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Purification and Identification of an Antifungal Agent from *Streptomyces* sp. KH-614 Antagonistic to Rice Blast Fungus, *Pyricularia oryzae*

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Abstract The actinomycete strain KH-614 possessed strong antifungal activity, especially antagonistic to the rice blast fungus, *Pyricularia oryzae*. Diaminopimelic acid (DAP) type and morphological and physiological characteristics, examined by scanning electron microscopy (SEM), indicated that KH-614 belonged to the genus *Streptomyces*. Antifungal agent produced by this strain was found to be most active, when the strain was cultured in the presence of glucose, polypeptone, and yeast extract (PY) medium for 6 days at 27°C. Based on the spectral report data, MS and NMR, the antifungal agent was identified as cyclo(L-leucyl-L-prolyl). According to the antimicrobial activity test measured by minimal inhibitory concentration (MIC), the cyclo(leu-pro) exhibited the activity against *Candida albicans* IAM 4905, *Mucor ramannianus* IAM6218, *Rhizoctonia solani* IFO 6218, *Aspergilus fumigatus* ATCC 42202, *Glomerella cingulata* IFO 9767, *Trichophton mentagrophytes* ATCC 18749, and *Trichophyton rubrum* ATCC 44766, the order of MIC values were 50, 12.5, 5, 50, 25, 5, 5 μg/ml, respectively. Specifically, cyclo(leu-pro) was one of the most effective elements against *Pyricularia oryzae* IFO 5994 with the MIC value of 2.5 μg/ml, thus indicating that cyclo(leu-pro) is a potential antifungal agent.

Key words: *Streptomyces* sp. KH-614, cyclo(L-leucyl-L-prolyl), *Pyricularia oryzae* IFO

Since the beginning of the 20th century, cyclic dipeptides (also known as 2,5 diketopiperazines, DKPs, 2,5 dioxopiperazines, DOPs) including cyclo(leu-pro) have been well known. In recent years, these compounds attracted more attention, because they have therapeutic antimicrobial activity [2, 5, 11, 13, 14, 17]. Many derivatives of these compounds with a DKP ring, such as amphomycin and bicyclomycin, exhibit antiviral properties (gliotoxins) and powerful antibiotic and antitumor activities [8]. A variety of cyclic dipeptides are found in protein and polypeptide hydrolysates, as well as in cultures of yeast, lichens, and fungi [7, 15, 16, 26]. The cyclic dipeptides have been shown to exist as a simple peptide or a larger molecular complex as well as in several enzymatically synthesized members of protist and plant kingdoms [5, 13]. Among the various types of dipeptide, cyclo(leu-pro) has been shown to be produced from *Rosellinia necatrix* [3]. Previously, we reported that cyclo(phe-pro) isolated from *Streptomyces* sp. AMLK-335 contained antimicrobial activity against four pathogenic microorganisms, such as *Bacillus subtilis* IAM 1069, *Micrococcus luteus* JCM 1464, *Staphylococcus aureus* TK 784, and *Saccharomyces cerevisiae* IFO 1008, with the MIC values of 50, 12.5, 0.4, and 50 μg/ml, respectively [19].

In the present study, we described purification and identification of the antifungal agent produced by *Streptomyces* sp. KH-614 and evaluated its antimicrobial effects.

The actinomycete strain KH-614 was isolated from a soil sample which was collected in Suwon, Korea, and grown in S medium and AV medium [19] to isolate antimicrobial antibiotic. The organism was grown at 28°C on a modified Bennett's agar [9] slant and stored at 4°C. To determine the genus of actinomycete strain KH-614, the type of 2,6-diaminopimelic acid (DAP), one of the cell wall components of actinomycete mycelia, was analyzed by using the methods of the International *Streptomyces* Project (ISP) suggested by Shirling and Gottlieb [22], and *Bergey's Manual of Systematic Bacteriology* [23]. The strain KH-614 was cultured on autoclaved tryptic soy broth [19] for 7 days at 28°C by a rotary-shaking incubator. The cultured broth was filtered through a Whatman No. 1 filter paper, washed with sterilized distilled water, and freeze-dried. The dried cells (20 mg) were placed into a tightly sealed captube (13×100 mm) containing 5 ml of 6 N HCl, and hydrolyzed in a boiling water bath for 18 h. The hydrolysate was filtered with a Whatman No. 1 filter paper and evaporated to dryness to remove the residual HCl. Then this residue was dissolved in 1 ml of distilled

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water and applied onto a TLC plate (10×10 cm, HFTLC Cellulose, Merck Co. WH, St., NJ, U.S.A.). Five μl of 0.01 M DL-DAP (Sigma Co. St, Louis, MO, U.S.A.) (meso- and LL-DAP isomers), and amino acids (alanine, glycine, and glutamate) were also applied on TLC as a standard [25].

