Effect of Triton X-100 on Compactin Production from *Penicillium citrinum*

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Abstract Glucose alone was found to be the most effective carbon source for producing compactin. An initial glucose concentration of 40 g/L gave the highest compactin concentration of 250 mg/L. Among the various nitrogen sources, when 5 g/L of pharmamedia and soybean meal as the sole nitrogen source were used, respectively, the compactin concentration was higher than 250 mg/L. Especially, in the case of the mixture of 6 g/L of pharmamedia and 8 g/L of soybean meal, the compactin concentration was 400 mg/L. To select the best surfactant for effective compactin production, various surfactants were investigated. When Triton X-100 was used, the maximum compactin concentration was 445 mg/L. With the initial concentration ranging from 1.5 to 2.0 g/L, the compactin concentration was the highest at 465-450 mg/L. The cell concentration was similar to that of the control without the addition of Triton X-100. On the other hand, when the above 4.0 g/L of Triton X-100 were used, the cell concentration decreased. Using the based results, the continuous fed-batch cultures by adding the Triton X-100 were carried out for 10 days in an air-lift bioreactor. When 1.5 g/L of Triton X-100 was added to the culture broth at 0, 4, and 8 days of culture, respectively, the compactin production was increased with the increase of culture time. The maximum compactin concentration after 10 days of culture was 1,200 mg/L, which was about 2.0-fold higher than that of the control without the addition of Triton X-100.

Keywords: compactin, *Penicillium citrinum*, surfactant, Triton X-100, oxygen uptake rate

INTRODUCTION

Compactin, 7-[1,2,6,7,8,8a-hexahydro-2-methyl-8-(2-methylbutyrloxy)naphthyl]-3-hydroxyheptan-5-olide (Fig. 1), was first isolated from *Penicillium brevicompactum* by Brown *et al.* as an antifungal metabolite [1]. Later, Endo *et al.* reported their isolation of compactin together with ML-236A and ML-236C as hypocholesteremic agent from *Penicillium citrinum*. Among the three kinds, ML-236B was more active than ML-236A and ML-236C [2]. Similar structural features as well as biological activities have also been reported for monacolin K [3] or mevinoline [4] that were isolated from *Monascus ruber* and *Aspergillus terreus*, respectively. Statines such as fluvastatin, atorvastatin, and cerivastatin using chemical semi- or full-synthesis methods were also developed [5]. Compactin is an intermediate of pravastatin sodium. Pravastatin sodium is produced by a two steps fermentation process. The first step is the production of compactin by *Penicillium citrinum*. The purified compactin from the
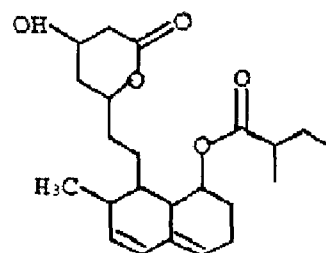

Fig. 1. Structure of compactin.

fermentation broth of *Penicillium citrinum* is changed to compactin sodium through the addition of sodium hydroxide. The second step, the beta-hydroxylation in the C6 position of compactin sodium, was carried out by *Streptomyces carbophilus*, *Nocardia* sp., or *Mucor hiemalis*. Pravastatin sodium is a competitive inhibitor of 3-hydroxy-3-methylgluaryl-coenzyme A (HMG-CoA) reductase (EC.1.1.1.34), which is the rate-limiting enzyme in cholesterol biosynthesis and is used as the cholesterol lowering medicine [6].

We are attempting to confirm the possibility of enhancing the production of compactin using an air-lift bioreac-

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tor, because air-lift bioreactors have many economical implications in regard to reactor construction, maintenance and scale-up as previously reported [7,8]. However, the ability of an air-lift bioreactors to supply oxygen is generally lower than that of the conventional type of bioreactors, *i.e.*, aeration and stirred tank bioreactor. Therefore, it is necessary to determine the optimal culture conditions that makes suitable for an air-lift bioreactor to operator.

In this paper, to determine the method for effective compactin production from *Penicillium citrinum* L-18065 using an air-lift bioreactor, first, the various carbon and nitrogen sources, and surfactant were investigated in flasks; second, using the results, the addition of the Triton X-100 to the continuous fed batch cultures was carried out in an air-lift bioreactor. Additionally, the relationship between the total oxygen amount consumed and the carbon source consumption was also investigated.

