Kor. J. Microbiol. Biotechnol.
Vol. 37, No. 3, 183–188(2009)

Isolation and Charaterization of Myxobacteria with Proteolytic Activity

Jaeyoung Kim, Jinwoo Chung[1], Kyungyun Cho[1], and Yongsub Yi*
Department of Herbal Medicine, Hoseo University, Asan 336-795, Korea
[1]Myxobacteria Bank, Department of Biotechnology, Hoseo University, Asan 336-795, Korea

Seven isolates showing strong proteolytic activity, KYC 1028, 1100, 1134, 1139, 1151, 1159, and 1182, were collected. Out of them, the broth of KYC 1134 and KYC 1139 showed the high proteolytic activity measured by azocazein. To determine 16S rDNA sequences for identification, 16S rDNA of seven isolates were amplified and compared with the 16S rDNA sequences of other myxobacteria at NCBI. It is evident from the phylogenetic tree that the isolates belong to the genus *Myxococcus*. Sharing high percentage similarity values with myxobacteria, the 16S rDNA sequences were involved in two species, *Myxococcus macrospores* and *M. Fulvus*. Biochemical characteristics of KYC 1134 broth, which showed the highest proteolytic activity, showed increased activity 8 times to seven days after culture, and protein production were increased gradually and stopped at five days. The broth had optimal temperature at 60°C for proteolytic activity, and stability of pH was ranged from pH 5 to 10, at 50°C and 60, respectively. To classify proteases being in the broth, ten inhibitors were determined and only bestatin showed 27% inhibition effect. The inhibition result demonstrates that the broth contains kinds of amino peptidases and other exopeptidases.

Key words: Myxobacteria, proteolytic activity, 16S rDNA

Proteases, which catalyze the cleavage of peptide bonds in proteins, offer the possibilities and potentials for their biotechnology application as well as commercial importance. The value of industrial enzymes, in the worldwide sales, is estimated over $ 1 billion per a year, and 75% of the industrial enzymes are hydrolytic enzymes market. Proteases represented one of the three largest groups of industrial enzyme and explained about 60% of the total enzymes market [6].

Most of sold proteases have been produced by bacteria to the genus *Bacillus* Commercial proteases of bacteria are mainly neutral and alkaline proteins [13].

Proteases have a long history of application in the food and detergent industries. Their application was developed to substitute toxic chemicals used in leather industry and to be suitable for use in the detergent industry. Also, they were genetically manipulated to generate new protease with desired properties which were developed for various application [13].

Myxobacteria are negative bacteria belonging to proteobacteria and have produced a rich source of secondary metabolites in the pharmaceutical and agrochemical industries [3, 5, 11]. To date, about 100 different natural products have been characterized and secondary metabolites are discovered for identification of novel compounds, such as epothilones, soraphen, myxochromid, chivosazol [1, 4, 7, 8, 11].

Proteolytic enzymes in myxobacteria have indicated considerable potential in application area, especially, for digestive diseases and food industry [3, 12, 18]. In Korea, myxobacteria studies are fundamental to application and have shown application potential. This work is to show application potential in study of proteolytic enzymes.

Media used for isolation of myxibacteria were CY medium (0.3% casitone, 0.1% yeast extract, 0.1% $CaCl_2 \cdot 2H_2O$), VY/2 medium (0.5% Baker's yeast, 0.1% $CaCl_2 \cdot 2H_2O$, 0.5 μg/mL cyanocobalamine, 1.5% agar), PDCY medium (0.5% casitone, 0.1% yeast extract, 0.1% $CaCl_2$, 0.24% potato dextrose, 1.5% agar). Formation of fruiting body was induced on WCX medium (10 mM 3-n-Morpholinopropanesulfonic acid [pH 7.6], 0.1% $CaCl_2 \cdot 2H_2O$, 300 μg/mL cycloheximide, 1.5% agar) [14].

