Fishery Technology 50 (2013) : 66 - 74

Chitinase Production in a Fed-batch Fermentation of Colloidal Chitin using a Mixed Culture of *Vibrio harveyi* and *Vibrio alginolyticus*

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Abstract

Endochitinases convert the chitin polymer to N-Acetyl glucosamine (GlcNAc) and chitooligosaccharides (COS) by random cleavage at internal points in the chitin chain. *Vibrio harveyi* and *V. alginolyticus* showed chitinase activity on Luria-Bertani chitin agar (LBC) containing 2% colloidal chitin at 26°C and 37°C and at pH 5.0, 7.0 and 9.0. *V. alginolyticus* showed higher and more specific chemotaxis than *V. harveyi* towards COS. When grown individually, chitinase production was detected by the end of 1st day of incubation in *V. alginolyticus* (134 U L^{-1}), whereas in *V. harveyi* production was lower (84 U L^{-1}) and detected later (2nd day). The fed-batch fermentation of colloidal chitin (4L of LBC broth) performed in a 10L Biostat® B plus bioreactor using a mixed culture of *Vibrio harveyi* and *Vibrio alginolyticus* with daily addition of 2% colloidal chitin (w/v), constant temperature (30°C), dissolved oxygen 20% and agitation 150 rpm, yielded a product with 990 units of chitinase and a specific activity of 1.250 U mg^{-1} by the end of three days of fermentation. The concentration of fermented product reduced to 10% of the original volume using a tangential flow concentrator within 30 min, led to a 3 fold increase in specific activity but a 54% decrease in total chitinase activity.

Keywords: Chitinase, colloidal chitin, shrimp, fermentation, fed batch, *Vibrio harveyi, V. alginolyticus*

Received 7 February 2012; Revised 2 October 2012; Accepted 26 October 2012

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Introduction

India exported 151 465 t of frozen shrimp in 2010-11 which accounted for 44.17% (USD 1261.81 million) of the total marine export earnings in US dollars in 2010-11 (MPEDA, 2011). Shrimp industries generate large amounts of shrimp shell waste (Suresh & Chandrasekaran, 1998). Efficient conversion of waste to by-products has direct impact on the economy and environmental pollution. Chemical treatment of shrimp waste for production of by-products such as chitin and chitosan generate large volume of waste. Enzyme-based processes can be promising alternative to either replace the chemical processes or act as catalysts that help reduce the quantities of chemicals consumed.

Chitin, a homopolymer of β-(1–4)-linked *N*-acetyl-D-glucosamine, is the second most abundant and renewable natural polymer (Muzzarelli, 1999) present as a structural polysaccharide in fungal cell walls, in the exoskeleton of arthropods, the outer shell of crustaceans and nematodes (Dahiya et al., 2006). Endochitinases (poly 1,4-N-acetyl-β-D-glucosaminide glycanohydrolase; EC 3.2.1.14) are enzymes that convert the chitin polymer to soluble compounds namely GlcNAc (N-Acetyl glucosamine) and chitooligosaccharides (Howard et al., 2003) by random cleavage at internal points in the chitin chain. Chitooligosaccharides (COS) are homo- or heterooligomers of *N*-acetylglucosamine and *D*-glucosamine that are produced using chitin or chitosan as a starting material, using enzymatic conversions, chemical methods or combinations thereof. Production of COS is of immense interest since these oligosaccharides are thought to have several important bioactivities (Aam et al., 2010).

COS produced using endochitinase showed antibacterial activity against those bacteria, that cause diarrhoeal and emetic syndromes in humans (Ortiz-Rodriguez et al., 2010). Other potential of COS include as anti-asthma drugs (Donnelly & Barnes 2004; Kawada et al., 2007), anti-fungal agents (Oliveira et al., 2008; Seyfarth et al., 2008) and ingredients in wound-dressings (You et al., 2004; Ribeiro et al., 2009). Additionally it can reduce metastasis of tumors (Nam et al., 2007; Shen et al., 2009), increase bone-strength in osteoporosis (Ratanavaraporn et al., 2009), inhibit chitinases in *Plasmodium* parasites and thereby prevent malaria (Shahabuddin et al., 1993), act as immune modulators (Kim et al., 2006), and have lowering effect on serum glucose levels in diabetics (Lee et al., 2003).

Microbial chitinases find application as a clean alternative for chitinous waste management from shrimp processing units. Production of chitinases from bacteria is advantageous over fungi as bacteria grow faster and do not pose problems associated with mycelia growth. Chitinase production by *Vibrio harveyi* and *Vibrio alginolyticus* (Suginta et al., 2000; Defoirdt et al., 2010) and crystal structure of *V. harveyi* chitinase A has been elucidated (Songsiriritthigul et al., 2008). *V. harveyi* and *V. alginolyticus* are suitable species for chitinase production from shrimp shells as they have the ability to attach to the chitinous shrimp shell and release chitinases as virulence factors (Aguirre-Guzman et al., 2004). *V. harveyi* is a known shrimp pathogen (Austin et al., 2003) and *V. alginolyticus* is the most commonly detected *Vibrio* in the shrimp culture systems (Rao & Surendran, 2010). The present study uses a mixed culture of *V. harveyi* and *V. alginolyticus* in a fed batch fermentation system to produce chitinase from colloidal chitin.

