



Isolation and identification of fibrinolytic protease-producing microorganisms from *Red Oncom* and *Gembus*, Indonesian fermented soybean cakes

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ABSTRACT

Aims: This study was to isolate, screen, and identify microorganisms from fermented food *Red Oncom* and *Gembus* that can produce fibrinolytic proteases.

Methodology and results: Forty-three isolates showed proteolytic activities on skim milk agar, while thirty-eight of them showed fibrinolytic activities both on a fibrin plate and a fibrinogen zymography. The isolates that showed activity in fibrin plate and fibrinogen zymography with lower molecular weight and considered as safe were chosen and identified as *Bacillus licheniformis* and *Bacillus pumilus* by using API CHB kit and 16S rRNA. The novel fibrinolytic microorganisms were referred to as *B. licheniformis* RO3 and *B. pumilus* 2.g.

Conclusion, significance and impact of study: *Red Oncom* and *Gembus* are potential sources of microbial fibrinolytic protease. This is the first time that *Red Oncom* and *Gembus* as Indonesian fermented foods based on soybean cake were shown having fibrinolytic microorganism. Microbial fibrinolytic enzymes from these fermented foods can be used for functional food formulation to prevent thrombosis and other related diseases.

Keywords: Microbial fibrinolytic enzyme, *Red Oncom*, zymography, *B. licheniformis*, *B. pumilus*

INTRODUCTION

Cardiovascular diseases are the leading cause of death in the world, including in Indonesia (WHO, 2011). Accumulation of fibrin in the blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular diseases (Peng *et al.*, 2005). Fibrin is the major protein component of blood clots, which are formed from fibrinogen by thrombin. Insoluble fibrin can be hydrolyzed to fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor and plasminogen-streptokinase complex (Collen and Lijnen, 2004).

Thrombolytic therapy by injection and oral thrombolytic agents to degrade the thrombus in the blood have been widely studied and practiced (Tough, 2005). Based on its mechanism of action, thrombolytic agents are classified into two types. The first is a plasminogen activator, such as tissue plasminogen activator (t-PA) and urokinase which activates plasminogen to plasmin and further hydrolyses fibrin (Collen and Lijnen, 2004). Other type for thrombolytic therapy is using plasmin-like proteins, which directly degrade fibrin in the blood clotting,

thus dissolve the thrombus quickly and perfectly. Lumbrokinase of earthworms and fibrolase of snake venom are known as plasmin-like protein (Mihara *et al.*, 1991). Although t-PA and urokinase are still widely used in thrombolytic therapy, its price and the side effects possibility of undesirable such as the risk of bleeding in the gut when taken orally (Peng *et al.*, 2005), prevent its normal utilization. This encourages study to find the source of thrombolytic agent that is cheaper and safer.

Fibrinolytic enzyme originated from microbes has attracted the attention of many researchers to be applied as a thrombolytic agent. Streptokinase from *Streptococcus hemolyticus* and Staphylokinase from *Staphylococcus* sp. are potential alternative plasminogen activator and had proven effective in thrombolytic therapy (Banarjee *et al.*, 2004). However, streptokinase is available in large quantities at high prices and during the production and purification is easily contaminated by other proteins that can cause antigenic effect. Furthermore, repeated use for a long time can induce allergies (Banerjee *et al.*, 2004).

Recently, many fibrinolytic enzymes have been identified from traditional fermented foods such as Japanese *Natto* (Fujita *et al.*, 1993), Chinese *Douchi* (Peng and Zhang, 2002), Korean *Doen-jang* (Choi *et al.*,

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2005), Korean *Cheonggukjang* (Jeong *et al.*, 2007) and Korean *Meju* (Jo *et al.*, 2011a). These interesting reports imply some fermented foods contain enzymes that can potentially prevent cardiovascular diseases. This fact opens opportunities to explore new sources of fibrinolytic enzyme from typical Indonesian fermented food. *Red Oncom* and *Gembus* are two of the typical Indonesian fermented foods made from fermented soy pulp. Until now there has been no research on the fibrinolytic enzyme from *Red Oncom* and *Gembus*, either isolation of bacteria producing enzymes or extraction of fibrinolytic enzyme present in *Red Oncom* and *Gembus*. The purpose of this study was to isolate, screen, and identify microbes from fermented food *Red Oncom* and *Gembus* that can produce fibrinolytic proteases.

