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Article

Isolation of Protease-producing Yeast, *Pichia farinosa* CO-2 and Characterization of Its Extracellular Enzyme

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A yeast strain, CO-2, was isolated as a producer of extracellular protease from a sample of bamboo by-product. The cells were spherical- to ovoid-shaped and 3.7-4.2×4.1-4.8 μm in size. The isolated strain grew at 20-45°C, in the pH range of 3.0-12.0. Optimal growth conditions were 35°C and pH of 6.0 for 14 h. The isolate was able to grow in up to 8.0%(w/v) NaCl and 9.0%(v/v) ethanol. Biochemical data obtained using the VITEK system were close to those of *Pichia farinosa* (exhibiting a 97% matching level). Based on molecular phylogenetic analysis of the 18S rDNA sequences and restriction enzyme analysis of the 18S rDNA-ITS1 region, this strain was identified as *P. farinosa*. Optimum pH and temperature of the crude protease preparation of *P. farinosa* CO-2 were pH 3.0 and 40°C, respectively. The enzyme retained more than 80% of its stability at pH ranging from 3.0 to 8.0. After heating at 60°C for 1 h, the enzyme exhibited 71% of its original activity.

Key words: 18S rDNA, extracellular protease, identification, molecular phylogeny, restriction enzyme analysis, *Pichia farinosa*, yeast

Proteases are degradative enzymes that hydrolyze peptide bonds between amino acids of other proteins. These enzymes are available commercially and are widely distributed in nature [Rao *et al*., 1998]. Proteases are commonly used in detergents, foods, pharmaceuticals, leather and textile manufacture, and waste treatment [Najafi *et al*., 2005]. In the natural world, proteases are important in the biocontrol mechanisms including mycoparasitism, competition, and antibiosis [Elad and Kapat, 1999].

In general, compared to proteases obtained from plants and animals, microbial proteases are extracellular in nature, and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme. Extracellular proteases in particular have also been commercially exploited for assistance with protein degradation in various industrial processes [Kurmar and Tagaki, 1999]. Mold and bacteria are well-known protease producers, of which *Trichoderma* [Elad and Kapat, 1999] and *Bacillus* [Genckal and Tari, 2006] are the most prominent sources.

In contrast, relatively little is known about yeast extracellular protease. Extracellular protease producing yeast include *Candida albicans* [Ruchel, 1981], *Candida humicola* [Ray *et al*., 1992], *Candida olea* [Nelson and Young, 1987], *Saccharomycopsis lipolytica* [Yamada and Ogrydziak, 1983], *Aureobasidium pullulans* [Chi *et al*., 2007], *Rhodotorula glutinis* [Kamada *et al*., 1972], and *Sporidiobolus ruineniae* [Kim, 2009]. Production of yeast proteases has so far been studied mainly for their implications in the beer and wine industries [Strauss *et al*., 2001]. However, because some yeast may be suitable for biological control of post-harvest diseases of fruits and grains, and as bioremediation agents, those species have been studied as enzyme producers for potential industrial exploitation [Buzzini and Vaughan-Martini, 2006]. Consequently, recent studies have been directed toward yeasts as possible producers of useful enzymes [Nakagawa *et al*., 2004].

As part of an investigation to identify biotechnologically interesting microorganisms for industrial application, we isolated and identified a yeast strain from bamboo by-products that produces extracellular proteases. In addition, characteristics of the obtained crude enzyme were investigated.

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Materials and Methods

Growth media and culture conditions. For all experiments, the isolated yeast strain was routinely

cultivated at 35°C in yeast extract-malt extract (YM) medium composed of 0.3%(w/v) yeast extract (Difco, Detroit, MI), 0.3%(w/v) malt extract (Difco, Detroit, MI), 0.5%(w/v) Bacto-peptone (Difco), and 1.0%(w/v) dextrose (Merck, Darmstadt, Germany). To avoid bacterial growth, 100 μg/mL penicillin-streptomycin solution (HyClone; Thermo Fisher Scientific, Logan, UT) was added. A single colony of an isolate was pre-inoculated in YM broth and incubated at 35°C with agitation (200 rpm) until the stationary phase was reached. The isolate (1.0%, v/v) was inoculated into a fresh YM broth and incubated statically at 20-50°C with 5°C increments for each culture, and pH of 2.0-13.0 with an increment of 1.0 between each culture. Required pH was established after sterilization and prior to inoculation by addition of 5 M HCl and 10 M KOH. Salt and ethanol tolerances were assessed at 35°C using YM broth supplemented with 0-15.0%(w/v) NaCl in increments of 1%, and 0-10.0%(v/v) EtOH in increments of 1%, respectively. Growth was monitored by measurement of optical density at 600 nm (OD_{600}) using a BioPhotometer 6131 spectrophotometer (Eppendorf AG, Eppendorf, Germany).