Materials and Methods

V. harveyi ATCC 14126 and *V. alginolyticus* ATCC 17749 were used in all experiments. *V. harveyi* was cultured on photobacterium agar and *V. alginolyticus* on marine agar. Colloidal chitin was prepared using chitin from shrimp shells (Sigma C7170) and concentrated HCl (Hsu & Lockwood, 1975). The bacteria were spotted on pre-set plates of Luria-Bertani chitin (LBC) agar (2% colloidal chitin, 3% NaCl, 1.5% agar; casein digest peptone 1%, yeast extract 0.5%, pH 7.0 ± 0.1) and incubated either at 26°C or 37°C for > 3 days. Chitinase production is indicated by zones of clearance around colonies. LBC agar plates with pH adjusted to 5.0, 7.0, 9.0 and 11.0 were used to study the influence of pH on chitinase activity.

Preparation of chitooligosaccharides: Chitooligosaccharides were prepared by adding chitinase to colloidal chitin (1 unit to 4 ml ratio) and placed on rotary platform to maintain chitin in suspension. After incubation for 2 h at 25°C, the suspension was centrifuged at 5000 x g and the supernatant was filtered and used as chitooligosaccharide solution. This was considered as 100% COS from which 20, 40 and 80% COS solutions were prepared by aqueous dilution (v/v).

Chemotaxis of *V. harveyi* and *V. alginolyticus* towards chitooligosaccharides: Sterile pre-set plates containing chemotactic medium (10 mM phosphate buffer, 1 mM ammonium sulphate, 1 mM magnesium sulphate, 0.1 mM potassium EDTA) (Shaw, 1995) solidified with 1.5% low melting agarose were used for studying chemotaxis. Five wells were cut aseptically with an average distance of 8 mm between the outer margins of central and peripheral wells and sealed with a thin layer of the agarose. In the central well (C), 200 µl of either *V. harveyi* or *V. alginolyticus* culture was loaded. In the first well 100 µl of distilled water was loaded whereas in wells 2, 3 and 4, 100 µl volumes of aqueous COS solutions of 20, 40 and 80% concentrations (v/v) were loaded respectively. The plates were incubated at 26°C and observed daily for chemotactic movement.

Chitinase assay: Chitinase activity of culture supernatant was quantified colorimetrically by measuring the release of reducing sugars (Monreal & Reese, 1969). Briefly, 0.5 ml of centrifuged culture supernatant was added to 2 ml of colloidal chitin (1.25% w/v of colloidal chitin in 200mM potassium phosphate buffer, pH 6.0 with 2mM calcium chloride), incubated at 25°C for 2 h; placed in boiling water for 5 min and cooled in chilled water. One unit of β-N-acetylglucosaminidase (New England Biolabs) was added to the cooled substrate (2.5 ml), incubated at 25°C for 30 min and centrifuged. One ml of the resultant supernatant was added to 2 ml of distilled water and 1.5 ml of colour reagent (3,5, dinitrosalicylic acid and sodium potassium tartarate), placed in boiling water for 5 min, cooled to ambient temperature and A_{540} was measured. The amount of NAG liberated was determined using the standard curve with known amounts of NAG. Chitinase from

Streptomyces griseus (Sigma C6137) was used as the positive control.

One unit (U) of chitinase liberates 1 mg of NAG from chitin h^{-1}at pH 6.0 at 25°C in a two step reaction with β-N-acetylglucosaminidase (2 h assay). The quantity of chitinase produced was expressed as U L^{-1} or U mg^{-1} of protein.

Protein content: The culture supernatant was centrifuged at 10 000 rpm for 10 min and protein was determined as per Bradford (1976) using Bradford reagent (Sigma-B6916) employing a standard 3.1 ml assay protocol. Absorbance at 595 nm was measured and protein concentration was derived from bovine serum albumin (BSA) standard curve. Protein content was expressed as mg ml^{-1}.

Culture density: Optical density at 600 nm (OD_{600}) was measured employing a visible spectrophotometer.

Bioluminescence assay: Luminescence was measured in *V. harveyi* grown in LBC broth (2% colloidal chitin, 3% NaCl, casein digest peptone 1%, yeast extract 0.5%). One ml aliquots were transferred to microfuge tubes and luminescence (Relative Luminosity Units, RLU) was measured as 10 sec recording using steady-GLO parameter in a luminometer (Modulus™, Turner Biosystems).