MATERIALS AND METHODS

Materials

Red oncom was obtained from the traditional markets in Bogor, West Java, whereas *Gembus* was obtained from the traditional markets in Semarang, Central Java. Growth media including skim milk agar (SMA) was purchased from Difco. Luria Bertani (LB) broth was made from yeast extract and tryptone were purchased from Oxoid. Fibrinogen from bovine plasma was purchased from Sigma. API 50CHB kit was purchased from BioMérieux.

Isolation of proteolytic bacterial strain

Heat pretreatment at 80 °C for 15 min was applied to some of the samples to avoid possible pathogen contamination. One-tenth gram of the sample was suspended in sterile physiological saline (0.85% NaCl). The suspension was plated on sterile skim milk agar (SMA), and then incubated at 37 °C for 48 h. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. At the end of incubation period, the protease colonies were picked, purified and sent for further fibrinolytic screening.

Fibrinolytic protease production

A loop of selected microorganisms was cultivated in 25 mL LB broth and incubated at 37 °C in a shaking incubator (120 rpm) for 48 h. At the end of incubation, the fermentation broth was centrifuged at 6000 g at 4 °C for 15 min. The clear supernatant was collected as source of enzyme.

Analysis of protease activity and protein determination

Protease activity was measured according to the Bergmeyer method (Bergmeyer *et al.*, 1983) with casein (1%) as the substrate. As much as 50 µL enzyme filtrate was mixed with 250 µL substrate and incubated at 37 °C for 10 min. Trichloroacetic acid (TCA) 0.1 M was added

and incubated at 37 °C for 10 min, and centrifuged at 4000xg for 10 min. The supernatant was mixed with Na₂CO₃ 0.4 M, followed by addition of Folin Ciocalteu reagent (1:2) and incubated further at 37 °C for 20 min. The reaction products were measured at λ 578 nm. Substrate solution without enzyme was used as control. One unit (U) of enzyme activity was defined as enzyme which produces 1 µmol of tyrosine per min. Protein concentration was analysed by Bradford's method (1976) using reagents consisted of 100 mg Coomassie brilliant blue (CBB) G-250 in 50 mL ethanol 95% and 100 mL phosphate acid 85% in 1L. Bovine serum albumin was used as the protein standard. Triplicate experiments were conducted for each measurement.

Screening of fibrinolytic bacterial strain

Fibrinolytic bacterial strain was screened by fibrin plate and fibrinogen zymography (Hwang *et al.*, 2007). Approximately 0.2% fibrinogen solution in 50 mM sodium phosphate buffer (pH 8) was mixed with 2% agarose solution along with 0.02 mL of a thrombin solution (100 NIH units). The solution was applied to a Petri dish and left for 1 h at room temperature to form a fibrin clot layer. Twenty µL of the enzymes was dropped onto a fibrin plate and incubated at 37 °C for 5 h. The activity of fibrinolytic enzyme was estimated by measuring the dimensions of the clear zone on the fibrin plate. Fibrinogen zymography was carried out in 12% polyacrylamide gels containing 0.1% fibrinogen. The enzymes were diluted in 5x sample buffer, which consisted of 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, and 0.1% bromophenol blue. Electrophoresis was carried out at 70 V and 50 A for 4 h until the bromophenol blue reached the bottom of the gel. After electrophoresis, the gel was soaked in 2.5% Triton X-100, for 1 h at room temperature for enzyme renaturation. The gel was washed with distilled water to remove Triton X-100, and then incubated with 50 mM phosphate buffer (pH 8) at 37 °C for 12 h. The gel was stained with Coomassie blue for 1 h and then destained. The clear bands, correspond to the areas where fibrinogen was digested.

Identification of microorganisms

Identification of microorganisms followed three steps. First is microbiology analysis i.e. Gram staining, spore staining, and morphological examination. Second is biochemical tests with API 50CHB kit for *Bacillus* spp. followed by identification using Apiweb™ software. Third is molecular identification for the best fibrinolytic bacterial strain. The selected microorganisms were enriched, and its 16SrRNAs was analyzed in order to identify the microorganisms at the species level. Amplification of 16SrRNA was performed with universal primers, 63F: 5'-CAG GCC TAA CAC ATG CAA GTC-3' and 1387R: 5'-GGG CGG WGT GTA CAA GGC-3', using a 2720 thermal cycler (Applied Biosystems). PCR products was isolated from the agarose gel and sequenced with a BidDye Terminator v3.1 cycle sequencing chemistry,

genetic analyzer 3730XL (Applied Biosystems). A sequence similarity search was performed using BLAST in the NCBI database.

