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Optimization of SOD Biosynthesis by Controlling Sucrose Concentration in the Culture of Carrot Hairy Root

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Abstract In order to optimize the carrot hairy root culture for SOD production, a fed-batch culture of hairy roots was performed in a bioreactor. Maximum SOD activity was obtained when the hairy roots were transferred to the MS medium containing 110 g/l concentration of sucrose. By controlling the sucrose concentration (70 g/l sucrose for growth and 110 g/l sucrose for production, respectively) in a two-stage fed-batch culture, 29 g/l of the hairy roots was obtained based on the final dry mass. The volumetrically determined SOD activity and productivity in the fed-batch culture were about 6 times higher than those from the flask culture containing sucrose at 30 g/l concentration.

Key words: Plant cell culture, carrot, hairy roots, SOD, fed-batch culture

Plant cell/tissue culture techniques are becoming increasingly important for the production of economically valuable bioproducts, such as enzymes, flavonoids, pigments, pharmaceuticals and cell mass by itself. In particular, hairy roots transformed by *Agrobacterium rhizogenes* have many advantages for the stable production of valuable products. Nevertheless, since hairy root cultures present a unique scale-up problem due to their morphological structure, a comparative research on hairy root cultures in a bioreactor has not yet been conducted as of this time. Taya *et al.* [22] reported that the growth of immobilized horseradish roots in a bubble column yielded the best performance. Buitelaar *et al.* [1] reported that the highest productivity of thiophene production in *T. patula* was obtained by using a bubble column reactor. Other reactor systems with good performances include the airlift [20], a rotating drum [12], a mechanically agitated reactor with isolated impeller [7], and a trickle-bed reactor in which medium is recycled and sprayed over the top [24].

Superoxide dismutase (SOD; EC 1.15.1.1) is a metalloenzyme that catalyses dismutation of superoxide radicals (O_2^-) to molecular oxygen and hydrogen peroxide. Since these radicals are known to be toxic byproducts of oxygen metabolism, which not only oxidize membrane fatty acids and proteins but damage DNA [3] as well, SOD has been widely used as an antioxidant [13], ingredient in cosmetics, and as an anti-inflammatory and radioactive drug [17]. SOD is produced in oxygen-tolerant microorganisms, higher plants, and animals [2, 4, 5]. Matkovics [16] examined the level of SOD in various higher plants and reported the high level of SOD in *Cecei paprika* and radish root. Carrot hairy roots can also produce a large quantity of SOD [10]. The biosynthesis of SOD in carrot hairy roots was stimulated by high concentration of sucrose and $CuSO_4$. However, since high concentration of sucrose and $CuSO_4$ inhibited the growth of hairy roots, the two-step culture method that separates growth and biosynthesis was suggested for maximum production of SOD.

Fed-batch culture is an efficient culture technique to achieve high performances of biomass and metabolites [11, 14, 23]. This operation is suitable for achieving high yields of desired products at the end of fermentation. Specific growth rate is one of the most important process parameters that represent the dynamic behavior of cells in the fermentation process. However, due to a lack of the sensing system, cell mass has not been directly used as a measured variable for determining specific growth rate in controlling the fermentation process. In the plant cell/tissue culture system, conductometry is widely used as an indirect method for the cell mass determination [6, 18, 19]. In general, the correlation between conductivity changes and cell growth was empirically predetermined during the cultivation process at the specific culture condition. We developed a new method for *in situ* estimation of the mass

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of hairy roots [9]. Using this method, the cell mass was analyzed by monitoring the medium conductivity and the nitrogen concentration during cultivation. Also, specific growth rate can be easily determined and used as a control variable.

In this study, a high density culture of hairy roots and induction of SOD biosynthesis was conducted through a fed-batch cultivation in a bioreactor to maximize SOD production in carrot hairy roots.

MATERIALS AND METHODS

Hairy Roots and Maintenance

Carrot hairy roots were obtained from Chunnam National University. The roots were subcultured every 10 days in 500-ml Erlenmeyer flasks (working volume: 150 ml) by using a Murashuge and Skoog's (MS) medium containing 30 g/l sucrose that was maintained at 27°C and at 120 rpm in the dark. The pH of the medium was adjusted to 5.8 with 2 M of NaOH, followed by autoclaving at 121°C for 15 min.