For isolation of myxobacteria and morphological observation, myxobacteria were collected from soil and the

*Corresponding author
Tel: 82-41-540-5979, Fax: 82-41-541-5979
E-mail: yongsub@hoseo.edu

pieces of rotted plant inoculated on WCX medium spreading *E. coli* MC4100 at 32°C for two weeks [15]. Vegetable cells of them were observed on PDCY medium plate at 32°C for 4 days, and fruiting body and spores were observed on WCX medium spreading *E. coli* MC4100 at 32°C for 5 days. The morphological characteristics of them were monitored by phase-contrast microscopy for cells and spores (Nikon E600, Nikon, Japan). Fruiting body and colony edge were monitored by anatomy microscopy (Nikon SMZ1000, Nikon, Japan).

For phylogenetic analysis, genomic DNA was isolated using DNA extraction kit (QIAGEN, Germany) and 16S rDNA sequences were amplified. The primer sequences were designed for sense primer, 5'-GAGTTTGATCCTGGC-TGAG-3' and antisense primer, 5'-AGAAAGGAGGTG-ATCCAGCC-3'. PCR reaction was as followed; 1 cycle of denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and additional extension at 72°C for 10 min. The homology search of the 16S rDNA sequences was conducted using BLAST program at NCBI. Multiple alignments of sequenced nucleotides were carried out using Clustal W [19]. Neighbor joining trees were constructed in MEGA 3.0 using bootstrapping at 1000 bootstrap trials through Kimura-2 parameters [10].

For determination of protease activity, all myxobacteria were obtained from Myxobacteria Bank (Hoseo University, Asan, Korea). Myxobacteria firstly were cultured on PDCY medium with skim milk. The isolates that showed the clear zone were collected, and then the collected isolates were inoculated to produce protease in 300 mL of CY media at 120 rpm for 7 day at 25°C [17]. The cultured broth was centrifuged at 11000 x g for 30 min at 4°C. The supernatant was used to prepare protease by ammonium sulfate precipitation. The precipitated pellet was centrifuged at 1100 × g for 30 min at 4°C and dissolved in 50 mM sodium phosphate buffer [pH 7]. The dissolved protein were dialyzed and kept at the 4°C until use. Protein were measured by Bradford method [3].

To determine enzyme activity at temperature and pH, the dissolved protein was determined from 30 to 80°C by 2% azocasein and determined from pH 3 to 10 at 40, 50, and 60°C. Thermal stability also was determined by pre-incubating at 50 and 80°C for 24 hours. The specific type of protease was determined according to the method of ProteSEEKER™ kit (Geno-technology, USA). The reaction mixture was determined at 574 nm against the blank and calculated the inhibition as follows;

$$\% \text{ Protease activity present} = \frac{\text{test}}{\text{O.D. control}} \times 100$$

$$\% \text{ Protease activity inhibition} = 100 - \% \text{ protease activity present}$$

The inhibtions activity was determined by following eleven inhibitor; antipain-dihydrochloride (papain, trypsin and plasmin inhibitor), aprotinin (seine proteases inhibitor), bestatin (amino-peptidases and other exopeptidases inhibitor: not carboxypeptidases), chymostatin (α, β, γ, δ chymotrypsin inhibitor), E-64 (papain and cysteine proteases inhibitor), EDTA-Na_2 (metalloproteases inhibitor), leupeptin (serin and cysteine proteases inihibitor), pepstatin (aspartic proteases inhibitor), AEBSF (serine proteases inhibitor), phosphoramidon (thermolysin, collagenase and other metalloendoproteinases inhibitor), PMSF (serine, cysteine proteases inhibitor).

Only isolates confirmed as myxobacteria were determined proteolytic activity on medium supplemented with skim milk (Fig. 1). Out of five hundred isolates, only seven isolates, KYC 1028, 1100, 1134, 1139, 1151, 1159, and 1182, showed clear zone and were determined proteolytic activity by azocazein. Two isolates, KYC 1134 and 1139, showed high value in proteolytic activity and better 8 to 13 times in growth than other five isolates. That is, the isolates showing good growth indicated high value in proteolytic activity.

