Application of protease producing bacterial culture for deproteination of shrimp shells as an alternative to chemical deproteination by NaOH

M. S.Girase[1], V. B.Gaikwad[2], S. N. Patil[3], S. S. Borase[4]

[1]Dept. of Microbiology, K. S. K. W. Arts, Com. & Sci. College, CIDCO, Nashik. Maharashtra, India.
[2]Principal, K. T. H. M. College, Nashik. 422005. Maharashtra, India.
[3]Department of Biotechnology K. T. H. M. College, Nashik. 422005. Maharashtra, India.
[4]Lokmanya Tilak Mumbai Municipal Corporation College, Sion, Mumbai.

***Abstract*:** Deproteination and demineralization are the two critical steps in the recovery of chitin from shrimp shells. Chitin is conventionally recovered from shrimp shells using NaOH and HCl for deproteination and decalcification respectively. Use of these chemicals cause environmental problems during disposal of waste water of the chitin recovery unit and sometimes may cause denaturation of chitin.To overcome this, in the present study for deproteination of shrimp shells, protease producing bacterial culture was checked for its efficiency to remove protein from white shrimp(*Litopenaeus vannamei*) shells. Elemental analysis of head and abdominal part of the shrimp shell was done to find out chitin rich fraction. Efficient organism, capable of more deproteination efficiency (%) was identified as *Vibrio metschnikovii* strain Xmb057 and after antibiotic sensitivity checking it was found to be sensitive for majority of commonly prescribed antibiotics. For measuring deproteination efficiency (%), dry weight of shells and Nitrogen content of shrimp shells by Kjeldahl methodwas measured daily after drying. Protein Nitrogen removed was calculated by subtracting chitin Nitrogen from total Nitrogen. To check presence of amino acids in recovered protein, broth was hydrolyzedand analyzed for amino acid contentby Ultra Performance Liquid Chromatography (UPLC).

***Index terms:Litopenaeus vannamei*, Protease, deproteination, chitin recovery**

---

1. INTRODUCTION

Aquaculture is the fastest developing business in the world.In development of any country fisheries plays very important role. It is an important source of livelihood for a considerable section of population. In last few years, aquaculture has become a profitable commercial business. In India at present about 1.57 lac hectare is in actual shrimp farming. Average production of shrimp is 660 Kg/hectare/year. In year 2010-11 in India about 1.45 lac tones of shrimps were produced, of which 50% are cultured shrimps. Maharashtra has 720 km coastline area. For shrimp farming at present out of around 12445-hectare available area, 1056-hectare area is utilized (Sadafule et al, 2012). In recent year fishery is not a profession of only low income group or socially backward communities, but many industrialists and technologists are involved in this profession. This profession can provide employment to large group. (Duangpaseuth et al, 2007).

India is second largest producer of aquaculture after China and supplies maximum shrimp to USA. In total shrimp export only 27% is captured shrimp while rest is cultured shrimp consisting of mainly Vannamei and black shrimp (Nair, 2015).During processing of shrimps, head, shell and tail portions of shrimps are removed. They constitute about 50% of the total volume of raw materials (Islam et al,2004).Up to 1980s this waste was dumped in the sea by pipelines or by boats and it was considered as returning of nutrients in the sea. Now a day it is realized that due to accumulation of waste containing organic matter dissolved Oxygen, sediment quality, benthic and epi-benthic eco-systems are adversely affected. It also reduces clarity of sea water hence light penetration is affected and ultimately

phytoplankton population reduces. In reduced dissolved Oxygen condition various harmful compounds like methane, Ammonia and Hydrogen Sulphide are released (Mazik et al, 2005).

Crustacean waste is mainly composed of 60% of the organic material due to shells and carapace of crabs, prawns and lobsters (Pfeiffer, 2003).This waste can affect surrounding area and a wider coastal zone at different ecosystem levels; it can reduce the biomass, decrease the biodiversity of phytoplankton, zooplanktons and so can alter the food webs. Due to these adverse effects on marine ecosystem, development in aquaculture, conservation and management of coastal environment has become a great challenge. Such large volume of biomass can cause environmental problems due to dumping (Ioannis and Aikaterini,2008).

Now a dayfrom shrimp shells recovery of chitin is doneusing NaOH, HCl and H_2O_2 for de-proteinization, demineralizationand decolourization respectively. This chemical extraction process results in environmental problems during disposal of waste and due to mixing with NaOH, recovered protein cannot be used as a protein supplement in feed or fodder. Another problem is the possible denaturation of the chitin. (Troger and Niranjan,2010).

