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Isolation of *Streptomyces* sp. YU100 Producing Extracellular Phospholipase D

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Abstract Soil samples were screened for actinomycete strains capable of producing phospholipase D, and a strain, *Streptomyces* sp. YU100, showing a high transphosphatidylation activity was isolated. This strain secreted phospholipase D in a culture broth after 12 h of cultivation, and its productivity continued to increase for 36 h of fermentation. In addition, its transphosphatidylation rate of phosphatidylcholine to phosphatidylserine was almost 68% within 1 h. The morphological and chemotaxonomical characteristics showed that this strain could be classified as a number of the *Streptomycetaceae* family, particularly due to the spiral form of its spore chain consisting of 60–70 smooth spores (0.75×1.0 μm) on an aerial mycelium, FA-2c type of fatty acid profile in the cell wall, and LL-DAP component in the cell wall peptidoglycan. A phylogenetic analysis of the 16S rDNA provided a clue that the strain YU100 was actually a member of the genus *Streptomyces*, because the determined sequence exhibited a higher homology with *Streptomyes* sp. ASB27, *S. peucetius* JCM9920, and *S. griseus* ATCC10137. A dendrogram based on the 16S rDNA sequences also showed a phylogenetic relationship between the strain YU100 and these strains. However, the strain YU100 has not yet been assigned to a particular species, because of absence of any other classified species with a high matching score.

Key words: Phospholipase D, transphosphatidylation, *Streptomyces* sp., phylogeny, 16S rDNA

For industrial utilization of waste lecithin (phosphatidylcholine; PC) from the soy-oil industry, its enzymatic conversion has been attempted to produce functional phospholipids including phosphatidylserine or phosphatidylethanolamine, which are known to improve brain functions, especially in the elderly [13]. Accordingly, microbial phospholipase D from *Streptomyces* species, including *S. hachijoensis* [16], *S. lydicus* [23], *S. antibioticus* [21], *S. chromofuscus* [7], and *Streptoverticillium cinnamoneum* [15], has been studied for the last two decades.

The enzyme phospholipase D catalyzes two reactions: one is the hydrolysis of lecithin to phosphatidic acid and choline, and the other is the transphosphatidylation of lecithin to functional phospholipids by interconversion of polar headgroups with serine, ethanolamine, or glycerol. The enzyme is widely distributed in animals, plants, and microorganisms [11]. Cabbage phospholipase D is the most abundant in nature, yet it shows a very low transphophatidylation activity [2, 31]. In contrast, microbial phospholipase D, especially from *Streptomyces* sp., is known to have a relatively higher transphosphatidylation activity than those enzymes from other organisms [9].

The current study attempted to isolate actinomycete strains producing extracellular phospholipase D from soil samples from the areas of Daegu and Gyongsan. Consequently, one *Streptomyces* species was isolated, and its cultural and morphological characteristics are presented herein along with a chemotaxonomic and phylogenetic study.

Materials and Methods

Reagents

The standards including L-α-phosphatidycholine (PC) (Type XVI-E, 99% pure, from fresh egg yolk), L-α-phosphatidyl-L-serine (PS) (98% pure, from soybeans), and DL-α,ε-diaminopimelic acid (DAP) (98% pure, containing LL-, DD-, and meso-isomers), as well as the choline oxidase and peroxidase, were purchased from Sigma Chemical Co., MO, USA. All other reagents were chemical pure grades.

Isolation of Actinomycetes Producing Phospholipase D

Soil samples were collected from the humus layer of the soil in Daegu and Gyongsan, Korea. The soil samples were

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pretreated by air-drying for 3 days. The serially diluted sample suspensions were then spread onto a Bennett's agar medium (1.0% glucose, 0.1% yeast extract, 0.2% peptone, and 0.1% beef extract, pH 7.0) containing nalidixic acid (20 μg/ml), and incubated for 3 weeks at 28°C. Single colonies of actinomycetes were then transferred into an egg yolk selection agar for the identification of lipolytic activity [27]. Those colonies exhibiting cloudy zones were then inoculated into an ISP 2 medium (0.4% yeast extract, 1.0% malt extract, and 0.4% glucose, pH 7.2) at 28°C for 5 days with shaking at 200 rpm, and the phospholipase D activity in the fermentation broth was checked.