MATERIALS AND METHODS

Microorganisms, Medium, and Culture Conditions

The strain used in this study was *Penicillium citrinum* L-18065. The composition of the agar slant medium used was as follows (g/L): glucose, 50; peptone, 20; $NaNO_3$, 2; $MgSO_4 \cdot 7H_2O$, 1; malt extract 10; yeast extract, 3. The composition of the first seed medium used in the seed culture was as follows (g/L): glycerol, 10; glucose, 20; yeast extract, 5; peptone, 5; $NaNO_3$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5. The composition of the second seed medium for flask or an air-lift bioreactor culture was as follows (g/L): sucrose, 30; glucose, 20; yeast extract, 5; soybean meal, 5; $NaNO_3$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5. For production of compactin in an air-lift bioreactor, the following medium was used (g/L): glucose, 40; pharmamedia (Traders protein, Texas), 6; soybean meal, 8; $NaNO_3$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5. All the media components were sterilized at 121°C and 1.2 atm for 20 min. The pH of the media was adjusted to 6.5 before sterilization. One looful of *Penicillium citrinum* L-18065 was transferred to the slant medium and cultured at 25°C for 10 days. Then, one looful of the slant culture of *Penicillium citrinum* L-18065 was inoculated into a 500-mL Erlenmeyer flask containing 50 mL of the first seed medium and cultured at 25°C for one day on a reciprocating shaker at 150 rpm. For the second seed, 5% of the first seed culture was inoculated into a 500-mL Erlenmeyer flask containing 50 mL of the second seed medium and cultured at 25°C for 2 days on a reciprocating shaker at 150 rpm. For the production of compactin, 10% of the second seed was inoculated into a 500-mL Erlenmeyer flask containing 50 mL of the basal medium and to an 3-L air-lift bioreactor containing 2 L of basal medium and was cultured at 25°C.

Air-lift Bioreactor

The schematic structure of an air-lift bioreactor was previously reported [7, 8]. Based on this design, an air-lift bioreactor was modified for the *Penicillium citrinum* L-18065 culture. An air-lift bioreactor is comprised of three parts, a conical bottom holding the sparger, a cylindrical middle section, and a top portion with a degassing zone. In order to increase the mixing characteristics, the draft tube was removed and three ring spargers were used. An air-lift bioreactor is provided with several ports-for measuring the dissolved oxygen concentration and foam inside the bioreactor, for the removal of exhaust gas, and for the addition of antifoam agents and feeding medium. There is also a sampling port at the bottom of the cylindrical section. The temperature of an air-lift bioreactor contents can be controlled by circulating water through a glass jacket. An air-lift bioreactor can be steam-sterilized in an autoclave. An air-lift bioreactor was operated for 10 days. The dissolved oxygen concentration in the culture broth was measured by a DO sensor (Marubishi MDL-300, Japan). In order to maintain the DO concentration at 20-30%, oxygen-enriched air (30%) was supplied throughout the experiment and the oxygen concentration was controlled by the oxygen concentrator.

Cell Concentration

The cell concentration was measured by the packed cell volume (PCV) method, because the natural nitrogen source such as soybean meal, gluten meal, fishmeal, and pharmamedia contain insoluble components that do not dissolve completely in the fermentation broth, and it is not useful to use the optical density or dry cell weight.

Oxygen Uptake Rate

The oxygen uptake rate (OUR) was calculated by the oxygen balance method that directly determines, the amount of oxygen consumed into the air-lift bioreactor for a set time interval.

Total Oxygen Amount Consumed

The total oxygen amount consumed by time was calculated as follow:
$SOUR (t) = SOUR (t-1) + 1/2[OTR (t-1) + OUR (t)] dt$
$SOUR (t)$: total oxygen consumed by time, t (mmol/L). $OUR (t)$: oxygen uptake rate at t (mmol L^{-1} h^{-1}). $OUR (t-1)$: oxygen uptake rate at $t-1$ (mmol L^{-1} h^{-1}). $SOUR (t-1)$: total oxygen consumed by time, $t-1$ (mmol/L).

