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Enhancing the sporulation of *Streptomyces kasugaensis* by culture optimization

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Abstract−To be an effective microbial biocide, *Streptomyces kasugaensis* should be converted into spore during cultivation process for successful long-term storage. By statistical design methods, culture conditions including medium components and operating parameters were optimized and more than 100 times increase in spore yield was achieved. Addition of spent culture fluid (100 ppm), EDTA (30 ppm), mycophenolic acid (32 ppm) with combination of pH upshock (5.5 to 8.5) increased total viable cell and spore conversion rate, resulting in 1.6×10^7 (spore/mL) in 5 days of culture in a fermenter. This result provides a practical method for obtaining high spore number for commercial production of *Streptomyces kasugaensis* as a microbial pesticide.

Key words: Biocontrol Agent, Sporulation, Statistical Experimental Design, *Streptomyces kasugaensis*

INTRODUCTION

The overuse of chemical pesticides to control or prevent plant diseases causes soil pollution and has harmful effects on human beings. To reduce the use of the chemicals, one possibility is to utilize active microorganisms. It is ideal if the microorganisms that produce biological pesticides stably inhabit the environment as non-dominant species but maintain their effectiveness [1]. 'Biocontrol' is defined as organisms whose specific biological roles as anti-fungal, anti-insect, and anti-microbial activities are uniquely pathogenic to detrimental organisms in terms of farming, livestock production and human health [2,3]. Therefore in-situ biological viability of 'biocontrol' is an essential factor to be an effective 'biocontrol'.

Streptomycetes, in which 70-80% of antibiotics are produced, are Gram-positive bacteria characterized by their versatile ability to produce secondary metabolites as well as their endospore formation. These endospores are resilient structures capable of surviving desiccation, heat, oxidizing agents, and UV and radiation. These qualities allow for long-term storage and relatively easy commercialization of biocontrol agents [4-6]. Although the sporulation of *Bacillus* sp. has been reported previously, the strategies for sporulation of *Streptomyces* sp. during high density cultivation have received less attention and have not yet been examined in detail [7,8].

In this study, we report the optimization of culture conditions that promote sporulation of *Streptomyces ksaugaensis* determined by a statistical experimental design method. Under optimum conditions, more than 100 times increase in spore yield was achieved.

MATERIALS AND METHODS

1. Strain, Cultivations and SCF (Spent Culture Fluid) Preparation

S. kasugaensis isolated from soils was used as a source biocontrol agent. Spores were harvested by adding 10 mL of 0.85% saline on the solid media containing agar (15 g/L) and cultivated at 30 °C for 2-3 weeks. After cultivation for 7 days in the liquid media, cells were stored at −70 °C until use. The culture medium for the formulation of submerged spores was composed of 1 g/L yeast extract, 1 g/L beef extract, 2 g/L casamino acid, and 10 g/L dextrose. The stocked solution (1%) was inoculated at 30 °C in 250 mL Erlenmeyer flasks containing 50 ml Bennett medium. The fermentation conditions were as follows: 1 L working volume in a 2.5 L jar fermenter, temperature 30 °C, initial pH 6.0, agitation speed 250 rpm and aeration rate 1.0 vvm. The Bennett medium was supplemented with different concentrations of mycophenolic acid, SCF, amino acid, and ethylenediamine tetra acetic acid (EDTA) after sterilization.

SCF was prepared as follows. After cultivation of *S. kasugaensis* for 7 days, suspended cells were disrupted by sonication (Ultrasonication, Branson sonifier 450, U.S.A, 100-150W). Cell debris was removed by rough filtration by using cotton and then centrifuged at 8,000 rpm for 15 min at 4 °C. The supernatant was filtered by ultrafiltration (Amicon ultrafiltration cell, MWCO; 100,000 Da). By using chroloform (1 : 1 v/v) extraction, SCF was obtained in solid form.

2. Optimization of Media Composition

Among the seven factors for increasing cell growth and sporulation in the submerged culture, the significant components were ultimately selected based on spore number by using the Plackett-Burman design [9], which was used to select the component that efficiently increased the number of spores. Table 1 gives the high and low concentrations of the 7 components. The P-B design experiment was composed of 15 runs: 12 runs containing the combinations of high and low concentrations of each component and 3 runs with the average of high and low value of each component as a control.

