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Kinetic and Technical Studies on Large-Scale Culture of *Rhodiola sachalinensis* Compact Callus Aggregates with Air-Lift Reactors

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Abstract: Compact callus aggregates (CCA) of *Rhodiola sachalinensis*, spherical, smooth-surfaced clumps of 2–8 mm in diameter displaying some level of tissue differentiation, were successfully cultured in 10 dm^3 and 100 dm^3 air-lift reactors. High salidroside yields of 60·0 mg dm^{-3} were obtained, which were 10-fold the dispersed cell cultures. The salidroside accumulation was found to be growth-associated due to the differentiated structure of CCA. No 'foaming' was observed since the broth remained almost clear throughout the culture cycle. The size of CCA conformed to normal distribution with average diameters varying from 3·1 mm to 3·6 mm during the culture. The depositing velocity of CCA in culture broth was small enough to be readily retained in suspension, therefore avoiding the clogging of the reactors. The significant increase in solid hold-up of the culture system was suggested to contribute to the variation of $k_L a$ during the culture. © 1998 SCI

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Key words: *Rhodiola sachalinensis*; salidroside; plant cell culture; aggregates; air-lift reactor; oxygen transfer coefficient

NOTATION

d	Diameter of aggregates (mm)
$\bar{d}$	Average diameter of aggregates (mm)
E_i	Weight percentage in each size fraction
$k_L a$	Oxygen transfer coefficient (min^{-1})
u_g	Gas flow rate (m s^{-1})
u_t	Depositing velocity of single aggregates (m s^{-1})
w_i	Dry weight of CCA captured in each size fraction (g)
μ	Specific growth rate (day^{-1})
μ_L	Viscosity of broth (cP)
ρ_L	Specific gravity of broth (g dm^{-3})
ρ_s	Specific gravity of CCA (g dm^{-3})
σ	Standard deviation (mm)

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1 INTRODUCTION

Suspension cultures of higher plant cells may provide viable alternatives for the production of a number of high-value secondary metabolites *in vitro*.[1] The biosynthetic potential of cultured cells has been exploited for the production of a number of metabolites but with limited commercial success,[2] low yields of the metabolites and genetic instability of the cell lines being the major constraints.[3] It is generally accepted that secondary metabolite production by plant cells is often associated with tissue differentiation.[4] In most cases a higher level of tissue differentiation means more secondary metabolites accumulated.[2] For this reason, highly differentiated organ cultures including root,[5] embryo[6] and shoot cultures[7] have been developed. However, differentiated cultures do pose technical problems in conventional bioreactors, in fact no bioreactor has hitherto

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been developed for the efficient culture of plant organs in large volume.[8]

In the process of callus induction several states of calli may occur on the explants, of which the callus composed of compact aggregates was often neglected when the main purpose was the initiation of the dispersed cell cultures. However, this kind of compact callus aggregate (CCA) shows evidence of partial morphological differentiation,[9] and may have greater potential than dispersed cells for the production of a variety of compounds. In previous work, the suspension culture of CCA of *Rhodiola sachalinensis* was established.[10] *Rhodiola sachalinensis* is a precious traditional Chinese herb containing the specifically effective constituent—salidroside. As a drug of 'source of adaptation to environment' in Chinese traditional medicine, it can effectively enhance the body's ability to resist anoxia, microwave radiation and fatigue, and to delay aging.[11,12] Studies with suspension cultures of *Rhodiola sachalinensis* CCA in shake flasks demonstrated that a high salidroside content of 0·48% in cultured CCA was obtained, almost 10 times as high as that of dispersed cells,[10] suggesting that CCA suspension culture may be more economical than the dispersed cell culture for the production of salidroside. However, the commercial feasibility of this new culture technique depends on the success of large-scale culture in bioreactors. At present, even the large-scale cultures of the conventional dispersed cells in reactors remain problematic. Proper oxygen transfer and efficient mixing of these cultures are generally hampered by their tendency to foaming and flotation, shear sensitivity and non-Newtonian rheological properties at high cell density (>10 g dm^{-3}). Immobilization has been suggested as a better way to solve these problems since the immobilized cells grow naturally in tissues.[13] CCA can to some extent be regarded as the self-immobilized plant cell clumps, therefore they may have the same advantages as the immobilized plant cell culture.

In this paper, we report the kinetic studies of large-scale cultures of *Rhodiola sachalinensis* CCA in 10 dm^3 and 100 dm^3 air-lift reactors, after that the characteristics of the CCA suspension culture system, including the properties of broth, aggregates, as well as the mass transfer capacity, were investigated.

