# ANTIMICROBIAL EFFECTS OF PALM KERNEL CAKE PROTEIN HYDROLYSATES (KESAN ANTIMIKROB HIDROLISAT PROTEIN DEDAK ISIRUNG KELAPA SAWIT)

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ABSTRACT

Disc clearing zone test and minimum inhibitory concentration (MIC) assay were used to evaluate antimicrobial effects of palm kernel cake (PKC) hydrolysates against a range of Gram positive and Gram negative bacteria. Trypsin and pepsin were separately used to prepare the protein hydrolysates. Both PKC protein hydrolysates, the trypsin hydrolysed hydrolysates (TH) and pepsin hydrolyzed hydrolysates (PH), showed positive inhibitory against Bacillus cereus according to disc clearing zone test but not against other organisms tested. The MICs of both TH and PH on Bacillus cereus are 800 μg/ml and 2400 μg/ml respectively. The molecular weight of PKC protein hydrolysates responsible for the inhibitory effect were lower than 30 kDa as determined by tris-tricine SDS-PAGE technique. PKC protein hydrolysates are high in amino acid of arginine which may contribute to the inhibitory effect.

***Keywords**: PKC protein hydrolysate, antimicrobial,* Bacillus cereus, *tris-tricine SDS-PAGE*

ABSTRAK

Ujian cakera zon cerah dan asai kepekatan rencatan minimum (MIC) digunakan untuk mengkaji kesan antibakteria hidrolisat protein dedak isirung kelapa sawit (PKC) ke atas bakteria Gram positif dan bakteria Gram negatif. Enzim tripsin dan pepsin digunakan untuk menghidrolisis protein PKC. Menurut ujian cakera zon cerah, kedua-dua hidrolisat cernaan tripsin (TH) dan hidrolisat cernaan pepsin (PH) menunjukkan kesan perencatan ke atas Bacillus cereus. Namun tiada kesan perencatan terhadap bakteria yang lain. Nilai MIC bagi TH dan PH masing-masing adalah 800 μg/ml and 2400 μg/ml. Berat molekul hidrolisat protein PKC yang memberi kesan perencatan didapati adalah kurang daripada 30 kDa yang ditentukan dengan teknik SDS-PAGE dalam sistem penimbal tris-trisin. Hidrolisat protein PKC tinggi dalam asid amino arginin yang dipercayai memberi kesan perencatan.

***Kata kunci**: hidrolisat protein PKC, antimikrob,* Bacillus cereus*, SDS-PAGE tris-trisin*

## INTRODUCTION

Recently, there is a growing pressure on the food industry to reduce its reliance on synthetic chemical preservative, and also interest in using natural alternative materials due to the increasing bacteria resistances to many classes of antibiotics. Many reports have been published on the occurrence and characterization of low-molecular-mass antimicrobial peptides from a wide variety of organisms (Zasloff 2002). Some studies have shown that antimicrobial peptides act in synergy with today's commonly used antibiotics, and do so against multi-drug resistant bacteria (Spellberg et al. 2004). Therefore, even if the peptides cannot be used on their own, they can be administered in combination with today's antibiotics and improve their potency.

Antimicrobial peptides have been proposed to achieve their bactericidal effect in different fashions. In all cases, an initial interaction with the outer and/or inner membranes of bacteria is necessary. Antimicrobial peptides interfere with membrane function to cause bacterial cell death. Membrane disruption can occur by a number of different mechanisms. Certain models for example the barrel-stave, toroidal pore, and carpet models. Not all antimicrobial peptides are thought to exert their major action on membranes. An increasing number of peptides are being described that act on intracellular targets in bacteria, inhibiting

protein, or cell-wall synthesis, interact with DNA or RNA or inhibit some sort of enzymatic activity (David et al. 2006). From a wide range of antimicrobial peptides, they could be classified into seven different groups. One of them is peptide with particular and unusual amino acid composition that contributes to antimicrobial effect. For example, those peptides with a high content of tryptophan and arginine residues (Vogel et al. 2002). Glycopeptides, another class of peptides which has gradually becoming popular and attracted considerable interest in the field of antimicrobial.

