Isolation and screening of protease producing halotolerant bacteria from fermented freshwater fishes

Viraxay Bandavong[1,3], Kridsada Unban[2], Apinan Kanpiengjai[2] and Chartchai Khanongnuch[2,*]

Abstract

A total of 45 halotolerant bacterial isolates were isolated from 38 samples of fermented freshwater fishes using modified MRS agar containing 20% (w/v) NaCl. Screening for protease production on modified MRS agar supplemented with 20% (w/v) NaCl and 0.5% (w/v) casein found that only 8 bacterial isolates formed clear zone and the isolates VK31 formed the widest clear zone following by isolate VK20. Confirmation of protease production in liquid culture found that the enzyme activities produced by both isolates were found 4.41±0.02 and 3.35±0.04 U/mL, respectively. Based on the 16S DNA nucleotide sequence analysis, the isolate VK20 and VK31 were identified to be *Virgibacillus halodenitrificans* VK20 and *Kushneria avicenniae* VK31, respectively. Time course of protease production at 37°C for 3 days showed the maximum protease activity of 5.42±0.02 and 4.19±0.03 U/mL by *V. halodenitrificans* VK20 and *K. avicenniae* VK31, respectively.

Keywords: Halophiles, Protease, Siamese Mud Carp, Isolation, *Kushneria* sp.

1. Introduction

Fish sauce is a popular seasoning sauce in Asian countries particularly in Southeast Asia. It is called by different names such as *nampla* in Thailand and Lao PDR, *patis* in Philippines, *budu* in Malaysia, *nouc-nam* in Vietnam, and *yu-lu* in China (Lopetcharat *et al.,* 2001). Fish sauce imparts good taste to local food preparation and supplements protein in daily meal. Regarding the unique characteristics, fish sauce has been distributed to the Japanese and European markets. However, distinctive and fishy odors of fish sauce become a limitation for its uses (Fukami *et al*., 2002).

[1]Division of Food Science and Technology, School of Agro-Industry, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, 50100 Thailand.

[2]Division of Biotechnology, School of Agro-Industry, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, 50100 Thailand.

[3]Division of Food Science and Technology, Faculty of Agriculture and Forestry Resources, Souphanouvong University, Luang Prabang, Lao PDR.

*Corresponding author, e mail: chartchai.k@cmu.ac.th Tel. +66-53-948261 Fax. +66-53-948206

The commercial fish sauce normally uses anchovy, *Stolephorus* sp., as raw material and takes 6–12 months for fermentation period. Fish sauce fermentation is mainly occurred naturally from fish protein degradation catalyzes by the proteolytic enzymes which is mainly from two sources, one from the visceral organs of fish which is called “endogenous proteases” and the other from halotolerant microbes grew during fermentation (Lopetcharat *et al*., 2001). The recent finding of fish sauce fermentation development mostly targets on either the shortening of fermentation period or flavor development. Investigation the factors influencing on fermentation process can be categorized into 3 concepts, the first is applying acid in fish protein hydrolysis, the second is using commercial proteolytic enzyme or improve the internal protease release and the last, using halotolerant microbes capable of protease producing. Using of acids in fish protein hydrolysis can reduce the fermentation to only 2 months and improve the soluble amino acid in fish sauce final products, but the loss of fish sauce sensory properties is observed (Gildberg *et al.,* 1984). Addition of exogenous enzyme is one alternative to improve fish sauce fermentation (Beddows and Ardeshir, 1979). However, flavor of fish sauce fermented using only commercial protease is different from natural fermentation and also increase the production cost. Utilizing of either halotolerant or hallophillic microorganism to improve the fish sauce fermentation process still being in the process of research, no practical use of the selected microbes as seed culture in industrial scale. Halophilic bacteria are salt-loving bacteria that distribute in hypersaline environment. They grow in a wide range of salinities, even at 20–30% (w/v). Moderately halophilic bacteria grow either in the absence of or in up to 20% (w/v) NaCl environment, it is an interesting group from a biotechnological point of view (Tanasupawat *et al*., 2011). Several protease-producing halophilic/halotolerant bacteria from fish sauce have been reported such as *Halobacillus* sp. SR5-3 (Namwong *et al*., 2006), *Virgibacillus* sp. SK37 (Sinsuwan *et al*., 2007; Phrommao *et al*., 2010) and *Virgibacillus* sp. SK33 (Sinsuwan *et al*., 2010). Most of them are the bacterial isolates found in fish sauce fermented using seawater fish as substrate.