2 MATERIALS AND METHODS

2.1 Plant material and culture method

The CCA suspension culture of *Rhodiola sachalinensis* was established as described previously.[10] The medium for maintaining CCA suspension cultures was Murashige and Skoog salts supplemented with 3% (w/v) sucrose, 0·3 mg dm^{-3} *a*-naphthaleneacetic acid (NAA) and 3 mg dm^{-3} 6-benzylaminopurine (BAP). The pH of the medium was adjusted to 5·8 before sterilization (121°C, 30 min). The stock cultures were maintained in 500 cm^3 Erlenmeyer flasks containing 200 cm^3 medium. Flasks were placed on a gyratory shaker at 120 rpm in the dark at 24°C. Subculture was carried out every 10 days by decanting all the medium from the flask and adding fresh medium.

Two stainless air-lift reactors with permanent water jackets were employed for CCA suspension cultures. The working volumes of the two reactors were 10 dm^3 and 100 dm^3, respectively. A water bath (Polystat, Bioblock Scientific Co.) was used for temperature control by circulating water through the jacket of the reactors. The mixing and aeration was achieved using sterile gas from an air pump through a flow meter and an air filter. CCA aggregates were inoculated at a density of 1·5% (w/v) and cultured for 30 days. The aeration rate was kept at 0·1 vvm in the culture systems of the two reactors. The on-line monitoring of pH, dissolved oxygen (DO) concentration and temperature of the cultures were carried out by connecting the pH electrode (Ingold), DO electrode (Ingold) and temperature electrode to the Celligen Cell Culture System (New Brunswick Scientific Co., Edison, NJ). The conductivity of the medium was also measured on-line using the conductivity electrode (Shanghai, China), then the biomass (gDW dm^{-3}) was estimated indirectly from the medium conductivity.[14] The samples (50 cm^3) were withdrawn at 5-day intervals from the cultures. To compensate for the volume loss caused by sampling, 50 cm^3 sterilized water was added after each sampling.

2.2 Analytical procedures

The samples drawn from the cultures were filtered on a nylon filter (50 μm pore size). The filtrate was collected for analysis of specific gravity, viscosity and sugar content. The biomass aggregates were washed three times with non-ionic water, then the specific gravity, size, water content and salidroside content measured. The salidroside content of CCA was determined using a Waters HPLC system. The collected aggregates were dried for 24 h at 80°C in an oven, then 0·5 g of dry aggregates were extracted with 20 cm^3 of methanol and the suspension was sonicated at 125 W for 30 min. The extracts were filtered through the 0·45 μm membrane filters and 10 mm^3 of solution was injected. The HPLC system was equipped with a Waters μBondapak ODS column (3·9 mm × 300 mm, 10 μm) and a UV detector (Waters Co., USA) at 276 nm. A mobile phase mixture of water (80%) and methanol (20%) at a flow rate of 1·0 cm^3 min^{-1} was used.

The size distribution of CCA was detected using a set of standard sieves with pore size of 1, 2, 3, 4, 5, 6, 7 and 8 mm respectively. The dry weight of CCA captured in each size fraction (w_i) was measured and the weight per-

centage in each size fraction (E_i) was calculated as follows:

$$E_i = \frac{w_i}{\sum_{i=1}^{8} w_i} \quad j = 1, 2, \ldots, 8 \tag{1}$$

The average diameter of CCA ($\bar{d}$/mm) was therefore calculated as:

$$\bar{d} = \sum_{i=1}^{8} E_i d_i, \quad d_i = 0{\cdot}5, \ldots, 7{\cdot}5 \text{ mm} \tag{2}$$

The on-line measurement of oxygen transfer coefficient ($k_L a$) was carried out by a dynamic method using nitrogen and air exchange.

The viscosity of the broth was detected using a viscosity meter at 24°C. The specific gravity of the aggregates was measured using a 25 cm^3 pycnometer bottle according to the method of Jensen *et al.*[15]

3 RESULTS AND DISCUSSION

3.1 Kinetics of CCA suspension cultures

Data from the suspension cultures of *Rhodiola sachalinensis* CCA in the 10 dm^3 and 100 dm^3 air-lift reactors are shown in Fig. 1. Both the cultures displayed a relatively slow growth curve with an almost growth-associated pattern for salidroside accumulation. The maximum biomass attained was 13·12 gDW dm^{-3} in the 10 dm^3 reactor at day 26 (Fig. 1(a)) and 13·90 gDW dm^{-3} in the 100 dm^3 reactor at day 28 (Fig. 1(b)). The time for peak salidroside accumulation (0·446% for 10 dm^3 culture and 0·410% for 100 dm^3 culture) corresponded to that for the maximum biomass attained. Both the biomass and salidroside content in large-scale cultures were comparable to those in shake flasks.