Bioreactor experiment using *V. harveyi*: Biostat® A plus (Sartorius Stedim biotech, Germany) was used to grow *V. harveyi* in 1 L of LBC broth (pH 7.5) using 2% inoculums. The bioreactor conditions maintained were temperature 26°C, pH 7.5, pO_2 10%, air flow 0.6 L min^{-1}, agitation at 150 rpm. The culture conditions were continuously monitored using μDCU panel software. Dissolved oxygen level was measured by using the Oxyferm FDA 225 DO probe (Hamilton) and oxygen concentration was adjusted at a flow rate of 2 L min^{-1}. pH was measured using the Easyferm plus K8 200 probe (Hamilton). HCl (0.4M) and NaOH (0.4M) were provided to maintain pH. Antifoam B emulsion was used to knock out foam. Samples were drawn aseptically at daily intervals through the sample port and analysed for chitinase activity, cell density, protein content and bioluminescence.

Bioreactor experiment using *V. alginolyticus*: Bioflo® 2000 (New Brunswick Scientific, USA) was used to grow *V. alginolyticus* in 1 L of LBC broth (pH 7.5) using 2% inoculums. The bioreactor conditions maintained were temperature 26°C, pO_2 10%, air flow 0.6 L min^{-1}, oxygen flow rate 2 L min^{-1} and stirring at 180 rpm. Samples were drawn aseptically at daily intervals and analysed for chitinase activity, cell density, protein content and bioluminescence.

Fed-batch fermentation experiment: Biostat® B plus (Sartorius Stedim biotech, Germany) was used for the fed batch. Media used was 4 L of LBC broth (pH 7.5) containing 3% NaCl, 0.3% KH_2PO_4, 0.03% $MgSO_4$ and 2% (w/v) colloidal chitin. Overnight cultures of *V. harveyi* and *V. alginolyticus* grown separately in shake flasks, were used as inoculums at a final level of 5% (100 ml of each culture). The bioreactor conditions maintained were temperature 30°C, pO_2 20%, air flow rate and oxygen flow rate on auto mode and stirring at 150 rpm. The culture conditions were continuously monitored using μDCU panel software. Dissolved oxygen level was measured by using the Oxyferm FDA 425 DO probe (Hamilton) and pH was measured using the Easyferm plus K8 425 probe (Hamilton). Colloidal chitin was fed daily at 2% (w/v) level. Samples were drawn aseptically daily analysing for chitinase activity, cell density, protein content and bioluminescence.

Partial purification of chitinase: Partial purification of chitinase was performed using Tangential flow concentrator (Sartorius Sartojet, Germany) fitted with Sartocon slice cassette of 50 kDa pore size. The fermented material from the Biostat® B plus bioreactor was siphoned and centrifuged at 5000 x g for 20 min to remove cells and chitin. The supernatant was pumped through the tangential flow concentrator. The temperature was maintained at <4°C during all operations. The low molecular weight (<50 000 dalton) proteins pass into permeate whereas the proteins with molecular weight above 50 kDa are retained in the retentate. The concentrator was run for 30 min or until the volume of the retentate reached to about 10% of the starting volume. The proteins in the retentate were precipitated by 35 – 80% saturated ammonium sulphate, centrifuged at 12000 x g, 30 min, 4°C) and dissolved in 5 ml of 20 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl. The extracted protein was dialyzed using regenerated cellulose tubing (MWCO: 12000–14000) against 20 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl (overnight, three changes). The partially purified chitinase was assayed for activity and stored at -20°C.

Results and Discussion

Chitin agar screen: *V. harveyi* and *V. alginolyticus* showed chitinase activity on LBC agar at 26°C and 37°C by the end of three days of incubation (Fig. 1). *V. harveyi* showed a relatively greater zone of clearance (Fig. 1a) whereas *V. alginolyticus* swarmed over a wider area on the chitin agar (Fig. 1b). *V. alginolyticus* and *V. harveyi* showed chitinase activity at pH 5.0, 7.0, 9.0 by the end of third day of incubation. At pH 11.0, *V. harveyi* showed chitinase activity by the end of the sixth day with a smaller zone of clearance.

Chemotaxis of *V. alginolyticus* and *V. harveyi* towards COS: *V. alginolyticus* (Fig. 2) and *V. harveyi* showed positive chemotactic mobility towards chitooligosaccharides. The distance migrated by both the *Vibrio* species increased with the increase in the COS concentration. The distance migrated by *V. harveyi* was 7 , 17 and 22 mm for distilled water, 20% COS and 40% COS, respectively. Increase in COS concentration to 80% did not result in any further increase in chemotactic migration. In the case of *V. alginolyticus*, the distance migrated was 2, 20, 23 and 26 mm for distilled water, 20% COS, 40% COS and 80% COS, respectively. *V. alginolyticus* showed greater positive and specific chemotaxis towards COS than *V. harveyi*. Li & Roseman (2004) investigated the chemotactic effect of disaccharides from chitin on several strains of *Vibrio* and suggested that (GlcNAc)n was essential for signaling the presence of chitin in the microenvironment. Hirano et al. (2011) demonstrated that GlcNAc-GlcN also functioned as an effective chemo-attractant in *V. alginolyticus*.

