Protease Production by Newly Screened Strain Isolated from Carnivorous Plants

[1]Seok-Cheol Cho, Wan-Young Yoon

Abstract: ***Carnivorous plants have various mechanisms for uptake the nutrients of insects. Among them some parasiticbacteria produce protease which has the role of digest the insects. Protease producingbacteriawere isolated from four kinds of carnivorous plant. Selective media including skim milk or sodium caseinate were used for select protease activity. Nine bacteria were identified by 16s rRNA partial sequencing analysis method andfinally choose the eight bacteria which have protease activity. Eight bacteria cultivated at the commercial media for optimizing cell growth and protease activity. All eight isolated strains from carnivorous plants showed higher cell density in R2A broth. In R2A broth, effect of culture temperature for cell growth and protease activity of isolated strains was tested. Temperature range of 20-42°Call strains cultured well and appeared protease activity for 24 hrs incubation. But maximum cell growth and enzyme activity were gained at 37°C in strain C4. In order to optimize the protease production, cell growth profile and enzyme activity of C4 strain were investigated during incubated at 37°Cfor 36 hrs. Logarithmic cell growth occurred at 6 hrs and maximum protease activity was gained at 24 hrs but suddenly decreased at 27 hrs. Optimum protease activity gained at C4 strain incubated at 37°C for 24 hrs and protease activity maintained at the rage of 30-40°C and decreased suddenly at the below 20°C. Compared with temperature, pH condition was more affected to protease activity, so application of this enzyme must be considered the pH condition. Protease activity did not change at the range of pH 6.0-7.0. Storage temperature of 4°Cand 25°C rarely affected protease activity for 1 month but activity was rapidly decreased at 30°C. Further study on the protease which produced by newly isolated strain in the area of enzyme purification and application to cosmetics for removing dead skin were needed.***

Keywords: ***Carnivorous plant, microparasite, protease, Bacteria Parasitic, rRNA partial sequencing***

I. INTRODUCTION

Granular trichomes are specialized hairs;kind of modified surface cell found on about 30% of all vascular plants and is responsible for a significant portion of a plant's chemistry [1, 2]. In carnivorous plants glandular trichomesdeveloped at insectivorousleafand secreted digestive enzyme which attract insects for capturing anddigesting [3, 4, 5]. Carnivorous plantsreseed world widely classifiedabsorption type, trap type, pitfall type and adhesion type by shape and structure of insectivorous leaf and by style of capturing insects. *Sarracenia* and *Nepenthes* species are typical pitfall type which have pocket structure at the basal area of leaves and *Pinguicula*and *Drosera*species captured the insects by mucosa material which secreted at the trichomes in surface organ[6]. Among various organs of carnivorous plants, insectivorous organs and mechanisms are very attractive point of study [7]. Protease have important role in the various industrial field and occupy the nearly 60% of enzyme market [8, 9]. Carnivorous plants captureinsects and digest it for their survive energy. They have some special organ for attract and entrap the insects, finally digest and uptake the nutrient of insects for energy of life. For digest the insectscarnivorous plants have the glandular cells which secrets the enzyme or organic acids and uptake the nutrient itself[10, 11]. Some carnivorous plants have microparasite secrets the proteaseand assist the digestion of insect. Protease produced by microparasite expects industrial application for its higher activity [12]. In this study protease producing bacteriaparasitic in carnivorous plant werescreened and isolated. Optimizing the protease activity, culture conditions of screened strain were investigated. One of them shows the considerably lower similarity compared withregisteredbacteria in KCCM(Korean Culture Center of Microorganisms). It means more studies were needed for application of protease which produced by newly isolated bacteriaparasitic in carnivorous plant.

II. MATERIALS AND METHOD

2.1 Materials

Two types of carnivorous plants were used for screening new bacteria producing protease. Two Pitfall typeplants (Sarracenia and Nepenthes ventricosa) and two flypaper trap type plants (Pinguiculavulgarisand Droserarotundifolia) were purchased in flower shop located in Cheongju si, chungbuk province Korea.