To examine the spore chain morphology, the KH-614 was incubated for 14 days on a yeast extract-malt extract agar (ISP medium 2) (4.0 g yeast extract, 10.0 g malt extract, 4.0 g dextrose, 20.0 g agar, and 1 l H_2O, adjusted to pH 7.3 before autoclaving). The spore chain morphology of the strain KH-614 was examined by using light (×400 magnification) and scanning electron microscopies (SEM) (Model S-800, Hitachi Co.; Shinzyuku, Tokyo, Japan). According to the method reported by Williams and Davies [24], the SEM specimen was prepared. The morphological categories suggested by Pridham *et al.* [18], two categories of *Rectiflexibles* and *Spirales*, were employed for evaluating

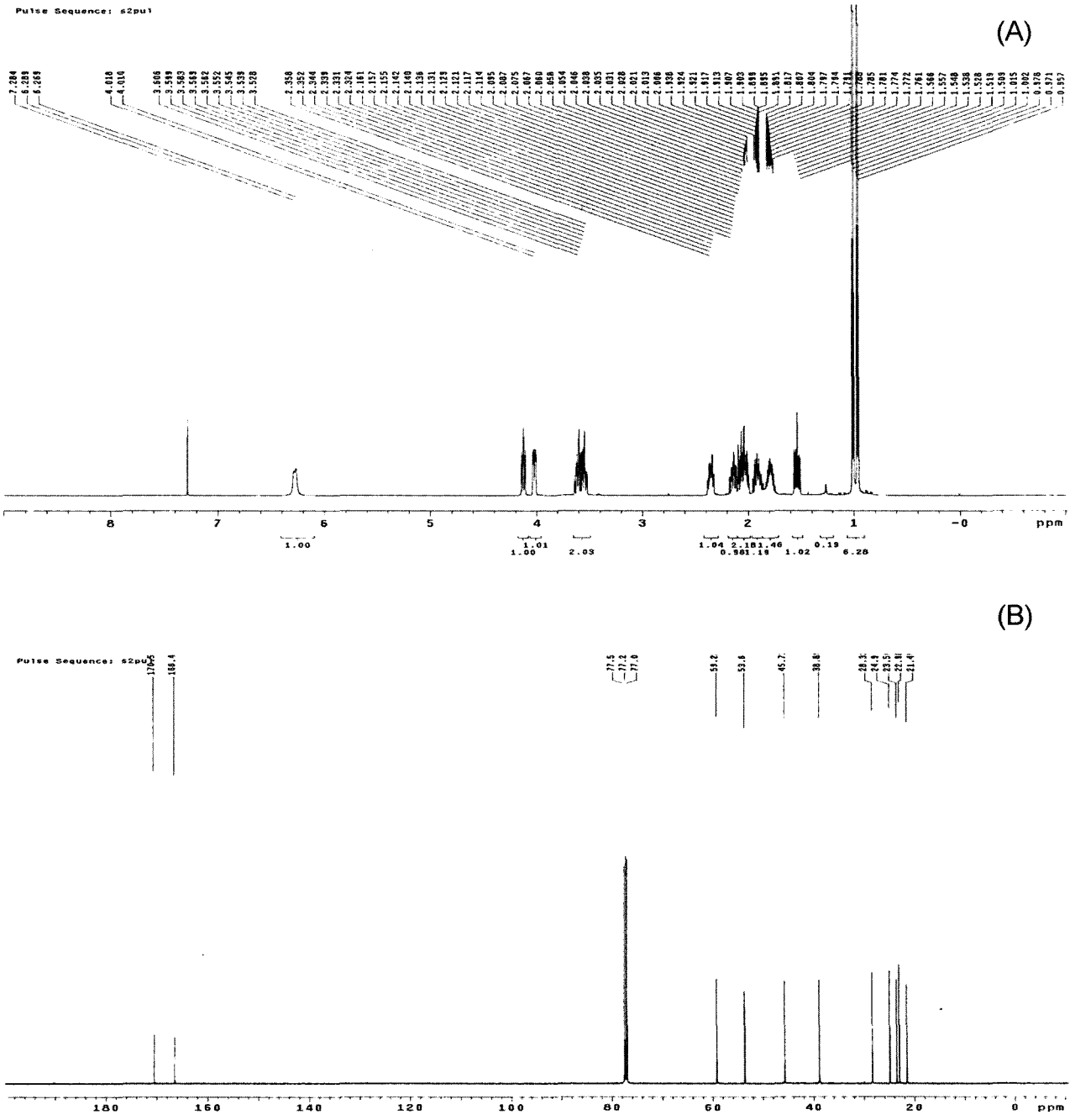

Fig. 1. ^{1}H-NMR spectrum (A) and ^{13}C-NMR spectrum (B) of the cyclo(L-leucyl-L-prolyl) isolated from *Streptomyces* sp. KH-614.

the spore chain morphology. The KH-614 cell wall hydrolysates were subjected to TLC cellulose plate analysis, and LL-DAP was found to be present in the cell wall. The KH-614 spore chain forms were a rectifiable type observed by light microscopy. Under SEM, the KH-614 had long and straight spores of cylindrical forms on aerial mycelia. The spore surface ornamentation was nodule shaped which did not belong to any of the five groups classified by Dietz and Mathews [4]. Special structures, such as a zoospore or a sporangium, were found in KH-614. Based on the type of DAP in cell wall and its morphological characteristics, KH-614 appeared to belong to the genus *Streptomyces*.

To identify the antibiotics, the antimicrobial antibiotic in the culture fluid (35-l) was applied to a Diaion HP-20 column. After washing with water and 25% methanol, the column was eluted with absolute methanol. The eluate was concentrated *in vacuo* to a small volume and extracted with ethyl acetate (5-l) at pH 7.0. The ethyl acetate layer was then concentrated to a small volume and again extracted with