British Journal of Pharmacology and Toxicology 1(1): 50-55, 2010
ISSN: 2044-2467

Submitted Date: May 20, 2010 Accepted Date: June 01, 2010 Published Date: June 20, 2010

Production, Isolation and Characterization of Exotoxin Produced by *Bacillus cereus* NCIM-2156 and *Bacillus licheniformis* NCIM-5343

Snehal G. Kashid and Jai S. Ghosh
Department of Microbiology, Shivaji University, Kolhapur 416004. MS. India

Abstract: The primary objective of this investigation was to bring in lime light the importance of the exotoxin produced by *B. cereus* and *B. licheniformis*, which otherwise are overshadowed by the 2 other exotoxins of bacterila origin viz. staphylococcal and clostridial toxins. The investigation has been with 2 known and identified strains viz. *B. cereus* NCIM-2156 and *B. licheniformis* NCIM-5339 were found to be potent exotoxin producers. The organisms showed all the 3 activities like proteolytic, phospholipolytic and hemolytic. The organisms were grown on simple substrates like skimmed milk powder and egg yolk emulsion. The organisms also showed strong fibrinolytic activity on blood clots. Both the organisms are known saprophytes in an environment like soil. Therefore, foods which are not handled with proper hygienic and sanitation care have all the probability of getting contaminated with these types of organisms and will contain the potent exotoxins.

Key words: Exotoxin, fibrinolysis, hemolytic, proteolytic, phospholipolytic

INTRODUCTION

The term "toxin" means the toxic material or product of plants, animals, microorganisms (bacteria, viruses, fungi, rickettsiae or protozoa), or infectious substances, or a recombinant or synthesized molecule, whatever their origin and method of production (Kenneth, 2008). It simply means it is a biologically produced poison." Toxins are poisonous products of organisms; unlike biological agents, they are inanimate and not capable of reproducing themselves. Toxins produced by microorganisms are important virulence determinants responsible for the pathogenicity of the pathogen and/or invasion of the host immune response (Kenneth, 2008).

Bacterial toxins: Bacterial toxins are by-products produced by pathogenic microbes that have taken up residence in the body. Bacterium can enter a host by various means, such as consuming contaminated food or water. Bacteria can also be introduced through mucous membranes, either by direct contact with the source or as a consequence of breathing in air-borne bacteria. The type of bacterial toxins released depends on the species of invading bacteria. Toxigenesis, or the ability to produce toxins, is an underlying mechanism by which many bacterial pathogens produce disease (Kenneth, 2008). There are two main types of bacterial toxins, lipopolysaccharides which are cell associated and are often referred as endotoxins, and the other type is the exotoxins, which may be proteins, lipoproteins etc (Kenneth, 2008).

***Bacillus cereus*:** *Bacillus cereus* produces both the types of toxins (Cecilie *et al*., 2005). The majority of *B. cereus* strains appear to be capable of producing either diarrhoeal or emetic toxin (Beattie and Williams, 1999; Rusul and Yaacob, 1995). There are several factors that affect the growth of this organism (ICMSF, 1996; Johnson, 1984; Lake *et al.*, 2000; Murray *et al*., 1996; EFSA, 2004; Jenson and Moir, 2003).

B. licheniformis (Kramer and Gilbert ,1989) associated food poisoning resembles that of *C. perfringens*, with an incubation period of 6-24 h and symptoms including diarrhea and abdominal pain. The optimal temperature for enzyme secretion is 37ºC. It can exist in spore form to resist harsh environs or in a vegetative state when conditions are good.

Bacillus licheniformis produces a lipopeptide called lichenysin. Lichenysin is a cyclic lipopeptide and belongs to the most effective biosurfactant discovered so far (Yakimov *et al.*, 1995) which contained the following amino acids (Glu, Asp, Val, Thre, Leu) connected by a lactone linkage (Jenny *et al*., 1991).