2.2 Methods

2.2.1 Screening of microparasite

For screening of strain which secret protease and symbiotic relation with carnivorous plants at the same time.Two kinds of sampling methods were applied. Sampling the insectivorous organand smashed in sterilized water by atomizer or swap the mucilages of carnivorous plants and diluted by sterilized water for selection ofthe bacteria. 0.3%(w/v)beef extract(BD difco, New Jersey, USA) and 0.5%peptone(BD difco, Maryland, USA) was dissolved in distilled water used for growth of bacteria. Nutrient agar(BD difco, New Jersey, USA) was used for viable cell countand 0.5% sodium caseinate(Daejung, Ansan, Korea)and 0.5% skim milk powder(Sigma-aldrich Co., St. Louis, MO, USA)were added respectivelyon the growth media for detecting protease producing bacteria.

Revised Manuscript Received on January 03, 2019.

Seok-Cheol Cho, Division of Food & Pharmaceutical Science and Engineering, Seowon University, Cheongju, Korea.

Wan-Young Yoon, Corresponding author, Department of Clinical Exercise Physiology, Seowon University, Cheongju, Korea.

2.2.2Isolation of protease producing bacteria

Colonies with clear zone in nutrient agar plate containing skim milk or white rings in plate containing sodium caseinate were picked by toothpicks and transferred same plates and incubated at 37℃ for 24hrs. Colonies showed same clear zone, white ring and morphology transferred three times. Picked strainscultivated in growth media again and confirmed clear zone or white ring.

2.2.3Identification of protease producing bacteria

Identification of protease producing bacteria was carried by 16s rRNA partial sequencing analysis and species were identified by eztaxon data server (http://eztaxon-e.ezbiocloud.net/)

Table 1. Composion of commercialized media

Name	Components	g/L*
Nutrient broth	BeefExtract	3.0
	Peptone.	5.0
R2A broth	Yeast Extract	0.5
	Proteose Peptone No. 3	0.5
	Casamino Acids	0.5
	Dextrose	0.5
	Soluble Starch	0.5
	Sodium Pyruvate	0.3
	Dipotassium Phosphate	0.3
	Magnesium Sulfate	0.05
YM (Yeast Mold) broth	Yeast Extract	3.0
	Malt Extract	3.0
	Peptone	5.0
	Dextrose	10.0
SD (Sabouraud Dextrose) broth	Peptic Digest of Animal Tissue Pancreatic Digest of Casein Dextrose	5.0 5.0 40.0
MRS broth	Proteose Peptone No. 3	10.0
	Beef Extract	10.0
	Yeast Extract	5.0
	Dextrose	20.0
	Polysorbate 80	1.0
	Ammonium Citrate	2.0
	Sodium Acetate	5.0
	Magnesium Sulfate	0.1
	Manganese Sulfate	0.05
	Dipotassium Phosphate	2.0

* Resolve in 1L distilled water

2.2.4Cultivation of protease producing bacteria

Identified bacteria(strain C4) cultivated at five commercialized media(BD difco, New Jersey, USA) for cell growth and production of protease. Standing culture was carried at same media for 24hrs at 30℃ for seed culture. Main culture carried in erlenmeyer flask for 24 hrs at 30℃ and check the cell mass and protease activity.

Verifying the relationship between culture temperatures and protease activities, R2A broth was used and strain S4 were cultivated at 20-42℃.Composition of commercialized growth media applied in this experiment arranged in Table 1.

Microbial growth was analyzed by absorbance at 600nm and viable cell count. Fermented broth was centrifuged(Labogene, GYROZEN Co., Ltd, Daejeon, Korea) by 3000×g for cell down and diluted by distilled water at the same amount of broth. Absorbance of fully mixed cell suspensions wasmeasured. Absorbance was measured using UV/Vis spectrophotometer (Optizen POP, Mecasys Co., Ltd, Daejeon, Korea) andmicrobial growth and protein digestive activity in various media were tested at 20-42℃.

2.2.5Protease activity of crude protease in culture broth

Supernatant of centrifuged culture broth was used as crude protease for measuring the protease activity [13, 14]. 3ml of 0.5% caseinsolution (Sigma-aldrich Co., St. Louis, MO, USA) mixed with 2ml of crude protease and react 10 min at 37℃. Terminate the reaction by adding 5ml of tricholoacetic acid (Waco Pure Chemical Industry., Ltd, Osaka, Japan)and measured the absorbance at 560nm.Protease from bacillus sp. (P3111, Sigma-aldrich Co., St. Louis, MO, USA)which activity was already known was used for standard curve. For obtain the specific activity of crude protease, amounts of remained crude proteinswhich total activity were verified were analyzed by BCA(Bicinchoninate) method. Mixture ofbicinchoninic acid and 4% copper sulfite (Sigma-aldrich Co., St. Louis, MO, USA) at the ratio of 49:1 was prepared. 950μ l of mixture and 50μ l of crude protease were well mixed and incubation at 37℃ for 30 min. After reaction absorbance was measured at 560nm. Bovine serum Albumin(Sigma-aldrich Co., St. Louis, MO, USA) was used for standard curve and pH was controlled phosphate buffer. Specific activity of crude protein was calculated the equation of protease activity U in crude protease/mg protein in crude protease.