Flask Culture

Hairy roots were cultured in 250-ml Erlenmeyer flasks (working volume: 50 ml) by using a Murashuge and Skoog's (MS) medium containing 30 g/l sucrose that was maintained at 27°C and at 120 rpm in the dark. The pH of the medium was adjusted to 5.8 with 2 M NaOH, followed by autoclaving at 121°C for 15 min.

Control experiments were conducted under the same condition. Effect of sucrose was investigated in the flask culture by varying the initial sucrose concentration from 10 to 130 g/l. Three replicates of flasks were taken for analysis in the flask culture, and results are expressed as mean values of three determinations with a deviation of less than 10%.

Bioreactor Operation

Bioreactor experiments were conducted in a modified 2.0 liter bubble column reactor (Newbrunswick, BioFlo reactor, New Brunswick, U.S.A.). To reduce the shear stress, a stainless mesh was placed on the sparger, and the growing region and the mass transfer region were separated by the mesh. Stirring at 200 rpm was conducted during addition of substrate. In order to eliminate evaporative losses in the reactor, humidified air was introduced into the reactor. *In situ* measurement of the medium conductivity was carried out using a conductivity meter (OM-1A, Toa Electronics, Co., Tokyo, Japan) with a conductivity cell (CG 201-PL). The airflow rate was 1.0 vvm and initial volume was 1.0 liter. The experiments were performed at 27°C in the dark. Hairy root culture in a bioreactor was started using MS medium containing 70 g/l sucrose. For the fed-batch culture in a bioreactor, MS medium containing 150 g/l sucrose and 400 g/l sucrose were fed for the growth of hairy roots and for the production of SOD, respectively. Six samples were taken from the entire part of the reactor (up, down, side, and inside portions of the reactor) to ensure reliable SOD assay.

Feeding Methods

Two-stage fed-batch culture which separates growth and production stage was performed in a bioreactor by controlling the sucrose concentration. The operation policy of growth was to maintain a maximum specific growth rate during the cultivation. Since the specific growth rate of the carrot hairy roots was correlated with the sucrose concentration in MS medium, maximum specific growth rate could be obtained at optimum sucrose concentration level. First, the maximum specific growth rate and optimum sucrose concentration were identified from the experiments such as flask culture. By monitoring the specific growth rate and the sucrose concentration during cultivation in a bioreactor, the maximum specific growth rate and the optimum sucrose concentration were updated whenever the observed specific growth rate was found to be greater than the maximum specific growth rate. Thus, in order to maintain the maximum specific growth rate, the substrate was fed into the reactor at a value of the optimum sucrose concentration. Even though the substrate was fed to maintain the optimum sucrose concentration, the operation mode was switched from the growth condition to the SOD induction condition when the specific growth rate decreased. At the SOD induction condition, calculated substrate volume to maintain the optimal sucrose concentration (110 g/l) was fed as a pulse lasted only for a minute.

Analytical Methods

Fresh hairy root weight was measured after washing with distilled water and vacuum filtrating through Whattman No. 2 filter paper. Dry weight was measured after drying the roots at 80°C for 2 days. During the cultivation in a bioreactor, the dry weight was estimated from a change in medium conductivity and nitrogen concentration [9].

Sucrose concentration was measured colorimetrically by the DNS method [15]. Ammonium and nitrate concentrations were measured colorimetrically by Berthlot [21] and Brucine [25] methods, respectively.

For determination of SOD activity, 0.15 g of fresh weight in 1 ml 50 mM phosphate buffer (pH 7.8) were homogenized using sonicator for 3 min. The homogenates were centrifuged at 12,000 rpm for 10 min, and the supernatant was used for the SOD activity assay. SOD was assayed by the inhibition of nitroblue tetrazolium reduction with O_2^- that was generated by the xanthine-xanthine oxidase system [10]. One unit of SOD was defined as the

amount of enzyme that inhibited the reduction by 50% under the assay condition.

