Research Article

Production, Isolation and Characterization of Exotoxin Produced by *Bacillus subtilis*, *Bacillus megaterium* and *Proteus vulgaris* and its Significance in Food Poisoning

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ABSTRACT

Bacillus species are ubiquitous bacteria commonly present in soil, water, milk and milk product and *Proteus valgaris* is normal flora of humans. These organisms produce variety of exotoxins, proteases and other extracellular enzymes which are not only responsible for food spoilage and food poisoning but also act as virulence factors. Exotoxins were isolated from known Gram positive strains of *Bacillus subtilis* NCIM 2010, *Bacillus megaterium* NCIM 2087 and Gram negative *Proteus vulgaris* NCIM 2027. All these organisms could be cultivated on simple substrate like skimmed milk powder and egg yolk emulsion, *Bacillus subtilis, Bacillus megaterium* produced heat stable exotoxin at 37°C and pH 6, 7 of growth respectively which was having strong proteolytic and haemolytic activity. *Proteus vulgaris* exotoxin showed proteolytic, lipolytic and haemolytic activities at 37°C and pH 6. *Bacillus subtilis* showed beta haemolysis while *Bacillus megaterium* showed alpha haemolysis on blood agar. *Bacillus spp.* are known saprophytes, therefore, food contaminated with these organism not only cause food spoilage, but also food poisoning due to exotoxins. It is therefore, essential to maintain proper hygienic condition during food handling and processing.

Keywords: exotoxin, caeseinase, lecithinase, haemolysis, skimmed milk, egg yolk.

INTRODUCTION

A toxin [Latin *toxicum,* poison] is a substance, such as a metabolic product of the organism that alters the normal metabolism of host cells with deleterious effects on the host.[1] Toxins are biological infectious agent; they are inanimate and not reproducing themselves. These substances produced by microorganism, Fungus, rikettsiae, or protozoa. And contributing in pathogenicity And/or invasion of host immune response, also called as virulence.[2]

Toxigenesis is the ability to produce toxins, through which many bacterial pathogens produce disease, there are two main types of bacterial toxins, lipopolysaccharides, which are associated with the cell wall of Gram-negative bacteria (endotoxin)[2] and the extracellular diffusible toxins are referred to as exotoxins.

However, in some cases, exotoxins are only released by lysis of the bacterial cell. Exotoxins are usually proteins, minimally polypeptides that act enzymatically or through direct action with host cells and stimulate a variety of host responses. However, some bacterial exotoxins act at the site of pathogen colonization and may play a role in invasion.[2]

As proteins, many bacterial toxins resemble enzymes in a number of ways. Like enzymes, they are denatured by heat, acid and proteolytic enzymes, they act catalytically, and they exhibit specificity of action.

The substrate (in the host) may be a component of tissue cells, organs or body fluid.[2]

Members of the *B. subtilis* group were reported to produce substances toxic to mammalian cells such as lichenysin A, from *B. licheniformis* connected to a fatal case.[3]

B. subtilis is not a human pathogen; it may contaminate food but rarely causes food poisoning.

B. subtilis produces the proteolytic enzyme subtilisin. *B. subtilis* spores can survive the extreme heat during cooking. And responsible for spoilage of milk or dairy product, quantitative assessment showed strain of *Bacillus subtilis* and also responsible for causing ropiness, sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough.[4]

Bacillus subtilis does produce an extracellular heat stable toxin like amylopsin which is toxic to sperms and effect on sperm motility,[3] subtilisin, although subtilisin has very low toxigenic properties,[5] this proteinaceous compound is capable of causing allergic reactions in individuals who are repeatedly exposed to it.[6] The subtilin protein has the bactericidal effect on many Grams positive and certain Gram negative bacteria.[7] This study is taken with the objective of isolation and characterization of exotoxins from *Bacillus subtilis* with two other and to assess the proteolytic, lipolytic and haemolytic activity of it.[8]