2.2.6 Statistical analysis

The cell growth and enzyme activity of the culture broth were shown as an average±SD for three or more repeated experiments. For the statistical processing, analysis of variance was performed using the SPSS software package (Version 22.0, SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test ($p < 0.05$) was performed.

III. RESULTS AND DISCUSSION

3.1 Newly screened microparasite

Four kinds of carnivorous plants have 3.4×104-3.6×105cfu/g(or cfu/cm2)in their surface of pitfall and trap. There was no remarkable difference of cell populations in tested carnivorous plants. Pick up the five colonies showed clear zone in sodium caseinate containing plate and four colonies in skim milk containing plate are isolated(Fig. 1). One colony waspicked upineight carnivorousplants each other and two colonies were choosed in Pinguiculavulgaris.

Table2.Identification results of nine isolated bacteria

No	Name	Strain	Similarity (%)
C1	Bacillus cereus	ATCC 14579(T)	100.00

C2	Pseudomonas koreensis	Ps9-14(T)	100.00
C3	Bacillus thuringiensis	ATCC 10792(T)	100.00
C4	Chryseobacterium taiwanense	BCRC 17412(T)	98.16
C5	Microbacterium testaceum	DSM 20166(T)	99.31
S1	Burkholderia arboris	R-24201(T)	99.54
S2	Pseudomonas koreensis	Ps 9-14(T)	100.00
S3	Pseudomonas beteli	ATCC 19861(T)	99.69
S4	Chryseobacterium taiwanense	BCRC 17412(T)	98.16

Finally nine strains (C1-C5, S1-S4)were identified andeight strains showover 99% similarities with well-knownbacterialikeBacillus cereus, Bacillus thuringiensis,Microbacterium testaceum and Pseudomonas koreensis. C4 and S4 were verified as a same strain and showed 98.16% similarity which mean itexpected to be a new strain. Fig 2 organized the results of 16s rRNA partial sequencing analysis.

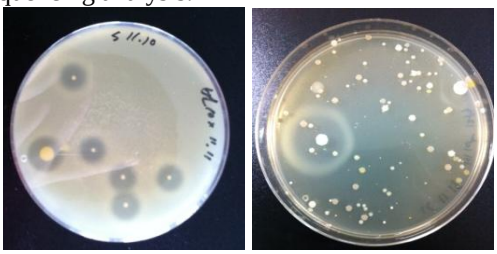

Figure1.Clear zone in selective agar plate containing skim milk (left) and sodium caseinate