In the present study instead of using NaOH, protease producer and chitinase deficient culture was used for deproteination of shrimp shells to make the process environment friendly and to retain the use of recovered proteins as supplement in feed and fodder.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals usedfor present study were of analytical grade and obtained from Hi Media, Bio-Era, Sigma and Merck.

2.2 Shrimp shells

Single species fresh white shrimp (*Litopenaeus vannamei*) shells were procured from shrimp processing plants located near Thane, Maharashtra, India. Head and abdomen part of shrimp shell was collected in separate bags. Shells were transported to the laboratory by packing in polythene bags and kept in ice. Shells were thoroughly washedwith tap water to remove adhering flesh part of the shrimp, sand or other impurities. Finally washed with distilled water and air dried for further analysis.

2.3 Analysis of shrimp shells

2.3.1 Moisture content - Moisturecontent of was determined by drying 3 gshrimp shellsin porcelain crucible at 105^0Cin hot air oven for 24 h., cooled in desiccators and weighted. (Mahmoud *et. al*.,2007)Moisture content was determined by finding weight difference before and after drying using formula

$$\text{Moisture (\%)} = \frac{\text{(Weight of sample before drying(g)-Weight of sample after drying(g))}}{\text{Weight of sample before drying(g)}} \times 100$$

2.3.2 Ash content - Ash content wasdetermined by igniting 2g dried shrimps at 550 ± 25^0Cin porcelain crucible for 2h, cooled in desiccators and weighted(AOAC-941.12,2016). % Ash content is determined by formula

$$\text{Ash (\%)} = \frac{\text{Weight of ash(g)}}{\text{Weight of dried sample(g)}} \times 100$$

2.3.3 Total Nitrogen content - Total Nitrogen content of shrimp waste was determined by Kjeldahl method (AOAC-920.87,2016) by weighing 1g of sample in digestion flask. To this 0.7g. Mercury Oxide and 15g. Anhydrous Sodium Sulphate and 25ml Conc. H_2SO_4. Digestion flask was kept in inclined position with gentle shaking for 1 to 2 hours. Digested mixture was cooled and volume adjusted to 200ml with distilled water in round bottom flask. On addition of 25g of NaOH flask was connected to condenser with tip of condenser immersed in 50ml of 0.1N Standard HCl. Heated till all ammonia was distilled. After distillation titrated with 0.1N standard NaOH. Nitrogen content was determined by formula

$$\text{Nitrogen content (g)} = \frac{[(N_1V_1)-(N_2V_2)]X1.4002X200}{W \times 100}$$

V_1-Volume of 0.1N standard HCl

V_2-Volume of 0.1N standard NaOH used for back titration
N_1- Normality of standard HCl
N_2- Normality of standard NaOH
W-Mass of sample in gram
For estimation of chitin Nitrogen - 5.0 g shrimp shells were boiled in 125ml of 3% NaOH for 15 min. to chemically deproteinize shrimp shells and washed with hot distilled water till neutral pH and chitin Nitrogen was estimated by Kjeldahl method as described above.(Arafat, 2015).
Protein Nitrogen of shrimp shells= (Total Nitrogen – Chitin Nitrogen) X 6.25
Total Protein % was estimated by formula
Total Protein % =Protein Nitrogen X6.25
2.3.4 Lipid content - Lipidcontent of dried shells was determined by hydrolyzing 5g. powdered shells with 50ml 6N HCl at 110^0C till it dissolves completely in 250ml round bottom flask in oil bath. Extracted with 50ml 1:1 (v/v) Chloroform: Methanol mixture 3 times.Each time organic solvent layer was retained. all organic solvent fractions were passed through Whatman filter paper no. 1 into a preweighed container suitable for rotary evaporator. Evaporation was done at 40^0C. (Shahidi, 2001)
Lipid content was determined by formula

$$\text{Lipid (\%)} = \frac{\text{Weight of lipid extracted}}{\text{Weight of sample}} \times 100$$

2.4Enrichment and screening of Protease producing bacteria.
Soil samples from fish market, slaughter house, mushroom production plant, marine sediment and water samples from Lonar Lake, Arabian Sea were used as a source of protease producer bacteria. Samples were transported in an ice box in a chilled condition (4^0C) and analyzed within 24 h. after collection. Enrichment was done in a medium containing shrimp protein extract (prepared by mixing 10.00g shrimp shells with distilled water and filtrate volume adjusted to 100mlwas used for preparation of medium) containing 0.5% Yeast Extract, 0.5% KH_2PO_4, 0.5%NaCl, 0.1%$MgSO_4.7H_2O$,pH was adjusted to 7.0 (Waldeck et al, 2006).Screening of protease producer organism was done by growing cultures on gelatin agar containing 1% gelatin, 0.1%Yeast extract, 0.01%KH_2PO_4, 0.01% $(NH_4)_2HPO_4$, 0.005% $MgSO_4,5H_2O$, 0.01% $CaCl_2$agar 2.5% and observation of zone of clearance on addition of acidified $HgCl_2$prepared by adding 20ml 6N HCl to 100ml 15% $HgCl_2$ solution.