Utilization of freshwater fishes such as Siamese Mud Carp as substrate for fish sauce is found in the local area away from the sea. However, there is no report described the fermentation process of Siamese Mud Carp using the pure culture of proteolytic halophiles. In addition, there is no report described the halotolerant in fish sauce produced from freshwater fish as raw material. Finding for the halophiles capable of highly active in fish protein degradation is possibly helpful for development of Siamese Mud Carp fish sauce fermentation in the future.

This paper described the isolation and screening of the protease-producing halophilic bacteria from fermented freshwater fishes. Molecular identification and preliminary investigation of protease production by the selected microbes are also described.

2. Materials and Methods

2.1 Preparation and enrichment of halotolerant bacteria

Siamese Mud Carp or related species freshwater fishes were used as a source of halotolerant bacteria. Twenty one samples were collected from various places in northern Thailand and 17 samples were collected from Khan and Mekong rivers, Luang Prabang, Lao PDR. All samples were mixed with sea salt in the ration of 3:1 of fish: salt, they were fermented at room temperature (25–35°C) for 2–4 weeks to enrich the halotolerant bacteria.

2.2 Isolation and screening of protease-producing bacteria

Halotolerant bacteria were isolated from fermented fish samples prepared as described above. All samples were diluted with 0.85% (w/v) NaCl and spread on modified MRS agar medium containing percent weight by volume (w/v) 0.5 peptone, 0.1 yeast extract, 0.1 sodium glutamate, 0.2 di-ammonium citrate, 0.25 sodium acetate, 0.05 Tween80, 0.2 KCl, 0.01 $MgSO_4.7H_2O$, 0.1 K_2HPO_4, 0.036 $FeCl_2.4H_2O$, 0.01 $MnSO_4.H_2O$, 1.5 agar, pH 7.0, supplemented with NaCl to obtain the final concentration of 20% (w/v), and incubated at 37°C for 5–8 days. Distinctive of bacterial colony grew on modified MRS agar were picked and purified by streaking on NA agar plates with 20% (w/v) NaCl until obtaining the pure culture of bacterial isolates.

Plate screening for selection of protease producing halotolerant bacteria was conducted on modified MRS medium supplemented with 0.5% (w/v) casein. Colonies of bacterial isolates surrounded by clear zone after overlay with 5% (w/v) TCA were selected and further purified by streak plate technique on NA agar plates with 20% (w/v) NaCl. Each of the selected isolates was transferred on the MRS plate and incubated at 37°C for 3 days, and then the diameter of halo around the colony was measured.

2.3 Confirmation of protease production

A loopful pure culture of the selected strains were inoculated in 250 mL Erlenmeyer flask containing 50 mL protease production medium with 0.5% (w/v) casein as of the same medium, incubated at 37°C (200 rpm) for 7 days. Five milliliter of culture broth was aseptically sampling at 1, 3, 5 and 7 days cultivation and the cell-free supernatant was recovered by centrifugation at 12,000×*g*, 4°C for 10 min. The clear supernatant was used as crude enzyme for protease activity assay by method described by Hiraga et al. (2005).