According to unpublish data, PKC peptides are glycopeptides. This probable potential of antimicrobial effect encouraged this research to be carried out. Further studies on the isolation and identification of new PKC bioactive peptides are considered highly challenging but also highly profitable.

## MATERIALS AND METHODS

### PKC PROTEIN PREPARATION

Palm kernel cakes (PKC) were supplied by Felda Kernel Products Sdn. Bhd. (Pandamaran, Klang). Protein of ground PKC (0.2 μm) were extracted by using 1M sodium hydroxide at 30$^{o}$C for 1 hour with the substrate: solvent ratio of 1:20. The solid and liquid phases were centrifuged. Supernatant were collected and precipitated at isoelectric point (pH range between 3.0-4.5). Precipitated proteins were separated by centrifugation. The proteins were freeze-dried. Protein content was determined by Kjeldahl method.

### PKC HYDROLYSATES PREPARATION

Preparation of PKC protein hydrolysates was carried out according to Niberring et al. (2001). A solution of 1% dry PKC protein at pH 2 was incubated with pepsin (1:100 w/w) at 37$^{o}$C, and a solution of 1% dry PKC protein at pH 8 was incubated with trypsin (1:100 w/w) at 37$^{o}$C. After 6 hours, the reactants were heated at 95$^{o}$C for 5 minutes to inactivate enzyme used, and rapidly cooled to room temperature. The solutions were ultrafiltered using vivaflow membrane (MW cutoff = 10 kDa) (Sartorius, Germany) against water with 5 water changes and materials collected were freeze-dried. pH of the ultrafiltered protein hydrolysates were

adjusted to 7.0±0.2 and sterilized through a cellulose acetate 0.2 μm membrane filter (Sartorius, Germany). The protein content of pepsin or trypsin PKC protein hydrolysates was determined by using Kjeldahl method.

## CULTURE PREPARATION

*Bacillus cereus, Escherichia coli O157, Listeria monocytogenes, Salmonella typhimurium* and *Staphylococcus aureus* stock cultures were obtained from Institute of Medical Research (IMR). All cultures were grown in tryptic soy broth (TSB) and sub-cultured for every 4 weeks to maintain viability. Working cultures were obtained by inoculating a loopful of culture into TSB and inoculated for 24 hours at 37$^{o}$C. After incubation, the cultures were diluted to ca. 5.0 log CFU/ml.

## DISC CLEARING ZONE TEST FOR ANTIBACTERIAL ACTIVITY

Disc clearing zone test (NCCLS 2008) was conducted using sterile petri dishes containing nutrient agar (2.8% w/v). The bacterium was spread over the plates. A sterile blank paper disc (0.625cm in diameter) was placed on the agar. Freeze-dried PKC protein hydrolysates (range of 0.5-2% w/v) in 10mM Tris-HCl buffer (pH7.4) was added to one of the disks. Only Tris-HCl buffer was added to the control disc. The plate was incubated at 37$^{o}$C for 24 hours. A transparent ring around the paper disc signified antibacterial activity.

## MICRO-BROTH DILUTION ASSAY

Sterile 96-well microtiter plates with a well capacity of 300 μl were used for micro-broth dilution assay. A total volume of 250 μl which consisting 125 μl of double strength TSB, 100 μl of filtered hydrolysates and 25 μl of inoculum (ca. 5.0 log CFU/ml) was used to determine minimum inhibitory concentrations of PKC protein hydrolysates. Microtiter plates were covered with a sterile lid and incubated for 24 hours at 37$^{o}$C and absorbance (630nm) of each well was read at 0, 3, 6, 12, 24 hour with a microtiter plate spectrophotometer (VersaMax Microplate Reader). The micro-broth dilution assays (NCCLS 2008) were performed in triplicate.

## TRIS-TRICINE SDS-PAGE

SDS-PAGE in tris-ticine buffer was performed according to the method of Ronald et al. (2005). Peptides standard was bought from GenScript Corporation, USA.

## AMINO ACID ANALYSIS

Amino acid profile of PKC glycopeptides were determined by using RP-HPLC accroding to the method of Othman (2001).

