Isolation of *Sporidiobolus ruineniae* CO-3 and Characterization of Its Extracellular Protease

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An extracellular protease-producing yeast strain, designated as CO-3, was isolated from fermented tea. The cells were spherical- to ovoid-shaped and 6.8-7.4×7.4-9.1 μm in size. Optimal growth conditions were 25-30°C and pH of 5.0-6.0. The isolate was able to grow in up to 4%(w/v) NaCl and 5%(v/v) ethanol. This strain was identified as *Sporidiobolus ruineniae* based on the internal transcribed spacer regions including 5.8S rDNA sequence and partial D1/D2 domain of 26S rDNA sequence analysis. Optimal activity conditions of the crude protease fraction of *S. ruineniae* CO-3 were pH of 7.0 at 50°C. Protease production reached maximum when 1.0%(w/v) xylose, 1.0%(w/v) yeast extract, and 0.3%(w/v) K_2HPO_4 were used as the sole sources of carbon, nitrogen, and mineral, respectively.

Key words: *extracellular protease, identification, molecular phylogeny,* Sporidiobolus ruineniae*, yeast*

Proteases are one of the most important industrial enzymes, accounting for approximately 60% of the total global enzyme sales [Rao *et al*., 1998]. They 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]. Although proteases are widely distributed in nature, microorganisms are the most suitable resources for industrial production of protease. Protease-producing microorganisms can easily be cultivated in a large scale, once their biochemical and physical characteristics and physiological functions are established [Deshpande, 1998].

In particular, extracellular proteases produced by microorganisms are important for the hydrolysis of proteins in cell-free environments, which enables the cells to absorb and utilize the hydrolytic products [Kalisz, 1988]. Extracellular proteases from different microorganisms including bacteria, molds, and yeasts have been characterized [Griffin *et al*., 1992; Elad and Kapat, 1999; Patel *et al*., 2005], although comparatively little is known about the yeast extracellular protease [Ray *et al*., 1992; Strauss *et al*., 2001]. Nevertheless, some yeast species have been studied as enzyme producers for potential industrial exploitation, because they may be suitable for the biological control of post-harvest diseases of fruits and grains, and as bioremediation agents [Buzzini and Vaughan-Martini, 2006]. Extracellular protease-producing yeast include *Candida albicans* [Ruchel, 1981], *Candida humicola* [Ray *et al*., 1992], *Candida olea* [Nelson and Young, 1987], *Saccharomycopsis lipolytic* [Yamada and Ogrydziak, 1983], *Aureobasidium pullulans* [Chi *et al*., 2007], and *Rhodotorula glutinis* [Kamada *et al*., 1972].

As part of an investigation to identify microorganisms for biocontrol applications, we isolated and identified a yeast strain from the fermented tea that produces extracellular enzymes. In addition, the crude enzyme was characterized.

Materials and Methods

Growth media and culture conditions. For all experiments, the isolated yeast strain was routinely cultivated at 30°C in YM medium composed of 0.3%(w/

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Abbreviations: CMC, carboxymethyl cellulose; ITS, internal transcribed spacer; PCR, polymerase chain reaction; TCA, trichloroacetic acid; YM, yeast extract-malt extract

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v) yeast extract (Difco, Detroit, MI), 0.3%(w/v) malt extract (Difco), 0.5%(w/v) Bactopeptone (Difco), and 1.0%(w/v) dextrose (Merck, Darmstadt, Germany). To avoid bacterial growth, 100 μg/mL^{-1} penicillin-streptomycin solution (HyClone; Thermo Fisher Scientific, Logan, UT) was added. A single colony of an isolate was preinoculated in the YM broth and incubated at 30°C with agitation (200 rpm) until the stationary phase was reached. The isolate (1%, v/v) was inoculated into a fresh YM broth and incubated statically at 10-40°C with 5°C increments for each culture, and pH of 2.0-12.0 with an increment of 1.0 between each culture. The required pH was established after sterilization and prior to inoculation by the addition of 5 M HCl and 10 M KOH. Salt and ethanol tolerances were assessed at 30°C using YM broths supplemented with 0-15%(w/v) NaCl in increments of 1%, and 0-10%(v/v) EtOH in increments of 1%. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a BioPhotometer 6131 spectrophotometer (Eppendorf AG, Eppendorf, Germany).