Allergic sensitivity produced by the inhalation of high concentrations of enzyme preparations from *Bacillus subtilis* was found in three workers with mainly bronchial disease. They gave strong immediate reactions to prick tests and immediate, followed by late, mainly asthmatic reactions to inhalation tests. Precipitins were found in the sera of the affected subjects, but even more often in controls.[9]

101 *Bacillus* strains representing 7 *Bacillus* species were tested for production of heat-stable toxins. Strains of *B.*

firmus and *B. simplex* were found to produce novel heat-stable toxins, which showed varying levels of toxicity. *B. cereus* strains (18 out of 54) were positive for toxin production. Thirteen were of serovar H1, and it was of interest that some were of clinical origin. Two were of serovars 17B and 20, which are not usually implicated in the emetic syndrome. Strains of *Bacillus cereus* can produce a heat-stable toxin.[10] Partial purification of the novel *B. megaterium, B. simplex* and *B. firmus* toxins showed they had similar physical characteristics to the *B. cereus* emetic toxin, cereulide.[11]

Proteus vulgaris is a Gram negative short rod shape bacteria, non spore forming, and non capsulated, motile, Some Proteus spp. Is normal flora of vaginal and human Intestinal tract, commonly known as opportunistic pathogen and, following *Escherichia coli*, is the Leading cause of Gram-negative bacteria urinary tract infections[12]. *Proteus mirabilis* accounts for 97% of Proteus urinary tract infections.[13] Under certain circumstances, Proteus spp. can also cause other infections, such as bacteremia, wound infection, and pneumonia.[14]

MATERIALS AND METHODS

Microorganism used and Growth medium

In this works the study was carried out on the toxins produced by *Bacillus subtilis* NCIM 2010 and *Bacillus megaterium* NCIM 2087and *Proteus vulgaris* NCIM 2027. The growth curve of *Bacillus subtilis* and *Bacillus megaterium* NCIM-5343, *and Proteus vulgaris* were carried out at 30°C in cZapek dox medium.

Determination of caseinase and lecithinase activity of *Bacillus subtilis, Bacillus megaterium* and *Proteus vulgaris*

Toxin production

The 24 hrs old culture of *Bacillus subtilis, Bacillus megaterium and Proteus vulgaris* grown on nutrient agar was inoculated in 5ml sterile saline separately. The respective suspension of each organism was separately added in sterile 100ml Nutrient *Broth*. Thus 4 flasks of nutrient broth containing the inocula were incubated at time intervals of 6, 12, 18 & 24 hours incubated on rotary shaker at 37°C with 120 R.P.M speed.

Acetone precipitation

After each time interval the whole broth was taken and cell free filtrate containing the crude exotoxin was precipitated by using cold acetone. Equal amount of acetone as that of the broth was used for the precipitation. The mixture was allowed to incubate at freezing temperature for about 18-24 hours. After incubation the mixture was re-centrifuged at 8000 rpm for 30 min at 4°C. The resulted pellet was dissolved in 5 ml of phosphate buffer at 25 mM (pH 7).

The obtained acetone precipitate (in solution) was added into dialysis bag for dialysis against 25mM phosphate buffer at pH 7. The obtained exotoxin preparation was concentrated against crystals of sucrose and kept in the refrigerator at 5°C overnight for further purification. This was then used for study of caseinase (protease) and lipolytic (i.e. Lecithinase) activity.

Procedure for caseinase (proteolytic) and lecithinase activity

In each plate of milk agar and egg yolk agar 3 wells of 5 mm diameter were prepared for each sample of specific time intervals. In each 3 wells of milk agar plate & egg emulsion agar each sample of specific time interval was added in 30 µl amount and they were incubated for 24 hours at 37°C. After 24 hours of incubation zone of hydrolysis of casein on milk agar plate was measured. For each sample 3 readings were taken and their mean was calculated. For Lecithinase (phospholipolytic) activity zone cannot be measured directly. So, soap test was carried out by using saturated solution of $CuSO_4$.