2.4 Bacterial identification

Genomic DNA of selected bacterial strains was isolated using method of Nishiguchi et al. (2002). The 16S rDNA was amplified using polymerase chain reaction (PCR) by using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGA CTT-3'), and the genomic DNA as the template under the following condition 94°C for 2 min; 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 90 s; and hold at 72°C for 5 min. The PCR product was purified using GF-1 96-well PCR Clean-up Kit (Vivantis, Oceanside, CA, USA) and sequenced using 3730XL DNA sequencer (Applied Biosystem, USA). Online similarity searches were performed using the BLAST algorithm of GenBank. Multiple sequence alignment was carried out with the CLUSTALX program, version 1.81. Phylogenetic tree was constructed by MEGA4 software (Tamura *et al.,* 2007). The taxonomic studies of selected bacterial isolates were determined as described in Bergey's Manual of Systematic Bacteriology. Gram staining was performed after 24 h of the bacterial cultivation at 30°C on nutrient agar. Endospore formation was assessed with spore-staining method using malachite green (Schaeffer and Fulton, 1933). Colony formation was assessed by spread plate technique on nutrient agar and enrichment media. Determination of temperature and pH for growth was performed at 10, 20, 25, 30, 37 and 45°C and at pH 3, 4, 5, 6, 7, 8, 9 and 10.

2.5 Time course of protease production

Time course of protease production by two selected strains, *V. halodenitrificans* VK20 and *K. avicenniae* VK31 were studied in protease production medium containing 0.5% (w/v) peptone, 1% (w/v) $MgSO_4.7H_2O$, 0.2% (w/v) KNO_3, 0.0005% (w/v) ferric citrate, 1% (w/v) glycerol, and 15% (w/v) NaCl (Sinsuwan *et al*., 2010) and incubated at 37°C with constant shaking at 100 rpm, for 3 days. Growth was monitored by measuring the absorbance at 660 nm and the number of viable bacterial cell was determined by spread plate technique on NA supplemented with 20% NaCl. Crude extracellular protease was collected by centrifugation at 12,000×*g* for 10 min, at 4°C (RC 28S, Sorvall Co., Newtown, Conn., U.S.A.). Subsequently, it was dialyzed against 100 volumes of 20 mM Tris-maleate (pH 7.0) twice using the dialysis membrane with molecular weight cut-off (MWCO) 10 kDa (Pierce Chemical Co., Rockford, Ill., U.S.A.) at 4°C overnight. The protease activity was determined.

2.6 Protease assay

Protease activity was determined by the method of Sinsuwan *et al.,* (2010). Briefly, 0.5 mL of enzyme was added to 3.0 mL 0.6% casein (w/v) in 0.02 M sodium phosphate buffer, pH 7.5 containing 1% (w/v) NaCl. The reaction mixture was incubated at 50°C for 20 min and stopped by adding 3.2 mL TCA mixture (0.1 M trichloroacetic acid, 0.2 M sodium acetate and 0.3 M acetic acid), followed by keeping the reaction mixture at room temperature for 30 min.

The precipitates were removed by filtration through Whatman-1 filter paper and the absorbance of the filtrate was measured at 660 nm (Specord 200, Analytikjena, Germany). One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the assay condition. Total protein in the supernatant was measured by the Lowry method (Lowry *et al.,* 1951) using bovine serum albumin (BSA) as standard protein.

3. Results and Discussion

3.1 Isolation of halotolerant bacteria

From 38 samples of fermented fish collected from domestic markets in northern Thailand and Lao PDR, 45 halotolerant bacterial isolates were capable of growth on MRS agar supplemented with 20% (w/v) sodium chloride. Twenty five isolates were obtained from the sample collected from northern Thailand and 23 isolates were from the samples collected from Khan River and Mekong River in Lao PDR.