2.5 Selection of potent protease producer culture

Selection of potent protease producer was done in two stages: production of protease enzyme and measurement of protease activity by measuringClearance Zone measurement technique. For protease production, isolates were grown in gelatin broth. In 50ml of gelatin broth cultures were inoculated with overnight culture of protease producing organism. After 24 hours' incubation at 30^0C broth was centrifuged at 10,000rpm for 15minutes. 250 µl centrifuged cell free broth was added in a wells of 10 mm diameter in skim milk agar plates in triplicates and incubated for 16-18 hours.After incubation remaining supernatant was removed from the well by micro-pipette.Colonies showing largest clearance zone diameter were selected(Mahmoud et al,2009).

2.6 Selection of protease positive and chitinase negative cultures

For chitin recovery from shrimp shells during deproteination step there should be no chitin hydrolysis. Therefore, culture should be Protease producer but chitinase deficient. Chitinase deficient colony was selected by growing culture on colloidal chitin agar(Murthy and Bleakley,2012). (Moist colloidal chitin:20.00;$(NH_4)_2SO_4$:0.02; K_2HPO_4:0.7; KH_2PO_4:0.3; $MgSO_4$:0.5; $FeSO_4.7H_2O$:0.01, $ZnSO_4$:0.001; $MnCl_2$:0.001; pH-7; Agar:20; d/w:1L) Colloidal chitin was prepared as per method given by Jamialahmadi (2011).Colony failed to show clearance on colloidal chitin agar was considered as chitinase deficient.Thus,potent protease producer andchitinase negative culture was selected by for deproteination of shrimp shells.

2.7 Identification and Antibiotic sensitivity of organism

2.7.1 Identification using VITEK 2 systems Version:07.01.

Potent protease producer and chitinase non producer organism was identified using VITEK 2 systems Version:07.01.by card type AST-N280. Analysis time was 4hours.

VITEK 2 system test card includes 46 fluorimetric tests including pH change detection, derivatives to detect presence of aminopeptidase and aminosidase, 16 fermentation detection tests, 2 carboxylase tests and 6 miscellaneous tests. In instrument test card is filled automatically by a vacuum, sealed and inserted in incubator mode at 35.5^0C. In 0.45% saline bacterial suspension of 0.5 McFarland turbidity standard was used.During incubation kinetic fluorescence was measured for every 15 minutes. Results were interpreted by database of the instrument and were obtained automatically. After analysis test cards are automatically discarded in a waste container.

Bacterial suspension having 0.5 McFarland turbidity standard was diluted in 0.45% saline up to $1.5x10^7$CFU/ml. In the VITEK 2, cards were filled automatically, sealed and loaded in the instrument and incubated for reading.

Depending on bacterial culture combination of antibiotics were selected for antibiotic sensitivity testing. (Ligozzi et al,2002) Minimum Inhibitory Concentration (MIC) of total 19 antibiotics were done to check antibiotic sensitivity of selected organism.

2.7.2 Identificationbased on 16s rDNA sequence

Potent protease producer and chitinase non producer organism was identified based on 16s rDNA sequence. Genomic DNA was isolated from the bacterium. The ~1.3 kb/1.5kb, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase.The PCR product was sequenced bi-directionally. The sequence data was aligned and analyzed to identify the bacterium and itsclosest neighbors.

The PCR product size was ~1.5 kb.

Primers used for PCR for amplification of 16 S rDNA were

16s Forward Primer: 5’ – AGHGTBTGHTCMTGNCTCAS – 3’

16s Reverse Primer: 5’ – TRCGGYTMCCTTGTWHCGACTH – 3’

PCR was performed using ABI 3500 Genetic Analyzer with a cycle of 96°C for 5 min for Initial Denaturation, 96°C for 30 secondsfor Denaturation, 50 °C for 30 seconds for Hybridization and 60 °C for 1.30 min for elongation.

2.8 Deproteination of shrimp shells

2.8.1 Deproteination of shrimp shells using protease producing culture

For deproteination inoculums was prepared by inoculating one loopful of (Protease positive and chitinase negative) cultures in 50ml Nutrient broth and incubated at 30^0C for 24 h. After 24 h. cell count is around 10^8 CFU/ml. Small pieces of shrimp shells were prepared Using grinder. 10 g shrimp were added in 200ml Deproteination media of composition-KH_2PO_4-0.5%, NaCl-0.5%, Yeast extract-0.5%, $MgSO_4$-0.5%, $CaCl_2$-0.1% was used for removal of proteins.

