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PRODUCTION, CHARACTERIZATION, APPLICATION OF CHITOLOGIOSACCHARIDES HYDROLYSATE WITH PARTIALLY PURIFIED CHITOSANASE ENZYME FROM MARINE ISOLATE *BREVIDOMONAS DIMINUTA*

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Keywords:

Antimicrobial properties, Enzyme synthesis, Chitosanase, chitooligosaccharide hydrolysate, *Brevidomonas* species

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ABSTRACT: Microbial chitosanase has received greater attention for producing chitooligosaccharides. In this study, chitooligosaccharide hydrolysate was developed using the chitosanase enzyme, and its bioactivity was evaluated. Marine muddy samples proved to be a source of isolating novel chitosan-degrading bacteria when compared to the other samples collected. A chitosan-degrading marine bacterium was isolated, and the characterization of its extracellular enzyme, chitosanase, was studied. The molecular characterization and 16S rDNA sequence evolutionary relatedness of the isolate were carried out. The organism was identified as *Brevidomonas diminuta*, and its sequence was deposited. The production of chitosanase enzyme was significantly induced by the chitosan substrate, while it reached its peak after 72 hours. pH and temperature have been verified to be ideal for enzyme synthesis at 6.5 and 30 °C, respectively. Utilizing a culture medium containing xylose as the substrate at a level ranging from 1.0 to 1.5% significantly enhanced the enzyme production. The molecular weight of the enzyme, 43 KDa, was ascertained through SDS-PAGE. The pathogen's growth was suppressed by the chitooligosaccharide hydrolysate enzyme. It has promising antimicrobial properties, exhibiting a MIC_{50} of 0.2 µg/ml against the test pathogens.

INTRODUCTION: Chitosan has gained increased attention and has been extensively studied due to its diverse applications in different scientific disciplines. Chitosan has diverse applications, but its insolubility in water, high viscosity, and high molecular weight limits the biomedical uses of chitosan [1]. Chitooligosaccharides derived from chitosan exhibit a wide range of functional characteristics, such as solubility in water, lower molecular weight, antitumor activity, and antimicrobial activity.

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The enzymatic conversion of chitosan to chitooligosaccharides is the more advantageous method over the physical chemical methods due to its environmental compatibility, mild condition, and low cost [2, 3]. Chitosanase enzymes are widely used for the cleavage of chitosan into oligosaccharides and were categorised into three classes based on their cleavage specificity.

Chitosanases are members of glycosyl hydrolase groups that target the β1, 4glycosidic linkage in chitosan to depolymerize into chitosanoligo-saccharides. Chitosanases are classified into five glycosyl hydrolase (GH) families, such as GH5, GH8, GH46, GH75, and GH 80, based on amino acid sequences. The majority of bacterial and fungal chitosanses are grouped into the GH 45 and GH 75 families, respectively [4, 5, 6]. Chitosanolytic enzymes have been gaining attention due to their

capacity to depolymerize chitosanoligomers. The demand for new chitosanase enzymes with potential catalytic activity is increasing for various industrial productions. The use of chitosanoligosaccharide derived from chitosanase hydrolysis is restricted by elevated expenses associated with production and purification as well as the limited availability of the enzymes in sufficient quantity [7, 8] COS product usage is limited; this could be overcome by isolating the chitosanolytic strain, which utilizes a readily available, reliable source to reduce production costs [9, 10].

MATERIALS AND METHODS:

Screening of Chitosanase Organisms: The marine samples (muddy and dry soil) were gathered from various regions of Tamil Nadu and Pondicherry. These samples were obtained from a depth of 10–15 cm and subsequently transported to the laboratory in sterile conditions to undergo further processing. The collected soil samples were weighed and then mixed with 5 ml of marine water. Then the suspension mixture (0.1 ml) was spread and plated on the Chitosanase Detection Agar plates (CDA-plates) (CDA) plates (basal medium M9 with 0.5%, 1% and 2% substrate chitosan) and incubated for 3-5 days at 30 °C. The presence of chitosanase activity on the plates was indicated by the formation of clear zones [11, 12].