Determination of Enzyme Activity

The hydrolytic activity of phospholipase D was determined by measuring the amount of choline released from PC, as described in previous reports [6, 23]. The reaction mixture containing 0.1 ml PC emulsion (10 ml of distilled water was added to 500 mg of PC dissolved in 1 ml of diethyl ether and then sonicated on an ice bath), 0.1 ml of 0.1 M sodium citrate buffer (pH 6.0), 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 7.5% Triton X-100, and 0.1 ml of the enzyme was incubated at 37°C for 10 min. The released choline was treated with choline oxidase and peroxidase, colorized with 4-aminoantipyrine, and then assayed spectrophotometrically at 500 nm. One unit of enzyme was defined as the amount required to release 1 μmol of choline from PC at 37°C for 1 min.

The transphosphatidylation reaction was carried out at 25°C in 2.0 ml reaction solution containing 1 μmol of PC in diethyl ether, 2 mmol of L-serine in 0.2 ml of 0.2 M sodium acetate buffer (pH 6.0), and 0.2 ml of the enzyme solution, and it was stopped by adding 0.12 ml of 1.6 M citric acid. The reaction mixture was then extracted by the addition of diethyl ether:ethanol (4:1, v/v), and loaded on a high performance thin layer chromatography (HPTLC) plate (Silicagel 60 F_{254}, Merck KGaA, Germany) [5, 26]. After developing with chloroform:methanol:7 N ammonium hydroxide (130:60:8, v/v), the spots were visualized by iodine vapor.

Analysis of Morphological and Cultural Characteristics

In order to determine the actinomycete genus, several cultural characteristics were determined by the procedures of the International *Streptomyces* Project (ISP), as suggested by Shiring and Gottlieb [25], and Bergey's Manual of Systematic Bacteriology [12]. To examine the spore morphology using scanning electron microscopy (SEM) (S-4100, Hitachi, Japan), the strain was cultivated on an ISP 2 agar medium (1.0% malt extract, 0.4% yeast extract, 0.4% glucose, 2.0% agar, pH 7.2) at 30°C for 21 days. The specimens for SEM were prepared according to the method of Williams and Davies [29], and the spore chain morphology observed was then evaluated, based on the categories developed by Pridham *et al.* [17].

Analysis of DAP Isoform in Cell Wall Peptidoglycan

The form of DAP isomer in the cell wall was determined by cellulose thin layer chromatography (TLC) according to the method of Yamada and Komagata [30]. A freeze-dried biomass (ca. 20 mg) was hydrolyzed with 1 ml of 6 N HCl in a screw capped bottle at 121°C for 30 min. After filtering the hydrolysates through filter paper, the filtrate was dried under a vacuum until the odor of HCl was no longer detected. The residue was then redissolved in 0.1 ml of distilled water and loaded onto a cellulose TLC plate (20×20 cm, Merck KGaA, Germany) with 0.01 M DL-α,ε-DAP standard. The plates were developed in methanol:distilled water:10 N HCl:pyridine (80:26.25:3.75:10, v/v), and visualized with 0.2% ninhydrin solution in acetone.

Analysis of Fatty Acid Methyl Esters (FAMEs)

To examine the fatty acid pattern in the cell wall, their methyl esters (FAMEs) were prepared using alkaline methanolysis according to the protocol of Miller and Berger [14], after being cultivated on a trypticase soy broth at 28°C for 48 h with shaking at 125 rpm. The FAMEs extracted with hexane/methyl-*t*-butyl ether (1:1, v/v) were subjected to gas chromatography (HP 5890A, Hewlett-Packard Co., CA, U.S.A.) equipped with a flame ionization detector and methyl silicone-fused silica capillary column (25 m×0.2 μm). The FAMEs were identified by their retention times using the Microbial Identification System software package (Sherlock verision: 2.95, MIDI Inc., DE, U.S.A.), and expressed as percentages relative to the total peak area.

