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Spore Inoculum Optimization to Maximize Cyclosporin A Production in *Tolypocladium niveum*

Lee, Mi-Jin[1], Han-Na Lee[1], Kyuboem Han[2], and Eung-Soo Kim[1]*

[1]*Department of Biological Engineering, Inha University, Incheon 402-751, Korea*
[1]*Hanson Biotech Co., Ltd., Daejeon 305-811, Korea*

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The cyclic undecapeptide, cyclosporin A (CyA), is one of the most commonly prescribed immunosuppressive drugs. It is generated nonribosomally from a multifunctional cyclosporin synthetase enzyme complex by the filamentous fungus *Tolypocladium niveum*. In order to maximize the production of CyA by wild-type *T. niveum* (ATCC 34921), each of three culture stages (sporulation culture, growth culture, and production culture) were sequentially optimized. Among the three potential sporulation media, the SSMA medium generated the highest numbers of *T. niveum* spores. The SSM and SM media were then selected as the optimal growth and production culture media, respectively. The addition of valine and fructose to the SM production medium was also determined to be crucial for CyA biosynthesis. In this optimized three-stage culture system, 3% of the spore inoculum generated the highest level of CyA productivity in a 15-day *T. niveum* production culture, thereby implying that the determination of an appropriate size of *T. niveum* spore inoculum plays a critical role in the maximization of CyA production.

Keywords: *Tolypocladium niveum*, cyclosporin A, spore inoculum

Cyclosporin A (CyA) is a major secondary metabolite generated by an aerobic filamentous fungus, *Tolypocladium niveum* [2, 20]. Although CyA was initially developed as an antifungal antibiotic by the Sandoz Pharmaceutical Company in the early 1970s [7], it is currently prescribed as one of the most important immunosuppressive drugs for the treatment of organ transplantees, as well as patients with autoimmune diseases, including AIDS, owing to its superior T-cell specificity and low levels of myelotoxicity [3, 21]. CyA is a cyclic undecapeptide compound, which harbors some unusual amino acids, including α-aminobutyric acid and (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (MeBmt), which is generated *via* a nonribosomal biosynthetic pathway by a multifunctional cyclosporin synthetase enzyme complex [13]. It has been fairly well documented that filamentous fungi, including *T. niveum*, undergo unique physiological and morphological differentiation in liquid cultures, to which the onset of secondary metabolite biosynthesis is closely related [10, 19, 23]. Similarly to the majority of fungal secondary metabolites, CyA is not generally produced during the vegetative mycelial growth stage, but is generated only in the later pellet production stage [13], thereby implying that *T. niveum* culture stages must be optimized in order to maximize the production of CyA. Although it has been determined that three separate *T. niveum* culture stages (sporulation culture, growth culture, and production culture) occur sequentially in the production of CyA, each *T. niveum* culture stage still requires further optimization [1, 8, 16]. Moreover, inoculum size, a critical factor in fungal metabolite productivity [11, 26], has never before been assessed in a CyA-producing *T. niveum* culture system. Therefore, we briefly describe herein the selection of an optimal *T. niveum* culture medium, in addition to the most appropriate spore inoculum size for optimal CyA productivity.

In order to select the medium that generates the highest numbers of *T. niveum* spores, equal amounts of *T. niveum* stock solution purchased from ATCC 34921 was inoculated into plates, each of which contained one of the three most commonly utilized fungal spore media: PDA (potato dextrose agar 39 g in 1 l of distilled water), MYA (malt extract 20 g, yeast extract 4 g, and agar 20 g in 1 l of distilled water) [4], and SSMA (glucose 50 g, Bacto peptone 10 g, KH_2PO_4 5 g, KCl 2.5 g, and agar 20 g in 1 l of distilled water) [5]. After 10 days of incubation at 28°C, the spores on each plate were harvested with 5 ml of 20% glycerol solution, followed by spore counting with a hemacytometer and an inverted microscope (Samwon Scientific, Co., Korea). As is shown in Fig. 1, among the three tested media, SSMA generated the highest numbers of *T. niveum* spores,