Isolation of yeast from bamboo by-products. The bamboo by-product sample was obtained from AGRO KOREA, Guri, Korea. Ninety milliliters of sterile 0.85%(w/v) NaCl was added to 10 g of the bamboo by-product sample and homogenized for 2 min in a Happy Dream DMP-900 blender (Dreamtech, Bucheon, Korea). The homogenate was diluted serially ten-fold with 0.85% NaCl (representing 10^1-10^8 cells) spread onto Luria-Bertani agar consisting of 0.5%(w/v) yeast extract (Difco), 1.0%(w/v) Bacto-tryptone (Difco), and 1.0%(w/v) NaCl (Bioshop Canada, Burlington, Ontario, Canada) for isolation of bacteria, YM agar for isolation of yeast, and Potato Dextrose agar (Neogen, Lansing, MI) for isolation of mold. Individual colonies, which were selected randomly according to their different color and morphological characteristics, were purified by single colony isolation after triple re-streaking on the appropriate agar. Each final pure culture was suspended in 20%(v/v) glycerol and frozen at −80°C until needed.

Detection of extracellular protease production. Extracellular protease production was ascertained using a direct agar plate assay. A single colony was inoculated on 1.0%(w/v) skim milk-YM agar plates [Amoozegar *et al*., 2006] and incubated for 2-3 days at optimum growth temperature. Protease production was observed by the direct appearance of hydrolysis halos surrounding the colonies.

Characteristics of the isolated yeast strain. Morphological, physiological, and biochemical characteristics of the bamboo by-product isolate were determined as previously described [Barnett *et al*., 2000]. Morphology of the cells recovered from YM medium was determined using a Model BX51 phase-contrast microscope (Magnification: ×1,000 Olympus, Tokyo, Japan). Enzymatic, acidification, alkalinization, assimilation, inhibition, and precipitation tests utilized a VITEK 2 Compact 60 apparatus (bioMérieux, Hazelwood, MO). Experiments were performed in duplicate with inoculum that was subcultured at least once under the same test conditions.

18S rRNA gene sequencing and phylogenetic analysis. PCR amplification and DNA sequencing of the 18S rRNA gene were performed for identification of the isolate. Total genomic DNA was extracted using the GeneAll™ genomic DNA extraction kit (GeneAll™, GenEx™Genomic Sx; GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. PCR amplifications were performed as previously described [White *et al*., 1990; Fell *et al*., 2000]. The 18S rDNA region was amplified using primers NS1 (5'-GTAGTCAT ATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCA CCTACGGA-3'). The PCR reaction mixture consisted of 10 μL of 2× Prime Taq Premix Solution (GeNet Bio, Cheonan, Korea) containing of 1 U/μL Prime Taq DNA polymerase, 4.5 mM $MgCl_2$, 0.5 mM dNTP, 0.1% gelatin, 1 μL of 10 pM primers, and 1 μL of genomic DNA template, prepared to a final reaction volume of 20 μL. Amplification was carried out with the Model PC708 Program Temp Control System (ASTEC, Tokyo, Japan) using an initial denaturation at 93°C for 3 min, followed by 30 cycles of denaturation at 93°C for 1 min/cycle, primer annealing at 57°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. Amplified PCR products were analyzed by 0.8%(w/v) agarose gel electrophoresis and purified using Wizard® SV Gel and a PCR clean-up system (Promega, Madison, WI), according to the manufacturer's instructions. Sequencing of the amplified DNA fragments was contractually performed by Solgent (Daejeon, Korea; http://www.solgent.co.kr).

The acquired sequences were used for the BLAST gene homology search (http://www.ncbi.nlm.nih.gov/BLAST/) with the publicly available 18S rDNA sequences. Identification was assigned to the generic level [Altschul *et al*., 1997]. The 18S rDNA sequences of the isolated strain were aligned with sequences of related organisms obtained from GenBank [Thompson *et al*., 1997] using the CLUSTAL X multiple sequence alignment program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/). Phylogenetic analysis was performed using PHYLIP software (http://evolution.genetics.washington.edu/phylip.html) [Felsenstein, 1985] and a phylogenetic tree was constructed by the neighbor-joining method using the TreeView software (http://taxonomy.zoology.gla.ac.uk/

rod/treeview.html) [Page, 1996]. Reproducibility of the branching pattern was validated using the bootstrap analysis.

