

*Purification and characterization of
microbial protease produced
extracellularly from Bacillus subtilis FBL-1*

Article

Accepted Version

Si, J.-B., Jang, E.-J., Charalampopoulos, D. and Wee, Y.-J.
(2018) Purification and characterization of microbial protease
produced extracellularly from Bacillus subtilis FBL-1.
Biotechnology and Bioprocess Engineering, 23 (2). pp. 176-
182. ISSN 1226-8372 doi: <https://doi.org/10.1007/s12257-017-0495-3> Available at <https://centaur.reading.ac.uk/78941/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

Published version at: <http://dx.doi.org/10.1007/s12257-017-0495-3>

To link to this article DOI: <http://dx.doi.org/10.1007/s12257-017-0495-3>

Publisher: Springer

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

[Click here to view linked References](#)

1
2 1 **Purification and characterization of microbial protease produced**
3
4
5 2 **extracellularly from *Bacillus subtilis* FBL-1**
6
7
8
9 3

10
11
12 4 **Jin-Beom Si, Eun-Ju Jang, Dimitris Charalampopoulos, Young-Jung Wee**
13
14
15
16 5

17
18
19 6 Jin-Beom Si, Eun-Ju Jang, Young-Jung Wee*
20
21

22
23 7 Department of Food science and Technology, Yeungnam University, Gyeongsan, Gyeongbuk
24
25

26 8 38541, Republic of Korea
27
28

29 9 Tel: +82-53-810-2951; Fax: +82-53-810-4662
30
31

32
33 10 E-mail: yjwee@ynu.ac.kr
34
35
36
37 11

38
39
40 12 Dimitris Charalampopoulos
41
42

43 13 Department of Food and Nutritional Sciences, University of Reading, Whiteknights, P.O. Box
44
45
46

47 14 226, Reading, RG6 6AP, United Kingdom
48
49
50
51 15

52
53
54 16 **Abstract** An ammonium sulfate precipitation of fermentation broth produced by *Bacillus*

55
56
57 17 *subtilis* FBL-1 resulted in 2.9-fold increase of specific protease activity. An eluted protein
58
59
60
61
62
63
64
65

1
2 18 fraction from the column chromatographies using DEAE-Cellulose and Sephadex G-75 had
3
4
5 19 94.2- and 94.9-fold higher specific protease activity, respectively. An SDS-PAGE revealed a
6
7
8
9 20 band of purified protease at approximately 37.6 kDa. Although purified protease showed the
10
11
12 21 highest activity at 45°C and pH 9.0, the activity remained stable in temperature range from
13
14
15
16 22 30°C to 50°C and pH range from 7.0 to 9.0. Protease activity was activated by metal ions
17
18
19 23 such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Ca^{2+} and K^+ , but 10 mM Fe^{3+} significantly inhibited enzyme
20
21
22 24 activity (53%). Protease activity was inhibited by 2 mM EDTA as a metalloprotease inhibitor,
23
24
25
26 25 but it showed good stability against surfactants and organic solvents. The preferred substrates
27
28
29 26 for protease activity were found to be casein (100%) and soybean flour (71.6%).
30
31
32
33 27
34
35

36 28 **Keywords:** protease; *Bacillus*; metalloprotease; organic solvent; purification
37
38
39
40 29
41
42

43 30 **1. Introduction**

44
45
46
47 31
48
49

50 32 Proteases (E.C.3.4.21-24) catalyze the cleavage of peptide bond in protein molecules
51
52
53 33 resulting in smaller fragments such as peptides and/or amino acids. They are distributed
54
55
56
57 34 broadly in nature and a wide variety of microorganisms. Proteases are usually divided into
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

35 two groups, exopeptidases or endopeptidases, depending on their site of hydrolysis.

36 Exopeptidases break the peptide bonds formed between the end amino acid and the rest of

37 peptide chain, but endopeptidases hydrolyze the peptide bonds found within the polypeptide

38 or protein. There was an attempt to classify proteases based on structural features of enzyme

39 active center, rather than origin, specificity, or physiological action [1]. Proteases are then

40 divided into four classes based on the type of functional group present at the active site and

41 their mechanism of action: 1) serine protease, 2) aspartic protease, 3) cysteine/thiol protease,

42 and 4) metalloprotease [1, 2].

43 Proteases are extensively used in a variety of industries, including detergent, leather,

44 pharmaceuticals, food, textile, bakery, soy-processing, peptide synthesis, and X-ray film. The

45 estimated value of worldwide sales of enzymes has been over 3 billion U.S. dollars, and the

46 market for proteases accounts for approximately 60% of the total worldwide sale of enzymes

47 [3-6]. Proteases have been isolated, purified, and identified in living organisms and bacteria.

48 Microorganisms are good source of proteases due to a number of advantages; 1) the broad

49 biochemical diversity, 2) the rapid growth, 3) the limited space required for cell cultivation,

50 and 4) the ease at which the enzymes can be genetically manipulated to generate new

51 enzymes for various applications [3]. Some bacteria, yeasts, and fungi are able to produce

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

52 proteases, but only those microorganisms that produce enough amounts of extracellular
53 proteases are of industrial importance [7]. Microbial proteases are widely different not only in
54 their functions but also in their properties.

55 Recently, most of the industrial processes are carried out at harsh conditions, where the
56 enzymes are unstable under extremely high temperature, high or low pH, high concentration
57 of organic solvents and detergents, but only a limited class of proteases is recognized as
58 commercial resource. Alkaline serine proteases such as subtilisin Carlsberg, subtilisin BPN',
59 and Savinase are the major application as detergent industrial source, and some
60 metalloproteases are usually used in brewing and therapeutic industry [6].

