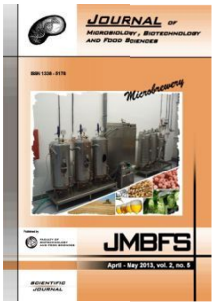

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PROTEOLYTIC AND FIBRINOLYTIC ACTIVITIES OF HALOPHILIC LACTIC ACID BACTERIA FROM TWO INDONESIAN FERMENTED FOODS

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ABSTRACT

Exploration of fermented foods as sources of fibrinolytic enzymes is increased in the last decades. Terasi and Jambal roti is Indonesian traditional fermented fish products, which were famous in Java Island. Both are important products in Indonesian dishes, especially in Java. Investigation on halophilic lactic acid bacteria using MRS and M-17 agar obtained seventy four isolated strains. Their proteolytic and fibrinolytic activities were determined using skim milk agar and plasminogen-free fibrin plate. Twenty five isolates showed protease activities, while only four of them secreted fibrinolitic enzyme. The highest proteolytic and fibrinolytic activity was shown by TB1 strain, which is identified as *Bacillus coagulans*. The 16s rDNA is still in investigating to confirm the TB1 strain identity.

Keywords: Fibrinolytic, Indonesian, fermented fish, Terasi, Jambal roti

INTRODUCTION

Applications of enzymes in industrial and medicine have become extensively increase in the recent decades. The applications of enzyme such proteases are dominant among others enzymes. In leather, food, meat, detergent and tenderization industries, the protease is irreplaceable. Moreover, the data showed that more than 50 % retailed enzyme in the world is dominated by protease **(Rao *et al*., 1998).** Besides that, protease is also used in medical diagnosis, pharmaceuticals, and biomolecular applications as well. Proteases account for about 60% of the total amount of the enzymes sale in the world with two third of this amount, being of microbial origin **(Johnvesly and Naik, 2001).** Hence, among all commercial enzymes, protease is the most marketable enzyme.

Fibrinolytic enzyme is well known as a sub class of proteinase, which has an ability to degrade fibrin. In the past decades, this enzyme generally isolated from various animals such as lizards, worms and snake (**Mihara, 1991; Maruyama *et al*., 1992, Cho *et al*., 2003).** Recently, some bacteria and fungi are also known to produce those enzymes. Many researchers believe that enormous sources of fibrinolytic enzyme are waiting to be found. This enzyme is distributed and could be found in microorganisms that exist in several fermented foods for instance Natto, Skipjack shiokara, Kimchi and Armillariela mellaare **(Sumi *et al*., 1987; Sumi *et al*., 1995; Noh *et al*., 1999; Kim and Kim, 1999).**

Traditional fermented foods are a potential source of fibrinolytic microorganisms. The original Indonesian fermented food that have explored for its fibrinolytic activity was tempe **(Joo *et al*., 2002**). Following the success of isolating fibrinolytic bacteria from several traditional fermented foods and considering that Indonesia has a lot of fermented foods, it is important to explore another fermented food such Terasi and Jambal roti. Terasi and Jambal roti are Indonesian traditional fermented fish products, which were famous in Java Island. Both are an important products in Indonesian dishes, especially in Java. Terasi is shrimp fermented product in paste form. Terasi is a flavour enhancer in almost all Javanese food. While, Jambal roti is salt fermented catfish named Jambal (*Pangasius* sp.). The objective of this research was to explore halophilic lactic acid bacteria producing protease and fibrinolytic enzyme from two Indonesian fermented foods.

MATERIAL AND METHODS

Materials

All fermented fish products from Indonesia (Table 1) were purchased and bought from home made industry in several areas in East Java province, Indonesia. All chemicals were purchased from Sigma (USA). Microbial growth media, MRS, M17 and Skim Milk Agar were purchased from Oxoid (England).

Isolation of bacteria

One gram of samples were added to 9 ml of 1%NaCl sterile water in tubes. The tubes were then serially diluted (10^{-3} to 10^{-5}). One ml of each dilution was spreaded onto MRS and M-17 media contained 10 % NaCl. Plates were incubated at 35 °C for 24-48 h. Each different colony was purified in agar slant to obtain single pure colony.

Proteolytic assay

Proteolytic activity was detected using Skim milk agar (SMA) medium. Isolates were directly spotted onto skimmed milk-agar plates and incubated at 35ºC for 24 h. The proteolytic activities were indicated by the formation of a clear zone around colonies.

