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Research Article

STUDIES ON CHITINOLYTIC BACTERIA IN INTESTINAL TRACT OF MARINE FISHES AND SHRIMPS

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ABSTRACT

The contribution of the gut microbiota in production of digestive enzymes, regarding endogenous enzyme activities in fish seems pertinent in both marine and fresh water fishes. Chitinase are ubiquitous in nature, being found in eukaryotes, prokaryotes, archaea and viruses (Suzanne *et al*., 2001). It is believed that stomach chitinase have an indirect digestive function, helping to breakdown the exoskeleton of prey, which allows other digestive enzymes access to soft inner tissue (Fange and Grove, 1979; Lindsay, 1984; Clark Quayle *et al*., 1998). Chitosan is one of the most abundant biomasses on earth chitooligosaacharides from chitosan polymer have become a remarkable resource for the development of functional foods, artificial skin, medicine and other materials, For this study 5 fishes were collected from the Cuddalore and Pazhaiyar fish market. From the fish, intestine were taken and homogenized with the distilled water and then used for the further process. to study the enzyme activity of Chitinase by optimization parameters by using various nitrogen source, different pH and determining the stability of lipase enzyme by immobilization technique. The isolates were selected based on cellular morphology, growth conditions and biochemical tests. The different *Bacillus* species were screened by using Hicrome *Bacillus* for *Pseudomonas* cetrimide agar is used. The results obtained in the present study revealed that chitinase producers shows promising and good source of chitinase.

INTRODUCTION

Microorganisms excrete a wide variety of Chitinolytic enzymes, which are also found in mammalian systems. commercially they are very important and isolated from various living sources such as fishes, Shrimps, Insects, animals, bacteria and fungi. Chitinase are ubiquitous in nature, being found in eukaryotes, prokaryotes, archaea and viruses (Suzanne *et al*., 2001).It is believed that stomach chitinase have an indirect digestive function, helping to breakdown the exoskeleton of prey, which allows other digestive enzymes access to soft inner tissue (Fange and Grove, 1979; Lindsay, 1984; Clark Quayle *et al*., 1998). Chitosan is one of the most abundant biomasses on earth chitooligosaacharides from chitosan polymer have become a remarkable resource for the development of functional foods, artificial skin, medicine and other materials attemped by methods such as the treatment of chitosan polymer by chemicals or enzyme synthetic production of chitooligosaccharide from as the treatment of chitosan polymer.

MATERIALS AND METHODS

Isolation and identification

Fresh marine fish were collected, washed with water, surface sterilized with alcohol, and again washed with sterile saline. The intestine of the fish is taken by dissection process.The intestine is homogenized by using mortar and pestle. The homogenized sample is used for serial dilution technique. By using basic microbiological techniques. The chitinolytic bacteria was isolated by using Minimal salt agar and then further it was screened and its phenotypic character was studied by using Hicrome Bacillus agar for Identifying *Bacillus* species and Cetrimide agar for *Pseudomonas* species. The isolates was identified based on cellular morphology, Gram staining, endospore staining and biochemical tests and further confirmed by molecular methods (16sR RNA)

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Optimization of Chitosanase

Effect of pH on Chitosanase Production

The isolates such as *SSPZ11, SSPZ15, and KPP20 were* inoculated in chitosan minimal salt broth at different pH ranges from pH 4 to 9 and incubated at 37°C for 48 hours. The chitosanase production was observed in spectrophotometer at OD of 560nm.

Effect of Various Nitrogen Source on Chitosanase Production

The isolates such as *SSPZ11, SSPZ15, KPP20* were inoculated in the chitosan minimal salt broth supplemented with various nitrogen source such as tryptone, peptone, yeast extract, beefextract, ammonium chloride, 40% ureaincubated at 37^{O}C for 48 hours. The chitosanase activity was observed in spectrophotometer at OD of 560nm.

Genome Sequencing

The following cultures SSPZ11, SSPZ15, KPP20 were subjected for genome sequencing and the sequencing was done in PACE Microbial technology. Puducherry.