Isolation of yeast from fermented tea. The fermented tea sample was obtained from Boseong, Korea. Ninety milliliters of sterile 0.85%(w/v) NaCl was added to 10 g of the fermented tea 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) Bactotryptone (Difco), and 1.0%(w/v) NaCl (Bioshop Canada, Burlington, Ontario) for the isolation of bacteria, YM agar for the isolation of yeast, and Potato Dextrose agar (Neogen, Lansing, MI) for the isolation of mold. Individual colonies, selected randomly according to their different color and morphological characteristics, were purified by the 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 required.

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 the optimum growth temperature. Protease production was observed by the direct appearance of the hydrolysis halos surrounding the colonies.

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

PCR and DNA sequencing. To identify the isolate, PCR amplification and DNA sequencing of the ITS regions (ITS 1 and ITS2) including the 5.8S rRNA gene and D1/D2 domain of the 26S rRNA gene were performed. 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 5.8S-ITS rDNA region was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The 26S rDNA D1/D2 region was amplified using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). The PCR reaction mixture consisted of 10 μL of 2× Prime Taq Premix Solution (GeNet Bio, Cheonan, Korea) consisting of 1 U/μL^{-1} 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 was 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. The 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).

Phylogenetic analysis. The acquired sequences were used for the BLAST gene homology search (http://www.ncbi.nlm.nih.gov/BLAST/) with the publicly available 5.8S-ITS rDNA, and 26S rDNA D1/D2 sequences. Identification was assigned to the generic level [Altschul *et al*., 1997]. The 5.8S-ITS rDNA and the 26S rDNA D1/D2 sequences of the isolated strain were aligned with the 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 with 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 complete 5.8S-ITS rDNA sequence and partial 26S rDNA D1/D2 sequence of the isolated strain have been deposited in GenBank under the accession number EU547494.

Assay of protease activity. The culture supernatants used as the source of crude enzyme were prepared by centrifugation at 12,000 rpm and 4°C for 5 min. The protease activity of the enzyme was assayed as described previously [Hagihara *et al*., 1958] using casein as a substrate. The crude enzyme solution (1.0 mL) was added to an equal volume of 0.6% Hammarsten casein (BDH Biochemical, Poole, UK) in 0.1 M phosphate buffer, pH 7.0. After incubation at 50°C for 10 min, the reaction was stopped by adding 5 mL of 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, and the precipitate was removed by centrifugation at 10,000 rpm for 5 min. The 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 before the addition of the substrate. One unit of the protease activity was defined as the amount of enzyme that liberated a digestion product not precipitated by TCA equivalent to 1 μg mL^{-1} min^{-1} of tyrosine under the assay conditions. All assays were carried out in triplicate, and the data represent averages of three determinations.

Effect of pH and temperature on protease activity. The conditions for obtaining maximum protease production were studied by assaying the enzyme at different pHs and temperatures, and the relative enzyme activities were calculated. All determinations were performed in triplicates. The effect of pH on the protease activity of the crude enzyme was determined by incubating 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)] using the aforementioned assay conditions. For the pH stability determination, enzyme solutions were preincubated in the absence of the substrate at the desired pH (pH 3.0-11.0) at 4°C for 24 h. After incubation, the residual enzyme activity (%) was measured under the optimum conditions of the assay. The effect of temperature on the protease activity was determined by incubating the reaction mixture at 20-70°C. To determine the thermal stability, the residual activity (%) of the enzyme was measured under the optimal assay conditions after preheating of the enzyme solution at different temperatures for different time periods.

Culture conditions for growth and protease production. To optimize the culture conditions for maximum production of protease, the influences of carbon, nitrogen, and mineral sources were assessed. To test the effects of different carbon sources on the protease production, dextrose in YM medium was individually substituted with 1.0%(w/v) each arabinose, CMC, dextrose, fructose, galactose, glucose, glycerol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, soluble starch, sucrose, and xylose. The effects of different nitrogen sources on protease production were studied by individually supplementing a liquid medium containing 1.0%(w/v) dextrose with 1.0%(w/v) complex nitrogen sources (beef extract, casein, malt extract, peptone, skim milk, soytone, tryptone, urea, urea base, and yeast extract) and 1.0%(w/v) inorganic nitrogen sources [$(NH_4)H_2PO_4$, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, KNO_3, and $NaNO_3$]. To determine the effect of mineral sources on the protease production, 0.3%(w/v) each of $CaCl_2$, $CaCO_3$, $CoCl_2$, $CuSO_4$, $FeSO_4$, K_2HPO_4, KCl, KH_2PO_4, $MgSO_4$, $MnSO_4$, NaCl, and $ZnCl_2$ were individually added as mineral sources to 1.0%(w/v) each xylose and yeast extract broth media. The effects of the exposure duration of the mineral sources on *S. ruineniae* CO-3 growth and protease production were also determined under the previously determined optimal growth conditions. Culture supernatants were recovered periodically, and the protease activity was determined under the optimal conditions of the assay.