GenBank accession number. The partial 18S rDNA sequence of the isolated strain has been deposited in GenBank under the accession number EU106163.

Amplification and restriction enzyme analysis. As described by Dlauchy *et al*. [1999], yeast identification at the species level was performed according to the restriction fragment patterns of PCR-amplified 18S rRNA-coding DNA with the neighboring internal transcribed spacer (ITS) 1 region. Total DNA extraction and PCR amplification were performed using the method described above. The 18S rDNA-ITS1 region was amplified using primers NS1 (5'-GTAGTCATATGCTTG TCTC-3') and ITS2 (5'-CGCTGCGTTCTTCATCGAT GC-3') [White *et al*., 1990]. The PCR product was digested with four different restriction enzymes, *Hae*III, *Msp*I, *Alu*I, and *Rsa*I (Promega, Madison, WI) according to the manufacturer's instructions and conditions; 1 μL of PCR product (1 μg/μL) was digested in a 20 μL final volume of digestion mixture consisting of 0.5 μL restriction enzyme (10 U/μL), 0.2 μL of acetylated bovine serum albumin (10 μg/μL), and 2 μL of RE 10× buffer, depending on the restriction enzyme used. Fragments were separated by electrophoresis on 2% agarose gels with a molecular size standard. After electrophoresis, the gels were stained with ethidium bromide and photographed under transilluminated UV light. Restriction fragment patterns generated by enzyme digestion were compared with results reported by Dlauchy *et al*. [1999].

Assay of protease activity. Culture supernatants used as the source of crude enzyme were prepared by centrifugation (Centrifuge 5415R, Eppendorf AG, Eppendorf, Germany) at 12,000 rpm (13,362×g) and 4°C for 5 min. Using casein as a substrate, protease activity of the enzyme was assayed in the manner previously described [Hagihara *et al*., 1958]. The crude enzyme solution (1.0 mL) was added to an equal volume of 0.6% Hammarsten casein (BDH Biochemical, Poole, UK) in 0.05 M citrate buffer, pH 3.0. After incubation at 40°C for 10 min, the reaction was stopped by addition of 5 mL of trichloroacetic acid (TCA) mixture (0.11 M TCA, 0.22 M sodium acetate, 0.33 M acetic acid). The mixture was kept at room temperature for 30 min; precipitate was removed by centrifugation at 10,000 rpm (9,279×g) for 5 min. Absorbance of the recovered supernatant was examined at 280 nm. The blank was run in the same manner, except that the enzyme solution was mixed with the TCA prior to addition of the substrate. One unit of protease activity was defined as the amount of enzyme that liberated a digestion product not precipitated by TCA equivalent to 1 μg/mL/min of tyrosine under the assay conditions. All assays were carried out in triplicate. Data represent averages of three determinations.

Effect of pH and temperature on crude protease activity. Conditions for obtaining maximum protease activity were studied by assay of the enzyme at different pHs and temperatures; relative enzyme activities were calculated. All determinations were performed in triplicates. Using the aforementioned assay conditions, the effect of pH on protease activity of the crude enzyme was determined by incubation of the reaction mixture at pH 3.0-11.0 [0.05 M citrate buffer (pH 3.0-6.0), 0.1 M phosphate buffer (pH 7.0-8.0), 0.1 M sodium bicarbonate buffer (pH 9.0-11.0)]. To determine pH stability, enzyme solutions were pre-incubated in the absence of the substrate at the indicated pH (pH 3.0-11.0) at 4°C for 24 h. After incubation, residual enzyme activity (%) was measured under optimum conditions of the assay.

The effect of temperature on protease activity was determined by incubation of the reaction mixture at temperatures ranging from 20 to 75°C in 0.05 M citrate buffer (pH 3.0) for 10 min. Thermostability was determined by incubation of the enzyme solution for different time periods at temperatures ranging from 30 to 60°C. After the enzyme solution was rapidly cooled to 4°C, residual activity (%) of the enzyme was measured under optimum conditions of the assay.

Results and Discussion

Isolation of protease-producing yeast from bamboo by-products. In order to obtain a strain that might serve as a new source of protease, two strains of yeast were isolated from bamboo by-product samples obtained in Guri, Korea, and screened for protease production. Growth was evident only on YM agar plates supplemented with 100 μg/mL of penicillin-streptomycin after approximately 2-3 days of incubation. Protease production by the isolate was tested using its ability to grow on YM agar supplemented with 1.0%(w/v) skim milk. Development of halo zones around colonies was indicative of extracellular protease production. One yeast strain that produced protease (designated as strain CO-2) was selected for further investigation.

