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Purification and Characterization of Bacterial Chitinase isolated from Crustacean Shells

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ABSTRACT

Four Chitin degrading bacterial strains vibrio aestuarianus, Flavobacterium, Shewenella and Exiguobacterium were isolated from crustacean shells. The strains were confirmed by Biochemical analysis, FAME-GC analysis and 16s rDNA sequencing. The chitinase was purified by a two step chromatographic method and characterized. The enzyme was purified to homogeneity by 6.75 fold with 46% recovery after ion exchange chromatography followed by gel filtration chromatography. The purified enzyme revealed a single band on SDS-PAGE gel with a molecular mass of 24 kDa. It showed an optimum pH at 6.0. The optimum temperature for enzyme activity was 40 °C. The maximum activity was observed with a 2% substrate concentration of colloidal chitin. The enzyme was strongly inhibited by Fe2+ and K+ while enhanced by Zn2+ and Ca2+. Thus the purification of microbial chitinase from shell waste could be effectively utilized for the manufacturing of many chitin derived products.

Keywords: *Chitinase, Crustacean shells, FAME-GC, ion exchange chromatography, gel filtration chromatography, Characterisation.*

INTRODUCTION

Chitin is a major structural polysaccharide of fungi and of arthropods such as insects and crustaceans, and is thus abundant in nature, second only to cellulose. Chitin is a polymer of N-acetyl-D-glucosamine, and represents an important potential source of renewable biomass. The recycling of chitin waste, especially from the sea food industry, is therefore of considerable economic and environmental significance. However, chitin is a rather intractable polymer, as it is insoluble in water and organic solvents. Chitinases that produced by different micro-organisms have received increased attention due to their wide range of biotechnological applications, especially in the production of chitooligosaccharides and N-acetyl D-glucosamine, biocontrol of pathogen and pests, preparation of sphaeroplasts and protoplasts from yeast and fungal species, and bioconversion of chitin waste to single cell protein. Therefore, application of chitin-hydrolyzing enzymes (chitinases) is expected for effective utilization of this abundant biomass.

Chitinase is widely distributed in bacteria, actinomycetes, and plants[1,2,3,4]. Chitinase-producing marine bacteria play an important role in the degradation of chitin in the oceans[5,6]. Chitinases from marine bacteria have been isolated and their properties reported[7,8,9,10,11,12]. Among gram-negative bacteria, chitinolytic activity has been described for strains from the genera *Aeromonas*, *Alteromonas*, *Enterobacter*, *Pseudomonas* , *Serratia*, *Ewingella*, and *Vibrio*. Although studies on chitinolytic activity in *Vibrio* and *Pseudoalteromonas* sp, have shown that soluble oligosaccharides liberated by the action of extracellular chitinase on chitin elicit the induction of expression of a number of proteins, little is known at present about the genetic regulation of chitinolytic enzyme expression in gram negative bacteria.

According to Rheinheimer[13], the most active bacteria are those of *Pseudomonas* and *Vibrio* genera and also actinomycetes of the *Micronomospora* genus.

Thus there is considerable interest in the development of an efficient bioconversion process based on the exploitation of chitinases. Marine bacteria are excellent sources of chitinases[14], and may be considered to be the natural agents for the catabolic conversion of crustacean chitin into useful molecules for applications in biotechnology and medicine. Bacteria from the family Vibrionaceae frequently occur in the marine environment[14], and are potentially suitable sources of enzymes for the recycling of crustacean chitin. Bacteria typically possess multiple, usually inducible, chitinases[7,15] and chitobiases[9]. The chitinases isolated from a single bacterial strain often appear to be heterogeneous with respect to substrate specificity and molecular size and may, in addition, be subjected to posttranslational modification including proteolytic processing[16,17]. Moreover, extensive studies are required on the maximum utilization of chitinous wastes for production of chitinases and biomass. Hence, this paper explains the isolation, identification, purification and characterization of chitinase from four bacterial strains isolated from marine crustacean shells.

