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Studies on Screening and Characterization of Chitinolytic Bacteria from Shrimp Farming Ponds

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Abstract

Shrimp are swimming, decapods crustaceans classified in the order Cardiea, found widely around the world in both fresh and salt water. Asia has always led world production of cultivated shrimp with a market value of billions of US dollars per year. Shrimp aquaculture is one of the fastest growing agricultural industries, with more than 10% average growth in last decade. The extensive farming of shrimp in ponds has widely increases the chitinolytic microorganisms due to the abundance nature of chitinous wastes. *Bacillus* species are well known for their extracellular chitinase enzyme production and industries majorly uses *Bacillus subtilis* for the production of various enzymes. *Bacillus* is rod shaped bacterium with protective endospore that can withstand extreme conditions. They are obligate aerobes or facultative anaerobes in which few are pathogenic and others are free living. The present study reports The Screening and characterization of chitinolytic bacteria from various shrimp ponds. From 11 different samples 40 bacterial species were isolated and screened. Two bacterial species KK2 and VMP were selected for the optimization of chitinase enzyme using various parameters such as carbon, nitrogen and pH. In order to identify the bacteria species using molecular methods two isolates KK2 and VMP were selected for genome sequencing.

Article Info

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Keywords

Chitinase, Hicrome Bacillus agar, *Bacillus* sps, colloidal chitin

Introduction

Chitosan is an amino polysaccharide composed mainly of 2-amino-2-deoxy-Dglucose units also known as glucosamine (GLcN) linearly linked by beta-1,4glycosidic linkages with varying degrees of acetylation. Generally, more than 80% of glucosamine units are deacetylated. The hydrolysis of this molecule promotes breakage of the polysaccharide chain with consequent release of oligosaccharide and monosaccharide. The production of oligosaccharide from chitosan has been the focus of several studies in the pharmaceutical, chemical, food and medical areas, due to their functional properties. Inspite of their abundance in nature. The commercial utilization of chitin and chitosan has remained undeveloped for a long time. The insolubility of chitin in common solvent limits the utilization of chitin as a natural resource. Chitinase are ubiquitous in nature, being found in eukaryotes, prokaryotes, archaea and viruses (Suzanne *et al.*, 2001). Chitosan is one of the most abundant biomasses on earth chito-oligosaacharides from chitosan polymer have become a remarkable resource for the development of functional foods, artificial skin, medicine and other

materials attempted by methods such as the treatment of chitosan polymer by chemicals or enzyme synthetic production of chito-oligosaccharide from as the treatment of chitosan polymer.

Shrimp chitin can be extracted by chemical, physical, enzymatic or microbiological methods. The use of corrosive chemicals such as strong acids or bases makes the process of chitin extraction ecologically unacceptable. Recovery of chitin by biotechnological process from crustacean shell waste consists of two fundamental steps: protein degradation or demineralization with HCl, ethanol has wide application in preparation of colloidal chitin (OlfaGhorbel-Bellaaj *et al.*, (2012).

Materials and Methods

Isolation and identification

Various samples from different shrimp ponds were collected from various sites of Cuddalore &Pondicherry and bought to the laboratory for processing. These samples were subjected for serial dilution technique and spread plate method was performed. Chitosan minimal salt agar medium used to isolate the Chitinolytic producing bacteria and then further it was screened and its phenotypic character was studied by using Hicrome Bacillus agar for Identifying *Bacillus* species. The isolates was identified based on cellular morphology, Gram staining, endospore staining and biochemical tests, and further confirmed by molecular level taxonomy.

Screening of chitinase utilizers

Different types of bacteria were isolated from various shrimp ponds by serial dilution method and they were screened for the production of chitinase enzyme. Sterile production medium called chitosan minimal salt agar medium was inoculated with the organism. After incubation period, the clear zone was formed. Formation of zone around the organism was considered as positive [chitinase utilizers]

Optimization of chitinase

Effect of pH on chitinase production

The isolates such as GUW1,PPS2 and VMP were inoculated in chitosan minimal salt broth at different pH ranges from pH 4 to 9 and incubated at 37℃ for 48 hours. The chitinase production was observed in spectrophotometer at OD of 560nm.

Effect of various nitrogen source on chitinase production

The isolates such as PSS3, PPS5 were inoculated in the chitosan minimal salt broth supplemented with various nitrogen source such as Casein, peptone, yeast extract, beef extract, ammonium chloride, 40% urea incubated at 37℃ for 48 hours. The chitosanase activity was observed in spectrophotometer at OD of 560nm.

Effect of various carbon sources on chitinase production

The isolates such as PSS3 and PPS5 were inoculated in the chitosan minimal salt broth supplemented with various nitrogen sources such as Glucose , Sucrose, Lactose, Maltose, Mannitol, Glycerol incubated at 37 ℃ for 48 hours. The chitinase activity was observed in spectrophotometer at OD of 560nm.

Genome sequencing

The following cultures KK2 and VMP were subjected for genome sequencing and the sequencing was done in PACE Microbial technology. Puducherry.

16srRNA isolation, amplification, sequencing and treeing programme protocol

1. Preparation of template DNA It is important to use a pure cultured bacterium for identification. Colonies are picked up with a sterilized toothpick, and suspended in 0.5ml of sterilizes saline in a 1.5ml centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5ml of InstaGene Matrix (Bio-Rad, USA). Incubated 56°C for 30 min and then heated 100°C for 10 min. After heating, supernatant can be for PCR.