The 16S rDNA sequences of the above seven isolates were determined for identification. The amplified 16S rDNA sequences were about 1530 base pairs in length and compared with the 16S rDNA sequences of other myxobacteria at NCBI. The evolutionary tree was inferred by bootstrap

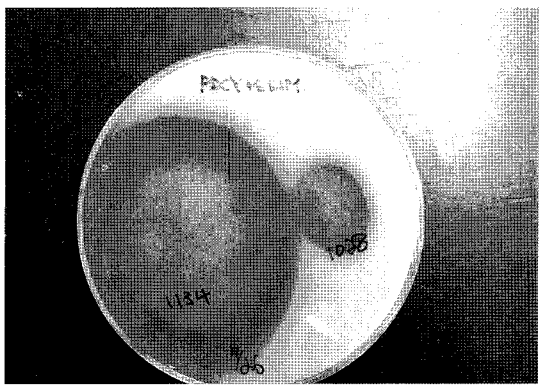

Fig. 1. Assay of proteolytic activity on PDCY medium with skim milk.

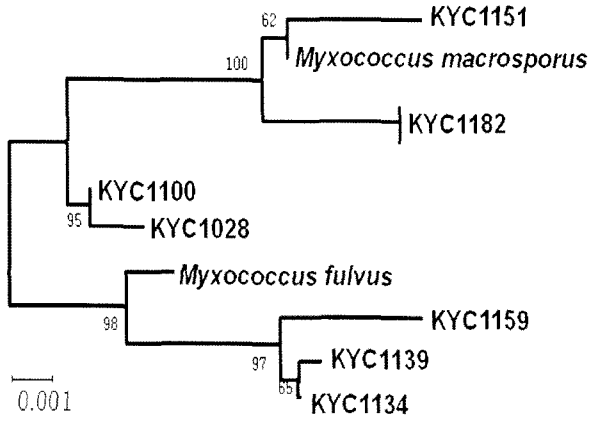

Fig. 2. Phylogenetic tree of myxobacteria showing proteolytic activity.

analysis of the neighbour-joining method based on 1000 re-samplings from the evolutionary distance data corrected by Kimura's two-parameter model [8, 15]. The CLUSTAL W program was used for multiple alignment and phylogenetic analysis [18]. It is evident from the phylogenetic tree that the isolates belong to the genus *Myxococcus* (Fig. 2). Sharing high percentage similarity values with myxobacteria, the 16S rDNA sequences of seven isolates were involved in two species, *Myxococcus macrospores* and *M. Fulvus*. Four isolates, KYC 1028, 1100, 1151, and 1182, formed a group sharing high homology of 99% with *M. macrospores*. Three isolates, KYC, 1134, 1139, and 1159, formed another group sharing high homology of 99% with *M. Fulvus* (Fig. 2). Though sharing homology of 98% in the 16S rDNA sequences, above two groups were divided having distinct nucleotides. For morphological identification of myxobacteria, the above seven isolates were observed by phase-contrast microscopy and anatomy microscopy (Fig. 3). All the isolates showed the morphological characteristics of myxobacteria.

To determine biochemical characteristics, KYC 1134 was cultured and centrifuged to collect the cultured broth. Total protein and proteolytic activity were determined during the culture. Proteolytic activity and protein production of the broth was increased 10 times after seven days, and protein production was increased higher after five days (Fig. 4). From relationship of protein production and the proteolytic activity of the broth, it could be seen that protease production was correlated with growth.

The broth had optimal temperature at 60°C for proteolytic activity, and stability of pH was ranged from pH 5 to 10, at 50°C and 60, respectively. The above results showed

