture from days 2 to 14 (6.22–19.59 U/mg protein), followed by a decline occurring from days 16 to 20 (12.84–7.77 U/mg protein). CAT activity achieved a maximum value of 19.59 U/mg of protein after 14 days, a value higher than that of the control (16.12 U/mg protein) (Table 3).

Previous reports have shown that, depending on the plant species, the tested tissue type and culture conditions can either stimulate or inhibit the activities of several antioxidant enzymes [27–29]. In this study, we have demonstrated for the first time, the relationship between physiological oxidative response and suspension culture of zedoary cells in a bioreactor.

In the published literature, it has been established that curcumin exerts a profound antioxidant effect [30, 31]. Curcumin and its derivative (tetrahydrocurcumin) can induce antioxidant enzymes, including glutathione peroxidase, glutathione-S-transferase [32], glutathione peroxidase, glutathione reductase, catalase, and others [33]. Our data, therefore, suggest that the accumulation of curcumin in zedoary cells, which evidenced maximum values in this study of 9.69% (for ethanol) or 9.42% (for Soxhlet), can also be associated with the highest levels of increase in the activities of POD (0.63 U/mg protein), SOD (16.60 U/mg protein), and CAT (19.59 U/mg protein) at the 14th day of culturing. The induction of such detoxifying enzymes by curcumin illustrates the potential value of this compound as a protective agent against oxidative stress. More detailed studies to clarify the molecular mechanisms underlying the induction of enzymes by these antioxidants are currently underway [32].

In conclusion, the results presented in this communication indicate that the growth of zedoary cell suspension cultures in bioreactor systems equipped with three impellers and a working volume of 5 l is possible. Scale-up protocols will need to be designed and tested in the future. The results, however, are promising, and demonstrate for the first time, the production of essential oil and curcumin from a zedoary suspension culture. Further studies should focus on the optimization of cell suspension growth and the large-scale production of secondary metabolites.

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