3. Assays: Number of Vegetative Cells, Spores and Antagonistic Activity

The vegetative cells and spores of the cultivation broth were measured under 50 °C heat shock condition every 10 min from 0 to 40 min. Measurements were also made by ultrasonication at the same time intervals as the control. Sporulation was observed in different initial pH of 5, 6, 7, 8, 9 at 250 rpm for 7 days at 30 °C in a shaking incubator. A substitute of expensive beef extract in the Bennett's medium consisting of corn oil, corn steep liquor, and soybean meal,

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Table 1. Concentration of factors used in P-B design

Factors / Condition	A Yeast extract (g/L)	B Soybean meal (g/L)	C Casamino acid (g/L)	D Dextrose (g/L)	E Initial pH	F Temp. (°C)	G Working volume (mL)
–	0.2	0.2	0.4	2	5	26	10
0	1	1	2	10	6	30	50
+	1.8	1.8	3.6	18	7	34	90

which were added for industrial usage, was added to the medium. The pellet of *S. kasugaensis* was dispersed by a 3-4 order ultrasonication (duty cycle; 30, output control; 4), and the vegetative cells were counted after appropriate serial dilution. The spores were counted by the same method after heat shock of 50 °C, 15 min. Samples were taken from the cultures every 24 hrs. Cell morphology was observed by an optical microscope (BX41, Olympus, Japan) and scanning electron microscope (S-4200, Hitachi, Japan).

Antagonistic activity of *S. kasugaensis* against *Fusarium oxysporum*, which caused Fusarium wilt and damping-off of the seed was measured. *S. kasugaensis* was cultured for 7 days and the culture broth was centrifuged (9,000 rpm, 4 °C, 30 min) for crude separation. After equilibration by deionized water, the supernatant was passed through HP-20 (Sigma Chemical Co.) and 30, 50, 100% isopropanol was added with a 20 mL/min flow rate, to sequentially elute each sample.

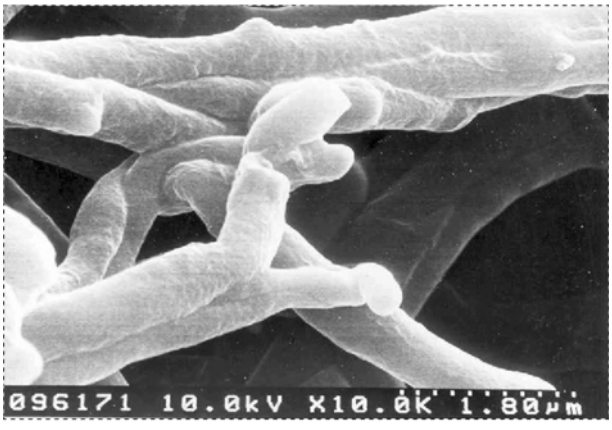

Fig. 1. Scanning electron photo of the *S. kasugaensis*; (a) a black hyphae at 96 hr (b) spores emerging from the pellet surface at 168 hr.

RESULTS AND DISCUSSION

1. Cultivation and Antagonistic Activity

S. kasugaensis was cultivated in Bennett's medium for 10 days. The morphological changes were monitored by light microscope. *S. kasugaensis* formed a pellet, which showed up black, hyphae. Also spores were observed emerging from the pellet surface. In addition, thinning and fracturing of the hyphae was observed with the SEM as submerged spores were released from the hyphae (Fig. 1). *S. kasugaensis* has a differentiation process that differs from other *streptomycetes*, which forms spores by septation [5].

After adsorption on a Diaion HP-20 column, the supernatant of *S. kasugaensis* was gradiently eluted by isopropanol. Antifungal substances were released at 50% isopropanol (Fig. 2). *S. kasugaensis* extracellularly secreted antifungal substances against *F. oxysporum*.