diethyl ether (2-l). The ether layer was completely evaporated, and residue was then dissolved in a small volume of methanol which was then applied to a Sephadex LH-20 column (2.0×90 cm), and eluted with absolute methanol. The active fractions were evaporated *in vacuo*, and further purified by preparative TLC (silica gel 60 GF 254, Merck Co.; WH, St., NJ, U.S.A.), using solvent system; n-butanol:acetic acid:H_2O=4:2:2. High-performance liquid chromatography (HPLC, Hewlett-Packard 1100, HP Co.; Palo Alto, CA, U.S.A.) was performed by using a diode-array detection system equipped with a μ-bondapak C18 column (10 μm, 150 mm×3.9 mm, Waters Co.; Milford, MA. U.S.A.) at room temperature. The system was operated at a flow rate of 0.5 ml/min with a methanol:water (80:20) solvent mixture. The purified compound was identified by GC-MS, ^{1}H- and ^{13}C-NMR, UV, and FT-IR analyses. The active antifungal compound was isolated from pure concentrated powder of the strain KH-614 using solvent extraction and chromatography, and the final yield was 37 mg from 50-l Jar fermenter^{-1} with a purity of 98% by HPLC. The structure of the purified compound was analyzed by GC-MS, ^{1}H- and ^{13}C-NMR, UV, and FT-IR. Figure 1 explains NMR data of cyclo(leu-pro), providing the identity of the compound with the authentic compound (Lot No. 0535159, Bachem, AG; Bubendorf, Swiss). Figure 2 shows the structure of cyclo(leu-pro).

Production of antimicrobial antibiotics from the KH-614 preculture was conducted by using PC II (10 g dextrose, 2 g polypeptone, 1 g yeast extract, 1 g meat extract, 0.5 g asparagines, 0.1 g thiamine ·HCl, and 1 l H_2O, adjusted to pH 7.0 before autoclaving) for 2 days, and the main culture by PY medium [5 g dextrose, 3 g polypeptone, 2 g yeast extract, 5 g meat extract, 10 g soluble starch, 10 g glycerol, 1 g casein (from milk), 2 g $CaCO_3$, 0.01 g thiamine·HCl and 1 l H_2O, adjusted to pH 7.0 before autoclaving]

