

PAPER • OPEN ACCESS

## Antioxidant and antifungal activities of collagen hydrolysates from skin of milkfish (*Chanos chanos*) hydrolyzed using various *bacillus* proteases

To cite this article: E Kusumaningtyas *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **278** 012040

View the [article online](#) for updates and enhancements.

# Antioxidant and antifungal activities of collagen hydrolysates from skin of milkfish (*Chanos chanos*) hydrolyzed using various *bacillus* proteases

E Kusumaningtyas<sup>1\*</sup>, M Nurilmala<sup>2</sup> and D Sibarani<sup>3</sup>

<sup>1</sup>Indonesian Research Institute for Veterinary Sciences, Bogor, Indonesia

<sup>2</sup>Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Bogor, Indonesia

<sup>3</sup>Faculty of Pharmacy, Pancasila University, Jakarta, Indonesia

\*E-mail: enikusuma@yahoo.com

**Abstract.** The skin of fish contains collagen which can be used as a material for food, pharmacy or cosmetics. Collagen from fish is a good source of bioactive peptides which are produced through enzymatic hydrolysis and may be beneficial for human health. This research was conducted to investigate antioxidant and antifungal activities of milkfish (*Chanos chanos*) collagen which was hydrolyzed by *Bacillus licheniformis* and *Bacillus thuringiensis* proteases. The collagen was isolated from milkfish skin and the hydrolysis was done using *B. licheniformis* protease and crude protease from *B. thuringiensis* at alkaline condition, 55°C for 30 and 60 minutes. The hydrolysates obtained were assayed for antioxidant activities using ABTS and DPPH methods and antifungal activities against *Candida albicans*. The results showed that generally, collagen hydrolyzed using *B. thuringiensis* protease both 30 and 60 minutes showed higher antioxidant activities than of that hydrolyzed using *B. licheniformis* protease. The hydrolysates also reduced *C. albicans* at least for 5 log cycles. The results indicated that collagen hydrolyzed using *B. thuringiensis* protease is very potent as antioxidant and antifungal against *C. albicans* in future pharmaceutical product.

**Keywords:** antifungal, collagen, skin

## 1. Introduction

Fish is an essential source of food which provides bioactive natural products such as proteins and peptides. The bioactive compound is not only present in fish meat, but also in fish processing by-product such as skin and bones. The skin and bones are commonly discarded and create a new problem in the environment as a problem in the form of environmental pollution. In fact, the waste contains useful protein which represents a large amount source of bioactive peptide. Collagen is a fish protein which is abundantly available in the skin, bones and scales of fish. Collagen and its hydrolysates are important in cosmetics, pharmacy and food industry [1]. Bioactive peptides derived from collagen are commonly used as functional food in many countries. Some are used as antioxidant, cryoprotectant and moisturizing ingredient in the cosmetics product [2, 3].

The active peptide is commonly bound in the natural protein and is released by chemical or enzymatic hydrolysis. Chemical method uses acid and/or alkaline to hydrolyze collagen. The method is suitable for



industrial scale due to its inexpensiveness and ease to perform, but sometimes it produces non-specific cleavage peptide bond, uncontrolled process, low in homogeneity and decreasing of nutritional quality or bioactivity of the hydrolysate [4]. Enzymatic hydrolysis would produce hydrolysates containing bioactive peptides. A suitable protease is essential to obtain peptides with strong bioactivities. The enzymes commonly used to produce bioactive peptide from fish skin protein are proteinase K, collagenase, alcalase and trypsin. Previous studies revealed that hydrolysis of skin fish protein using Alcalase® or trypsin produce hydrolysates with higher antioxidant activity compare to that of hydrolyzed by other enzymes such as papain,  $\alpha$ -chymotrypsin or pepsin [5].

Alcalase is produced by *Bacillus licheniformis* with optimum pH levels of 7-9. Hydrolysis of Cuttlefish skin gelatin hydrolysate using alcalase showed high antioxidant activity which indicated by  $\beta$ -carotene bleaching, scavenging activity to DPPH radical, reducing power activity and inhibition of lipid peroxidation. The activities were relatively stable in various condition such as pH range 7-9, gastrointestinal digestion stimulation or heating treatment up to 100°C for 4 hours [6]. Protease *Bacillus thuringiensis* has been used to produce antibacterial and antioxidant peptide fraction from horse milk protein [7]. Substrate, enzyme, ratio of enzyme-substrate and duration of hydrolysis determine the activities of the peptide or hydrolysate product. Amino acid sequence in the natural protein determine the sequence and the activity of the peptide produced during hydrolysis. Application of both protease, from *B. licheniformis* and *B. thuringiensis*, in skin fish hydrolysis enable to produce hydrolysate with different bioactivities.

