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ORIGINAL ARTICLE

Bacillus subtillis RTSBA6 6.00, a new strain isolated from gut of *Helicoverpa armigera* (Lepidoptera: Noctuidae) produces chymotrypsin-like proteases

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KEYWORDS

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Protease inhibitor

Abstract Exploring bacterial communities with proteolytic activity from the gut of the *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) insect pests was the purpose of this study. As initial efforts to achieve this goal here we report the isolation of new *Bacillus subtillis* RTSBA6 6.00 strain from the gut of *H. armigera* and demonstrated as proteases producer. Zymographic analysis revealed 12 proteolytic bands with apparent molecular weights ranging from 20 to 185 kDa. Although some activity was detected at acidic pH, the major activity was observed at slight alkaline pH (7.8). The optimum temperature was found to be 35 °C with complete loss of activity at 70 °C. All proteases were completely inactivated by PMSF (phenylmethylsulfonyl fluoride) and TPCK (N-tosyl-L-phenylalanine chloromethyl ketone), suggesting that proteases secreted by *B. subtillis* RTSBA6 6.00 belong to serine proteases class with chymotrypsin-like activity. The occurrence of protease producing bacterial community in the gut of the *H. armigera* advocates its probable assistance to insect in proteinaceous food digestion and adaptation to protease inhibitors of host plants.

1. Introduction

Gut bacterial flora contributes to insect reproduction, nutrition, pheromone production, pesticide degradation, detoxification of plant allelochemicals and competitive exclusion (Lundgren and Lehman 2010; Tartar et al., 2009; Dillon and Dillon 2004; Reeson et al., 2003). Gut bacterial flora possesses metabolic properties that are absent in insects (Douglas, 1992). Gut bacterial flora increases the host insect survival by improving digestion efficiency and providing digestive enzymes or vitamins (Dillon and Dillon, 2004). In termites, gut bacterial flora secretes cellulases and lignases which provide glucose and fatty acid to their host as energy source (Breznak and Brune, 1994).

The digestion related role of gut bacterial flora in lepidopteron insect, which includes some of the most damaging

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agricultural and forest pests worldwide, is gaining attention from researchers because it is found that digestive enzymes of some lepidopteron insects might be derived from the gut bacterial flora (Broderick et al., 2004; Terra et al., 1996). In crickets and cockroaches the digestion of food by gut associated bacteria has been demonstrated (Santo Domingo et al., 1998; Gijzen et al., 1994). Several gut bacterial strain with serine protease activity from velvet bean caterpillars were found to contribute in protein-digestion and that could assist in adaptation to protease inhibitors is reported earlier (Visôtto et al., 2009).

Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) is a devastating insect pest of many important agricultural crop plants like cotton, chickpea, pigeon pea, tomato, sunflower, okra, corn, etc. (Parde et al., 2010; Patankar et al., 2001). Microbial diversity in the gut of *H. armigera* has been explored using traditional culturing and culture independent method. Predominance of *Bacillus* species in the fore gut and hind gut of *H. armigera* was revealed using traditional culturing method (Mishra and Tandon, 2003). However culture independent methods revealed the presence of more variable gut bacterial flora in the field population of *H. armigera* (Xiang et al., 2006). Recently bacterial diversity in the gut of *H. armigera* and its response to different host plant was reported (Gayatri et al., 2012).

Although presence of bacterial flora and diversity in the gut of *H. armigera* are revealed, the involvement of gut bacterial flora in the digestion of proteinaceous food is not clear. Here we isolated a new bacterial strain *Bacillus subtillis* RTSBA6 6.00 from the gut of fourth instar *H. armigera* larvae which demonstrated as proteases producer with chymotrypsin-like activities.

In this paper, we identified and characterized 12 protease isoforms by electrophoretic separation followed in-gel visualization on polyacrylamide gels. Quantitative estimations and studies of biochemical properties were performed using standard enzyme assays.

2. Material and methods

2.1. Sample collection

Fourth instar larvae of *H. armigera* were collected from chick pea field, Maliwada, Aurangabad (MS), India. The larvae were immobilized by chloroform and surface sterilized by 0.1% sodium hypochlorite and were dissected under aseptic conditions in laminar air flow to remove the gut.