Fibrinolytic assay

The strain was grown in 200 ml in stated media and harvested by centrifuging at 12,000 rpm for 15 min. The supernatant was a sample for fibrinolytic activities assay. Fibrinolytic activity was determined by plasminogen-free fibrin plate followed **(Astrup and Müllertz, 1952)** method. This media consisted of a fibrinogen solution (2.5 ml of 1.2% (w/v) human fibrinogen (Sigma) in 0.1 M sodium phosphate buffer, pH 7.4), 10 U of thrombin solution (Sigma), and 1% agarose. fibrin plates were heated at 80°C for 30. Blank disk (6 mm diamater) whic was impregnated with samples were put onto fibrin plate. After incubated at 35°C for 24 h. The activity of the fibrinolytic enzyme was determined by measuring the clear zone diameter.

Strain characterization

Only the best strain would be selected for strain characterization to identify the isolate. Isolate characterization was conducted by two combined method. Morphological and biochemical characterizations were studied to obtain the strain identity.

RESULTS AND DISCUSSION

Proteolytic activity

The data showed that all of samples contained at least three different halophilic lactic acid bacteria (Table 1.). Seventy four strain isolated from all samples by MRS and M17 medium with suplemented by 10% NaCl. Seventen strains were recovered in TB using both medium. JB contained fewest isolated strain. The isolated strains of TB sample were about twice as can be found in JB. The low amount of screened isolates in samples could be due to the inappropriate medium for bacteria growth. MRS always showed a higher amount of halophilic lactic acid bacteria than M-17 medium. Total isolates, which were successfully isolated by MRS are forty nine in all samples. It is about twice higher than M-17 which only recover twenty five isolates. From this data, It is obvious that M17 apparently is inappropriate media to isolate halophilic lactic acid bacteria from terasi and jambal roti. Several mediums, could be applied in order to isolate bacterial from fermented fish. JCM No. 377 agar medium and Nutrient agar with 15% NaCl were reported as a good medium for fermented fish **(Tanasupawat *et al*., 2011; Deejing *et al*., 2005)**. This finding illustrated that MRS medium can be used to isolate halophilic lactic acid bacteria from fermented fish product with relatively higher isolated strain than other medium. This result corroborated **Kobayashi *et al*., (2003)** research, that MRS agar suplemeted with 10 % NaCl is a good medium for isolating bacteria in terasi.

Table 1 Proteolytic and fibrinolytic bacteria from two fermented fish

Name	Geographic sources	Samples Code	Medium	strain obtained	proteolytic strain	Fibrinolytic strain
Terasi	Pamekasan	TP	MRS	7	2	1
			M-17	4	-	-
Terasi	Tuban	TT	MRS	9	3	-
			M-17	3	1	-
Terasi	Sidoarjo	TS	MRS	9	2	1
			M-17	4	2	-
Terasi	Banyuwangi	TB	MRS	11	2	2
			M-17	6	2	-
Terasi	Medan	TM	MRS	8	4	-
			M-17	3	2	-
Jambal Roti	Bandung	JB	MRS	5	3	-
			M-17	5	2	-

All of 49 isolates from MRS and 25 isolates from M-17 mediums were assayed for their proteolytic activity. Enzyme activity was measured by a ratio of clear zone diameter to a colony diameter. The result showed that only about one third of the isolates secreted protease. All samples contained for at least two proteolytic halophilic lactic acid bacteria. Even though in TP samples, proteolytic bacteria failed to grow in M-17, It still obtained two proteolytic strain from MRS medium. we also knew that the amount of bacterial strain was not correlated with proteolytic bacteria. For examples, the amounts of isolated strain in TM were not as many as TB, however, Its amount of proteolytic bacteria was higher. The highest proteolytic activity was found at TB1 isolate. TB1 produced extra cellular protease with 14 mm of clear zone diameter (Figure 1). Hence, TB1 was selected for strain characterization assay.