Production of chitinase by *V. harveyi* in Biostat® **A plus bioreactor:** *V. harveyi* was grown in a 2L Biostat® A plus bioreactor using 1 L of LBC broth and the results are depicted in Fig. 3. Constant temperature (26°C), pH (7.5), dissolved oxygen (10%) and agitation (150 rpm) were maintained in the bioreactor. Chitinase production was detected only at the end of two days of incubation (84 U L^{-1}), which increased to 175 U L^{-1} by the end of 5th day. Suginta et al. (2000) reported 221 U L^{-1} of chitinase production by *V. carchariae* (*V. harveyi*). Maximum bioluminescence was observed at the end of first day (>10^7 RLU) and thereafter rapidly decreased to 12 931 RLU by the end of 5th day. The bioluminescence production and chitinase production appear to be inversely related. The extracellular protein content increased gradually from 0.04mg ml^{-1} (1st day) to 0.14 mg ml^{-1} (5th day). The specific activity of chitinase showed a steady increase; the values being 0, 0.7 and 1.25 U mg^{-1} of protein at the end of 1st, 2nd and 5th day of incubation, respectively. The results show that, under controlled conditions, chitinase production in *V. harveyi* ATCC 14126 appears only after 2 days of incubation during the stationary phase, and decreasing bioluminescence is an indicator for chitinase production.

Production of chitinase by *V. alginolyticus* in Bioflo® **2000 bioreactor:** *V. alginolyticus* was grown in a 2L New Brunswick Bioflo® 2000 bioreactor using 1 L of LBC broth at pH 7.5, 26°C, 10% DO and 180 rpm agitation. The results are depicted in Fig. 4. *V. alginolyticus* produced chitinase by the end of 1st day of incubation (134 U L^{-1}), which increased to 175 U L-1 by the end of 5th day of incubation.

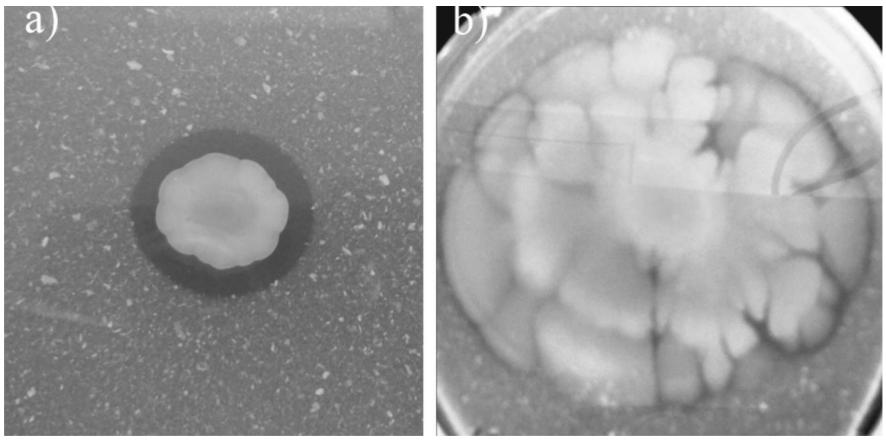

Fig. 1. Chitinase activity on Luria-Bertani chitin (LBC) agar;
a) *Vibrio harveyi* b) *Vibrio alginolyticus*

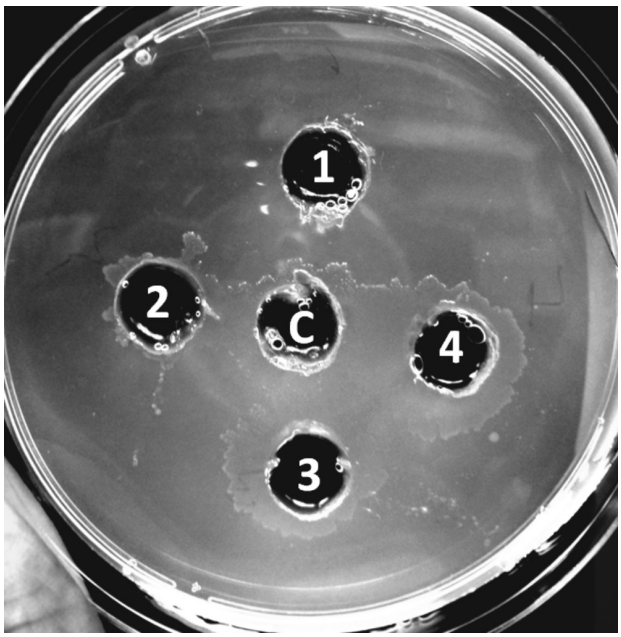

Fig. 2. Chemotaxis of *Vibrio alginolyticus* towards chitooligosaccahides (COS)

Centre well C : 200 µl *Vibrio alginolyticus* culture; well 1: 100 µl distilled water; well 2 : 100 µl of 20% COS solution; well 3 : 100 µl of 40% COS solution; well 4 : 100 µl of 80% COS solution.