This research used the collagen of milkfish (*Chanos chanos*) to produce collagen hydrolysates. Hydrolysis was performed using *B. licheniformis* and *B. thuringiensis* proteases. The antioxidant and antifungal activity of the hydrolysates were investigated and evaluated.

## 2. Materials and Methods

### 2.1. Collagen extraction

Fish skin was washed to discard the fat and other contaminants and then cut into 3 x 3 cm pieces and washed by water 5 times. The clean skin was soaked in acetic acid 1.5% with ratio of fish skin to acetic acid of 1:2 (g/mL) for 24 hours. The fish skin was cleaned using running water until it is free of acid. The fish skin was then soaked in aquadest with the ratio of fish skin to aquadest of 1:2 (g/mL), then shaken and incubated at 50°C until the fish skin fused. The fish skin suspension was filtered using Whatman no 41 paper. The filtrate obtained was kept in the refrigerator until used.

### 2.2. Microorganisms

*B. thuringiensis* was isolated from horse milk from Bogor, Indonesia. *Candida albicans* was obtained from BBLitvet Culture Collection (BCC) BCC F059.

### 2.3. Enzyme preparation

Alcalase from *B. licheniformis* (Novozyme Corp.) was used for collagen hydrolysis. Another protease was extracted from *B. thuringiensis*. The enzyme was extracted and isolated using ammonium sulfate precipitation. *B. thuringiensis* was inoculated in luria bertani broth (Difco, becton dickinson and Co, US) which added with 0.05 % (w/v) skim milk (Difco, Becton Dickinson and Co, US). The suspension was then incubated at 37°C. After 24 hours incubation, the suspension were centrifuged at  $3500 \times g$  for 20 minutes. Supernatant was taken and added with ammonium sulfat 50% (w/v) and then stored at 4°C overnight. The crude enzyme was harvested by centrifugation at  $10,000 g$ , 4°C for 20 minutes. The precipitate obtained was then dried and diluted in phosphate buffer saline (10 mM, pH 7.4 (1:2 (w/v) if it is used directly or stored at -20°C if it will be used later. Quick start™ Bradford protein assay (Bio-Rad Inc) was used for analyzing protein concentration of the crude enzyme. Five  $\mu\text{L}$  of serial dilution of 2.5-25  $\text{mg mL}^{-1}$  bovine serum albumin was mixed with 95  $\mu\text{L}$  Bradford solution to obtain curve standard. Deionized water was used as blank. Protein concentration was determined based on the absorbance at  $\lambda$  600 nm.

Enzyme activity assay was performed from reaction of 50  $\mu\text{L}$  enzyme, 250  $\mu\text{L}$  casein (w/v) and 250  $\mu\text{L}$  PBS 0.05 M pH 7, and then incubated at 37 °C for 10 minutes. Trichloroacetic acid (TCA) 0.2 M (Sigma-Aldrich, USA), 500  $\mu\text{L}$  was added into the mixture followed by incubation at 37 °C for 10 minutes and centrifugation at 2000 x g for 10 minutes. Supernatant was taken and then added with 1250  $\mu\text{L}$  0.4 M  $\text{Na}_2\text{CO}_3$  and 250  $\mu\text{L}$  reagent folin ciolcateau (Sigma-Aldrich) followed by incubation at 37 °C for 20 minutes. Enzyme activity was determined based on the absorbance at  $\lambda$  578 nm (spectrophotometer UV-Mini 1240 Shimadzu) [8]. One unit (IU) enzyme is amount of enzyme needed for producing 1  $\mu\text{mol}$  tyrosine per minute.