Determination of hemolytic activity of Bacillus subtilis, Bacillus megaterium and Proteus vulgaris by Cyamethemoglobin method

This method was used for the calculation of hemolytic activity (i.e. Hb content) of exotoxin of *Bacillus* subtilis, *Bacillus megaterium & Proteus vulgaris by* Cyanmethemoglobin method & standard for the determination of blood Hemoglobin was according to the recommendations of the international committee for standardization in Hematology (ICSH).

Separation of blood cells and plasma

Blood with anticoagulant (Heparin 500mg for 200-250ml blood) was diluted with sterile saline (to avoid haemolysis and to adjust the cell density) in 1:10 proportion.

(1ml blood + 9ml saline) 0.2ml amount of this diluted blood was centrifuged at 5000 rpm for 10-15 minutes in cold centrifuge.

The pellet and supernatant were referred as pellet no.1 & supernatant no .1 (plasma) was kept separately.

The pellet was again suspended in saline (no hemolysis should be there) and centrifuged at 5000 rpm for 10-15 minutes.

Supernatant was discarded & the pellet was taken for hemolytic activity. This was referred as pellet no.2 (10mg).

3 tubes each containing 5ml broth of each organism at specific time interval was taken & these tubes were centrifuged at 5000rpm for 10minutes.

Cell free broth containing exotoxin was taken & pellet was discarded. Supernatant was added with 10mg of pellet no.2 i.e. blood cells.

The mixture was incubated in water bath for 15 minutes at 37°C and centrifuged at 10,000rpm for 5-10 minutes.

The Heme contents in supernatant were checked as per the Cyamethemoglobin method of Dacie and Lewi.

Effect of pH and temperature on exotoxin production of *Bacillus subtilis*, *Bacillus megaterium* and *Proteus vulgaris*

Optimum pH and temperature for exotoxin production (caseinase) were determine by performing the standard assay at different temperature ranging from 10°C to 50°C and at different pH in range of 5 to 9.

After incubation, exotoxin precipitated by acetone and dialysis against phosphate buffer, caseinase and lecithinase of purified exotoxin activity was checked as same as above.

Determination of protein content of *Bacillus subtilis*, *Bacillus megaterium* and *Proteus vulgaris* exotoxin

Protein content in exotoxin produced by *Bacillus subtilis, Bacillus, megaterium* and *Proteus vulgaris* was determined by Lowry method.[17]

Electrophoresis

The purity of exotoxin was checked by SDS-PAGE, by using 12.5 % polyacrylamide gel.[19] The bands were visualized by silver staining technique. The molecular mass of exotoxin of *Bacillus subtilis, Bacillus megaterium* and *Proteus vulgaris* was determined on a calibrated scale with standard marker enzyme (Phosphorylase b 98 kDa, Bovine Serum Albumin 66 kDa, Oval albumin 43 kDa, Carbonic Anhydrase 29 kDa, Soya bean Trypsin Inhibitor 20 kDa).

RESULTS AND DISCUSSION

Species of Bacillus can grow at high temperature and capable to produce Exotoxin at that temperature therefore foods cooked at high temperature not guarantee to be safe for consumption.

Organism *Bacillus subtilis* and *Bacillus megaterium* had 2 h Lag phase followed by 11 h exponential phase shows that exotoxins produced within short time 2h. While *Proteus vulgaris* shows very short lag of 1.5 h followed long exponential phase.

Exotoxin produced at 12h incubation by *Bacillus subtilis* and *Bacillus megaterium* shows strong Caseinase activity while lecithinase activities at lesser extent indicate that production of toxins of bacillus spp. mainly start in exponential phase, and continued in stationary phase also Figure No.1 and 2. Exotoxin produced at 6h incubation by *Proteus valgaris* shows strong caseinase and lecithinase activities indicate that they produce Exotoxins at exponential phase of growth Figure No.3. Exotoxin produced by *Bacillus subtilis* after 24h of incubation (stationary phase) shows significant Haemolytic activity Figure No.4, but *Bacillus megaterium does not* shows *significant* haemolytic activity Figure No.5, while Exotoxin produced by *Proteus valgaris* in exponential phase shows strong haemolytic activity Figure No.6, all organism produced exotoxin in specific growth condition of pH and Temperature, *Bacillus subtilis produced exotoxin at 37°C and pH 6 shows activity at high temperature also, Proteus valgaris* produced exotoxin at 37°C and pH 6 while *Bacillus megaterium* at 37°C and pH 7 Figure No.7 and 8.

