ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science **2019** Vol. 29. No. 10. 1126~1135 DOI : https://doi.org/10.5352/JLS.2019.29.10.1126

Isolation of the Protease-producing Yeast *Pichia anomala* CO-1 and Characterization of Its Extracellular Neutral Protease

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Received August 16, 2019 / Revised October 4, 2019 / Accepted October 5, 2019

From a sample of bamboo byproduct, the protease-producing yeast strain CO-1 was newly isolated. Strain CO-1 is spherical to ovoid in shape and measures 3.1 - 4.0×3.8 - 4.4 μm. For the growth of strain CO-1, the optimal temperature and initial pH were 30°C and 4.0, respectively. The strain was able to grow in 0.0 - 15.0%(w/v) NaCl and 0.0-9.0%(v/v) ethanol. Based on a phylogenetic analysis of its 18S rDNA sequences, strain CO-1 was identified as *Pichia anomala*. The extracellular protease produced by *P. anomala* CO-1 was partially purified by ammonium sulfate precipitation, which resulted in a 14.6-fold purification and a yield of 7.2%. The molecular mass of the protease was recorded as approximately 30 kDa via zymogram. The protease activity reached its maximum when 1.0%(w/v) CMC was used as the carbon source, 1.0%(w/v) yeast extract was used as the nitrogen source, and 0.3%(w/v) $MnSO_4$ was used as the mineral source. The protease revealed the highest activity at pH 7.0 and 30°C. This enzyme maintained more than 75% of its stability at a pH range of 4.0-10.0. After heating at 65℃ for 1 hr, the neutral protease registered at 60% of its original activity. The protease production coincided with growth and attained a maximal level during the post-exponential phase.

Key words : Characterization, extracellular neutral protease, identification, optimization, *Pichia anomala*

Introduction

Protease is one of the important commercial enzymes, accounting for approximately 60% of the total market sales in the world. It has numerous applications in detergents, food processing, medical applications, and leather processing [26]. In addition, protease is important in biocontrol mechanisms, including mycoparasitism, competition, and antibiosis [9]. On the basis of pH range in which enzyme activity is optimum, protease is categorized as acid, neutral and alkaline protease. Unlike other proteases, neutral protease hydrolyzes hydrophobic amino acid bonds at neutral pH, thereby decreasing the bitter taste of protein hydrolysates. Thus, neutral protease process has been discovered to be efficient in food industry [29, 33]. Although many different proteases have been characterized until now, some of them are not sufficient to meet most of the industrial applications [17].

Protease is obtained from all organisms. Especially, protease-producing microorganisms are the most appropriate resources for use in industrial production and can be commercially exploited. The microorganisms can easily be cultivated in a large scale, once their biochemical and physical characteristics and physiological functions are established [26]. Extracellular protease from different microorganisms including bacteria, mold, and yeast has been characterized [2, 9, 24]. Despite reports on yeast protease, comparatively little is known about the characteristics of extracellular protease. Nevertheless, some yeast has been studied as protease producers for potential industrial development, because they may be suitable for the biological control of post-harvest diseases of grains and fruits, and as bioremediation agents [5]. *Candida humicola* [28], *Yarrowia lipolytica* [13], and *Sporidiobolus ruineniae* [18] were studied with regard to variability of the characteristics of extracellular protease, according to the medium composition, pH, and temperature.

The aim of the present work was to identify industrially interesting microorganism for biological control applications. Accordingly, I isolated and identified a yeast strain from bamboo-by product that produces extracellular enzymes. In addition, characteristics of the obtained crude enzyme were investigated.

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Materials and Methods

Growth medium and culture condition

Cultivations were carried out in YM medium composed of 0.3% (w/v) yeast extract (BioShop, USA), 0.3% (w/v) malt extract (BioShop), 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. To determine the culture conditions, the yeast cells was inoculated in YM broth and cultivated at 30°C with agitation (200 rpm) for 18-20 hr. Then, the culture suspension (1.0%, v/v) was transferred into an YM broth and incubated statically at 20-40°C, and pH of 2.0-12.0. Salt and ethanol tolerances were evaluated at 30°C using YM broth added with 0-15.0% (w/v) NaCl, and 0-10.0% (v/v) ethanol, respectively. The growth of yeast cells was checked by determination of optical density at 600 nm (OD_{600}) using a spectrophotometer (BioPhotometer 6131, Eppendorf AG, Eppendorf, Germany).