RESULTS

Proteolytic activity of several microorganisms

Isolation of microbes from *Red oncom* and *Gembus* revealed 43 isolates, 30 isolates from *Red Oncom* and 13 isolates from *Gembus* that produce protease enzyme characterized by their ability to produce clear zones on

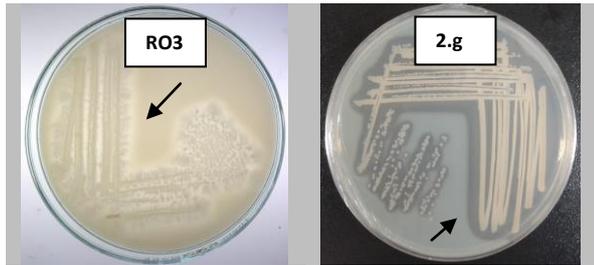


Figure 1: Proteolytic activity of several microorganisms.

the SMA plate. Figure 1 shows the clearing zone observed from the best proteolytic microorganism when grown in SMA. Isolates tested were: RO3 and 2.g.

Fibrinolytic and fibrinogenolytic activity of several microorganisms

Fibrinolytic activity on the fibrin plate showed that 40 isolates were able to produce a clear zone. The activity of fibrinolytic enzyme was estimated by measuring the dimensions of the clear zone on the fibrin plate (Table 1). Fibrinogenolytic activity of a protease was also analyzed *in situ* with zymography method. The substrate used was fibrinogen 0.02%. Protease activity and protein concentration loaded into the gel were about 0.01-0.53 mU and 0.34-1.59 µg. Microbial protease RO1-19 were isolated from fresh *Red Oncom* while ROa-k were isolated from heated *Red Oncom*. Isolates 1-7.g were from fresh *Gembus*, while a-f.g were from heated *Gembus* (Figure 2). Of the 43 proteolytic isolates, we found that 38 isolates showed fibrinogenolytic activity and some of them have different pattern of fractions with various molecular weight (Table 2).

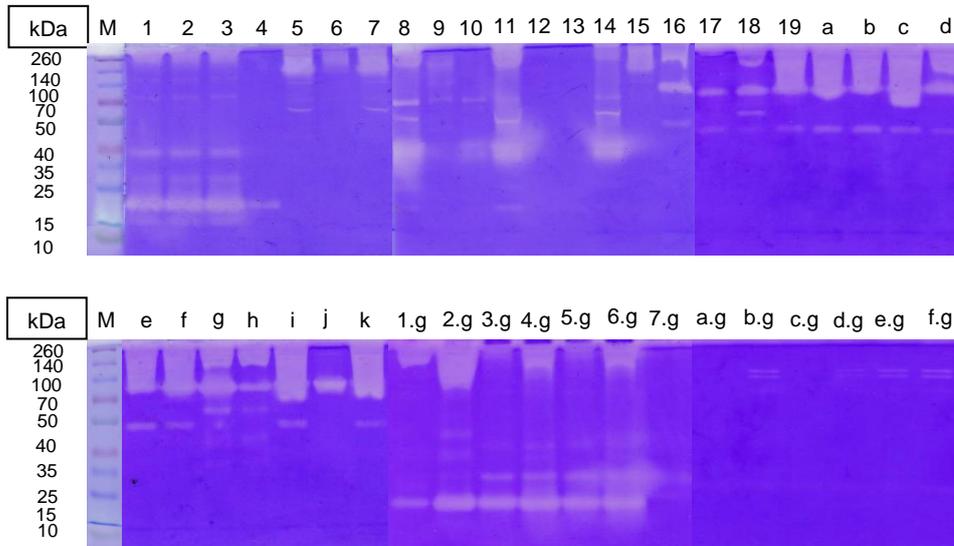


Figure 2: Fibrinolytic activity analyzed by zymography. M, Spectra™ Multicolor Broad Range Protein Ladder (Fermentas); 1-19 refer to enzymes from isolates RO1-19; a-k refer to enzyme from isolates ROa-k (*Red Oncom*); 1.g-7.g and a.g-f.g refer to enzyme from isolates 1.g-7.g and a.g-f.g (*Gembus*).