It has been observed that most the studies involving these 2 important toxins, have been to study the clinical manifestations and to some extent to determining the chemical nature. In this study, it has been attempted to characterize the biochemical properties of these toxins *in vitro*.

MATERIALS AND METHODS

This research was carried out from June 2009 to March 2010.

Corresponding Author: Dr. Jai S. Ghosh, Assistant professor, Department of Microbiology, Shivaji University, Kolhapur 416004. MS. India, Ph. +91 9850515620

Table 1: Composition of nutrient agar medium

Sr.no.	Components	Percentage (%)
1	Peptone	1.0
2	Yeast extract	1.0
3	NaCl	0.5
4	Agar	2.5

Table 2: Composition of Mineral base medium

Sr.no.	Composition	Percentage (%)
1	Sodium nitrate	0.20
2	Dipotassium hydrogen phosphate	0.10
3	KCl	0.05
4	Glucose	1.00
5	Yeast extract	0.02
6	Agar	2.50

Microorganism and growth medium: The organisms used in this study were *Bacillus cereus* NCIM 2156 and *Bacillus licheniformis* NCIM 5343. The organisms could be easily cultivated on nutrient agar and mineral based medium. The compositions of these 2 media are as in Table 1 and 2:

The organisms were maintained on nutrient agar medium but all other studies were carried out in the mineral base medium. Therefore, it was decided to see the growth pattern in the mineral base medium. This was done by growing the organisms in liquid medium and recording the absorbance values at 530 nm.

Determination of caseinase and lecithinase activity of *Bacillus cereus* and *Bacillus licheniformis* exotoxin:
Toxin production: The 24 h old culture of *Bacillus cereus* and *Bacillus licheniformis* grown on nutrient agar were separately inoculated in sterile 100 ml mineral base liquid medium which were incubated at 30ºC on a rotary shaker with a speed of 150 r.p.m. for 6, 12, 18 and 24 h.

Step 2; Acetone precipitation: After each incubation time interval the medium was centrifuged at 3220xg for 10 min and cell free medium containing the crude exotoxin was precipitated by using cold acetone for 18 h. Equal amount of acetone as that of the broth was used for the precipitation. After precipitation the mixture was centrifuged at 6400xg for 30 min at 4ºC. The residue was dissolved in 5 ml of 25 mM phosphate buffer at pH 7.0.

The was concentrated against crystals of sucrose and kept in the refrigerator at 5ºC. Such a concentrate was then used for study of caseinase (protease) and phospholipolytic (i.e. Lecithinase) activity.

Caseinase (Proteolytic) and phospholipolytic (Lecithinase) activity: In each plate of milk agar (the composition of which is as shown in Table 3. Three cups of 3 mm diameter were prepared. In each of these cups the 20 µl of the above precipitate was added and they were incubated for 24 and 48 h at 37ºC respectively. Zone of hydrolysis of casein on milk agar plate was measured. This was repeated 3 times to get a standard deviation less than 10.

Table 3: Composition of milk agar

Components	Quantity
Skimmed milk powder	1%
Agar	3%
pH	7

Table 4: Composition egg yolk powder agar

Components	Quantity
Egg powder	0.5%
NaCl	0.5%
Agar	3%
pH	7

To check the lecithinase activity the procedure was the same except that in place of milk agar, egg yolk powder agar (the composition of which is as in Table 4) was used. The egg yolk powder was commercial grade used for making similar medium. The zones were measured by the soap test using $CuSO_4$ solution.

Determination of Hemolytic activity of exotoxion of *Bacillus licheniformis* and *Bacillus cereus* by Cyanmethemoglobin method: This method was used for the calculation of hemolytic activity (i.e. Hb content) of exotoxin of *Bacillus cereus* and *Bacillus licheniformis* and was according to the recommendations of the International Committee For Standardization in Hematology (ICSH).