Isolation of yeast strain 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. The homogenate was diluted with 0.85% NaCl (10^1-10^8 cells). The suspension was spread onto YM agar and cultivated for 24 hr at 30°C. Colonies were picked randomly, through their different color and shape, and pure cultured by subsequent streaking on YM agar.

Screening of extracellular protease-producing yeast strain

A direct agar plate assay was used for ascertainment of extracellular protease production. Isolated strains were inoculated in YM agar containing skim milk (1.0%, w/v) [2] and cultivated for 24 hr at 30℃. Protease production was detected by formation of transparent halos surrounding the colonies on the plates. Positive yeast strains were used to the assay of protease activity.

Characteristics of a newly isolated yeast strain

Morphological, cultural, and biochemical characteristics of the bamboo by-product isolate were determined as previously described [3]. Morphology of cells recovered from YM medium was determined using a phase-contrast microscope (Model BX51, Olympus, Tokyo, Japan). Biochemical characteristics of yeast isolates were obtained according to the VITEK 2 system (VITEK 2 Compact 60 apparatus, bioMérieux, Hazelwood, MO) and the method explained by Barnett et al. [3].

PCR and DNA sequencing of the 18S rRNA gene

Total genomic DNA of the strain was isolated using the genomic DNA extraction kit (GeneAll™, GeneAll Biotechnology, Seoul, Korea). Polymerase chain reaction (PCR) was performed as previously described [10, 34]. The primers NS1 and NS8 were used. The PCR mixture consisted of 10 µl of 2× Prime Taq Premix Solution (GeNet Bio, Cheonan, Korea) containing 1 U/µl Prime Taq DNA polymerase, 0.5 mM dNTP, 4.5 mM $MgCl_2$, 0.1% gelatin, 1 µl of 10 pM primers, and 1 µl of template, prepared to a final reaction volume of 20 µl. PCR was completed with a total of 30 cycles in the Thermal Cycler (Model PC708 Program Temp Control System, ASTEC, Tokyo, Japan). The amplification program was proceed with an initial denaturation (93°C) for 3 min followed by 30 cycles of denaturation (93℃) for 1 min, primer annealing (57°C) for 1 min, and extension (72℃) for 2 min. PCR was finished with a final extension (72℃) for 5 min and the amplified product was cooled (4℃). Sequencing of amplified DNA fragment was contractually accomplished by Solgent (Daejeon, Korea).

Phylogenetic analysis

The collected DNA sequences were applied for the BLAST gene homology search with the publicly available 18S rDNA sequences. Identification was assigned to the generic level [1]. The 18S rDNA sequences of the isolate were aligned with the sequences of related species obtained from GenBank [31] using the CLUSTAL X multiple sequence alignment program. Phylogenetic analysis was achieved using PHYLIP software [11] and a phylogenetic tree was built by the neighbor-joining method using TreeView software [25].

GenBank accession number

The partial 18S rDNA sequence of the isolate has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers EF427893.

Assay of protease activity

Protease activity with casein as the substrate was established by the modified method of Hagihara et al. [15]. The enzyme solution (1.0 ml) was supplemented to an equal vol-

ume of 0.6% Hammarsten casein (BDH Biochemical, Poole, UK) in 0.1 M phosphate buffer (pH 7.0) and incubated for 10 min at 30℃. The reaction was then stopped by supplement of 5 ml of trichloroacetic acid (TCA) mixture (0.11 M TCA, 0.33 M acetic acid, 0.22 M sodium acetate). The mixture was kept at 20-25℃ for 30 min, and the precipitate was removed by centrifugation (10,000 rpm) for 5 min. Then, optical density of the recovered supernatant was determined at 280 nm. The blank was run in the same manner, except that the enzyme solution was blended with the TCA before 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. The protein content of the enzyme preparation was estimated by Lowry et al. [23].