2.2. Isolation, cultivation and identification of bacteria

The gut was homogenized in 5 ml of peptone broth and 1 ml was spread on blood base agar plate. The plate was incubated at 37 °C for 24 h. Pure bacterial culture was isolated by using repeated pour and streak plate methods. The bacterial isolates were identified by Fatty Acid Methyl Ester (FAME) based analysis (Buyer, 2003).

2.3. Detection of proteases by agar-plate method

Primary screening of bacteria in the gut homogenate was done by skimmed milk casein hydrolysis on the solid media containing 1% skimmed milk, 0.25% yeast extract, 0.5% casein, 0.1% dextrose and 3.8% agar. Cultures were inoculated on the media plate and incubated for 24 h at 37 °C (Sharmin et al., 2005).

2.4. Protease production from B. subtillis RTSBA6 6.00

The pure culture of *B. subtillis* RTSBA6 6.00 was cultured in skim milk broth. The skimmed milk broth was prepared by mixing all ingredients except skimmed milk together in Milli-Q water. The pH of broth was adjusted to alkaline condition before sterilization at 121 °C for 15 min. Skimmed solution was separately sterilized at 121 °C for 15 min. These solutions were mixed aseptically before use. The culture was incubated at room temperature on shaker for 48 h. The cells were harvested by centrifugation at 6000 rpm, 4 °C for 20 min. The supernatant was used as crude enzyme source for further studies. The protein concentration was estimated by Folin–phenol reagent (Lowry et al., 1951) using bovine serum albumin as standard.

2.5. Dot-blot assay

The supernatant isolated was screened for gelatinolytic activity by using gelatin coating on X-ray film as substrate (Pichare and Kachole, 1994). The principle of this technique is that the X-ray film has a layer of gelatin and as we put a drop of protease on the film, it hydrolyzes gelatin and forms clear transparent spot against dark background, hence proteases present in sample are detected. Twenty microliters of protease extract was spotted on X-ray film at 37 °C and the extent of gelatin hydrolysis was monitored visually.

2.6. Protease assay

Protease activity was assayed using the azo-casein (Sigma) as substrate. Mixture containing 250 µl 0.5% azo-casein (prepared in 0.1 M Tris–HCL pH 7.8), 250 µl buffer and 100 µl of crude enzyme extract was incubated for 30 min at 37 °C. Three hundred microliters of trichloroacetic acid (TCA, 5% w/v) was added to the mixture to stop the reaction and centrifuged at 10,000 rpm for 10 min at 4 °C. Equal volume of 1 N NaOH was added to the supernatant and the activity was measured at 440 nm. The blank used for this assay was prepared by incubating a mixture of the crude enzyme extract, and buffer for 30 min at 37 °C, followed by the addition of TCA and azo-casein.

2.7. Gelatin-zymography

For gelatin zymography, 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970). 0.1% gelatin (from porcine skin; Sigma) was incorporated in the gel. Ninety micrograms of crude protein was loaded on gel with standard molecular weight markers (Genei). After electrophoresis, gel was washed twice at room temperature in a solution of 2.5% Triton-X 100 for 30 min. After gentle washing with Milli-Q water the gel was incubated in 0.1 M Tris–HCL (pH 7.8) at 37 °C for over night. The gel was stained with 0.2% coomassie blue R-250 and the zones of proteolysis were detected.

2.8. Effect of pH on protease activity

The effect of different pH on the activity of proteases was examined by using standard azo-casein assay as described

previously. Proteolytic pattern of proteases at different pH was observed on 10% gelatin zymography. After electrophoresis, the gel was cut and the stripes of gel were washed twice at room temperature in a solution of 2.5% Triton-X 100 for 30 min. Each stripe of gel were incubated in 0.1 M phosphate buffer for pH 4.8 and 5.8, 0.1 M Tris–HCL pH 7.8 and 0.1 M Glycine–NaOH pH 9.8 and 0.1 M Tris–NaOH 11.8, respectively, at 37 °C for overnight and bands of gelatin degradation were visualized.

