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Optimization of Herbicidin A Production in Submerged Culture of *Streptomyces scopuliridis* M40

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Herbicidin A is a potent herbicide against dicotyledonous plants as well as an antibiotic against phytopathogens. In this study, fermentation parameters for herbicidin A production in submerged culture of *Streptomyces scopuliridis* M40 were investigated. The herbicidin A concentration varied with the C/N ratio. High C/N ratios (>4) resulted in a herbicidin A production of more than 900 mg/l, whereas maximally 600 mg/l was obtained at ratios between 1 and 3.5. In 5-L batch fermentation, there was a positive correlation between the oxygen uptake rate (OUR) and herbicidin A production. Once the OUR increased, the substrate consumption rate increased, leading to an increase in volumetric productivity. Mechanical shear force affected the hyphal morphology and OUR. When the medium value of hyphal size ranged from 150 to 180 μm, high volumetric production of herbicidin A was obtained with OUR values >137 mg O_2/l·h. The highest herbicidin A concentration of 956.6 mg/l was obtained at 500 rpm, and coincided with the highest relative abundance of hyphae of 100–200 μm length and the highest OUR during cultivation. Based on a constant impeller tip speed, which affects hyphal morphology, herbicidin A production was successfully scaled up from a 5-L jar to a 500-L pilot vessel.

Keywords: *Streptomyces scopuliridis* M40, herbicidin A, oxygen uptake rate, hyphal length, scale-up

Introduction

Weeds pose a serious problem in organic horticulture production, causing severe yield losses as well as labor consumption [1]. Weeds spread rapidly, compete for nutrients and water, and suppress crop plant growth. Crop yield is negatively correlated with weed population; thus, weed management is critical to agriculture. Chemical herbicides are primarily used to control these troublesome plants; however, herbicides pose health risks to humans, can persist in the soil, and are related to weed resistance due to excessive use [2, 3]. Alternatively, bioherbicides using phytopathogenic microorganisms or microbial herbicidal compounds have been developed, and they have several advantages; namely, high specificity of target weed, absence of residue in the environment, and reduction of herbicide-resistant weed populations [4].

Multiple studies on herbicidal compounds produced by microorganisms have been reported. Actinomycetes are gram-positive soil bacteria that generally show filamentous and branching growth [5]. *Streptomyces* spp. of actinomycetes are famous producers of antifungal, antibacterial, and antiviral agents, pigments, and even herbicides [6–10]. Many herbicidal compounds were reported from secondary metabolites of *Streptomyces* spp., such as herbicidins A and B from *S. saganonensis* and *S. scopuliridis* [6, 11], blasticidin S from *S. griseochromogenes* [12], and nigericin, hydantocidin, and geldanamycin from *S. hygroscopicus* [13, 14]. Glufosinate-

ammonium, derived from *S. hygroscopicus*, was successfully commercialized in 1984 and is a top 10-ranked herbicide in the global market.

A herbicidin-producing microorganism was previously isolated in the Republic of Korea and was identified as *S. scopuliridis* KR-001 [15]. Herbicidin A, derived from the culture broth of *S. scopuliridis* KR-001, was found to inhibit plant germination as well as to have herbicidal activity against several weed species, including *Echinochloa oryzoides*, *Digitaria ciliaris*, *Abutilon theophrasti*, and *Amaranthus retroflexus* [16]. In addition, when EP4C (sodium *bis*(2-ethylhexyl) sulfosuccinate) was used as an adjuvant, the herbicidal efficacy of herbicidin A was comparable to that of glufosinate-ammonium (active ingredient 540 g/ha) [17]. These results indicated that this compound has great potential as a natural herbicide.

One of the criteria for industrialization of herbicidin A is the cost-effectiveness of its production. Mass production of herbicidin A in a conventional industrial bioreactor can cut the production cost. There are many studies on the physiochemical parameters of production of secondary metabolites by *Streptomyces* spp., such as nutritional composition optimization [18, 19], morphological analysis [20, 21], bioreactor hydrodynamics on physiology of the strain [22], and dissolved oxygen tension [23]. However, because of the complex developmental life cycle of *Streptomyces* spp., successful process development depends upon empirical research. To our best knowledge, there are no reports on process development for herbicidin A production in submerged culture of *S. scopuliridis* with conventional industrial equipment.