S. kasugaensis culture broth was obtained at 24 h and 120 h. After heat treatment at 50 °C for 10-40 min, the viable cell number was counted. As shown Table 2, spores were more stable than the vegetative cells to heat treatment and the vegetative cells dramatically decreased after being heat treated at 50 °C for 10 min. In addition,

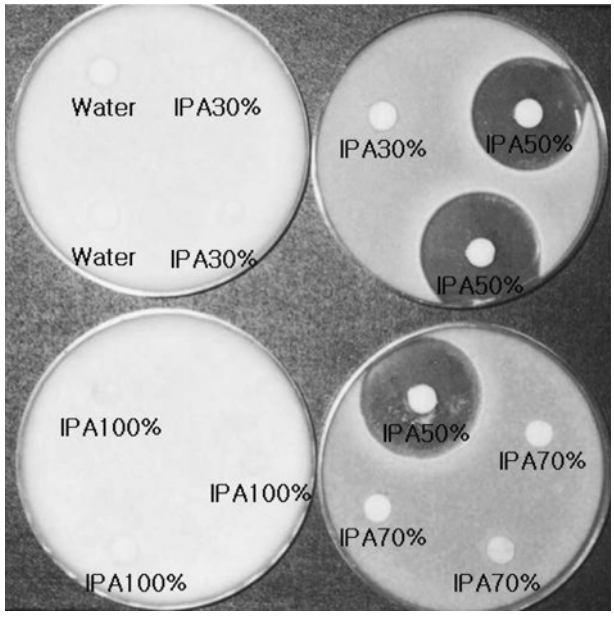

Fig. 2. Antifungal activity of each fractions from Diaion HP-20 column chromatography against *F. oxysporum*.

Table 2. Effect of heat treatment on the survival of submerged spores and vegetative hyphae treated by heating at 50 °C

Culture time / Heating time	Vegetative mycelia ($\times 10^6$/mL) (24 hr)	Spore ($\times 10^6$/mL) (120 hr)
Control	5.50	6.25
10 min	0.0006	0.06
20 min	n.d*	0.016
30 min	n.d	0.013
40 min	n.d	0.005

*n.d; not determined

Table 3. Effect of initial pH for sporulation and mycelia formation of *S. kasugaensis* (cfu/mL)

Initial pH	Mycelia cell number ($\times 10^7$)	Spore cell number ($\times 10^5$)
5	1.39±0.050	2.55±0.13
6	2.62±0.012	38.4±4.9
7	1.24±0.059	6.0±1.0
8	0.21±0.025	0.0317±0.0076
9	0.12±0.015	0.00375±0.00025

spores were more resistant to ultrasonication and the viable cell number was high after 30 sec of treatment. Cell distribution was essential for viable cell number counting because *S. kasugaensis* grew in the form of a pellet. Therefore, heat (50 °C, 10 min) and ultrasound (30 s) can be used to discriminate between vegetative cells and spores.

2. Medium Optimization to Increase Spore Conversion

After 5 days cultivation, the viable cell number and sporulation were high at low initial pH (pH 5-6). However, sporulation was low at high pH. Final pH changed to approximately 7 in all cases. Initial pH of the Bennett medium, which is usually used for sporulation of *streptomycetes*, is 5.6-5.8 (Table 3). In addition, sporulation of *streptomycetes* induced well at a low initial pH [10].

Most industrial media are composed of complex components such as yeast extract, peptone, soy bean meal, casein etc. Therefore, it is difficult to determine the C/N ratio of these components. A statistical experimental design, named the Plackett-Burman experimental design (PBD), is very useful in identifying the important nutrients for cell growth or enzyme production [11-13]. A statistical experimental design, PBD (Plackett-Burman Experimental Design) was applied to find positive factors for high cell density cultivation of *S. kasugaensis*. Main factors were selected by PBD for optimization of *S. kasugaensis* sporulation. As shown in Table 1, the PBD method was composed of 15 culture flasks: twelve flasks containing a combination of high and low concentrations of each factor, and three control flasks containing a mean value of each factor. Spore number was used as response for calculation of the main effect (Fig. 3). Dextrose at a high concentration increased the spore number. However, high pH and working volume decreased the spore number of *S. kasugaensis*. The optimum dextrose concentration was 30 g/L for increasing spore formation (Table 4).

DPA and divalent cation Mg^{2+} enhanced sporulation about 3 times (Table 5). DPA incorporated with a divalent cation increased the cell wall hardness, protease synthesis and dehydration during sporulation.