3.2 Screening of protease-producing bacteria

The result from plate screening on MRS agar supplemented with 0.5% (w/v) casein showed that among 45 bacterial isolates, only 8 isolates showed the halo surrounding their colonies after overlay with TCA solution. Those were isolates VK2, VK4, VK8, VK13, VK14, VK20, VK31 and VK32. The isolates VK2, VK4, VK8 and VK14 were Gram-positive cocci, isolate VK20 were Gram-positive short rod, while the remaining VK13, VK31, VK32 were Gram-negative short rod. Among 8 isolates, the isolate VK20 and VK31 were the most interesting to be used as the protease producing microbes regarding the most widely clear zone formed after grew on MRS medium with 0.5% (w/v) casein. The halo formed by the isolate VK20 and VK31 was demonstrated in Figure 1.

Confirmation of protease producing isolates was investigated by culture the selected strains of halobacterium in enzyme production medium containing 20% (w/v) NaCl at 37°C (100 rpm) for 3 days. The protease produced by the halotolerant isolated bacteria was found in range of 0.01–4.41 U/mL culture broth (Figure 2). The isolate VK31 and VK20 originally obtained from fermented Siamese Mud Carp produced the maximal protease activities up to 4.41 and 3.35 U/mL, respectively, after cultivation at 37°C, 150 rpm for 5 days. This result is corresponding to the halo formed in the step of plate screening.

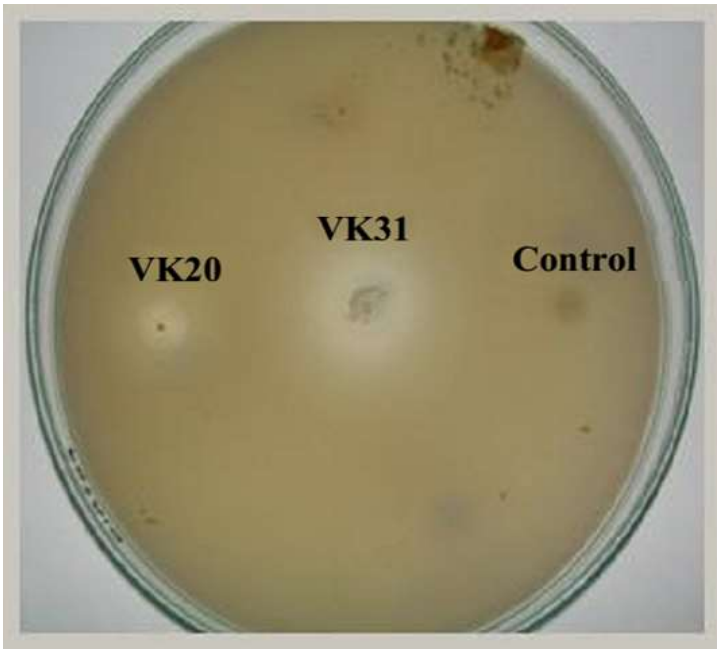

Figure 1. Halo formation of the selected bacterial isolate VK31 and VK20 in MRS agar supplemented with 20% (w/v) NaCl and 0.5% (w/v) casein compare to non-selected isolate VK3 (control) after cultivation at 37°C for.