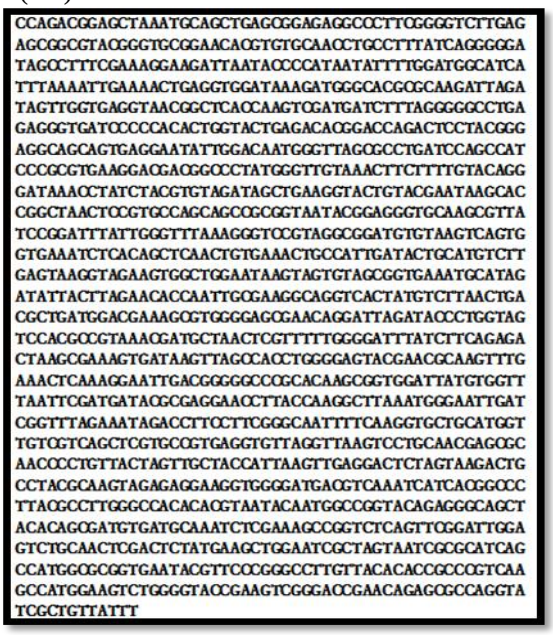

Figure2.16s rRNA gene sequence of strain S4 isolated from carnivorous plant

3.2 Optimize the cell growth and Protease activity

Among the five commercial enrichment media, cell growth of isolated strains was higher in R2A, YM and MRS media.As nutrient broth and SD broth are lack of mineral and carbon source, isolated strains show weak growth and naturally lower protease activity. Higher cell growth and enzyme activity were verified in R2A broth, so further fermentation research about isolated strains were carried in R2A broth. R2A broth was developed to study bacteria which normally inhabit potable water and tend to be slow-growing [15]. Alleight isolated strains from carnivorous plantsshowed higher cell density in R2A broth. In R2A broth, effect of culture temperature for cell growth and protease activity of isolated strainswas tested.Temperature range of 20-42℃all strains cultured welland appeared protease activity for 24 hrs incubation. Butmaximumcell growth and enzyme activity were gained at 37℃in strain C4.

Table3.Cell growth and protease activities of eight isolated strain

	cell growth(O.D. at 600nm)			
Temp.	25℃	30℃	37℃	42℃
C1	$0.60^{a1)}$±0.05	1.00^{b}±0.04	1.00^{b}±0.03	0.65^{a}±0.03
C2	0.50^{a}±0.03	1.20^{b}±0.11	1.24^{b}±0.05	0.51^{a}±0.03
C3	0.60^{a}±0.03	1.01^{b}±0.09	1.21^{b}±0.04	0.61^{a}±0.04
C4	0.61^{a}±0.04	1.12^{b}±0.08	1.32^{c}±0.09	0.62^{a}±0.04
C5	0.55^{a}±0.02	1.01^{b}±0.05	1.25^{c}±0.10	0.51^{a}±0.06
S1	0.50^{a}±0.05	1.20^{b}±0.04	1.59^{c}±0.05	0.50^{a}±0.05
S2	0.43^{a}±0.07	1.20^{b}±0.06	1.00^{c}±0.06	0.54^{a}±0.05
S3	0.45^{a}±0.07	1.19^{b}±0.04	0.89^{c}±0.04	0.49^{a}±0.03
	enzyme activity (specific activity U/mg crude protein)			
C1	18.8^{a}±0.66	28.2^{b}±0.55	27.1^{b}±0.49	15.5^{c}±0.55
C2	12.5^{a}±1.00	21.0^{b}±0.41	25.0^{c}±0.87	16.3^{d}±0.67
C3	12.3^{a}±1.01	20.5^{b}±0.28	24.6^{c}±1.97	13.6^{a}±0.98
C4	16.4^{a}±1.88	27.6^{b}±1.00	30.6^{c}±1.98	14.8^{a}±0.87
C5	14.8^{a}±1.69	22.4^{b}±1.14	23.5^{b}±0.54	14.8^{a}±0.54
S1	16.7^{a}±1.74	26.3^{b}±1.23	30.4^{c}±1.00	14.2^{a}±0.99
S2	15.1^{a}±1.55	22.3^{b}±0.69	26.5^{c}±1.05	11.4^{d}±0.94
S3	14.3^{a}±1.25	21.7^{b}±0.85	25.6^{c}±1.11	11.1^{d}±0.48

1) Means with different letters within a row are significantly different from each other determined by Duncan's multiple range tests. α=0.05

In order to optimize the protease production, cell growth profile and enzyme activity of C4 strain were investigated during incubated at 37℃for 36 hrs. Logarithmic cell growth occurred at 6 hrs and maximum protease activity was gained at 24 hrs but suddenly decreased at 27 hrs. Fermentation time controlled carefully does not exceed 27 hrs for enzyme activity.

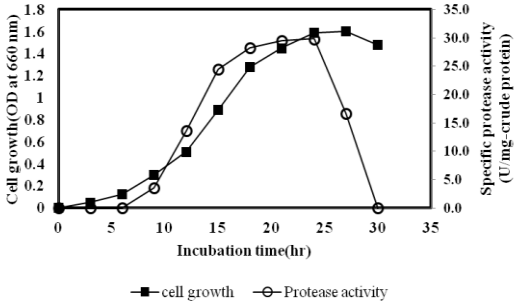

Figure3.Fermentation profile of strain S4 isolated from carnivorous plant

For characterization of protease which produced by C4 strain, stability of crude protein containing culture broth was tested. Stability depend on reaction temperature, pH and storage time was studied. Protease activity maintained at the rage of 30-40 ℃ and did not declined above 40 ℃ .But,protease activity decreased suddenly at the below 20℃ (Fig. 4, A). Change of protease activity in culture broth with pH was confined at the range of pH 4.0-9.0. Protease activity did not changed at the range of pH 6.0-7.0, rapidly decreased at acidic and basic pH range (Fig. 4, B).Compared with temperature, pH condition was more affected to protease activity, so application of this enzyme must be considered the pH condition.