61 We have successfully isolated and examined *B. subtilis* FBL-1 to produce potential
62 protease [7, 8]. One of the possible objectives of purifying and characterizing a bacterial
63 protease has been the production of enzymes for commercial purposes. Therefore, in the
64 present study, a bacterial protease was produced by *B. subtilis* FBL-1, which was then
65 purified and characterized by ammonium sulfate precipitation, column chromatographies, and
66 SDS-PAGE. In addition, enzymatic properties of the purified protease were further
67 investigated to characterize the effects of enzyme activity and stability on organic solvents,
68 detergents, temperature, pH, oxidizing agents, and reducing agents.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

69

2. Materials and Methods

71

2.1. Bacterial strain

73

74 *Bacillus subtilis* FBL-1 KCCM 43196 isolated from soil was procured by Food
75 Bioengineering Laboratory in Yeungnam University, Daegu, South Korea [7, 8]. Stock
76 cultures were preserved in 1.5 mL sample tubes containing 50% (v/v) glycerol at -70°C until
77 use. In order to activate cultures, strains were inoculated into tryptic soy broth (TSB; BD,
78 Sparks, MD, USA) and then grown at 37°C and 200 rpm for 15 h.

79

2.2. Production of protease

81

82 The cells from stock cultures were inoculated to sterile 100 mL growth medium (TSB) and
83 dispensed into 250 mL Erlenmeyer flasks, followed by incubation at 37°C for 15 h. This was
84 then inoculated aseptically at 1.5% (v/v) into 250 mL Erlenmeyer flask containing 100 mL
85 production medium, which were incubated on a shaking incubator (VS-8480SF; Vision

1
2 86 Scientific Co., Daejeon, Korea) at 37°C and 200 rpm. The production medium was composed
3
4
5
6 87 of 32.4 g/L, yeast extract 15.0 g/L, KH₂PO₄ 1.0 g/L, and MgSO₄·7H₂O 0.6 g/L. After 36 h of
7
8
9 88 cultivation, cells were centrifuged at 13,000 × g and 4°C for 15 min with a high-speed
10
11
12
13 89 refrigerated centrifuge (Supra 21K; Hanil Scientific Inc., Gimpo, Korea). The supernatant
14
15
16 90 was collected and used as a crude enzyme preparation.
17
18
19
20
21
22

23 92 **2.3. Protease activity assay**

24
25
26
27
28
29
30
31
32

33 95 Protease activity was measured using casein as a substrate by the modified Folin &
34
35
36 96 Ciocalteu's method [9]. A 20 μL of the enzyme was mixed with 500 μL of 0.5 M glycine-
37
38
39
40 97 NaOH buffer (pH 9.0) containing 1% (w/v) casein and incubated at 40°C for 10 min with
41
42
43
44 98 control. The enzyme reaction was stopped by addition of 2 mL of 10% (w/v) trichloroacetic
45
46
47 99 acid. The mixture was incubated at room temperature for 15 min, followed by centrifuged at
48
49
50
51 100 16,000 × g for 15 min. The supernatant was mixed with 2.5 mL of 0.5 M Na₂CO₃ and then
52
53
54 101 500 μL of 20% (v/v) 2 N Folin & Ciocalteu's reagent was added. The mixture was incubated
55
56
57 102 at 40°C for 10 min then the absorbance of mixture at 660 nm was measured by a
58
59
60
61
62
63
64
65

1
2 103 defined as the amount of enzyme required to liberate 1 μ g of tyrosine per minute under the
3
4
5 104 standard assay conditions.
6
7
8

9 105

10 11 12 106 **2.4. Measurement of protein concentration** 13 14

15
16 107

17
18
19 108 Protein concentration was determined by the BCA (bicinchonic acid) method using bovine
20
21
22 109 serum albumin as a standard [10]. During chromatographic purification steps, the protein
23
24
25
26 110 content of each fraction was monitored by measuring the absorbance at 280 nm.
27
28
29

30 111

31 32 33 112 **2.5. Enzyme purification** 34 35

36
37 113

38
39
40 114 Culture supernatant was subjected to ammonium sulfate precipitation for purification of
41
42
43 115 protease. Ammonium sulfate fractions of 30-80% were collected by centrifugation at 13,000
44
45
46
47 116 $\times g$ and 4°C for 60 min, and the pellet was dissolved in a minimum amount of 0.1 M Tris-
48
49
50 117 HCl buffer (pH 7.0). The protein was dialyzed against the same buffer to remove the residual
51
52
53
54 118 salt at 4°C overnight with changing buffer solution. The dialysate was loaded onto a DEAE-
55
56
57 119 Cellulose column (15 \times 300 mm), which was equilibrated with 0.1 M Tris-HCl buffer (pH
58
59
60

1
2 120 7.0). Proteins were eluted with a linear gradient of NaCl (0-0.5 M) dissolved in the same
3
4
5 121 buffer, and each fraction of 2.0 mL was collected. The column was washed with the same
6
7
8
9 122 buffer until the absorbance of effluent at 280 nm reached zero. Enzyme activity and protein
10
11
12 123 concentration of each fraction were measured. After then, the resultant fractions showing
13
14
15
16 124 protease activity were loaded onto a Sephadex G-75 column (15 × 300 mm), which was
17
18
19 125 equilibrated with 100 mM Tris-HCl buffer (pH 7.0). The fractions of 1 mL each were
20
21
22
23 126 collected, and enzyme activity and protein concentration were measured. All purification
24
25
26 127 procedures were carried out at 4°C.

28
29
30 128 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5% stacking
31
32
33 129 gel and a 12% resolving gel was used to determine the purity and molecular weight of the
34
35
36
37 130 enzyme by the method of Laemmli [11]. Protein bands were visualized by silver staining
38
39
40 131 method. Molecular weight of the purified enzyme was estimated by comparing the relative
41
42
43
44 132 mobility of standard molecular weight marker protein (Bio-rad, Hercules, CA, USA).