2.8.2 Amino acid analysis of protein obtained after deproteination

2.8.2.1 Analysis of a. a.

Broth obtained after deproteination of shrimp shells after 7 days was evaporated to dryness. Dry weight of protein was recorded as a yield of protein. Separation, comparison of the amino acids byWATERS Acquity (make) UPLC by using Mobile Phase A: Accq Tag Ultra eluent A1andMobile Phase B: Accq Tag Ultra eluent B. To 1ml of the sample 4ml of methanol was added and incubated overnight at -20˚C. After overnight incubation sample was centrifuged and the supernatant was taken for evaporation. Evaporated sample completely under N_2 gas at 60˚C using dry bath. After evaporation, the pellet was dissolved in 0.1N HCL and transferred into a clean HPLC vial. To 50 ml broth tubes, 2ml of 6N HCL was added and the HPLC vial was placed into the tube. And the broth tube was sealed with parafilm. The tube was placed in the dry bath at 60˚ C under N_2 gas for 15 minutes, to maintain inertness. Then the temperature was increased to 110˚ C and incubated for 24 hrs. To this pellet 80 ul of Borate buffer, 20 ul of Accq Tag ultra-reagent was added and incubated for 10mins at 55˚ C. After incubation 1ul is loaded on to the instrument, which is quantified using a Sigma standard.Flow rate was 0.7ml/min, Column temperature: 55˚ C and Detectors (PDA): 260nm.Run method was as shown in the table1.

Table1Run method for amino acids by WATERS Acquity (make) UPLC.

Minutes	Mobile Phase A	Mobile Phase B
0.0	99.9	0.1
0.54	99.9	0.1
5.74	90.9	9.1
7.74	78.8	21.2
8.04	40.4	59.6
8.05	10.0	90.0
8.64	10.0	90.0
8.73	99.9	0.1
9.50	99.9	0.1

3. RESULTS AND DISCUSSION

3.1Analysis of shrimp waste

In the research by Gopakumar(2002) chitin content of shrimps, Squilla and Crab shell is15-20, 12-16 and 13-15% respectively. The main aim of this research is to recover chitin using microbial culture therefore in the present study shrimp shells which has maximum chitin content were selected as a raw material for recovery of chitin. During shrimp processing for export head and abdomen shell parts are separated by automatic machine. In the present study both parts were initially separately analyzed to find out moisture, ash, protein, lipid, chitin and pH content as shown in Table 2.Main product obtained from shrimp processing industrial waste is chitin. In the analysis as shown in table 2, it was observed that as compared to head part, abdominal shell portion contains more chitin, hence for deproteination study abdominal shell part was used for further study.

Table 2.Analysis of shrimp processing industry waste

Fraction of shrimp waste	Moisture content (%)	Ash content (%)	Protein content[1] (%)	Lipid content[2] (%)	Chitin Content[3] (%)	pH	Mass Balane Error[4]
Shell Without Head	77.0±0.99	25.27±0.09	36.69±0.28	2.333±0.24	29.12±0.18	8.3-8.6	6.59
Head	81.6±0.67	29.86±0.59	38.58±0.47	3.765±0.67	22.74±0.35	8.2-8.6	5.055

[1]Protein content from protein Nitrogen x 6.25.
[2]Lipid content by Hexane extraction.
[3]Chitin content from chitin Nitrogen x 14.25.
[4]Mass Balance Error =100 - (% ash + % protein + % lipid +% chitin)

3.2 Screening of Protease positive and chitinase negative culture.

In primary screening 51protease producer organisms were isolated on gelatin agar plate. Out of this 15 cultures were selected in secondary screening using cell free extracts on gelatin agar. Only 5 cultures protease producer were chitinase non- producers. These 5 cultureswere used for shrimp deproteination study and analyzed for deproteination efficiency (DP%). From these 5 cultures as shown in Table 3 only 2 culturesshowed significantand nearly same removal of protein and decrease in weight of shrimpshells due to deproteination.

Table 3 Dry weight of shells after deproteination and % removal of protein.

Culture	Mean dry weight of shrimp shells after deproteination	Mean % removal of protein
LL-17	2.44	80.19
LL-19	2.45	80.16
LL-20	3.00	68.89
LL-21	3.54	40.62
FM-2	3.83	58.18

3.3 Identification of culture

3.3.1 VITEK 2 systems based identification

Both organisms (LL-17 and LL-19) wereGram negative rods. LL-17 was identified using VITEK 2 Version:07.01. systems. After analysis time of 4 hoursLL-17 was identified byas *Sphingomonas paucimobilis*.