Results and Discussion

Isolation of protease-producing yeast from fermented tea. During a search for yeast with potential biocontrol agents, a single strain of yeast was isolated from the samples of fermented tea collected in Boseong, Korea. Growth was evident only on the YM agar plates supplemented with 100 μg/mL^{-1} of penicillin-streptomycin after approximately 3 days of incubation. Protease production by this isolate was tested using its ability to grow on the YM agar supplemented with 1.0%(w/v) skim milk. The development of halo zones around the colonies was indicative of the extracellular protease production. The yeast strain was designated as CO-3 and was used for the subsequent experiments.

CO-3 characterization. Isolate CO-3 was characterized morphologically, physiologically, and biochemically as previously described [Barnett *et al*., 2000]. After incubation for 2-3 days at 30°C on YM agar medium, all developed colonies displayed an entire margin and convex elevation, and were opaque, non-glistening, pink-to-red colored,

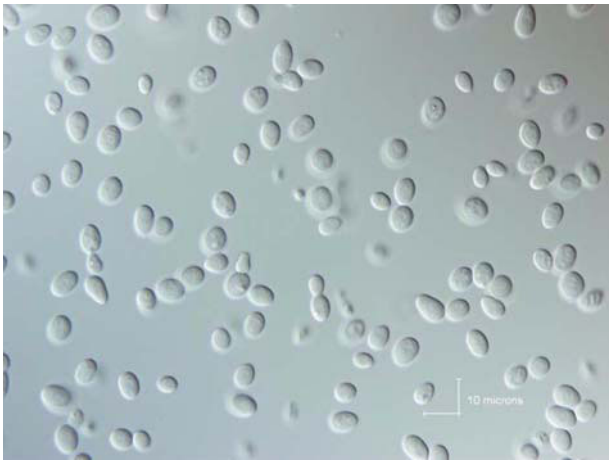

Fig. 1. Phase-contrast photomicrograph of strain CO-3. Cells were photographed after grown in YM broth for 2-3 days at 30°C.

and circular. Microscopic examination revealed spherical- to ovoid-shaped cells, 6.8-7.4×7.4-9.1 μm in size, which occurred singly or with buds (Fig. 1).

Table 1 summarizes the effects of temperature, pH, NaCl, and EtOH on the growth of CO-3. Strain CO-3 grew between 15 and 35°C, with the optimal growth evident at 25-30°C. Growth was not observed at 40°C, and was markedly diminished at below 15°C and above 35°C. The pH range of the medium for the cell growth was from 2.0-12.0. The isolate grew well at pH 3.0-12.0 with the optimum growth occurring at 5.0-6.0; no growth was observed at pH 2.0. Therefore, in the subsequent experiments, the pH of the medium was kept at 5.0-6.0. Isolate CO-3 grew in the presence of 0-4%(w/v) NaCl and 0-5%(v/v) EtOH, but was the most exuberant when both compounds were absent.

Biochemical characteristics of CO-3 are summarized in Table 2. CO-3 was positive in the assimilation test for acetate, citrate, D-glucose, D-mannose, D-raffinose, D-sorbitol, L-glutamate, L-malate, L-proline, L-sorbose, nitrate, and sucrose. Positive acidification results were obtained for D-mannitol, D-mannose, methyl-β-D-glucopyranoside, raffinose, sorbitol, and sucrose. Positive results were also evident in the alkalinization test for argin dihydrolase and urease, enzymatic test for alanine arylamidase, L-leucine arylamidase, L-proline arylamidase, phenylalanine arylamidase, and tyrosine arylamidase, and precipitation test for esculin hydrolysis. All inhibition tests gave negative results.