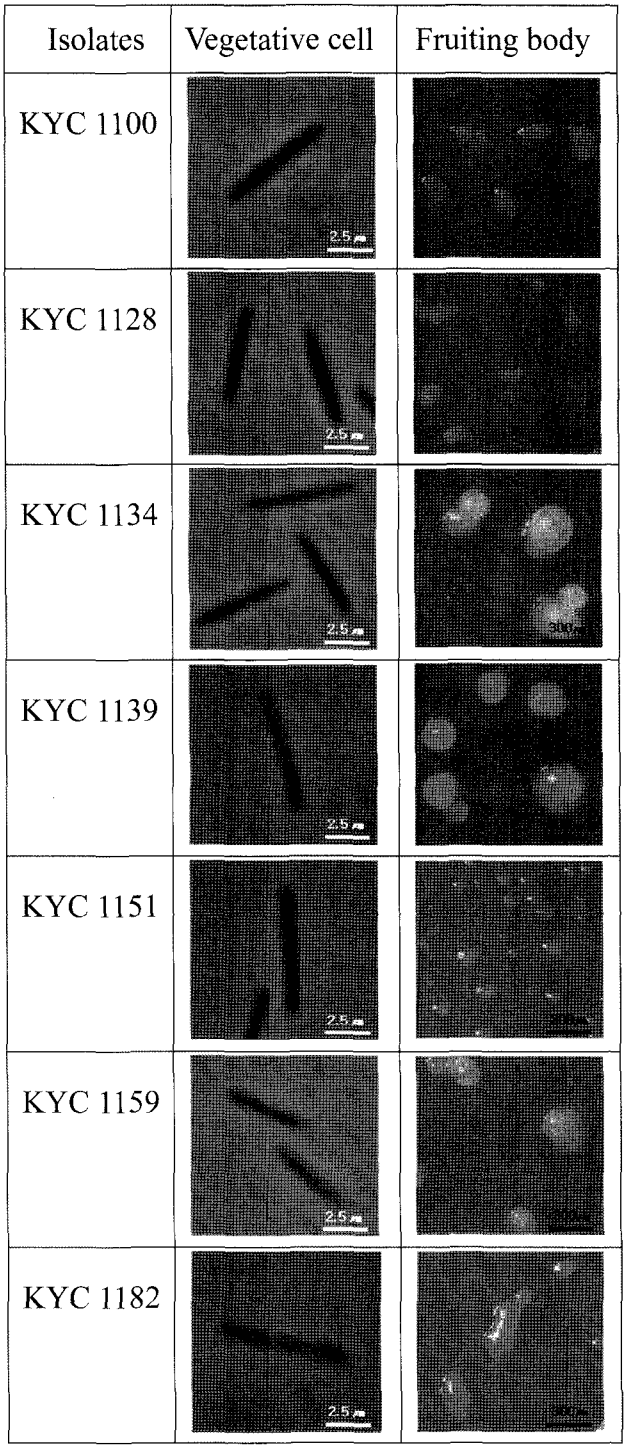

Fig. 3. The morphological characteristics of myxobacteria showing proteolytic activity by phase-contrast microscopy and anatomy microscopy.

that the broth was stable in a wide range of pH. The broth activity was decreased to 50% after 6 hours pre-incubation at 50°C and stayed 30% from the initial to 24 hours by pre-incubation at 80°C. The broth retained 30% of activity after

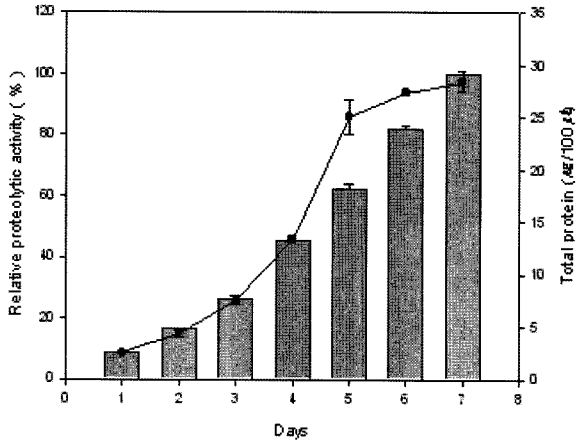

Fig. 4. Relationship of protein production and proteolytic activity of KYC1134. Total protein (-●-), relative proteolytic activity (bar chart).

Table 1. Effect of protease inhibitors

Substrate	Inhibition [%]
Control	0
Bestatin	27
AEBSF	0
PMSF	0
Chymostatin	0
Aprotinin	0
E-64	0
Phosphoramidon	0
Pepstatin	0
Leupeptin	0
Antipain dihydrochloride	0
EDTA-Na_2	0

24 hours incubation both at 50°C and 80. Eleven protease inhibitors were determined to classify proteases being in the broth. Ten inhibitors, Aprotinin, PMSF, E-64, Chymostatin, AEBSF, Phosphoramidon, Pepstatin, Leupeptin, Antipain, dihydrochloride, EDTA-Na_2, were ineffective as inhibitors, and only bestatin showed 27% inhibition effectiveness (Table 1). The inhibition result could suggest that the broth contain kinds of amino peptidases and other exopeptidases.