Quantification of Chitosanase Activity: The selected strains were grown on broth Chitosanase Detection Agar plates (CDA-plates) (CDA) media for 5 days at 30 °C. The enzyme analysis was carried out with the supernatant, and the strains were selected based on the chitosanase enzyme activity. Quantitative estimation of chitosanase activity was determined by reducing the sugar liberated, and the protein content of the crude extract was checked by DNS and Lowry method, respectively [13, 14].

Molecular Characterization and Identification of the Organisms: The chosen strains were identified by their biochemical, physical, and morphological characteristics. Genomic DNA from microbial isolates that produce chitosanase was isolated using STE buffer and chloroform extraction. Agarose gel electrophoresis was then used to determine quality and quantity, followed by staining with Ethidium Bromide at a concentration 0.5 µg/ml. The 16S rDNA was amplified using forward primer 8F (5´AGAGTTTGATCCTGGCTCAG-3´) and reverse primer 1942 R (5´-GGTTACCTTGTTACGACTT-3´). The Qiaquick PCR purification kit (QIAGEN, USA) was employed to purify the PCR product [15]. For sequencing reactions, the same forward and reverse primers were used for amplification of the 16S rDNA region with the DNA Sequencer (ABI-PRISM 3730, Applied Bio systems). The amplified regions were compared with the Genbank database using BLAST, and a phylogenetic tree was then constructed using the Neighbor-joining (NJ) method [16].

Optimization of Chitosanase Production: The production medium (CDA) was prepared with different concentrations of substrate (chitosan): 0.5%, 0.6%, 0.8%, 1%, 1.5%, and 2%. For temperature standardization, the inoculated culture was checked at 27°C, 30°C, 45°C, and 55°C for 8 days. After selecting the temperature and pH, various carbon sources (glycerol, glucose, xylose, sucrose, D-glucosamine, starch, and fructose) and nitrogen sources (such as yeast extract, peptone, and ammonium nitrate (NH_4NO_3) and ammonium chloride (NH_4Cl) were checked for the isolate's maximum enzyme activity [18].

Partial Purification and Determination of the Molecular Mass of the Enzyme: Ammonium sulphate was added gradually, and the proteins precipitated were separated by centrifugation, later the pellet was dissolved in phosphate buffer (pH 7.0). The remaining supernatant was again added with ammonium sulphate for 100% (w/v) saturation. The fractions containing precipitated proteins were further analysed using SDS-PAGE technique [19].

Application of Chitosanase Enzyme Hydrolysate:

Preparation of Chitooligosaccharide hydrolysate: The chitosan was hydrolysed with a partially purified chitosanase enzyme. The enzyme solution of 1 ml in 1 ml of 0.05 mM acetate buffer (pH 5.0) was mixed with chitosan (1 ml of 1%) and incubated at 55 °C for 30 minutes. The termination of the reaction was done by heating the mixture for

5 minutes at boiling temperature, and then the mixture was cooled to add 0.25 M NaOH.
The mixture was then centrifuged at 1,000 x g for 20 minutes to collect the chitooligosaccharide supernatant[20.] The mixture was added to concentrated alkali, resulting in precipitate formation and then the aliquots were lyophilized.

Solubility Test: The solubility of the chitooligosaccharide hydrolysate prepared was assessed at room temperature in different solvents with concentrations of 5 mg/ml, including ethanol, water, ethyl acetate, glacial acetic acid, and diethyl ether [21].

Antimicrobial Activity of Chitooligosaccharide Hydrolysate: The multidrug-resistant pathogens *Staphylococcus aureus*, *Pseudomonas* sp., and *Escherichia coli* were obtained from CMC, Vellore, and KMCH Laboratories, Coimbatore, Tamil Nadu. The broth dilution method was used to analyse the MIC and MBC values for each strain. The chitooligosaccharide hydrolysate samples were diluted to achieve the final concentrations of 0.25, 0.5, 1, 2, 4, 5, 10, 15, 20, mg/ml. The final MIC was regarded as the lowest concentration, which produced no visible turbidity even after incubating for 48 hours. The MBC value was determined, and the test dilution, which showed no visible turbidity, was sub-cultured on agar plates [22].