*Corresponding author
Phone: 82-32-860-8318; Fax: 82-32-872-4046;
E-mail: eungsoo@inha.ac.kr

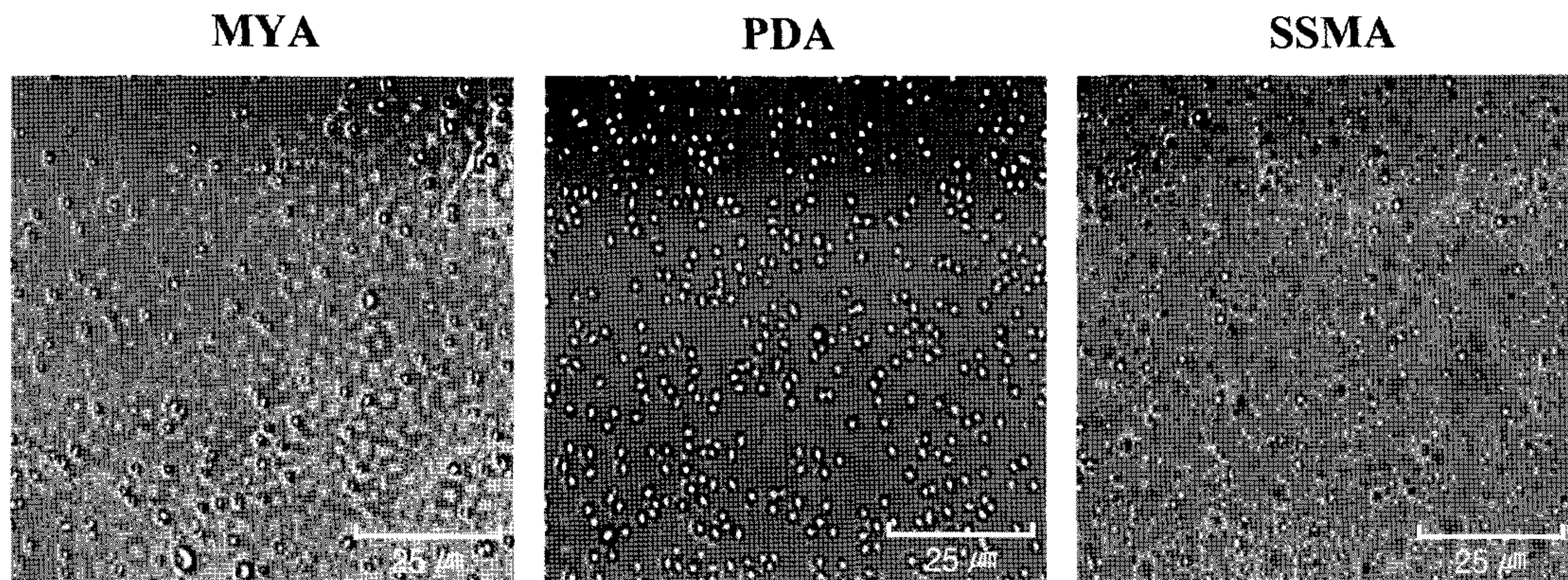

Fig. 1. Photographs of *T. niveum* spores from sporulation media.

approximately 10^9 per ml. This is approximately 100-fold higher than those seen with the MYA (5.5×10^7 spores/ml) and PDA (3.2×10^7 spores/ml).

In order to select the medium that provides maximal mycelial growth, the four most-commonly used fungal growth media were tested [12, 14, 19]. The total spores harvested from each SSMA plate in 5 ml of 20% glycerol solution were inoculated into 50 ml of each growth media, followed by 5 days of incubation at 28°C with constant shaking at 200 rpm. Microscopic observations of each culture broth showed that the spores inoculated in both FC1 [glucose 80 g, tryptone 40 g, urea 2 g, $NaNO_3$ 3 g, KH_2PO_4 2 g, KCl 0.5 g, $MgSO_4$ 0.5 g, and $FeSO_4\cdot7H_2O$ solution (10 g/l) 1 ml in 1 l of distilled water] failed to generate filamentous mycelia growth over 5 days of culture (Fig. 2). Although some of the spores from the MGP medium developed into filamentous mycelial forms, a significant portion of the spores remained ungerminated (Fig. 2). Unlike the three other growth media utilized, most of the spores inoculated into the SSM (glucose 50 g, Bacto peptone 10 g, KH_2PO_4 5 g, and KCl 2.5 g in 1 l of distilled water) [5] medium were successfully germinated into confluent filamentous mycelia, which suggests that SSM