Amounts of genome information about myxobacteria in *Myxococcus xanthus* was opened to researchers who study myxobacteria and several interested genes were cloned and characterized the function [2-4, 7]. Out of the several interested genes, proteases have been paid attention for industrial application, for examples, demands of protease were increased in cheese making industry due to insufficiency of world supply. In general, proteases produced by

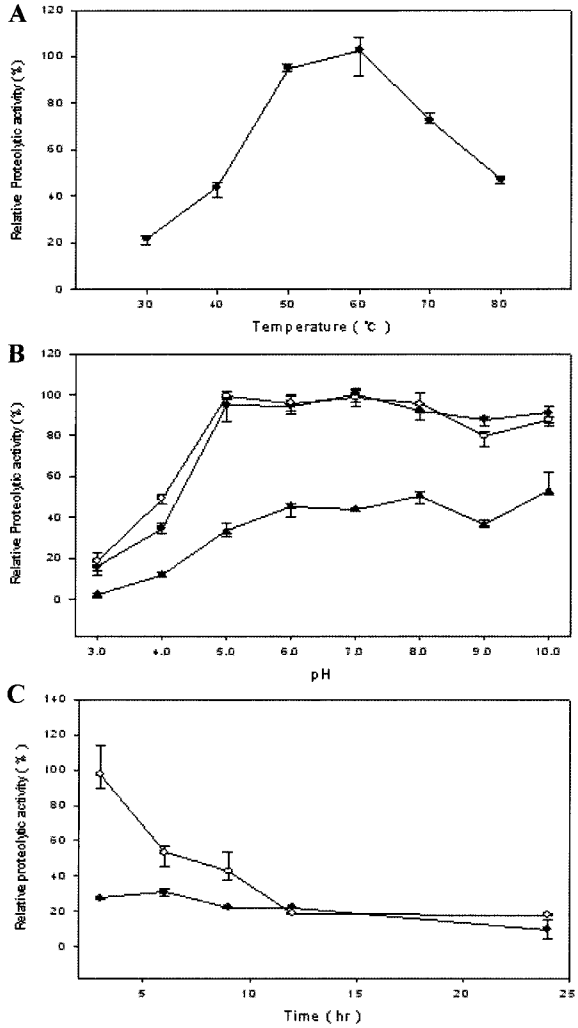

Fig. 5. Stability of temperature (A), pH (B), and time (C) of KYC 1134. A. activity was assayed in 50mM sodium phosphate buffer (pH 7.0). B. activity was assayed at 40°C (-▲-), 50°C (-●-), 60°C (-○-) using azocasein as a substrate and sodium 50 mM acetate buffer between pH 3 and 5, 50 mM tris-HCl buffer between pH 6 and 8, 50 mM glycine-NaOH buffer between pH 9 and 10. c, thermal stability was determined by pre-incubating at 50°C (-○-), 80°C (-●-) for 24 hours.

animal were insufficient to supply to the world. The reason has prompted research to both microbial and recombinant rennin. Several protease genes were cloned and recombinant protein expressed for industrial application. Myxobacteria are good resource to produce protease and known to contain amounts of amino peptidases and other exopeptidases at the base of genomic database [11, 14].

REFERENCE

1. Bollag, D.M., P. A. McQueney, J. Zhu, O. Hensens, L. Koupal, J. Liesch, M. Goetz, E. Lazarides, and M. Woods.

1995. Epothilones, a new class of microtubule-stabilizing agents with Taxol-like mechanism of action. *Cancer Res.* **55**: 2325-2333.