Partial purification of protease

The isolate CO-1 was incubated in optimum medium [1.0% (w/v) carboxymethyl cellulose (CMC), 1.0% (w/v) yeast extract, and 0.3% (w/v) $MnSO_4$, pH 7.0] for 24 hr at 30°C. The culture broth was centrifuged (12,000 rpm) for 10 min at 4°C. The enzyme from the cell-free supernatant was precipitated by ammonium sulfate (up to 80% saturation) and kept overnight at 4°C. The precipitate was collected by centrifugation at 10,000 rpm for 30 min, dissolved in 0.1 M phosphate buffer (pH 7.0), and then dialyzed against the same buffer to eliminate residual ammonium sulfate. This dialyzed soluble fraction was used for protease activity.

Zymogram (activity staining)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% (w/v) polyacrylamide gel containing 1% gelatin (w/v) was conducted as described by Laemmli [21]. After electrophoresis was completed, zymogram was completed as explained by Bernal et al. [4] with a minor modification. The gel with separated proteins was washed for 30 min at 4℃ in 0.1 M phosphate buffer (pH 7.0) containing 2.5% Triton X-100. Then, the washed gel was soaked at 30°C for 1 hr in pre-warmed 0.1 M phosphate buffer (pH 7.0) and was stained with Coomassie Brilliant Blue R-250 (Sigma, USA). Areas of protease activity occurred as non-stained transparent bands on a dark blue background. The molecular mass of the proteases was recorded by comparing the bands of standard molecular mass markers for 220, 97, 66, 45, 30, 20.1 and 14.3 kDa (Amersham Biosciences, UK).

Effect of various carbon, nitrogen and mineral sources

To investigate the effects of various carbon sources on the protease activity, dextrose in YM broth was individually replaced with 1.0% (w/v) each carbon sources as shown in the Table 3. The effects of various nitrogen sources were examined by individually supplementing a broth containing 1.0% (w/v) CMC with 1.0% (w/v) complex nitrogen sources and 1.0% (w/v) inorganic nitrogen sources (Table 4). To study the effect of various mineral sources, 0.3% (w/v) each mineral sources were individually added to 1.0% (w/v) each CMC and yeast extract broth (Table 5).

Effect of different pH and temperature on protease activity and stability

Conditions for obtaining maximal protease activity were studied by assay of the enzyme at different pH and temperatures, and the relative enzyme activities were calculated. Assays were repeated three times, and values presented are averages of the three experiments. For determination of the effect of pH on protease activity, the pH of the reaction mixture containing buffer and 0.6% (w/v) Hammarsten casein was varied over the range of 3.0-11.0. Buffers used were 0.05 M citrate buffer (pH 3.0-5.0), 0.1 M phosphate buffer (pH 6.0-8.0), and 0.1 M sodium bicarbonate buffer (pH 9.0-11.0). pH stability was tested by 24 hr pre-incubation of the enzyme solutions in the absence of the substrate at different pH values ranging from 3.0 to 11.0 at 4°C. The residual activities (%) of enzyme were determined immediately after this treatment using the standard method described above. The effect of temperature on protease activity was measured by incubation of the reaction mixture at temperatures ranging from 20 to 75℃ in 0.1 M phosphate buffer (pH 7.0) for 10 min. To ascertain the temperature stability, the enzyme reaction was carried out at optimal condition after pre-incubating of the enzyme solution at various temperatures (30-70℃) for 1 hr. And then remaining activity (%) of the enzyme was assayed immediately.

Time course of growth and protease production

The growth and protease production was also studied under the previously determined optimal conditions. The culture broth was inoculated in optimum medium and incubated at 30℃ with agitation (200 rpm). Culture supernatants were recovered periodically, and cell growth and

protease activity were monitored as described above.

Results and Discussion

Isolation of protease-producing yeast strain

Two yeast strains were isolated from bamboo by-product samples obtained in Guri, Korea, and screened according to their ability to produce extracellular protease based on testing for formation of transparent halos. Growth was evident only on YM agar plates supplemented with 100 μg/ml of penicillin-streptomycin after approximately 24 hr of incubation. One strain that produced protease (strain CO-1) was selected.