2. PCR Add 1µl of template DNA in 20µl of PCR reaction solution. Use 518 F/800 R primers for bacteria, and then perform 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragment are amplified about 1,400bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

518 F	5’CCAGCAGCCGCGGTAATACG 3’
800 R	5’TACCAGGGTATCTAATCC 3’

Purification

Products remove unincorporated PCR primers and dNTPs from PCR product by using Montage PCR clean up kit (Millipore).

Sequencing

The purified PCR products of approximately 1,400bp were sequenced by using the primers (785 F 5' GGA TTA GAT ACC CTG GTA 3' and 907 R 5' CCG TCA ATT CCT TTR AGT TT 3'). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product were resolved on an Applied BioSystems were model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Phylogenetic tree construction

The culture sequence obtained were subjected to BLAST analysis, the phylogenetically similar type strains sequences and other phylogenetic related sequence were selected from the Gen Bank and they were subjected to multiple sequence alignment and then align sequences were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6. In the tree the number at the nodes indicate levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbour- joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.005 substitutions per site.

Results and Discussion

Various water and sediment soil samples from different sites were processed and identified using various tests and the results are shown below (Table 1–9):

Screening for chitinolytic bacteria using chitosan minimal salt agar medium

40 Chitinase producing bacterial islolates were isolated from various water and sediment soil samples using Chitosan minimal salt agar by the appearance of Zone of clearance around the bacterial colonies (Chitin utilization).

Optimization of chitinase enzyme at different parameters

Chitinase enzyme at different pH

The isolates such as GUW1 and PPS5 were inoculated in Chitinase minimal salthbroth at different pH 4to 9 and incubated at 37°C for 48 hours. The chitinase production was observed in spectrophotometer at OD 560nm.

Results of genome sequencing

The following cultures KK2 and VMP were subjected for genome sequencing and the results were identified by molecular method such as, KK2 –*Bacillus cereus* and VMP-*Photobacterium ganghwense*. The genome sequenced isolates were submitted to NCBI-GENBANK and the culture name and accession number of KK2and VMP are MH173816 and MH173815 respectively.

Table.1 Distribution of chitinase producing bacterial isolates

S.NO	SAMPLE	SAMPLING AREA	NUMBER OF ISOLATES
1	Pond Water	GunduUppalavadi (Cuddalore)	5
2	Sediment soil	Pudhukuppam	5
3	Sediment soil	Periyasamy street	4
4	Sediment soil	Parangipettai	4
5	Pond Water	Parangipettai	2
6	Sediment soil	Thazhanguda	3
7	Pond Water	Kandakkadu	4
8	Sediment soil	Alapakkam	2
9	Sediment soil	Chidambaram	5
10	Pond Water	Metupalayam	5
11	Sediment soil	Marakkanam	1

Table.2 Gram staining and Spore staining results

S.NO	ISOLATES	GRAM STAINING	SPORE STAINING	MORPHOLOGY ON HICROME BACILLUS AGAR
1	GUW1	Gram positive rods	Spore forming	Yellowish green to green
2	GUW2	Gram positive rods	Spore forming	Yellow mucoid
3	GUW3	Gram positive rods	Spore forming	Yellowish green to green
4	GUW5	Gram positive rods	Spore forming	Yellow mucoid
5	GUW6	Gram positive Rods	Spore forming	Light green to green
6	PSS1	Gram positive rods	Spore forming	Light blue, large, flat with irregular margins
7	PSS2	Gram positive rods	Spore forming	Light blue large, flat with irregular margins
8	PSS3	Gram positive rods	Spore forming	Yellowish green to green
9	PSS4	Gram positive rods	Spore forming	Light blue, large, flat with irregular margins
10	PSS5	Gram positive rods	Spore forming	Yellow mucoid
11	PKS1	Gram positive rods	Spore forming	Yellow mucoid
12	PKS2	Gram positive rods	Spore forming	Yellow mucoid
13	PKS3	Gram positive rods	Spore forming	Light blue, large,flat with irregular margin
14	PKS4	Gram positive rods	Spore forming	Light blue, large,flat with irregular margins
15	PPS1	Gram positive rods	Spore forming	Pink small raised
16	PPS2	Gram positive rods	Spore forming	Yellow mucoid
17	PPS3	Gram positive rods	Spore forming	Yellow mucoid
18	PPS5	Gram positive rods	Spore forming	Yellow mucoid
19	PPW1	Gram positive rods	Spore forming	Yellow mucoid
20	PPW3	Gram positive rods	Spore forming	Yellow mucoid
21	TGS1	Gram positive rods	Spore forming	Light blue, large, flat with blue center
22	TGS3	Gram positive rods	Spore forming	Yellowish green to green
23	TGS4	Gram positive rods	Spore forming	Yellowish green to green
24	KKW1	Gram positive rods	Spore forming	Yellow mucoid
25	**KK2**	Gram positive rods	Spore forming	Light blue, large, flat with blue center
26	KKW3	Gram positive rods	Spore forming	Pink small raised
27	KKW4	Gram positive rods	Spore forming	Yellowish green to green
28	APS1	Gram positive rods	Spore forming	Pink small raised
29	APS2	Gram positive rods	Spore forming	Yellow mucoid
30	CHDS1	Gram positive rods	Spore forming	Yellow mucoid
31	CHDS2	Gram positive rods	Spore forming	Yellow mucoid
32	CHDS3	Gram positive rods	Spore forming	Yellow mucoid
33	CHDS4	Gram positive rods	Spore forming	Yellowish green to green
34	CHDS5	Gram positive rods	Spore forming	Light green to green
35	MPS1	Gram positive rods	Spore forming	Yellow mucoid
36	MPS2	Gram positive rods	Spore forming	Yellow mucoid
37	MPS3	Gram positive rods	Spore forming	Pink small raised
38	MPS4	Gram positive rods	Spore forming	Pink small raised
39	MPS5	Gram positive rods	Spore forming	Yellow mucoid
40	**VMP**	Gram negative rods	Non spore forming	-