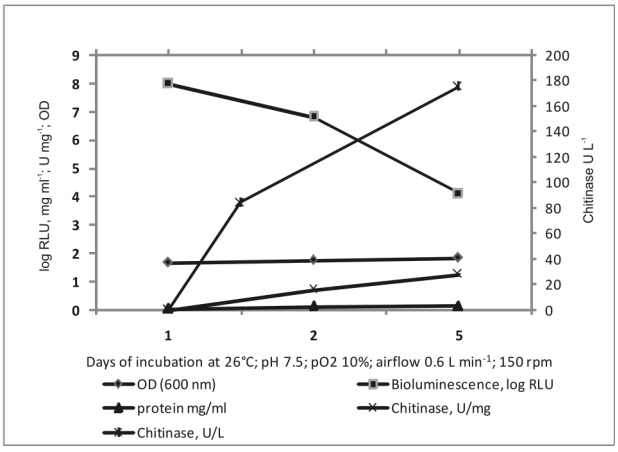

Fig. 3. Production of chitinase by *Vibrio harveyi* in 2L Biostat® A plus bioreactor

The extracellular protein content increased marginally from 0.06 mg ml^{-1} (1st day) to 0.08 mg ml^{-1} (5th day). The maximum specific activity of *V. alginolyticus* chitinase was obtained at the end of 1st day of incubation. Suginta et al. (2000) reported a maximum specific activity of 0.78 U mg^{-1} of protein for *V. alginolyticus* which was observed after 4 days in shake flask culture. The higher specific activity of the product in the bioreactor might be due to better control of fermentation conditions. pH of the bioreactor culture initially dropped from 7.5 to 7.01 (1st day) and later steadily increased to 7.59 (2nd day) and finally reached 8.16 (5th day).

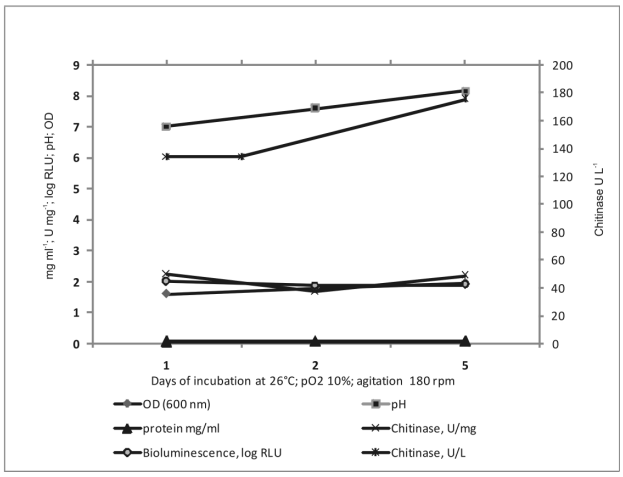

Fig. 4. Production of chitinase by *Vibrio alginolyticus* in 2L Bioflo® 2000 bioreactor

Comparison of chitinase production by *V. alginolyticus* and *V. harveyi* Chitinase production was detected by the end of 1st day of incubation in *V. alginolyticus* (134 U L^{-1}) whereas chitinase production was detected later (2nd day) and lower (84 U L^{-1}) in *V. harveyi*. Initial (1st day) extracellular protein production was higher in *V. alginolyticus* (0.06 mg ml^{-1}) than in *V. harveyi* (0.04 mg ml^{-1}) but on subsequent days the extracellular protein production was higher in *V. harveyi* (0.12 mg ml^{-1}after 2 days and 0.14 mg ml^{-1}after 5 days). The specific activity of *V. alginolyticus* chitinase was maximum at the end of 1st day (2.23 U mg^{-1} of protein) whereas the specific activity of *V. harveyi* chitinase attained a maximum value of 1.25 U mg^{-1} of protein at the end of 5th day of incubation. The results show that *V. alginolyticus* ATCC 14126 is a better candidate species for chitinase production as it produces chitinase rapidly and higher quantities than *V. harveyi* ATCC 14126. Although the temperature (26°C) and dissolved oxygen (10%) were similar in both the bioreactors, differences existed in pH and agitation. *V. alginolyticus* was grown at a higher agitation (180 rpm) and under uncontrolled pH conditions and these might have influenced chitinase production (Donderski & Trzebiatowska, 2000).

Fed-batch fermentation experiment: A fed batch fermentation with mixed culture of *V. harveyi* and *V. alginolyticus* was performed to assess their synergistic potential for chitinase production. *V. harveyi* and *V. alginolyticus* were grown simultaneously in a Biostat® B plus bioreactor and the results are depicted in Fig. 5. Temperature (30°C), dissolved oxygen (20%) and agitation (150 rpm) were maintained in the bioreactor whereas pH was uncontrolled but monitored. Colloidal chitin was fed daily at 2% (w/v) level; the volume of colloidal chitin was adjusted to 200 ml with sterile distilled water before addition.