Characteristics of a newly isolated strain CO-1

The isolated CO-1 was characterized morphologically, culturally, and biochemically, as previously described [3]. Morphological and cultural properties of the strain CO-1 are shown in Table 1. After incubation for 24 hr at 30°C on YM agar, all developed colonies displayed a smooth margin and convex elevation, and were opaque, non-glistening, circular and white-to-cream colored. Microscopic examination revealed spherical- to ovoid-shaped cells, measuring 3.1-4.0× 3.8-4.4 μm, which occurred singly or with buds. The strain CO-1 grew at temperatures range of 20-40℃, with optimum growth evident at 30℃. This strain grew in a wide pH range of 2.0-11.0 with optimum growth occurring at 4.0. No growth was detected at pH 12.0. Therefore, in subsequent experiments, the pH of the medium was retained at 4.0. CO-1 grew in a NaCl concentration ranging up to 15.0% (w/v), and optimally at 1.0(w/v). In addition, it grew in the presence of 0-9.0%(v/v) ethanol, but showed the greatest exuberance in the absence of ethanol.

Table 1. Morphological and cultural characteristics of a newly isolated strain CO-1

Characteristics	Strain CO-1
Shape	Spherical to ovoid
Cell size (μm)	(3.1-4.0)×(3.8-4.4)
Vegetative production	Budding
Colony color	White to cream
Form	Circular
Elevation	Convex
Margin	Smooth
Opacity	Opaque
Brilliancy	No glistening
Temperature (°C) for:	
Optimum growth	30
Growth range	20-40
pH for:	
Optimum growth	4.0
Growth range	2.0-11.0
Growth in NaCl (%, w/v) at:	
Optimum growth	1.0
Growth range	0.0-15.0
Growth in ethanol (%, v/v) at:	
Optimum growth	0.0
Growth range	0.0-9.0

The VITEK system was used for further characterization of the isolated. Biochemical characteristics of CO-1 are displayed in Table 2. CO-1 was positive in the assimilation test for acetate, amygdalin, arbutine, citrate, lactate, galacturonate, gluconate, glucose, mannose, melezitose, raffinose, sorbitol, trehalose, turanose, erythritol, gentobiose, glucuronate, glycerol, glutamate, malate, proline, methyl-α-D-glucopyranoside, nitrate, and sucrose. Positive acidification results were obtained for glucose, mannitol, melezitose, lactate, maltose, maltotriose, mannose, palatinose, salicin, and sucrose. Positive results were also evident in the alkalinization test for arginine and pyruvate, enzymatic test for alkalin phophatase, leucine arylamidase, phenylalanine arylamidase, phosphatidylinositol phospholipase C, phosphoryl cholin, α-glucosidase, β-glucosidase, and β-xylosidase, and precipitation test for esculin hydrolysis. In particular, all inhibition tests showed positive results. The characteristics of *P. anomala* CO-1 differed slightly from those of *P. anomala* J121 [12], and *P. anomala* ATCC96603 [14], respectively.

Phylogenetic analysis of a newly isolated strain CO-1

The 18S rDNA sequences analysis was achieved for identification of CO-1. The 1,682 bp sequences obtained were aligned with all of the presently available sequences in the GenBank database. The sequence of CO-1 was highly homologous to *P. anomala*. The partial 18S rDNA sequence of CO-1 exhibited 99% identity with corresponding sequences of *P. anomala* (DQ520880), *P. anomala* GK1 (AY218895), and *P. anomala* (AB054562). A phylogenetic tree was formed based on the 18S rDNA sequences in order to indicate the comparative relationship between CO-1 and other related organisms. Fig. 1 indicates the phylogenetic position of CO-1 based on the almost full-length 18S rDNA sequences. CO-1 belonged to the genus *Pichia*, and was most closely connected to *Pichia anomala*. Thus, based on morphological, cultural, biochemical characteristics, and phylogenetic analysis, the yeast strain CO-1 was identified as *P. anomala*, and then

Table 2. Biochemical characteristics of a newly isolated strain CO-1

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

VITEK 2 system was used. +, positive; −, negative; (+), weakly positive; (−), weakly negative.