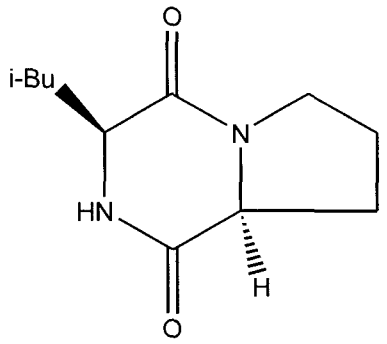

Fig. 2. Chemical structure of the isolated compound, cyclo(L-Leucyl- L-prolyl).

for 4 days. In the present study, media for test organisms were: Gram-positive bacteria [*Bacillus subtilis* IAM 1069 (NIHJ PCI 219P), *Staphylococcus aureus* IAM 1011, *Mycobacterium phlei* DIPH IFO 3518, and *Sarcina lutea*], Gram-negative bacteria (*Escherichia coli* and *Pseudomonas fluorescence* IAM 1201), yeast (*Candida albicans* IAM 4905 and *Cryptococcus neoformans* ATCC 13690), fungi (*Mucor ramannianus* IAM 6218, *Rhizoctonia solani* IFO 6218, *Glomerella cingulata* IFO 9767, *Colletotrichum gloeosporoides*, *Pyricular oryzae* IFO 5994, *Aspergilus fumigatus* ATCC 42202, *Trichophyton mentagrophytes* ATCC 18749, *Trichophyton rubrum* ATCC 44766), and algae (*Chlorella regularis* and *Chlorella* sp. A_2dl). The following media were used for each organism: bouillon agar (3 g dextrose, 10 g polypeptone, 5 g NaCl, 10 g meat extract, 10 g agar, and 1 l H_2O, pH adjusted to 7.0 before autoclaving) for *Staphylococcus aureus*, *Sarcina lutea*, and *Pseudomonas fluorescens*; glucose bouillon (3 g dextrose, 10 g polypeptone, 5 g NaCl, 10 g meat extract, 10 g agar, and 1 l H_2O, pH adjusted to 7.0 before autoclaving) for *Escherichia coli*; Arnon's medium (KH_2PO_4 1.0 g, $MgSO_4$·$7H_2O$ 1.0 g, $FeSO_4$·$7H_2O$ 0.005 g, A-5 solution 1.0 ml, yeast extract 5.0 g, glucose 20.0 g, agar 12.0 g, and 1 l H_2O, pH adjusted to 6.5 before autoclaving) for *Chlorella regularis*; Sabouraud medium (polypeptone 5.0 g, glucose 30.0 g, agar 11.0 g, and 1 l H_2O, pH adjusted to 7.0 before autoclaving) for *Candida albicans* IAM 4905; yeast-starch medium (yeast extract 2.0 g, soluble starch 10.0 g, agar 10.0 g, pH not adjusted) for *Mucor ramannianus* IAM 6218, and potato dextrose agar (PDA) (Difco Co.; Detroit, MI, U.S.A.) medium for the other organisms. The antimicrobial activity of cyclo(leu-pro) was assayed according to the conventional agar dilution method for each strain, such as glucose bouillon, bouillon, Arnon's A-5, Sabouraud, yeast-starch, and potato dextrose agar (PDA) (Difco, U.S.A.). The minimal inhibitory concentration (MIC) was determined by the conventional agar dilution method [11], which was defined as the lowest concentration that showed no visible microbial growth after 48 h of

Table 1. Antimicrobial activity of cyclo(leu-pro) produced by *Streptomyces* sp. KH-614.

Test microorganism	MIC (μg/ml)
Gram-Positive Bacteria	
Bacillus subtilis IAM 1069 (NIHJ PCI 219P)	>100
Staphylococcus aureus IAM 1011	100
Mycobacterium phlei DIPH IFO 3518	>100
Sarcina lutea	>100
Gram-Negative Bacteria	
Escherichia coli	100
Pseudomonas fluorescence IAM 1201	>100
Yeast	
Candida albicans IAM 4905	50
Cryptococcus neoformans ATCC 13690	>100
Fungi	
Mucor ramannianus IAM 6218	12.5
Rhizoctonia solani IFO 6218	5
Pyricularia oryzae IFO5994	2.5
Aspergillus fumigatus ATCC 42202	50
Glomerella cingulata IFO 9767	25
Colletotrichum gloeosporoides	100
Trichophyton mentagrophytes ATCC 18749	5
Trichophyton rubrum ATCC 44766	5
Algae	
Chlorella regularis	100
Chlorella sp. A2dl	>100

incubation. The assays were performed in triplicate for each experiment.