RESULTS AND DISCUSSION

Morphological and Physiological Characteristics

Minimal Salt Agar Medium

The chitinase producing bacterial strains were isolated from fish sample and different fish using minimal salt agar medium observed for clear zone.

Table no 1 Chitinolytic Bacteria Isolated from Various Fishes & Shrimp

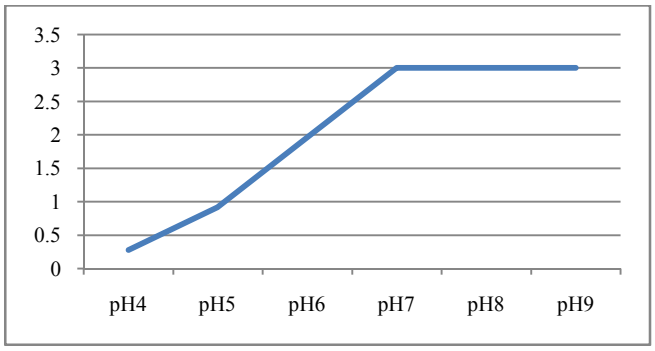
S.No	Sample (Fishes & Shrimp)	Sampling Area	Number of Isolates	Total Number of Isolates
1	*catla catla*	OT-cuddalore	7	
2	*Rastrelliger kanagurt*	Pazhaiyar	14	
3	*Paenus monodon*	Pazhaiyar	3	34
4	*Rastrelliger kanagurta*	Cuddalore	7	
5	*Paenus monodon*	Cuddalore	3	

Optimization of Chitosanase Enzyme at Various pH

The isolates such as SSPZ11, SSPZ15, KPP20 were inoculated in chitosan minimal salt broth at different pH 4to 9 and incubated at 37°c for 48 hours. The chitosanase production was observed in spectrophotometer at OD 560nm.

Table 3 Effect of Various pH on Chitosanase Production By Isolate SSPZ11

S.NO	Chitosan Minimal Salt Broth at Different pH	OD AT 560nm
1	4	0.2762
2	5	0.9163
3	6	0.9625
4	7	3.0000
5	8	3.0000
6	9	3.0000



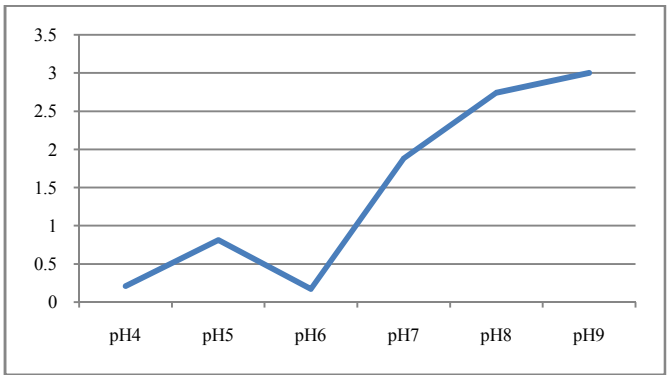
Graph 1 Effect of Various pH On Chitosanase Production By Isolate SSPZ11

Table 4 Effect of Various pH on Chitosanase Production By Isolate SSPZ15

S.NO	Chitosan Minimal Salt Broth at Different pH	OD AT 560nm
1	4	0.2086
2	5	0.8111
3	6	0.1719
4	7	1.8832
5	8	2.7419
6	9	3.0000