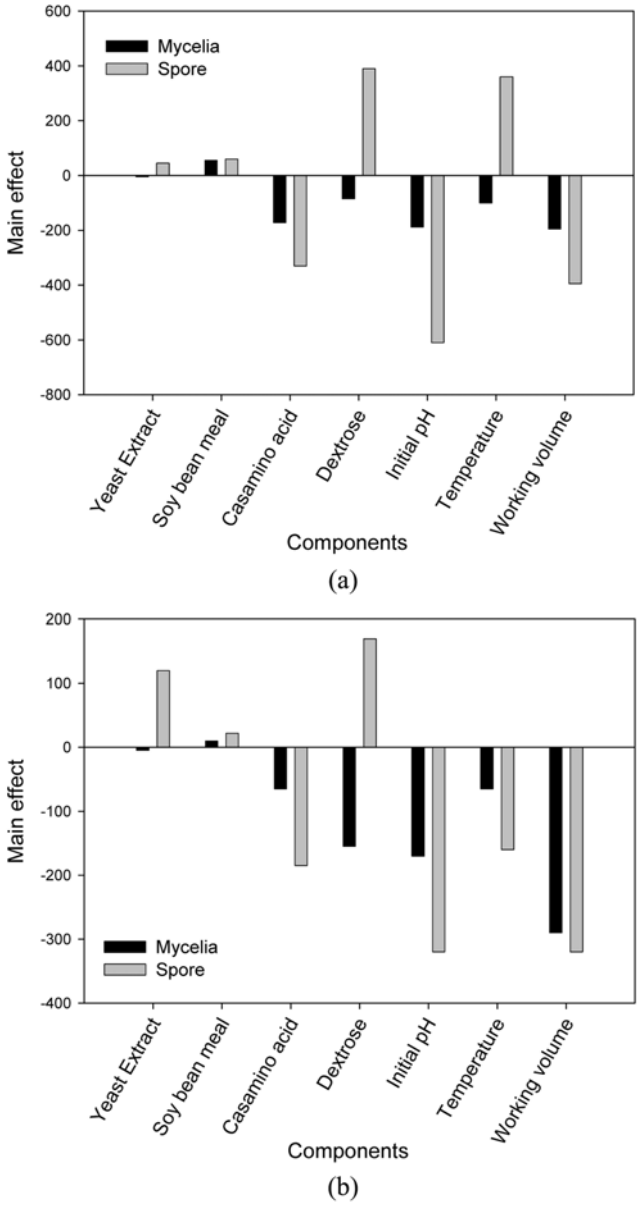

Fig. 3. Main culture components determined by Plackett-Burman Design (a) at 3 days and (b) at 5 days cultivation.

Mycophenolic acid (32 mg/L) was added to the medium at 0, 12, and 24 h, and the spore number was counted. In addition, at 12 h, during the early exponential phase, the spore number increased 10-fold in comparison with the control (Table 6). As mycophenolic acid inhibits IMP (inosine-5-monophosphate) dehydrogenase guanine triphosphate (GTP) level indirectly decreases and sporulation enhances [14-17].

Streptomycetes formed a pellet during submerged cultivation. The pellet formation was caused by high concentration of divalent cation, especially $MgCl_2$ [18]. During pellet formation, cell lysis occurred at the inner pellet due to undersupply of nutrients and oxygen [4,19]. A high concentration of $MgCl_2$ enhanced pellet formation and decreased cell number. However, a chelator, EDTA, enhanced sporulation and cell number. The results suggest that inhibition of pellet formation increased the total cell number and sporulation (Fig.