#### 2.4. Hydrolysis

Alcalase of *B. licheniformis* (2.4 U/g) was used to hydrolyze fish collagen with enzyme substrate ratio 1:20, at 55 °C, pH 8 for 30 and 60 minutes as recommended by producer. Fish collagen was also hydrolyzed using *B. thuringiensis* protease (0.67 U/g) with enzyme substrate ratio 1:20, 55 °C, pH 11 for 30 and 60 minutes. The reaction was stopped by putting the reaction in boiled water (95 °C) for 5 minutes. Lipid and insoluble protein from the hydrolysate were removed by centrifugation at 11.000 g for 15 minutes. The supernatant was collected, the pH was adjusted to around 7 and then filtered using 0.45  $\mu\text{m}$  membrane (Acrodisc LC 13 mm, 0.45  $\mu\text{m}$ , PVDF, Pall Life Sciences, USA).

#### 2.5. Antioxidant assay

Antioxidant assays was performed using ABTS (2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (Sigma-Aldrich, USA) and DPPH (2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, USA) methods. ABTS radicals was made by mixing the stock solution of 7.4 mM ABTS and 2.6 mM potassium persulfate, incubated in the dark at room temperature for 18 hours. The solution was diluted with deionized water to adjust the absorbance  $1.1 \pm 0.05$  at 405 nm. The fresh ABTS radicals, 200  $\mu\text{L}$ , was mixed with 100  $\mu\text{L}$  hydrolysate and incubated at room temperature for 15 minutes to allow the reaction. The absorbance at 405 nm of the mixture was recorded using microplate reader (Labsystems, original Multiscan Ex and Champaign, USA). Deionized water was added to the hydrolysate to replace ABTS as hydrolysate control and it added to ABTS to replace hydrolysate as ABTS control. Standard curve of vitamin C p.a was by made using serial concentration of the vitamin 2.5-15.5  $\mu\text{g/mL}$ .

DPPH was diluted in ethanol 96% until the absorbance at 540 nm reached  $1.1 \pm 0.05$ . The diluted DPPH was then used for assay. DPPH 200  $\mu\text{L}$  was added to 100  $\mu\text{L}$  hydrolysate and incubated at room temperature for 30 minutes to allow the reaction. The absorbance of the mixture was measured at  $\lambda$  540 nm. Serial concentration of vitamin C p.a (2-16  $\mu\text{g/mL}$ ) was used to generate a standard curve. The scavenging activity of the hydrolysates in ABTS and DPPH assay was calculated using the following equation (1):

$$\text{Scavenging activity (\%)} = 100 \times (\text{A}_0 - \text{A}_1) / \text{A}_0 \quad (1)$$

Where  $\text{A}_0$  was the absorbance of radical ABTS/DPPH,  $\text{A}_1$  was the final absorbance of hydrolysate minus initial absorbance (before mix with ABTS/DPPH). Result of the assays was expressed as means of three replication of the experiment.

#### 2.6. Antifungal assay

Briefly, 100  $\mu\text{L}$  of *C. albicans* suspensions of  $10^6$  CFU/mL was added to the micro tube containing 100  $\mu\text{L}$  hydrolysate and incubated at 37 °C for 1 hour to allow for the interaction between hydrolysate and *C. albicans* cells. The 100  $\mu\text{L}$  of mixed suspension was grown onto Sabouraud dextrose agar plate (BD Difco™, Becton Dickinson and Co, USA) and incubated at 37 °C for 24 hours. The viable colonies were counted. Each treatment was done in three replications.

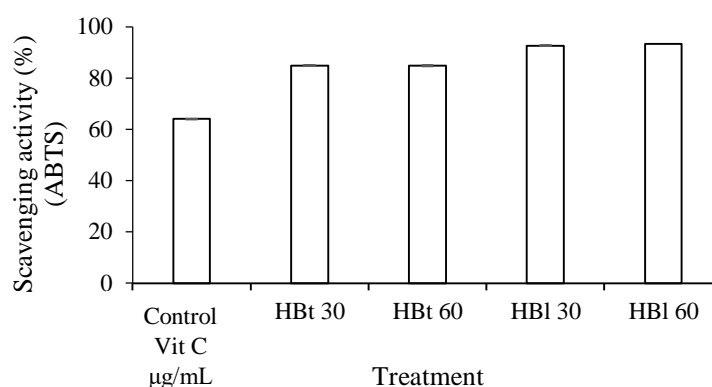
Minimum Fungicidal Concentration (MFC) assay was performed with some modification [9]. Briefly, 100  $\mu\text{L}$  of hydrolysate was prepared in 2-fold dilution series microtube. As much as 100  $\mu\text{L}$  *C. albicans* suspensions of  $10^6$  CFU/mL was then added to the well containing hydrolysate and incubated at 37 °C for 1 hour. The mixture (100  $\mu\text{L}$ ) was grown into Sabouraud dextrosa agar plate (BD Difco™, Becton Dickinson and Co, USA) and incubated at 37 °C for 24 hours. To ensure that there is no fungal growth,