Characteristics of strain CO-2. Isolated CO-2 was characterized morphologically, physiologically, and biochemically in the manner previously described [Barnett *et al*., 2000]. After incubation for 14 h at 35°C on YM agar medium, all developed colonies displayed a smooth margin and convex elevation, and were opaque, non-glistening, white-to-cream colored, and circular. Microscopic examination revealed spherical- to ovoid-

Table 1. Characteristics of the isolated strain CO-2

Characteristics	Strain CO-2
Shape	Spherical to ovoid
Cell size (μm)	(3.7-4.2)×(4.1-4.8)
Vegetative production	Budding
Colony color	White to cream
Form	Circular
Elevation	Convex
Margin	Smooth
Opacity	Opaque
Brilliancy	No glistening
Temperature (°C) for:	
Optimum growth	35
Growth range	20-45
pH for:	
Optimum growth	6.0
Growth range	3.0-12.0
Growth in NaCl (%, w/v) at:	
Optimum growth	0.0-2.0
Growth range	0.0-8.0
Growth in EtOH (%, v/v) at:	
Optimum growth	2.0
Growth range	0.0-9.0

shaped cells, 3.7-4.2×4.1-4.8 μm in size, which occurred singly or with buds.

Strain CO-2 grew at temperatures between 20 and 45°C, with optimal growth at 35°C; however, no growth was observed at 50°C. This strain grew in a wide pH range of 3.0-12.0, and optimally at 6.0. No growth was observed at pH 2.0. Therefore, in subsequent experiments, the pH of the medium was maintained at 6.0. Isolate CO-2 grew in a NaCl concentration ranging up to 8.0%(w/v), and optimally at 0-2.0%(w/v). In addition, it grew in the presence of 0-9.0%(v/v) EtOH, and optimally at 2.0%(v/v) EtOH. Morphological, cultural, and physiological characteristics of strain CO-2 are shown in Table 1.

The isolate was further characterized and identified using the VITEK system. Biochemical characteristics of CO-2 are summarized in Table 2. CO-2 was positive in the assimilation test for acetate, arbutine, citrate, D-galactose, D-glucose, D-mannose, D-sorbitol, erythritol, glycerol, L-glutamate, L-proline, methyl-α-D-glucopyranoside, and N-acetyl-glucosamine. Positive acidification results were obtained for D-mannitol and salicin. Positive results were also evident in the alkalinization test for argin dihydrolase, enzymatic test for L-leucine arylamidase and phenylalanine arylamidase, inhibition test for growth for 6.5% NaCl, kanamycin resistance, oleandomycin resistance, and polymyxin-β resistance. All precipitation tests showed negative results. Biochemical characteristics obtained using the VITEK system were close to those of *Pichia farinosa* (exhibiting a 97% matching level).

Molecular phylogenetic analysis based on 18S rDNA sequencing. Among many molecular methods, rDNA sequence analysis offers a generally rapid and useful means of speciation [Fell *et al*., 2000]. Therefore, molecular phylogenetic-based identification of the strain CO-2 was performed using rDNA sequence analysis. The 18S rDNA of strain CO-2 was amplified by PCR, and nearly complete sequences for 18S rDNA were determined. The 1,521 bp sequences obtained were aligned with all of the presently available sequences for the 18S rDNA in the GenBank database. CO-2 was determined to be a close relative of *Pichia farinosa*. The partial 18S rDNA sequence of the strain CO-2 displayed 99% identity with corresponding sequences of the *P. farinosa* strain DC3343 (AY227021), *P. farinosa* strain JCM10734 (AB054281), and *P. farinosa* strain JCM8895 (AB013513). A phylogenetic tree was constructed based on the 18S rDNA sequences to show the comparative relationship between the strain CO-2 and other related species. CO-2 was a member of the genus *Pichia*, and was most closely related to *P. farinosa* (Fig. 1).