Table 2 Morphological & Biochemical Characteristic Results of Various Isolates

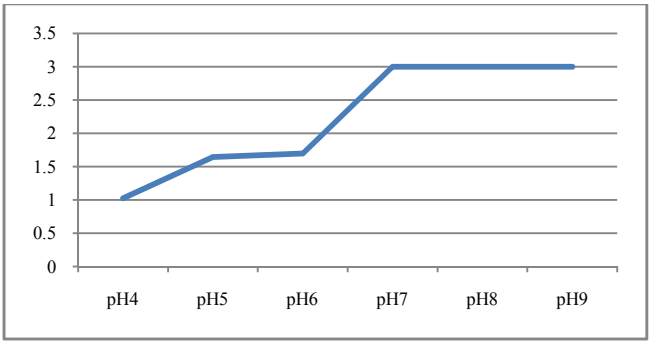
S.No	ISOLATE NO	Gram staining	Spore staining	INDOLE	MR	VP	CITRATE	UREASE	TSI	LIA	CATALASE	OXIDASE	MANNITOL MOTILITY TEST
1	GKT03	Gram negative rod	Non spore forming	-	+	-	+	+	A/A	K/K	+	+	Motile
2	GKT04	Gram negative rod	Non spore forming	-	+	-	+	+	K/A	K/K	+	+	Motile
3	GKTO5	Gram negative rod	Non spore forming	-	+	-	+	+	K/A	K/K	+	+	Motile
4	GKT06	Gram negative rod	Non spore forming	-	+	-	+	+	A/A	K/K	+	+	Motile
5	GKT07	Gram negative rod	Non spore forming	-	+	-	+	+	K/A	K/A	+	+	Motile
6	GKT08	Gram negative rod	Non spore forming	-	+	-	+	+	K/K	K/K	+	+	Motile
7	GKT09	Gram negative rod	Non spore forming	-	+	-	+	+	K/K	K/K	+	+	Motile
8	SSPZ08	Gram positive rod	spore forming	-	+	-	-	-	A/A	K/K	-	+	Non motile
9	SSPZ09	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
10	**SSPZ11**	Gram positive rod	spore forming	-	+	-	+	-	A/A	K/A	+	+	Non motile
11	SSPZ12	Gram positive rod	spore forming	-	+	-	+	-	K/A	K/K	+	+	Non motile
12	SSPZ13	Gram positive cocci	Non spore forming	-	+	-	-	-	A/A	A/K	+	+	Non motile
13	SSPZ14	Gram positive rod	spore forming	-	+	-	+	-	A/A	A/K	+	+	Motile
14	**SSPZ15**	Gram positive cocci	Non spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
15	SSPZ17	Gram positive cocci	Non spore forming	-	+	-	-	-	A/A	K/A	+	+	Non motile
16	SSPZ18	Gram negative rod	Non spore forming	-	+	-	-	-	A/A	K/A	+	+	Non motile
17	SSPZ19	Gram positive rod	spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
18	SSPZ20	Gram positive rod	spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
19	SSPZ21	Gram positive rod	Spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
20	SSPZ22	Gram positive cocci	Non spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
21	SSPZ23	Gram negative rod	Non spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
22	RKMJ13	Gram positive rod	spore forming	-	+	-	-	-	A/A	K/K	+	+	Motile
23	RKMJ14	Gram positive cocci	Non spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
24	RKMJ15	Gram positive rod	spore forming	-	+	-	+	-	A/A	K/A	+	+	Motile
25	KPP15	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
26	KPP17	Gram negative rod	Non Spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
27	KPP18	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
28	KPP19	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
29	**KPP20**	Gram positive cocci	Non spore forming	-	+	-	+	-	A/A	K/K	+	+	Motile
30	KPP21	Gram positive cocci	Non spore forming	-	+	-	-	-	K/A	K/A	+	+	Motile
31	KPP23	Gram negative rod	Non Spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
32	KKP20	Gram positive rod	Spore forming	-	+	-	+	-	A/A with gas production	K/K	+	+	Motile
33	KKP25	Gram positive rod	Spore forming	-	+	-	-	-	A/A	K/K	+	+	Non motile
34	KKP26	Gram positive rod	Spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile



Graph 2 Effect of Various pH on Chitosanase Production By Isolate SSPZ15

Table 5 Effect of Various pH on Chitosanase Production by Isolate KPP20

S.no	Chitosan Minimal Salt Broth At Different pH	OD AT 560nm
1	4	1.0248
2	5	1.6432
3	6	1.6970
4	7	3.0000
5	8	3.0000
6	9	3.0000