MATERIALS AND METHODS

Screening and isolation of Chitin degrading Bacteria

Exoskeletal bacterial isolates were obtained by abrasion of shell-disease lesion and non-lesion areas of both dorsal and ventral surfaces using a sterile scalpel. Each scraping was transferred into 600µl sterile 3.2%NaCl solution, homogenized briefly and spread-plated in triplicate onto MA and chitin agar as described by Vogan *et al*[18]. After incubation for 5-7 days at room temperature, clearance zone forming bacteria were selected as the chitinase producer. Pure cultures from randomly chosen colonies were placed on MA slopes and stored at 4°C until use. Those strains were selected for the production and characterization of chitinase. The isolates were identified through a) it's morphological and physiological properties according to Bergey's manual of Systematic Bacteriology. b) The nearly complete nucleotide sequence of 16S rRNA was determined using bacterial universal primers. The 16S rDNA sequence was compared to the sequences in the GenBank nucleotide database by using Basic Local Alignment Search Tool (BLAST) and c) FAME-GC analysis.

Enzyme production and extraction

For enzyme preparation , the strains were grown at 28°C in colloidal chitin medium consisting of (g/l): colloidal chitin,10; peptone,3; KNO_3,3; K_2HPO_4,0.7; $MgSO_4$,0.5; KCl,1.0; and cultured in 50 ml medium in 250ml conical flasks with shaking at 180rpm for 3 days. The culture fluid was centrifuged at 8,000xg for 20 mins. Chitinase was extracted from the above bacterial bran by ammonium sulfate precipitation upto 75% saturation and the precipitate was resuspended in a minimum volume of 20mmol/l citrate phosphate buffer (pH 5.8). The precipitate obtained after ammonium sulfate fractionation was extensively dialysed against the same buffer for 24hrs at 4°C with continuous stirring and occasional changes of the buffer[19.] The resultant dialysate was chitinase crude extract and was subjected to further purification.

Purification of Chitinase

The dialysate obtained above was concentrated by lyophilisation and loaded on top of the DEAE-Sephadex A-50 (Pharmacia) column equilibrated with dialysis buffer. The procedure was carried out at 4°C. The concentrated active fractions from anion exchange chromatography were loaded onto a Sephadex G-100 column with 50 mmol/l Sodium acetate buffer (pH 4.6) containing 0.1 mol/l NaCl. The enzyme was eluted in 2ml fractions with a linear gradient from 0.2 to 1.0 mol /l 20mM bis tris buffer (pH7.0) at a flow rate of 1ml/min in a sequential manner.

Each fractions were analysed for enzyme activity and protein content.. The active fractions were pooled and concentrated by lyophilisation. The purified samples were stored at 20°C for further studies.

Chitinase assay

The strains were preinoculated in TSA for 24hrs and thereafter inoculated into minimal salt agar media and the activity was analysed every 24 hrs till it reaches a decline phase. The enzyme activity was also

measured with crude culture supernatant, after ammonium sulfate precipitation and the pooled elutes obtained after purification steps (ion-exchange and gel filtration chromatography).

Chitinase activity was determined colorimetrically by detecting the amount of GlcNAc released from a colloidal chitin substrate[20]. The reaction mixture consisted of 0.3ml of crude enzyme and 0.2ml of colloidal chitin. The reaction was performed at 37°C for 30min. The mixture was boiled for 10min, chilled and centrifuged to remove insoluble chitin. The resulting adduct of reducing sugars was measured by DNSA method[21]. GlcNac was used as the standard. One unit of Chitinase activity is defined as the amount of enzyme that released 1 μmol of GlcNAc from colloidal chitin per minute. The yield of the enzyme was also measured by calculating the specific activity and fold purification of the enzyme.

Protein estimation

The concentration of protein present in crude culture supernatant, after ammonium sulfate precipitation and the pooled elutes obtained after purification steps (ion-exchange and gel filtration chromatography) were determined by the method of Bradford[22] using Bovine Serum Albumin (BSA) as the standard. The reaction mixture consists of 0.2 to 1.0 ml of standard in 0.1N NaOH. The test solution contains 0.2 ml of each sample. To the solution, 5ml of CBB R250 was added and incubated for 10 minutes. The absorbance was measured colorimetrically at 595nm.