Sugar was near to exhausted at day 25 in these two cultures, after which the biomass remained almost constant and salidroside concentration started to decrease. Since the biomass was measured indirectly on the basis of medium conductivity, the actual decline of biomass during the culture could not be detected because of the continuous decrease of medium conductivity. In fact the biomass level would start to decline after the depletion of sugar at day 25 or so instead of remaining constant. The direct measurement of biomass of 11·45 gDW dm^{-3} in the 10 dm^3 reactor and 12·83 gDW dm^3 in the 100 dm^3 reactor at day 30 confirmed the decline of biomass.

DO was measured on the basis of the percentage of the saturated concentration in the medium at 24°C. Figure 1(a) shows that DO decreased continuously over the first 25 days from 84% to 56% and subsequently rose to 68% by day 30. In the 100 dm^3 reactor, DO increased during the first 4 days to 85% and then dropped slightly to 60% at the end of the culture (Fig. 1(b)). In these two culture systems, aeration was maintained at 0·1 vvm during the whole culture period, demonstrating that the low level of gas supply was enough to keep the DO value above 50% and provide an adequate supply of oxygen for CCA growth. The pH of the medium declined during the first 8–10 days then increased gradually again, consistent with other plant cell cultures.

To further investigate the kinetics of CCA growth and salidroside accumulation, the specific growth rate (μ) and specific salidroside production rate were calculated using the mid-point slope method based on the data obtained above. The results presented in Fig. 2 show that exponential growth occurred during days 4–10 for both the two culture systems with an average

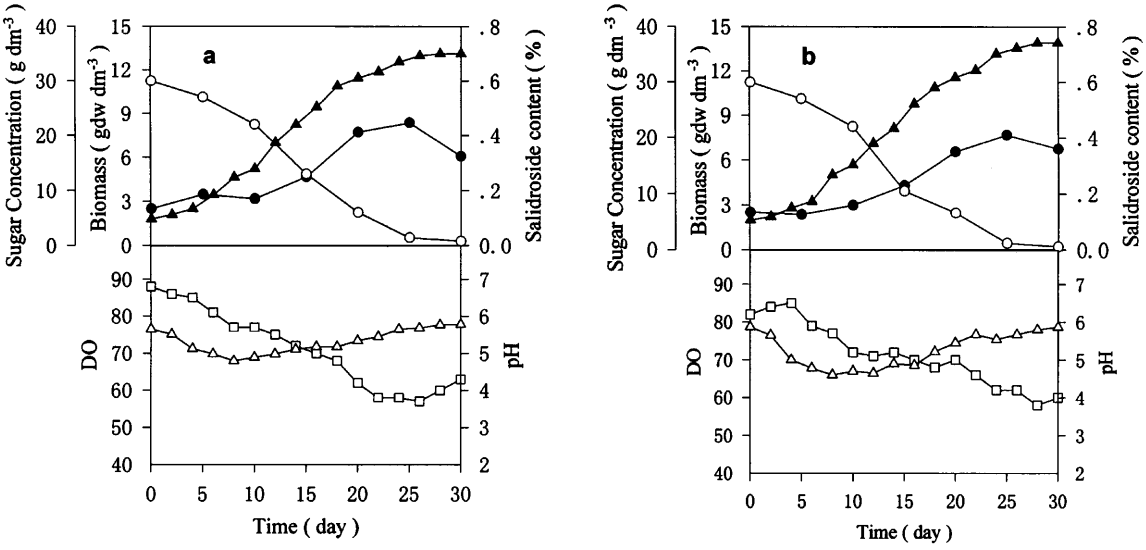

Fig. 1. Time course profiles of biomass (▲), sugar content (○), salidroside content (●), DO (□) and pH (△) during suspension cultures of *Rhodiola sachalinensis* CCA in 10 dm^3 (a) and 100 dm^3 (b) air-lift reactors.