45
46
47 133

50 134 **2.6. Effect of pH on protease activity and stability**

51
52
53
54 135

55
56
57 136 The effect of pH on protease activity was measured at different pH values. The pH of the
58
59
60

1
2 137 reaction mixture was adjusted to the desired values by using 0.1 M of buffers containing 1%
3
4
5 138 (w/v) casein as a substrate as follows; citric acid buffer (pH 3.0 to 5.0), phosphate-citrate
6
7
8
9 139 buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and glycine-NaOH buffer (pH 9.0 to
10
11
12 140 12.0). The pH stability of protease was determined by pre-incubation in the above mentioned
13
14
15
16 141 buffers at room temperature for 30 min and 60 min. The relative activity of enzyme was
17
18
19 142 quantified under the standard assay conditions.
20
21
22

23 143

26 144 **2.7. Effect of temperature on protease activity and stability**

27
28
29
30 145

31
32
33 146 The effect of temperature on protease activity was carried out by incubation of reaction
34
35
36 147 mixture at different temperatures ranged between 30°C and 60°C in 0.5 M glycine-NaOH
38
39
40 148 buffer (pH 9.0) containing 1% (w/v) casein as a substrate. Thermal stability of the protease
41
42
43
44 149 was determined by pre-incubation of protease at 30-70°C for 30 min and 60 min. The relative
45
46
47 150 activities were quantified under the standard assay conditions.
48
49

50
51 151

54 152 **2.8. Substrate specificity**

55
56
57 153
58
59
60
61
62
63
64
65

1
2 154 Substrate specificity of the protease was determined using different substrates. The reaction
3
4
5
6 155 mixtures were prepared by adding 1% (w/v) of casein, bovine serum albumin (BSA), soybean
7
8
9 156 flour, and gelatin in 0.5 M glycine-NaOH buffer (pH 9.0). The enzyme activity was
10
11
12
13 157 determined as described above.

14
15
16 158
17
18
19 159 **2.9. Effect of metal ions on protease activity**
20
21

22
23 160
24
25
26 161 The effect of metal ions on enzyme activity was investigated by incubating the reaction
27
28
29
30 162 mixture with NH_4Cl , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$,
31
32
33 163 KCl , CaCO_3 , and ZnCl_2 at concentrations of 1 mM and 5 mM for 30 min and 60 min at room
34
35
36 164 temperature, respectively. The enzyme activity measured under the absence of metal ions was
37
38
39
40 165 considered as 100%.

41
42
43 166
44
45
46
47 167 **2.10. Effect of detergents, oxidants, and reductants on protease activity**
48
49

50
51 168
52
53
54 169 The enzyme solution was incubated at room temperature for 30 min in 0.5 M glycine-NaOH
55
56
57 170 buffer (pH 9.0), containing 1% (v/v) Tween 20, 1% (v/v) Tween 80, 1% (v/v) Triton X-100, 1
58
59
60

1
2 171 mM and 10 mM sodium dodecyl sulfate, 2% (v/v) H₂O₂, and 2% (v/v) 2-mercaptoethanol.

3
4
5 172 Esterase activity was assayed by the spectrophotometric method and compared with the

6
7
8
9 173 activity of the enzyme in the absence of detergent. Protease activity was measured and

10
11
12 174 compared with the proteolytic activity of the enzyme in the absence of surfactants, oxidants,

13
14
15
16 175 and reductants.

17
18
19 176

20 21 22 177 **2.11. Effect of organic solvents on protease activity**

23
24
25
26 178

27
28
29 179 The enzyme solution was incubated at room temperature for 30 min in in 0.5 M glycine-

30
31
32
33 180 NaOH buffer (pH 9.0), containing 25 or 50% (v/v) of 1-butanol, benzene, *n*-hexane, 2-

34
35
36 181 propanol, dimethyl sulfoxide (DMSO), and ethyl alcohol. Tween 80 was used as an

37
38
39
40 182 emulsifier for water-immiscible solvents. Protease activity was measured and compared with

41
42
43 183 the proteolytic activity of the enzyme in the absence of organic solvents.

44
45
46
47 184

48 49 50 185 **2.12. Effect of inhibitors on protease activity**

51
52
53
54 186

55
56
57 187 The effect of several inhibitors on protease activity was investigated by incubating the

1
2 188 enzyme solution with ethylenediaminetetraacetic acid (EDTA, 2 mM and 10 mM),
3
4
5 189 phenylmethylsulfonyl fluoride (PMSF, 1 mM), and diisopropyl fluorophosphates (DIFP, 0.1
6
7
8
9 190 mM). The purified enzyme was pre-incubated with each inhibitor at room temperature for 30
10
11
12
13 191 min and then the residual activity was measured under the standard assay condition.
14
15
16 192

19 193 **3. Results and Discussion**

26 195 **3.1. Purification of protease**

23 194
24
25
26 195
27
28
29
30 196
31
32
33 197 *B. subtilis* FBL-2 was cultivated in optimized medium for 36 h. The crude enzyme
34
35
36
37 198 preparation was subjected to 30-80% ammonium sulfate precipitation, followed by dialyzed
38
39
40 199 against 0.1 M Tris-HCl buffer (pH 7.0) at 4°C overnight by changing the fresh buffer every 4
41
42
43
44 200 h. The dialyzate was loaded onto DEAE-Cellulose column at a flow rate of 2 mL/min. As
45
46
47 201 shown in Fig. 1A, the fractions showing high protease activity (Fractions 5 to13) were pooled,
48
49
50
51 202 desalted, concentrated, and loaded again onto Sephadex G-75 column. Fractions 15 to 38
52
53
54 203 showed high protease activity (Fig. 1B). Purification factors and recoveries at each step are
55
56
57 204 shown in Table 1. The enzyme was purified 94.89-fold with a yield of 2.3% from the crude
58
59
60
61
62
63
64
65

1
2 205 extract, and the specific activity was increased to 3378.1 U/mg-protein.
3
4