## RESULTS AND DISCUSSION

### PROTEIN CONTENT OF PKC HYDROLYSATES

PKC protein with the protein content of 60.83±2.05% was hydrolyzed using pepsin and trypsin respectively. After going through ultrafiltration system with cutoff point of 10000 Da, the retentate and filtrate portions of both pepsin and trypsin PKC hydrolysates were freeze-dried and the protein contents were determined. The results were presented in Table 1. Apparently the trypsin was capable of hydrolyzing PKC protein better than the pepsin.

TABLE 1. Protein content of both pepsin (PH) and trypsin (TH) hydrolyzed PKC protein hydrolysates

| Samples | Protein content (%) |
|---|---|
| PH (without ultrafiltration) | 50.60 ± 8.91 |
| PH(>10 kDa) | 59.44 ± 4.23 |
| PH (<10 kDa) | 20.36 ± 0.16 |
| TH (without ultrafiltration) | 69.25 ± 5.57 |
| TH (>10 kDa) | 74.49 ± 0.98 |
| TH (<10 kDa) | 32.07 ± 1.89 |

### DISC CLEARING ZONE TEST

PKC protein hydrolysates have significant antibacterial effect on *Bacillus cereus*, though no effect was shown on other bacteria tested. The diameter of inhibitory zone at different

concentrations of PKC protein hydrolysates on *Bacillus cereus* is presented in Table 2. Meanwhile, the inhibitory zone of both pepsin and trypsin hydrolyzed PKC protein hydrolysates at different concentrations on *Bacillus cereus* is illustrated in Figure 1. Trypsin hydrolyzed PKC protein hydrolysates (without ultrafiltration) and trypsin hydrolyzed PKC protein hydrolysate (<10 kDa) at concentration of 1% showing better inhibition effect on *Bacillus cereus* with the diameter of inhibitory zone of 14mm compared to other portions of hydrolysate. Only 2% pepsin hydrolyzed PKC protein hydrolysate (<10 kDa) showed positive inhibitory zone on *Bacillus cereus*. According to our unpublish data, PKC protein hydrolysates are found to be glycopeptides. Vancomycin and teicoplanin are members of the glycopeptides antibiotic family which are in clinical use today for bacterial infections caused by Gram-positive pathogens (Yong 2002). Glycopeptides antibiotics bind to the C-terminal sequence -Lys-D-Ala-D-Ala of the bacterial cell-wall precursors, thereby inhibiting transglycosylation and transpeptidation, and ultimately leading to cell death (Thomas et al. 1999). This could be one of the reasons for the PKC protein hydrolysates to have the inhibitory effect on Gram positive bacteria, *Bacillus cereus*.

TABLE 2. Diameter of inhibitory zone at different concentrations of PKC protein hydrolysates on *Bacillus cereus*

| Samples | Diameter (mm) |
|---|---|
| 0.5% TH (without ultrafiltration) | 8 |
| 1.0% TH (without ultrafiltration) | 14 |
| 1.0% TH (>10 kDa) | 10 |
| 1.0% TH (<10 kDa) | 14 |
| 2.0% PH (<10 kDa) | 12 |

Note:

TH=Trypsin hydrolyzed PKC protein hydrolysate

PH=Prypsin hydrolyzed PKC protein hydrolysate

**Figure 1**

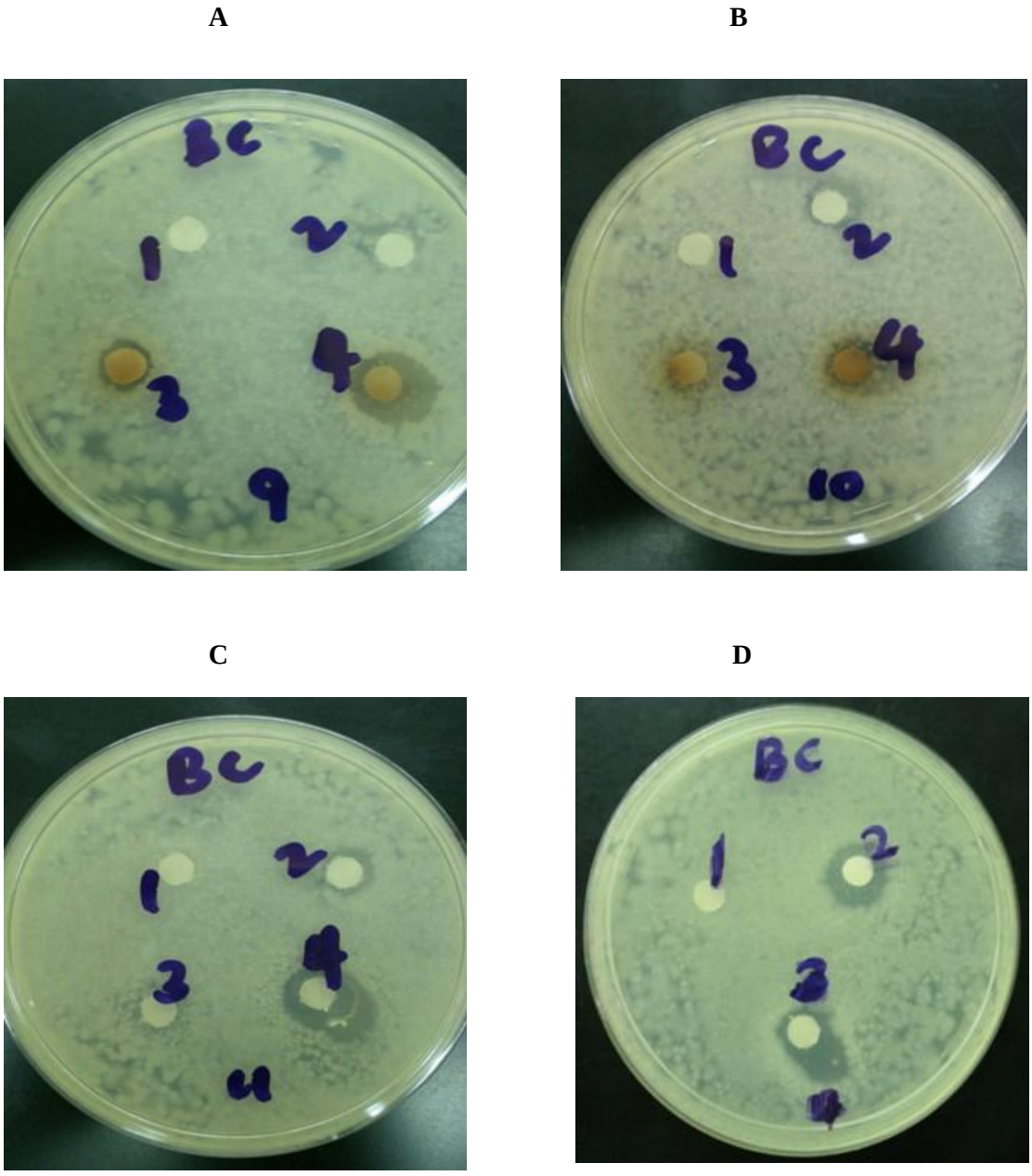


FIGURE 1. Growth of *Bacillus cereus* was inhibited by PKC protein hydrolysates. **A**, zone inhibitory of trypsin hydrolyzed PKC protein hydrolysate (without ultrafiltration), **B**, zone inhibitory of trypsin hydrolyzed PKC protein hydrolysate (>10 kDa) and **C**, zone inhibitory of trypsin hydrolyzed PKC protein hydrolysate (<10 kDa) which tested at 0.5% and 1.0% (w/v) on disc no. 3 and disc no. 4 respectively. Meanwhile, **D**, zone inhibitory of pepsin hydrolyzed

PKC protein hydrolysate (<10 kDa) against *Bacillus cereus* at concentration of 2% (w/v) on disc no.3. For disc no. 1 is the negative control (10mM tris-HCl buffer, pH 7.4) and disc no. 2 is the positive control (70% methanol).

MICRO-BROTH DILUTION ASSAY

The Minimum inhibitory concentrations (MIC) of PKC hydrolysates to restrict the growth of *Bacillus cereus* were showed in Table 3. Trypsin hydrolyzed PKC protein hydrolysate (without ultrafiltration) has the lowest MIC on *Bacillus cereus* with 800 μg/ml. Ultrafiltrated trypsin hydrolyzed PKC protein hydrolysates have higher MIC probably due to the lost of some peptides which contributed to inhibitory effect during ultrafiltration. Overall, trypsin hydrolyzed PKC protein hydrolysates have lower MIC than pepsin hydrolyzed PKC protein hydrolysates. Trypsin hydrolysates contained a greater quantity of small molecular weight peptides which may have contributed to greater antimicrobial activity (Kellie 2004). There was no significant inhibitory effect of pepsin hydrolyzed PKC hydrolysates (>10 kDa) on *Bacillus cereus* according to Disc Clearing Zone test, however, micro-broth dilution assay did showing a positive inhibitory effect at the concentration of 2400 μg/ml.