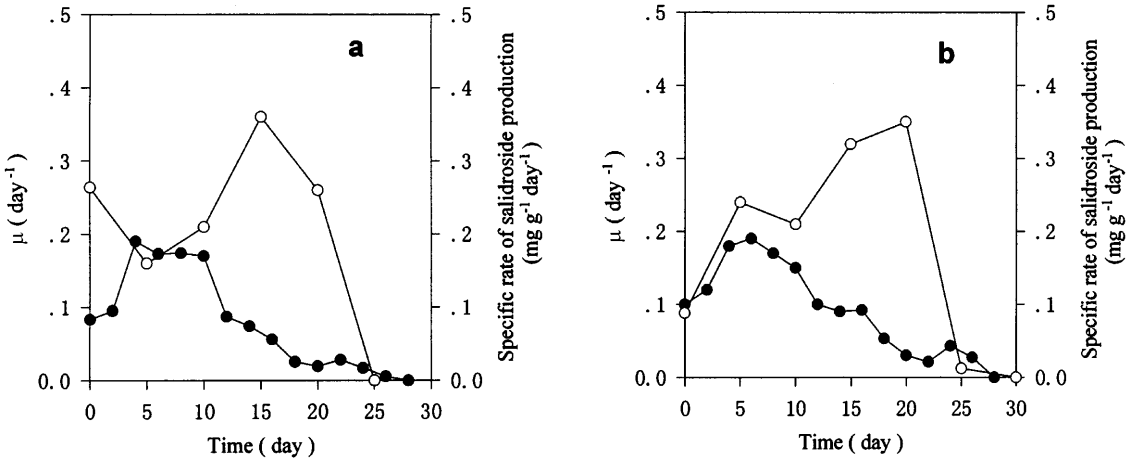

Fig. 2. Specific growth rate (●) and specific salidroside production rate (○) during the suspension culture of *Rhodiola sachalinensis* CCA in 10 dm^3 (a) and 100 dm^3 (b) air-life reactors.

value of $\mu = 0{\cdot}17\ day^{-1}$ in the 10 dm^3 reactor and $0{\cdot}19\ day^{-1}$ in the 100 dm^3 reactor. Subsequently, the specific growth rate decreased continuously till the end of the culture period. This growth pattern was similar to those of conventional plant cell cultures. However, the pattern for salidroside accumulation differed greatly from that of dispersed cell cultures of *Rhodiola sachalinensis*. Figure 2 shows that the salidroside production in the CCA suspension culture followed CCA growth. The maximum specific rate of salidroside production in 10 dm^3 and 100 dm^3 cultures were $0{\cdot}36\ mg\ g^{-1}\ day^{-1}$ (at day 15) and $0{\cdot}35\ mg\ g^{-1}\ day^{-1}$ (at day 20) respectively, corresponding to the marked decrease in specific growth rate. In contrast, with dispersed cell cultures salidroside production mainly occurred during the stationary phase.[10] This growth-associated pattern of product accumulation in CCA suspension culture may be related to the differentiated structures of the CCA, which allowed the synthesis of secondary metabolites to occur throughout the growth cycle instead of during the stationary phase. In the self-immobilized cell culture of *Solanum aviculare* developed by Tsoulpha and Doran,[16] the same pattern for secondary metabolite accumulation was found.

Kinetic analysis of CCA growth and secondary metabolite production should take into account mass transfer limitations within the aggregates. Despite the relatively low metabolic rate of plant cells, the effects of nutrient diffusion on CCA cell activity cannot be ignored. However, the result observed above demonstrated that the CCA growth was not obviously affected by the diffusion limitation. In viability testing experiments with a 2,3,5-triphenyltetrazolium chloride (TTC) method[17] almost all the cells within the CCA of various sizes were stained red, indicating the absence of nutrient transfer limitation. This unusual finding was also reported by Doran and colleagues in self-immobilized cell cultures.[18] To explain this phenomenon each CCA should be considered as an independent biological unit instead of a simple combination of individual cells. Tracheary development in CCA was observed in this work, indicating that differentiation might provide channels for improved transport of oxygen and other nutrients. Moreover, as cells in CCA were mitotically related, it is very likely they possess plasmodesmata, small cytoplasmic channels between plant cells that allow intracellular transfer of molecules. Plasmodesmata have been shown to occur in natural plant aggregates.[19] Thus it is possible that nutrients were transported efficiently within CCA through these channels, so preventing diffusion limitation.

The results of several further culture experiments with varying inocula are shown in Table 1. The final biomass obtained and salidroside contents of these three batch cultures were comparable to those discussed above, indicating that air-lift offers a practical process for product generation.

3.2 Characterization of CCA suspension culture

The CCA suspension culture was composed of compact aggregates, which exhibited the characteristics of a three-phase system when cultured in the air-lift reactor. Compared with commonly used three-phase systems such as the immobilized cell suspension culture, the CCA suspension showed increases in both the size and number of aggregates throughout the culture period. The technical characteristics of this new culture system were further investigated, as described below.