Procedure for testing Hemolytic activity of exotoxin of *Bacillus licheniformis* and *Bacillus cereus*:
Separation of blood cells and plasma: Blood with anticoagulant (Heparin) was diluted with sterile saline (to avoid hemolysis and to adjust the cell density) in 1:10 proportion and 0.2 ml amount of this diluted blood was centrifuged at 2200xg for 10 min at 4ºC. The sediment was washed with sterile saline (prevent hemolysis) and the final sediment was used to check hemolytic activity.

The acetone precipitate suspended in 25 mM phosphate buffer (200 µl) was added with 10mg of washed blood cells. The mixture was incubated in water bath for 15 min at 37ºC and centrifuged at 8900xg for 5 min. The Heme contents in supernatant were checked as per the method of Dacie and Lewis (1968).

Determination of protein content of *Bacillus cereus* and Bacillus licheniformis exotoxin: Protein content in exotoxin produced by *Bacillus cereus* and *Bacillus licheniformis* was estimated by Lowry method (Plummer, 1971).

Electrophoresis: The purity of exotoxin from *Bacillus cereus*, was checked by SDS-PAGE, by the method of Laemmli *et al.* (1970). The bands were visualised by silver staining technique. The molecular mass of exotoxin of *Bacillus cereus* was determined on a calibrated scale with standard marker enzyme (Phosphorylase b 98 kDa,

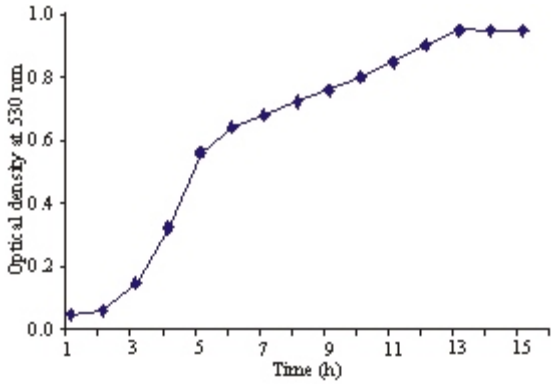

Fig. 1: Growth curve of *Bacillus licheniformis*

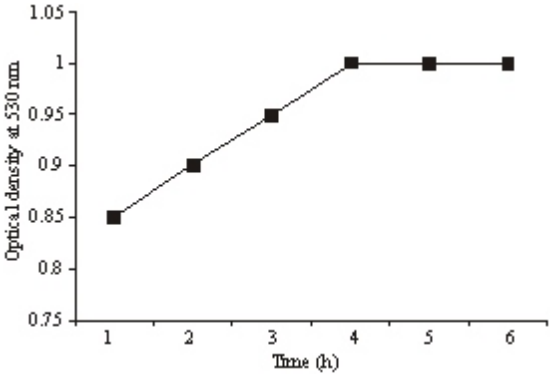

Fig. 2: Growth curve of *Bacillus cereus*

Bovine Serum Albumin 66 kDa, Oval albumin 43 kDa, Carbonic Anhydrase 29 kDa, Soya bean Trypsin Inhibitor 20 kDa).

Determination of caseinase and lecithinase (phospholipase) activity of exotoxin produced by mutants of *Bacillus licheniformis*:

Procedure for obtaining mutants of the strain of *Bacillus licheniformis*: The organism was grown on solid nutrient agar and the growth was exposed to U.V. rays having λ 256nm for 90 mins. This cured strain was checked for the caseinase, lecithinase and hemolytic properties of acetone-precipitated exotoxin. The method was as described above.

RESULTS AND DISCUSSION

The organism had a lag period of 2 h and followed by a exponential phase of 11 h. This implies that food contaminated with this organism would contain the toxin within a short period of 2 h (Fig. 1).

If one observes the growth pattern of this organism (Fig. 2) then one could see that an exposure of 1 h is sufficient to make the food unsafe for consumption.