Characterization of the purified enzyme

Molecular weight determination

Protein analysis was done by Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) with 10% gels [23]. The pure protein sample from each of the bacterial isolates obtained after gel filtration was run along with standard molecular markers such as BSA 66kDa, Glutamate dehydrogenase 55kDa, Ovalbumin 45kDa, Carbonic anhydrase 30kDa and Trypsin 21kDa

Effect of pH and temperature on Chitinase activity

Chitinase activity was assayed at different pH values (pH 5.0 to 9.0) and Optimum temperature was measured by incubating the reaction mixtures at different temperatures to assay the enzyme activity. Chitinase activity was assayed at different temperatures ranging from 10-50ºC at pH 5.5 in citrate phosphate buffer (50 mM).

Effect of substrate concentration

The effect of substrate concentration on chitinase activity was determined at different concentrations of chitin, varying between 0.5 mg ml^{-1} to 2.5 mg ml^{-1} (w/v).

Effect of salt ions

The effect of metal ions on enzyme activity was studied by incorporating these metal ions at 10 mM concentration such as KCl, $CaCl_2.2H_2O$, $ZnSO_4$ and $FeSO_4$ each at different volumes ranging from 0.2 – 1ml in reaction mixture

RESULTS AND DISCUSSION

Chitinase producing bacterial strains were isolated from waste crustacean shells collected from different places along the coastal areas of Chennai. All the isolates were identified to various genera representing species of *Vibrio, Flavobacterium, Shewenella* and *Exiguobacterium*. The chitin degrading strains were identified at the species level by basic biochemical tests, 16s rRNA sequencing and FAME-GC analysis. The results suggested that all the strains isolated were non-identical and thus identified as *Vibrio aestuarianus, Flavobacterium odoratus, Shewenella putrefaciens* and *Exiguobacterium.* The biochemical tests showed the pattern of gram staining, physiological and biochemical characteristics of the chitinolytic bacterial isolates (Table I).

The fame-gc analysis proved the identification of the strains at the species level. The table shows the analysis and identification of the strains by comparing the FAME pattern generated for the bacterial strains with the MIDI library. The search is based on the similarity index values obtained for the bacterial fame pattern. It is to be noted that the fame pattern for the strain *Exiguobacterium* doesn't match with any

of the entries present in the MIDI library. The similarity index as represented by the MIDI library is given (Table II).

Table I. Biochemical Characterisation

S. No.	Test	*Vibrio aestuarianus*	*Flavobacterium odoratus*	*Shewenella putrefaciens*	*Exiguobacterium*
01	Gram Staining	-ve	-ve	-ve	+ve
02	Spore formation	-ve	-ve	-ve	-ve
03	Nitrate reduction	+ve	-ve	+ve	-ve
04	Catalase	+ve	+ve	+ve	+ve
05	Oxidase	+ve	+ve	+ve	-ve
06	Gelatin hydrolysis	+ve	+ve	-ve	-ve
07	Citrate Utilisation	-ve	-ve	-ve	-ve
08	Methyl red	+ve	-ve	-ve	-ve
09	Indole production	-ve	-ve	-ve	-ve
10	Sugar fermentation				
a)	Sucrose	+ve	-ve	-ve	+ve
b)	Arabinose	-ve	-ve	-ve	-ve
c)	Xylose	+ve	-ve	-ve	-ve
d),	Mannitol	+ve	-ve	-ve	+ve

Their biochemical characteristics have been determined by routine analysis according to Bergey's manual of Systematic Bacteriology.

The physiological and biochemical characteristics have been summarized as follows.

i) *Vibrio aestuarianus* is a gram negative, non- spore forming bacteria which shows nitrate reductase, catalase, Oxidase and gelatinase positive. It shows negative results for Citrate utilization, indole production and fermentation of Arabinose.

ii) *Flavobacterium odoratus* is a gram negative bacteria which shows positive results for catalase, Oxidase and Gelatinase. However, it shows negative for all the other tests.

iii) *Shewenella putrefaciens* is a gram negative bacteria which shows positive results for Nitrate reductase, catalase, and Oxidase. However, it shows negative for all the other tests like gelatinase, citrate utilization, indole production and sugar fermentation.

iv) The novel *exiguobacterium* strain which is gram positive shows only catalase activity as positive whereas it showed negative results for all the tests.