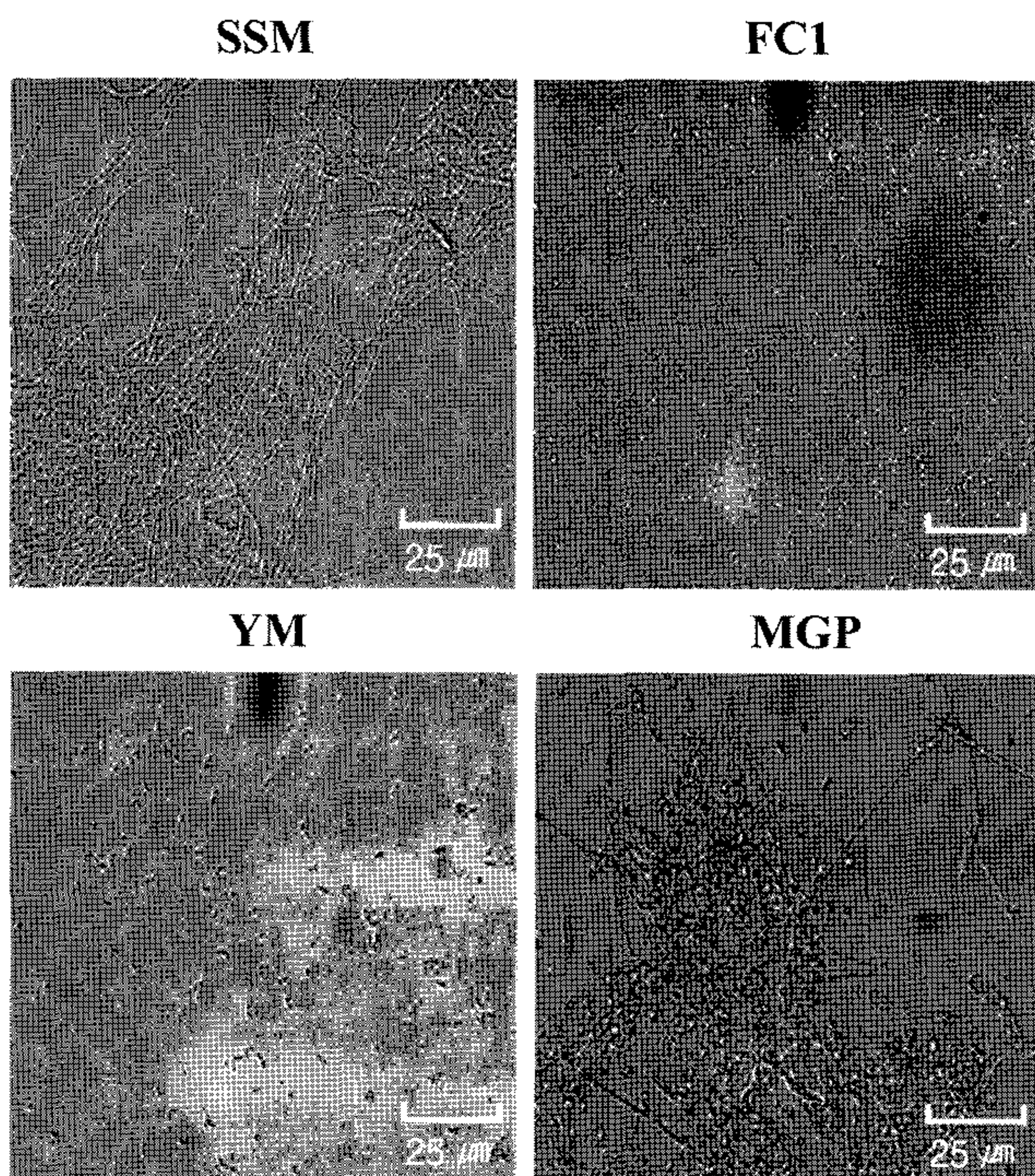

Fig. 2. Photographs of *T. niveum* mycelia from growth media.