In this study, a mutant strain of *S. scopuliridis* KR-001 was employed for herbicidin A production. The optimum C/N ratio of the fermentation medium was investigated in flasks, and fermentation parameters, including dissolved oxygen uptake rate and hyphal size distribution, were investigated under various agitation rates in 5-L jar fermenters. After gaining a full understanding of the characteristics of *S. scopuliridis* M40 with regard to herbicidin A production, the production was scaled up from a 5-L jar to a 500-L pilot vessel.

Materials and Methods

Microorganisms and Media

S. scopuliridis KR-001 [15], which was isolated from woodlands in Daejeon, Republic of Korea, was used in this study. For strain improvement, UV mutagenesis was performed according to the protocol of Miller [24] with slight modification. One hundred microliters of spore suspension of *S. scopuliridis* KR-001 containing 5×10^4 spores was spread onto Bennet's agar plates (KisanBio, Korea), followed by a 1 h incubation at 30°C. The plates were placed at a distance of 17 cm from a UV lamp (VL-4.LC; Vilber, France) (254 nm) for 45 sec when the mutant colony-forming rate reached 1%. All exposures were conducted in the dark to avoid any photoreaction in the production of mutants. The plates were incubated at 30°C for 120 h. The resulting mutant colonies were isolated, cultured in 500-ml baffled flasks containing 100 ml of Bennet's broth for 120 h, and compared for herbicidin A concentration. Mutant strains were maintained in 1.2-ml Cryovial tubes (Simport, Canada) at –70°C to avoid any genetic change due to successive culturing.

For herbicidin A production, the fermentation medium was composed of glucose (Duksan, Korea), corn flour (Ingredion Korea, Korea), and soybean meal (Gaemifood, Korea). The nutrients were tested in various concentration ratios to enhance herbicidin A production. Cultivations were performed in triplicates.

Cultivations

For seed cultures, *S. scopuliridis* M40 was thawed at 25°C and cultured in 500-ml baffled flasks containing 100 ml of Bennet's medium. The flasks were incubated on a rotary shaker (IS-971RF; Jeiotech, Korea) at 30°C and 180 rpm for 24 h.

For determination of the optimum ratios of carbon and nitrogen sources, 2% (v/v) of seed culture was inoculated into flasks containing 100 ml of fermentation medium with various C/N ratios and cultured at 30°C at 180 rpm for 120 h. The optimum ratio was used for subsequent herbicidin A production.

For small-scale fermentation, 2% (v/v) of seed culture was inoculated into a 5-L jar containing 3 L of fermentation medium and equipped with a dissolved-oxygen meter and a pH meter (BioCnS, South Korea). Cultivations were performed for 120 h at 30°C with agitation speeds of 300–600 rpm and an aeration rate of 1.0 vvm. For scaled-up 50-L pilot fermentation (BioCnS), 2% (v/v) of seed culture was poured into a vessel containing 30 L of fermentation medium. Cultivations were performed for 120 h at 30°C and at 150 rpm with an aeration rate of 1.0 vvm. For 500-L pilot-scale fermentation, seed cultures were grown for 24 h in a 50-L pilot vessel as described above. Then, the culture broth was added at 2% (v/v) to a 500-L vessel containing 300 L of fermentation medium. The pilot fermentation was carried out for 120 h at 30°C and at 90 rpm with an aeration rate of 1.0 vvm. Cultivations were performed in triplicates.

Analytic Methods

Herbicidin A was purchased from Santa Cruz Biotechnology, USA. Culture broth of *S. scopuliridis* M40 was centrifuged at 7,000 ×*g* (5810R, fixed angle type; Eppendorf, USA), serially diluted, and filtered through a 0.45-μm PTFE syringe filter (Whatman, USA). Herbicidin A was measured by HPLC (Waters Alliance e2695 system, Waters, USA) using an XBridge C_{18} column (4.6 × 250 mm, 5 μm; Waters) at 40°C with a photodiode array

detector. The mobile phase consisted of HPLC-grade distilled water (J.T. Baker, USA) and HPLC-grade methanol (J.T. Baker) at a ratio of 65:35. The flow rate and detection wavelength were 1 ml/min and 254 nm, respectively. A calibration curve was constructed by plotting the peak area of five different standards of herbicidin A (*i.e.*, 50, 100, 200, 400, and 800 mg/l) against the corresponding concentrations of standard solutions, showing the best linearity (R^2 = 0.999). With the calibration curve, the peak area of samples was converted into herbicidin A concentration.