Caseinase and lecithinase activity of purified (dialysed) exotoxin of *Bacillus subtilis*

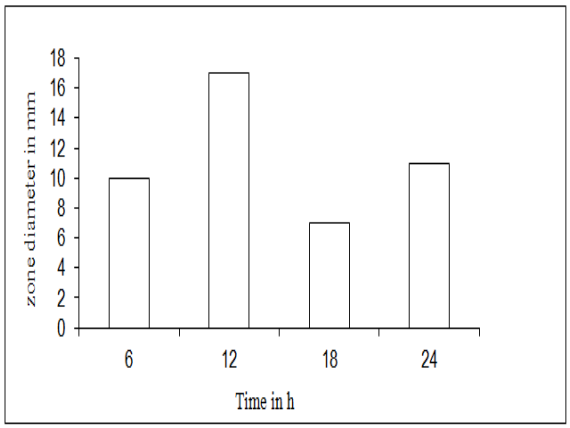

Figure 1: Caseinase and lecithinase activity of purified (dialysed) exotoxin of *Bacillus subtilis*. 24 hours. And maximum activity observed in exponential phase.

Caseinase and lecithinase activity of purified (Dialysed) exotoxin of *Bacillus megaterium*

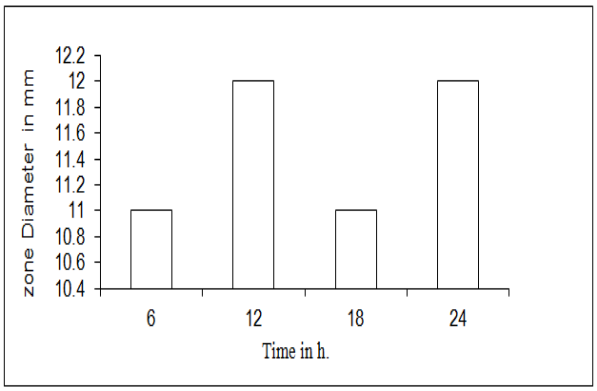

Figure 2: Caseinase and lecithinase activity of purified (Dialysed) exotoxin of *Bacillus megaterium* 24 hours. And maximum activity observed at 12 h & 24 h of incubation. This shows 12 mm zone of inhibition.

Caseinase and lecithinase activity of purified (Dialysed) exotoxin of *Proteus vulgaris*

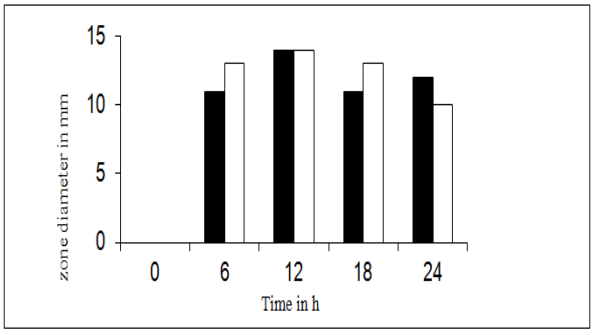

Figure 3: Caseinase and lecithinase activity of purified (Dialysed) exotoxin of *Proteus vulgaris* 24 hours. And maximum activity observed at 12 h, of incubation. This shows 14 mm zone of inhibition.