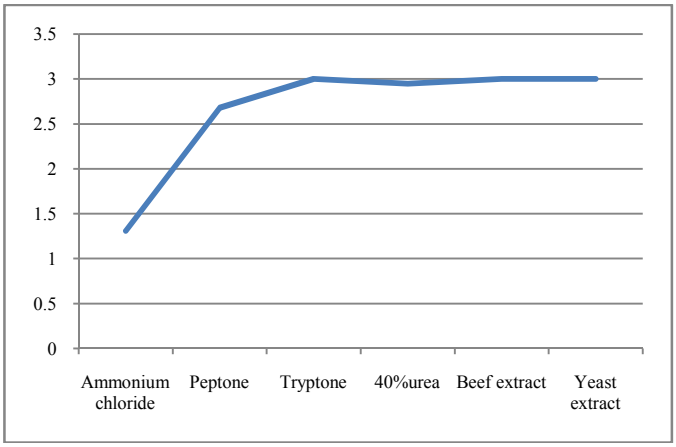
Graph 3 Effect of Various pH on Chitosanase Production by isolate KPP20

Optimization of Various Nitrogen Source on Chitosanase Production

The isolates such as SSPZ11, SSPZ15, KPP20 were inoculated in the chitosan minimal salt broth supplemented with various carbon source such as ammonium chloride, peptone, Tryptone, 40% urea, beef extract, yeast extract and incubated and incubated at 37°c for 48 hours. The chitosanase activity was observed in spectrophotometer at OD of 560nm.

Table 6 Effect of Various Nitrogen Source on Chitosanase Production by Isolate SSPZ11

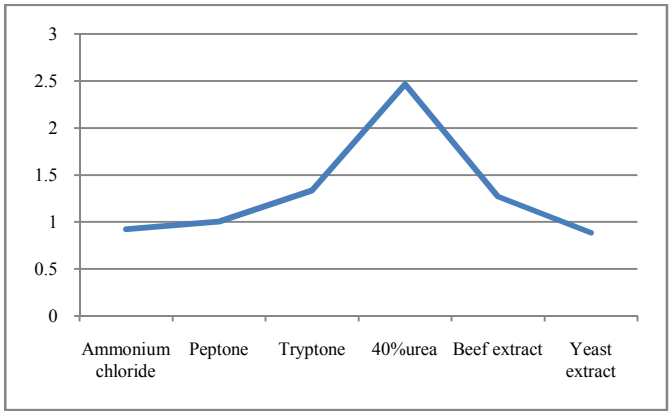
S.No	Chitosan Minimal Salt Broth With Various pH	OD AT 560nm
1	Ammonium chloride	1.3087
2	Peptone	2.6804
3	Tryptone	3.0000
4	40% urea	2.9456
5	Beef extract	3.0000
6	Yeast extract	3.0000



Graph 4 Optimization of Various Nitrogen Source on Chtosanase Production by Isolate SSPZ11

Table 7 Effect of Various Nitrogen Source on Chitosanase Production by Isoalte SSPZ15

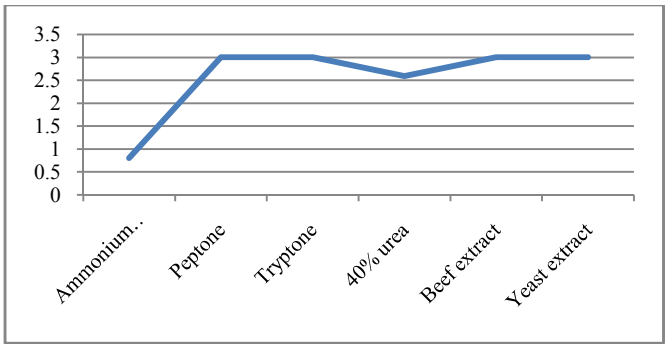
S.NO	Chitosan Minimal Salt Broth At Different Nitrogen Source	OD AT 560nm
1	Ammonium chloride	0.9209
2	Peptone	1.0025
3	Tryptone	1.3344
4	40% urea	2.4648
5	Beef extract	1.2694
6	Yeast extract	0.8840



Graph 5 Effect of Various Nitrogen Source on Chitosanase Production By Isoalte SSPZ15

Table 8 Effect of Various Nitrogen Source on Chitosanase Production by Isoalte KPP20

S.No	Chitosan Minimal Salt Broth At Different Nitrogen Source	OD AT 560 nm
1	Ammonium chloride	0.8026
2	Peptone	3.0000
3	Tryptone	3.0000
4	40% urea	2.5873
5	Beef extract	3.0000
6	Yeast extract	3.0000