As shown in Table 1, the antimicrobial activity of the cyclo(leu-pro) was investigated by the MICs; we found that MICs for all the organisms were >100 to 2.5 μg/ml. However, significant susceptibility against cyclo(leu-pro) was observed in five fungi, namely, *Mucor ramannianus* IAM 6218, *Rhizoctonia solani* IFO 6218, *Pyricularia oryzae* IFO5994, *Trichophyton mentagrophytes* ATCC 18749, and *Trichophyton rubrum* ATCC 44766 which exhibited MIC values of 12.5, 5, 2.5, 5, and 5 μg/ml, respectively. The MIC values for cyclo(leu-pro) against *Candida albicans* IAM 4905, *Aspergillus fumigatus* ATCC 42202, *Glomerella cingulata* IFO 9767 were 50, 50, and 25 μg/ml, respectively. *Colletotrichum gloeosporoides*, *Staphylococcus aureus* IAM 1011, *Escherichia coli*, and *Chlorella regularis* showed low level of resistance toward cyclo(leu-pro) with an MIC value of 100 μg/ml. However, other organisms showed the higher level of resistance toward cyclo(leu-pro) with an MIC value of >100 μg/ml.

An antagonistic bacterial strain KH-614 to rice blast fungus *P. oryzae* was isolated. The strain KH-614 was identified as *Streptomyces* sp. Generally, antagonistic microorganisms such as fluorescent *Pseudomonas* and *Bacillus* sp. have shown a broad antifungal spectra against soil pathogen [1, 6, 10], and cyclo(leu-pro) from the strain KH-614 exhibited a high antifungal activity against five fungus strains. Although the antimicrobial effect of the cyclic dipeptides cyclo(phe-pro), cyclo(tyr-pro), cyclo(trp-trp), cyclo(pro-trp), and cyclo(trp-pro) have previously been isolated from *Penicillium nigricans*, *Rosellinia necatrix*, *Aspergillus ochraceus*, and *Aspergillus fumigatus*, respectively, the antimicrobial effect of cyclo(leu-pro) has never been reported from the *Streptomyces* species. Furthermore, it was found that cyclo(leu-pro) significantly exhibited antifungal activity against *P. oryzae* which causes blast disease of rice. The results of this study demonstrated that cyclo(leu-pro) displayed effective protection against a wide range of fungal pathogens, including eyespot, scab, powdery mildews, leaf spot disease, club root, dallar spot, and grey mould. Further investigations to understand the biological activity of potential fungicides of the cyclo(leu-pro) are in need.

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REFERENCES

1. Bae, D. W., Y.-S. Kwak, J.-T. Lee, D.-Y. Son, S.-S. Chun, and H.-K. Kim. 2000. Purification and characterization of a novel antifungal protein from *Paenibacillus macerans* PM1 antagonistic to rice blast fungus, *Pyricularia oryzae*. **10:** 805-810.
2. Bodansky, M., G. F. Singler, and A. Bodansky. 1973. Structure of the peptide antibiotic amphomycin. *J. Am. Chem. Soc.* **95:** 2352-2357.
3. Chen, Y.-S. 1960. Studies on the metabolic products of *Rosellinia necatrix*. Isolation and characterization of several physiologically active neutral substances. *Bull. Agric. Chem. Soc. Jpn.* **24:** 372-377.
4. Dietz, A. and J. Mathews. 1971. Clasification of *Streptomyces* spore surfaces into five groups. *Appl. Microbiol.* **21:** 527-533.
5. Forster, M. O. and W. B. Saviole. 1974. Cyclopeptides from *Rocella funciformis*. *J. Chem. Soc.* **121:** 816-820.
6. Fravel, D. R. 1998. Role of antibiosis in the biocontrol of plant disease. *Annu. Rev. Phytopathol.* **26:** 75-91.
7. Han, D.-W., H. Suh, D.-H. Lee, B.-J. Park, K. Takatori, and J.-C. Park. 2002. Detection of oleic acid biodegradation by fungi. *J. Microbiol. Biotechnol.* **12:** 514-517.
8. Heinemann, B., M. A. Kaplan, R. D. Muir, and I. R. Hooper. 1977. Amphomycin, new antibiotic. *Chemotherapy* **3:** 1239-1244.
9. Lim, D.-S., S.-K. Yoon, M.-S. Lee, W.-H. Yoon, and C.-H. Kim. 1996. Isolation and identification of *Streptoverticillium* sp. NA-4803 producing antifungal substance. *Kor. J. Appl. Microbiol.* **24:** 664-670.
10. Mckeen, C. D., C. C. Reilly, and P. L. Pusey. 1986. Production and partial characterization of antifungal substances

antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. *Phytopathology* **76:** 136-139.
11. Milne, P., M. Graz, A. Hunt, H. Jamie, and G. Grant. 1999. Antimicrobial activity of selected cyclic dipeptides. *Pharmazie* **54:** 772-775.
12. National Committee for Clinical Laboratory Standards. 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A3. *Villanova*, PA:NCCLS.
13. Ovchinnikov, Y. A. and V. T. Ivanov. 1975. Conformational states and biological activity of cyclic peptides. *Tetrahedron* **31:** 2177-2209.
14. Ottenheijm, H. C. J., J. D. M. Herscheid, G. P. C. Kerkhoff, and T. F. Spende. 1976. Preparation of episulfide DKPS from Zn^{2+} catalysed cyclization procedure. *J. Org. Chem.* **41:** 3433-3438.
15. Park, H.-J., K.-O. Hwang, and E.-S. Kim. 2002. Construction of characterization of a recombinant biolumiscence *Streptomyces* for potential environmental monitoring. *J. Microbiol. Biotechnol.* **12:** 702-706.
16. Park, R.-D., K.-J. Jo, Y.-Y. Jo, Y.-L. Jin, K.-Y. Kim, J.-H. Shim, and Y.-W. Kim. 2002. Variation of antifungal activities of kitosans on plant pathogens. *J. Microbiol. Biotechnol.* **12:** 84-88.
17. PRASAD, C. 1995. Bioactive cyclic dipeptides. *Peptides* **16:** 151-164.
18. Pridham, T. G., C. W. Hesseltine, and R. G. Benedict. 1958. A guide for classification of streptomycetes according to selected groups placement of strains in morphological sections. *Appl. Microbiol.* **6:** 52-79.
19. Rhee, K.-H., K.-H. Choi, C.-J. Kim, and C.-H. Kim. 2001. Identification of *Streptomyces* sp. AMLK-335 producing antibiotic substance inhibitory to VRE (vancomycin-resistant enterococci). *J. Microbiol. Biotechnol.* **11:** 469-474.
20. Rhee, K.-H. 2002. Inhibition of DNA topoisomerase I by cyclo(L-prolyl-L-phenylalanyl) isolated *Streptomyces* sp. AMLK-335. *J. Microbiol. Biotechnol.* **12:** 1013-1016.
21. Rhee, K.-H. 2002. Isolation of characterization of *Streptomyces* sp. KH-614 producing anti-VRE (vancomycin-resistant enterococci) antibiotics. *J. Gen. Appl. Microbiol.* **48:** 321-327.
22. Shirling, E. B. and D. Gottlieb. 1966. Methods for charaterization of *Streptomyces* sp. *Int. J. Syst. Bacteriol.* **16:** 313-340.
23. Williams, S. T., M. E. Sharpe, J. G. Holt, R. Murray, G. E. Brener, D. J. Krieg, N. R. Mouldar, J. W. Pfennig, N. P. Sneath, H. A. and J. T. Staley. 1989. *Bergey's Manual of Systematic Bacteriology*, vol. 4.
24. Williams, S. T. and F. L. Davies. 1967. Use of a scanning electron microscope for the examination of actinomycetes. *J. Gen. Microbiol.* **48:** 171-177.
25. Yamada, K. and K. Kamagata. 1970. Taxonomic studies on coryneform bacteria. II. Principle amino acid in the cell wall and their taxonomic significance. *J. Gen. Appl. Microb.* **16:** 103-113.
26. Young, P. E., V. Madison, and E. R. Blout. 1976. Cyclic dipeptides. 15. Lanthanide-assisted ^{13}C and ^{1}H NMR analysis of preferred side-chain rotamers in proline-contaning cyclic dipeptide. *J. Am. Chem. Soc.* **98:** 5365-5371.