RESULTS AND DISCUSSION

Effects of Sucrose Concentration on SOD Biosynthesis

It was reported that SOD biosynthesis was increased by adding sucrose and $CuSO_4$ [10]. Optimal concentration of $CuSO_4$ to maximally produce SOD was reported to be 0.3 mM. To examine the possibility of two inducers (sucrose and $CuSO_4$) for enhancing the SOD production, the effects of various sucrose concentrations and 0.3 mM $CuSO_4$ on the biosynthesis of SOD were investigated. Two days after the hairy roots were transferred to new medium containing various sucrose concentrations and 0.3 mM $CuSO_4$, and the SOD activity was measured. As shown in Fig. 1, the highest SOD activity was obtained in MS medium containing 70 g/l sucrose and 0.3 mM $CuSO_4$. However, SOD activity was decreased at high sucrose concentration level containing 0.3 mM $CuSO_4$, suggesting that the addition of two inducers had a negative effect on the biosynthesis of SOD.

To confirm these results, various two-step culture methods were examined. Hairy roots were transferred to the new medium containing various concentrations of sucrose and $CuSO_4$ after 11 days of cultivation. As shown in Table 1, the cellular level of SOD was increased in proportion to the increase of sucrose concentration and the addition of $CuSO_4$. However, the total SOD activity was lower than the results obtained from the controlled sucrose concentration alone, since the growth of hairy roots was significantly inhibited by the addition of $CuSO_4$ to the broth. Thus, a maximum total SOD activity was obtained when the hairy roots were transferred to a high sucrose concentration (110 g/l) medium. Therefore, to develop a technology to enhance the production of SOD in the carrot hairy roots, further study on the high density culture technique of hairy roots is needed. In addition, studies on optimization of induction condition by controlling sucrose concentration are also required.

Fed-Batch Culture of Hairy Roots in Bioreactor

First, optimal culture period for the enhancement of SOD production was determined in MS medium containing

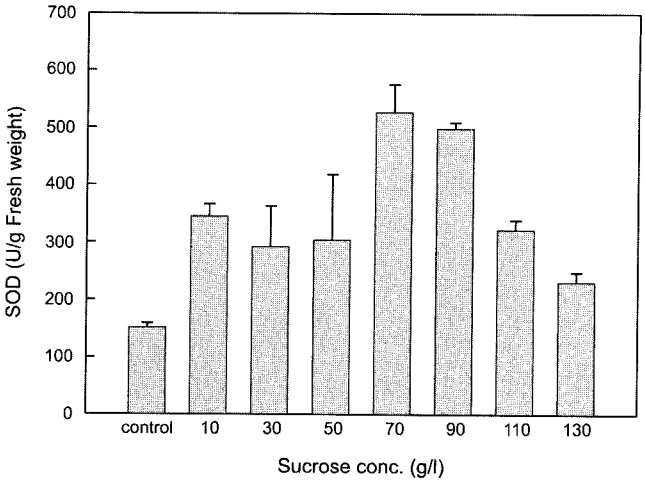

Fig. 1. Effect of sucrose concentration on the biosynthesis of SOD.
SOD activity was analyzed after 2 days by transferring to the new MS medium containing 0.3 mM $CuSO_4$ and various concentrations of sucrose.

110 g/l sucrose. SOD activity was increased and showed its maximum value (847 unit per gram of fresh weight) after 4 days as shown in Fig. 2. Based on the results derived from the flask culture, hairy roots cultivation in a bioreactor was started at the optimum sucrose concentration level (70 g/l).