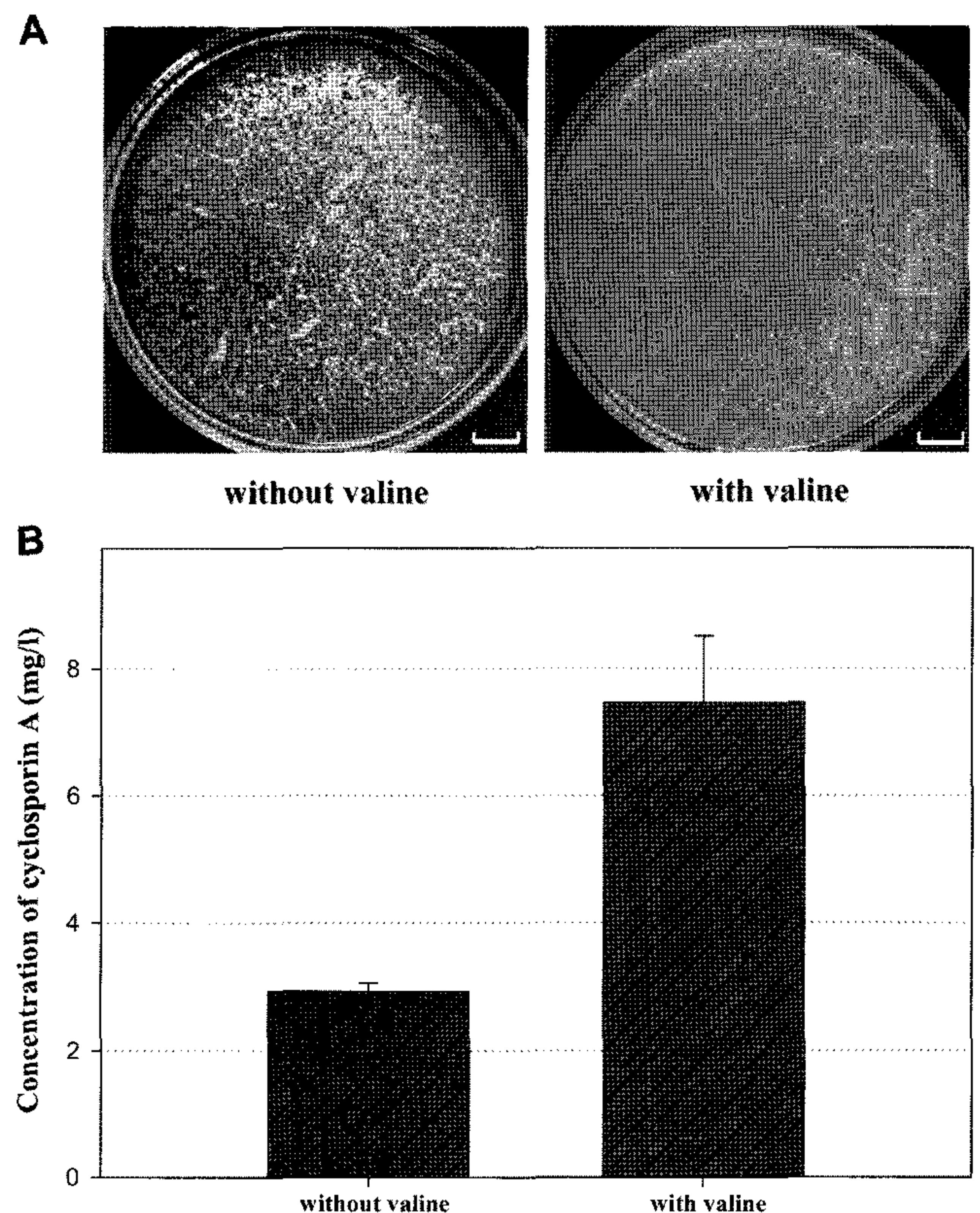

Fig. 3. *T. niveum* morphologies (**A**) and CyA productivities (**B**) with or without the addition of L-valine in production media.
The length of the marker bar in the figures represents one centimeter.

should be the most appropriate spore-germinating growth medium for *T. niveum* (Fig. 2).