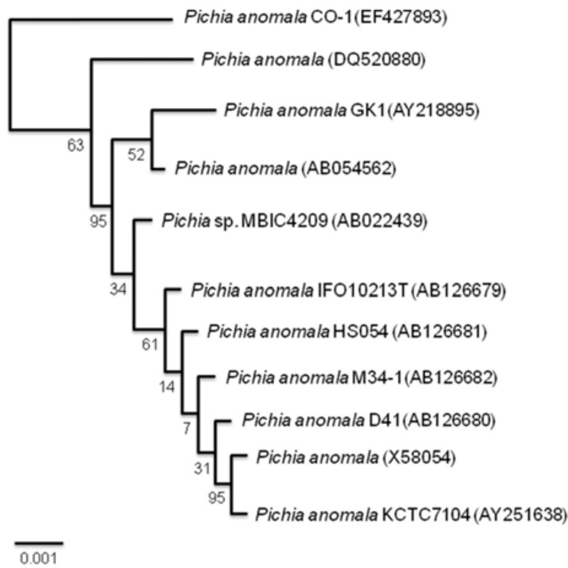

Fig. 1. Phylogenetic position of a newly isolated strain CO-1 based on 18S rDNA sequences GenBank accession numbers are given in parentheses. Scale bar corresponds to 0.001 subscriptions per nucleotide position. Numbers at nodes reveal levels of bootstrap support (%) determined from 100 resampled data.

we named *P. anomala* CO-1.

P. anomala is one of the interesting yeast species, and has shown potential for exploitation in environmental bioremediation, food fermentation, therapeutic protein production, and biofuel production [32]. Especially, anti-microbial activities of *P. anomala* make it an appropriate organism for biological control in the agricultural and food sectors [5].

Partial purification and zymogram of the extracellular protease

Partial purification of the extracellular protease from *P. anomala* CO-1 was carried out by ammonium sulfate precipitation. Approximately 14.6-fold purification of the crude enzyme was accomplished with a recovery of 7.2% and specific activity of 13.9 U/mg proteins (Table 6). The protease activity was visualized by zymogram using gelatin as a protease substrate. Zymogram revealed a clear hydrolysis band against dark background for both crude and partial purified enzymes at equivalent positions in SDS-PAGE (Fig. 2). The enzyme indicated a single band equivalent to an obvious molecular mass of 30 kDa. This value is slightly smaller than that studied for some protease from other yeast [24, 27, 28].

Effect of culture conditions on growth and protease activity

The growth and protease activity can also be influenced by the medium composition (carbon, nitrogen, and mineral sources). *P. anomala* CO-1 could utilize different carbon sources for growth (Table 3). The maximum effect on growth was observed with mannose. CMC was the significant carbon source for protease activity, followed by xylose. On the other hand, the growth of CO-1 increased in the presence of glycerol, mannitol, raffinose, soluble starch or sucrose as individual carbon sources, the activity of the target protease decreased. The varying results depending on the carbon

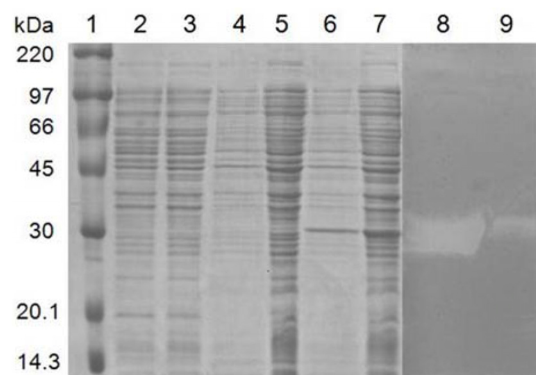

Fig. 2. SDS-PAGE and zymogram of the protease produced by *P. anomala* CO-1 Lane 1: molecular mass markers, lane 2, 3, 5 and 7: crude enzyme (culture supernatant), lane 4 and 6: partially purified protease (ammonium sulfate fractionation), lane 8: zymogram of crude enzyme, lane 9: zymogram of partially purified enzyme.