Graph 6 Effect of Various Nitrogen Source on Chitosanase Production IN KPP20

Chitosan Minimal Salt Agar

Plate 1 Shows the chitinase producing bacteria forming clear zone

Hicrome Bacillus Agar

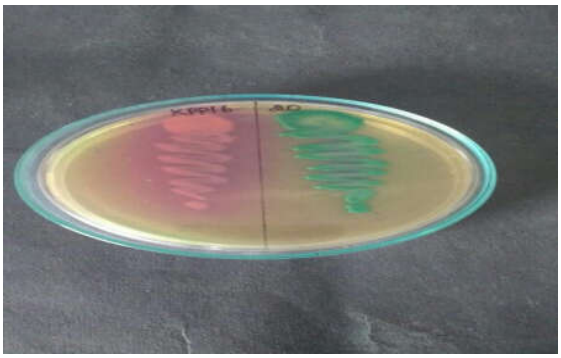


Plate 2 Shoes the growth of different *Bacillus* species forming colourful colonies

Cetrimide Agar Base

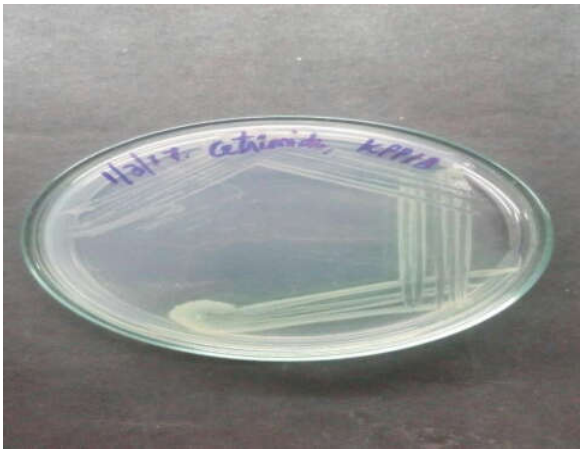


Plate 3 Shows the Pseudomonas species green colour formation in Cetrimide agar

Enzyme Immobilization

Plate 4 Shows the chitinase immobilized beads

Results of Genome sequencing

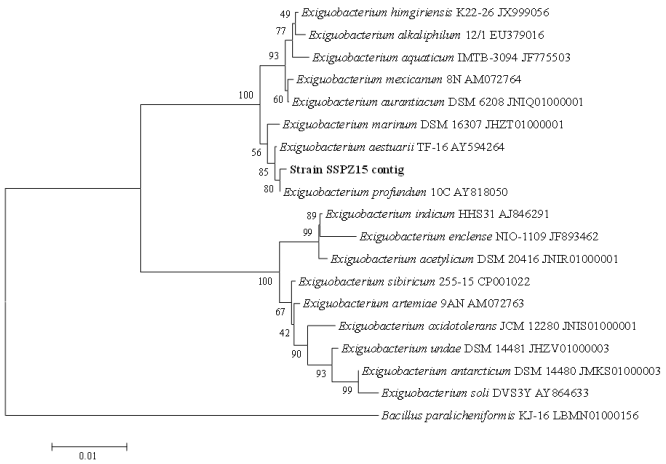
The following cultures SSPZ11, SSPZ15, KPP20 were subjected for genome sequencing and the results were identified by molecular method such as, SSPZ11-*Pseudomonas* sp, SSPZ15- *Exiguobacterium* sp, KPP20- *Pseudomonas* sp.