Table.3 Biochemical Reactions

S.NO	ISOLATES	INDOLE	MR	VP	CITRATE	UREASE	TSI	LIA	CATALASE	OXIDASE
1	GUW1	-	+	-	+	+	K/A	K/K	+	+
2	GUW2	-	+	-	+	-	A/A	K/K	+	+
3	GUW3	-	+	-	+	-	A/A	K/K	+	+
4	GUW5	-	+	-	+	-	A/A	K/K	+	+
5	GUW6	-	+	-	+	+	A/A	K/K	+	-
6	PSS1	-	+	-	+	-	K/A	K/K	+	+
7	PSS2	-	+	-	-	-	K/A	K/K	+	+
8	PSS3	-	+	-	+	-	K/A	K/K	+	+
9	PSS4	-	+	-	+	-	A/A	K/K	+	+
10	PSS5	-	+	-	-	-	K/A	K/K	+	+
11	PKS1	-	+	-	+	-	A/A	K/K	+	+
12	PKS2	-	+	-	+	-	A/A	K/K	+	-
13	PKS3	-	+	-	+	-	A/A	K/K	+	+
14	PKS4	-	+	-	+	-	K/A	K/K	+	+
15	PPS1	-	+	-	+	-	K/A	K/K	-	+
16	PPS2	-	+	-	-	-	A/A	K/K	+	-
17	PPS3	-	+	-	+	-	A/A	K/K	+	-
18	PPS5	-	+	-	+	-	K/A	K/K	+	+
19	PPW1	-	+	-	+	-	K/A	K/K	+	-
20	PPW3	-	+	-	+	-	K/A	K/K	+	-
21	TGS1	-	-		+	-	K/A	K/K	+	-
22	TGS3	-	+	-	+	+	K/A	K/K	+	-
23	TGS4	-	-	-	+	+	K/A	K/K	+	-
24	KKW1	-	+	-	+	-	K/A	K/K	+	-
25	**KK2**	-	+	+	-	-	K/A	K/K	+	+
26	KKW3	-	-	+	-	+	K/A	K/K	+	+
27	KKW4	-	+	-	+	+	K/A	K/K	+	+
28	APS1	-	+	+	-	-	K/A	K/K	-	+
29	APS2	-	-	+	+	+	K/A	K/K	+	+
30	CHDS1	-	-	-	-	-	A/A	K/K	+	+
31	*CHDS2*	-	-	-	-	-	K/A	K/K	+	+
32	*CHDS3*	-	-	-	-	-	A/A	K/K	+	+
33	CHDS4	-	-	-	-	-	K/A	K/K	+	+
34	CHDS5	-	+	-	-	-	K/A	K/K	+	+
35	MPS1	-	-	-	+	-	A/A	K/K	+	+
36	MPS2	-	-	-	-	-	A/A	K/K	+	-
37	MPS3	-	-	-	-	-	A/A	K/K	+	+
38	MPS4	-	-	-	-	+	K/A	K/K	+	+
39	MPS5	+	+	-	-	-	K/A	K/K	+	+
40	**VMP**	+	+	-	+	+	K/A	K/K	+	-

Table.4 Effect of various pH on chitinase production by isolate PSS3

Sl. NO.	Chitosan minimal salt broth at different pH	OD Value (560nm)
1.	4	0.016
2.	5	0.551
3.	6	0.410
4.	7	0.390
5.	8	0.658
6.	9	0.001

Table.5 Effect of various pH on chitinase production by isolate PPS5

Sl. NO.	Chitosan minimal salt broth at different pH	OD Value (560nm)
1.	4	0.012
2.	5	0.234
3.	6	0.252
4.	7	0.346
5.	8	0.112
6.	9	0.235

CHITINASE ENZYME AT DIFFERENT CARBON SOURCE

Table.6 Effect of various carbon source on chitinase production by isolate PSS3

Sl. NO.	Chitosan minimal salt broth at different Carbon sources	OD Value (560nm)
1.	Glucose	0.300
2.	Sucrose	0.989
3.	Lactose	0.697
4.	Maltose	0.142
5.	Mannitol	0.596
6.	Glycerol	0.736

Table.7 Effect of various carbon source on chitinase production by isolate PPS5

S. NO.	Chitosan minimal salt broth at different Carbon sources	OD Value (560nm)
1.	Glucose	0.427
2.	Sucrose	0.520
3.	Lactose	0.527
4.	Maltose	0.519
5.	Mannitol	0.015
6.	Glycerol	0.357