Table 3. Effects of various carbon sources on the growth and activity of the extracellular protease produced by *P. anomala* CO-1

Sources (1.0%, w/v)	Cell growth (OD_{600})	Relative enzyme activity (%)
Control	4.92	100.0
Arabinose	5.07	160.4
CMC	5.13	365.7
Dextrose	5.86	112.7
Fructose	5.92	136.1
Galactose	5.80	112.7
Glucose	6.01	109.0
Glycerol	5.98	96.7
Lactose	5.12	107.3
Maltose	5.86	108.1
Mannitol	5.88	90.8
Mannose	12.56	114.7
Raffinose	5.47	92.1
Rhamnose	4.85	106.7
Soluble starch	5.60	93.8
Sucrose	5.91	94.6
Xylose	5.56	187.0

source are consistent with a previous description that altered carbon sources have different effects on enzyme activity by various strains [6, 18]. Chi et al. [7] found that soluble starch, and corn starch supported protease activity while sucrose decreased activity. The influence of particular nitrogen sources on protease activity varies according to organism [18, 20]. The influences of various complex and inorganic nitrogen sources on the growth and protease activity are presented in Table 4. The highest growth was found in the presence of skim milk. Yeast extract had apparent effect on the activity of the extracellular protease. This result is consistent with *S. ruineniae* CO-3 [18]. However, malt extract and skim milk revealed decrease in the protease activity. The use of inorganic nitrogen sources had no outstanding influence on the protease activity. Chi et al. [7] found that $NaNo_3$ could be stimulatory for alkaline protease activity by *Aureobasidium pullulans*. 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 different mineral sources investigated, $CaCO_3$ had the excellent effect on the growth. $MnSO_4$ best enhanced the enzyme activity, followed by $CaCO_3$, $CuSO_4$. KH_2PO_4 supported the growth but decreased the enzyme activity. Ma et al. [24] reported $MnCl_2$ followed by $CuCl_2$ to be significant mineral sources

Table 4. Effects of various nitrogen sources on the growth and activity of the extracellular protease produced by *P. anomala* CO-1

Sources (1.0%, w/v)	Cell growth (OD_{600})	Relative enzyme activity (%)
Control	2.82	100.0
Complex nitrogen sources:		
Beef extract	6.51	226.7
Casein	2.93	106.6
Malt extract	11.41	96.8
Peptone	2.90	183.4
Skim milk	11.73	96.0
Soytone	10.50	162.0
Tryptone	5.68	245.8
Urea	2.33	206.1
Urea base	2.93	173.3
Yeast extract	5.99	418.2
Inorganic nitrogen sources:		
$(NH_4)H_2PO_4$	2.49	110.0
NH_4Cl	2.28	103.9
NH_4NO_3	1.83	115.1
$(NH_4)_2SO_4$	2.33	106.0
KNO_3	2.70	103.9
$NaNO_3$	2.35	110.6

Table 5. Effects of various mineral sources on the growth and activity of the extracellular protease produced by *P. anomala* CO-1

Mineral sources (0.3%, w/v)	Cell growth (OD_{600})	Relative enzyme activity (%)
Control	2.89	100.0
$CaCl_2$	2.82	129.4
$CaCO_3$	5.51	148.9
$CoCl_2$	1.20	111.7
$CuSO_4$	1.39	146.4
$FeSO_4$	2.46	77.1
K_2HPO_4	2.79	95.5
KCl	2.74	97.9
KH_2PO_4	2.92	98.3
$MgSO_4$	2.82	96.0
$MnSO_4$	2.10	222.3
NaCl	2.87	98.1
$ZnCl_2$	1.40	131.3

while $FeCl_3$ was poor for protease activity by *A. pullulans* 10. Part of the production cost of industrial enzymes is required to be the cost of the medium [16, 22]. Thus, it is meaningful to optimize the medium conditions for cost-effective enzyme production.

Effect of pH and temperature on the protease activity and stability

For determination of the characteristics of the protease produced by CO-1, influences of pH and temperature on the protease activity and stability were examined. A strong reliance on pH for extracellular enzyme activity is an important characteristic of most microorganisms [20]. Fig. 3

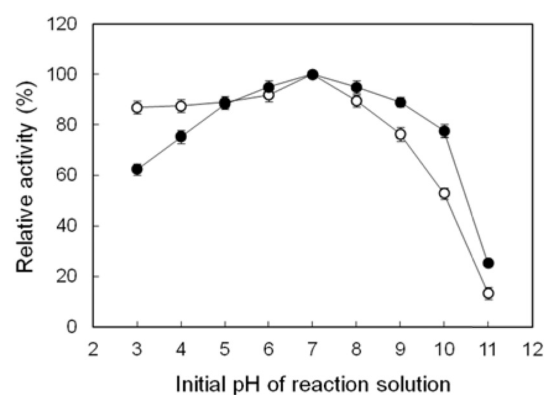

Fig. 3. Effect of pH on the extracellular neutral protease activity and stability Enzyme activity was recorded at the indicated pH (reaction solution) for 10 min at 30℃. The maximum activity of the enzyme was taken as 100%. The error bars represent standard deviations of the means of the three experiments. Symbols: ○, protease activity; ●, protease stability.