Phylogenetic analysis of CO-3. Among the many molecular methods, ribosomal DNA (rDNA) sequence analysis offers a generally rapid and a useful means of speciation [Fell *et al*., 2000]. Therefore, a molecular phylogenetic-based identification of the strain CO-3 was performed using the rDNA sequence analysis. Nearly complete sequences for ITS1-5.8S rDNA-ITS2 regions and D1/D2 domain of 26S rDNA were determined. The 2,879 bp sequences obtained were aligned with all the presently available sequences for the ITS1-5.8S rDNA-ITS2 region and D1/D2 domain of 26S rDNA in the GenBank database. CO-3 was determined to be a close relative of *S. ruineniae*. The ITS1-5.8S rDNA-ITS2 region sequence of both yeast were completely identical with those of *S. ruineniae* strains CBS 5811 (Genbank accession number AF444491) and JCM 8097 (AB030339). The partial D1/D2 domain of 26S rDNA sequence of the strain CO-3 displayed 99% identity with the corresponding sequences of *S. ruineniae* var. *ruineniae* (AF070438), *S. ruineniae* var. *coprophilus* (AF070434), and *S. ruineniae* strain IGC 5692 (AF0387128). A phylogenetic tree was constructed based on the ITS1-5.8S rDNA-ITS2 regions and D1/D2 domain of 26S rDNA sequences to show the comparative relationship between the strain CO-3 and other related species. CO-3 belonged to the genus *Sporidiobolus* and was most closely related to *S. ruineniae* (Fig. 2). Accordingly, we named the isolated strain *S. ruineniae* strain CO-3.

Extracellular protease properties of the crude enzyme. Although it is known that *Sporidiobolus* species produce extracellular enzymes, the characteristics of the proteases produced by this yeast grown under various conditions have not been analyzed [Brizzio *et al*., 2007].

Table 1. Characteristics of the isolated strain CO-3

Characteristics	Strain CO-3
Shape	Spherical to ovoid
Cell size (μm)	(6.8-7.4)×(7.4-9.1) μm
Colony color	Pink to red
Form	Circular
Elevation	Convex
Margin	Entire
Opacity	Opaque
Brilliancy	No glistening
Temperature (°C) for	
optimum growth	25-30
growth range	15-35
pH for:	
optimum growth	5.0-6.0
growth range	3.0-12.0
Growth in NaCl (%, w/v) at	
optimum growth	0.0-1.0
growth range	0.0-4.0
Growth in EtOH (%, v/v) at	
optimum growth	0.0-2.0
growth range	0.0-5.0

Table 2. Biochemical characteristics of the isolated strain CO-3

Biochemical characteristics[a]	Strain CO-3	Biochemical characteristics	Strain CO-3
Assimilation test:		Methyl-D-xyloside	-
2-Keto-D-gluconate	-	Methyl-β-D-glucopyranoside	+
Acetate	+	Myo-inositol	-
Amygdalin	-	N-acetyl-glucosamine	-
Arbutine	-	Palatinose	-
Citrate	+	Pullulan	-
D L-lactate	-	Raffinose	+
D-cellobiose	-	Salicin	-
D-galactose	(+)	Sorbitol	+
D-galacturonate	-	Sucrose	+
D-gluconate	-	Xylose	-
D-glucose	+	Alkalinisation test:	
D-mannose	+	Argin dihydrolase	+
D-melezitose	-	Arginine	(-)
D-melibiose	-	Pyruvate	(+)
D-raffinose	+	Urease	+
D-sorbitol	+	Enzymatic test:	
D-trehalose	-	Alanine Arylamidase	+
D-turanose	-	Ala-phe-pro-arylamidase	-
D-xylose	-	Alkalin phosphatase	-
Erythritol	-	Ellman	-
Gentobiose	-	Glycine Arylamidase	-
Glucuronate	-	L-aspartate arylamidae	-
Glycerol	(+)	L-leucine arylamidase	+
Lactose	-	L-lysine arylamidase	-
L-arabinose	-	L-proline arylamidase	+
L-glutamate	+	L-pyroglutamic acid arylamidase	-
L-malate	+	L-pyrrolydonyl arylamidase	-
L-proline	+	Phenylalanine arylamidase	+
L-rhamnose	-	Phosphatidylinositol phospholipase C	-
L-sorbose	+	Phosphoryl cholin	-
Methyl-α-D-glucopyranoside	-	PNP-N-acetyl-β-D-galactosaminidase 1	-
N-acetyl-glucosamine	-	tyrosine arylamidase	+
Nitrate	+	α-Galactosidase	-
Putrescine	-	α-Glucosidase	-
Sucrose	+	α-Mannosidase	-
Xylitol	-	α-Galactosidase	-
Acidification test:		β-Glucosidase	-
Amygdalin	-	β-Glucuronidase	-
Cyclodextrine	-	β-Mannosidase	-
D-galactose	-	β-N-acetyl-glucosaminidase	-
D-glucose	-	β-Xylosidase	-
D-mannitol	+	γ-Glutamyl transferase	-
D-mannose	+	Inhibition test:	
D-melezitose	-	Bacitracin resistance	-
D-ribose	-	Growth in 6.5% NaCl	-
D-tagatose	-	Kanamycin resistance	-
D-trehalose	-	Novobiocin resistance	-
Glycogene	-	O129	-
Inulin	-	Oleandomycin resistance	-
Lactate	-	Optochin resistance	-
Lactose	-	Polymyxin-β resistance	-
L-rhamnose	-	Precipitation test:	
Maltose	-	Esculin hydrolysis	+
Maltotriose	-	Tetrazolium RED	-