Table II. FAME-GC Analysis of the Isolates.

S. No.	Name of the Isolate	Similarity Index
1	*Vibrio-aestuarianus*	0.740
2	*Myroides-odoratus (Flavobacterium odoratum)*	0.470
3	*Shewanella- putrefaciens*	0.411
4	*Exiguobacterium*	No matches found

Further, the isolation of non-identical species were confirmed by the results obtained from 16s rRNA sequencing. The partial 16s rRNA sequences obtained were submitted to Genbank with accession numbers is shown (Table III).

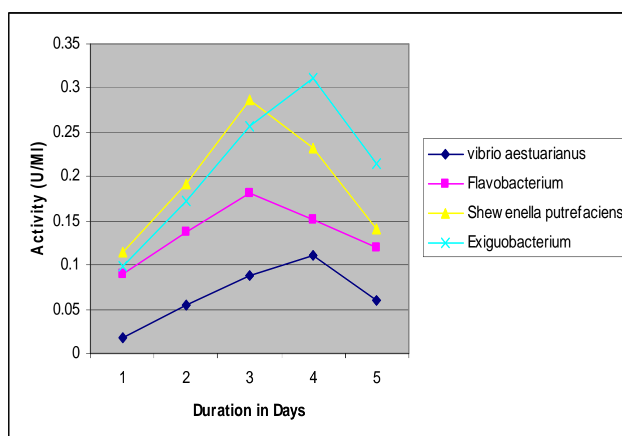
Table III. Accession number of partial 16s rRNA sequence submitted in Genbank.

S. No	Organism Name	Accession number
1	*Vibrio aestuarianus*	GQ906358
2	*Flavobacterium odoratus*	GU734766
3	*Shewenella putrefaciens*	GU734767
4	*Exiguobacterium strain*	GU734770

Bacteria produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. Chitins can vary by the arrangement of *N*-acetylglucosamine strands, degree of deacetylation, and presence of cross-linked structural components, such as proteins and glucans. We had expected some chitinases to be specialized for hydrolysis of particular types of chitin and others to be involved in degradation of all chitin shells. Bacterial chitinases belong to family 18 of the glycosyl hydrolases[24,25]. There is considerable complexity because many bacteria possess up to five or six separate chitinase genes[7,15,16,26] and as mentioned before there can also be multiple proteolytic forms. Watanabe *et al*[27] have classified bacterial chitinases into three groups (A, B, C) according to sequence similarities. The screening of bacterial species for chitinase activity revealed a marked variation in the capability to secrete elevated levels of enzyme(s) in response to chitin induction.

The pattern of enzyme production by each of the four strains inoculated in minimal chitin media at different hours of incubation till 120hrs was determined (Fig. 1). Results suggested that the strains *Flavobacterium odoratus* and *Shewenella putrefaciens* showed maximum activity on 3rd day [72 hours] of incubation whereas the other strains *Vibrio aestuarianus* and *Exiguobacterium strain* showed maximum activity on 4th day [72 hours] of incubation.

Fig. 1. Chitinase production by respective strains for an incubation period of 5 days.



Chitinase from all the six bacterial strains was purified by Ammonium sulphate precipitation and dialysis followed by a two step chromatographic technique- ion exchange chromatography followed by gel filtration chromatography. The over all purification summaries are presented (Table IV to Table VII).

Table IV. Purification of chitinase from *V. Aestuarianus*.

S.No	Fractions	Protein (mg)	Total Activity (u)	Specific Activity	Yield (%)	Fold
1	Crude	0.73	50.0	68.5	100	1
2	Ammonium sulfate fractionation	0.2	34.0	170.00	68	2.48
3	Ion exchange	0.06	27.0	450.0	54	6.6
4	Gel filtration	0.05	23.0	460.0	46	6.75

Table V. Purification of chitinase from *F. Odoratus*.

S.No	Fractions	Protein (mg)	Total Activity (u)	Specific Activity	Yield (%)	Fold
1	Crude	0.68	69.6	102.35	100	1
2	Ammonium sulfate fractionation	0.15	54.4	362.7	78.2	3.54
3	Ion exchange	0.08	32.9	412.0	4703	4.03
4	Gel filtration	0.05	23.5	470.0	33.8	4.60

Table VI. Purification of chitinase from *S. Putrefaciens*.