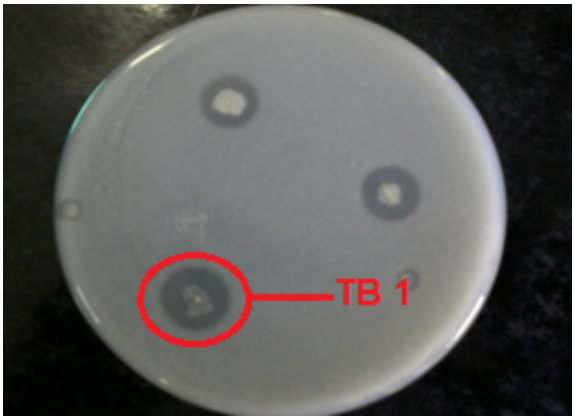

Figure 1 Proteolytic activity of TB 1 isolate

Fibrinolytic activity

It was clear from the data (Table 1.) that proteolytic bacteria was not automatically degrade fibrin. The amount of the fibrinolytic bacteria was quite few compared to proteolytic bacteria. Only four isolates showed capability to degrade fibrin. They were obtained from TP, TS and TB sample. All fibrinolytic bacteria were isolated by MRS medium. Fermented food is the common source of fibrinolytic bacteria. It is interesting to note, similar to proteolytic activity, TB1 also presented high fibrinolytic activity with 6.8 mm clear zone diameter (Figure 2.). Several fermented foods bacteria have been proved as fibrinolytic enzyme sources. These kinds of bacteria included *Bacillus subtilis* from Japanese fermented soyben called Natto, **(Yamashita *et al*., 2003)** and *Bacillus amyloliquefaciens* from chickpeas fermentation (**Wei *et al*., 2011**). *Bacillus subtilis* from Natto secretes the nattokinase that capable of acting as trombolysis in human blood vessel **(Sumi *et al*., 1990; Kim *et al*., 2008)**. It seems that *Bacillus* sp. is a potent source for fibrinolytic enzyme, although some of *Bacillus* such as *B. cereus* is responsible for foodborne illnesses **(Shin *et al*., 2011)**.

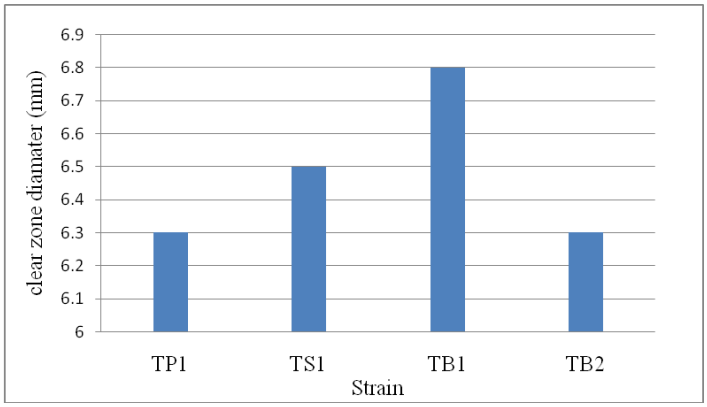

Figure 2 Clear zone diameter of fibrinolytic activities

Strain Characteristics

TB1 was characterized to obtain the identity of the strain. Morphological and Biochemical characterizations were conducted to clarify the strain of TB1. The results (Table 2.) were then used to classify the strain using Bergey's Manual of Systematic Bacteriology **(Claus and Berkeley, 1986).**

Table 2 Morphological and Biochemical characterization of TB1

Test	Result
BGP	+
Spore	+
FERMENTATION	
Glucose	+
Xylose	-
Mannitol	+
Lactose	-
Sucrose	-
Maltose	-
Arabinosa	+
GROWTH TEMPERATURES	
25^0C	+
37^0C	+
40^0C	+
45^0C	+
NaCl CONCENTRATION	
3%	+
4%	+
5%	+
7%	-
Nutrient Broth	+
MCA	-
TSI	A/A,H2S-
Citrate	-
Indol	-
MR	-
VP	+
CHARACTERISTIC TEST	
Catalase	+
Coagulase	-

Based on its characters, TB1 was identified as *Bacillus coagulans*. *Bacillus coagulans* is a sporogenic lactic acid bacteria that grows optimally in relatively high temperature. This bacterium also produces lactic acid as the primary fermentation product **(Su *et al*., 2011; Ou *et al*., 2011)**. From this result, it was clear that *B. coagulans* was considerly as potential protease producer. This result is in agreement with **Ram *et al*., (1994)**, who reported that *Bacillus coagulans*, when grown on casein would produce metalloprotease.

Bacillus is a common genus that produces protease in fish fermented food **(Yossan *et a*l., 2006; Ha *et al*., 2002)**. Bacillus can easily be isolated from food and environment. Most of Bacillus in Food are non toxic and even have a good impact in human health. **Fitzpatrick**`s research (2011) revealed that *B. coagulans* was not only effective as anti-inflammatory and immune-modulating effects but also anti-colitis and anti-diarhea. With this benefit, It seemed that *Bacillus coagulans* is a good source for proteolytic and fibrinolytic enzyme for food and pharmaceutical application.

CONCLUSION

Only few halophilic lactic acid bacteria which could produce proteolytic and fibrinolytic enzymes have successfully isolated from two Indonesian fermented fish. Based on Bergey‘s manul analysis, *Bacillus coagulans* is indicated as strain that was responsible for high proteolytic and fibrinolytic activity. To confirm the identity of isolates, 16S rDNA analysis will be performed for phylogenetic analysis, due to the potency of this isolate as a fibrinolytic source.

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