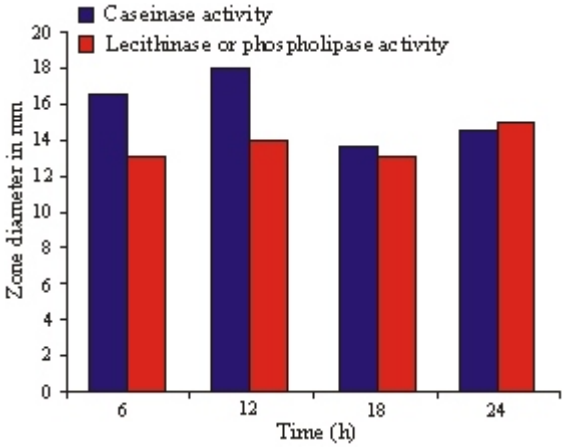

Fig. 3: Caseinase (proteolytic) and Lecithinase (phospholipase) activity of purified (dialysed) exotoxin *Bacillus cereus* after 24 h

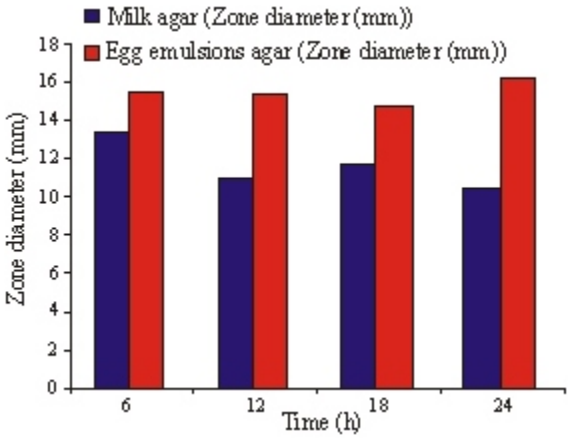

Fig. 4: Caseinase and phospholipolytic activity of purified (dialysed) toxin of *Bacillus cereus* after 48 h

Figure 3 shown that initially there is an increase in caseinase activity, which decreases slightly and then this is followed by a steady level of lecithinase activity (since the fluctuation in lecithinase activity is very small).

However, during the next 24 h (Fig. 4) it can be noted that there is again a slight dip in the lecithinase activity (starting from the 30th h), but a prominent increase in the caseinase activity.

It is very evident from Fig. 5 that the lecithinase activity is very prominent as compared to the caseinase activity. However in both the cases the fluctuation as a function of time is not so prominent. This is in contrast to that of *B. cereus*.

Figure 6 shown that by 30th h the lecithinase activity starts dipping making the caseinase activity prominent.

It can be observed that initially the lecithinase activity increases till the 18th h and then dips slightly,

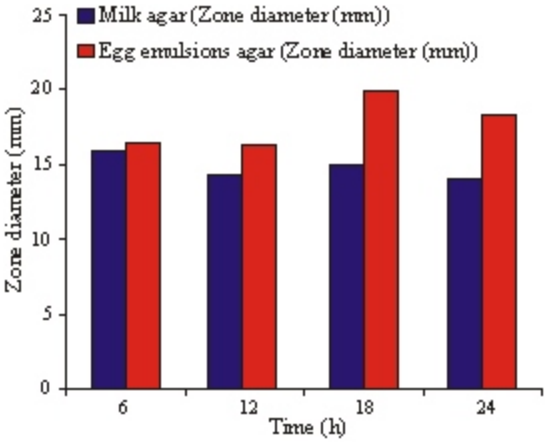

Fig. 5: Caseinase and phospholipolytic activity of purified (dialysed) toxin of *Bacillus licheniformis* after 24 h