plates were incubated for additional 24 hours. The minimum fungicidal concentration (MFC) was calculated as the lowest concentration of the hydrolysate which produce no visible fungal growth. Each treatment was conducted in three replications.

### 3. Results and Discussion

#### 3.1. Antioxidant activity

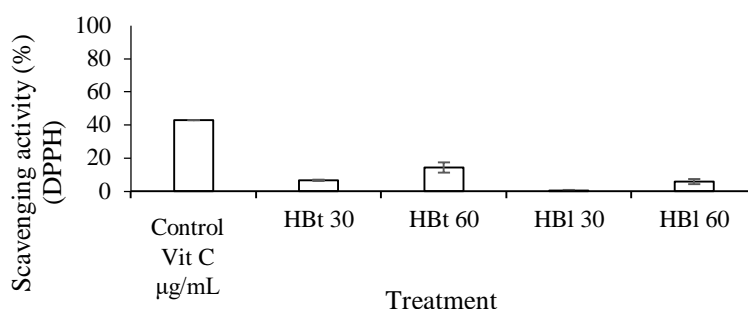
The antioxidant activity of fish skin collagen hydrolysate was obtained from the ability of the hydrolysate to scavenge free radicals. The ability of antioxidant to scavenge DPPH radicals may be due to the donation of hydrogen by hydrolysate while ABTS assay was revealed as an excellent tool for scavenging aqueous phase radicals [10]. The ABTS is commonly used for antioxidant assay dan suitable for both hydrophilic and lipophilic compound. High results of ABTS radical scavenging activity may indicated that the hydrolysates were tend to hydrophilic [11]. Scavenging activities of the hydrolysates were determined by the reduction of ABTS or DPPH absorbance after addition of hydrolysate. Scavenging activity 100% represents a complete scavenging of ABTS or DPPH radicals. The scavenging radical activity of the hydrolysate is shown in figure 1. Hydrolysates from collagen hydrolyzed by protease *B. licheniformis* 30 or 60 minutes have higher scavenging activities compared to collagen hydrolysates produced by *B. licheniformis* protease.



**Figure 1.** Scavenging activity collagen hydrolysates produced by protease of *B. thuringiensis* (Bt) and alcalase of *B. licheniformis* (BI) againts ABTS radical (per mg protein/mL). Value represent the mean  $\pm$  standard deviation (SD).

In DPPH assay (figure 2), hydrolysate Bt 30 and Bt 60 were more active than BI30 and BI60. A hydrolysis of 60 minutes showed higher scavenging activity than 30 minutes. This may be due to longer hydrolysis producing smaller molecules. Hydrolysate with lower molecular weight is more stable in temperature change [10]. Previously, it was reported that hydrolysates containing short chain water soluble peptide possess antioxidant properties [12]. Addition of hydrolysis time may produce smaller molecule with lower molecular weight which may have higher activities. The molecules are able act as electron donors and react with free radicals to form more stable substance and stop the chain reaction [13].

It reported that the hydrolysis of fish skin collagen using collagenase from *B. licheniformis* F11.4 has scavenging activity againts DPPH of around 30% for 30 minutes and 35% for 60 minutes of hydrolysis [14]. The result is higher than scavenging activity of collagen hydrolysate by alcalase of *B. licheniformis* in this research (0.47 and 5.79%).