Table 1: Fibrinolytic activities on fibrin plate.

Isolate	Fibrinolytic activities (cm)	Isolate	Fibrinolytic activities (cm)	Isolate	Fibrinolytic activities (cm)
RO1	0.90±0.10	RO16	0.47±0.15	1.g	0.90±0.00
RO2	0.87±0.15	RO17	0.37±0.12	2.g	1.45±0.07
RO3	1.00±0.10	RO18	0.33±0.06	3.g	0.90±0.14
RO4	None	RO19	0.30±0.00	4.g	1.00±0.00
RO5	1.23±0.25	ROa	0.50±0.00	5.g	0.70±0.14
RO6	0.97±0.12	ROb	0.37±0.12	6.g	1.00±0.00
RO7	1.23±0.15	ROc	0.33±0.06	7.g	0.45±0.07
RO8	1.13±0.32	ROd	0.50±0.10	a.g	0.50±0.00
RO9	0.67±0.15	ROe	0.30±0.00	b.g	0.50±0.00
RO10	0.47±0.15	ROf	0.30±0.00	c.g	0.35±0.07
RO11	1.35±0.35	ROg	0.80±0.14	d.g	0.30±0.00
RO12	None	ROh	0.30±0.00	e.g	0.30±0.00
RO13	None	ROi	0.33±0.14	f.g	0.35±0.07
RO14	0.87±0.21	ROj	0.35±0.07	--	--
RO15	1.17±0.06	ROk	0.35±0.07	--	--

Table 2: Fibrinolytic fractions with various molecular weight.

kDa Isolate	MW				
	15-25	30-50	60-80	100-140	>160
RO1	√	√	√	√	√
RO2	√	√	√	√	√
RO3	√	√	√	√	√
RO4	√	-	-	-	-
RO5	-	-	√	√	√
RO6	-	-	-	-	√
RO7	-	-	√	√	√
RO8	-	√	√	-	√
RO9	-	-	√	√	√
RO10	-	√	√	-	-
RO11	-	√	√	-	-
RO12	-	-	-	-	-
RO13	-	-	-	-	-
RO14	-	√	√	-	√
RO15	-	-	-	√	√
RO16	-	√	-	√	√
RO17	-	√	-	√	√
RO18	-	√	√	√	√
RO19	-	√	-	√	√
ROa	-	√	-	√	√
ROb	-	√	-	√	√
ROc	-	√	√	√	√
ROd	-	√	-	√	√
ROe	-	√	-	√	√
ROf	-	√	-	√	√
ROg	-	√	√	√	√
ROh	-	√	√	√	√
ROi	-	√	√	√	√
ROj	-	-	-	√	-
ROk	-	√	√	√	√
1.g	√	-	-	√	√
2.g	√	√	√	√	√
3.g	√	√	-	-	√
4.g	√	√	√	√	√
5.g	√	√	-	-	√
6.g	√	√	√	√	√
7.g	√	√	-	-	-
a.g	-	-	-	-	-
b.g	-	-	-	√	-
c.g	-	-	-	-	-
d.g	-	-	-	√	-
e.g	-	-	-	√	-
f.g	-	-	-	√	-

Microorganisms identification

The isolates with the best fibrinolytic activity were selected for microbial identification. Gram and spore staining identified that isolate RO1, RO2, RO3, RO16, RO17, RO18, RO19, ROa-k, 1-6.g and a-f.g, were *Bacillus* (Gram positive and have spore). Isolates RO1, RO2, and RO3 show similar fibrinogenolytic pattern *in situ*. Therefore only isolate RO3 was selected for further identification. Similarly, similar fibrinogenolytic pattern was shown by isolates RO16, RO17, and RO19. Therefore for the further identification only isolate 19 was selected. Among ROa-k isolates, isolates ROg and ROj were selected for identification. Among various isolates from *Gembus*, only 2.g was selected for further identification. The results of biochemical tests using API 50 CHB kit specifically for *Bacillus* spp., revealed that isolate RO3 was identified as *B. licheniformis* (99.9%), isolate RO19 as *B. cereus* 1 (71.7%), isolates ROg as *Brevibacillus laterosporus* (99.3%), isolate ROj as *B. cereus* 1 (40.5%), and isolate 2.g as *B. pumilus* (99.7%). Target of isolation of fibrinolytic bacteria was to find safe isolates. Therefore isolate RO3 and 2.g were used for further research. Molecular identification based on 16S rRNA gen was performed and isolate RO3 was confirmed as *B. licheniformis* (96%) and isolate 2.g was confirmed as *B. pumilus* (97%). The nucleotide sequences were deposited at GenBank with accession number AB968524 for isolate RO3 and AB968523 for isolate 2.g. The phylogenetic tree constructed on the basis of the sequences and presented in Figure 3. Fibrinolytic activity on the fibrin plate (Fig. 4) showed that isolate *B. licheniformis* RO3 and *B. pumilus* 2.g were able to produce a clear zone. Plasmin 20 mU was used as a positive control