Chitinase enzyme at different nitrogen source

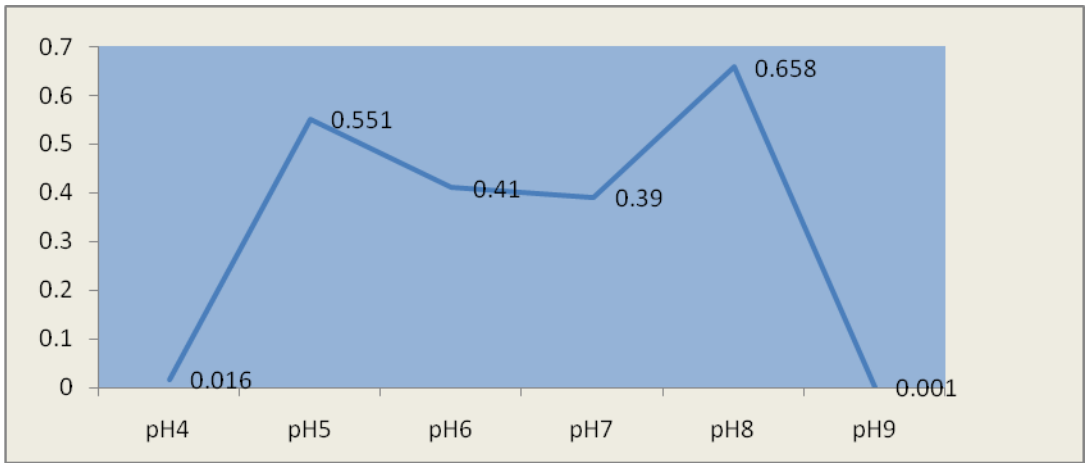
Table.8 Effect of various nitrogen source on chitosanase production by isolate PSS3

Sl. NO.	Chitosan minimal salt broth at different Nitrogen sources	OD Value (560nm)
1.	Peptone	1.374
2.	Ammonium Chloride	0.322
3.	Caesin	1.553
4.	Yeast Extract	1.652
5.	Beef Extract	0.339
6.	Urea	0.247

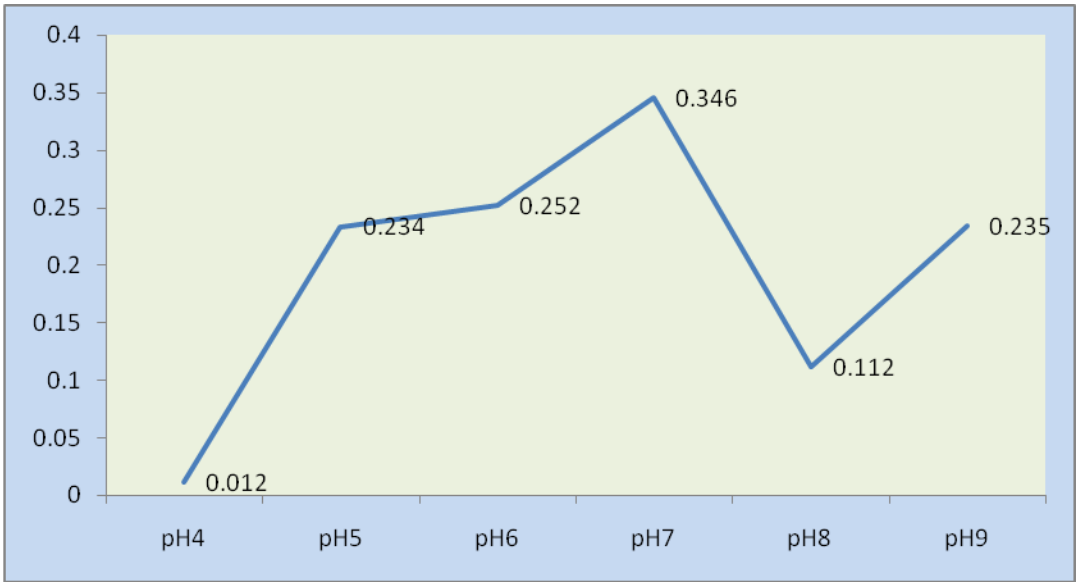
Table.9 Effect of various nitrogen source on chitinase production by isolate PPS5

S. NO.	Chitosan minimal salt broth at different Nitrogen sources	OD Value (560nm)
1.	Peptone	1.767
2.	Ammonium Chloride	0.690
3.	Caesin	0.995
4.	Yeast Extract	1.554
5.	Beef Extract	1.539
6.	Urea	0.645

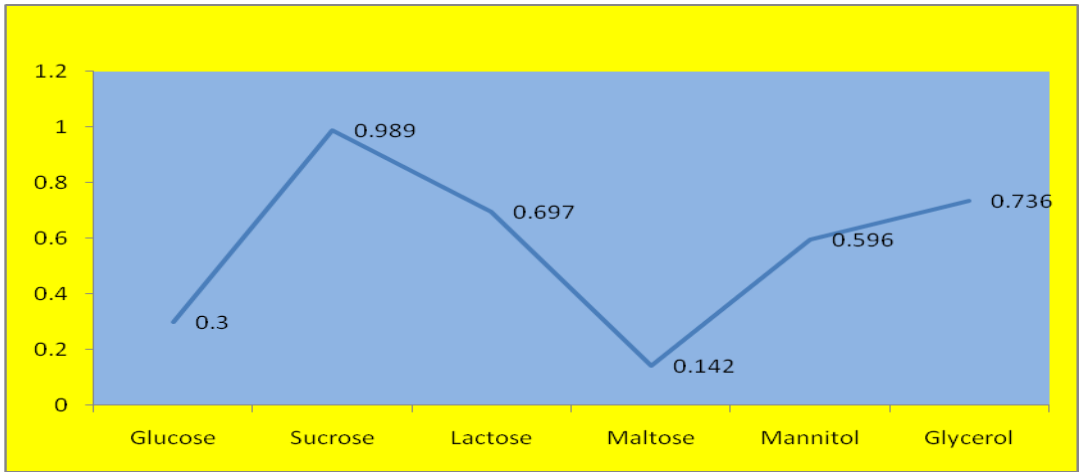
Graph.1 Effect of various pH on chitinase production by isolate PSS3