Table 4. Effect of initial concentration of dextrose for sporulation and mycelia formation (after 7 days of cultivation) of *S. kasugaensis* (cfu/mL)

Initial concentration (g/L)	Mycelia cell number ($\times 10^7$)	Spore cell number ($\times 10^5$)
Control	0.01±0.002	*n.d
5	0.74±0.200	0.29±0.010
10	1.18±0.080	1.22±0.191
20	1.70±0.076	2.29±0.525
30	1.03±0.058	7.05±0.423
40	1.53±0.391	2.91±0.140

*n.d; not determined

Table 5. Effect of DPA and divalent cations on the sporulation of *S. kasugaensis*

Enhancers of sporulation	Spore number (10^4 cfu/mL)
No addition	7.67±0.67
*DPA	7.25±0.23
DPA+$MnCl_2$	8.77±0.67
DPA+$MgCl_2$	14.83±0.25
DPA+$CaCl_2$	3.47±0.55
$CaCl_2$	2.03±0.21

*DPA; Dipicolinic acid, 8.25 mg/L
Concentration of divalent cations; 8.25 mg/L

Table 6. Effect of mycophenolic acid and its addition times on the sporulation of *S. kasugaensis*

Addition time (hr)	Average of spore forming unit (cfu/mL, $\times 10^5$)
No addition	1.78±0.46
0	1.67±0.76
6	2.78±0.26
12	12.33±1.0
18	3.05±0.13
24	1.50±0.25

Concentration of mycophenolic acid; 32 mg/L

4). During cultivation of *S. kasugaensis* in a jar excessive foam was generated, which caused the cells to be released from the jar and attach to the wall (data not shown). Therefore, it was difficult to count the exact cell number. To control excessive foam generation, an organic type antifoam agent, Antifoam 204 (Sigma Chemicals, St. Louis, MO), was added to the medium. When more than 500 mg/L of Antifoam 204 was added, vegetative cell growth was inhibited; however, sporulation slightly increased (data not shown). When *S. kasugaensis* was cultivated in a 2.5 L jar without controlling pH and DO, sporulation was affected by pH (Fig. 5). Spore number dramatically decreased at pH 4 and 8, and a high spore number was observed at pH 5 and 6. Also, Fig. 5 shows that viable cell number was decreased during pH up-shock. This result is similar to initial pH effect. We found that this strain was more affected by pH rather than DO and a weak acidic condition was important in sporulation. The effect of the sharp change of pH on sporulation was investi-

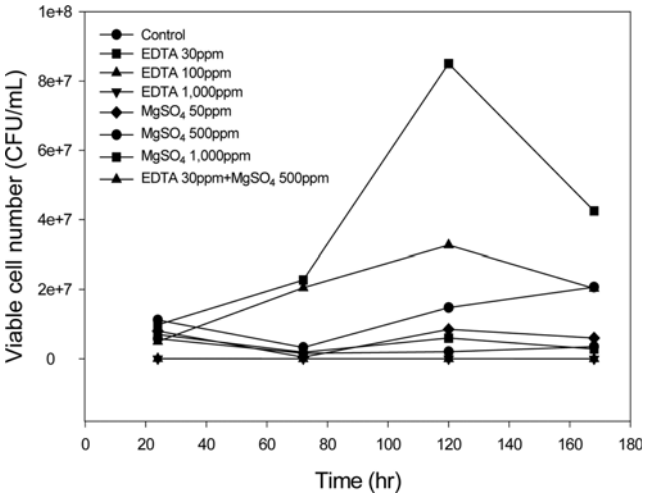

Fig. 4. Effect of EDTA and $MgSO_4$ on the sporulation of *S. kasugaensis*.

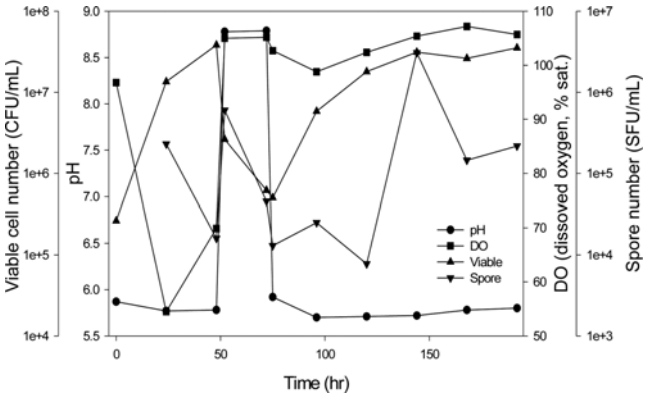

Fig. 5. Effect of pH up-shock on cell growth in batch fermentation of *S. kasugaensis*.