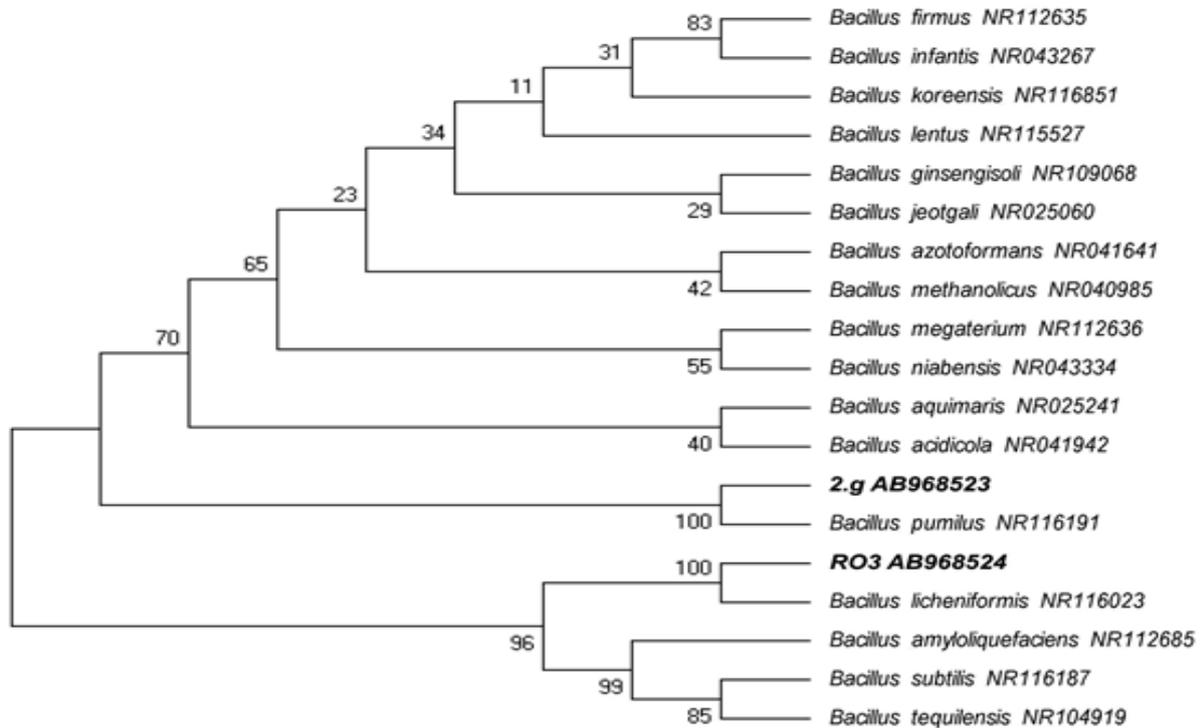


Figure 3: Phylogenetic tree of the strains based on 16S rRNA gene sequences.

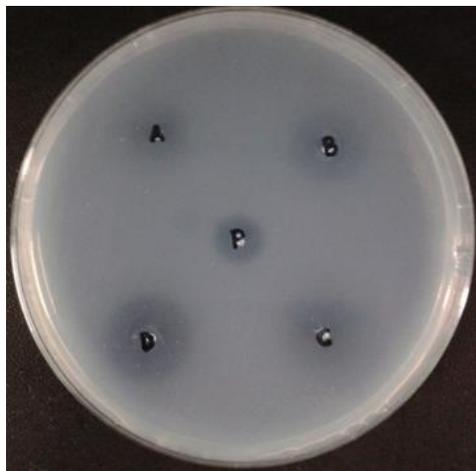


Figure 4: Fibrinolytic activity on fibrin plate. A, *B. licheniformis* RO3 on LB media; B, *B. licheniformis* RO3 on NB media; C, *B. pumilus* 2 g on LB media; D, *B. pumilus* 2.g on NB media; P, plasmin 20 mU.