2.9. Effect of heating on enzyme activity

The influence of heating on the catalytic activity of proteases was determined by pre-incubation for 10 min at different temperatures ranging from 20 to 70 °C, followed by cooling and measuring the remaining activity. Proteolytic stability of proteases at different temperature was observed by loading pre-incubated sample on 10% gelatin zymography as described previously.

2.10. Protease inhibition study

To determine the class, to which the proteases belong, extract was incubated with 5 mM 1,10-phenanthroline (containing 10 mM EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM *N*-tosyl-lysine chloromethyl ketone (TLCK) and 100 μM *N*-tosyl-*l*-phenylalanine chloromethyl ketone (TPCK) for 1 h at 37 °C. These samples with control (without inhibitor) were loaded on 10% SDS–polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gel was washed twice at room temperature in a solution of 2.5% Triton-X 100 for 30 min. The gel was incubated in 0.1 M Tris–HCL (pH 7.8) at 37 °C for overnight and stained.

3. Results and discussions

3.1. Cultivation and identification of B. subtillis RTSBA6 6.00

In the present study, the bacteria isolated from gut of fourth instar larvae of *H. armigera* were identified as Gram-positive, spore forming *B. subtillis* RTSBA6 6.00 by using FAME based analysis (Buyer, 2003). The *Bacillus* isolate was then screened for protease activity using skimmed milk casein containing agar. The proteolytic activity was detected by the presence of clear zone of hydrolysis of skimmed milk casein on agar plate (Fig. 1). This finding is analogous with Visôtto et al. (2009) where he found several bacterial strains including *B. subtillis* with proteolytic activity from the caterpillar gut on calcium caseinate agar. The pure culture of *B. subtillis* RTSBA6 6.00 was cultured in skim milk broth for protease production with optimum parameters as mentioned earlier (Qureshi et al., 2011; Pastor et al., 2001) and maximum protease production achieved in agreement with their findings. The resulting proteases extract shows gelatinolytic activity when spotted on gelatin coated X-ray film (Fig. 2).

3.2. Characterization of bacterial proteases by gelatin zymography

Efficacy of SDS–PAGE zymography for detection and characterization of microbial proteases was documented earlier (Choi et al., 2001; Kim et al., 1998). Zymography is an adaptable

Figure 1 Screening of *Bacillus subtillis* RTSBA6 6.00 for proteases activity. Isolate are streaked on agar plate containing skimmed milk casein, incubated for 24 h at 37 °C. The clear zone indicated the hydrolysis of skimmed milk casein.

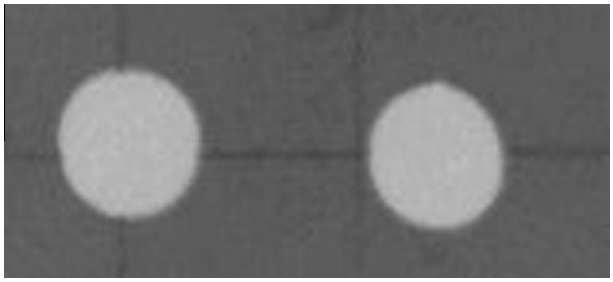

Figure 2 Dot-blot assay – gelatinolytic activity of serine protease of *Bacillus subtillis* RTSBA6 6.00.

two-stage technique involving protein separation by electrophoresis followed by in situ detection of proteolytic activity. Profile of the proteases activity of bacterial extract using non-denaturing 10% SDS–PAGE containing 0.1% gelatin is shown in Fig. 3. On a gelatin-containing zymogram, a clear area of gelatin hydrolysis was formed, whereas the undigested gelatin stained blue. The zone of gelatin clearing corresponded to a migration distance of proteases by comparison to the molecular weight standards used in the study. Generally *Bacillus* strains are well known for secreting various extracellular proteases with molecular weights that range from 15 to 100 kDa (Jeong and Han, 2001; Kim and Choi, 2000). Almost similar results were obtained in agreement to above findings in case of *B. subtillis* RTSBA6 6.00. The 12 protease activity protein bands were visualized on zymographic gel among those of the band no. 1 showed relatively slow mobility with the molecular weight of 185 kDa. Molecular weights of proteases (eight bands) which showed moderate mobility were 92.5, 83.5, 72.5, 60.2, 56.2, 45.7, 37.1 and 34.4 kDa whereas fast migrating proteases (three bands) were 22.0, 21.2 and 20.0 kDa, respectively.