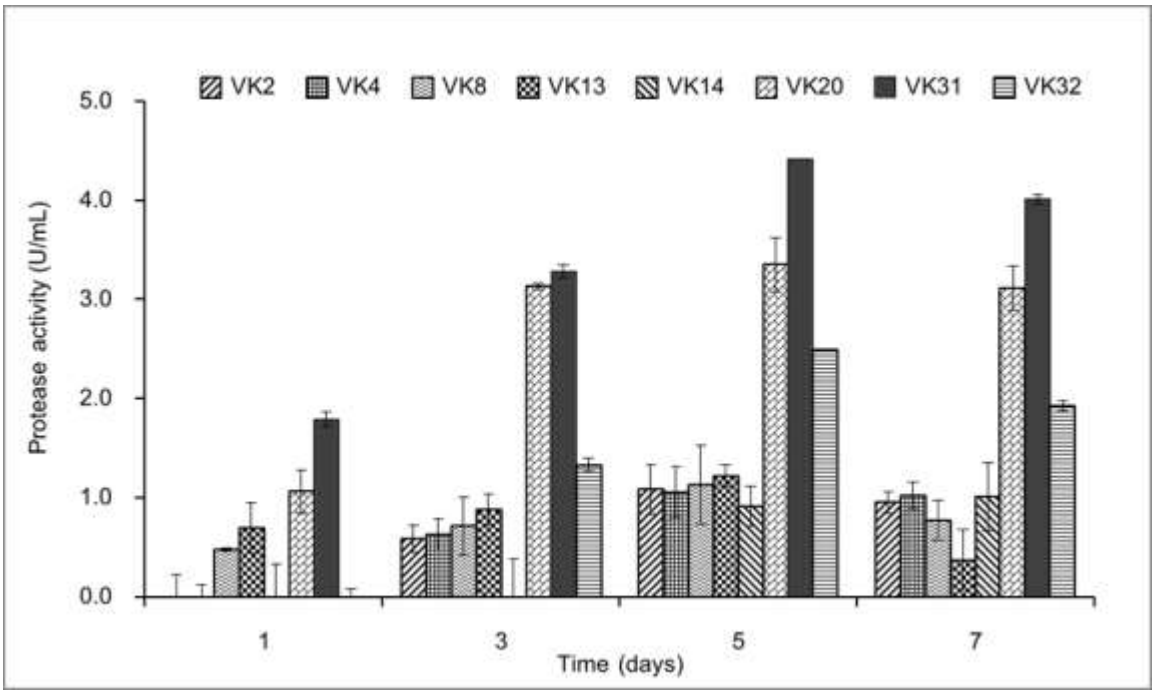

Figure 2. Profile of protease production of eight selected isolates in protease production medium containing 20% (w/v) NaCl and 0.5% (w/v) casein as substrate after cultivation at 37°C, 150 rpm for 7 days.

3.3 Bacterial identification

According to the morphological characteristics of the 8 selected protease producing isolates and 16S DNA sequence analysis, 4 isolates were identified to be included in the genus *Staphylococcus*, 2 isolates were in the genus *Kushneria* and the remaining 2 isolates were *Virgibacillus* and *Halomonas*.

The most interesting isolate with the high potential to be used as protease producing strains, VK31 and VK20, were identified to be *Virgibacillus halodenitrificans* and *Kushneria avicenniae,* respectively. The halophile *Virgibacillus* sp. is a Gram-positive bacterium that has been reported to be the important bacterium in fish sauce fermentation (Sinsuwan *et al*., 2010; Sonchai, 2011). Regarding the endospore forming characteristics of the bacteria in this genus, the actual original source of this bacterium might be from the sea salt used in the step of preparation of fermented fresh water fishes. However, our result revealed that the most interesting isolate is *Kushneria avicenniae* VK31 due to the highest protease activity was found from this bacterial isolate. In addition, most of the bacterial strains in this group are reported to be found in the mangrove area (Sánchez-Porro *et al*., 2009). It is non-spore forming bacteria, so, it is interesting as the source of this bacterium was fermented fresh water fish collected from Mae Lai sub-district, Phrae province, whish located far away from the sea. Our results may lead to the discovery of the novel species of the bacterial strain in this genus. However, more details of bacterial identification have to be further more investigated.

Moreover, our result revealed that the dominant bacterial isolates from fermented fresh water fish samples were the genus *Staphylococcus* spp., this does not out of the expected results as the bacteria in this genus are commonly known to tolerate and able to survive in high sanity condition (MacFaddin, 2002). Some are suggested to be pathogens causing the dermatitis in human or other animals. Particularly the isolate VK14 which identified to be *Staphylococcus saprophyticus,* the pathogenic bacteria caused urinary tract infectious disease in human (Schneider and Riley, 1996). Fortunately, these *Staphylococcus* spp. obtained from this experiment did not show any potential to be used as protease producer. The *Halomonas* sp. is also found to be common microorganism found in high salt condition (Ma *et al*., 2010) and some were isolated from fish sauce (Namwong *et al*., 2006). The closest relatives were *Halomonas avicenniae* MW2aT, *Halomonas marisflavi* DSM 15357T and *Halomonas indalinina* CG2.1T, with 95.7, 95.2 and 95.0% sequence similarities, respectively. (Sánchez-Porro *et al*., 2009).