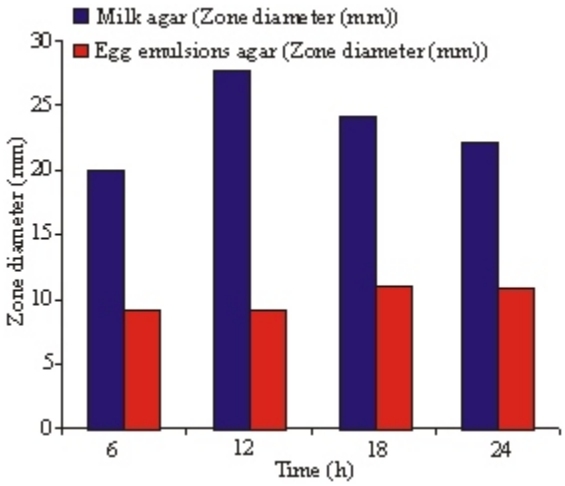

Fig. 6: Caseinase and phospholipolytic activity of purified (dialysed) toxin of Bacillus licheniformis after 48 h

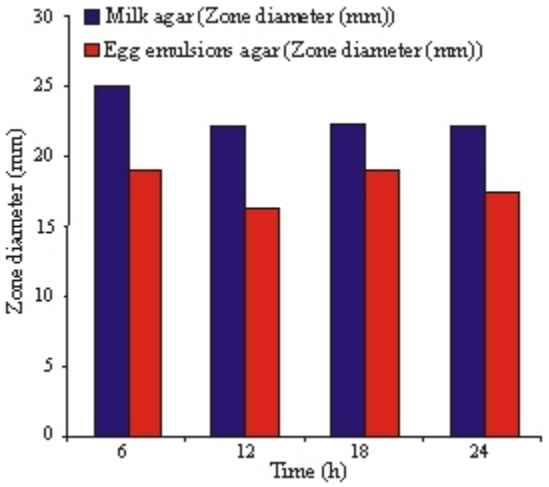

Fig. 7: Caseinase and phospholipolytic activity of purified (dialysed) toxin of UV exposed *Bacillus licheniformis* after 24 h

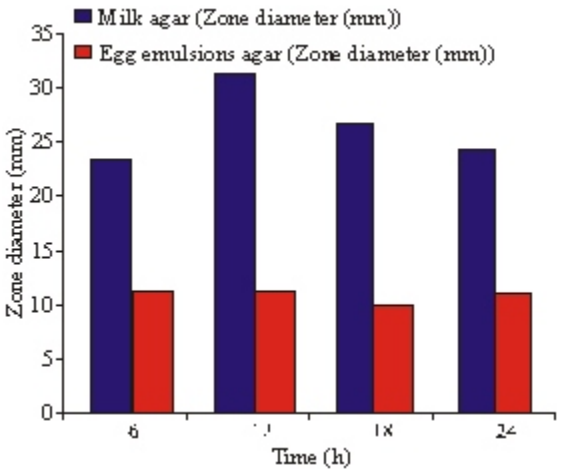

Fig. 8: Caseinase and phospholipolytic activity of purified (dialysed) toxin of UV exposed *Bacillus licheniformis* after 48 h

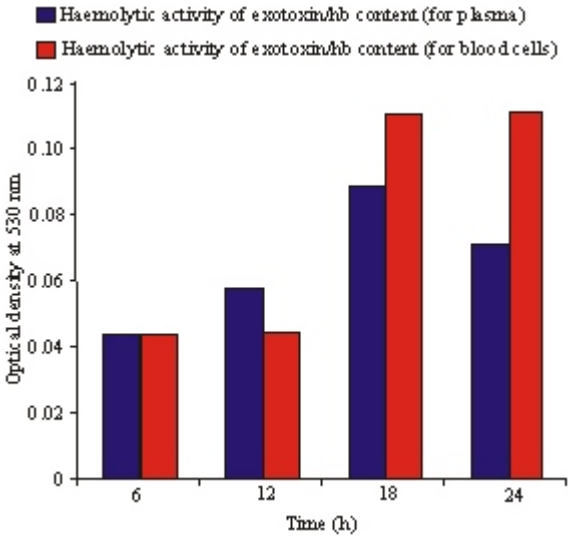

Fig. 9: Haemolytic activity of exotoxin of *Bacillus cereus* (Hb content)

whereas the caseinase activity do not show significant variation (Fig. 7).