Compactin Concentration

The compactin concentration was measured by HPLC (Shimazu, Japan). Three milliliters of culture broth was added to 2 mL of sodium hydroxide (0.1 N) and shaken on a reciprocating shaker at 150 rpm for 30 min at 30°C. The mixture was added to 5 mL of methanol and centrifuged at 3,000 rpm for 15 min. The supernatant was measured under the following conditions: mobile phase, methanol:acetic acid:water (80:0.1:19); flow rate, 1.0 mL/min; column, ODS (Shimazu, Japan); temperature, room

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Table 1. Effect of initial glucose concentration on the concentration of compactin, cell, and residual glucose

Glucose concentration (g/L)	Compactin concentration (mg/L)	Cell concentration (mL/10mL)	Residual glucose concentration (g/L)
20	190	1.5	0
40	250	2.2	5.2
60	230	2.3	11.5
80	210	2.4	19.8
100	185	2.5	30.3
120	160	2.6	40.0

Basal medium (g/L): Soybean meal, 0.5; yeast extract, 0.5; $(NH_4)_2SO_4$ 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; and KH_2PO_4 2.

Table 2. Effect of various nitrogen sources on the concentration of compactin and the cell

Nitrogen sources (5 g/L)	Compactin concentration (mg/L)	Cell concentration (mL/10 mL)
Peptone	340	2.5
Polypepton	269	2.4
Yeast extract	192	3.6
Pharmamedia	350	2.6
Corn steep liquor	250	2.3
Gluten meal	150	2.4
Fish meal	267	2.45
Soybean meal	360	2.5
Malt extract	275	2.3

* Basal medium (g/L): glucose, 40; $(NH_4)_2SO_4$ 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; and KH_2PO_4 2.

temperature; detection, UV detector (236 nm); pressure, 600 psi; injection volume, 10 μL.

Carbon Source Concentration

The glucose concentration was measured by the reducing sugar using the Somogyi-Nelson method.

RESULTS

Effect of Glucose Concentrations on the Compactin Production

In determining the optimal concentration of glucose for the effective compactin production, initial concentrations of 20, 40, 60, 80, 100, and 120 g/L were used for 4 days in a flask. As shown in Table 1, an initial glucose concentration of 40 g/L gave the highest compactin concentration, 250 mg/L. When 20 g/L of glucose was used, the glucose was entirely consumed. However, when 40, 60, 80, 100, and 120 g/L of glucose were used, the residual concentrations were 5.2, 11.5, 19.8, 30.3, and 40 g/L, respectively. When 20 g/L of glucose was used, the cell concentration was 1.5 mL/10 mL of culture broth. However, when other concentrations were used, the con-

centration ranged from 2.2 to 2.6 mL/10 mL of culture broth. The compactin yield using 20 g/L of glucose was 9.50 mg/consumed glucose. However, in the case of 40 g/L of glucose, the yield was 7.0 mg/consumed glucose. From the view-point of the yield and compactin concentration, although 20 g/L of glucose produced the highest yield, it would take a large amount of energy to evaporate water in the down-stream process due to the relatively low compactin concentration relatively. Thus, the optimum initial concentration of glucose was determined to be 40 g/L due to the fact that it gave the second highest yield.

Effect of Various Nitrogen Sources on the Compactin Production

For investigating the effect of various nitrogen sources on the compactin production and cell concentration, peptone, polypepton, yeast extract, pharmamedia, corn steep liquor, gluten meal, fishmeal, soybean meal, and malt extract were used. Batch cultures were carried out in a flask containing 50 mL of basal medium with 5 g/L of each nitrogen source for 4 days and the results are shown in Table 2. Among the various nitrogen sources, when 5 g/L of pharmamedia and soybean meal as a sole nitrogen source were used, respectively, the compactin con-
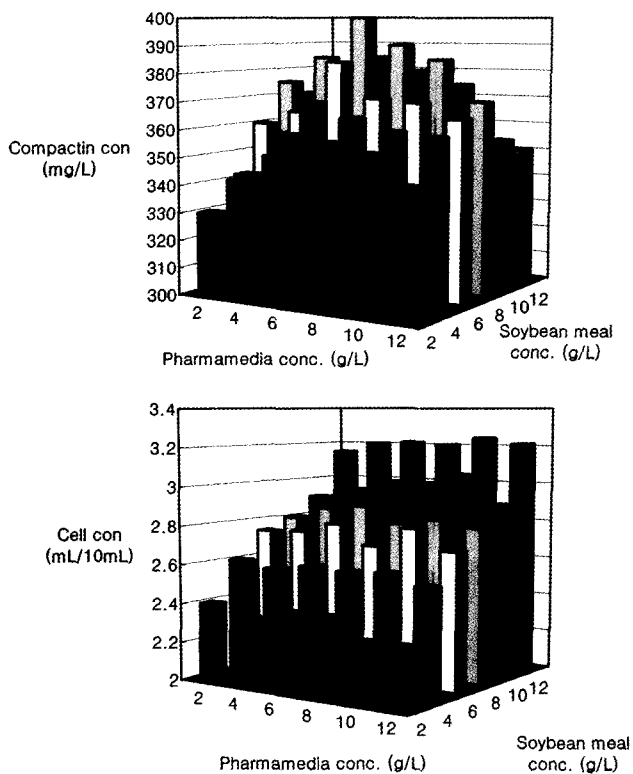
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Fig. 2. Effect of pharmamedia to soybean meal concentration ratio on the concentration of compactin and the cell. * Basal medium (g/L): glucose, 40; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; and KH_2PO_4, 2.0.