For the determination of dry cell weight, 10 ml of culture broth was filtered through dry filter paper (Whatman No. 2), washed twice with distilled water, and placed in an oven (VS-120203; Vision Co., Korea) at 70°C for 24 h. Dry cell weight was calculated as the difference between the weight of the filter paper before and after use.

The concentration of reducing sugars was determined by a dinitrosalicylic acid assay [25]. The reagent was prepared as follows: 0.25 g of 3,5-dinitrosalicylic acid (Sigma-Aldrich, USA), 75 g Rochelle salts (sodium potassium tartrate; Sigma-Aldrich), and 4 g NaOH (Sigma-Aldrich) were dissolved in 300 ml of distilled water. The reagent was purged with nitrogen gas prior to the assay. Culture broth of *S. scopuliridis* M40 was centrifuged at 7,000 ×*g* to remove the cells, and the supernatant was filtered through a 0.45-μm PTFE syringe filter (Whatman). One hundred microliters of filtered supernatant was added to 1 ml of the reagent. The reaction mixture was boiled in a water bath for 10 min, transferred to an ice bath to cool down, and placed at room temperature. Absorbance at 570 nm was determined using a spectrophotometer (UV-1800; Shimadzu Co., Japan).

Determination of the Oxygen Uptake Rate (OUR)

Oxygen is one of the important factors in aerobic fermentation and usually dissolved into the broth by air sparging. The material balance for dissolved oxygen in a liquid phase can be established as below [26]:

$$\frac{dC}{dt} = OTR - OUR$$

where dC/dt = the dissolved oxygen rate concentration, OTR = oxygen transfer rate from the gas to the broth, and OUR = oxygen uptake rate by microorganisms. Dissolved oxygen tension (DOT, the partial pressure of oxygen molecules dissolved in the broth) was measured by using a dissolved-oxygen meter. The OUR value was obtained by the dynamic method [26]. It was calculated as the slope of the plot of dissolved oxygen concentration during temporary interruption of the air supply to the bioreactor.

Morphological Analysis

The frequency distribution of hyphal lengths was determined by using a Microtrac S3500 SIA (Microtrac, USA) equipped with PartAnSI software (Microtrac). A strobe light and digital camera were used to capture images of the hyphal length of *S. scopuliridis* M40 passing through a sensing area. Images were digitalized, and the shape and size distribution were calculated on the basis of pixel size.

Statistical Analysis

Data were analyzed using the PASW software (Ver. 17; SPSS Inc., USA). Analysis of variance (ANOVA) tests were used to determine the significant differences between treatments at $p < 0.05$ using Tukey's HSD test.

Results and Discussion

Selection of a High Producer of Herbicidin A

S. scopuliridis KR-001 [15] was used as a parental strain for stepwise mutagenesis. UV radiation was used for mutagenesis as described in Materials and Methods. Eleven out of 530 colonies were obtained as herbicidin A-producing mutants (data not shown). The mutant strain showing the highest productivity was selected and named "M40." The herbicidin A concentration produced by *S. scopuliridis* M40 was 308.7 ± 23.3 mg/l, which was 3 times higher than that produced by *S. scopuliridis* KR-001 (103.9 ± 9.5 mg/l).

Optimum Ratio of Fermentation Medium for Herbicidin A Production

Medium composition is one of the factors critical to microbial metabolite production [27, 28]. Based on preliminary tests, glucose or corn flour was selected as a carbon source, and soybean meal was used as a nitrogen source. The complex corn flour medium had carbon and nitrogen contents of 76.43% and 7.84%, respectively, whereas the soybean meal consisted of 30.52% carbon and 54.69% nitrogen (Table 1). Herbicidin A production with corn flour as a sole carbon source was much higher than that with glucose (Fig. 1). Maximal herbicidin A production (1,073.0 ± 143.3 mg/l) was obtained when 5% corn flour and 1% soybean meal were used as sole carbon and nitrogen sources, whereas herbicidin A was produced at

Table 1. Composition of corn flour and soybean meal.