In the next 24 h lecithinase activity goes down rapidly and by 36th h the caseinase activity increases sharply, which is very vivid from Fig. 8.

In case of this organism the hemolysis is very prominent (Fig. 9) by the 18th h and it remains steady for 24th h. When the cells are very few as in plasma there is a significant drop in the hemolytic activity of the toxin.

By the 30th h the activity has started reducing (Fig. 10) and by 42nd h again there is a sharp rise. Protein content of exotoxin of *Bacillus cereus* after 24 h: -80 µg/ml. Protein content of exotoxin of *Bacillus licheniformis* after 24 h: -50 µg/ml.

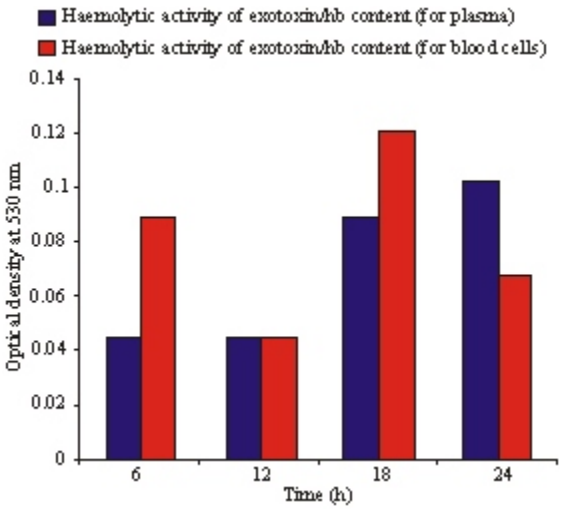

Fig.10: Haemolytic activity of exotoxin of *Bacillus licheniformis* (Hb content)

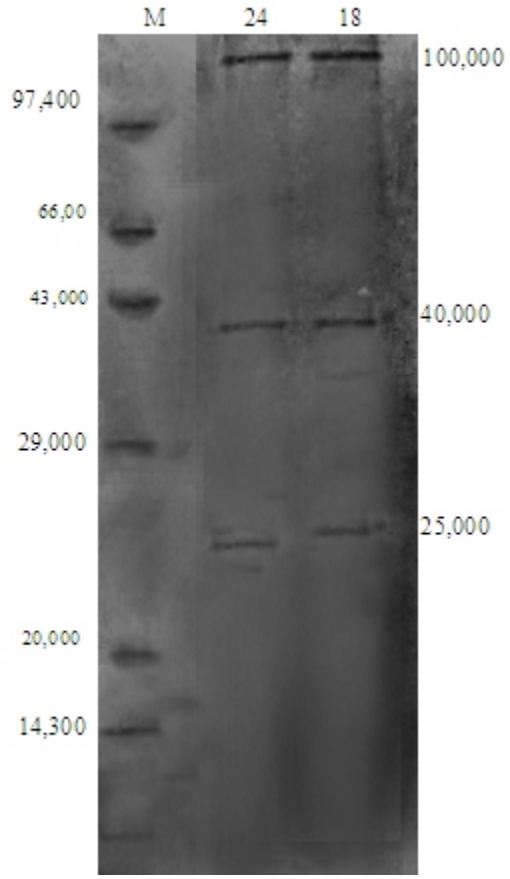

Fig. 11: Electrophoresis of exotoxin of *Bacillus cereus*

Electrophoresis: The results of SDS electrophoresis as shown in Fig. 11. In this there are 3 bands having molecular weight approximately 25, 40 and 100 kDa. These are the bands of the Hemolysin toxin, which consists of three components.