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

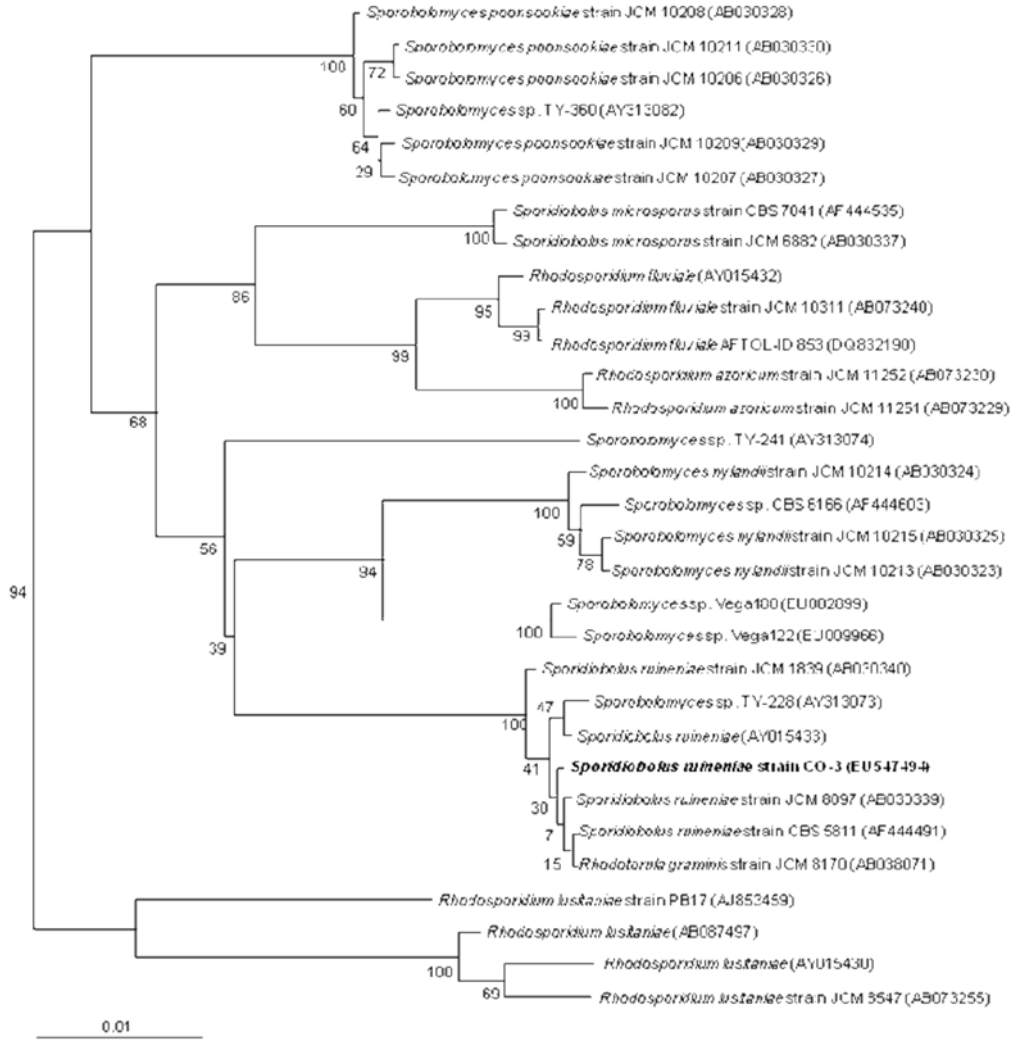

Fig. 2. Phylogenetic tree showing the relationship between the isolated strain CO-3 and other related species. The GenBank accession numbers are given in parentheses. The tree was constructed using the CLUSTAL-X and neighbour-joining method. Scale bar corresponds to 0.01 subscriptions per nucleotide position. Numbers at nodes indicate levels of bootstrap support (%) determined from 100 resampled data.