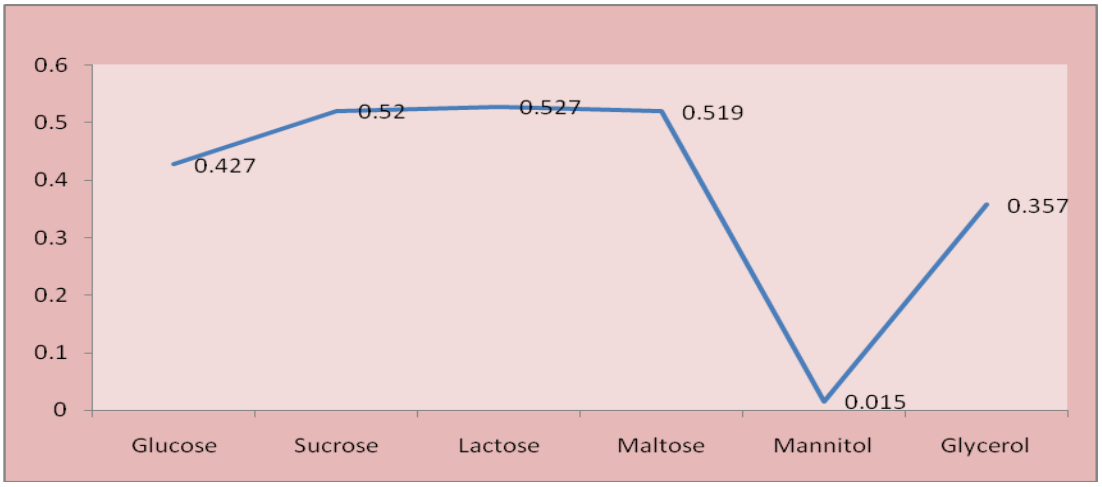
Graph.2 Effect of various pH on chitinase production by isolate PPS5



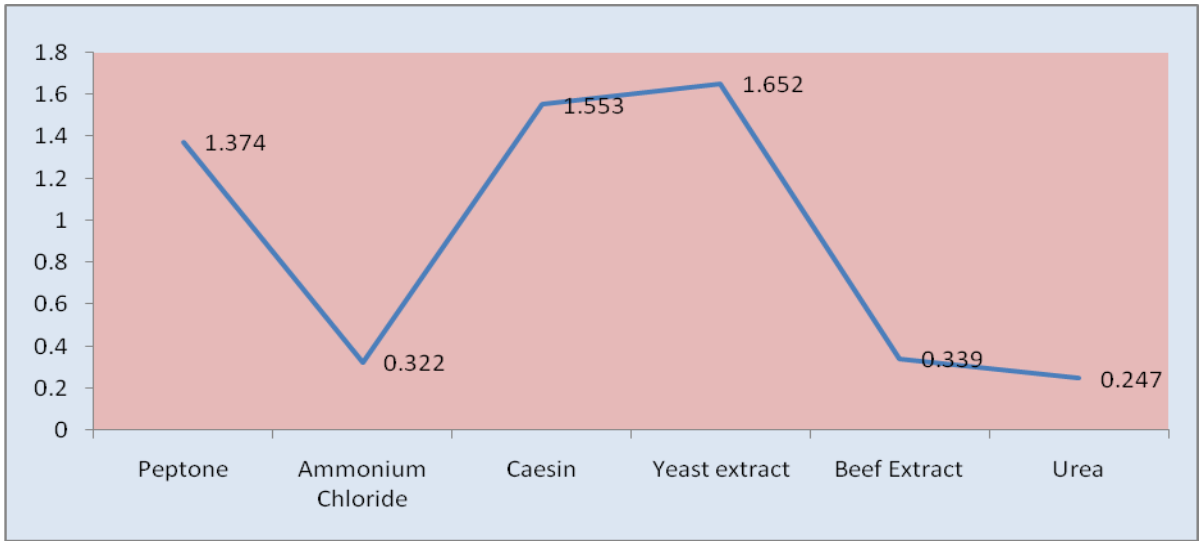
Graph.3 Effect of various carbon source on chitinase production by isolate PSS3



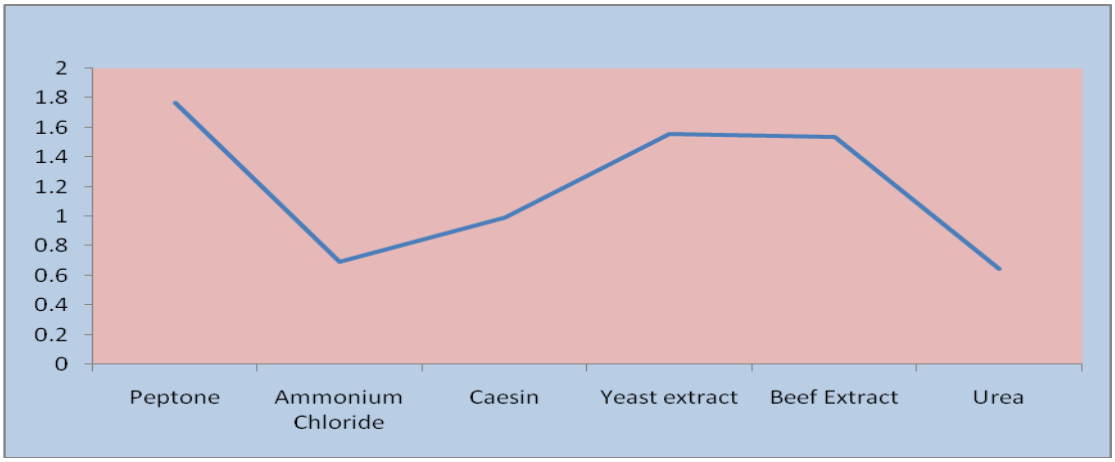
Graph.4 Effect of various carbon on chitinase production by isolate PPS5