S.No	Fractions	Protein (mg)	Total Activity (u)	Specific Activity	Yield (%)	Fold
1	Crude	2.8	278.5	99.5	100	1
2	Ammonium sulfate fractionation	1.2	223.5	186.25	80.25	1.9
3	Ion exchange	0.9	192.56	213.96	69.4	2.15
4	Gel filtration	0.6	160.8	268.0	57.75	2.70

Table VII. Purification of chitinase from *Exiguobacterium strain*.

S.No	Fractions	Protein (mg)	Total Activity (u)	Specific Activity	Yield (%)	Fold
1	Crude	0.75	84.0	112.0	100	1
2	Ammonium sulfate fractionation	0.2	62.0	310.0	73.8	2.8
3	Ion exchange	0.2	41.8	418.0	49.8	3.73
4	Gel filtration	0.08	37.6	470.0	44.8	4.2

For all the samples, the chitinase activity and protein concentration was determined for the crude supernatant and after ammonium sulfate fractionation. It is clear from the results that the specific activity and fold increased after each step of purification. The chitinase activity was measured for all the elutes obtained from ion exchange chromatography. The absorbance was read at 540nm. The specific activity was increased several fold after ion exchange chromatography. The protein concentration was determined at 660nm for all active fractions. The elutes which showed highest concentration of protein were pooled, concentrated and subjected to gel electrophoresis. Similarly after gel filtration process, the specific activity and fold increased for all the isolated chitinolytic bacterial strains. The chitinase of *v.aestuarianus* shows moderate yield with good purity. The chitinase of *s.putrefaciens* shows high yield with low purity.

The molecular weights of chitinases from marine bacteria are mostly around 60 kDa [7,11,12,28,29]. However, in this study the molecular weight of the pure chitinase was determined to be between 21KDa to 30 KDa for all the samples. Eventhough there is high chitinase activity among the strains under study, the yield and fold decreased upon purification. This reflects the practical problems of extracting and purifying chitinase from marine microbial strains.

The enzyme was active over a range of pH 5.0 – 8.0 (Fig. 2) with an optimum pH of 8.0 for *S. putrefaciens* as many marine bacterial chitinases showed broader pH optima[7,11] or were more active in neutral or slightly alkaline conditions[28,29]. The pH optimum of the chitinase produced by strains *V.aestuarianus, F.odoratus* and *Exiguobacterium* is similar to that of the chitinase from vibrio sp-98CJ11027[30] enteric bacterium *Ewingella americana*[31]and from kidney beans[32].

Fig. 2. Effect of pH on activity of Chitinase.