Restriction enzyme analysis of the PCR-amplified 18S rDNA-ITS1 region. The yeast isolate was rapidly identified by restriction enzyme analysis of the 18S rRNA-coding DNA with the neighboring ITS1 region [Dlauchy *et al*., 1999]. Variability in restriction fragment patterns enabled differentiation and identification of 169 strains of 128 yeast species. CO-2 was identified at the species level by amplification of the 18S rDNA-ITS1 region and subsequent restriction analysis with endonucleases *Hae*III, *Msp*I, *Alu*I, and *Rsa*I. Restriction fragment patterns of DNA fragments amplified from 18S rDNA-ITS1 with NS1 and ITS2 primers from CO-2 are shown in Fig. 2. The approximate length of the amplified product was 2.2 kb. Digestion of the PCR product with the enzyme *Hae*III yielded approximately 550, 300, and 150 bp sized fragments. Restriction products from *Msp*I included four bands of 1600, 700, 400, and 300 bp. In the case of *Alu*I, fragments of approximately 700, 350, 250, and 100 bp were observed. Fragments produced with *Rsa*I were 1000, 500, and 450 bp. After agarose gel electrophoresis on a 2% gel, fragments smaller than 50 bp could not be reproducibly visualized. Band patterns of CO-2 were identical to those of the *P. farinosa* type strain (*P. farinosa* var *farinosa* NCAIM999^T) obtained by others [Dlauchy *et al*., 1999] using the same four enzymes.

Therefore, based on morphological, cultural, physiological, and biochemical properties, as well as phylogenetic analysis of the 18S rDNA sequences and restriction enzyme analysis of the 18S rDNA-ITS1 region, the CO-2

Table 2. Biochemical characteristics of the isolated strain CO-2

Biochemical characteristics	Strain CO-2	Biochemical characteristics	Strain CO-2
Assimilation test:		N-acetyl-glucosamine	-
2-keto-D-gluconate	-	palatinose	-
acetate	+	pullulan	-
amygdalin	-	salicin	+
arbutine	+	Alkalinisation test:	
citrate	+	argin dihydrolase	+
D L-lactate	-	arginine	-
D-cellobiose	-	pyruvate	-
D-galactose	+	urease	-
D-galacturonate	-	Enzymatic test:	
D-gluconate	-	alanine arylamidase	-
D-glucose	+	ala-phe-pro-arylamidase	-
D-mannose	+	alkalin phosphatase	-
D-melezitose	-	ellman	-
D-melibiose	-	glycine arylamidase	-
D-raffinose	-	L-aspartate arylamidae	-
D-sorbitol	+	L-leucine arylamidase	+
D-trehalose	-	L-lysine arylamidase	-
D-turanose	-	L-proline arylamidase	-
D-xylose	-	L-pyroglutamic acid arylamidase	-
erythritol	+	L-pyrrolydonyl arylamidase	-
gentobiose	-	phenylalanine arylamidase	+
glucuronate	-	phosphatidylinositol phospholipase C	-
glycerol	+	phosphoryl cholin	-
lactose	-	PNP-N-acetyl-β-D-galactosaminidase 1	-
L-arabinose	-	tyrosine arylamidase	-
L-glutamate	+	α-galactosidase	-
L-malate	-	α-glucosidase	-
L-proline	+	α-mannosidase	-
L-rhamnose	-	β-galactosidase	-
L-sorbose	-	β-glucosidase	-
methyl-α-D-glucopyranoside	+	β-glucuronidase	-
N-acetyl-glucosamine	+	β-mannosidase	-
nitrate	-	β-N-acetyl-glucosaminidase	-
putrescine	-	β-xylosidase	-
sucrose	-	γ-glutamyl transferase	-
xylitol	(-)	Inhibition test:	
Acidification test:		bacitracin resistance	-
cyclodextrine	-	growth in 6.5% NaCl	+
D-mannitol	+	kanamycin resistance	+
D-ribose	-	novobiocin resistance	-
D-tagatose	-	O129	-
glycogene	-	oleandomycin resistance	+
inulin	-	optochin resistance	-
maltose	-	polymyxin-β resistance	+
maltotriose	-	Precipitation test:	
methyl-D-xyloside	-	esculin hydrolysis	-
methyl-β-D-glucopyranoside	-	tetrazolium RED	-
myo-inositol	-		

VITEK system was used. +, positive; -, negative; (+), weakly positive; (-), weakly negative.