Phylogenetic Analysis of 16S rDNA Sequences

The 16S rDNA of strain YU100 was amplified by polymerase chain reaction (PCR) in a 20 μl mixture containing 2–3 ng of chromosomal DNA, 1 μM of each primer (forward; CCGAATTCGTCGACAACAGAGTTTGATCCTGGCT-CAG, reverse; CCCGGGATCCAATCTTAAGGAGGTG-ATCCAAGCC), and 0.2 units of TaKaRa Ex Taq™ polymerase (Takara, Japan), using a thermal cycler (GeneAmp PCR System 2400, PE Applied Biosystems, CT, U.S.A.) which was programmed as follows: first denaturation of 2 min at 95°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 42°C, and 4 min at 72°C, and then one final cycle for extension at 72°C for 10 min [1, 18]. The amplified PCR products (1.5 kb) on the agarose gel were purified using a QIAEX II gel extraction kit (Qiagen Inc., Germany). The DNA fragment was inserted into a pGEM-T Easy vector (Promega Corporation, WI, U.S.A.) by T4 DNA ligase, and the ligated mixture was then used to transform *E. coli* XL-1 Blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*446 *thi relA*1 *lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn10 (*tet*^{r})]) [20]. Using the white colonies on LB media (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.2) containing 50 μg/ml ampicillin, 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG), and 0.04% 5-bromo-4-chloro-3-indolyl-D-galactopyranoside

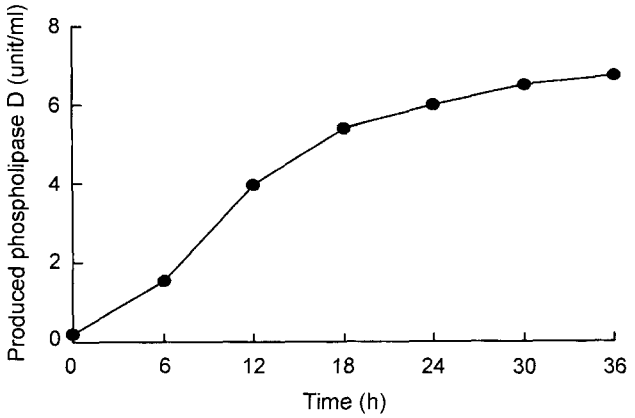

Fig. 1. Fermentation profile for the production of phospholipase D by strain YU100.
Strain YU100 was cultivated in 300 ml of a fermentation medium at 28°C. At 6 h interval, 10 ml was recovered, and the hydrolytic activity of phospholipase D was assayed in a culture broth.

(X-gal), the plasmid with the PCR product was isolated. Two PCR fragments of 0.9 kb and 0.6 kb in the isolated plasmid were subcloned again into a pUC19 vector by cutting with *Eco*RI enzyme, thereby transforming the same strain for nucleotide sequencing. The 16S rDNA sequence was read using a ABI PRISM 377 DNA Sequencer (PE Applied Biosystems, CT, U.S.A.) with a BIG Dye Terminator. The sequences were then analyzed by the BLAST search program (http://www3.ncbi.nlm.nih.gov/BLAST/). Dendrograms were constructed using the neighbor-joining method based on a distance matrix calculated using the Clustal W and Jalview software (http://www.ebi.ac.uk/~michele/jalview/) [19].

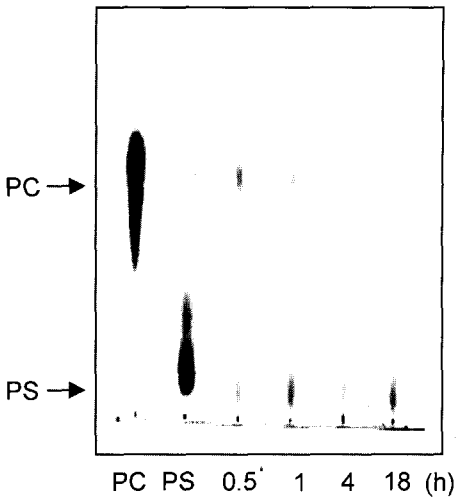

Fig. 2. Confirmation of transphosphatidylation activity of strain YU100.
After cultivating strain YU100 in a fermentation medium at 28°C for 3 days, the culture broth was subjected to a transphosphatidylation reaction using phosphatidylcholine and L-serine. The reaction mixture was then resolved on an HPTLC plate of Silica gel, as described in Materials and Methods. The numbers noted under the HPTLC plate indicate the reaction time. PC, phosphatidylcholine; PS, phosphatidylserine.