Figure 3 shows the profiles of the sucrose concentration and its specific growth rate of the first fed-batch culture. In the initial period, the specific growth rate was increased as the sucrose concentration was decreased. Optimum specific growth rate was obtained at 67 g/l sucrose concentration. To maintain an optimum specific growth rate, the fed-batch cultivation was started after 7 days of cultivation. The specific growth rate was increased by the first addition of substrate, although growth inhibition due to overfeeding of sucrose was observed. However, maximum specific growth rate was not well maintained during the cultivation period, even though the substrate addition slightly increased the specific growth rate. The fed-batch did not effectively perform during the growth phase, especially in the early period. The substrate was first added at 7 days of cultivation, and the second addition at 10.5 days, because of sucrose overfeeding at the first addition. It seemed that

Table 1. Effect of various two-step culture methods on the growth of hairy roots and SOD biosynthesis.

Culture condition of the second step	Fresh weight (g/l)	Dry weight (g/l)	SOD (U/g FW)	Total SOD (U/l)
30 g/l sucrose	445	20.0	194	86,427 (±4,300)
70 g/l sucrose	475	25.0	420	199,490 (±7,500)
110 g/l sucrose	418	22.6	515	215,582 (±10,000)
30 g/l sucrose and $CuSO_4$	318	15.0	538	170,621 (±8,500)
70 g/l sucrose and $CuSO_4$	288	18.2	587	169,436 (±8,000)

Hairy roots were cultivated in MS medium containing 30 g/l sucrose for 11 days and transferred in new MS medium. Samples were taken at the 15th day of cultivation. 0.3 mM $CuSO_4$ was used for the experiments.

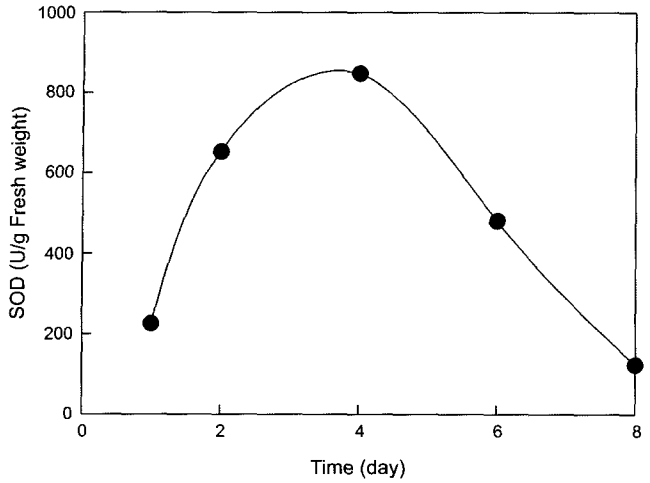

Fig. 2. Time course of SOD activity in MS medium containing 110 g/l sucrose.

the starting time and the feeding mode of the fed-batch culture were important to maintain the maximum specific growth rate during cultivation.

The second fed-batch culture was conducted, based on the data obtained from the first fed-batch. Figure 4 shows profiles of the sucrose concentration and the specific growth rate. Fed-batch started after 2 days of cultivation in order to maintain the observed specific growth rate. Since the specific growth rate was increased after the first substrate addition, the second addition was performed at 3 days of cultivation to maintain a newly determined specific growth rate. Maximum specific growth rate (0.33 day^{-1})

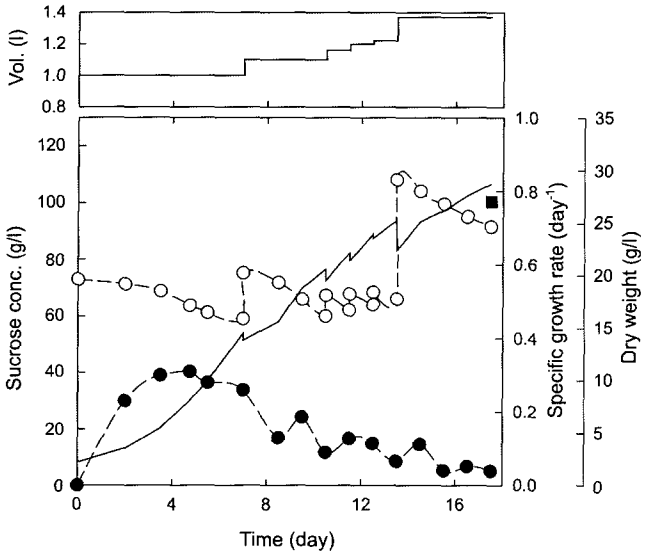

Fig. 3. Time courses of sucrose concentration, specific growth rate, dry weight, and culture volume in the first-batch culture.
○: sucrose, ●: specific growth rate, ■: final dry weight, —: estimated dry weight.