3.3.2 16S rRNA sequencing

Isolate number LL-19 was submitted to Chromous Biotech Private limited,Bengaluru.Based on sequencing studies the isolate LL-19 was confirmed as *Vibrio metschnikovii*strain Xmb057with 100% match having Accession No. KT986183.1.

Aligned Sequence Data of *Vibrio metschnikovii* strain Xmb057(shown as 1T in fig.1)(1420bp):

CATGCAGTCGAGCGGTAACAGGAAGAAAGCTTGCTTTCTTTGCTGACGAGCGGCGGACGG
GTGAGTAATGCCTGGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAA
TACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATCT
CCTGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT
CTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA
AGGCCTTCGGGTTGTAAAGTACTTTCAGTGGTGAGGAAGGGAGTGTGGTTAATAGCCATAT
TCTTTGACGTTAGCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAA

GTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAGTTGCATTTGAAACTGGCAGGCTAGAG
TACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAA
TACCGGTGGCGAAGGCGGGCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCTACTTGGAGGTTGT
GGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCG
CAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATCCTGCGGAGACGC
GGGAGTGCCTTCGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGTAATGG
TGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTC
ATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGCGGCG
AGCTAGCGATAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAAC
TCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGCAGGTA
GTTAACCTTCGGGAGGACGCTGCCACTTGT

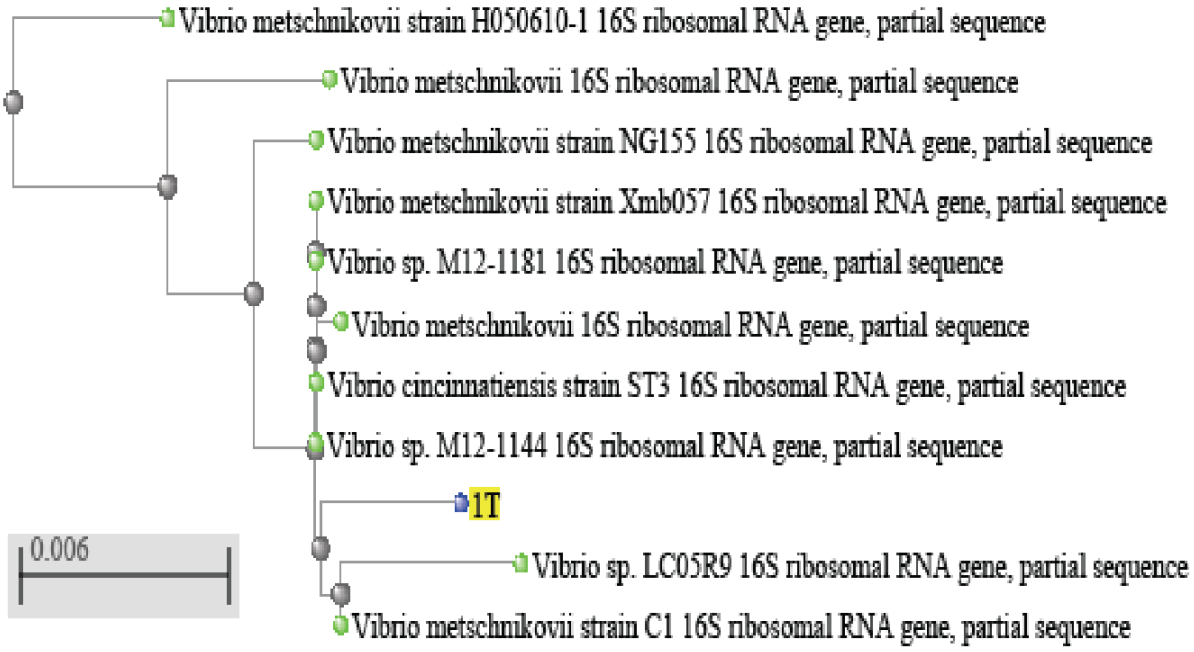

Figure 1Phylogenetic Tree of *Vibrio metschnikovii* strain Xmb057 (1T)

Among the two finally selected organism *Sphingomonas paucimobilis* and *Vibrio metschnikovii* strain Xmb057 showed nearly same deproteination. But the organism *Sphingomonas paucimobilis*is opportunistic pathogen capable of causing many infections in immunocompromised and healthcare associated persons. It can cause bacteremia, pneumonia and catheter associated infections with mortality rate of 5.5%. (Toh et al, 2011, Bayram et al, 2013). Due to the above mentioned risk associated with handling of *Sphingomonas paucimobilis* culture, it was considered to be unsuitable and hence not used for further shrimp shell deproteination experiments.