To determine the characteristics of the protease produced by CO-3, the influences of pH and temperature on activity and stability of the extracellular proteases recovered from the culture supernatants were investigated. The pH optimum for enzyme activity, as ascertained over a range of 3.0-11.0 using Hammarsten casein as a substrate, was 7.0 in 0.1 M phosphate buffer, with more than 60% of the maximal activity retained at pH 5.0-7.5 (Fig. 3). Acidic pH (3.0-4.0) and alkaline pH (8.0-11.0) only marginally affected enzyme activity.

The pH stability of protease was determined after pre-incubation for 24 h at 4°C in the buffers with varied pHs. The protease was very stable over the tested pH range, maintaining over 80% of its maximal activity between pH 5.0 and 7.0, and more than 50% of its activity at 4.5 and 8.0 (Fig. 4).

Temperature is one of the most important factors affecting the enzyme production [Chi and Zhao, 2003]. With a reaction time of 10 min, the optimum temperature for assaying the protease activity in the culture supernatant was 50°C, and more than 90% of the maximal activity was obtained at 40°C (Fig. 5). The protease still retained more than 50% of the maximal activity at 30 and 55°C.

Thermal stability studies showed that the protease retained more than 80% of the maximal activity when pre-incubated for 10 min at 40, 50, 55, and 60°C. After pre-incubation for 30 min at its optimum temperature of 50°C, the enzyme retained 65% of the maximal activity, then steadily decreased. At 60°C, the enzyme still retained about 55% of the initial activity after 30 min pre-incubation (Fig. 6). Pre-incubation for 60 min at 55 and 60°C rapidly inactivated the enzyme, retaining only 30 and 22% activities, respectively. Based on these experimental results, we determined that pH 7.0 and 50°C

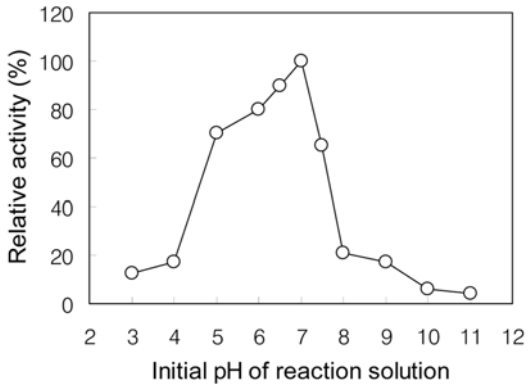

Fig. 3. Effect of initial pH on the activity of the extracellular protease produced by *S. ruineniae* CO-3. The enzyme activity was assayed at 50°C for 10 min in 0.05 M citrate (pH 3.0-6.0), 0.1 M phosphate (pH 7.0-8.0), and 0.1 M sodium bicarbonate (pH 9.0-11.0) buffer. The values are shown as percentages of the maximum activity of the enzyme observed at pH 7.0, which is taken as 100%.

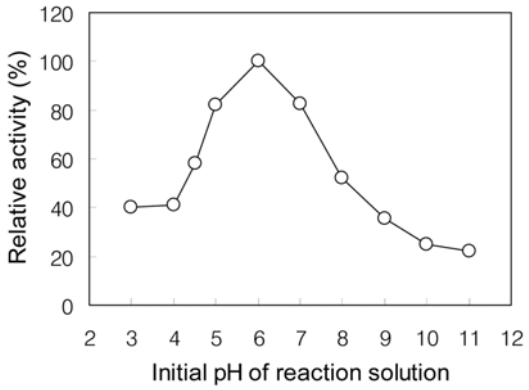

Fig. 4. pH stability of the extracellular protease produced by *S. ruineniae* CO-3. The residual activity was measured at 50°C for 10 min after 24 h pre-incubation of the enzyme at 4°C in various pH buffers: 0.05 M citrate (pH 3.0-6.0), 0.1 M phosphate (pH 7.0-8.0), and 0.1 M sodium bicarbonate (pH 9.0-11.0). Relative activity is expressed as a percentage of the maximum enzyme activity under the standard assay conditions.

were optimal for the protease activity.