RESULTS AND DISCUSSION

Isolation of Strain YU100 as Producer of Phospholipase D

In order to screen the strains producing phospholipase D from soil samples collected in the Daegu and Gyongsan areas, the colonies of actinomycetes were first isolated on a Bennett's agar plate, and more than 1,000 strains with high lipolytic

Table 1. Certain cultural characteristics of *Streptomyces* sp. YU100[1].

Medium[2]	Growth	Spore formation	Aerial mycelium	Substrate mycelium
ISP 2 agar	very well	-	white, poor	yellow
ISP 4 agar	well	gray, poor	white, well	white
ISP 5 agar	poor	-	-	pale yellow
ISP 7 agar	well	-	-	pale yellow
Glucose asparagine agar	very poor	-	white	pale yellow
Glucose nitrate agar	poor	-	white	white
Glucose peptone agar	poor	-	-	yellow
Nutrient agar	well	-	-	white
Tryptone yeast extract agar	well	-	-	white
Corn starch agar	well	gray, poor	white	yellow
Bennett's agar	very well	-	white, poor	pale yellow

[1]The cultural characteristics were observed after cultivation on each agar plate at 28°C for 7 days.
[2]ISP 2 agar, 0.4% yeast extract, 1% malt extract, 0.4% glucose, 2% agar; ISP 4 agar, 1% soluble starch, 0.2% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, 0.2% $CaCO_3$, 1.2% agar, 0.1 % (v/v) of trace salt's solution (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnCl_2 \cdot 4H_2O$, and 0.1% $ZnSO_4 \cdot 7H_2O$); ISP 5 agar, 0.1% glycerol, 0.1% L-asparagine, 0.1% K_2HPO_4, 2% agar, 0.1 % (v/v) of trace salt's solution; ISP 7 agar, 1.5% glycerol, 0.05% L-tyrosine, 0.1% L-asparagine, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 2% agar, 1% (v/v) of trace salt's solution; Glucose asparagine agar, 1% glucose, 0.05% L-asparagine, 0.05% K_2HPO_4, 1.5% agar; Glucose nitrate agar, 0.54% glucose, 0.15% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 2 ppm of thiamine, 2% agar; Glucose peptone agar, 1% glucose, 0.2% peptone, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1.5% agar; Tryptone yeast extract agar, 0.5% tryptone, 0.3% yeast extract, 2% agar; Nutrient agar, 0.5% peptone, 0.3% beef extract, 1.5% agar; Corn starch agar, 3% corn starch, 0.3% yeast extract, 1.6% agar.

activity (a cloudy zone on the egg yolk selection agar) were selected. Finally, these strains were tested for their phospholipase D activity in a culture broth. One strain, named YU100, with high hydrolytic activity and transphosphatidylation activity, was eventually chosen for further study.

Production and Reaction Profile of Phospholipase D from the Strain YU100

In the fermentative study, it was observed that the strain YU100 secreted phospholipase D in the culture broth after 12 h of cultivation on an ISP 2 medium, and as shown in Fig. 1, its productivity continued to increase for 36 h of fermentation. The enzyme activity finally reached nearly 7 units/ml of broth. In addition, the transphosphatidylation of PC to PS was also as high as 68% within 1 h (Fig. 2).

Morphological and Chemotaxonomical Characteristics of Strain YU100

To classify the species of the strain YU100, its cultural characteristics were examined on several media by the procedures of the International *Streptomyces* Project (ISP) [12, 25]. As shown in Table 1, the isolated strain YU100 grew well on ISP 2, ISP 4, ISP 7, nutrient agar, tryptone yeast extract agar, and corn starch agar media, as well as Bennett's agar medium, and exhibited tough leathery colonies, white-yellowish substrate mycelia, white aerial mycelia, poor spore formation, and nondiffusible pigment production on all the agar plates tested after 7 days of cultivation. A gray-colored aerial spore mass was rarely observed. Using SEM, the configuration of the spore chains of the strain YU100 grown on the ISP 4 agar was found to be a spiral form of spore chain, consisting of 60–70 smooth spores (0.75×1.0 μm) on an aerial mycelium (Fig. 3).

By the analysis of the cell wall hydrolysate on a cellulose TLC plate, the type of DAP in the cell wall peptidoglycan of the strain YU100 was identified as LL-DAP, which is typical for the *Streptomycetaceae* family.