shows that the maximum activity was observed at pH 7.0, with more than 75% of the maximal activity maintained at pH 3.0-9.0. The protease activity decreased above pH 10. This indicated that it is an extracellular neutral protease. The results obtained were in corresponded with those reported by other researchers [8, 18]. Contrary to this result, optimum protease activities were observed for *A. pullulans* 10 (pH 9) [24], *Pichia farinosa* (pH 3) [19], and *Cryptococcus* sp. S-2 (pH 5) [27]. The protease showed maximum stability at pH 7.0. It maintained more than 75% of the maximal activity between pH 4.0-10.0 (Fig. 3). This enzyme was stable within a broad range of pH which is in accordance with the results for *P. farinosa* CO-2 [19].

Temperature is one of the major factors affecting production of an enzyme [6]. The influence of temperature was examined by reaction of the enzyme at temperatures range from 20℃to 75℃, which showed that the protease activity was the highest at 30°C. It had more than 80% of the maximal activity between 20℃ and 50℃, while at 75℃ the enzyme activity was only 20% (Fig. 4). Similar result was detected by Rao et al. [27] for protease produced by *Cryptococcus* sp. S-2 that showed maximum activity at 30℃. This temperature is quite different from the optimal values reported for protease from *S. ruineniae* CO-3 with an optimal temperature at 50℃[18], *P. farinosa* CO-2 with an optimal temperature at 40℃[19], *C. buinensis* with an optimal temperature at 25℃[8], and *A. pullulans* with an optimum temperature at 45℃[24]. The thermos-stability was analyzed by pre-incubating the enzyme for 1 hr and the residual activity was evaluated (Fig. 4). The enzyme was stable up to 50°C and showed 60% activity at 65°C, indicating that it was relatively stable at high temperature. A similar result was studied by Rao et al. [27] for protease produced by *Cryptococcus* sp. S-2. The enzyme exhibited more than 80% residual activity at 30-50°C after 1 hr. According to these results, the protease seemed to have thermo-stability.

Cell growth and protease production during cultivation

Cultivation time is one of the significant factors to the protease production on an industrial application. The cell growth and extracellular neutral protease production by CO-1 was investigated under optimized conditions for 48 hr (Fig. 5). The highest biomass yield (OD_{600} = 2.97) was measured after 30 hr of incubation. Under the optimal conditions, maximum neutral protease activity was reached at 24 hr of the cultivation when the cell growth attained the post-exponential phase. The relationship between protease production and growth has also been reported in some published works with yeast strains. In *A. pullulans*, maximum protease production was found at the mid-exponential phase [7]. Although, *S. ruineniae* CO-3, optimal enzyme production took place during the early-stationary phase.

Yeast is able to produce enzymes with industrial significance; however, several yeast enzymes have been studied for potential applications [5, 28, 30]. *P. anomala* is known for production of several enzymes that have shown potential for exploitation as biotechnological commodities [32], whereas the characteristics of extracellular protease under various

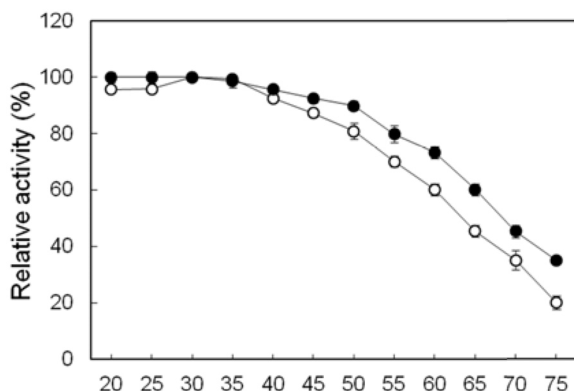

Fig. 4. Effect of temperature on the extracellular neutral protease activity and stability Enzyme activity was recorded at different temperatures (20-75℃) for 10 min in 0.1 M phosphate buffer (pH 7.0). The maximum activity of the enzyme was taken as 100%. The error bars represent standard deviations of the means of the three experiments. Symbols: ○, protease activity; ●, protease stability.