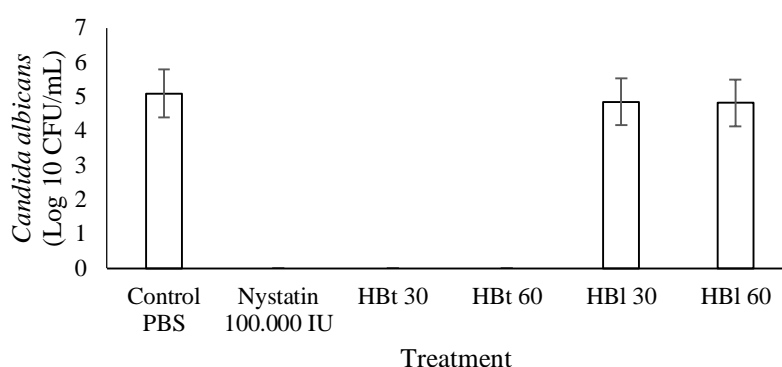
**Figure 2.** Scavenging activity collagen hydrolysates produced by protease of *B. thuringiensis* (Bt) and alcalase of *B. licheniformis* (Bl). againsts DPPH radical (per mg protein/mL). Value represent the mean  $\pm$  standard deviation (SD).

The scavenging activity of whole hydro lysates from Bt and Bl, generally showed that results of ABTS assay were higher than that of DPPH assay. This indicated that fish skin hydrolysates produced by *B. thuringiensis* protease and alcalase from *B. licheniformis* might produce more lipophilic antioxidant compound. Moreover, the hydrolysates varied in antioxidant activities. This might be due to the difference of amino acid in the hydrolysate. Protease hydrolyzed protein in different site of protein which may produce peptide with different length and amino acid composition. The amino acid composition affects the hydrophobicity and charge of the peptides which determine their bioactivities. It has been reported that antioxidant activities of hydrolysate influenced by amino acid composition [15]. In addition, hydrophobic and aromatic amino acid are also play an important role in the antioxidant activities of the hydrolysates [16].

### 3.2. Antifungal activities

*C. albicans* are human commensal fungi commonly found in skin, gastrointestinal tract, oropharynx and upper respiratory tract. Immuno-compromised patient increase significantly in the recent years. This fact followed by increasing incident of clinical disease associated with fungal infection which cause mortality. Resistance to antifungal has been observed in recent years and resulted in increased demand for new antifungal. Hydrolysate of fish skin which contains numerous bioactive peptide may be a choice to overcome the problem. Isolation and characterization of antimicrobial peptide or hydrolysate generated from fish processing by product were still lower than achievement of antioxidant research derived from the similar material [17]. In antimicrobial searching antifungal is less intensive compared to antibacterial peptide. Here the antifungal activities against *C. albicans* of fish skin hydrolysates were evaluated.

Antifungal activities of the fish skin hydrolysates is shown in figure 3. Hydrolysate produced by *B. thuringiensis* protease for 30 and 60 minutes of hydrolysis (Bt 30 and Bt 60) showed higher activity compare to Bl 30 and Bl 60 and decrease *C. albicans* by at least 5 log cycles. The result was similar to that which was observed in Nystatin 100.000 IU. Minimum fungicidal concentrations of the hydrolysates were 0.5 mg protein/mL for Bt 30 and 0.45 mg/mL for Bt 60.



**Figure 3.** Colony number of *C. albicans* treated by collagen skin fish hydrolysate (per mg protein/mL) produced by protease of *B. thuringiensis* (HBt) and alcalase of *B. licheniformis* (HBI). Value represent the mean  $\pm$  standard deviation (SD).

The result showed that the hydrolysate has the potential to develop as a new antifungal. Hydrolysate contains peptides which may be safer than chemical medicine as it degrades easily. The small cationic antifungal peptide may have a specific target and multifunction [18]. In addition, antifungal peptide or hydrolysate were varied in their structural and functional characteristic which very potent for developing the new generation of the novel drug [19].

#### 4. Conclusion

Collagen hydrolysates from the skin of milk fish *Chanos chanos* hydrolyzed using Bt and Bl proteases for 30 and 60 minutes (Bt 30, Bt 60, Bl 30, Bl 60) have the potential to act as an antioxidant and antifungal agent. The activity of hydrolysates to ABTS radicals were higher than their activity DPPH which leads to the possibility that the active molecule is hydrophilic. Hydrolysate produced Bt protease resulted in a high antifungal activity against *C. albicans* and decreased at least 5 log cycles.