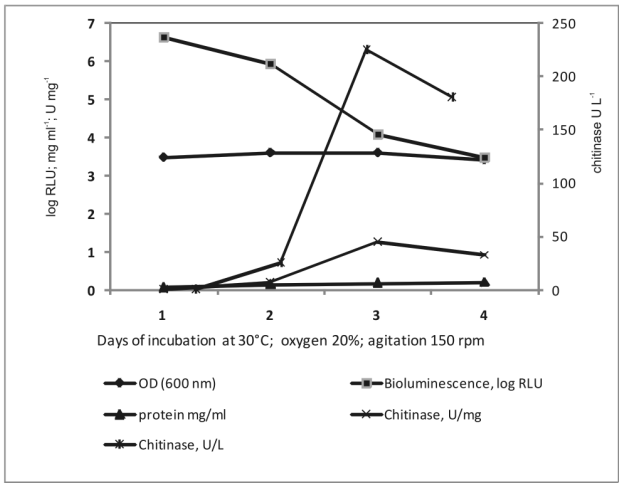

Fig. 5. Production of chitinase by mixed culture of *Vibrio harveyi* and *Vibrio alginolyticus* in Biostat® B Plus bioreactor

Chitinase production was detected only at the end of two days of incubation (25 U L^{-1}), which increased to 225 U L^{-1} by the end of 3rd day and decreased to 180 U L^{-1} by the end of 4th day (Fig. 5). Maximum bioluminescence was observed at the end of first day (4,218,143 RLU) and thereafter rapidly decreased to 2992 RLU by the end of 4th day. The bioluminescence production and chitinase production showed inverse relationship. Cross species induction of bioluminescence in *V. harveyi* by *V. alginolyticus* and the quorum sensing regulated expression of bioluminescence and chitinase production might have been the factors for lower production of chitinase during the initial days of fed-batch fermentation. Induction of intra- and inter-species bacterial communication for coordinated gene expression is mediated by extracellular molecules termed autoinducers in a cell density dependent regulation process called quorum sensing (Miller & Bassler, 2001). Three parallel quorum sensing systems based on three autoinducers viz., AI-1 (N-homoserine lactone), AI-2 (furanosyl borate diester) and CAI (cholera auto inducer) regulate gene expression (including bioluminescence) in *V. harveyi* (Nealson & Hastings 1979; Meighen 1991; Henke & Bassler, 2004; Jung 2011). Cross species induction of luminescence in *V. harveyi* by cell free culture fluid of several bacterial species including *V. alginolyticus* has been reported (Greenberg et al., 1979; Bassler et al., 1997).

The extracellular protein content increased gradually from 0.06 mg ml^{-1} (1st day) to 0.20 mg ml^{-1} (5th day). The maximum specific activity of chitinase was obtained at the end of 3rd day (1.25 U mg^{-1} of protein) of incubation. pH of the bioreactor culture initially showed a steady increase and finally reached 8.47 (4th day). The total units of chitinase produced by the mixed culture of *V. alginolyticus* and *V. harveyi* in the fed-batch system are given in Table 1. Maximum quantity of 990 units of chitinase with a specific activity of 1.25 U mg^{-1} of protein was obtained at the end of 3rd day and so in fed-batch day 3 would be the ideal time to harvest the culture for purification of chitinase.

Partial purification of chitinase was performed using Tangential flow concentrator fitted with Sartocon of 50 kDa pore size slice cassette as the chitinase secreted by *V. alginolyticus* and *V. harveyi* have a molecular weight above 50 kDa. The predominant chitinase secreted by *V. harveyi* has a molecular weight of 63 kDa (Suginta et al., 2000). Mass fingerprinting data of chitin binding proteins secreted by *Vibrio alginolyticus* strain 283 identified the 90, 65, and 47 kDa proteins as chitinases (Suginta, 2007).The molecular weights of chitinases C1 and C3 from *Vibrio alginolyticus* H-8 were estimated to be 81 kDa and 68 kDa, respectively (Ohishi et al., 1996). The fermented material (4600 ml) from the Biostat B plus ® bioreactor was concentrated to 10% of the starting volume (retentate) comprising mostly of high molecular weight proteins (> 50 kDa). This procedure is rapid and aids in the concentration of the fermented culture which enables further processing steps of enzyme purification, easier. The specific activity of chitinase in the retentate increased to 2.65 U mg^{-1} of protein which can be attributed to the filtering out of low molecular extracellular proteins but the main disadvantage was the higher loss in chitinase activity which dropped from 828 units to 382 units

Table 1. Chitinase production by mixed culture of *Vibrio alginolyticus* and *Vibrio harveyi* in the fed-batch system

Completed days of incubation	Total volume in Bioreactor* (ml)	Total chitinase activity (units)	Specific activity (U $mg^{-1)}$
1 day	4000	0	0
2 day	4200	105	0.179
3 day	4400	990	1.250
4 days	4600	828	0.900
Tangential Filtration Retentate	4600	382	2.650

* 200 ml of colloidal chitin solution was fed daily

(Table 1.). The loss in chitinase activity might be due to the denaturation of the enzyme during the pumping of the supernatant under pressure through the filter. Ammonium sulphate precipitation and dialysis of the retentate, marginally increased the specific activity (2.89 U mg^{-1}) but there was further loss in the total chitinase activity. The fed-batch fermentation of colloidal chitin using a mixed culture of *Vibrio harveyi* and *Vibrio alginolyticus* and daily addition of 2% colloidal chitin (w/v) yielded a product with 990 units of chitinase with a specific activity of 1.25 U $mg^{-1}$of protein by the end of three days of fermentation. Although the conditions of fermentation were different, *V. alginolyticus* when used singly produced relatively higher quantity of chitinase (134 U L^{-1}) and maximum specific activity (2.23 U mg^{-1} of protein) by the end of 1st day of incubation.