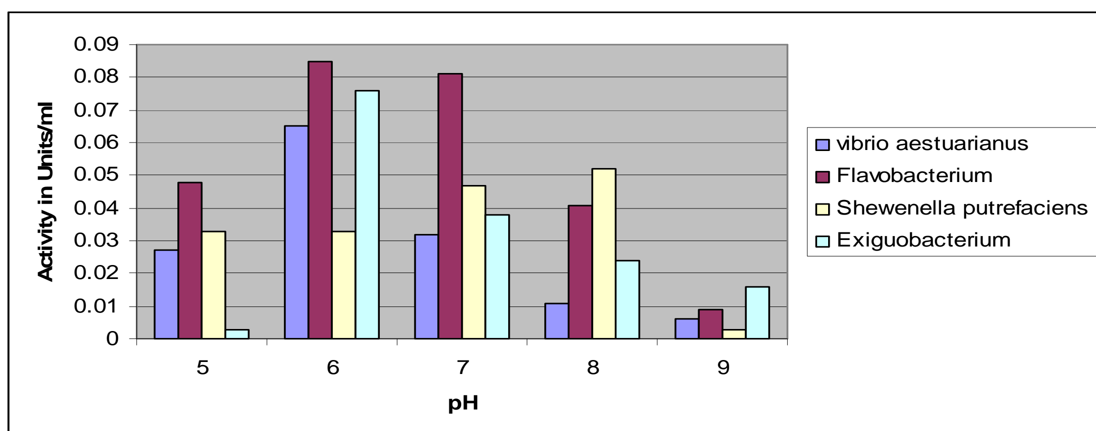
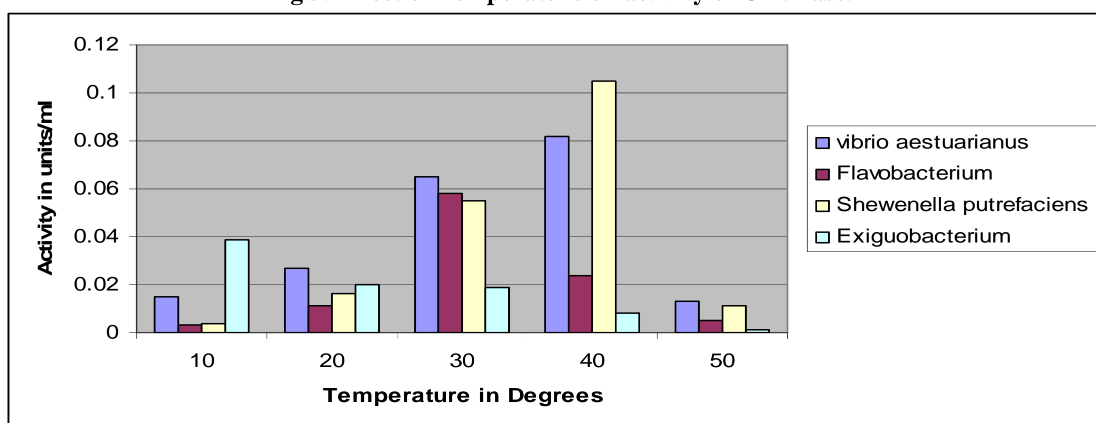


Fig.3. Effect of Temperature on activity of Chitinase.



The effect of temperature on activity of the enzyme was determined by subjecting them to various temperatures ranging from 10°C to 50°C (Fig. 3). The results indicated that the temperature optimum for this enzyme is 40°C for the strains *V.aestuarianus* and *S. putrefaciens.* These isolates showed a similar temperature optimum to those of other marine bacteria (*Aeromonas hydrophila* H-2330[7], *Alteromonas* sp. O-7[28], and *Pseudomonas aeruginosa* K-187[29]. However, the chitinase from *F.odoratus* and *Exiguobacterium* has the temperature optima at 30°C and 10°C respectively (Fig. 4). The optimum temperature comparison between the four isolates is given (Fig. 5). This was also in accordance with other reports in literature such as *Arthrobacter* sp. NHBN-10[33], *Vibrio alginolyticus* TK-22[34]. Chitinase from *Vibrio alginolyticus* TK-22 was stable at 40ºC for 30 min[34] and purified chitinase of *Vibrio* sp. P-6-1 was stable at 40ºC but completely inactivated at 55ºC in 30 min[35] .

Fig. 4. Effect of Substrate concentration on activity of Chitinase.

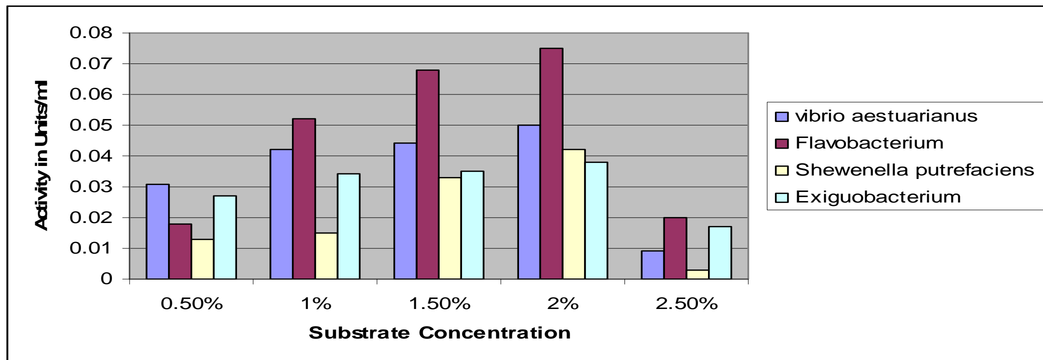


Fig. 5. Effect of 10mM $ZnSO_4$ on activity of Chitinase.