5 206 Molecular weight of the purified protease was verified by SDS-PAGE and silver staining
6
7
8
9 207 method. As shown in Table 1, after ammonium sulfate precipitation step, the recovery ratio of
10
11
12 208 protein and enzyme were as low as 2.5% and 7.1%, respectively. In addition, ammonium
13
14
15
16 209 sulfate precipitation resulted in more concentrated proteins other than protease. This low
17
18
19 210 amount of recovery for a target enzyme and low selectivity of ammonium sulfate
20
21
22
23 211 precipitation might result in denser protease band of crude extract than that of ammonium
24
25
26 212 sulfate fraction (Fig. 2). However, the protease was dramatically purified in the next
27
28
29
30 213 chromatographic separation steps, which was visualized in Fig. 2. By comparing the relative
31
32
33 214 mobility of standard marker proteins, molecular weight of the purified protease was estimated
34
35
36
37 215 to be approximately 37.6 kDa, which was similar to molecular weight of protease derived
38
39
40 216 from *B. subtilis* RKY3 (38 kDa) [12].
41
42
43
44 217
45
46

47 218 **3.2. Effect of pH on protease activity and stability**

48
49
50
51 219

52
53
54 220 The effect of pH on protease activity and stability was examined over a pH range from pH
55
56
57 221 3.0 to 12.0. As shown in Fig. 3A, enzyme showed the highest activity at pH 9.0 (glycine-

1
2 222 NaOH buffer), but it was declined rapidly beyond pH 9.0. As shown in Fig. 3B, the purified
3
4
5
6 223 enzyme was stable between pH 7.0 and 9.0 for 30 and 60 min, respectively. In addition, the
7
8
9 224 enzyme activity could be retained approximately 80% of its initial activity at pH 6.0 to 9.0
10
11
12 225 after incubation for 30 min. Similar results of pH effect on protease activity have been
13
14
15
16 226 reported, and the proteases produced by *B. subtilis* Y-108 [13], *B. tequilensis* P15 [14], *B.*
17
18
19 227 *cereus* SV1 [15], and *B. cereus* AK1871 [16] showed their optimum pH at 7.5 to 8.0 and high
20
21
22
23 228 pH stability at 7.0 and 9.0.

24
25
26 229

30 230 **3.3. Effect of temperature on protease activity and stability**

31
32
33 231

34
35
36
37 232 The effect of temperature on protease activity was investigated. As shown in Fig. 4A, the
38
39
40 233 protease activity was highest at 45°C, and the enzyme activity at 50°C was sustained with
41
42
43 234 98.5% of maximum activity. However, the protease activity was rapidly declined beyond
44
45
46
47 235 50°C. As shown in Fig. 4B, the purified protease could retain 100% relative activity at 30 to
48
49
50 236 50°C for 30 min and 60 min, respectively. Similar effects of temperature on protease activity
51
52
53
54 237 have been reported. For example, the protease produced by *B. subtilis* Y-108 showed its
55
56
57 238 optimum temperature at 50°C and thermal stability at 25 to 50°C [13]. The protease from *B.*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

239 *subtilis* RKY3 had its optimum temperature at 60°C, and its thermal stability was rapidly
declined above 40°C [12].

3.4. Substrate specificity

244 The purified protease was reacted with different substrates such as casein, BSA, soybean
245 flour, or gelatin. As shown in Table 2, casein was found to show the highest substrate
246 specificity (100%) to the purified enzyme, followed by soybean flour (71.6%) and BSA
247 (22.3%). However, the enzyme could not assimilate gelatin as a substrate. McConn *et al.* [17]
248 previously reported that a neutral protease derived from *B. subtilis* was active in hydrolyzing
249 casein but its ability to hydrolyze gelatin and egg albumin was only limited. This result
250 suggests that the purified protease form *B. subtilis* FBL-1 show similar aspect of *B. subtilis*
251 reported by McConn *et al.*.

3.5. Effect of metal ions on protease activity

255 The effect of metal ions on protease activity was shown in Table 3. The relative activity of

1
2 256 protease in the presence of 10 mM Mg²⁺, Ca²⁺, and Mn²⁺ were 41%, 30%, and 23%,
3
4
5 257 respectively. Though the enzyme was significantly inhibited by 10 mM Fe³⁺, Fe²⁺ led to
6
7
8
9 258 activation of protease activity. Similar effect of metal ions on protease activity has been
10
11
12 259 reported. Proteases produced by *B. tequilensis* P15 [14], *B. cereus* SV1 [15], *B. cereus*
13
14
15
16 260 AK1871 [16], *Bacillus* sp. B001 [4], *B. mojavensis* A21 [18] and *Bacillus* sp. AK.1 [19] were
17
18
19 261 activated by presence of Ca²⁺. Some earlier reports have also showed that thermal stability of
20
21
22
23 262 protease was improved in the presence of Ca²⁺ [20]. It may be explained by strengthening the
24
25
26 263 interactions inside protein molecules and by combining Ca²⁺ to autolysis site to prevent
27
28
29
30 264 autolysis and thermal unfolding [19, 21].
31
32

36 266 **3.6. Effect of surfactants, oxidants, and reductants on protease activity**

37
38
39
40 267

41
42
43 268 The effect of various chemicals such as surfactants, oxidants, and reductants on protease
44
45
46
47 269 activity was investigated. As shown in Table 4, the enzyme was stable in the presence of 1%
48
49
50 270 (v/v) nonionic surfactants like Tween 20, Tween 80, and Triton X-100. However, the enzyme
51
52
53
54 271 was inhibited by the presence of 1 mM SDS as an anionic surfactant and 69% of the enzyme
55
56
57 272 activity was inhibited by addition of 10 mM SDS. In addition, hydrogen peroxide (H₂O₂) and
58
59
60
61
62
63
64
65

1
2 273 2-mercaptoethanol inhibited the protease activity by 12% and 36%, respectively. Similar
3
4
5
6 274 effects of surfactants, oxidants, and reductants on protease activity have been reported.
7
8
9 275 Protease from *B. tequilensis* P15 was stable in the presence of nonionic surfactants such as
10
11
12 276 Tween 20, Tween 80, and Triton X-100, but it was inhibited in the presence of anionic
13
14
15
16 277 surfactant such as SDS by 47.4% [14]. However, the protease produced by *B. mojavensis* was
17
18
19 278 stable in the presence of high concentration SDS up to 1% (w/v) [21].
20
21
22
23 279