Table 3. Effect of various surfactants on the concentration of compactin and the cell

Surfactants (2 g/L)	Compactin concentration (mg/L)	Cell concentration (mL/10 mL)
Negamine 142-A	315	2.4
Span 180	405	2.3
Sodium dodecyl sulfate	220	1.4
Alrolene 65	310	2.2
Sunnix PP 4000	412	2.3
Triton X-100	445	2.1
Lecithin	200	1.3
Tween 80	410	2.2

* Basal medium (g/L): glucose, 40; pharmamedia, 8; soybean meal, 8; $MgSO_4 \cdot 7H_2O$, 0.1; and KH_2PO_4, 2.

tion. The optimal mixing ratio was 6 g/L of pharmamedia to 8 g/L of soybean meal, which gave a compactin concentration of 400 mg/L.

Effect of Various Surfactants on the Compactin Production

In order to investigate the effect of various surfactants on the compactin production and cell concentration, Negamine 142-A, Span 180, Sodium dodecyl sulfate, Alrolene 65, Sunnix PP 4000, Triton X-100, Lecithin, and Tween 80 were used. Batch cultures were carried out in a flask containing basal medium with 2 g/L of each surfactant concentration for 4 days. The results are shown in Table 3. When Triton X-100 was used, the compactin concentration was the highest at 445 mg/L. The cell concentration was similar to that of the other surfactants except in the case of sodium dodecyl sulfate and lecithin addition.

Effect of Triton X-100 Concentration on the Compactin production

In determining the optimal concentration of Triton X-100 for the effective compactin production, various Triton X-100 concentrations (from 0.5 to 4.0 g/L) were used for 4 days. As shown in Table 4, an initial Triton X-100 concentration of 1.5 to 2 g/L gave the highest compactin concentration of 465~450 mg/L. In the case of cell concentration, when 0.5 to 2.0 g/L of Triton X-100 were used, it was similar to that of the control without Triton X-100. On the other hand, when a concentration above 4.0 g/L of Triton X-100 was used, the cell concentration was decreased. Therefore, the optimal concentration of Triton X-100 for effective compactin production was 1.5~2.0 g/L.

Continuous Fed-batch Cultures in an Air-lift Bioreactor by Adding the Triton X-100

The continuous fed-batch cultures by adding surfactant such as Triton X-100 were carried out for 10 days in

centration was higher than 250 mg/L. The cell concentrations ranged from 2.0 to 2.6 mL/10 mL of culture broth. On the other hand, when the yeast extract as the sole nitrogen source was used, the cell concentration was the highest, but compactin concentration was only 192 mg/L. This suggests that pharmamedia and soybean meal might contain components that are effective for compactin production. Therefore, the pharmamedia and soybean meal as a nitrogen source were used in the following experiments.