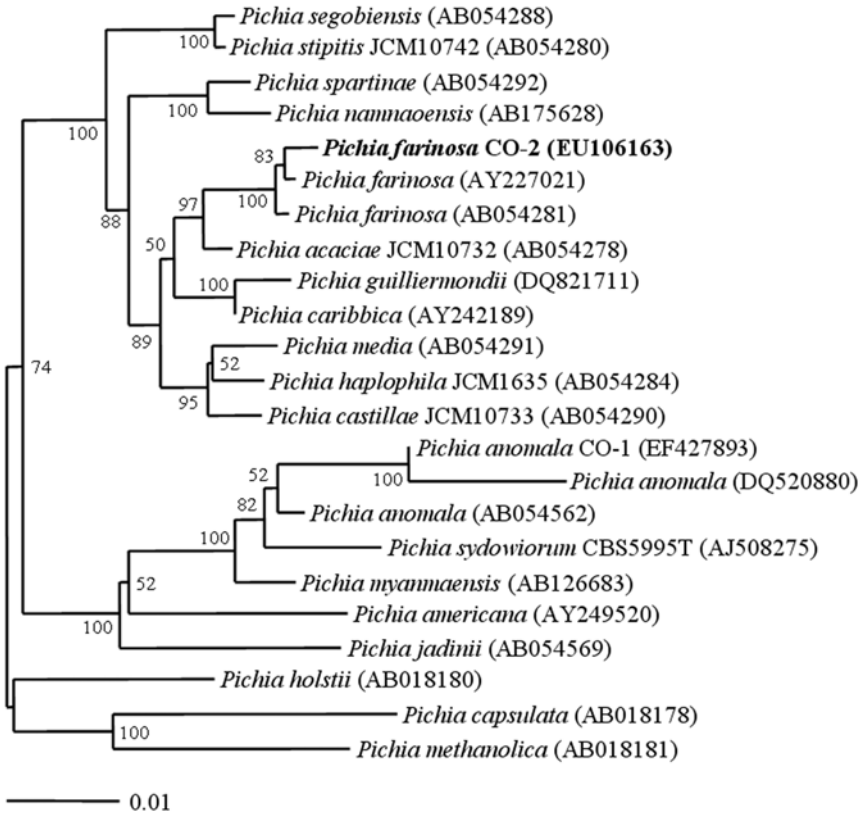

Fig. 1. Phylogenetic tree showing the relationship between the isolated strain CO-2 and other related species. GenBank accession numbers are given in parentheses. The tree was constructed using the CLUSTAL-X and neighbor-joining method. Scale bar corresponds to 0.01 subscriptions per nucleotide position. Numbers at nodes indicate levels of bootstrap support (%) determined from 100 resampled data.

yeast strain was identified as *P. farinosa*. Accordingly, we named the isolated strain *P. farinosa* strain CO-2.

Characterization of the extracellular crude protease. To determine the characteristics of the protease produced by CO-2, influences of pH and temperature on activity and stability of extracellular proteases recovered from the culture supernatants were investigated.

A strong dependence on extracellular pH for enzyme activity is an important characteristic of most microorganisms [Kurmar and Tagaki, 1999]. Fig. 3 shows that *P. farinosa* CO-2 protease had a relatively broad range of pH for its optimum activity. Optimum pH of protease activity, as ascertained over a range of 3.0-11.0 using Hammarsten casein as a substrate, was determined to be pH 3.0, and more than 80% of the maximal activity was retained at pH 4.0-9.0. Activity decreased above pH 10.

Relatively little is known about yeast extracellular proteases. Studies have characterized extracellular proteases produced by *S. lipolytica* [Yamada and Ogrydziak, 1983], *C. olea* [Nelson and Young, 1987], *C. humicola* [Ray *et al*., 1992], *A. pullulans* [Chi *et al*., 2007] and *S. ruineniae* [Kim, 2009]. Acid proteases have also been reported in *S. lipolytica* [Yamada and Ogrydziak, 1983] and *C. humicola* [Ray *et al*., 1992], which showed optimal activity in the pH range of 3.1-4.2. In contrast, *S. ruineniae* [Kim, 2009] and *A. pullulans* [Chi *et al*., 2007] showed optimum activity at neutral or alkaline pH. *C. olea* [Nelson and Young, 1987] secretes an acid and an alkaline protease. The acid protease is optimally active at pH 3.3, and the alkaline protease is optimally active at pH 8.0-9.0.

pH stability of the protease was determined after pre-incubation for 24 h at 4°C in buffers with varied pH (pH 3.0-11.0). After 24 h incubation at 4°C, the protease was very stable at pH 3.0-5.0, and showed maximum stability at pH 4.0. It retained more than 80% of the maximum activity between pH 3.0-8.0, and more than 70% of its activity at 3.0-11.0 (Fig. 4). pH stability was different from that of *S. ruineniae* [Kim, 2009].

Temperature is one of the most important factors affecting enzyme production [Chi and Zhao, 2003]. Activity of the protease was tested in the temperature range of 20°C to 75°C. With a reaction time of 10 min, optimum temperature for assay of protease activity in the culture supernatant was 40°C. The enzyme also displayed more than 80% of maximum activity at 20-75°C (Fig. 5).