Items	Content (%)	
	Corn flour	Soybean meal
Moisture	13.69	7.6
Crude protein	7.84	54.69
Crude lipid	1.13	0.62
Crude ash	0.91	6.57
Crude carbon	76.43	30.52
Total	100	100

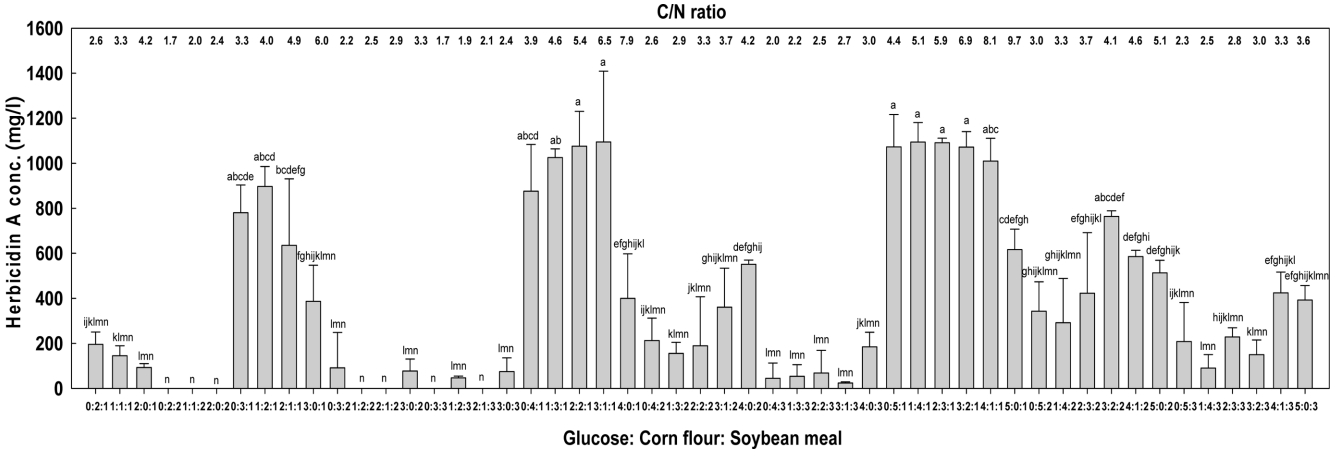

Fig. 1. Herbicidin A production depends on the ratios of glucose, corn flour, and soybean meal.
Cultivations were carried out in 500-ml baffled flasks for 120 h at 30°C and 180 rpm. Different letters indicate values are significantly different at $p < 0.05$ (Tukey's honestly significant difference).

616.5 ± 91.2 mg/l at 5% glucose and 1% soybean meal. However, there was a synergetic effect on herbicidin A production when corn flour and glucose were combined. Herbicidin A production at 4% of corn flour plus glucose was equivalent to that at 5% of corn flour or glucose alone. Considering the price of the two components is about the same, lowering the medium content is advantageous for cutting the production cost.

Changing the C/N ratios resulted in large differences in herbicidin A production (F = 31.8, df = 50,102, $p < 0.001$) (Table 2). High yields of herbicidin A were observed at C/N ratios ranging from 4.40 to 6.87, with concentrations higher than 1,000 mg/l, which was 3 times higher than that in Bennet's broth. Lower C/N ratios resulted in decreased herbicidin A production; for C/N ratios ranging from 1 to 3.5, herbicidin A production was generally less than 400 mg/l, indicating a negative correlation between the nitrogen and herbicidin A concentrations. The highest concentration of 1,094.9 ± 314.0 mg/l was obtained at a C/N ratio of 6.51, with a culture medium consisting of 3% glucose, 1% corn flour, and 1% soybean meal. The concentrations in our system were 354.7% higher than those with Bennet's broth (308.7 mg/l).

Batch Fermentation Patterns at Various Agitation Rates

To the best of our knowledge, this is the first report describing herbicidin A production in submerged culture of *Streptomyces* spp. in bioreactors. Herbicidin A production varied with agitation rates of 300–600 rpm, which had an impact on the DOT level during culture (Fig. 2). DOT, but not the herbicidin A concentration, increased with increasing agitation rates. At 300 rpm, the DOT level and herbicidin A concentration were lowest, although there was sufficient residual sugar throughout the culture. As agitation rates increased from 400 to 600 rpm, the DOT levels increased proportionally. Reducing sugars decreased with increasing herbicidin A. The highest concentration of herbicidin A of 956.6 ± 63.3 mg/l was observed at 500 rpm after 120 h, followed by 823.5 ± 87.9 mg/l at 400 rpm. DOT was the highest throughout culture at 600 rpm; however, the herbicidin A concentration (768.7 ± 14.7 mg/l) at this speed was lower than that at 400 and 500 rpm, although reductions in reducing sugar concentrations were similar at 400–600 rpm.