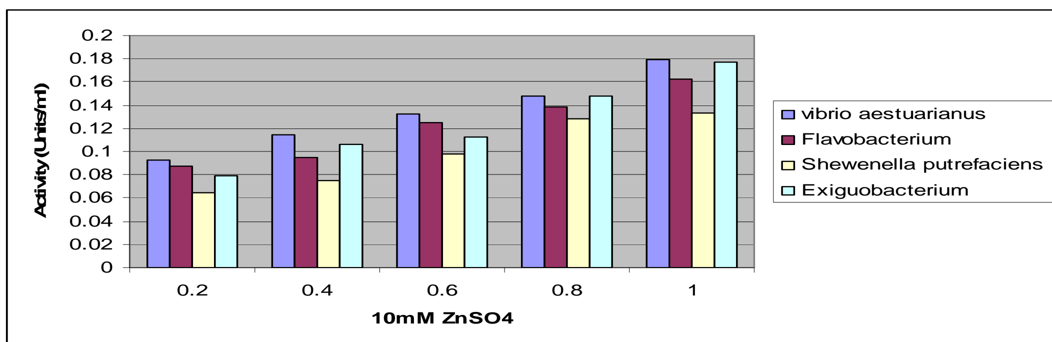


Fig. 6. Effect of 10mM KCl on activity of Chitinase.

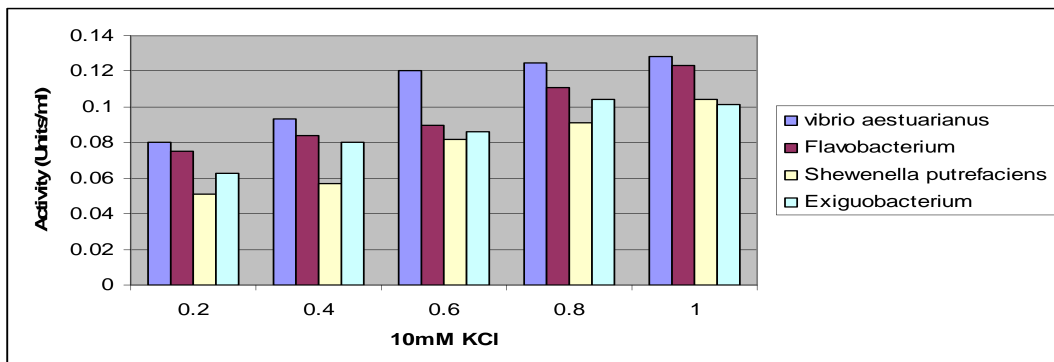


Fig. 7. Effect of 10mM $CaCl_2$ on activity of Chitinase.