Even though *Vibrio* spp. is well known as pathogen due to its ability to synthesize many toxins and hemolysins, but infections to human by *Vibrio metschnikovii* is a very rare event. Till now very negligible number of cases are reported (cholecystitis:1, septicemia:3 and diarrhea: few cases)(Linde et al, 2004). Taking into consideration these facts instead of *Sphingomonas paucimobilis*, *Vibrio metschnikovii* was selected as the final organism for shrimp shell deproteination.

3.3.3 Antibiotic sensitivity of *Vibrio metschnikovii*strain Xmb057.

To find out if by chance during application of *Vibrio metschnikovii* strain Xmb057 during shell deproteinationprocess, causes any infection, how can it be controlled, therefore antibiotic sensitivityof *Vibrio metschnikovii* strain Xmb057was done. It was observed that *Vibrio metschnikovii*strain Xmb057was sensitive to majority of commonly recommended antibiotics. Out of 17 antibiotics *Sphingomonas paucimobilis* showed sensitivity to 16 antibiotics as shown in table 4. From this result it could be inferred that, if during use of organism for deproteination of shrimp shells accidently it produces infection, it can be cured immediately using commonly used antibiotics and it will not pose a problem of drug resistance. Therefore,*Vibio metschnokovii* was considered as anefficient and safe organism for deproteination of shrimp shells.

Table 4. Antibiotic sensitivity of *Vibrio metschnikovii* strain Xmb057

Antibiotic	**Sensitivity**	**Antibiotic**	**Sensitivity**
Ampicillin	R	Meropenem	S
Ampicillin/Clavulanic	S	Amikacin	S
Piperacillin/tazobactum	S	Gentamycin	S
Cefuroxime	S	Ciprofloxacin	S
Cetriaxome	S	Tigeccyclin	S
Cefoperazone/Sulbactum	S	Nitrofurantoin	S
Cefepime	S	Ccolistin	S
Etrappenem	S	Trimethoprim	S
Imipenem	S		

"R"- Resistant, "S"-Sensitive

3.3.4 Protein recovery and amino acid analysis

Recovery of proteins

After deproteination treatment broth was evaporated in hot air oven at 60^{o}C till constant weight in a pre-weighed glass beaker to measure recovered proteins. From 20 g shrimp shells deproteination, on evaporation 6.99 g of protein was recovered. In shrimp shells around 37 g% protein is present. Using *Vibrio metschnikovii* strain Xmb057 organism 34.95 g protein was recovered. This is 94.46% recovery of the proteins.

Amino acid profile

After deproteination treatment 1ml recovered broth was subjected to acid hydrolysis for analysis of amino acids as shown in fig. 2 presence of amino acids was detected using standard amino acids.

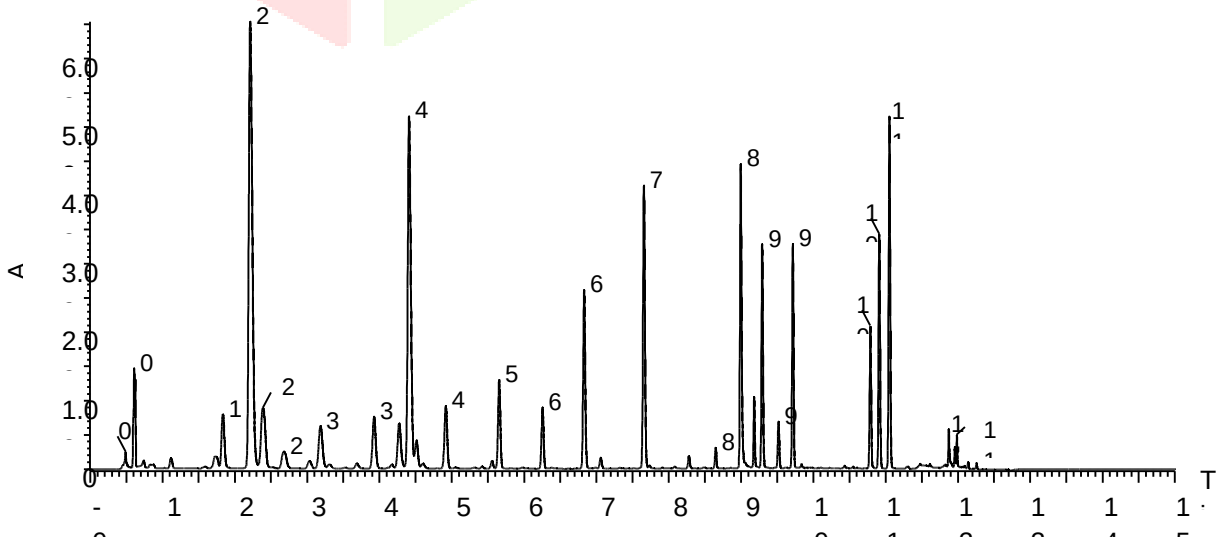

Figure 2. Chromatogram of amino acids after hydrolysis recovered proteins from shrimp shells.