2. Bradford, M. M. 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
3. Gass. J., J. Ehren, G. Strohmeier, I. Isaacs, and C. Khosla. 2005. Fermentation, Purification, Formulation, and Pharmacological Evaluation of a Prolyl Endopeptidase from *Myxococcus Xanthus*: Implication for Celiac Sprue Therapy. *Biotechnology and Bioengineering* **92**: 674-684.
4. Gerth, K., N. Bedorf, H. Irschik, G. Höfle, and H. Reichenbach. 1994. The soraphens: a family of novel antifungal compounds from *Sorangium cellulosum* (*Myxobacteria*). I. Soraphen A1 alpha: fermentation, isolation, biological properties. *J. Antibiot.* **47**: 23-31.
5. Gerth, K., S. Pradella, O. Perlova, S. Beyer, and R. Müller. 2003. Myxobacteria: proficient producers of novel natural products with various biological activities-past and future. *J. Biotechnol.* **106**: 233-253.
6. Godfrey, T., and S. West. 1996. Industrial enzymology, 2nd ed., Macmillan Publishers Inc., New York, p. 3.
7. Irschik, H, R. Jansen, K. Gerth, G. Höfle, and H. Reichenbach. 1995. Chivosazol A, a new inhibitor of eukaryotic organisms isolated from myxobacteria. *J. Antibiot.* **48**: 962-966.
8. Jansen, R., H. Irschik, H. Reichenbach, V. Wray, and G. Höfle. 1994. Disorazoles, highly cytotoxic metabolites from the Sorangicin-producing bacterium *Sorangium cellulosum*, strain So ce12. Liebigs *Ann. Chem.* **8**: 759-773.
9. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111-120.
10. Kumar, S., K. Tamura, and M. Nei. 2004. Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinfor.* **5**: 150-163.
11. Perlova, O., J. Fu, S. Kuhlmann, D. Krug, A. F. Stewart, Y. Zhang, and R. Müller. 2006. Reconstitution of the Myxothiazol Biosynthetic Gene Cluster by Red/ET Recombination and Heterologous Expression in *Myxococcus Xanthus*. *Appl Environ Microbiol.* **72**: 7485-7494.
12. Poza, M., M. Prieto-Alcedo, C. Sieiro, and T. G. Villa. 2004. Cloning and Expression of *clt* Genes Encoding Milk-Clotting Proteases from *Myxococcus xanthus* 422. *Appl Environ Microbiol.* **70**: 6337-6341.
13. Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande. 1998. Molecular and biotechnological aspects of microbial proteases. *Mirobiology and Molecular Biology Review.* **62**: 597-635.
14. Reichenbach, H. 2004. The *Myxococcales*. In: G.M. Garrity, Editor, *Bergey's Manual of Systematic Bacteriology*, Springer-Verlag, New York, p. 1059–1143.
15. Reichenbach, H. and M. Dworkin. 1992. The myxobacteria. In Balows A., H.G. Trer, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Procaryotes, 2nd ed., Springer Verlag, New York, p. 3416-3478.
16. Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* **4**: 406-425.
17. Sarath, G., R. S. Motte, and F. W. Wagner. 1989. Protease assay Method. In. proteolytic enzyme: a practical approach, R. J. Beynon and J.S. Bond, eds., Oxford: IRL Press, p. 25-55.
18. Shan, L., T. Marti, L. M. Sollid, M. G. Gary, and C. Khosla 2004. Comparative biochemical analysis of three bacterial prolyl endopeptidases. *Biochem J.* **383**: 311-318.
19. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**: 4876-4882.

(Received Sep. 5, 2008/Accepted Aug. 7, 2009)

국문초록

Myxobacteria의 Proteolytic Activity 특성

김재영 · 정진우[1] · 조경연[1] · 이용섭*
호서대학교 화장품 과학과, [1]생명공학과

Proteolytic 활성을 나타내는 균주 KYC 1028, 1100, 1134, 1139, 1151, 1159, 1182 등 7개 균주를 선발하였고, 이중 KYC 1134와 KYC 1139이 azocazein을 이용한 proteolytic 활성 조사에서 높은 활성을 나타내었다. 선발된 7개 균주의 동정은 16S rDNA 염기서열를 조사하였으며, NCBI에서 myxobacteria의 16S rDNA와 비교분석 하여 Myxococcus속 *M. macrospores*와 *M. Fulvus*에 높은 상동성을 나타내었다. 활성이 가장 높은 KYC 1134배양액의 생화학적 특성은 배양 7일까지 활성이 10배 증가하고, 단백질 생산도 함께 증가하였다. 배양액의 적정 proteolytic 활성 온도는 60°C이고, pH 5에서10까지 활성이 안정적이었다. 배양액의 proteases의 종류를 확인하기 위하여 11개의 inhibitors를 조사하여 bestatin만이 억제효과를 나타내어 amino peptidases와 exopeptidases의 종류가 배양액에 존재하는 것으로 보인다.