The results show that, for chitinase production, single culture of *V. alginolyticus* is better than mixed culture with *V. harveyi*. *V. harveyi* might down regulate the expression of chitinase by *V. alginolyticus*. The concentration of fermented product to 10% of the original volume was achieved within 30 min by tangential flow concentrator but the major drawback was loss in activity.

Acknowledgements

B. Madhusudana Rao was supported by the National Agricultural Innovation Project (NAIP-Component-I), Indian Council of Agricultural Research (ICAR) and Central Institute of Fisheries Technology (CIFT), Cochin, India. We thank the University of North Carolina, Pembroke (UNCP) for providing facilities.

References

Aam, B.B., Heggset, E.B., Norberg, A.L., Sorlie, M., Varum, K.M. and Eijsink, V.G.H. (2010) Production of chitooligosaccharides and their potential applications in medicine. Mar. Drugs 8: 1482-1517

Aguirre-Guzman, G., Ruíz, H.M. and Ascencio, F. (2004) A review of extracellular virulence product of vibrio species important in disease of cultivated shrimp. Aquaculture Res. 35: 1395–1404

Austin, B., Pride, A.C. and Rhodie, G.A. (2003) Association of a bacteriophage with virulence in *Vibrio harveyi*. J. Fish Dis. 26: 55–58

Bassler, B.L., Greenberg, E.P. and Stevens, A.M. (1997) Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. 179: 4043-4045

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Dahiya, N., Tewari, R. and Hoondal, G.S. (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl. Microbiol. Biotechnol. 71: 773-782

Defoirdt, T., Ruwandeepika, H.A.D., Karunasagar, I., Bon, N. and Bossier, P. (2010) Quorum sensing negatively regulates chitinase in *Vibrio harveyi*. Environ. Microbiol. Rep. 2: 44-49

Donderski, W. and Trzebiatowska, M. (2000) Influence of physical and chemical factors on the activity of chitinases produced by planktonic bacteria isolated from Jeziorak lake. Polish J. Environ. Stud. 9: 77-82

Donnelly, L.E. and Barnes, P.J. (2004) Acidic mammalian chitinase - a potential target for asthma therapy. Trends. Pharmacol. Sci. 25: 509–511

Greenberg, E.P., Hastings, J.W. and Ulitzer, S. (1979). Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Arch. Microbiol. 120: 87-91

Henke, J.M. and Bassler, B.L. (2004). Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. J. Bacteriol. 186: 6902-6914

Hirano, T., Aoki, M., Kadokura, K., Kumaki, Y., Hakamata, W., Oku, T. and Nishio, T. (2011) Heterodisaccharide 4-O-(N-acetyl-b-D-glucosaminyl)-D-glucosamine is an effective chemotactic attractant for Vibrio bacteria that produce chitin oligosaccharide deacetylase. Lett. Appl. Microbiol. 53: 161–166

Howard, M.B., Ekborg, A.N., Weiner, R.M. and Hutcheson, S.W. (2003) Detection and characterization of chitinases and other chitin-modifying enzymes. J. Ind. Microbiol. Biotechnol. 30: 627–635

Hsu, S.C. and Lockwood, J.L. (1975) Powdered chitin agar as a selective medium for enumeration of Actinomycetes in water and soil. Appl. Microbiol. 29: 422-426

Jung, K. (2011) Tuning communication fidelity. Nat. Chem. Biol. 7: 502-503

Kawada, M., Hachiya, Y., Arihiro, A. and Mizoguchi, E. (2007) Role of mammalian chitinases in inflammatory conditions. Keio. J. Med. 56: 21–27

Kim, H.M., Hong, S.H., Yoo, S.J., Baek, K.S., Jeon, Y.J. and Choung, S.Y. (2006) Differential effects of chitooligosaccharides on serum cytokine levels in aged subjects. J. Med. Food. 9: 427–430

Lee, H.W., Park, Y.S., Choi, J.W., Yi, S.Y. and Shin, W.S. (2003) Antidiabetic effects of chitosan oligosaccharides in neonatal streptozotocin-induced noninsulin-dependent diabetes mellitus in rats. Biol. Pharm. Bull. 26: 1100–1103

Li, X. and Roseman, S. (2004) The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor-kinase. PNAS, 101: 627–631