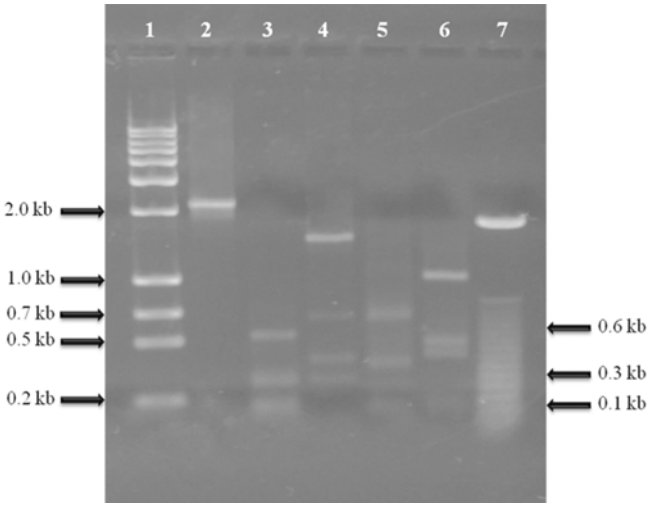

Fig. 2. Amplification and restriction analysis of the 18S rDNA-ITS region of yeast with four restriction endonucleases. Oligonucleotide primers NS1 and ITS2 were used to amplify the fragment. An enzymatically amplified fragment was digested with *Hae*III, *Msp*I, *Alu*I, and *Rsa*I, and separated on 2% agarose. Lane 1: DNA marker (Vivagen); lane 2: PCR amplified 18S rDNA-ITS; lane 3: PCR product digested with *Hae*III; lane 4: PCR product digested with *Msp*I; lane 5: PCR product digested with *Alu*I; lane 6: PCR product digested with *Rsa*I; lane 7: 50 bp DNA Step Ladder (promega)

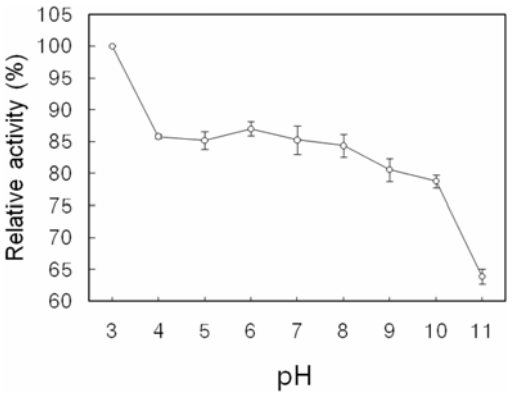

Fig. 3. Effect of pH on activity of the extracellular protease produced by *P. farinosa* CO-2. Enzyme activity was measured at the indicated pH (reaction solution) for 10 min at 40°C. The buffer used was 0.05 M citrate (pH 3.0-6.0), 0.1 M phosphate (pH 7.0-8.0), and 0.1 M sodium bicarbonate (pH 9.0-11.0). Values are shown as percentages of the maximum activity of the enzyme observed at pH 3.0, which is taken as 100%.

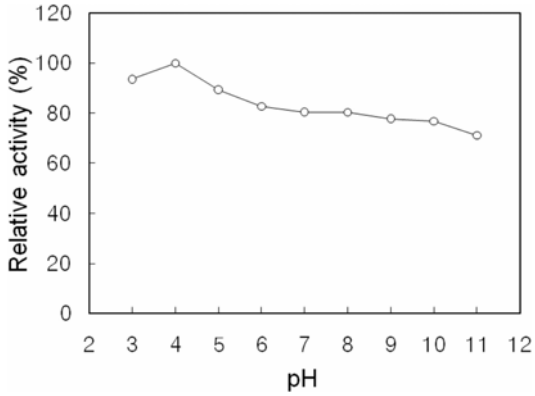

Fig. 4. pH stability of the extracellular protease produced by *P. farinosa* CO-2. Enzyme solution was pre-incubated at 4°C for 24 h at the given pH (pre-incubation solution) values, and residual activity was measured at 40°C for 10 min. The following buffers were used: 0.05 M citrate (pH 3.0-6.0), 0.1 M phosphate (pH 7.0-8.0), and 0.1 M sodium bicarbonate (pH 9.0-11.0). Relative activity is expressed as a percentage of the maximum enzyme activity under optimum conditions.