Graph.5 Effect of various nitrogen on chitinase production by isolate PSS3



Graph.6 Effect of various nitrogensource on chitinase production by isolate PPS5



Chitosan minimal salt agar medium

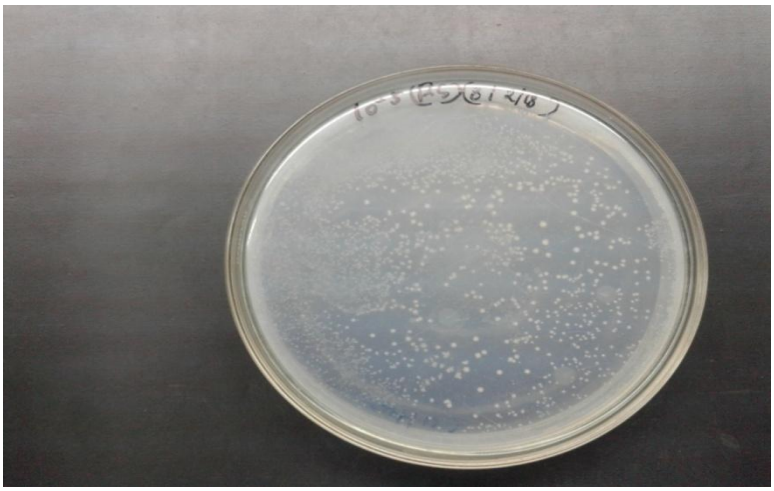


Fig.1 Shows the Chitinase producing bacteria forming clear zone

HICHROME BACILLUS AGAR

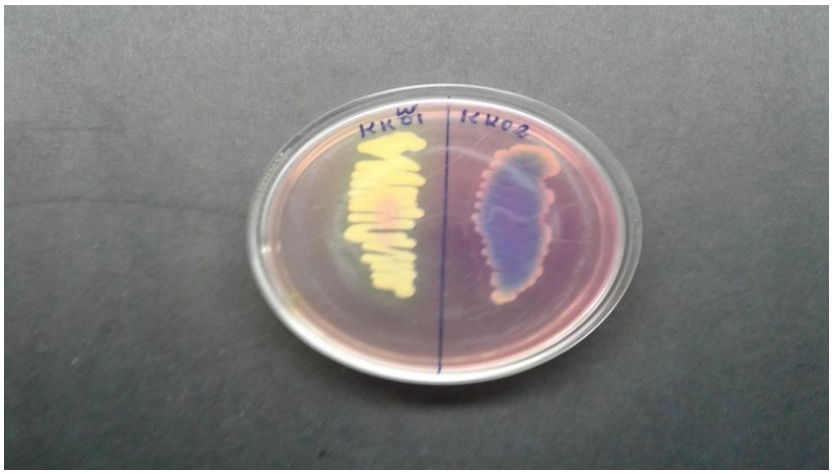


Fig.2 Shows the growth of different *Bacillus* species forming colourful colonies