Haemolytic activity of exotoxin of *Bacillus subtilis*

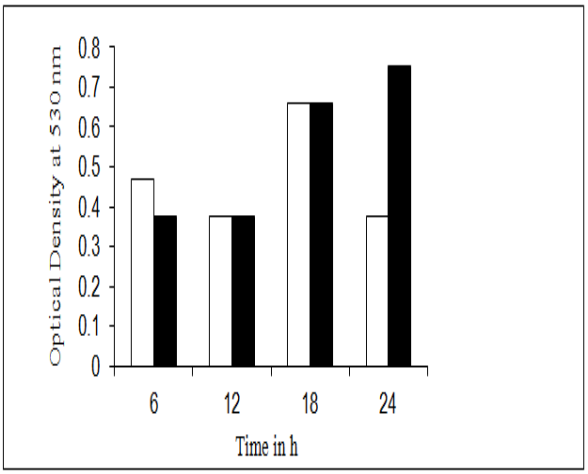

Figure 4: Haemolytic activity of exotoxin of *Bacillus subtilis* (Hb content) by DRABKIN'S method. Maximum hemolytic activity observed at 24 h incubation.

Haemolytic activity of exotoxin of *Bacillus megaterium*

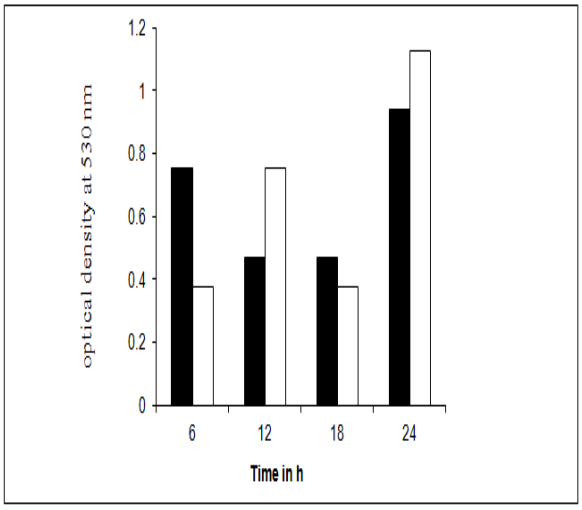

Figure 5: Haemolytic activity of exotoxin of *Bacillus megaterium* (Hb content) by DRABKIN'S method. Maximum hemolytic activity observed at 24 h incubation.

Haemolytic activity of exotoxin of *Proteus valgaris*

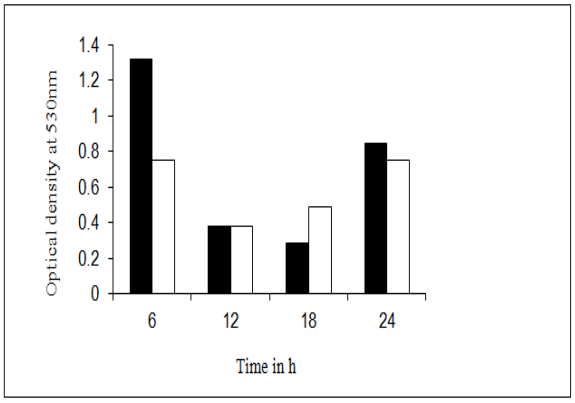

Figure 6: Haemolytic activity of exotoxin of *Proteus vulgaris* (Hb content) by DRABKIN'S method. Maximum hemolytic activity observed at 6 h incubation.

Effect of pH on exotoxin production.

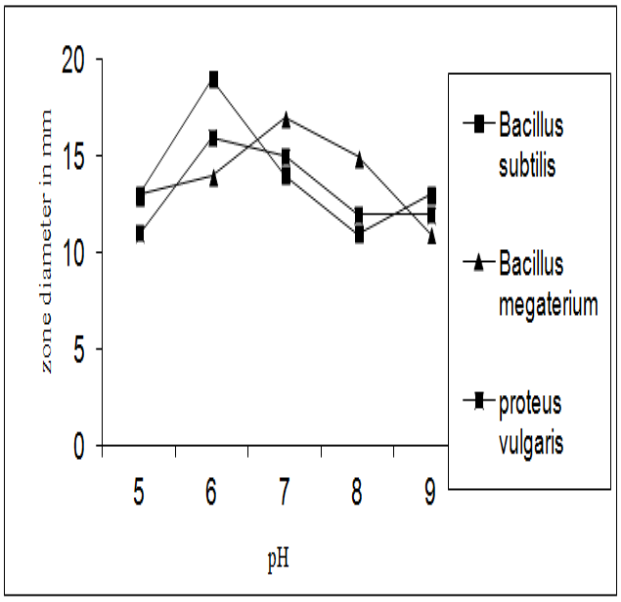

Figure 7: Effect of pH on exotoxin production of *Bacillus subtilis*, *Bacillus megaterium and Proteus vulgaris* which shows optimum pH for *Bacillus subtilis 6.8*, *Bacillus megaterium 7.0 and Proteus vulgaris*