#### References

- [1] Arvanitoyannis I S and Kassaveti A 2008 Fish industry waste: treatments, environmental impacts, current and potential uses *Int J.Food Sci.Technol.* **43** 726-745
- [2] Chen X, Peng M, Li J, Tang B, Shao X, Zhao F, Liu C, Zhang X, Li P, Zhang Y and Song X 2017 Preparation and functional evaluation of collagen oligopeptide-rich hydrolysate from fish skin with the serine collagenolytic protease from *Pseudoalteromonas* sp. SM991 *Scientific Report* **7** 1-13
- [3] Wang X, Yu H, Xing R and Li P 2017 Characterization, preparation and purification of marine bioactive peptides *Biomed. Res. Int.* **5** 1-16
- [4] Armin M K and Marzieh M N 2018 Bioactive peptides derived from fish by-product collagen *Int J. Environ. Sci. Nat. Res.* **13** 555-559
- [5] Yi J, Gobba C D, Skibsted L H and Otte J 2017 Angiotensin-I converting enzyme inhibitory and antioxidant activity of bioactive peptides produced by enzymatic hydrolysis of skin from grass carp (*Ctenophayngodon idella*). *Int J. Food Prop.* **20** 1129-1144
- [6] Jridi M, Lassoued I, Nasri R, Ayadi M A, Nasri M and Souissi N 2014 Characterization and potential use of cuttlefish skin gelatin hydrolysates prepared by different microbial proteases *Biomed. Res. Int.* **4** 1-14
- [7] Kusumaningtyas E, Widiastuti R, Kusumaningrum H D and Suhartono M T 2016 Bioactivities and analysis of peptides of Sumbawa horse milk generated by *Bacillus thuringiensis* protease *JITV* **21** 244-254
- [8] Bergmeyer H U, Bergmeyer J and Grassl M 1983 *Methods of Enzymatic Analysis* (Germany: Verlag Chemie, Weinheim)
- [9] Keepers T R, Gomez M, Celeri C, Nichols W W and Krause K M 2014 Bactericidal activity, absence of serum effect and time-kill kinetics of Ceftazidime-Avibactam against  $\beta$ -lactamase-

- producing Enterobacteriaceae and *Pseudomonas aeruginosa* Antimicrob Agents Chemother **58** 5297-5305
- [10] Dey S S and Dora K C 2014 Antioxidative activity of protein hydrolysate produced by alcalase hydrolysis from shrimp waste (*Penaeus monodon* and *Penaeus indicus*) *J. Food Sci. Technol.* **51** 449-457
- [11] Klomklao S and Benjakul S 2018 Protein hydrolysates prepared from viscera of skipjack tuna (*Katsuwonus pelmanis*): antioxidative activity and functional properties *Turkish J. Fish. Aquatic Sci.* **18** 69-79
- [12] Je J Y, Park P J and Kim S K 2005 Antioxidant activity of peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate *Food Res. Int.* **38** 45-50
- [13] Chi C, Cao Z, Wang B, Hu F, Li Z and Zhang B 2014 Antioxidant and functional properties of collagen hydrolysates from Spanish mackerel skin as influenced by average molecular weight *Molecules* **19** 11211-11230
- [14] Baehaki A, Suhartono M T, Sukarno, Syah D and Setyahadi 2016 Antioxidant activity of collagen hydrolysates from fish skin with a microbial collagenase *Res. J. Pharm. Biol. Chem. Sci.* **7** 1677-1681
- [15] Chalamaiah M, Dinesh kumar B, Hemalatha R and Jyothirmayi T 2012 Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: a review *Food Chem.* **135** 3020-3038
- [16] Chi C F, Hu F Y, Wang B, Li Z R and Luo H Y 2015 Influence of amino acid compositions and peptide profiles on antioxidant capacities of two protein hydrolysates from skipjack tuna (*Katsuwonus pelamis*) dark muscle *Marine Drugs* **13** 2580-2601
- [17] Di B R, Mullen A M, Bolton D, Kerry J, O'Neill E, Hayes M 2012 Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions *Meat Sci.* **90** 226-235
- [18] Matejuk A, Leng Q, Begum M D, Woodle M C, Scaria P, Chou S T and Mixson A J 2010 Peptide-based antifungal therapies against emerging infections *Drugs of the Future* **35** 197-205
- [19] Bondaryk M, Staniszewska M, Zielinska P, Urbanczyk-Lipkowska Z 2017 Natural antimicrobial peptide as inspiration for design of a new generation antifungal compound *J. Fungi* **3** 1-36