Meighen, E.A. (1991) Molecular biology of bacterial bioluminescence. Microbiol. Rev. 55: 123-142

Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. Ann. Rev. Microbiol. 55: 165-199

MPEDA (2011) Marine products exports from India during 2010-11. http://www.mpeda.com (Accessed 02 January 2012)

Monreal, J. and Reese, E.T. (1969) The chitinase of *Serratia marcescens*. Can. J. Microbiol. 15: 689-696

Muzzarelli, R.A. (1999) Native, industrial, and fossil chitins. In: Chitin and Chitinases (Jolles, P. and Muzzarelli, R., Eds), Basel, Switzerland

Nam, K.S., Kim, M.K. and Shon, Y.H. (2007) Inhibition of proinflammatory cytokine-induced invasiveness of HT-29 cells by chitosan oligosaccharide. J. Microbiol. Biotechnol. 17: 2042–2045

Nealson, K.H. and Hastings, J.W. (1979) Bacterial bioluminescence: Its control and ecological significance. Microbiol. Rev. 43: 496-518

Ohishi, K., Yamagishi, M., Ohta, T., Suzuki, M., Izumida, M., Sano, H., Nishijima, M. and Miwa, T. (1996) Purification and properties of two chitinases from *Vibrio alginolyticus* H-8. J. Ferment. Bioeng. 82: 598-600

Oliveira, E.N.Jr., El Gueddari, N.E., Moerschbacher, B.M., Peter, M.G. and Franco, T.T. (2008) Growth of phytopathogenic fungi in the presence of partially acetylated chitooligosaccharides. Mycopathologia. 166: 163–174

Ortiz-Rodríguez, T., de la Fuente-Salcido, N., Bideshi, D.K., Salcedo-Hernandez, R. and Barboza-Corona, J.E. (2010) Generation of chitin-derived oligosaccharides toxic to pathogenic bacteria using ChiA74, an endochitinase native to *Bacillus thuringiensis*. Lett. Appl. Microbiol. 51: 184-190

Rao, B.M. and Surendran, P.K. (2010) Genetic heterogeneity of non-O1 and non-O139 *Vibrio cholerae* isolates from shrimp aquaculture system: a comparison of RS-, REP- and ERIC-PCR fingerprinting approaches. Lett. Appl. Microbiol. 51: 65–74

Ratanavaraporn, J., Kanokpanont, S., Tabata, Y. and Damrongsakkul, S. (2009) Growth and osteogenic differentiation of adipose-derived and bone marrow-derived stem cells on chitosan and chitooligosaccharide films. Carbohydr. Polym. 78: 873–878

Ribeiro, M.P., Espiga, A., Silva, D., Baptista, P., Henriques, J., Ferreira, C., Silva, J.C., Borges, J.P., Pires, E., Chaves, P. and Correia, I.J. (2009) Development of a new chitosan hydrogel for wound dressing. Wound Repair Regen. 17: 817–824

Seyfarth, F., Schliemann, S., Elsner, P. and Hipler, U.C. (2008) Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and N-acetyl-D-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata*. Int. J. Pharm. 353: 139–148

Shahabuddin, M., Toyoshima, T., Aikawa, M. and Kaslow, D.C. (1993) Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. Proc. Natl. Acad. Sci. USA. 90: 4266–4270

Shaw, C.H. (1995) Agrobacterium tumifaciens chemotaxis protocols. In : Agrobacterium Protocols: Methods in molecular biology. (Gartland, K.M.A. and Davey M,R. Eds.), Humana Press Inc. Totowa, NJ, 44: 29-36

Shen, K.T., Chen, M.H., Chan, H.Y., Jeng, J.H. and Wang, Y.J. (2009) Inhibitory effects of chitooligosaccharides on tumor growth and metastasis. Food Chem. Toxicol. 47: 1864-1871

Songsiriritthigul, C., Pantoom, S., Adeleke, H., Aguda, A.H., Robinson, R.C. and Suginta, W. (2008) Crystal structures of *Vibrio harveyi* chitinase. A complexed with chitooligosaccharides: Implications for the catalytic mechanism. J. Structural Biol. 162: 491-499

Suginta, W., Robertson, P.A.W., Austin, B., Fry, S.C. and Fothergill-Gilmore, L.A. (2000) Chitinases from Vibrio: activity screening and purification of chiA from *Vibrio carchariae*. J. Appl. Microbiol. 89: 76-84

Suginta, W. (2007) Identification of chitin binding proteins and characterization of two chitinase isoforms from *Vibrio alginolyticus* 283. Enzyme Microb. Technol. 41: 212-220

Suresh, P.V. and Chandrasekaran, M. (1998) Utilization of prawn waste for chitinase production by the marine fungus *Beauveria bassiana* by solid state fermentation. World J. Microbiol. Biotechnol. 14: 655–660

You, Y., Park, W.H., Ko, B.M. and Min, B.M. (2004) Effects of PVA sponge containing chitooligosaccharide in the early stage of wound healing. J. Mater. Sci. Mater. Med. 15: 297-301