3.3. Effect of pH and heating on bacterial protease activity

In our study we found that, proteases isolated from *B. subtillis* RTSBA6 6.00 showed optimum activity in slightly alkaline pH

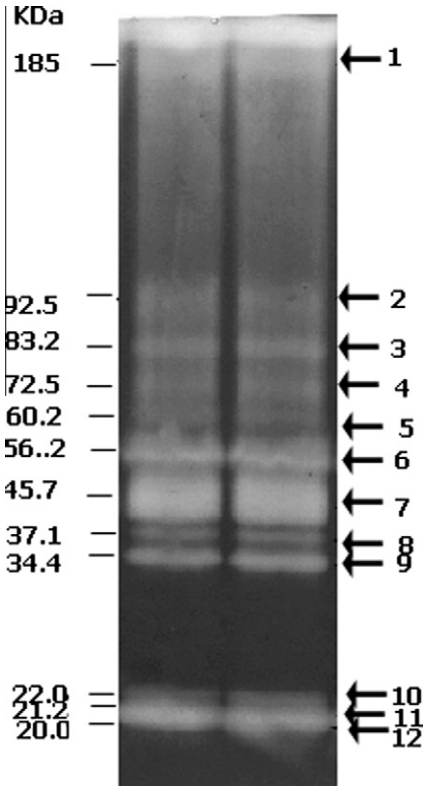

Figure 3 Non-denaturing 10% gelatin SDS–PAGE of proteases produced by *Bacillus subtillis* RTSBA6 6.00.

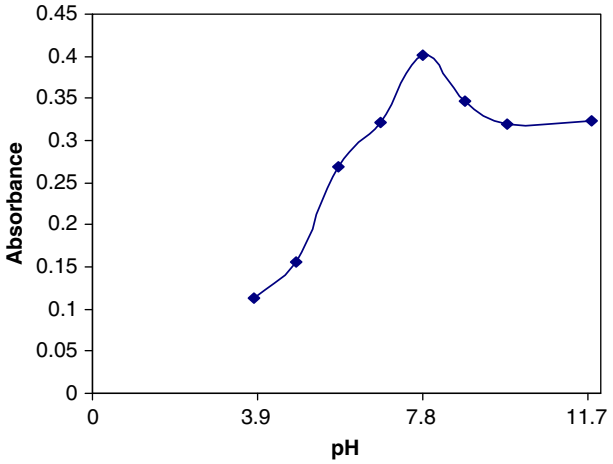

Figure 4 Effect of different pH values on proteolytic activity of *Bacillus subtillis* RTSBA6 6.00. Samples were assayed by standard procedure using azo-casein as substrate.

(7.8) as shown in Figs. 4 and 5. This parameter assessed under standard assay conditions and zymographically. These proteases had a relatively low activity in acidic pH range from 3.8 to 5.8. After pre-incubating at different temperatures, i.e. 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C at pH 7.8 for 10 min, proteases were found to be stable at temperature range from 20 to 60 °C with optimum activity at 35 °C. A rapid decrease in enzyme activity was detected above 60 °C with negligible activity at 70 °C indicating inactivation of proteases as

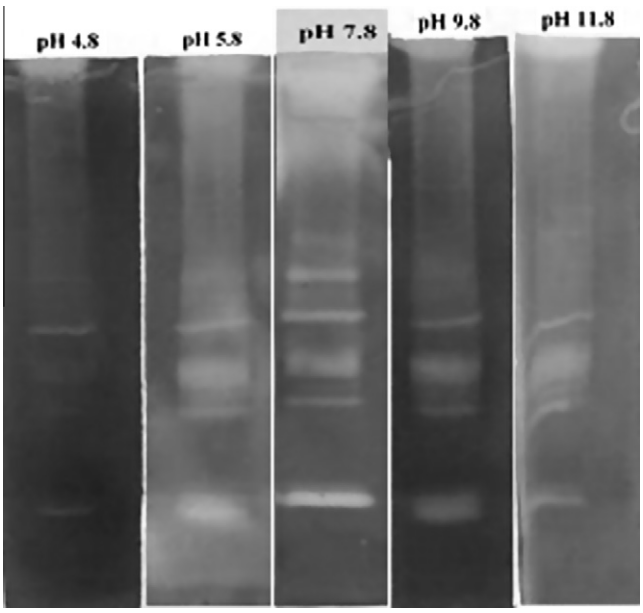

Figure 5 Non-denaturing 10% gelatin SDS–PAGE showing the influence of pH on the proteolytic pattern of *Bacillus subtillis* RTSBA6 6.00.