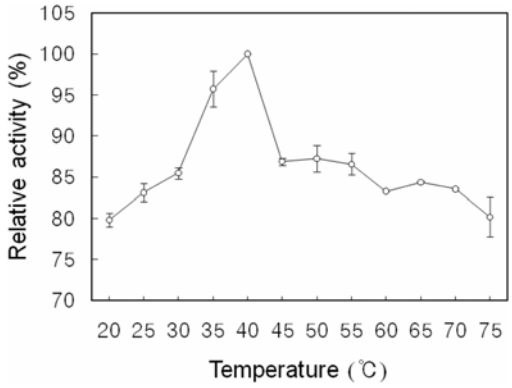

Fig. 5. Effect of temperature on activity of the extracellular protease produced by *P. farinosa* CO-2. Enzyme activity was measured at various temperatures (20-75°C) for 10 min in 0.05 M citrate buffer (pH 3.0). Values are shown as percentages of the maximum activity of the enzyme observed at 40°C, which is taken as 100%.

These results are in accordance with those of previous work, where a temperature optimum of 40-42°C was reported for *C. olea* [Nelson and Young, 1987] proteases, and 45°C was reported for *A. pullulans* [Chi *et al*., 2007]. Proteases from *S. ruineniae* [Kim, 2009] showed temperature optima of 50°C, whereas *C. humicola* protease [Ray *et al*., 1992] showed optimum activity at 22°C.

Thermostability studies showed that the crude enzyme was entirely stable at 30-60°C, and that more than 70% of its activity was retained after 1 h. Because it maintained 89.2, 83.1, 79.5, and 71.0% residual activity at 30, 40, 50, and 60°C, respectively, after 1 h of incubation, the enzyme was relatively thermostable (Fig. 6).

On the other hand, protease from *S. ruineniae* [Kim, 2009] exhibited only 55% residual activity at 60°C after 30 min. Pre-incubation for 60 min at 60°C rapidly inactivated the enzyme, retaining only 22% activity. Based on these experimental results, we determined that

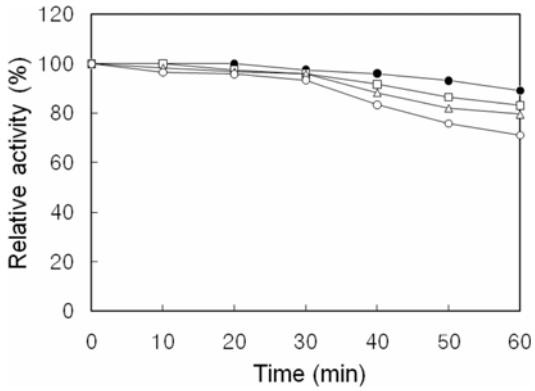

Fig. 6. Thermostability of the extracellular protease produced by *P. farinosa* CO-2. Enzyme solution was incubated for different time periods under the indicated temperatures, and residual activity was measured at 40℃ for 10 min. Relative activity is expressed as a percentage of the maximum enzyme activity under optimum conditions. Symbols: ○, 60°C; △, 50°C; □, 40°C; ●, 30°C

pH 3.0 and 40°C were optimal for protease activity.

Most studies of *P. farinosa* have focused on killer toxin production and halotolerant characteristics [Suzuki, 1999; Suzuki *et al*., 2001]. *P. farinosa* has also been reported to produce exocellular β-glucosidase [Drider *et al*., 1993], whereas extracellular protease from *P. farinosa* remains uncharacterized. Furthermore, characterization of extracellular protease produced by *P. farinosa* CO-2 has yielded important information on its optimal growth conditions, as well as the effect of pH and temperature on crude protease activity. It has many good properties that other yeasts do not have, such as fast growth, broad growth range of pH and temperature, and thermal stability of the extracellular protease. Results from the present study on growth and protease production of *P. farinosa* CO-2 demonstrate its strong potential for use in industrial biocontrol technology. Ray *et al*. [1992] and Gonzalez-Lopez *et al*. [2002] reported on variability of the characteristics of extracellular protease, which was produced by yeasts, according to the composition of the growth medium, pH, and temperature. Accordingly, in cases of *P. farinosa* CO-2 isolated from the current study to determine the presence of any of the characteristics described above, ongoing studies have been conducted with regard to the effect of culture conditions on growth and protease production. In addition, because the current enzyme is an unpurified, crude enzyme, it was examined following the purification for the presence of variants of extracellular protease produced by *P. farinosa* CO-2. Clarification of biochemical characteristics in greater detail will be essential.

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