26 280 **3.7. Effect of organic solvents on protease stability**

27
28
29

30 281
31
32
33 282 The relative activity of protease after exposure to organic solvents is shown in Table 5.
34
35
36 283 Enzymes are generally inactivated in the presence of organic solvents such as 1-butanol,
37
38
39
40 284 benzene, *n*-hexane, 2-propanol, dimethyl sulfoxide, or ethyl alcohol. However, the protease
41
42
43 285 from *B. subtilis* FBL-1 was rarely inhibited by water-immiscible solvent such as *n*-hexane
44
45
46
47 286 even at 25% and 50%. In addition, the enzyme was quite stable in the presence of 1-butanol
48
49
50
51 287 and DMSO at 25% and 50%. Benzene and 2-propanol at 50% significantly lowered the
52
53
54 288 enzyme activity to 69.4% and 42.8%, respectively. According to the previous studies, the
55
56
57 289 protease from *Aeromonas veronii* PG01 [22] was inhibited in the presence of DMSO at 50%,
58
59
60
61
62
63
64
65

1
2 290 the enzyme from *B. pumilus* 115B [23] was stable in the presence of hexane and benzene, the
3
4
5
6 291 protease from *B. tequilensis* P15 [14] was unstable in the presence of hexane, and the enzyme
7
8
9 292 from *B. cereus* AK187 [16] was significantly unstable in the presence of butanol.
10

11
12
13 293

16 294 **3.8. Effect of inhibitors on protease activity**

17
18
19 295
20

21
22
23 296 The purified protease derived from *B. subtilis* FBL-1 was completely inhibited by the EDTA
24
25
26 297 as a metalloprotease inhibitor (Table 6). The enzyme activity was almost reduced to 11% and
27
28
29
30 298 3.67% in the presence of 2 mM and 10 mM EDTA, respectively, but it was nearly not
31
32
33 299 inhibited in the presence of PMSF and DIFP. Serine residue in the active site of the
34
35
36
37 300 proteinases is irreversibly acylated by PMSF or DIFP, which results in inactivation of the
38
39
40 301 enzymes. Therefore, the reagents such as PMSF or DIFP are serine protease inhibitors [24].
41
42
43 302 Cysteine proteases are generally inactivated with oxidative agents, metal ions, or alkylating
44
45
46
47 303 agents. However, inhibition of metalloproteases is achieved with chelating agents (EDTA) or
48
49
50 304 sodium dodecyl sulfate [25]. Therefore, the results obtained here suggest that the purified
51
52
53
54 305 protease derived from *B. subtilis* FBL-1 should be considered to be a metalloprotease.
55

56
57 306
58

1
2 307 **4. Conclusion**
3
4
5

6 308
7
8
9 309 The protease from *B. subtilis* FBL-1 was purified and characterization for industrial
10
11
12 310 application. The enzyme showed pH stability from 7.0 to 9.0 and thermostability from 30°C
13
14
15
16 311 to 50°C. The protease activity was strongly activated by divalent metal ions. EDTA as a metal
17
18
19 312 chelator almost inhibited protease activity, but no inhibition was observed when DIFP was
20
21
22
23 313 added, suggesting that the protease from *B. subtilis* FBL-1 might be classified into a neutral
24
25
26 314 metalloprotease. In addition, the enzyme activity could be highly stable even in the presence
27
28
29
30 315 of nonionic surfactants, reducing agents, or organic solvents. It's stability against various
31
32
33 316 chemicals makes this enzyme a potential biocatalyst for industrial applications. These study
34
35
36
37 317 efforts need to get more knowledge on metalloproteases in *B. subtilis* for potential industrial
38
39
40 318 applications such as brewing and grain starch isolation industries.
41
42
43

44 319
45
46
47 320 **References**
48
49
50
51 321

- 52
53
54 322 1. Hartley, B. S. (1960) Proteolytic enzymes. *Annu. Rev. Biochem.* 29: 45-72.
55
56
57 323 2. Liao, C. H. and D. E. McCallus (1998) Biochemical and genetic characterization of an
58
59
60
61
62
63
64
65

1
2 324 extracellular protease from *Pseudomonas fluorescens* CY091. *Appl. Environ. Microbiol.*
3
4
5
6 325 64: 914-921.
7
8
9 326 3. Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande (1998) Molecular and
10
11
12 327 biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62: 597-635.
13
14
15
16 328 4. Deng, A., J. Wu, Y. Zhang, G. Zhang, and T. Wen (2010) Purification and
17
18
19 329 characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001.
20
21
22
23 330 *Bioresour. Technol.* 101: 7111-7117.
24
25
26 331 5. Godfrey, T. and J. Reichelt (1984) *Industrial Enzymology: The Application of Enzymes*
27
28
29 332 in Industry. The Nature Press, New York.
30
31
32
33 333 6. Li, Q., L. Yi, P. Marek, and B. L. Iverson (2013) Commercial proteases: present and
34
35
36 334 future. *FEBS Lett.* 587: 1155-1163.
37
38
39
40 335 7. Kim, M., J. B. Si, and Y. J. Wee (2016) Identification of a newly isolated protease-
41
42
43 336 producing bacterium, *Bacillus subtilis* FBL-1, from soil. *Microbiol. Biotechnol. Lett.* 44:
44
45
46 337 185-193.
47
48
49
50 338 8. Kim, M., J. B. Si, L. V. Reddy, and Y. J. Wee (2016) Enhanced production of
51
52
53 339 extracellular proteolytic enzyme excreted by a newly isolated *Bacillus subtilis* FBL-1
54
55
56 340 through combined utilization of statistical design and response surface methodology.
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

341 *RSC Adv.* 6: 51270-51278.

342 9. Folin, O. and V. Ciocalteu (1927) On tyrosine and tryptophane determinations in proteins.
343 *J. Biol. Chem.* 73: 627-650.

344 10. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D.
345 Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk (1985)
346 Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150: 76-85.

347 11. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head
348 of bacteriophage T4. *Nature* 227: 680-685.