gated because sporulation of *S. kasugaensis* was mainly affected by pH. *S. kasugaensis* was cultivated with pH control 7 and 5.6-6.0 for 2 days and pH down-shock (pH 3.4-4.0, 1 day) and up-shock (pH 8.6-9.0) were applied, respectively. The pH up-shock was more efficient rather than down-shock, where the spore number increased 2 times and the conversion ratio was high. The pH down-shock was less efficient because *S. kasugaensis* sporulated in a weak acidic condition (data not shown). This result means that a spore is more pH-sensitive than a vegetative cell because of its intracellular acidic condition. In other words, pH-shock is effective for spore formation in *Streptomyces* sp. [20].

Streptomyces sp., *Bacillus* sp., and yeast secreted and accumulated several signal factors during submerged cultivation [4]. Especially, A or C-factor of these factors in culture broth which *Streptomyces* sp. secreted induced sporulation [21]. SCF containing A-factor was obtained according to the method described in the materials and methods section and added to medium with/without amino acid. When SCF was solely added to the medium, the spore number increased at 500 ppm of SCF. However, when 10 ppm of proline was added simultaneously, spore number increased significantly and the sporulation ratio increased to 12.7% at 100 ppm of SCF (Table

Table 7. Effect of SCF and proline concentration on spore number

Additive (concentration, ppm)	CN (cfu/mL, $\times 10^7$)*	SN (cfu/mL, $\times 10^4$)**	Conversion rate (%)***
No addition	1.46	0.025	0.002
SCF (100)	1.38	0.100	0.007
SCF (500)	1.34	6.250	0.463
SCF (100)+Proline (10)	0.54	68.333	12.719
Proline (10)	1.28	7.825	0.611

*CN; Viable cell number

**SN; Spore cell number, $\times 10^4$

***Conversion rate (%)=(Spore number/Mycelia cell number)×100

7). We suggested that *S. kasugaensis* also secreted a signal factor, which induced sporulation in the medium. Furthermore, when amino acids were added to SCF, total cells increased 3.5 times and the spore number also increased 5 times in case of arginine after 7 days of cultivation (Fig. 6). The effect of SCF, DPA, $MgCl_2$, EDTA, and mycophenolic acid on cell growth was tested and the results are summarized in Table 8. Combinations 4, 9, and 15 show high total cells and spores. The highest number of spores was observed at combination 15, which increased 3 times compared with control. To enhance sporulation, first the pellet formation was inhibited for promotion of sporulation so that other factors such as SCF and amino acid could be applied. Combination 15 and pH up-shock were simultaneously applied to the fermenter cultivation. After 4 days, the spore number reached 1.6×10^7 (spore/mL) and the conversion ratio was 17%. In the optimum condition, both the viable cell number and the number of spores increased (Fig. 7).