3.2.1 Viscosity of culture broth

The viscosity of the broth increased slightly from 0·97 cP to 1·03 cP during the 10 dm^3 culture period. Compared with dispersed cell cultures, the variation of viscosity found in the CCA suspension was small. Cultured CCA did not secrete as large amounts of metabolites such as proteins, peptides and polysaccharide as dispersed cells, so that the broth was clear even at the late phase of the culture. This phenomenon is worthy of special attention, since in most plant cell suspension

TABLE 1
The Results of Suspension Cultures of *Rhodiola sachalinensis* CCA with Varying Inocula in Air-Lift Reactors

Reactor volume (dm^{-3})	*Inoculum* ($gDW\ dm^{-3}$)	*Culture time* (*day*)	*Biomass* ($gDW\ dm^{-3}$)	*Salidroside content* (%)	μ (*exponential phase*) (day^{-1})
	0·75	35	12·88	0·45	0·19
10	1·24	32	13·19	0·44	0·18
	2·34	24	13·78	0·46	0·15
	0·52	38	12·12	0·40	0·22
100	1·10	33	13·55	0·38	0·23
	2·10	26	13·90	0·41	0·20

cultures, foam build-up is a common problem in the late phase of culture processes due to the excretion of metabolites into the broth by aged cells.[20] Adverse consequences of foaming include reduction in the working volume of the reactor, contamination from foam overflow and loss of productivity, thus elimination of foaming has been a key issue for large-scale plant cell cultures. However, foaming was not observed in large-scale CCA suspension culture. This may be associated with the differentiated structure of CCA, since foaming is also absent in other differentiated tissue cultures (e.g. hairy root culture[21]). Plant tissue differentiation would prevent the secretion of metabolites, so keeping culture medium clear. This phenomenon enabled separation of biomass from broth to be readily achieved by precipitation of aggregates.

3.2.2 Properties of CCA

The CCA of *Rhodiola sachalinensis* were pale yellow and compact with a well-rounded surface. Viewed under a microscope the cells in each aggregate were closely associated with meristemic cells that occurred mainly at the periphery of the aggregates. The variation of water content (denoted by ratio of fresh weight to dry weight) and specific gravity of CCA throughout the culture in the 10 dm^3 reactor are shown in Fig. 3.

The CCA in the air-lift reactor tended to accumulate water especially during the last week of the culture, from an initial ratio of fresh weight to dry weight of 21·5 to 32 at the end of the culture. The specific gravity of CCA decreased from $1{\cdot}018 \times 10^3$ g dm^{-3} to $1{\cdot}011 \times 10^3$ g dm^{-3} throughout the culture, which may be related to the increase in water content of CCA.

The size distribution of CCA (Fig. 4) was mostly concentrated within the range 2–5 mm diameter which conformed to the normal distribution during the cultures, although the average of CCA's diameter increased from an initial 3·24 mm to a maximum of 3·56 mm at day 21 in the 10 dm^3 reactor culture, and to a maximum of 3·35 mm at day 6 then decreased to 3.10 mm at day 28 in the 100 dm^3 reactor culture (Fig. 5). It was worth noting that the size of the aggregates cultured in the 100 dm^3 reactor was smaller than in the 10 dm^3 reactor, which may result from the higher shear stress in the 100 dm^3 reactor than in the 10 dm^3 reactor.

Since CCA suspensions are composed to large compact aggregates, the air-life reactors used for large-scale CCA suspension culture are possibly clogged by aggregates accumulating at the bottom when aggregates deposit faster than the flow of liquid in the downcomer. However, in our culture experiments with a 10 dm^3 and a 100 dm^3 reactor, no reactor clogging occurred, which should be associated with the low depositing velocity of CCA in reactors. The depositing velocity of a single CCA can be calculated according to Stock's equation, as listed in Table 2.

Because the specific gravity of CCA was close to that of the culture broth, the depositing velocity of CCA was small enough for them to be readily retained in suspension even at low gas flows. In such cases the clogging of the reactor would be easily avoided.