349 12. Reddy, L. V. A., Y. J. Wee, and H. W. Ryu (2008) Purification and characterization of an
350 organic solvent and detergent-tolerant novel protease produced by *Bacillus* sp RKY3. *J.*
351 *Chem. Technol. Biotechnol.* 83: 1526-1533.

352 13. Yang, J. K., I. L. Shih, Y. M. Tzeng, and S. L. Wang (2000) Production and purification
353 of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme*
354 *Microb. Technol.* 26: 406-413.

355 14. Bose, A., V. Chawdhary, H. Keharia, and R. B. Subramanian (2014) Production and
356 characterization of a solvent-tolerant protease from a novel marine isolate *Bacillus*
357 *tequilensis* P15. *Ann. Microbiol.* 64: 343-354.

- 1
2 358 15. Manni, L., K. Jellouli, R. Agrebi, A. Bayouhdh, and M. Nasri (2008) Biochemical and
3
4
5
6 359 molecular characterization of a novel calcium-dependent metalloprotease from *Bacillus*
7
8
9 360 *cereus* SV1. *Process Biochem.* 43: 522-530.
- 10
11
12 361 16. Shah, K., K. Mody, J. Keshri, and B. Jha (2010) Purification and characterization of a
13
14
15
16 362 solvent, detergent and oxidizing agent tolerant protease from *Bacillus cereus* isolated
17
18
19 363 from the Gulf of Khambhat. *J. Mol. Catal. B-Enzym.* 67: 85-91.
- 20
21
22
23 364 17. McConn, J. D., D. Tsuru, and K. T. Yasunobu (1964) *Bacillus Subtilis* neutral proteinase.
24
25
26 365 I. a zinc enzyme of high specific activity. *J. Biol. Chem.* 239: 3706-3715.
- 27
28
29
30 366 18. Haddar, A., R. Agrebi, A. Bougatef, N. Hmidet, A. Sellami-Kamoun, and M. Nasri (2009)
31
32
33 367 Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21:
34
35
36
37 368 purification, characterization and potential application as a laundry detergent additive.
38
39
40 369 *Bioresour. Technol.* 100: 3366-3373.
- 41
42
43
44 370 19. Smith, C. A., H. S. Toogood, H. M. Baker, R. M. Daniel RM, and E. N. Baker (1999)
45
46
47 371 Calcium-mediated thermostability in the subtilisin superfamily: the crystal structure of
48
49
50
51 372 *Bacillus* Ak.1 protease at 1.8 Å resolution. *J. Mol. Biol.* 294: 1027-1040.
- 52
53
54 373 20. Sellami-Kamoun, A., A. Haddar, H. Ali Nel, B. Ghorbel-Frikha, S. Kanoun, and M.
55
56
57 374 Nasri (2008) Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

375 in commercial solid laundry detergent formulations. *Microbiol. Res.* 163: 299-306.

376 21. Beg, Q. K. and R. Gupta (2003) Purification and characterization of an oxidation-stable,
377 thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb.*
378 *Technol.* 32: 294-304.

379 22. Divakar, K., J. D. A. Priya, and P. Gautam (2010) Purification and characterization of
380 thermostable organic solvent-stable protease from *Aeromonas veronii* PG01. *J. Mol.*
381 *Catal. B-Enzym.* 66: 311-318.

382 23. Abd Rahman, R. N. Z. R., S. Mahamad, A. B. Salleh, and M. Basri (2007) A new organic
383 solvent tolerant protease from *Bacillus pumilus* 115b. *J. Ind. Microbiol. Biotechnol.* 34:
384 509-517.

385 24. Jaouadi, B., B. Abdelmalek, D. Fodil, F. Z. Ferradji, H. Rekik, N. Zaraï, and S. Bejar
386 (2010) Purification and characterization of a thermostable keratinolytic serine alkaline
387 proteinase from *Streptomyces* sp. Strain AB1 with high stability in organic solvents.
388 *Bioresour. Technol.* 101: 8361-8369.

389 25. Powers, J. C., J. L. Asgian, O. D. Ekici, and K. E. James (2002) Irreversible inhibitors of
390 serine, cysteine, and threonine proteases. *Chem. Rev.* 102: 4639-4750.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

391 **Figure Legends**

392
393 Fig. 1. Chromatograms obtained by (A) DEAE-Cellulose ion-exchange chromatography and
394 (B) Sephadex G-75 gel filtration chromatography. Both columns were equilibrated with 100
395 mM Tris-HCl buffer (pH 7.0).

396
397 Fig. 2. SDS-PAGE of the purified protease produced by *B. subtilis* FBL-1. Lane 1, molecular
398 weight marker proteins; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4,
399 DEAE-Cellulose fraction; lane 5, Sephadex G-75 fraction.

400
401 Fig. 3. Effect of pH on (A) protease activity and (B) stability. The buffer systems used were
402 as follows: 0.1 M citric acid buffer for pH 3.0-5.0, 0.1 M phosphate-citrate buffer for pH 5.0-
403 7.0, 0.1 M Tris-HCl buffer pH 7.0-9.0, and 0.1 M glycine-NaOH buffer for pH 9.0-12.0. The
404 highest enzyme activity was considered as 100%, and error bars showed standard deviations
405 of triplicate samples.