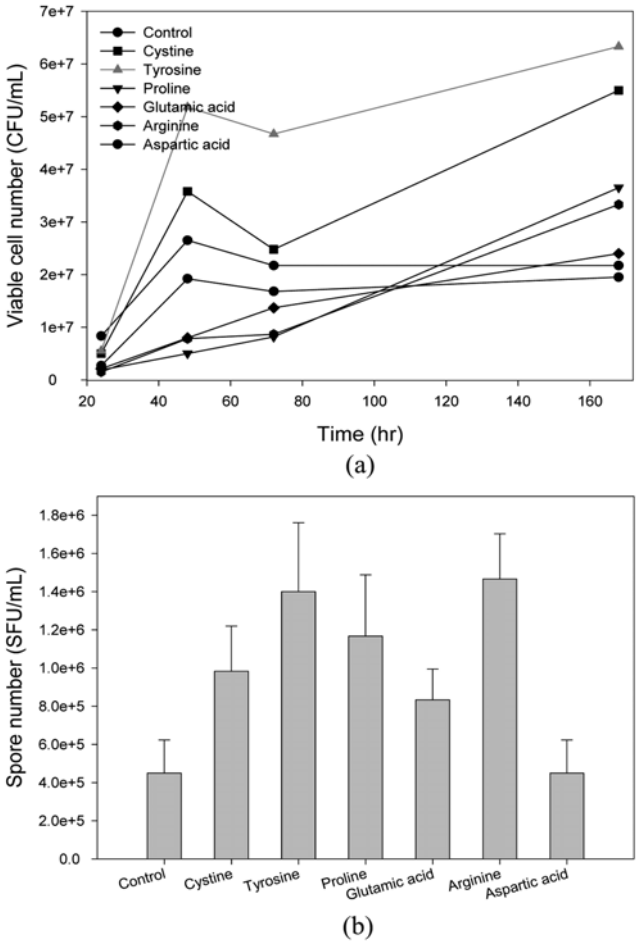

Fig. 6. Effect of amino acids (a) on cell growth and (b) on spore number.

Table 8. Effect of combination SCF and chemical agents on cell growth at 7 days

Runs	Combination***	MCN (cfu/mL, $\times 10^7$)*	SN (cfu/mL, $\times 10^6$)**	Conversion rate (%)
1	Control	2.42 (±0.029)	0.65 (±0.250)	2.69
2	D′	2.75 (±0.087)	0.73 (±0.208)	2.66
3	SCF	0.45 (±0.050)	0.12 (±0.013)	2.74
4	EDTA	4.83 (±0.289)	1.15 (±0.260)	2.38
5	M·A	2.25 (±0.391)	1.30 (±0.304)	5.78
6	D′+SCF	0.77 (±0.226)	0.15 (±0.013)	1.89
7	D′+EDTA	2.22 (±0.306)	0.63 (±0.153)	2.86
8	D′+M·A	0.55 (±0.132)	0.13 (±0.010)	2.33
9	SCF+EDTA	6.83 (±0.289)	1.35 (±0.087)	1.97
10	SCF+M·A	0.50 (±0.150)	0.11 (±0.035)	2.20
11	EDTA+M·A	2.30 (±0.132)	1.13 (±0.202)	4.93
12	D′+SCF+EDTA	3.12 (±0.076)	1.30 (±0.180)	4.17
13	D′+SCF+M·A	1.31 (±0.153)	0.58 (±0.144)	4.43
14	D′+EDTA+M·A	1.60 (±0.397)	0.58 (±0.153)	3.64
15	SCF+EDTA+M·A	5.17 (±1.607)	1.42 (±0.375)	2.74
16	D′+SCF+EDTA+M·A	0.92 (±0.202)	0.26 (±0.023)	2.85

*MCN; Mycelia cell number, $\times 10^7$

**SN; Spore cell number, $\times 10^6$

***M·A; 32 mg/L mycophenolic acid, D′; 8.25 mg/L DPA+8.25 mg/L $MgCl_2$, EDTA; 30 mg/L ethylenediaminetetraacetic acid, SCF; 500 mg/L spent culture fluid

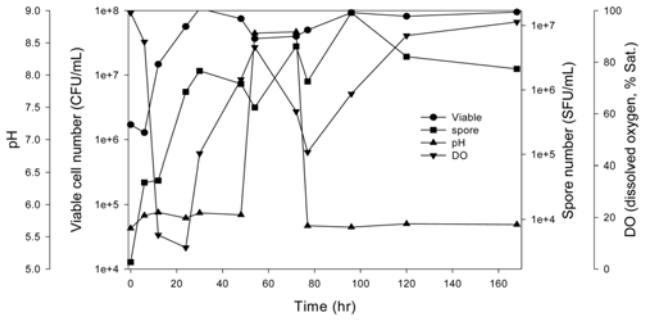

Fig. 7. Time profile of batch cultivation under optimized condition.

CONCLUSIONS

S. kasugaensis excretes antifungal substances against *F. oxysporum* which causes damping-off on plants and forms an endospore in self-defense. Endospores are resilient structures capable of surviving desiccation, heat, oxidizing agents and UV radiation. This property allows spores suitable for long-term storage and, hence, provides advantages for commercialization of microbial pesticide.

In this study, we reported the optimum culture conditions that promote sporulation of *Streptomyces ksaugaensis* by a statistical experimental design method for increasing the spore number. Significant increase in the conversion rate and final spore yield could be achieved under optimized conditions. This method can provide a practical means for the commercial production of *Streptomyces ksaugaensis* as a microbial pesticide.

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