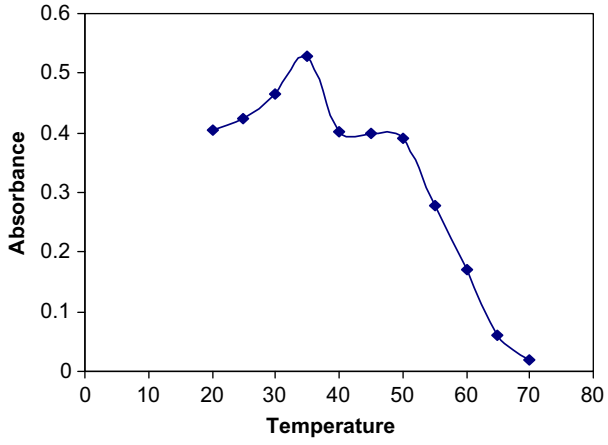

Figure 6 Effect of different temperatures on proteolytic activity of *Bacillus subtillis* RTSBA6 6.00. Samples were assayed by standard procedure using azo-casein as substrate mentioned in material and methods.

shown in Figs. 6 and 7. These results were in agreement with Pastor et al. (2001) who found maximum protease activity at alkaline pH, also our findings were similar with the finding of Abu Sayem et al. (2006) who demonstrated that, at temperatures 70 °C, the proteases lost its activity rapidly.

Numerous proteases present in the alkaline environment of gut of *H. armigera* which are proteolytic arsenal utilized in proteinaceous food digestion (Patankar et al., 2001; Mazumdar-Leighton et al., 2000; Gatehouse et al., 1997). The presence of bacteria producing several proteases with optimal activity in alkaline region could be advantageous to *H. armigera*. Here it might be possible that these bacterial proteases contribute to proteolytic arsenal of *H. armigera*.

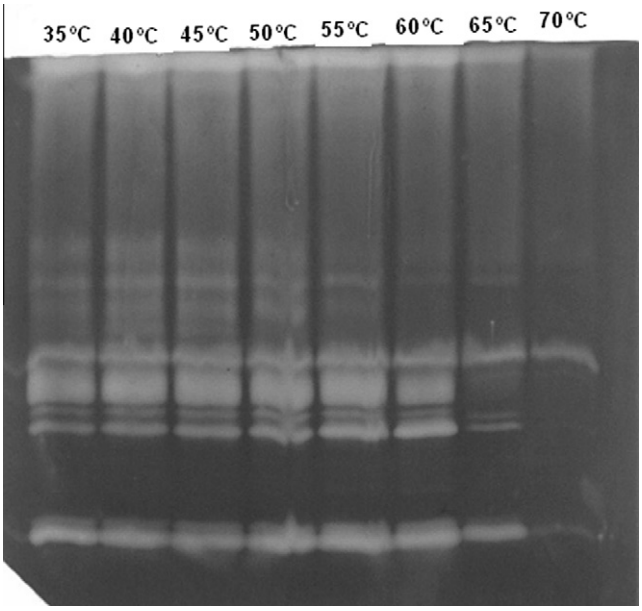

Figure 7 Non-denaturing 10% gelatin SDS–PAGE showing the influence of temperature on the proteolytic pattern of *Bacillus subtillis* RTSBA6 6.00.