3.4 Time course of protease production

Protease productions by *K. avicenniae* VK31 and *V. halodenitrificans* VK20 were investigated by cultivation in liquid medium which was modified by using the following nutrients; soybean, casein, yeast extract, at 0.5% (w/v) instead of casamino acids, at 37°C for 3 days. Both isolates produced significant amount of protease at 30 h of growth. Maximum protease production of VK31, VK20 (5.42±0.02 and 4.19±0.03 U/mL) were found after 5 days cultivation (Figure 3) whereas the viable cell number was corresponding to the enzyme activity produced from both strain (Figure 4). Regarding the capability of protease production by *K. avicenniae* VK31 and *V. halodenitrificans* VK20, both are expected to be used as pure culture inoculum and protease sources for fish sauce fermentation aiming either to reduce the fermentation time or accelerate of fish protein degradation.

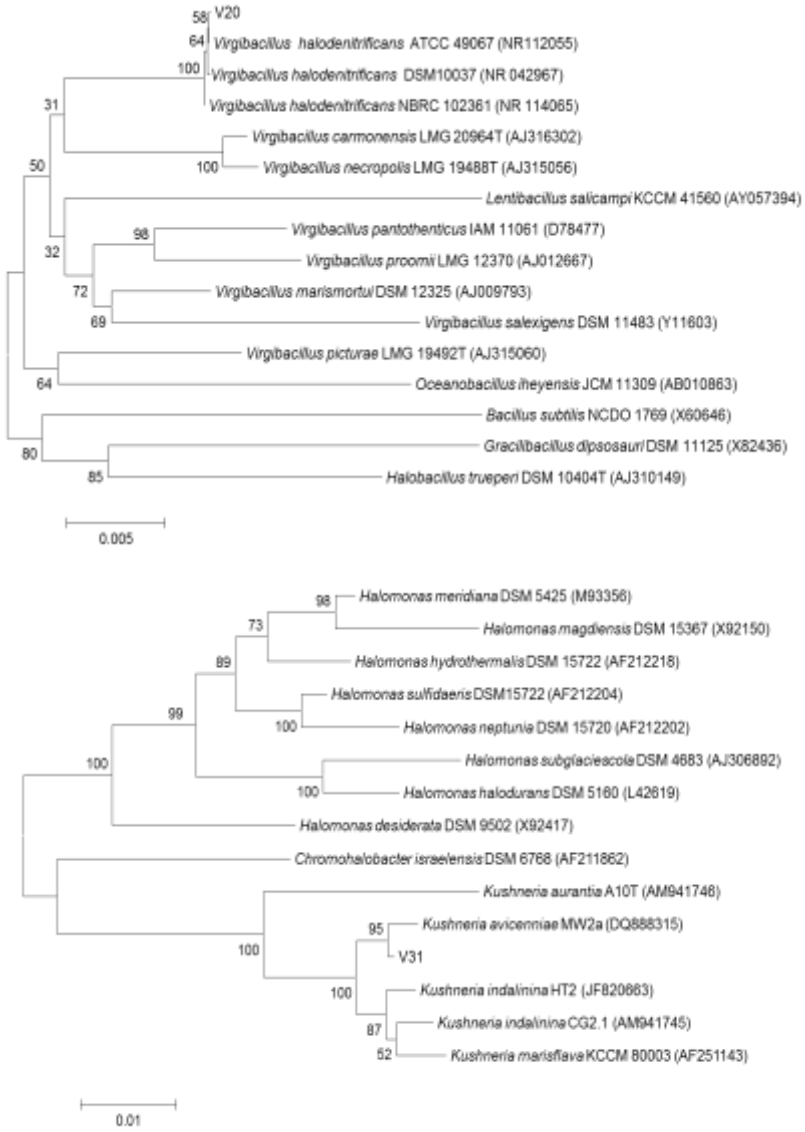

Figure 3. Phylogenetic tree of the isolate VK31 and VK20 comparing to the related species.