3.3 Oxygen transfer capacity of the culture system

The CCA suspension culture systems were characterized by large compact aggregates and clear broth. Through-

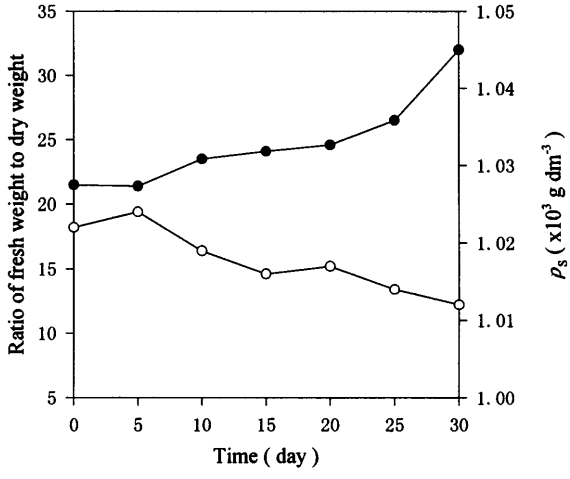

Fig. 3. The ratio of fresh weight to dry weight (●) and specific gravity (○) of CCA throughout the culture in the 10 dm^3 air-lift reactor.

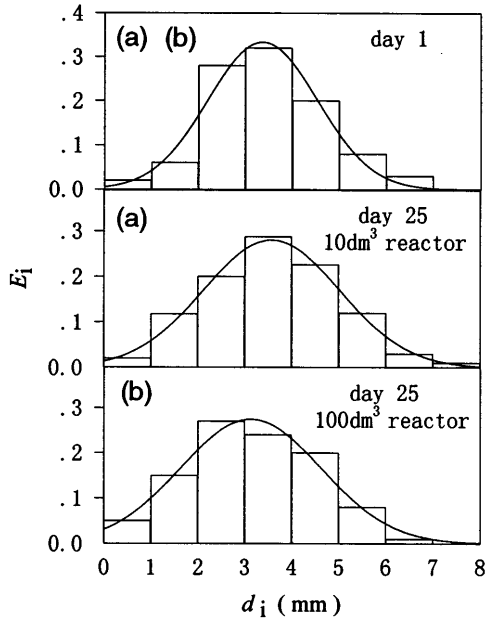

Fig. 4. Size distribution of CCA in suspension culture in 10 dm^3 (a) and 100 dm^3 (b) air-life reactors. Data determined at day 1 and day 25 respectively. The size distribution conforms to Normal distribution with average diameter $\bar{d} = 3{\cdot}28$ mm, standard deviation $\sigma = 1{\cdot}18$ mm at day 1, $\bar{d} = 3{\cdot}56$ mm, $\sigma = 1{\cdot}40$ mm at day 25 for the 10 dm^3 reactor culture and $\bar{d} = 3{\cdot}13$ mm, $\sigma = 1{\cdot}49$ at day 25 for the 100 dm^3 reactor culture.

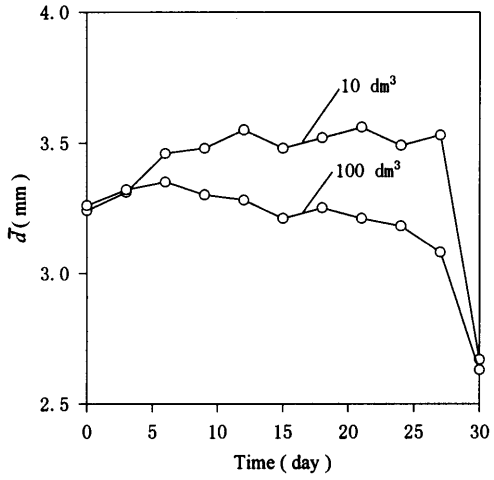

Fig. 5. The variation of CCA size over the culture period in 10 dm^3 and 100 dm^3 air-lift reactors.

out the culture period, the solid hold-up of the system increased significantly with cell growth, which would affect the mass transfer characteristics of the culture system. This is especially true for oxygen, since oxygen was the only nutrient supplied continuously during culture and its equilibrium concentration would be low in aqueous media, so that the oxygen transfer rate in the culture system would be much lower than that for other substrates. The oxygen transfer coefficient ($k_L a$) was used to characterize the mass transfer capacity of the culture system. To determine $k_L a$ on-line, the reactor was considered simply as a completely mixed vessel.