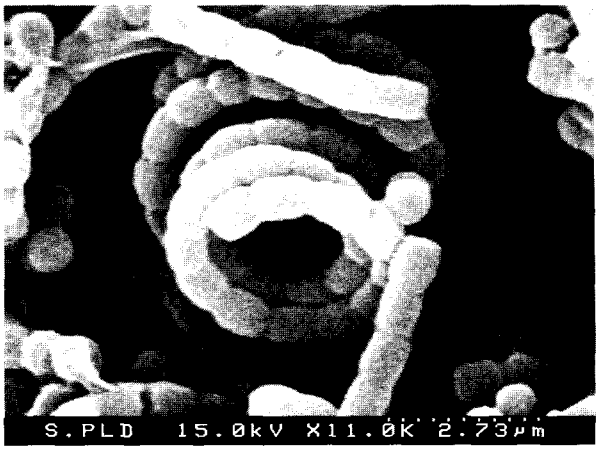

Fig. 3. Scanning electron microphotograph of the spore chain of strain YU100.
After cultivation on an ISP 2 agar medium at 28°C for 21 days, a photograph of the spore chain was taken by scanning electron microscopy.

Using a FAMEs analysis, the fatty acid profile of the strain YU100 was confirmed to be FA-2c type (Table 2). According to the classification of Kropenstedt [10], this kind of fatty acid profile is also a common feature in the *Streptomycetaceae* family. Therefore, it was concluded that the strain YU100 belonged to the *Streptomycetaceae* family.

Phylogenetic Analysis of Strain YU100 Using 16S rDNA Sequence

Even though the 16S rDNA of the genus *Streptomyces* are highly conserved, some regions are variable, therefore, the 16S rDNA sequences in these regions were compared as a powerful tool for deducing the phylogenetic relationships within the *Streptomycetaceae* family [18, 28]. Thus, the sequence of a 1.5 kb fragment of 16S rDNA from the strain YU100 was amplified using forward and reverse primers, and the sequences were determined and then compared. In a BLAST analysis, the determined 16S rDNA sequence of

Table 2. Summary of morphological and chemotaxonomical characteristics of the strain YU100.

Morphological characteristics	Strain YU100
Colony shape[1]	tough, leathery
Spore chain morphology[1]	spiral chain consisting of 60–70 spores
Spore size[1]	0.75×1.2 μm
Spore surface ornamentation[1]	smooth
Color of spore mass[1]	gray
Pigmentation of substrate mycelium[2,3]	yellow-brown
Melanin pigment production[2]	negative
Diffusible pigments[3]	negative
Chemotaxonomical characteristics	
Diaminopimelic acid	LL-DAP iso 16,
Fatty acids	Anteiso-15/17, type 2c

[1]Both 14 day-old and 21 day-old cultures on an ISP 2 agar were observed by light microscopy as well as scanning electron microscopy.
[2]The pigment was observed after incubation on an ISP 7 agar for 4 days.
[3]The pigment was detected after incubation on an ISP 5 agar for 14 days.