CONCLUSION

The exotoxin produced by *Bacillus cereus* and *Bacillus licheniformis* show caseinase (proteolytic), Lecithinase (phospholipase) activities. These also show hemolytic activity. The organisms were strongly proteolytic in nature. The significant lecithinase activity of *Bacillus licheniformis* goes to prove one fact as to why these organisms some time show a neutropenic leukemiea. Such type of syndromes can be attributed to the fact of high lecithinase activity of the exotoxin of this organism.

When the strain of *B. licheniformis* was cured by UV exposure 90 min, it is evident that the phospholytic activity decreased from 24-48 h of incubation. It showed stronger proteolytic activity. The toxin from *B. cereus* showed 3 components having molecular weights 25,40 and 100 kDa. This is a potent food poisoning organism. However, the exotoxin is highly thermolabile and rarely shows lethal effect.

ACKNOWLEDGMENT

The authors are very grateful to the Department of Microbiology, Shivaji University for extending all the facilities for completion of this work.

REFERENCES

Beattie, S.H. and A.G. Williams, 1999. Detection of Toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. J. Appl. Microbiol., 82: 677-682.

Cecilie, F., P. Rudiger, S. Peter, H. Vıctor and G. Per Einar, 2005. Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. Appl. Environ. Microbiol., 71: 1178-1183.

Dacie, J.V. and S.M. Lewis, 1968. Practical Hematology. 4th Edn., J and A, Churchill, UK, pp: 37.

EFSA (European Food Safety Authority - Scientific Panel on Biological Hazards), 2004. Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. EFSA J., 175: 1-48.

International Commission on Microbiological Specification for Foods (ICMSF), 1996. Microorganisms in Foods. In: Roberts, T. A., A.C. Baird-Parker, and R.B Tompkin, (Eds.), Vol: 5, Characteristics of Microbial Pathogens. Published by Blackie Academic and Professional, London.

Jenny, K., O. Kappeli and A. Fiechter, 1991. Biosurfactants from *Bacillus licheniformis*: Structural analysis and characterization. Appl. Microbial. Biotechnol., 36: 5-13.

Jenson, I. and C.J. Moir, 2003. *Bacillus cereus* and other *Bacillus* Species. In: A.D. Hocking, G. Arnold, I. Jenson, K. Newton and P. Sutherland, (Eds.), Foodborne Microorganisms of Public Health Importance. 6th Edn., Australian Institute of Food Science and Technology (NSW Branch), Sydney, Australia, pp: 379-406.

Johnson, K.M., 1984. *Bacillus cereus* foodborne illness-An update. J. Food Protect., 47(9): 145-153.

Kenneth, T., 2008. Online Text Book of Bacteriology, University of Wisconsin, Madison, US.

Kramer, J.M. and R.J. Gilbert, 1989. *Bacillus cereus* and other *Bacillus* Species In: Doyle, M.P., (Ed.), Food Borne Bacterial Pathogens. Marcel Dekker, pp: 21-70.

Lake, R.J., M.G. Baker, N. Garrett, W.G. Scott and H.M. Scott, 2000. Estimated number of cases of foodborne infectious disease in New Zealand. NewZeal. Med. J., 113: 278-281.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Murray, D.L., C.A. Earhart, D.T. Mitchell, D.H. Ohlendorf, R.P. Novick and P.M. Schlievert, 1996. Localization of biologically important regions on toxic shock syndrome toxin-1. Infect. Immun., 64: 371-374.

Plummer, D.T., 1971. An Introduction to Practical Biochemistry. 3rd Edn., Tata McGraw Hill Publication, Bombay,

Rusul, G. and N.H. Yaacob, 1995. Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA. Int. J. Food Microbiol., 25: 131-135.

Yakimov, M.M., K.N. Timmis, V. Wray and H.L. Fredrickson, 1995 Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS 50. Appl. Environ. Microbiol., 61: 1706-1713.