The $k_L a$ varied unusually over the culture cycle in these two culture systems, rising sharply at the initial culture phase then decreasing (Fig. 6). The maximum $k_L a$ occurred at day 7 in the 10 dm^3 reactor culture, corresponding to 14·5% of solid hold-up (biomass: 5·2 gDW dm^{-3}) (Fig. 6(a)). In the 100 dm^3 reactor, the value of $k_L a$ remained the highest during day 7 to day 14, then dropped sharply (Fig. 6(b)). Even at day 21, the $k_L a$ was still greater than at day 1 at low gas flow rate (Fig. 6(a)) or very close to day 1 (Fig. 6(b)), indicating that a good mass transfer capacity was retained during CCA culture. Since the viscosity of the culture broth varied only slightly and its value was low, it was assumed that the viscosity variation had no significant effect on $k_L a$. On the other hand, the solid hold-up of the system increased markedly from 4·5% to 32·8% in the 10 dm^3 reactor culture, and from 0·47% to 34·1% in the 100 dm^3 reactor, which may account for the variation pattern of $k_L a$ in the CCA suspension culture.

Figure 7 shows the effect of the solid hold-up of the culture system on $k_L a$ in the 10 dm^3 reactor. The same tendency for $k_L a$ variation in Fig. 7(a) as in Fig. 6(a) was found. Increases in $k_L a$ corresponding to increases in solid hold-up from 0 to 15·6% were followed by a marked decreases in $k_L a$ due to the high solid hold-up attained in the reactor. Figure 7(b) shows more clearly the variation of $k_L a$ with respect to solid hold-up. Since in the experiment shown in Fig. 7 the broth (medium) remained the same, only varying the solid hold-up, the initial increase in $k_L a$ could have resulted from the enhancement of mixing of the system caused by the stirring of the aggregates added. However, when the solid

TABLE 2
The Calculated Depositing Velocity of Single CCA of *Rhodiola sachalinensis* at Different Culture Periods in 10 dm^3 Air-Lift Reactor

Day	ρ_L ($\times 10^3$ $g\ dm^{-3}$)	ρ_s ($\times 10^3$ $g\ dm^{-3}$)	μ_L (*cp*)	u_t($\times 10^{-2}$ $m\ s^{-1}$)			
				$d = 2$ (*mm*)	$d = 4$ (*mm*)	$d = 6$ (*mm*)	$d = 8$ (*mm*)
1	1·010	1·018	0·97	1·80	7·18	16·1	28·7
15	1·007	1·015	0·99	1·76	7·04	15·8	28·2
25	1·004	1·011	1·01	1·51	6·04	13·6	24·1

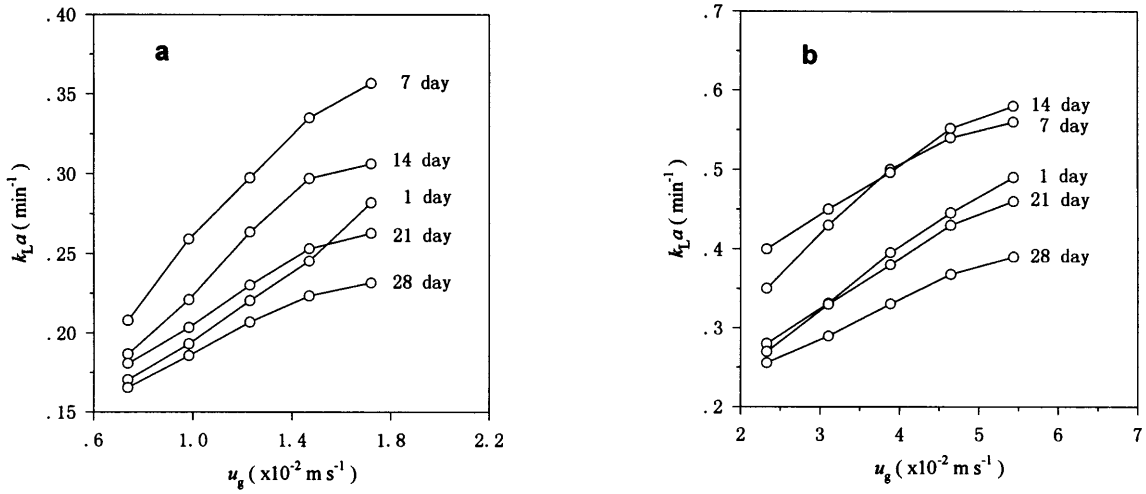

Fig. 6. The variation of oxygen transfer coefficients ($k_L a$) throughout the suspension culture of CCA in 10 dm^3 (a) and 100 dm^3 (b) air-life reactors.