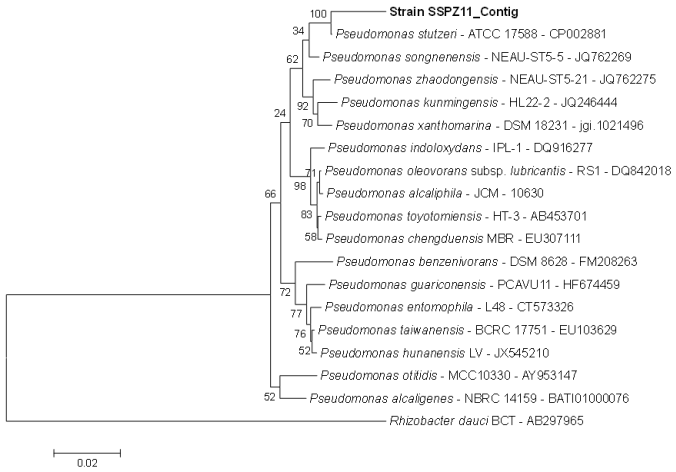
Sequencing

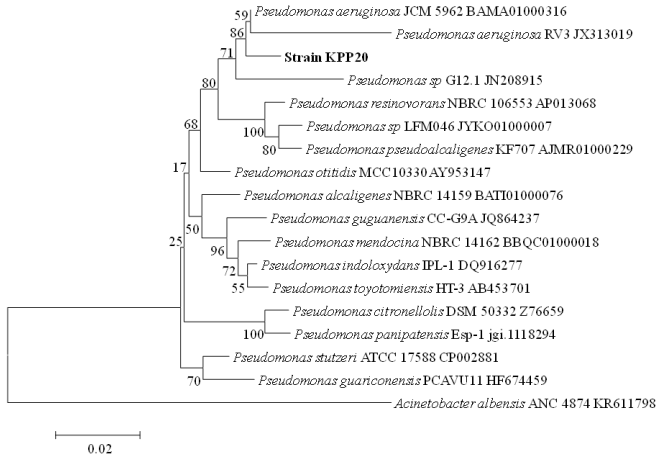
The purified PCR products of approximately 1,400bp were sequenced by using the primers (785 F 5' GGA TTA GAT ACC CTG GTA 3' and 907 R 5' CCG TCA ATT CCT TTR AGT TT 3'). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product were resolved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







The following cultures SSPZ11, SSPZ15, KPP20 were subjected for genome sequencing and the results were identified by molecular method such as, SSPZ11-*Pseudomonas* sp, SSPZ15- *Exiguobacterium* sp, KPP20- *Pseudomonas* sp.

DISCUSSION

The aim of my present study was Isolation and Characterization of chitosanase producing *bacteria* from gut of fish and shrimp. For this study 5 fishes and shrimp were collected from the Cuddalore and Pazhaiyar fish market. From the fish, intestine were taken and homogenized with the distilled water and then used for the further process. The present study was the isolation of chitosanase producing species from various fish samples and shrimp. Out 5 different samples 34 different species were isolated and identified.

The fish 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 24 hours. After incubation period, the colony morphology and the phenotypic characteristics were observed. 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 occur around the organism which indicates the organism are chitosan utilizers.

Optimization of the chitosanase enzyme was carried out by different pH (4,5,6,7,8,9) and different nitrogen source such as tryptone, 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 following cultures SSPZ11, SSPZ15, KPP20 were subjected for genome sequencing and the results were identified by molecular method for 16srRNA by BLAST and found to be, SSPZ11-*Pseudomonas* sp, SSPZ15- *Exiguobacterium* sp, KPP20-*Pseudomonas* sp.

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 bacterial sp is more predominant in the gut of fish and intestine 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 fir fyrther 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 Isolation and characterization of chitosanase producing microorganism from various homogenized fish gut samples. 34 bacterial species were isolated and screened. Three bacterial species (*SSPZ11, SSPZ15* and *KPP20)* showed higher production of chitosanases, the optimization of chitosanases at various pH, nitrogen source, was standard. To confirm the bacterial species it was further indentified by genome sequencing methods.

The culture *SSPZ11, SSPZ15* and *KPP20* isolated from shrimp and fish was sequenced in PACE Microbial technology Puducherry. The sequencing was interpreted by performing BLAST and aligned data sequence was produced. The isolate *SSPZ11* was identified as *Pseudomonas* sp. The isolate SSPZ15 was identified as *Exiguobacterium* sp and KPP20 was identified as *Pseudomonas* sp in the present investigation the presence of considerable population of bacterial consortia found in the gut of fishes and shrimp 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 *Pseudomonas* sp shows higher amount of Chitinase activity.

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