Screening and Preliminary Optimization of Protease in *Morganella Morganii* VBS1 Strain Isolated from a Gastropod, *Cerithidea cingulata*

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ABSTRACT: : Background and objective: *Production of bacterial protease enzymes is the more significant area of research due to their potential industrial applications. The objective of the present study was to identify a potent bacterial strain from the gut of a mangrove associated gastropod, Cerithidia cingulata and preliminary optimize protease production in a solid media.* ***Materials and Methods:*** *The gut associated bacterial samples were screened for potential protease production. The potent isolate was biochemically characterized and the enzyme was optimised for salinity and pH.* ***Results:*** *Based on the biochemical test and 16S rRNA gene sequence, the potential bacterial stain was identified as Morganella morganii. Protease production was more at pH7 and salinity at 3% concentration.* ***Conclusion:*** *The protease production was more at pH7 and salinity at 3% concentration. Further optimization of this enzyme production is needed for this bacterial species.*

Key Words: *gastropod, bacterial strain, Morganella morganii, protease, optimization*

INTRODUCTION

The gut micro-flora may be categorized as either autochthonous (indigenous) or allochthonous (transient) depending upon its ability to colonize and adhere to the mucus layer in the digestive tract[1, 2]. The bacterial flora of the gastrointestinal tract in general represents a very important and diversified enzymatic potential and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal[3]. Selected microorganisms including bacteria, fungi and yeasts have been globally studied for the bio-synthesis of economically viable preparations of various enzymes for commercial applications[4].

Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale[5, 6]. Proteases from marine microorganisms have received increased attention due to their inherent stability at different pH values, temperature and salinity. Microbial protease is predominantly extracellular and can be secreted in the fermentation medium. Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms and are essential for cell growth and differentiation. Kar and Ghosh[7] reported the enzyme producing bacteria in the gastrointestinal tracts of *Labeo rohita* and *Channa puncatus.* Balaji *et al*.[8] undertaken a study to isolate and characterize the protease producing potent bacteria from gastro intestinal tract of fresh water fish *Cyprinus carpio.* Esakkiraj *et al*.[9] investigated the extracellular protease production by *Basillus cereus* isolated from the intestine of fish *Mugil cephalus.* Geethanjali and Anitha Subash[10] optimized the protease production by *Bacillus subtilis* isolated from mid gut of fresh water fish, *Labeo rohitha*. The objective of present study was to screen and isolate protease producing bacteria from intestinal tract of gastropod, *Cerithidea cingulata* collected from Vellar estuary.

MATERIALS AND METHODS

Source of sample

The gastropod, *Cerithidae cingulata* was collected from mangrove forest of Vellar estuary of South East coast of Tamil Nadu, India. Collected samples were placed individually in the pre-sterilized polythene bags, sealed and kept in a portable ice box and transported to the laboratory for further bacteriological analysis.

Bacterial strain isolation and identification

The gastropod was washed with sterile distilled water and break the outer shell, intestine were dissected using sterile dissected set. One gram of gastropod intestine was taken and crushed then inoculated in to 100 ml of sterile nutrient broth. Dilution was made by taking 1 ml of enriched sample and transferred in to 5 ml of NaCl (physiological saline). The sterially tenfold dilutions of intestinal bacterial sample was spread in to nutrient agar plate and incubated for 24 hrs. Morphology dominant four isolates were selected for further

studies. Bacterial isolates were compared by colony morphology and colour[11]. The four bacterial isolates were purified and inoculated to nutrient broth separately for further analysis.

Gram staining

The smear was prepared and air dried then flooded with Gram's crystal violet for 1 minute. After washing with water Grams iodine was added for 1 minute. Followed by washed with water and decolourise with Grams decolouriser until no further violet colour comes off. Finally the counter stain, 0.5% Saffranine was added for about 1 minute. After washing with distilled water, the slide was observed under oil immersion. The lipase- producing bacterial strain was identified by standard procedures described in Bergy's manual of determinative bacteriology[12].

Indole production test

Using a sterile loop the organism was inoculated in peptone water in a test tube and incubated for 37ºC for 24 hours. After incubation, few drops of Kovac's reagent was added to the test tube. On adding the Kovac's reagent to the test tube there is red colour formation on the surface of medium indole positive. In the test tube there is no red colour formation indole negative.

Citrate utilization test

This test was used to determine the ability of an organism to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source. Using a sterile loop the organism was inoculated into the Simmon's citrate agar slant tubes by means of the stab and streak method and incubated 37ºC for 24 hours. The colour change and growth indicates that the organism has utilize citrate as the sole source of carbon. No growth and no colour change indicates that the organism has not utilized citrate.

Methyl red test

The test organism was inoculated into the MR broth in a test tube. The tubes were incubated at 37ºC for 48 hours. After incubation 5 drops of methyl red reagent was added to the test tube. The inoculated test tube developed a stable red colour, the organism is methyl red positive, remained in yellow in colour, the organism methyl red negative.

Voges proskauer test

The test organism was inoculated into the VP broth in a test tube. The tube was incubated at 35°C for 24 hours. After incubation 5% α-napthol and 40% potassium hydroxide was added. The tubes were gently shaken to expose the medium to atmospheric oxygen and tubes were left undisturbed for 10-15 minutes. The tube inoculated with organism developed red colour at the surface of the broth (within 15 minutes), the organism is VP positive. The tube inoculated with organism remained yellow in colour, the organism is VP negative.