Relatively little is known about the yeast extracellular protease. Studies have characterized the extracellular proteases produced by *C. olea* [Nelson and Young, 1987] and *S. lipolytica* [Yamada and Ogrydziak, 1983]. *C. olea* secretes an acid and an alkaline protease. The acid protease is optimally active at pH 3.3 and 42°C, with the alkaline protease being optimally active at pH 8.0-9.0 and 40°C. Both enzymes are inactivated at temperatures above 46°C. *S. lipolytica* CX161-1B, grown at pH 3.2, produces three extracellular proteases that are active at pH 3.1-4.2 [Yamada and Ogrydziak, 1983].

Effects of culture conditions on growth and protease production. The growth and production of proteases can also be influenced by the composition of the growth medium. Appropriately, the effects of the carbon, nitrogen, and mineral sources on CO-3 protease production were examined. *S. ruineniae* CO-3 could utilize a wide range of carbon sources for growth (Table 3). Xylose was the best carbon source for protease production, followed by arabinose, as compared to the control cultures. Although the growth of CO-3 increased in the presence of glycerol, mannitol or sucrose as individual carbon sources, the production of the target protease decreased by more than 60%. On the other hand, the use of lactose, rhamnose, and soluble starch as carbon sources had no significant effect on the production of protease with respect to the control. The varying results depending on the carbon source are

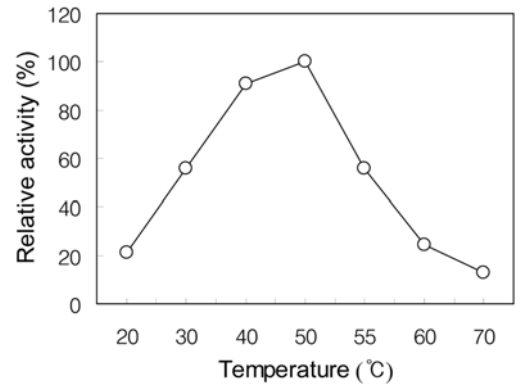

Fig. 5. Effect of temperature on the activity of the extracellular protease produced by *S. ruineniae* CO-3. The enzyme activity was assayed at various temperatures for 10 min in 0.1 M phosphate buffer (pH 7.0). The values are shown as percentages of the maximum activity of the enzyme observed at 50°C, which is taken as 100%.

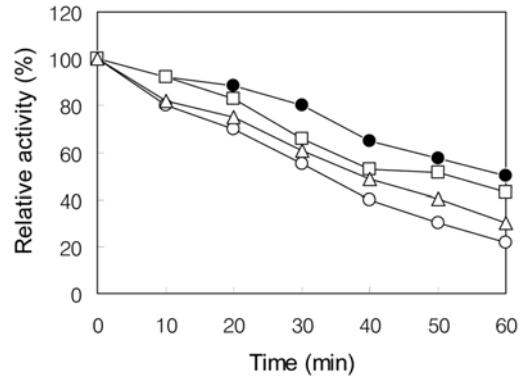

Fig. 6. Thermal stability of the extracellular protease produced by *S. ruineniae* CO-3. The enzyme reaction was carried out at optimal conditions after preheating the enzyme solution at the indicated temperatures for different time periods. Relative activity is expressed as a percentage of the maximum enzyme activity under the standard assay conditions. Symbols: ○, 60°C; △, 55°C; □, 50°C; ●, 40°C

Table 3. Effects of various carbon sources on the cell growth and activity of the extracellular protease produced by *S. ruineniae* strain CO-3

Sources (1.0%, w/v)	Cell growth (OD_{600})	Relative enzyme activity (%)
Control	3.389	100.0
Arabinose	8.265	119.6
CMC	4.463	97.4
Dextrose	8.920	68.0
Fructose	8.967	88.5
Galactose	9.001	76.5
Glucose	8.839	67.9
Glycerol	9.566	54.3
Lactose	3.792	107.4
Maltose	6.191	99.3
Mannitol	9.256	52.6
Mannose	8.976	69.1
Raffinose	7.149	92.4
Rhamnose	4.766	107.9
Soluble starch	4.220	102.5
Sucrose	9.440	56.9
Xylose	8.475	125.2

consistent with a previous description that different carbon sources have disparate influences on the extracellular enzyme production by different yeast strains [Chi and Zhao, 2003].