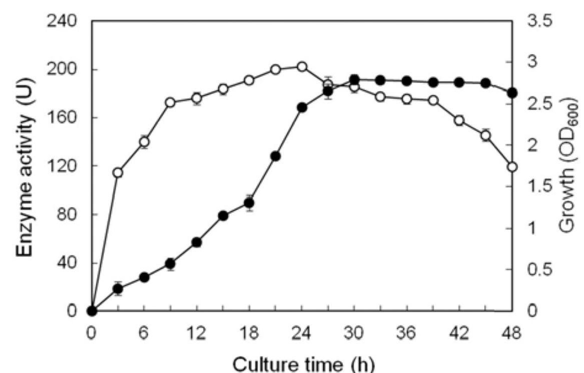

Fig. 5. Time course of the cell growth and extracellular neutral protease production during cultivation Cells were grown aerobically in optimum medium at 30°C. Samples were withdrawn at 3 hr interval for the measurement of cell growth (OD_{600}) and protease activity (U). The error bars represent standard deviations of the means of the three experiments. Symbols: ○, Enzyme activity; ●, cell growth.

Table 6. Purification of the extracellular neutral protease from *Pichia anomala* CO-1

Purification step	Total Protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	224.7	212.4	0.95	100	1
Ammonium sulfate precipitation	1.1	15.3	13.9	7.2	14.6

conditions have not been intensively studied. More importantly, the present study is the first, to the best of our knowledge, to characterize the extracellular neutral protease produced by *P. anomala* CO-1. In this study, the isolated strain has a tendency to exhibit fast growth with broad growth range of pH, temperature and NaCl concentration, broad range of pH and temperature for optimal protease activities, and pH and thermal stability for extracellular protease activities. Based on these merits, *P. anomala* CO-1 could be an efficient and economical microorganism with potential applications in industrial production. Accordingly, determination of the influence of culture conditions on growth and protease production is another area of *P. anomala* CO-1 research worthy of future investigation. In the future, statistically designed experiment will be applied for optimal culture conditions in biotechnological processes.

Acknowledgment

This work was supported by a grant from Research year of Inje University in 2016.

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초록 : 세포 외 중성 단백질분해효소를 생산하는 *Pichia anomala* CO-1의 분리 동정 및 효소 특성

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세포 외로 단백질분해효소를 생산하는 효모 균주 CO-1을 대나무 부산물에서 분리하였다. CO-1은 원형 또는 타원형(3.1-4.0×3.8-4.4 μm)으로, 생장을 위한 최적 온도는 30℃, 초기 pH는 4.0이었다. 그리고 최대 15.0% (w/v)의 NaCl과 9.0%(v/v)의 ethanol 농도에서 생장하였다. 형태적, 생리·생화학적 특성 및 18S rRNA 유전자 염기서열을 통한 계통분석을 이용하여 동정을 실시한 결과 *Pichia anomala*로 판명되었다. *P. anomala* CO-1 단백질분해효소를 부분 정제한 결과 수율은 7.2%였으며, 정제 전에 비해 약 14.6배 정제되었다. Zymogram으로 측정한 효소의 분자량은 약 30 kDa으로 확인되었다. 본 균주는 배지 중에 탄소원과 질소원, 무기염으로 1.0%(w/v) CMC와 1.0%(w/v) yeast extract, 0.3%(w/v) $MnSO_4$를 사용하였을 경우 가장 높은 단백질분해효소 활성을 나타내었다. *P. anomala* CO-1이 생산하는 단백질분해효소의 최적 활성 pH와 온도는 각각 7.0과 30℃였다. 또한 본 효소는 pH 4.0-10.0에서 75%의 안정성을 나타내었으며, 65℃에서 1시간 가열하여도 60% 전후의 활성을 유지하였다. 균주의 효소 생산은 생육과 비례하였으며 대수증식기 후반에 최대의 효소 생산을 나타내었다.