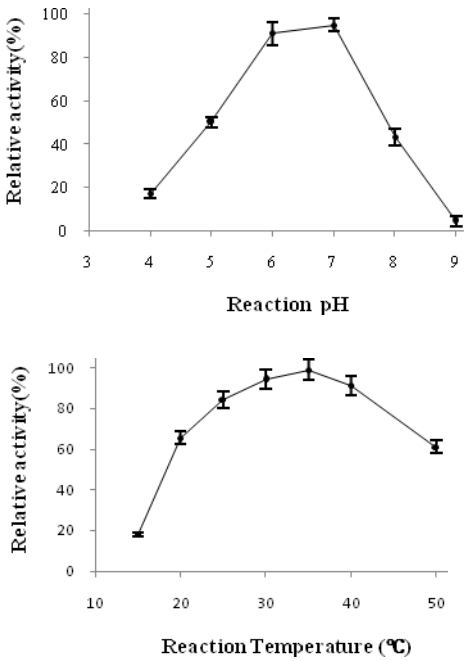

Figure 4.Protease activity profile depend on the temperature (A) and pH (B)

Stabilities of protease activity were tested at 4℃, 25℃ and 30℃. Storage temperature of 4℃ and 25℃rarely affected protease activity for 1 month but activity was rapidly decreased at 30℃. Storage temperature of protease was critically controlled below 30℃ for stable distribution.

ACKNOWLEDGMENT

Patented C4 strain was supportedby the Goldleben Co., Ltd. in Korea.

REFERENCES

1. Joris JG, Bernardus CJ, Juan MA, Rocio EB, Robert CS,Merjin RK. 2012. Plant glandular trichomes as targets for breeding or engineering of resistence to hervores. Int. J. Mol. Sci. 13, 17077-17103.
2. Alain Tissier. 2012. Glandular trichomes: what comes after expressed sequence tags? The Plant Journal, 70, 51-68.
3. Lee HJ, Kim IS. 2009. Development of the granular trichomes in trapping leaves of *Drosera* species. Korean J. Microscopy. 39(1), 57-64.
4. Fahn A. 1990. Plant anatomy. 4th ed. Pergamon Press, Oxford. 172-179.
5. Graham LE, Graham JM, Wilcox LW. 2006. Plant biology. 2nd ed. Pearson Prentice Hall. Upper Saddle River. 190-208.
6. Baek KY, Kim IS. 2008. Structure features of the glandular trichomes in leaves of carnivorous *Drosera anglica* huds. Korean J. Microscopy. 38(1), 21-28.
7. Juniper BE, Robins RJ, Joel DM. 1989. The carnivorous plants. Academic press, London, 3-341.
8. Dastager SG, Dayanand A, Li WJ, Kim CJ, Lee JC,Park DJ,Tian XP,Raziuddin QS. 2008. Proteolytic activityfrom an alkali-thermotolerant *Streptomyces gulbargensis sp*. nov.Curr. Microbiol. 57, 638-642.
9. Rao MB, Tanksale AM, Ghatge MS,Deshpande VV.1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62, 597-635.
10. Kim ES, Oh SE, Yu SC. 1998. Ultra structure and activity pattern of peroxidase in secretory trichomes of *Drosera capensis*. Kor. J. Electron Microsc. 28, 399-414.
11. Serna L, Martin C. 2006. Trichomes: different regulatory networks lead to convergent structures. Trends Plant Sci. 11, 274-280.
12. Kumar CG, Takagi H. 1999. Microbial alkaline proteases : From a bioindustrial viewpoint. Biotechnol. Adv. 17, 561-594.
13. Shin JE, Kang SM. 2012. Treatment effects of hair using hydrolysate of egg white by *Bacillus subtilis* K-54 crude protease. Kor. J. Aesthet. Cosmetol. 10(4), 783-790.
14. Joo HS, Choi JW. 2012. Purification and characterization of a novel alkaline protease from *Bacillus horikoshii*. J. Microbiol. Biotechnol. 22(1), 58-68.
15. Sandle T. 2004. An approach for the reporting of microbiological results from water systems. PDA J. Pharm. Sci. Technol. 58(4), 231-237.