The influence of specific nitrogen sources on protease production differs from organism to organism, although complex nitrogen sources are usually used for protease production [Kurmar and Tagaki, 1999]. The influences of various complex and inorganic nitrogen sources on the protease activity are presented in Table 4. Malt extract and peptone were replaced with various complex and inorganic nitrogen sources at a nitrogen concentration of 1.0%(w/v). Yeast extract had the most pronounced effect on the production of the extracellular protease when compared to the control, whereas addition of casein and malt extract produced little effect on the production. Among the inorganic nitrogen sources tested, $(NH_4)H_2PO_4$ had a profound effect on the production of the enzyme with maximal enzyme activity as compared to the control. The remaining complex and inorganic nitrogen sources stimulated the extracellular protease production by CO-3. The complex nitrogen sources supported even better extracellular protease production (Table 4).

The effects of various mineral sources on the growth of the isolate and the protease activity of the crude enzyme are summarized in Table 5. Among the various mineral sources tested, K_2HPO_4 best enhanced the enzyme

Table 4. Effects of various nitrogen sources on the cell growth and activity of the extracellular protease produced by *S. ruineniae* strain CO-3

Sources (1.0%, w/v)	Cell growth (OD_{600})	Relative enzyme activity (%)
Control	9.036	100.0
Complex nitrogen sources:		
Beef extract	9.119	436.4
Casein	9.306	125.3
Malt extract	9.370	145.0
Peptone	8.879	333.4
Skim milk	10.306	220.8
Soytone	9.634	483.3
Tryptone	9.515	526.7
Urea	1.180	309.3
Urea base	8.672	355.2
Yeast extract	9.593	828.3
Inorganic nitrogen sources:		
$(NH_4)H_2PO_4$	8.419	226.5
NH_4Cl	8.085	182.7
NH_4NO_3	8.333	203.8
$(NH_4)_2SO_4$	8.511	186.2
KNO_3	8.298	143.6
$NaNO_3$	8.317	164.5

Table 5. Effects of various mineral sources on the cell growth and activity of the extracellular protease produced by *S. ruineniae* strain CO-3

Mineral sources (0.3%, w/v)	Cell growth (OD_{600})	Relative enzyme activity (%)
Control	7.875	100.0
$CaCl_2$	8.988	80.3
$CaCO_3$	9.880	102.8
$CoCl_2$	1.127	154.9
$CuSO_4$	8.191	205.3
$FeSO_4$	3.463	125.2
K_2HPO_4	8.773	298.9
KCl	8.900	113.1
KH_2PO_4	8.959	141.5
$MgSO_4$	9.017	79.2
$MnSO_4$	1.330	143.4
NaCl	8.725	107.0
$ZnCl_2$	1.439	141.4

production, followed by $CuSO_4$. $CaCl_2$ and $MgSO_4$ supported the growth but decreased the enzyme production.

Protease activity and cell growth during cultivation. To determine the effect of cultivation time on the protease production and cell growth, the activity and growth profiles of the supernatant from *S. ruineniae* CO-3 grown

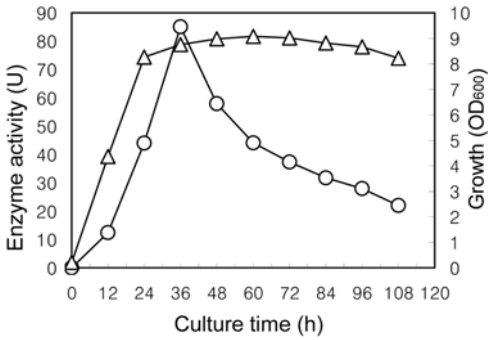

Fig. 7. Growth and extracellular protease production of *S. ruineniae* CO-3 during cultivation. CO-3 was grown aerobically in optimum media at 30°C. Culture supernatants was prepared periodically and protease activity was determined at 50°C for 10 min. ○, Enzyme activity (U); △, cell growth ($OD_{600\ nm}$)

under optimized conditions were studied for 120 h (Fig. 7). CO-3 required over 24 h to attain the stationary phase, and the highest biomass yield (9.07 absorbance at 600 nm) was recorded after 60 h of incubation. Under the optimal conditions, maximum extracellular protease activity was detected at the early stationary phase after 36 h of cultivation, decreasing thereafter over the remaining 84 h of cultivation.

Our collective results revealed that *S. ruineniae* is the main fermenting microorganism in the fermented tea sample examined. More importantly, the present study is the first, to the best of our knowledge, to characterize the extracellular protease produced by *S. ruineniae*. The present study on the growth and the protease production of *S. ruineniae* strain CO-3 has a high potential to be developed into the industrial biocontrol technology.

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