Fig. 2 shows the positive correlation between volumetric productivity and the corresponding OUR. When the OUR increased from 33 to 178 mg O_2/l·h, the volumetric

Table 2. ANOVA results for the effect of the ratios of glucose to corn flour to soybean meal on herbicidin A production.

	df	SS	MS	*F* value	*P* value
Ratios	50	21,493,038.8	429,860.8	31.8	<0.001
Residuals	102	1,385,416.8	13,582.5		
Corrected total	152	22,878,455.6			

df, degrees of freedom; *SS*, Sum of squares; *MS*, mean square.

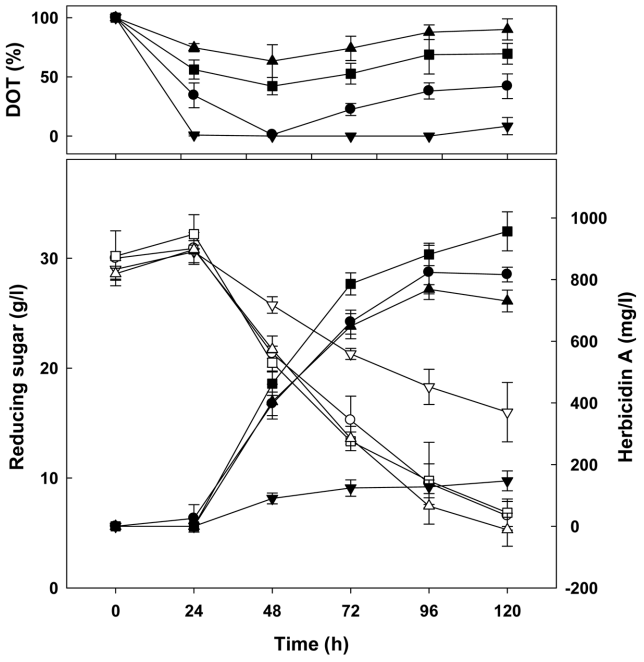

Fig. 2. Herbicidin A production profiles at various agitation rates (*i.e.*, 300, 400, 500, and 600 rpm).
Cultivations were carried out in 5-L jar fermenters for 120 h at 30°C. Open symbols indicate reducing sugar concentration, and closed symbols indicate either herbicidin A concentration or dissolved oxygen tension (DOT). Agitation rate: 300 rpm (▽, ▼); 400 rpm (○, ●); 500 rpm (□, ■); 600 rpm (△, ▲).