Effect of Temperature on exotoxin production

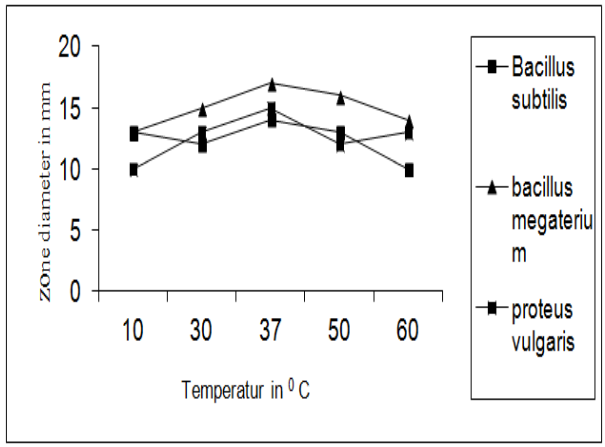

Figure 8: Effect of Temperature on exotoxin production of *Bacillus subtilis*, *Bacillus megaterium* and *Proteus vulgaris* Shows optimum temp.37°C for all three organisms

Protein estimation

Protein content of exotoxin of *Bacillus subtilis* after 24 hours 0.272 mg/ml, *Bacillus megaterium* after 24 hours 0.496 mg/ml, and exotoxin of *Proteus vulgaris* after 24 h 0.700 mg/ml.

Electrophoresis

The result of SDS electrophoresis as shown in fig. *B. subtilis* shows single band having approximate molecular weight 15.5 KDa, *B. megaterium* shows two bands having molecular weight 15 KDa, 28.5 KDa and *P. vulgaris* two bands having approximate molecular weight 60 KDa, 54 KDa. Figure 9.

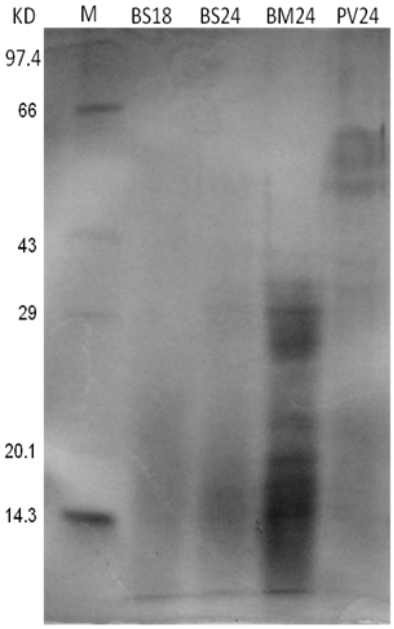

Figure 9: Electrophoresis

CONCLUSION

Exotoxin produced by *Bacillus subtilis* NCIM 2010, *Bacillus megaterium* NCIM 2087 and *Proteus vulgaris* NCIM 2027 show strong caseinase activity (proteolytic) while *Proteus vulgaris* shows lecithinase activity, all the three shows heamolytic activity.

Enzyme activity (protease) of exotoxin produced by *Bacillus subtilis* shows strong proteolytic activity while *Bacillus megaterium* and *Proteus vulgaris* shows proteolytic activity at lesser extent .The exotoxin production observed at optimum pH and temperature

Exotoxin produced by *Bacillus subtilis* shows single component having approximate molecular weight 15.5 KDa, and *Bacillus megaterium*, exotoxin shows two component having approximate molecular weight 15 KDa, 28.5 KDa and *Proteus vulgaris* exotoxin shows two component having approximate molecular weight 60KDa, 54 KDa however exotoxin are highly thermolabile.

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