TABLE 3. Minimum inhibitory concentrations (MIC) of PKC protein hydrolysates on *Bacillus cereus*

| Samples | MIC (μg/ml) |
|---|---|
| TH (without ultrafiltration) | 800 |
| TH (>10 kDa) | 800 |
| TH (<10 kDa) | 800 |
| PH (>10 kDa) | 2400 |
| PH (<10 kDa) | 2400 |

Note:

TH=Trypsin hydrolyzed PKC protein hydrolysate

PH=Pepsin hydrolyzed PKC protein hydrolysate

TRIS-TRICINE SDS-PAGE

Molecular weight of both pepsin and trypsin hydrolyzed PKC protein hydrolysates were determined by using tris-tricine SDS-PAGE. The molecular weight of PKC protein hydrolysates responsible for the inhibitory effect were lower than 30 kDa. There were 2 bands could be seen clearly in the samples of trypsin hydrolyzed PKC hydrolysate (without ultrafiltration), trypsin hydrolyzed PKC hydrolysate (>10 kDa), pepsin hydrolyzed PKC hydrolysate (without ultrafiltration) and pepsin hydrolyzed PKC hydrolysate (>10 kDa) with the molecular weight of 15.14 kDa and 28.84 kDa. While bands that smaller than 10 kDa could not be seen clearly in hydrolysate portions of smaller than 10 kDa for both type of enzyme hydrolyzed PKC hydrolysates probably due to the low purity of the sample hydrolysates and sugar portions of glycopeptides. Sugar component would contribute to the band swelling and tailing problems (Zhu & Zhou 2005) of the gel SDS-PAGE. Special glycopeptides staining solution is needed to overcome the aforesaid problem. Figure 2 showed the molecular weight bands of all portions of both pepsin and trypsin hydrolyzed PKC protein hydrolsates.

**Figure 2**

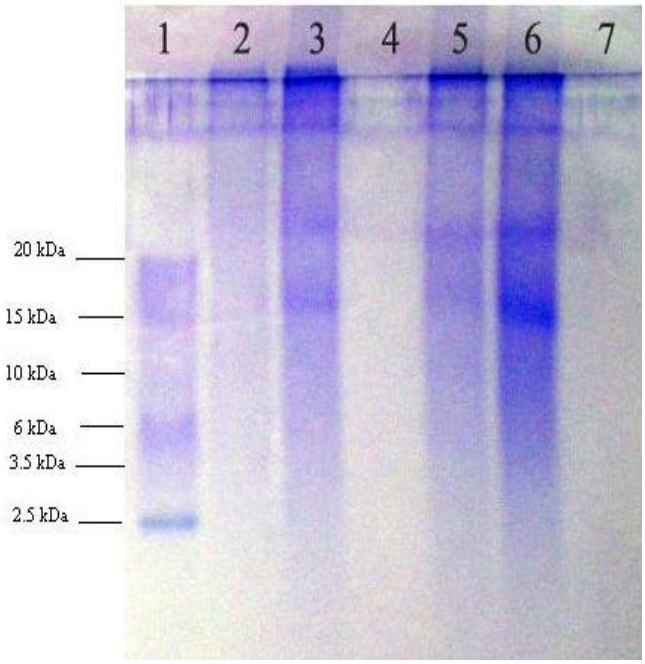


FIGURE 2. Tris-tricine gel electrophoresis of PKC protein hydrolysates. Lane 1, peptides standard. Lane 2, trypsin hydrolyzed PKC protein hydrolysate (without ultrafiltration)

fractions, lane 3, trypsin hydrolyzed PKC protein hydrolysate (>10 kDa) fractions, lane 4, trypsin hydrolyzed PKC protein hydrolysate (<10 kDa) fractions, lane 5, trypsin hydrolyzed PKC protein hydrolysate (without ultrafiltration) fractions, lane 6, pepsin hydrolyzed PKC protein hydrolysate (>10 kDa) fractions and lane 7, pepsin hydrolyzed PKC protein hydrolysate (<10 kDa) fractions.