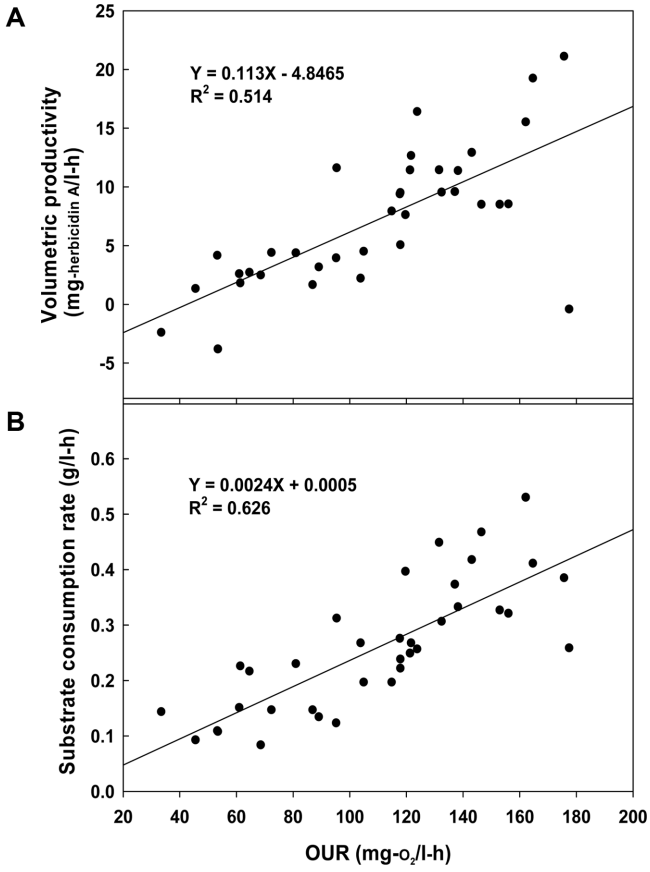

Fig. 3. Correlation between oxygen uptake rate and volumetric productivity (**A**), and between oxygen uptake rate and substrate consumption rate (**B**).
The linear line was generated via a simulation of experimental data.

productivity increased linearly (R^2 = 0.514) (Fig. 3A). The substrate consumption rate was positively correlated with an increasing OUR (R^2 = 0.626) (Fig. 3B). These results suggested that an increased OUR resulted in an increase in the substrate consumption rate, leading to an increase in volumetric productivity.

The biophysical parameters, DOT and OUR were closely correlated with the production of microbial metabolites. As the agitation rate increased in the 5-L bioreactor, the DOT level increased, indicating an improvement of OTR. In submerged culture of the aerobic microorganisms, oxygen availability had a significant effect on metabolite production, as high DOT improved the final yield. When dissolved oxygen was maintained at saturation levels, the production of cephamycin C in the culture of *S. clavuligerus* was much higher than that when the DOT level was controlled at 50% [29]. A reduction in DOT resulted in a significant decrease in natamycin production by *S. natalensis* [30]. In the log phase of submerged culture of *S. scopuliridis* M40, DOT levels were low owing to massive consumption of dissolved oxygen for biomass production, regardless of the agitation rate. OTR values might be lower than OUR values. In the stationary phase, when *S. scopuliridis* M40 growth stopped, the oxygen supply was balanced with the oxygen demand for herbicidin A production. As the OUR increased with the elevation of the agitation rate from 300 to 500 rpm, the herbicidin A concentration increased proportionally. At 600 rpm, the OUR value plunged below that observed at 400 and 500 rpm after 72 h, resulting in a lower concentration of herbicidin A. These results suggested that the OUR value is a critical factor for herbicidin A productivity.

Size Distribution of Hyphal Morphology

Few pellets were formed in submerged culture of *S. scopuliridis* M40. Based on the length, hyphal morphology was classified into five groups: 0–100, 100–200, 200–300, 300–500, and 500–1,000 μm. The relative abundances of hyphal lengths differed with agitation rates, indicating that mechanical force affected hyphal morphology in submerged