Violet red bile glucose agar

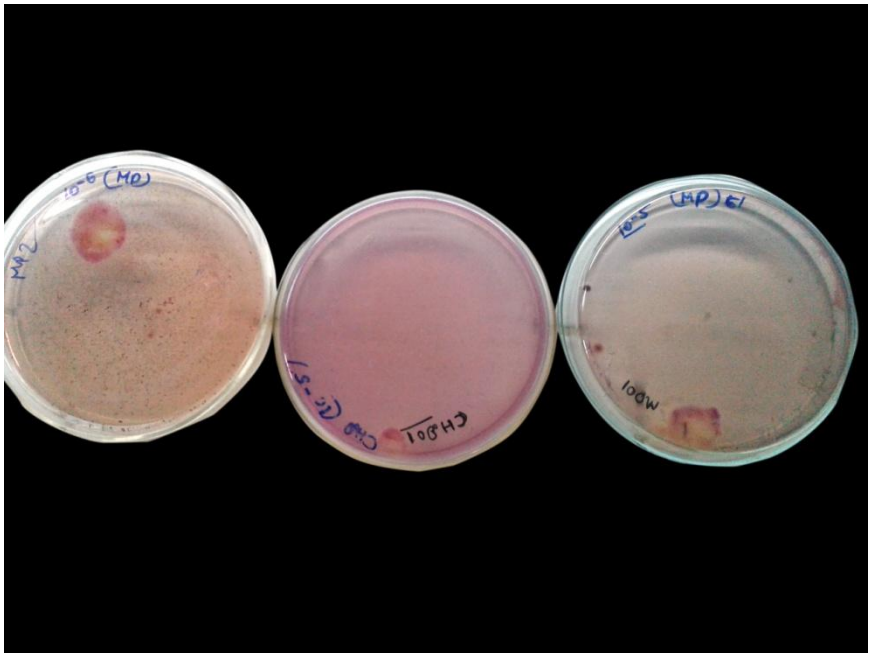


Fig.3 Shows the Growth of *Photobacterium* sps

Enzyme immobilization

Fig.4 Shows the Immobilization of Enzymes of Isolate VMP

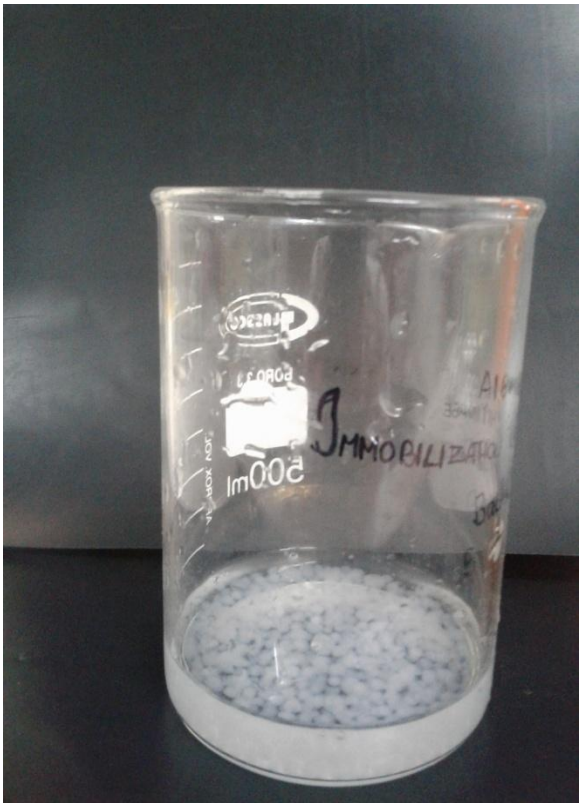


Fig.5 Shows the Immobilization of Enzymes of Isolate PPS2(*Bacillus megaterium*)

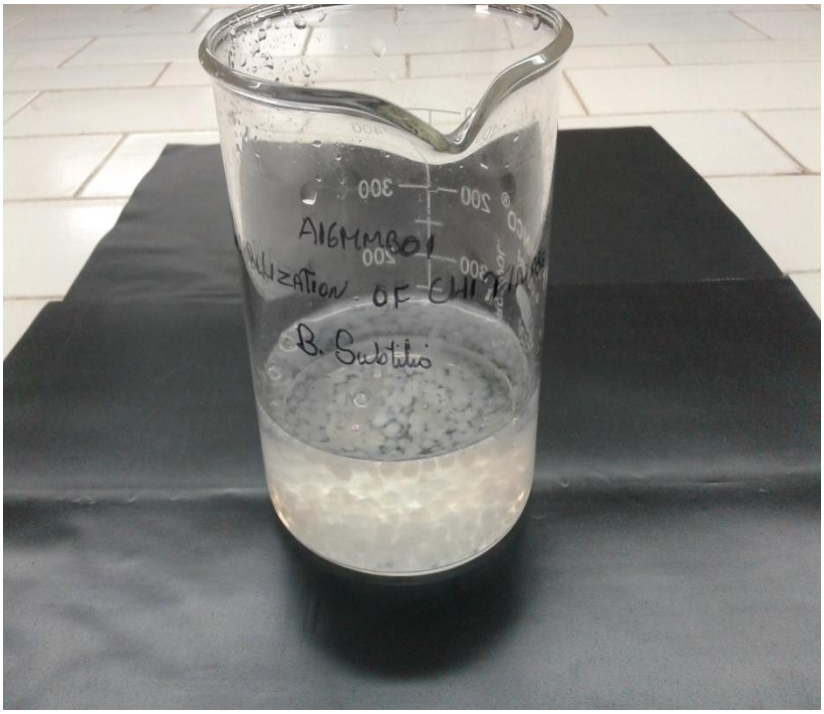
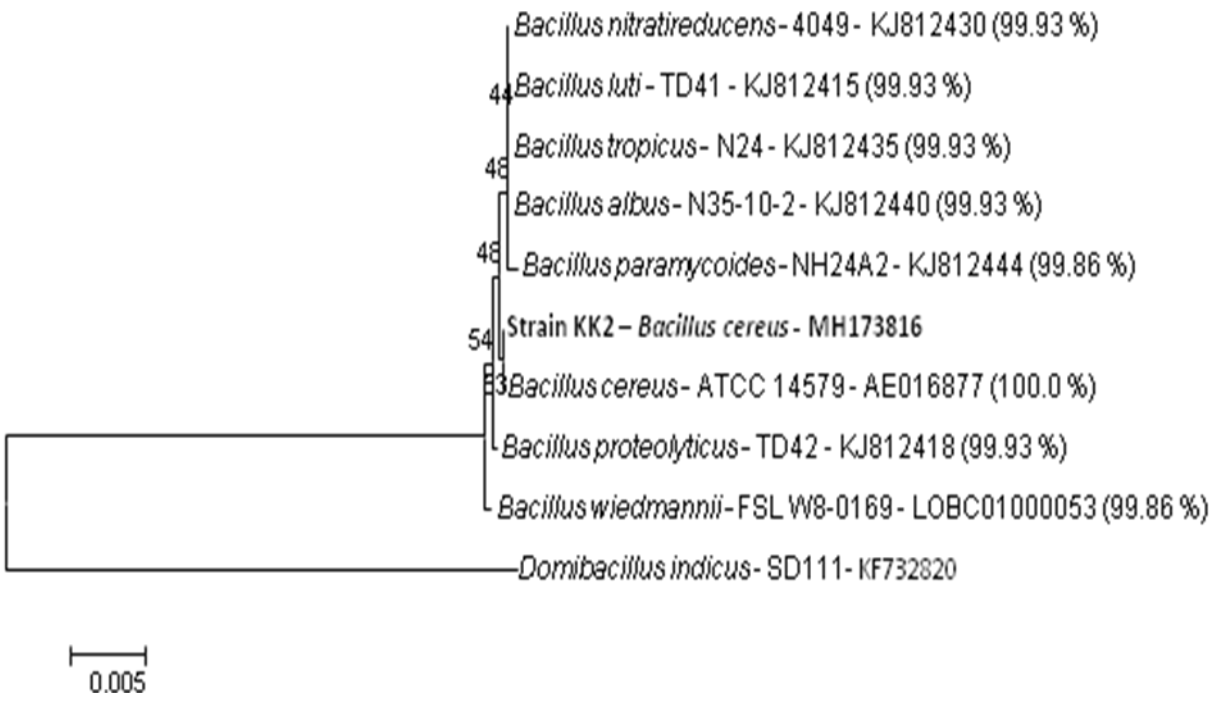