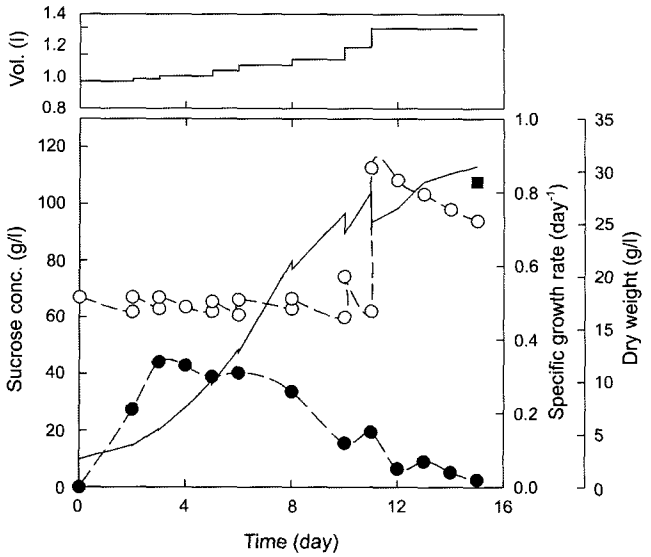

Fig. 4. Time courses of sucrose concentration, specific growth rate, dry weight, and culture volume in the second fed-batch culture.
○: sucrose, ●: specific growth rate, ■: final dry weight, —: estimated dry weight.

was obtained at 66 g/l sucrose concentration level. Thus, a fed-batch culture was actually performed to maintain the maximum specific growth rate. However, the maximum specific growth rate was not maintained after 8 days, even though the optimal sucrose concentration (66 g/l) was maintained by adding the substrate. It seemed that sufficient feeding of other nutrients was also important to maintain a high specific growth rate of hairy roots during the cultivation. After 11 days of cultivation, the operation mode was switched from the growth condition to the SOD production condition. Figure 4 shows also the time course of dry weight estimated by conductivity. Final dry weight (29.0 g/l) was slightly lower than an estimated final dry weight (30.5 g/l). Howerer, since error between measurement and estimation was 5.1%, hairy roots weight estimated by conductivity can successfully be correlated to the fed-batch system. The results derived from the fed-batch cultures in a bioreactor are summarized in Table 2. In the second fed-batch, 29 g/l of the final dry weight and 537,396 U/l of the total SOD activity were obtained. SOD productivity from the second fed-batch was approximately 24% higher than that from the first fed-batch. High final dry weight and intracellular SOD activity were obtained through a fed-batch culture in a bioreactor. The total volumetric SOD activity (537,396 U/l) and productivity (35,826 U/l·day) were about 6 times higher than those from the flask culture containing 30 g/l sucrose. Therefore, it can be said that a two-stage fed-batch culture method by controlling the sucrose concentration is indeed an efficient technique for producing SOD in carrot hairy roots.

Table 2. Results from the fed-batch cultures of carrot hairy roots in a bioreactor.

Parameter	First fed-batch	Second fed-batch
Fresh weight (g/l)	160	175
Dry weight (g/l)	27	29
SOD (U/g FW)	1,495	1,505
Total SOD (U/l)	504,649	537,396
Productivity (U/l day)	28,837	35,826
Final volume (l)	1.37	1.40

Samples were taken at the 17.5th days of cultivation in the first fed-batch and 15th days in the second fed-batch, respectively.

In general, the growth of hairy roots in a bioreactor is highly influeuced by the shear stress and mixing condition. In this study, a bubble column type reactor that separates the growing region and the mass transfer region by installing a mesh on the impeller was used. Hence, a shear stress was reduced and a good mixing condition was maintained, and an active hairy root growth condition was obtained throughout the entire region of the reactor.

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