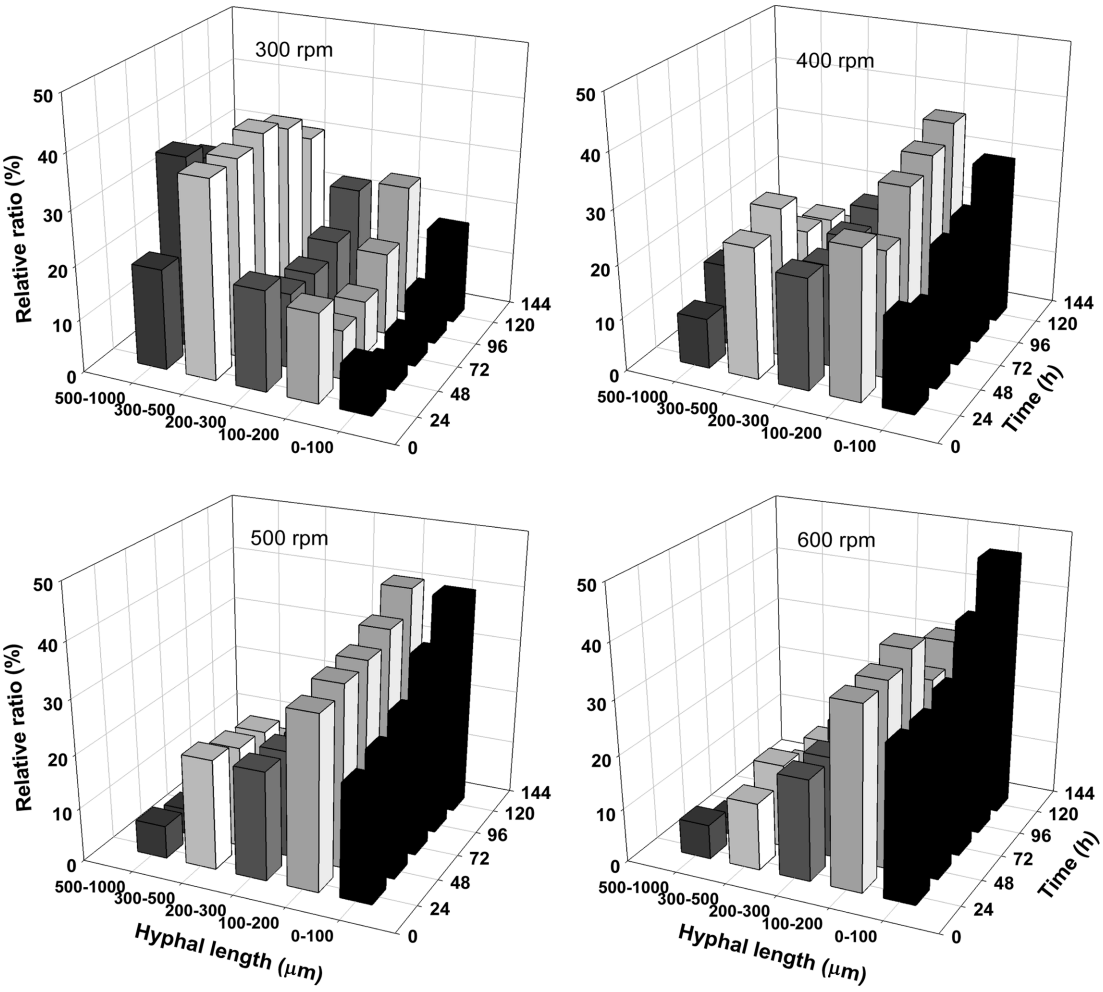

Fig. 4. Distribution of hyphal lengths under various agitation rates (*i.e.*, 300, 400, 500, and 600 rpm).

culture of *S. scopuliridis* M40 (Fig. 4). High mechanical shear force caused fragmentation of pellets or clumps, resulting in pellet disruption. At 400 rpm, the relative ratio of hyphal lengths of 0–100 μm accounted for maximally 30% throughout the culture, while it comprised at least 30% at 600 rpm. The relative abundance of 100–200 μm was highest at 500 rpm, accounting for more than 32.2%. The ratio gradually increased from 23.7% to 36.6% at 400 rpm, while it decreased between 96 and 120 h at 600 rpm. The concentration of herbicidin A depended on the relative ratio of hyphal lengths. Hyphal lengths in the ranges of 0–100 or >200 μm were not favorable for herbicidin A production. Considering that the highest production of herbicidin A was obtained at the highest relative abundance of 100–200 μm on the basis of morphology, the agitation rate of 500 rpm was best for herbicidin A production. Correlations between the medium value of hyphal size

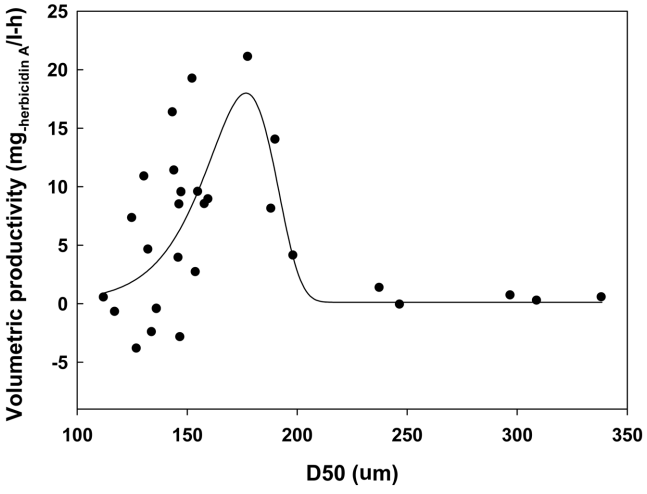

Fig. 5. Mutual relationships between the medium value of hyphal size distribution (D50) and the volumetric productivity of herbicidin A.

Table 3. Scale-up of herbicidin A fermentation from a 5-L jar to a 500-L pilot.