Quantitative data of amino acids present in protein recovered from shrimp shells using *Vibrio metschnikovii* strain Xmb057 is as shown in Table5.

Table 5 Amount of amino acids after hydrolysis recovered proteins from shrimp shells.

Sr.no.	Name	ug/ml of sample
1	Phospho serine	2742.041
2	Hydroxy Proline	7302.53
3	Histidine	2729.311
4	Aspargine	1200.186
5	Glutamine	166.8432
6	3-Methyl-Histidine	4597.942
7	Taurine	388.778
8	1-Methyl-Histidine	146.2007
9	Serine	5445.031
10	Glycine	16142.72
11	Ethylamine	116.3688
12	Aspartic acid	10811.26
13	b-Alanine	218.1954
14	Sarcosine	6892.554
15	Threonine	217.8726
16	Alanine	3618.053
17	GABA	15659.27
18	aAAA	1979.067
19	Proline	127.061
20	OH-Lysine-1	30127.2
21	OH-Lysine-2	118.3636
22	Alpha Amino Butyric Acid	144.3896
23	Ornithine	1264.487
24	Cystine	821.7798
25	Lysine	4143.803
26	Tyrosine	12165.18
27	Methionine	2727.69
28	Valine	12377.48
29	NorValine	57.62205
30	Isoleucine	8813.257
31	Leucine	14603.17
32	Phenylalanine	18420.99
33	Beta Amino Iso Butyric Acid	22941.71
34	Arginine	6491.505

Poultry feed should contain all essential amino acids which are not synthesized by bird. These essential amino acids must be present in feed to act as a building block essential for synthesis of bird structural and functional proteins at all physiological stages of growth of bird. Even if a single amino acid is not present in the feed it may limit the growth of bird and is considered as a limiting factor. Along with all essential amino acid poultry feed should contain nonessential amino acids so that there is no conversion of essential amino acids into nonessential amino acids(Todd J. Applegate, 2008).

Dozier et al. (2008) analyzed the amino acid requirement of Broiler chicken using studies conducted by National Research Council. In general, during early stages of growth of bird amount of amino acids required is more as compared to later stages of growth.

Table 6. Amino acid requirement of broiler chickenDozier et al (2008).

Amino acid	% of diet requirement
Total sulfur amino acids	0.70 - 0.94
Methionine	0.50 - 0.62
Lysine	0.97 - 1.36
Threonine	0.67 - 0.84
Isoleucine	0.70 - 0.91
Valine	0.82 - 1.03
Arginine	1.04 - 1.47

For proper growth a set of amino acids should be present in the feed. From the table 5 and table 6 it can be concluded that all the essential amino acid required for broiler chicken are present in protein fraction recovered from the shrimp shells using *Vibrio metschnikovii* strain Xmb057.

Thus the present study suggests the possible use of *Vibrio metschnikovii* strain Xmb057 for deproteination of shrimp shells and use of protein recovered during deproteination of shrimp shells, as a supplement for poultry feed.

ACKNOWLEDGMENT – Authors are thankful to BCUD, Savitribai Phule Pune University, Pune for financial support in the form of Minor research project for this reseach work.

REFERENCES

[1] AOAC, Edition 2016. Method no.-920.87, Official Methods of Analyses, Association of Official Analytical Chemists, Washington, DC, Ch-32, 1-14.

[2]AOAC, Edition 2016, Method no.-941.12, Official Methods of Analyses, Association of Official Analytical Chemists, Washington, DC, Ch-43, 1-2.

[3]Bayram, N., Devrim, I., Apa H., Gulfidan, G., Turkyilmaz, H. N., Gunay, I., 2013Sphingomonas paucimobilis infection in children:24 case reports**,** Mediterranean Journal of Hematology and Infectious diseases5(1), 1-5.

[4]Dozier, W.A., III, M.T. Kidd, and A. Corzo. 2008. Amino acid responses of broilers. J. Appl. Poultry Research 17:157-167.