In order to optimize CyA productivity in a final 15-day production culture, the most-commonly used SM production medium [glucose 30 g, $(NH_4)_2SO_4$ 10 g, KH_2PO_4 0.75 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.1 g, trace element solution 1 ml (trace element composition; $ZnSO_4 \cdot 7H_2O$ 4,400 mg, $MnCl_2 \cdot 4H_2O$ 180 mg, $NaMoO_4$ 25 mg, $CuSO_4 \cdot 5H_2O$ 80 mg, $FeSO_4 \cdot 7H_2O$ 5,000 mg in 1 l of distilled water), and H_2SO_4 2 ml in 1 l of distilled water] [5] was selected and further modified. First, glucose was replaced by fructose in order to minimize catabolite repression as well as oxygen limitation in the pellets formed during the production culture stage [18], because no CyA production was observed in the presence of glucose (data not shown). Second, 6 g/l of L-valine were further incorporated into the SM media in order to increase the precursor flux toward CyA biosynthesis. As some amino acid precursors, including L-valine, which is a necessary precursor substrate amino acid for CyA biosynthesis, are also critical for the primary metabolism of *T. niveum*, the CyA productivity was expected to be increased as the result of the addition of extra L-valine into the production medium [15]. Although addition of other amino acids necessary for CyA precursor substrates were also previously evaluated for CyA overproduction, none of these amino acids failed to stimulate the CyA biosynthesis probably due to the catabolite repression of nitrogen sources introduced by extra addition of these amino acids [15]. However, it still remains to be determined how extra addition of only L-valine, but not the other CyA precursor substrates, into the *T. niveum* culture induced variable CyA productivity. Using this modified SM production medium, *T. niveum* was shown to generate approximately 8 mg/l of CyA in a 50-ml flask culture incubated for 15 days at 28°C with constant shaking at 200 rpm, as shown by the results of quantitative analysis *via* reverse-phase HPLC (Fig. 3).

It has been previously documented that inoculum size in fungal cultures might influence spore germination, mycelial