Effect of Pharmamedia to Soybean Meal Concentration Ratio on the Compactin Production

To determine the optimal mixing ratio of pharmamedia and soybean meal to give a high level of compactin production, batch cultures were carried out for 4 days in a flask. A total 36 kinds of nitrogen sources were tested. The results are shown in Fig. 2. When the pharmamedia concentration was raised from 2 to 6 g/L, the compactin concentration increased, but it did not improved at the above 8 g/L. The pharmamedia concentration slightly affected the cell concentration. When the soybean meal concentration was raised from 2 to 8 g/L, the compactin concentration was increased, but it did also not improved at the above 10 g/L. The cell concentrations were also increased with increase of the soybean meal concentra-

Table 4. Effect of Triton X-100 concentration on the concentration of compactin and the cell

Triton X-100 concentration (g/L)	Compactin concentration (mg/L)	Cell concentration (mL/10 mL)
0.5	415	2.3
1.0	446	2.4
1.5	465	2.4
2.0	455	2.3
2.5	435	2.2
3.0	420	2.15
3.5	400	2.05
4.0	320	1.6
4.5	310	1.5

* Basal medium (g/L): glucose, 40; pharmamedia, 8; and soybean meal, 8; $MgSO_4 \cdot 7H_2O$, 0.1; and KH_2PO_4, 2.

3-L air-bioreactor containing 2.0 L of initial working volume. The aeration was fixed at 1.2 vvm in each case. The feeding rate of glucose was 15 g L^{-1} day^{-1} from 3 to 9 days of culture. For preventing the nitrogen source starvation during the compactin production, the mixture of 6 g/L of pharmamedia and 8 g/L of soybean meal was added to the culture broth after 5 days of culture. 1.5 g/L of Triton X-100 was added to the culture broth at 0, 4, and 8 days of culture, respectively.

The concentration of the cell, pH, OUR, residual glucose and compactin production are shown in Fig. 3. In the case of the conventional culture, the glucose consumption was increased with the increase of the culture time up to 4 days of culture, but from 6 days of culture it began to decrease, and finally, hardly consumed up to the end of culture. In the case of cell concentration, it was increased with the increase of culture time up to 4 days. However, the decrease was begun after 5 days of culture, and finally, it was 1.6 mL/10 mL of culture broth after 10 days of culture. The pH was 6.6 after 6 days of culture and was 8.2 after 10 days of culture. The oxygen uptake rate was increased with the increase of culture time up to 3 days of culture, however, the rate was decreased by half after 10 days of culture. The compaction production was also increased with the increase of culture up to 4 days of culture, however, did not increased after 8 days of culture.

On the other hand, when Triton X-100 was added to the culture broth during culture, the glucose concentration consumed after 10 days of the culture was 158 g/L. The value was about 1.63 fold higher than that of the control, in which Triton X-100 was not added. In the case of cell concentration, the increase began from the initial time of culture, and after 3 days of culture was similar to that of end of the culture. The pH was in the range between 6.0 and 6.5 during the culture. The oxygen uptake rate was increased with the increase of culture time up to 3 days of culture and then it leveled off during the culture. The compactin production was increased
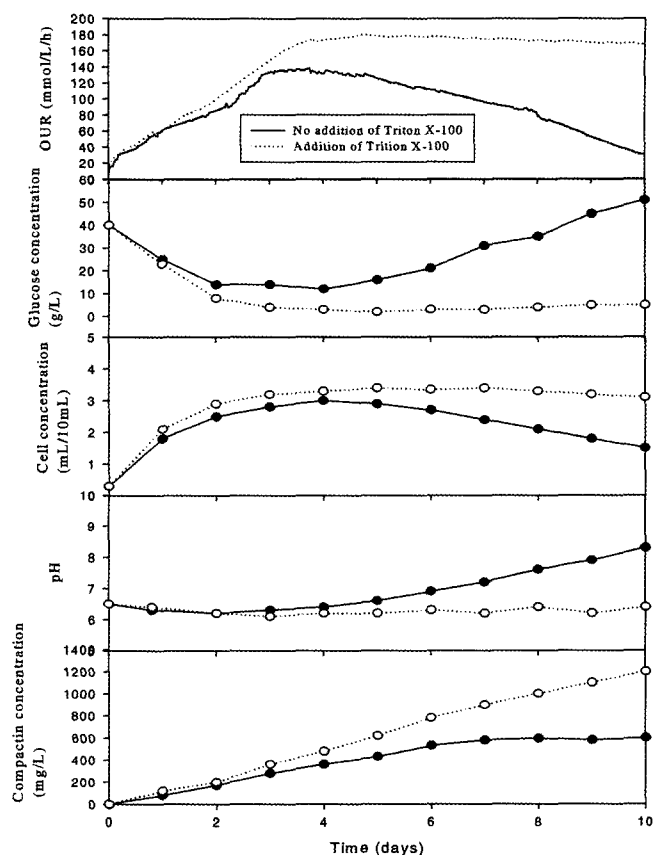
Fig. 3. Comparison of OUR, the concentration of glucose, cell, and compactin and pH in the culture with and without the addition of Triton X-100.