406
407 Fig. 4. Effect of temperature on (A) protease activity and (B) stability. The highest enzyme

1
2 408 activity was considered as 100%, and error bars showed standard deviations of triplicate
3
4
5
6 409 samples.
7
8
9 410

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

411 Table 1. Summary of the purification step for the proteolytic enzyme from *Bacillus subtilis*

412 FBL-1

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	7,073.0	251,794.5	35.6	100	1
(NH ₄) ₂ SO ₄ (30-80%)	175.4	17,795.3	101.5	7.1	2.85
DEAE-Cellulose	3.9	12,937.1	3,352.4	5.1	94.17
Sephadex G-75	1.7	5,669.9	3,378.1	2.3	94.89

413

414

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

415 Table 2. Substrate specificity of the purified protease from *Bacillus subtilis* FBL-1

Substrates (1%, w/v)	Relative activity (%)
Casein	100 ± 7.4
Bovine serum albumin	22.3 ± 1.3
Soybean flour	71.6 ± 4.3
Gelatin	0

416

417

1
2 418 Table 3. Effect of metal ions on protease activity
3
4

Metal ions	Relative activity (%)	
	1 mM	10 mM
None	100 ± 1.7	100 ± 1.6
Mg ²⁺ (MgSO ₄)	118 ± 12.9	141 ± 4.4
Fe ²⁺ (FeSO ₄)	119 ± 8.1	110 ± 3.6
Fe ³⁺ (FeCl ₃)	125 ± 4.7	47 ± 3.5
Mn ²⁺ (MnCl ₂)	123 ± 3.3	123 ± 6.5
NH ⁴⁺ (NH ₄ Cl)	105 ± 2.9	108 ± 4.6
Ca ²⁺ (CaCl ₂)	116 ± 0.8	130 ± 3.3
Ca ²⁺ (CaCO ₃)	106 ± 5.8	112 ± 2.1
K ⁺ (KCl)	107 ± 3.2	116 ± 3.0
Zn ⁺ (ZnCl)	104 ± 1.7	93 ± 4.2

419

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

420 Table 4. Effect of surfactants, oxidants, and reductants on protease activity

Surfactants, oxidants, or reductants	Concentration	Relative activity (%)
None	-	100 ± 4.6
Tween 20	1% (v/v)	77 ± 1.2
Tween 80	1% (v/v)	88 ± 1.0
Triton X-100	1% (v/v)	77 ± 2.9
Sodium dodecyl sulfate (SDS)	1 mM	86 ± 2.1
	10 mM	31 ± 0.7
H ₂ O ₂	2% (v/v)	88 ± 2.9
2-Mercaptoethanol	2% (v/v)	64 ± 3.7

421

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

422 Table 5. Effect of organic solvents on protease activity

Solvents	Relative activity (%)	
	25% (v/v)	50% (v/v)
None	100 ± 2.4	100 ± 2.4
1-Butanol	77.6 ± 3.1	77.4 ± 5.8
<i>n</i> -Hexane	102.3 ± 3.5	100 ± 8.1
Benzene	66.1 ± 3.5	30.6 ± 1.0
2-Propanol	75.8 ± 4.1	57.2 ± 2.4
Dimethyl sulfoxide (DMSO)	79.3 ± 3.2	73.1 ± 1.6
Ethyl alcohol	87.6 ± 3.8	61.2 ± 3.6

423

1
2 424 Table 6. Effect of inhibitors on protease activity
3
4

Inhibitors	Concentration (mM)	Relative activity (%)
None	-	100 ± 2.94
Ethylene-diaminetetraacetic acid (EDTA)	2	11 ± 0.47
	10	3.67 ± 0.47
Diisoprophyl fluorophosphahte (DIFP)	0.1	86.33 ± 3.68
Phenylmethly sulfonyl fluoride (PMSF)	1	84.67 ± 6.13

25
26
27 425
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

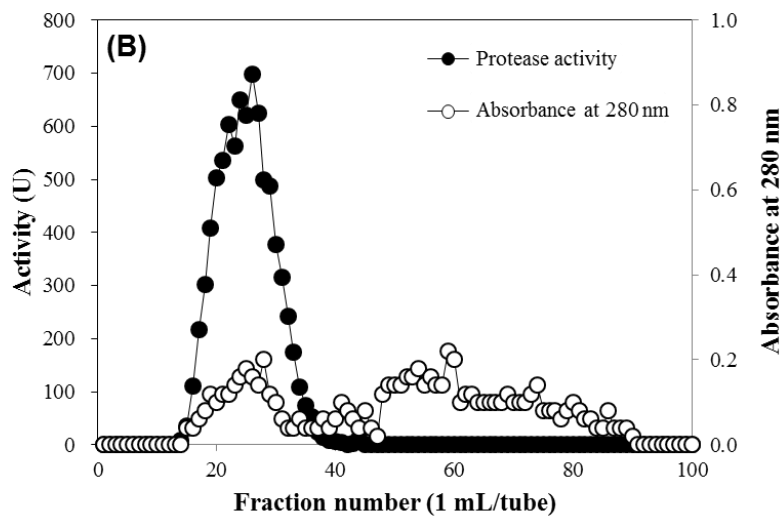
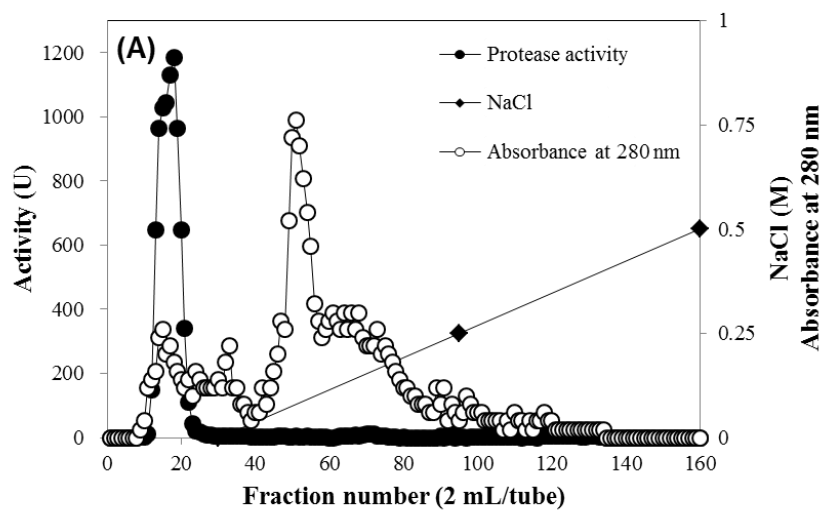