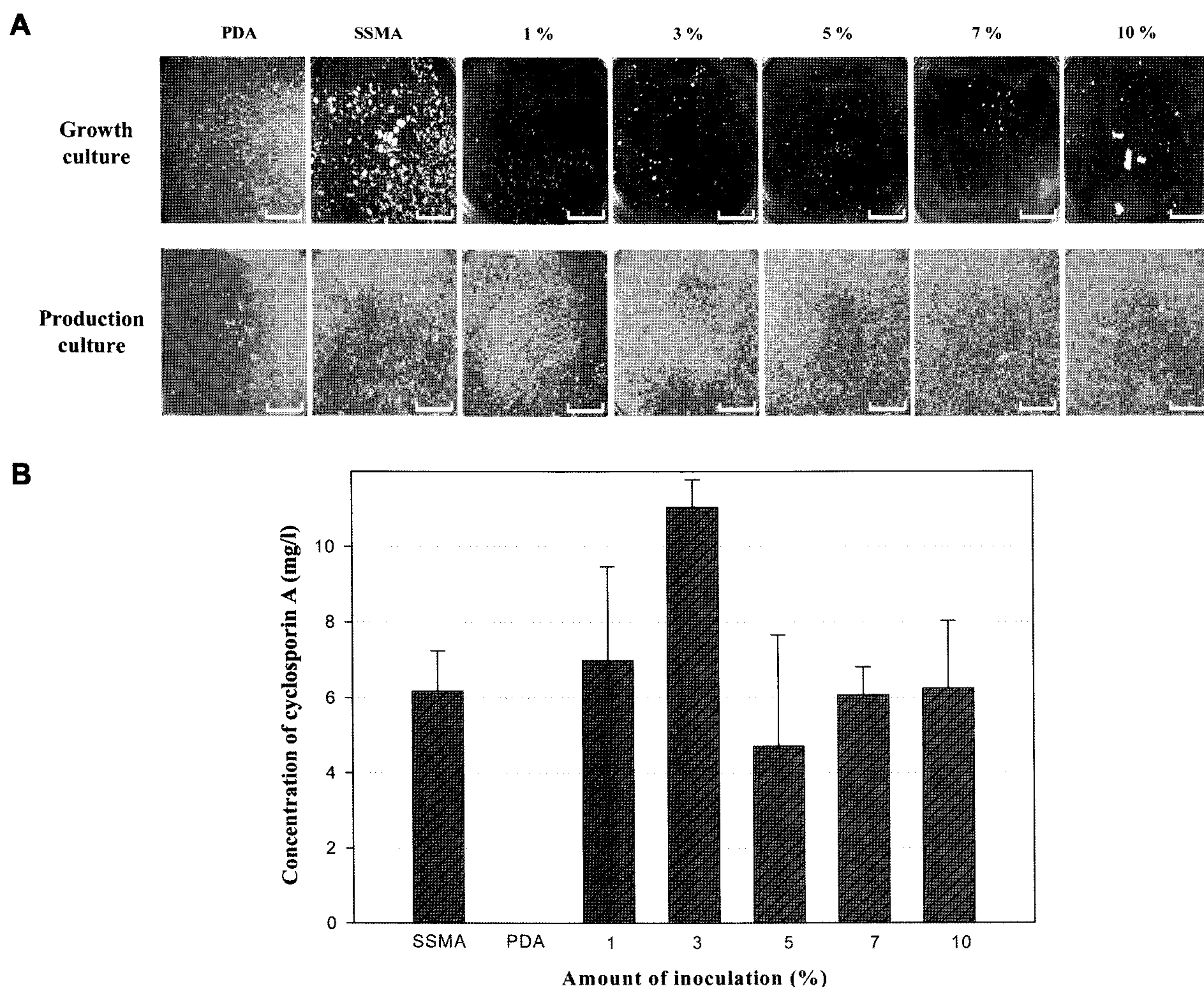

Fig. 4. *T. niveum* morphologies of growth and production cultures (**A**) and CyA productivities (**B**) depending on inoculum size. The length of the marker bar in the figures represents one centimeter.

growth, and pellet physiology [9, 18, 20]. In addition, pellet size and shape are also thought to be important with regard to secondary metabolite production as the result of the transfer limitation of both oxygen and nutrients in the fungal liquid culture [6, 17]. In order to further improve CyA productivity in this optimized three-stage culture system, the *T. niveum* spore inoculum size was finally optimized. One, three, five, seven, and 10% (v/v) of each *T. niveum* spore stock solution, each containing approximately 10^9 spores per ml, were individually inoculated into SSM growth medium, followed by production culturing and HPLC analysis. Although all of the culture systems tested here generated an average of approximately 5–6 mg/l of CyA, the culture inoculated with 3% of spore stock solution generated the highest CyA productivity, of more than 10 mg/l (Fig. 4). Less than 3% of *T. niveum* spore inoculation in the production culture apparently induced a prolonged lag phase resulting in delayed mycelial growth, which eventually lowered CyA productivity. However, more than 3% of spore inoculation appeared to stimulate germination too profoundly in a fixed culture volume, thereby resulting in the limitation of both oxygen and nutrients in the *T. niveum* production culture. In conclusion, 3% of the spore inoculum in this optimized three-stage culture system generated the highest CyA productivity in a 15-day *T. niveum* production culture, thereby implying that the determination of an appropriate *T. niveum* spore inoculum size plays a critical role in the maximization of CyA production.

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