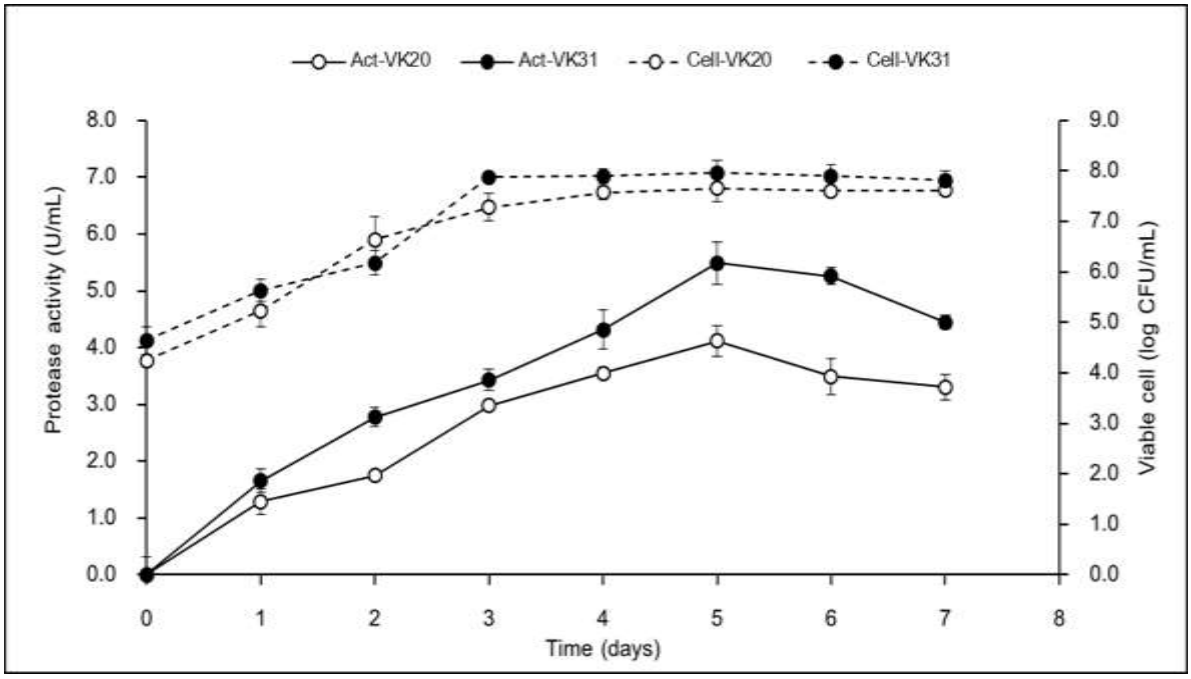

Figure 4. Time course of protease production by *K. avicenniae* VK20 and *V. halodenitrificans VK31* in enzyme production medium using 0.5% (w/v) casein as substrate at 37°C, 150 rpm for 7 days.

4. Conclusion

Forty five halotolerant bacterial isolates were isolated from 38 fermented freshwater fishes and 2 isolates were selected as protease producers and further identified to be *K. avicenniae* VK20 and *V. halodenitrificans* VK31, respectively. Both strains produced the satisfactory quantity of protease activity and expected to be used as pure culture starter in fish sauce fermentation using Siamese Mud Carp as substrate.

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