## AMINO ACID ANALYSIS

Inhibitory effect of PKC protein hydrolysates on *Bacillus cereus* depends on several reasons. Special amino acid composition would contribute to antimicrobial effect as well (Vogel et al. 2002). Some of the most active native antibacterial peptides, at least those against selected Gram-negative pathogens, belong to the proline-rich peptide family (Laszlo et al. 2005). In this study, enzyme pepsin and trypsin were used to hydrolyze PKC protein are due to their special cleavage bonds. Pepsin cleaves proteins at the amino acids phenylalanine, tyrosine and tryptophan. While trypsin cleaves bonds associated with the amino acids lysine and arginine (Kellie 2004). According to Table 4, PKC protein hydrolysates are high in arginine, moderate in lysine, phenylalanine and low in tyrosine and tryptophan. Significant amount of arginine, lysine and phenylalanine probably contribute to the inhibitory effect of PKC protein hydrolysates on *Bacillus cereus*. The actual contributing factors for the inhibitory effect are remained unknown, further studies are understudy for conformation.

Table 4. Amino acid content of PKC protein hydrolysates

| Amino acid | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Essential amino acid: | | | | | | |
| Phenylalanine | 6.87 | 7.43 | 1.16 | 7.49 | 8.14 | 2.20 |
| Histidine | 2.80 | 2.92 | 0.35 | 2.89 | 2.76 | 0.42 |
| Isoleucine | 5.85 | 6.57 | 1.57 | 6.69 | 6.43 | 1.92 |
| Leucine | 10.66 | 11.33 | 2.59 | 11.36 | 11.97 | 3.49 |
| Lysine | 4.24 | 3.67 | 0.93 | 3.81 | 3.34 | 1.21 |
| Methionine | 3.07 | 4.43 | 0.98 | 3.00 | 3.63 | 2.31 |
| Cysteine | ND | ND | ND | ND | ND | ND |
| Tyrosine | 0.22 | 0.17 | 0.06 | 0.09 | 0.14 | 0.05 |
| Threonine | 3.13 | 5.19 | 0.63 | 5.19 | 5.34 | 1.07 |
| Tryptophan | 0.05 | 0.09 | 0.01 | 0.04 | 0.04 | 0.02 |
| Valine | 12.49 | 13.79 | 3.47 | 14.03 | 15.57 | 5.54 |
| Nonessential amino acid: | | | | | | |
| Alanine | 6.40 | 7.02 | 1.87 | 6.81 | 6.92 | 2.95 |
| Arginine | 16.27 | 19.89 | 5.16 | 20.06 | 21.78 | 3.48 |
| Aspartic acid | 12.04 | 13.47 | 1.80 | 13.19 | 14.36 | 4.01 |
| Glutamic acid | 23.99 | 26.14 | 3.84 | 25.44 | 28.25 | 5.61 |
| Glycine | 6.13 | 6.43 | 1.37 | 6.59 | 7.55 | 2.65 |
| Proline | 5.06 | 4.69 | 1.32 | 4.63 | 4.23 | 0.70 |
| Serine | 5.39 | 5.75 | 1.40 | 5.72 | 6.57 | 2.34 |

Note:

A= Trypsin hydrolyzed PKC protein hydrolysate (without ultrafiltration)

B= Trypsin hydrolyzed PKC protein hydrolysate (>10 kDa)

C= Trypsin hydrolyzed PKC protein hydrolysate (<10 kDa)

D= Pepsin hydrolyzed PKC protein hydrolysate (without ultrafiltration)

E= Pepsin hydrolyzed PKC protein hydrolysate (>10 kDa)

F= Pepsin hydrolyzed PKC protein hydrolysate (<10 kDa)

ND=Not detected

## CONCLUSION

PKC protein hydrolysates have a significant inhibitory effect on *Bacillus cereus* probably due to the existence of carbohydrate component and high content of arginine. Further studies are needed for confirmation.

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