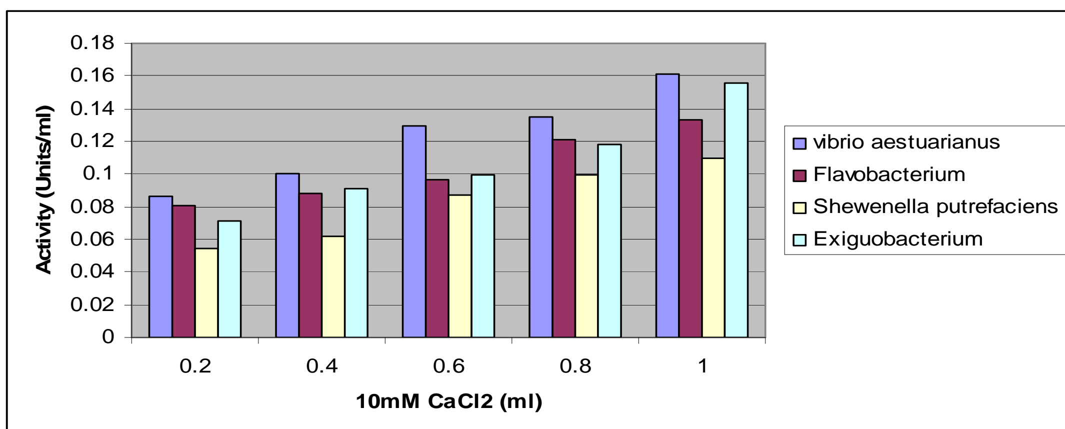
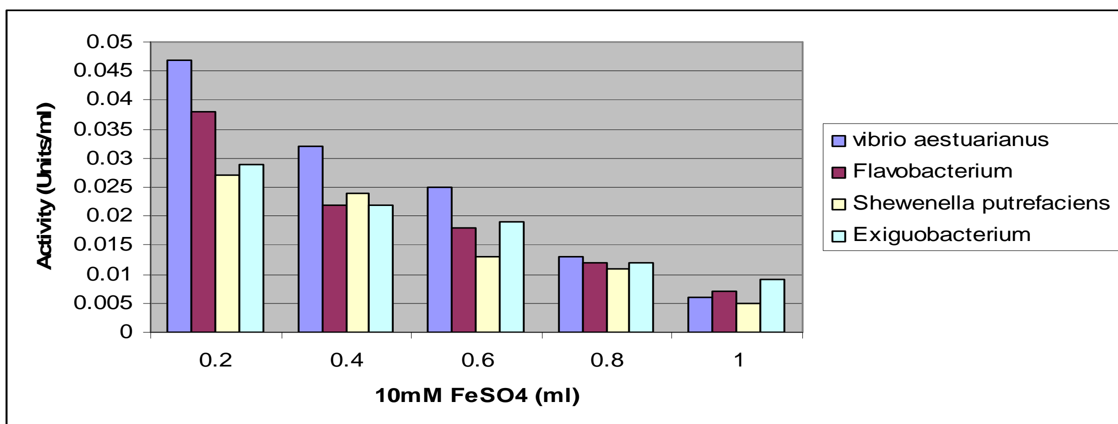


Fig. 8. Effect of 10mM $FeSO_4$ on activity of Chitinase.



The effect of substrate concentration on the activity of the enzyme was determined by assessing the enzyme activity at different concentration of substrate ranging from 0.5% to 2.5% of Colloidal chitin. Results suggested that 2.0% colloidal chitin has high specificity for chitinase from all the samples. Hence, 2% Colloidal chitin was the ideal substrate concentration for the maximum activity of Chitinase of all the isolated chitin degrading bacterial cultures (Fig. 4).

Among the different salt ions analyzed at different concentration, the $ZnSO_4$ was the best activator for the Chitinase activity among all the bacterial chitinase samples followed by KCl and $CaCl_2$ whereas, $FeSO_4$ was showing the most inhibitory activity on the Chitinase activity. The results of effect of different concentrations of a 10mM solution of Zinc Sulphate, Potassium Chloride, Calcium chloride and Ferrous Sulphate are shown (Fig. 5 to Fig. 8). Other chitinases from *A. hydrophila* H-2330[7], *Alteromonas* sp. O-7[28], *E. americana*[31], *P. aeruginosa* K-187[29]), and *Fusarium chlamydosporum*[36] were also inhibited by Fe2+, Fe3+ and/or Cu2+. Chitinase from *Alteromonas* sp. strain O-7[37] was activated by Na+ and Ca2+. The inhibition of chitinase by Fe2+ and Cu2+ could be related to the residues of aspartic and glutarmic acid in chitinases. It has been shown that these amino acids in the active sites of chitinases bind to certain divalent cations, thereby possibly inhibiting chitinases[38].

CONCLUSION

The capability of this chitinase to hydrolyze colloidal chitin efficiently, broad pH activity and stability makes the enzyme industrially significant for biotechnological applications, especially in production of chitobiose and N-acetyl D-glucosamine. The chitinolytic strains described here were assessed also for their ability to suppress the growth of several phytopathogenic fungi in vitro, and no enrichment techniques were used to specifically select chitinolytic bacteria. Though the study has to be extended to media optimization and production in large scale bioreactors, this finding enhances the potential of protein modification research that could substantially improve the function of chitinases in the production of chitin or chitosan derived products and also towards host plant defense.

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