[5]Duangpaseuth, S., Das, Q., Chotchamlong, N., Ariunbaatar, J., Khunchornyakong, A., Prashanthini, V., Jutidamrongphan, W.,2007. ED78.20 Industrial Waste Abatement and Management, AIT, School of Environment, Resource & Development, Seafood Processing, Term Project 2,1-23.

[6]Gopakumar K.2002.Textbook of fish processing technology, Indian council of agricultural research New Delhi, 467-483.

[7]Ioannis, S. A., Aikaterini, K.,2008. Fish industry waste: treatments, environmental impacts, current and potential use, International Journal of Food Science and Technology, 43, 726–745.

[8]Islam, S., Khan, S., Tanaka, M., 2004. Waste loading in shrimp and fish processing effluents: potential source of hazards to the coastal and near shore environments, Marine Pollution Bulletin 49, 103–110.

[9]Jamialahmadi K., Behravan J., Fathi Najafi M., Tabatabaei, Y., Shahverdi A. R., Faramarzi M. A. 2011Enzymatic production of N-Acetyl –D-Glucosamine from chitin using crude enzyme preparation of Aeromonas sp. PTCC1691, Biotechnology 10(3) 292-297.

[10]Linde H. J., Kobuch R., Jayasinghe S., Reischi U., Lehn, N., Kaulfuss S., Beutin, L.2004 Vibrio metschnikovii, a rare cause of wound infection. Journal of Clinical Microbiology, Oct. 42(10),4909-4911.

[11]Ligozzi, M., Bernini, C., Bonora, M. G., de Fatima, M., Zuliani, J., & Fontana, R. (2002). Evaluation of the VITEK 2 system for identification and antimicrobial susceptibility testing of medically relevant gram-positive cocci. Journal of clinical microbiology, 40(5), 1681-1686.

[12]Mahmoud N. S., Ghaly A. E., Arab F., 2007, Unconventional Approach for Demineralization of Deproteinized Crustacean Shells for Chitin Production, American Journal of Biochemistry and Biotechnology 3 (1): 1-9.
[13]Mahmoud, M. A., Al-Agamy, M. H. M., El-Loboudy, S. S. and Ashour, M. S.2009 Purification and characterization of neutral protease from Bacillus subtilis strain N. Egypt. J. Microbiology. 22(1);131-143.
[14]Mazik, K., Burdon, D., Elliott M.,2005. Seafood-waste disposal at sea- a scientific review. Report to the Sea Fish Industry Authority, Institute of Eustuarine and coastal studies, University of Hull. Reference No.-YBB088,1-62.
[15]Murthy N.K.S., Bleakley B.H.2012. Simplified Method of Preparing Colloidal Chitin Used for Screening of Chitinase- Producing Microorganisms. The Internet Journal of Microbiology. 10 (2).
[16]Nair L., 2015, Indian seafood market- Opportunities for innovation and Collaboration in fisheries and aquaculture. Fishery in India, MPEDA,1-78.
[17]Pfeiffer, N., 2003. Disposal and Re-utilization of fish and fish processing waste (including aquaculture wastes). Marine RTDI Desk studies 2001-02. Ref. No. DK/01/003, 61-62.
[18]Sadafule, N. A., Salim, S. S., Pandey, S.K., 2012. An economic analysis of shrimp farming in the coastal districts of Maharashtra, Centre for Fisheries and Rural Development); Director - Ambition Group (Fish and Fisheries Business and Consultancy), Panvel, Dist. Raigad -Maharashtra, India, 42-54.
[19]Shahidi, 2001. Current protocols in Food Analytical Chemistry, D1.1.1-D1.1.11, Copyright 2001 by John Wiley and Sons, Inc.
[20]Todd J. A. 2008. Protein and Amino Acid Requirements for Poultry Feed Management Education Project funded by the USDA NRCS CIG program.1-11.
[21]Toh H. S., Tay, H. T., Kuar W. K., Weng T. C., Tang H. J., Tan C. K.,2011. Risk factor associated with Sphingomonas paucimobilis infection, Journal of Microbiology, Immunology and infection, 44(4):89-295.
[22]Troger C., Niranjan K. 2010. Sustainable chitin extraction and chitosan modification for application in the food industry, Proceeding of InternationalFoodInnovation.Oct.2010.
[23]Waldeck, J., Daum, G., Bisping, B., Friedhelm. 2006. Isolation and Molecular Characterization of Chitinase – Deficient Bacillus licheniformis Strains Capable of Deproteinization of Shrimp Shell Waste to Obtain Highly Viscous Chitin, Applied and Environmental Microbiology, 72(12,):7879–7885.