Screening of extracellular enzyme production

The protease activity from isolated bacterial strain was followed by Cheng *et al*.[13]. To assess the protease production, all the bacterial isolates were inoculated on skim milk agar plates and sterilized by autoclaving at 121° C for 20 min. Approximately 20 ml mixture was poured into the sterile petriplates and allowed to solidify at room temperature. The isolates were screened for the production of extracellular protease. In brief, cultures were streaked as single streak on skim milk agar plates and incubated at 37º C for 24 to 48 hrs. Presence of zone of clearance surrounding the culture streak was taken as a measure of protease production.

Optimization of protease production in solid medium

To optimize the salinity for protease production, the skim milk agar plates were prepared at the range of 1, 2, 3, 4 and 5% salinity and incubated at 30°C for 24hrs. After the incubation is over, the clear zones were formed on skim milk agar plates and the clear zone was measured. To optimize the pH for protease production, the skim milk agar plates were prepared at the pH level of 4, 5, 6, 7 and 8 then incubated at 30°C for 24hrs. After the incubation is over, clear zone was formed on skim milk agar plates and the clear zone was measured.

Molecular level identification of the bacterial strain

Genomic DNA was extracted from the nutrient broth by the standard protocol[14]. The 16SrRNA gene was amplified by the universal primers[15]. The cleaned up PCR product was sequenced by outsourcing (Eurofins, Bangalore). The raw 16S rRNA gene sequence of the isolate was edited using BioEdit[16] and done BLAST in NCBI database.

RESULT AND DISCUSSION

Fifteen bacterial colonies of yellow, red and white were observed from gastropod gut sample. From all the bacterial strains, only one potent protease producing bacterial strain was selected as slight rod white gram

negative and designated as *Morganella morganii* VBS1 strain (Plate 1) based on the other biochemical and molecular characterization. Among the five different salinity concentrations, 3% salinity was showed higher zone (6mm) of clearance and the result is given in Fig.1. Among the five pH conditions, pH7 showed higher zone (8mm) of clearance and the result is shown in Fig.2.

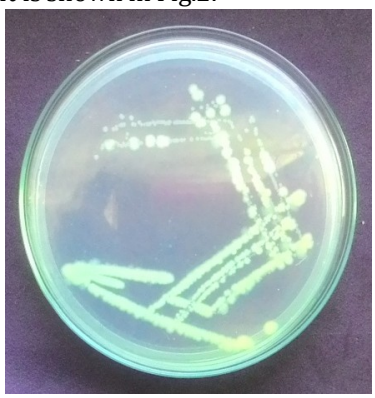

Plate 1. *Morganella morganii* VBS1 strain

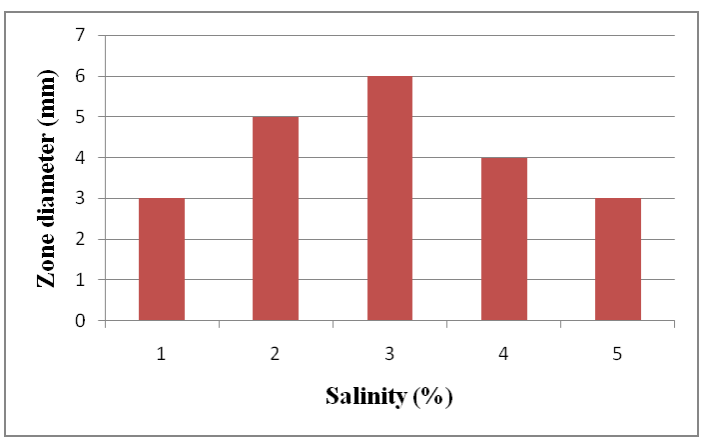

Fig. 1. The zone of enzyme activity at different salinity concentration at solid medium

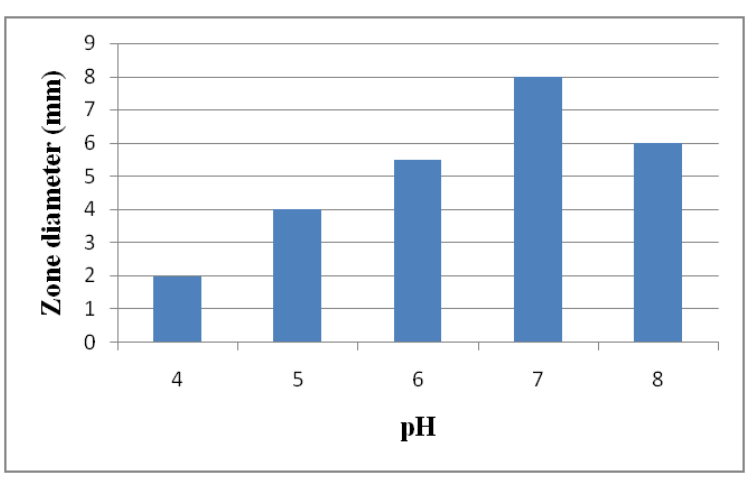

Fig. 2. The zone of enzyme activity at different pH at solid medium

In the present study, to investigate whether the concentration of NaCl affects the production of extracellular protease by *M. morganii*, it was grown in a medium added with varying concentration of NaCl (1.0 to 5.0%) as shown in Fig.2. Extracellular protease production was maximum in the medium with 3% NaCl, it indicated that *M. morganii* is moderately halophilic. As per the previous studies the bacterial protease production was considerably more at the salinity concentration of 10% in *vibrio* and *Bacillus*[17]. Some research have proved that the production was more at pH 7-10 in *Bacillus subtilis* and *Bacillus proteolyticus*[18, 19]. In this study we have observed more protease production in pH 7.

CONCLUSION

In this investigation, the gastropod gut bacteria, *M. morganii* VBS1 strain showed higher protease production at pH7 and salinity 3% condition. This is a preliminary study and further optimization of protease production with carbon source, temperature and other physical parameters are warranted.

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