with the increase of culture time. The maximum compactin concentration after 10 days of culture was 1,200 mg/L, which was about 2.0 fold higher than that of the control, in which Triton X-100 was not added. This indicates that the energy source consumption was increased by the increase of oxygen consumption using the Triton X-100 during compactin production in the culture of *Penicillium citrinum* L-18065.

DISCUSSION

In order to select the best strain for producing compactin, we previously investigated *Penicillium citrinum*, *Penicillium brevicompactum*, *Paecilomyces* sp., *Trichoderma pseudokoningi*, *Trichoderma longibrachiatum*, *Hyphomyces* sp., *Doratomyces* sp., *Gymnoascus* sp., *Pleurotus* sp., and *Eupenicillium* sp. Among the various strains, in the case of *Penicillium citrinum* the compactin production was much higher than that of other strains. Therefore, strain development using *Penicillium citrinum* was carried out and L-18065 was screened by UV and X-ray irradiation (data not shown).

For the efficient production of compactin in the culture of *penicillium citrinum* L-18065 using an air-lift bioreac-

tor, various carbon, nitrogen sources, and surfactants were first investigated in a flasks. In order to investigate the effect of various carbon sources on the compactin production and cell concentration, glucose, fructose, galactose, sucrose, maltose, lactose, starch, and glycerol were used as the sole carbon source. Batch cultures were carried out in a flask for 4 days. The cell concentrations were between a range of 2.0 to 2.5 mL/10 mL of culture broth. On the other hand, when the glycerol was used, the cell concentration was 4.5 mL/10 mL of the culture broth. Among the various carbon sources, the highest compactin concentration, 250 mg/L, was obtained particularly when 40 g/L of glucose was used (Table 1).

Nitrogen is one of the major components of living materials and plays a key role in biological regulation. Nitrogen sources can govern cellular growth, formation of products, and cellular enzymes. The regulatory effects of nitrogen on the enzyme production that are involved in nitrogen assimilation, catabolism of nitrogenous compound, and formation of glutamate have been reviewed [9]. Regarding antibiotic production in *actinomyctes*, many authors have reported that the type and concentration of various nitrogen sources in a growth medium have some influence [10,11]. Choi *et al.* previously applied the nitrogen sources such as gluten meal and pharmamedia in an air-lift bioreactor for the effective tylosin production from *Streptomyces fradiae* T 1555 because the nitrogen sources contained various amino acids. These were also a cheap and commercially available source in the fermentation process [7]. We therefore investigated the various nitrogen sources for their effectiveness on compactin production. Among the various nitrogen sources, when pharmamedia or soybean meal as the sole nitrogen source was used, the highest compactin production was gave. Especially, in the case of the mixture of pharmamedia and soybean meal, the compactin production was increased more than that of a sole nitrogen source. The apparent viscosity in the culture broth was similar although the phmarmedia concentration was increased (data not shown). In this case, it was similar to that of the culture of tylosin-producing *Streptomyces fradiae* T 1555 using pharmamedia medium [7]. On the other hand, in the case of the soybean meal medium, the apparent viscosity was increased with the increase of soybean meal concentration. It was also similar to that of culture of the cephamycin–producing *Streptomyces* sp. P6621 U-12-2 using soybean meal (data not shown)