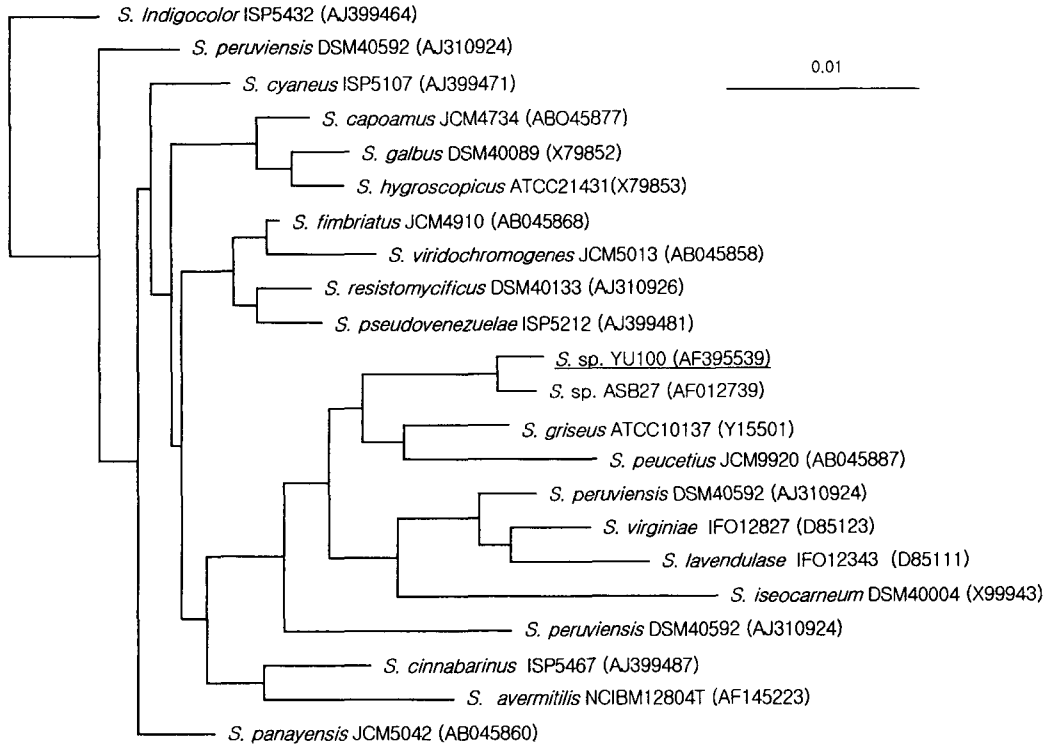

Fig. 4. Phylogenetic tree of *Streptomyces* species based on 16S rDNA sequence.
The dendrogram showing the phylogenetic relationships between *Streptomyces* sp. YU100 and other *Streptomyces* species was prepared by the neighbor-joining method in the Jalview program [16]. The scale bar on the rooted tree indicates a 0.01 substitution per nucleotide position.

strain YU100 (GenBank accession number, AF395539) showed 99% homology with that of *Streptomyes* sp. ASB27 (GenBank accession number; AF012739), 97% with that of *S. peucetius* JCM9920 (AB045887), and 97% with that of *S. griseus* ATCC10137 (Y15501). A dendrogram constructed on the basis of the 16S rDNA sequences also showed that there was a close phylogenetic relationship between *Streptomyces* sp. YU100, *S. peucetius*, and *S. griseus* (Fig. 4). This suggests that the strain YU100 is actually a member of the genus *Streptomyces*. However, it is still very difficult to assign a species, because no other species with similar taxonomic characteristics has yet been found.

Table 3. Fatty acid composition of total membrane lipid extracts from the strain YU100.

Fatty acid[1]	%	Fatty acid	%
$C_{14:0}$ iso	9.79	$C_{14:1}$ trans 9/Cis 9	2.13
$C_{15:0}$ iso	6.70	$C_{15:0}$ ante-iso	45.04
$C_{15:0}$	1.84	$C_{16:0}$ iso	12.34
$C_{16:0}$ ante-iso 2OH	1.29	$C_{16:0}$	6.38
$C_{17:1}$ ante-iso C	1.62	$C_{17:0}$ iso	2.88
$C_{17:0}$ ante-iso	7.66	$C_{17:0}$ cyclo	1.77
$C_{17:0}$ iso 3OH	0.57	$C_{14:1}$ trans 9/cis 9	2.13

[1]The description of the fatty acids is noted as the carbon numbers and number of double bonds. The type of branched-chain is also classified as an iso or ante-iso form.

Industrial Application of *Streptomyces* sp. YU100

Until now, several *Streptomyces* species have been investigated for their potential industrial application in the production of functional phospholipids [7, 15, 21-23]. Compared to these actinomycete strains, the isolated strain YU100 exhibited some typical cultural, morphological, chemotaxonomical, and phylogenetic characteristics, as confirmed by the analysis of the 16S rDNA sequences, spore-formation type, and FAME pattern.

Additionally, the final enzyme production of the strain YU100 reached 7 units/ml of broth after 36 h of fermentation, which is much higher than the production of *Streptoverticillium cinnamoneum* (1.85 unit/ml) [4] and that of *S. antibioticus* or its recombinant *Escherichia coli* strain (ca. 5 unit/ml) [8]. *Streptomyces* sp. YU100 would also be much more competitive in the production of phospholipase D than another *Streptomyces* sp. (8.3 unit/ml) [26] and *S. lydicus* (11.5 unit/ml) [24]. Accordingly, the strain YU100 appears to be promising as a new source of phospholipase D for the production of functional phospholipids.

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