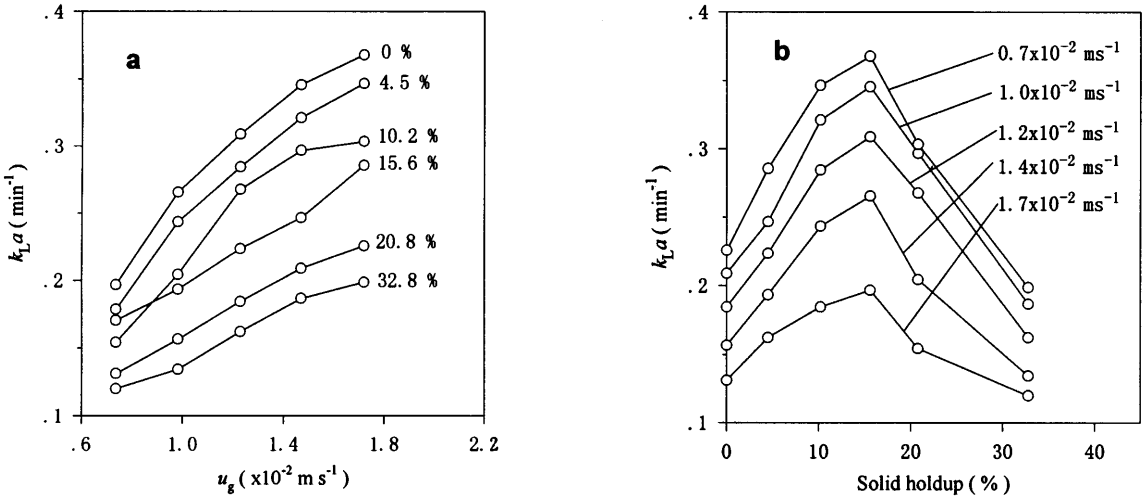

Fig. 7. The variation of $k_L a$ (a) with the gas flow rate at the solid hold-ups of 10%, 4·5%, 10·2%, 15·6%, 20·8% and 32·8%, respectively in the 0 dm^3 reaction; (b) with the solid hold-up at gas flow rates of $0{\cdot}7 \times 10^{-2}$ m s^{-1}, $1{\cdot}0 \times 10^{-2}$ m s^{-1}, $1{\cdot}2 \times 10^{-2}$ m s^{-1}, $1{\cdot}4 \times 10^{-2}$ m s^{-1} and $1{\cdot}7 \times 10^{-2}$ m s^{-1}, respectively in the 10 dm^3 reactor.

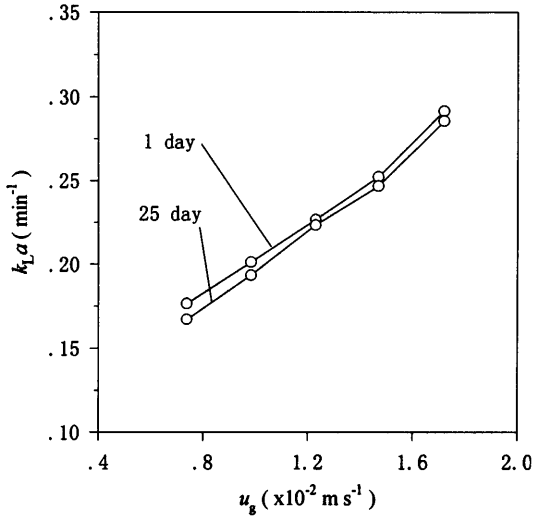

Fig. 8. The $k_L a$ measured in the broth collected after 1 day and 25 days of culture in the 10 dm^3 reactor.

hold-up of the system was high, the mixing of the vessel might be affected, which would weaken the mass transfer capacity, causing a reduction of $k_L a$. The result (Fig. 7) confirmed that the marked increase in solid hold-up contributed to the $k_L a$ variation pattern observed in the CCA culture process. Figure 8 shows that the age of the culture did not affect the $k_L a$, although the $k_L a$ in fresh medium was slightly higher than in 25-day-old broth.

4 CONCLUSIONS

Suspension cultures of *Rhodiola sachalinensis* CCA were successfully carried out in both a 10 dm^3 and a 100 dm^3 air-lift reactor. High levels of biomass and salidroside content were obtained. Salidroside accumulation followed CCA growth, and could probably be attributed to the organized and differentiated structure of CCA.

The growth of CCA was not significantly affected by nutrient transfer limitation.

The suspension cultures in the air-lift reactor exhibited a unique three-phase system, in which both the size and number of aggregates increased throughout the culture period. Advantages over conventional dispersed cell cultures included an absence of foaming, good mass transfer capacity throughout the culture periods and easy separation of biomass from broth. Variation of $k_L a$ was probably due to the significant increase in solid hold-up of the system.

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