3.4. Synthetic protease inhibitor study

Proteases from *B. subtillis* RTSBA6 6.00 were characterized using the specific proteinase inhibitors, PMSF and 1,10-phenanthroline (containing 10 mM EDTA). PMSF generally inhibits proteases which require serine residue at their active site whereas 1,10-phenanthroline (containing 10 mM EDTA) inhibits proteases which require a divalent metal ion at their reaction center (Salamone and Wodzinski, 1997). Here we observed that in the presence of 1 mM PMSF complete inhibition was occurred on gelatin zymography whereas no significant inhibition was occurred in the presence of 5 mM 1,10-phenanthroline (Fig. 8). From the above results it is suggested that these proteases belong to serine type family. These serine proteases further characterized using TLCK and TPCK. TLCK inhibits serine proteases with trypsin like activity, which cleaves after positively charged residues whereas TPCK inhibits chymotrypsin-like activity, which cleaves after large hydrophobic residues in protein. Complete inhibition by 100 μM TPCK, confirms chymotrypsin-like activity whereas 100 μM TLCK failed to inhibit these protease suggesting the absence of trypsin like proteases.

Imperative role of chymotrypsin-like serine proteases in insect physiology was documented earlier (Broehan et al., 2010; Kanost and Gorman, 2008; Yu et al., 2003; Lee et al. 2002; Kim et al., 2002). *H. armigera* found to feed on agricultural crop rich in protein and depend mainly on serine proteinases, particularly trypsin and chymotrypsin-like enzymes for its digestion (Srinivasan et al., 2006). The occurrence of proteolytic bacteria in the gut of *H. armigera* leads to hypothesize that proteolytic bacterium might be involved in assimilation of protein in the gut of host insect. Previous studies showed that gut bacteria assist to host insect by inducing necessary enzymes for food digestion (Kaufman and Klug, 1991; Santo Domingo et al., 1998).

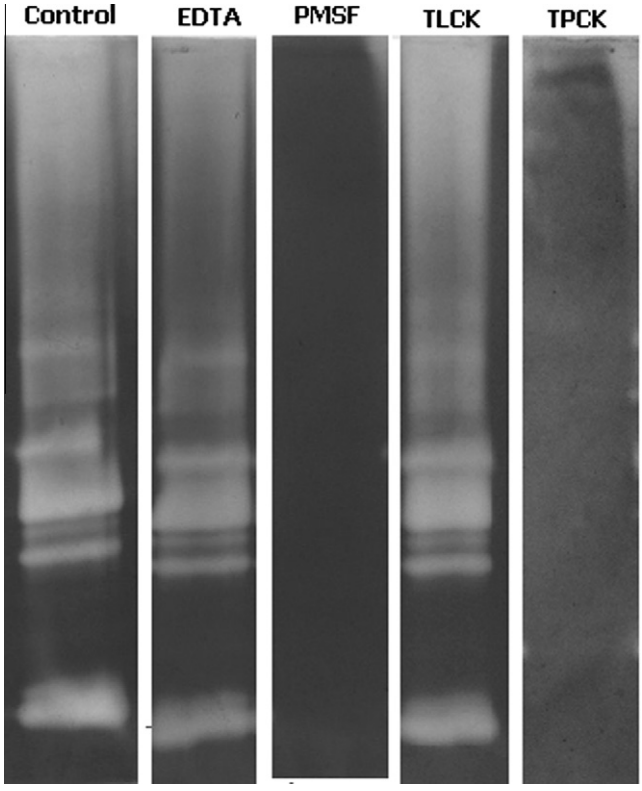

Figure 8 Effect of protease inhibitors on the proteolytic profile of *Bacillus subtillis* RTSBA6 6.00. Proteolytic assay were performed in the absence (control) or presence of each of the following protease inhibitors: 5 mM 1,10-phenanthroline (containing 10 mM EDTA), 1 mM PMSF, 100 μM TLCK and100 μM TPCK.

Various host plants found to secrete antagonist molecules like protease inhibitors to control devastating pest, *H. armigera* (Jouanin et al., 1998; Schuler et al., 1998), but insect insect pests were found to adapt these defense strategies of plant (Srinivasan et al., 2006; Brito et al., 2001; Paulillo et al. 2000; Lopes et al., 2004). This versatile adaptation to host plant protease inhibitors and possible contribution of bacterial proteolytic enzymes are the further area of utmost research interest. Detailed investigations of gut bacterial flora and deciphering possible role of protease secreting bacteria in gut host will be the future viewpoint of this study.

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