DISCUSSION

The genus *Bacillus* from fermented foods was reported to produce strong fibrinolytic enzyme, such as *Bacillus natto* from *Natto* (Fujita *et al.*, 1993), *B.*

amyloliquefaciens DC-4 from *Douchi* (Peng and Zhang, 2002), *B. amyloliquefaciens* MJ5-41 from *Meju* (Jo *et al.*, 2011a), *B. amyloliquefaciens* LSSE-62 from Chinese soybean paste (Wei *et al.*, 2011), *Bacillus* sp. DJ-2 from *Doen-jang* (Choi *et al.*, 2005), *Bacillus licheniformis* KJ-31 from *Jeot-gal* (Hwang *et al.*, 2007), and *Bacillus coagulans* form *Terasi*, Indonesian fermented fish (Prihantono *et al.*, 2013).

In this study, *Red Oncor* and *Gembus* samples were screened for microorganisms showing fibrinolytic and fibrinogenolytic activities. Among 43 isolates which grown on SMA, thirty-eight isolates showed fibrinolytic and fibrinogenolytic activity both on fibrin plate and fibrinogen zymography. Some of them were bacilli species, such as *B. cereus*, *Brevibacillus laterosporus*, *B. licheniformis*, and *B. pumilus*. Two isolates that showed activities in fibrin plate and fibrinogen zymography with lower molecular weight and considered as safe were chosen and identified as *Bacillus licheniformis* and *Bacillus pumilus*. The two novel fibrinolytic microorganisms were referred to as *B. licheniformis* RO3 and *B. pumilus* 2.g.

Research conducted by Olajuyigbe and Ajele (2008) succeeded in isolating *B. licheniformis* Lbbl-11 from "iru", a traditionally fermented African locust bean condiment that can produce extracellular protease. Research conducted by Hwang *et al.* (2007) succeeded in isolating *B. licheniformis* KJ-31 from Korean traditional *Jeot-gal* that can produce fibrinolytic

enzymes. *Bacillus* strains such as *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens* and *B. licheniformis* are the common bacilli species isolated from *Cheonggukjang*, a Korean soybean fermented food (Kim *et al.*, 2003; Kwon *et al.*, 2004; Kim *et al.*, 2009; Jo *et al.*, 2011b).

Result of fibrinogen zymography showed that all fibrinogen degrading enzymes were produced in different patterns. Particularly, *B. licheniformis* RO3 and *B. pumilus* 2.g showed several fractions. Research on the *B. licheniformis* CH3-17, this microorganism secreted six fibrinolytic proteins into the culture medium which can be observed by zymography conducted with the culture supernatant (Kim *et al.*, 2009). *B. subtilis* secretes several proteases into the culture medium, including alkaline protease (subtilisin, encoded by *apr*), neutral protease (encoded by *npr*), bacillo-peptidase F (encoded by *bpr*), Epr (extracellular protease, encoded by *epr*), Mpr (extracellular metalloprotease, encoded by *mpr*), and Vpr (extracellular serine protease, encoded by *vpr*). Among them, subtilisin and neutral protease are the most important enzymes and are responsible for >90% of the total extracellular protease activity (Choi *et al.*, 2004).

The genus *Bacillus* can easily be isolated from food and environment. Most of them are non-toxic and have a good impact in human health such as *B. pumilus* JB-1 that was isolated from Korean *Cheonggukjang* is an immuno-stimulating strain (Kwon *et al.*, 2004). Presently we are conducting media optimization for producing fibrin degrading enzyme from *B. licheniformis* RO3 and *B. pumilus* 2.g.

CONCLUSION

Red oncom and *Gembus* are potential sources of microbial fibrinolytic protease. We found 38 isolates that could produce fibrinolytic proteases. A few isolates were identified as *Bacillus* spp. Isolate RO3 was confirmed as *B. licheniformis* and isolate 2.g as *B. pumilus*.

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