Fig.6 Shows the Immobilization of Enzymes of Isolate GUW1 (*Bacillus subtilis*)

COLLOIDAL CHITIN

Fig.7 Shows the Preparation of Colloidal Chitin.

Results of Genome sequencing



The aim of the present study was Isolation and Characterization of chitosanase producing Bacteria From Shrimp farming ponds. For this study about 11 Pond water and Sediment soil samples were collected from the Cuddalore and Puducherry. The sample were collected in

a sterile container bought to the laboratory and then used for the further process.

The present study was the isolation of chitosanase producing Bacterial species from various samples. Out 11 different samples 40 different isolates identified. The samples were serially diluted and 0.1ml of diluted sample was transferred in the Minimal salt agar medium incubate the plates at 37°c for 48 hours. After incubation period, the colony morphology and the phenotypic characteristics were observed.

The chitosanase producing isolates were identified by chitosan minimal salt medium. The isolates were streaked in the chitosan minimal salt agar medium and incubate for 37 °C for 48 hours. After incubation period, the zone formation occurs around the organism which indicates the organism are chitosan utilizers.

The optimization of the chitosanase enzyme was carried out by different pH (4,5,6,7,8,9) different carbon sources such as Glucose, Sucrose, Lactose, Maltose, Mannitol. Glycerol and different nitrogen source such as casein, peptone, yeast extract, beef extract, ammonium chlorite, 40% urea. Enzyme immobilization technique was done by using sodium alginate and calcium chloride. The immobilized chitosanase beads were formed. The cultures KK2 AND VMP were subjected for genome sequencing and the results were identified by molecular method for 16srRNA by BLAST and found to be, KK2– *Bacillu scereus* and VMP – *Photobacterium ganghwense.*

Izvekova*et al.*, reported that bacteria associated specifically with gastrointestinal tract of riverine and pond water fishes examined and exhibited for enzymatic activity such as amylolytic, proteolytic and lipolytic activity among bacterial population, this implements the feeding habits fishes, being the herbivore fish species, may harbor proteolytic, amylolyitc and lipolytic bacterial consortium in the gut and adapt themselves forming symbiotic relationship and provides ecological niches for these organisms.

Chitosan has a number of commercial and possible biomedical uses. It can be used in agriculture as a seed treatment and biopesticide, helping plants to fight off fungal infections. In winemaking it can be used as a fining agent, also helping to prevent spoilage. In industry, it can be used in a self-healing polyurethane paint coating. In medicine, it may be useful in bandags to reduce bleeding and as an antibacterial agent; it can also be used to help deliver drugs through the skin.

From these studies it is very clear different *Bacillus* sps is more predominant in the shrimp ponds and marine sediments, Today most of the country relies on microbial enzymes for commercial exploitation chitosanases have diverse role in day life, for example chitosanases are employed in various industries like beverages, health, medicine. These isolates can be used for further studies and the gene responsible for chitosanase production can be identified, cloned and expressed to get increased production of chitosanase.

Significant and impact of the study:

The present study determines Screening and characterization of chitosanase producing microorganism from various Shrimp farming ponds samples. 40 bacterial species were isolated and screened. Three bacterial species (PSS3and PPS5*)* showed higher production of chitosanases, the optimization of chitosanases at various pH, nitrogen source, Carbon source was standard. To confirm the bacterial species it was further identified by genome sequencing methods. The degradation of marine chitinous wastes can be used for the preparation of colloidal chitin and identification of chitinolytic strains has bioprospecting future.

The culture KK2 and VM P isolated from shrimp farming ponds was sequenced in PACE Microbial technology Puducherry. The sequencing was interpreted by performing BLAST and aligned data sequence was produced. The isolateKK2 was identified as *Bacillus cereus.* The isolate VMP was identified as *Photobacterium gamghwense*. In the present investigation the presence of considerable population of bacterial consortia found in the shrimp ponds were highly remarkable in chitinase production. The genome sequencing results provides a detailed information on Chitinolytic bacteria which is signified in Phylogenetic tree under different genera. In our studies *Bacillus* is the only genera shows higher amount of Chitinase activity.

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