	Fermenters		
	5 L	50 L	500 L
Agitation rate (rpm)	500	150	90
Final herbicidin A concentration (mg/l)	956.6 ± 63.3	944.6 ± 52.4	1,001.5 ± 79.4
Volumetric productivity (mg/l·h)	7.97 ± 0.53	7.87 ± 0.44	8.35 ± 0.66

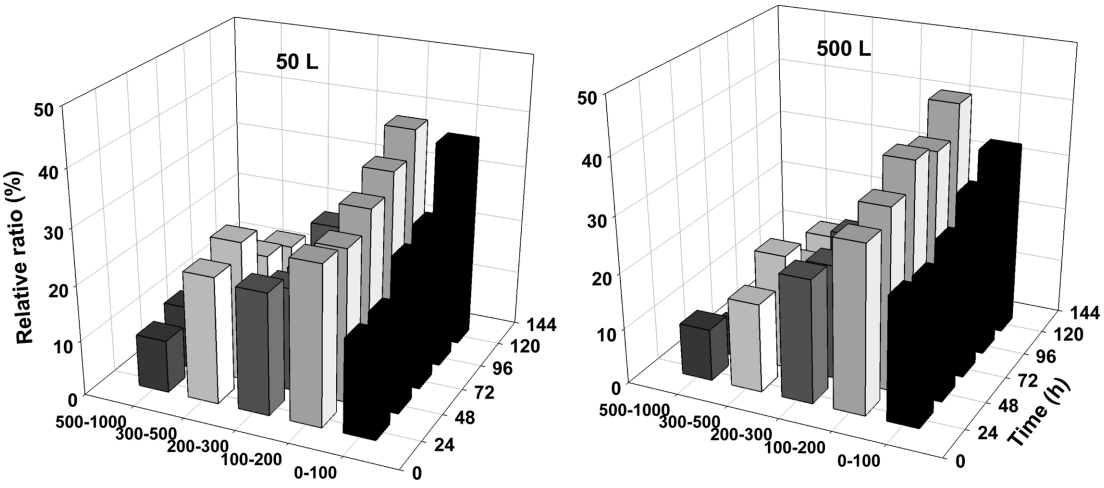

Fig. 6. Distribution of hyphal lengths in 50-L and 500-L bioreactors.

distribution (D50) and volumetric productivity further supported the importance of hyphal morphology (Fig. 5). High volumetric production of herbicidin A was obtained when D50 ranged between 150 and 180 μm. Our finding was similar to that of Tamura *et al*. [31], who indicated that tylosin production was dependent upon mycelial morphology in submerged culture of *S. fradiae*. They classified the morphology of *S. fradiae* into three types: free filament, entangled filament, and pellet. In order to maximize the productivity of tylosin from *S. fradiae*, either a small mycelial length or a small pellet was induced by controlling the mechanical shear force.

Scale-Up from a 5-L Jar to a 500-L Pilot Vessel

The production was scaled up from a 5-L jar to a 500-L pilot vessel based on a constant impeller tip speed, which had an effect on oxygen supply and hyphal length of *S. scopuliridis* M40. An agitation rate of 500 rpm in a 5-L jar corresponded to 150 rpm in a 50-L pilot and 90 rpm in a 500-L pilot, respectively (Table 3). Comparable yields of herbicidin A were obtained in the 50-L and 500-L systems, with concentrations of 944.6 ± 52.4 and 1,001.5 ± 79.4 mg/l, respectively. Volumetric productivity in a 500-L pilot was slightly higher than that in a 5-L jar, indicating successful scale-up.

Successful scale-up is essential for cost effectiveness. There have been multiple reports on scale-up strategies with constant oxygen transfer coefficient [32], constant gassed power [33], constant respiratory quotient values [34], and constant impeller tip speed [35]. Among these strategies, constant impeller tip speed was selected for scaling up in our study because the morphological character of *S. scopuliridis* M40 was important for herbicidin A production. Hyphal morphology, including hyphal length distribution, in the 50-L and 500-L pilot bioreactors was similar to that in the 5-L jar agitated at 500 rpm (Fig. 6). The relative abundance of 100–200 μm accounted for at least 28.8% throughout the cultivation. Considering the high productivity of herbicidin A at the high relative abundance of 100–200 μm, morphological control was successful in the 50-L and 500-L pilot bioreactors.

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