Fig. 1

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

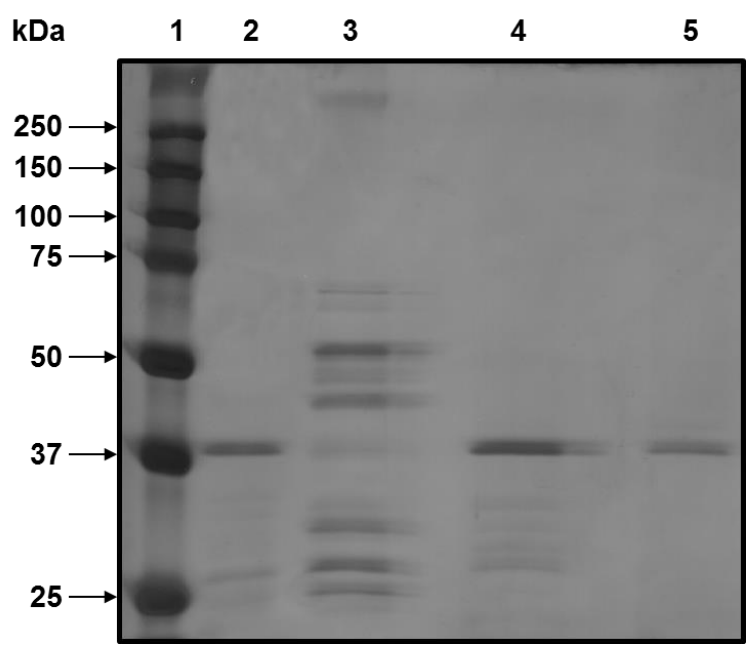


Fig. 2

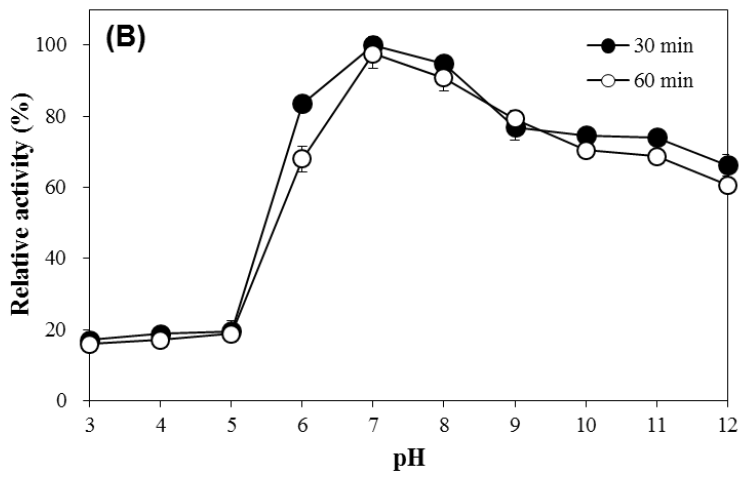
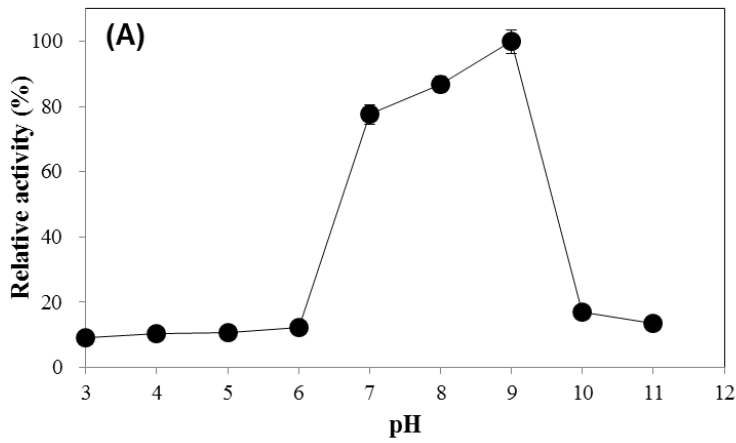
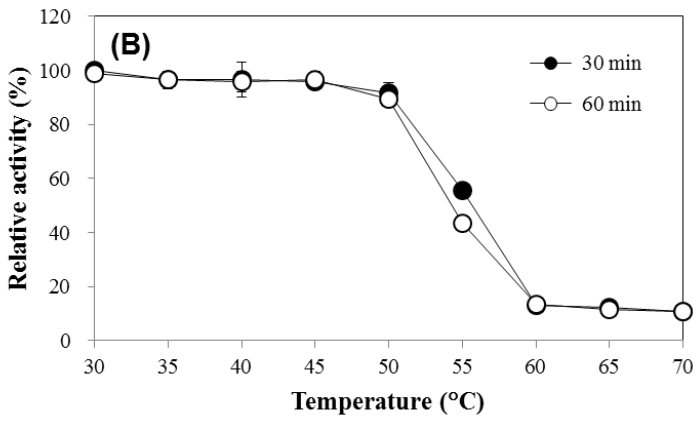
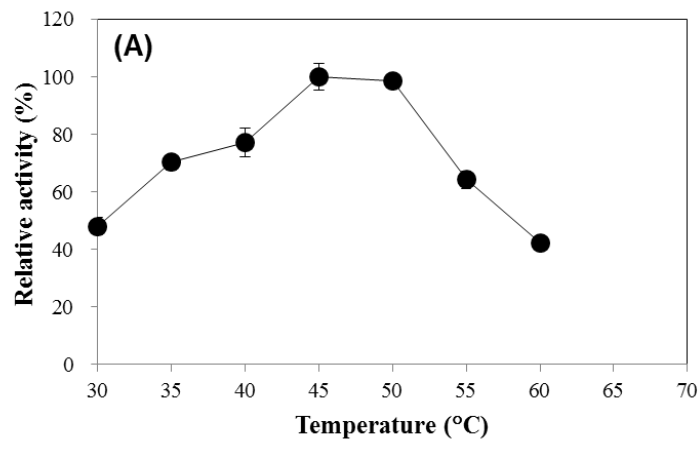


Fig. 3



437

438

439 Fig. 4