Many surfactants have been used with microorganism cultures for a number of different applications. For example, nonionic surfactants were used in order to remove surface proteins and in order to solubilize the bacterial membranes [12,13]. They were also used to enhance the transport of bacteria through porous media and to wash bacterial cultures prior to experimentation [14,15]. Kinoshita *et al.* used the polyoxyethylene nonyl phenol ether as the antisporulating reagent for the effective production of AICA-riboside in the culture of *Bacillus megaterium* MA-336 [16]. In the case of the synthetic media for lactic acid–producing *Lactobacillus* and *Lactococcus*, Tween 80 was used, because it appeared to improve the bacterial

growth rate and to shorten the cultivation time [17]. Lonvaud-Funel *et al.* also showed that the presence of Tween 80 in the growth medium induced marked differences in the fatty acid composition of *Oenococcus oeni* [18]. Choi *et al.* previously used the Lecithin as the surfactant for the effective tylosin production when rapeseed oil as the sole carbon source was used in the culture of *Streptomyces fradiae* T 1555. This indicated that the tylosin production was increased by the increase of oil consumption by using surfactant in the production medium [19]. Recently, we found that precipitates such as lipid or protein were produced during the culture of *Penicillium citrinum* L-18065; the energy source was barely consumed from the middle of the culture; and the compactin production did not increase in the end. This indicates that the lipid or protein substance was attached to the mycelial surface, and therefore, oxygen consumption was decreased. In order to dissolve these precipitates such as lipid or protein in the culture of *Penicillium citrinum* L-18065, various surfactants were investigated. Among the various surfactants, when Triton X-100 was used, the compactin production was higher than that of the other surfactants. Especially, in the case of 0.15 to 2.0 g/L of surfactant concentration, the compactin production was increased. However, there are still some problems to remove surfactant in the purification process of compactin.

The Triton X-100, a nonionic surfactant that has a high binding affinity for hydrophobic species, is very effective in binding to and solubilizing phospholipids from inner-membrane and outer-wall fragments of *E. coli* [20].

Using the results, the continuous fed-batch cultures using Triton X-100 were carried out in an air-lift bioreactor. The DO concentration was similar to that of the addition of Triton X-100 because the oxygen-enriched air was supplied in order to maintain DO concentration at 20~30% throughout the experiment with oxygen concentrator by automation. The total amount of oxygen consumed up to 10 days of culture was 36, 101.5 mmol/L, which was about 1.62 fold higher than that of the control without the addition of Triton X-100. The maximum compactin concentration after 10 days of culture was 1,200 mg/L, which is about 2.0 fold higher than that of control without addition of Triton X-100. This indicates that the compactin production was related to the amount of oxygen consumed.

Fig. 4 shows the relationship between the total amount of oxygen consumed and glucose consumption. The correlation between the total amount of oxygen consumed and glucose consumption was measured from 0 day to 10 days of culture. When the total amount of oxygen consumed was compared with glucose consumption, a significant correlation was observed with a correlation coefficient corresponding to 0.95. The changes in the total amount of oxygen that is correlated with the glucose consumption will help to develop a new glucose feeding strategy based on the OUR for the overproduction of compactin using an air-lift bioreactor.

It is difficult to culture aerobic fungi in an air-lift bioreactor because of the mycelial morphology, increase in apparent viscosity due to mycelial growth or medium,
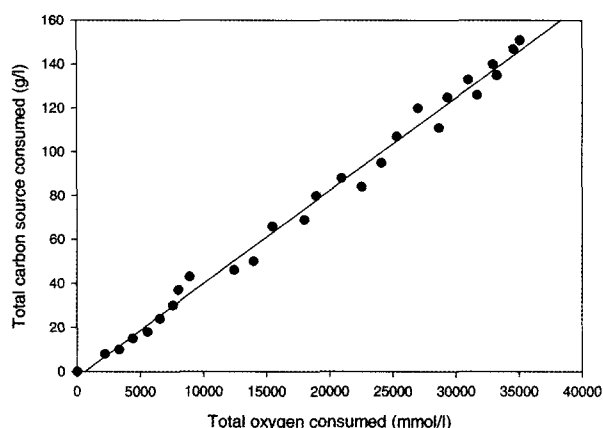
Fig. 4. Relationship between the total oxygen consumed and carbon source consumption.

and unplanted mixing. However, the compactin production in the culture of *penicillium citrinum* L-18065 by adding Triton X-100 was similar to that of jar fermentor (data not shown). In the case of single cell protein production from petroleum or methanol, an air-lift bioreactor of which the volume was over than 1,000 m^3 has been applied [21]. This suggests that an air-lift bioreactor has a possibility for the production of compactin using *penicillium citrinum* L-18065. The combination of the high yield compactin producer, simple, and less expensive production medium and air-lift bioreactor may lead to realize a large scale production of compactin.

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