Statistical Analysis: The experiments were conducted in triplicate, and average values are presented. All data were expressed as mean ± standard deviation, and the statistical significance was analysed using the SPSS software package.

RESULTS AND DISCUSSION:

Screening of Chitosan-enzyme-producing Organisms: The marine soil samples collected were spread and plated on CDA. Growth was observed only in CDA plates with 0.5% and 1% of chitosan after 3 days **Fig. 1.**

FIG. 1: CHITOSANASE PRODUCTION OF THE ISOLATE INDICATING THE CLEAR ZONE FORMATION ON THE CDA MEDIA

TABLE 1: MICROORGANISMS ISOLATED FROM DIFFERENT SOIL SAMPLES ON CDA PLATES

Soil Samples	No. of samples	Isolates	
		Bacteria	
		ZP	NZP
Sea fish wastes dumped beach soil (Pondicherry)	2	1	21
Sea muddy soil (Pondicherry)	3	**8**	14
Sea water (Pondicherry, Cuddalore)	8	-	13
Beach soil (Pondicherry, Cuddalore)	8	1	14
Total	21	10	52

Keynote: NZP* Non-zone Producers; ZP* Zone Producers

Growth was observed on 0.5% and 1% chitosan plates [22, 23]. The chitosanase-producing organism was isolated from a marine soil sample, forming a clear zone of halos on 2% colloidal chitosan, but no growth was seen [24, 25].

In our present investigation, no growth was observed with 2.0% chitosan since medium viscosity, limited oxygen availability, and a high concentration of chitosan might inhibit the growth [26, 27]. Among all the 21 samples taken for studies, the marine mud sample was shown to be the most prominent source for the isolation of chitosanase producers **Table 1**.

Quantification of Chitosanase Activity: The marine isolates B44 and B44a enzyme activities were carried out with D-glucosamine as the standard 31. On the 5thday, B44 (1.241 U/ml) and B44a (1.224 U/ml) showed their maximum chitosanase activity level at 35 ºC **Table 2**. The activity of the enzyme was at its maximum when

the chitosanase activity was detected, but it was lower when compared to 4.8 U/ml of *Bacillus cereus* reported in earlier findings [28, 29, 30].

TABLE 2: CHITOSANSE ACTIVITY OF THE ISOLATES

Isolates	Biomass (g/L)	Specific activity (U/ml)
B3	1.4 ± 0.41	1.122
B63	2.07 ± 0.32	0.985
B46 (B44a)	1.97 ± 0.62	1.224
B44	2.11 ± 0.31	1.241

Identification of the Isolates: The muddy samples showed higher significance for chitosanolytic organisms than the dry soil [31, 32]. On the contrary, the present exploration has given chitosanolytic organisms were isolated in dry than in muddy soil. The bacterial isolates with the highest chitosanase activity were named B44 and B44a. The bacterial isolates B44 and B44a were gram-negative rods subjected to biochemical characteristics as shown in accordance with Bergey's manual of determinatives for bacteriology [33], the isolates were *Brevidomonas* sp. and *Pseudomonas* sp., respectively **Table 3.**

TABLE 3: BIOCHEMICAL CHARACTERISTICS OF THE BACTERIAL ISOLATES

Characteristics	B44	B44a
Colony shape	Round	Round
Colony colour	White	White
Colony texture	Moist	Moist
Gram stain	-	-
Shape of the cell	Rod	Rod
Spore	-	-
Pigment	-	+
Motility	+	+
Catalase	+	+
Urease	+	-
Oxidase	+	+
Indole	+	-
Voges-Proskauer	+	-
Methyl red test	-	-
Citrate	+	+
D-Glucose	+	-
Glycerol	-	-
Sucrose	-	-
Hydrolysis of casein	+	-
Hydrolysis of starch	+	-
Hydrolysis of nitrate	+	+

Identification of the Isolate: Nucleotide sequencing of B44 and B44a was submitted to the Genbank database with accession numbers KR063699 and KR873425, respectively (https://www.ncbi.nlm.nih.gov/nuccore/KR063699, https://www.ncbi.nlm.nih.gov/nuccore/KR873425).

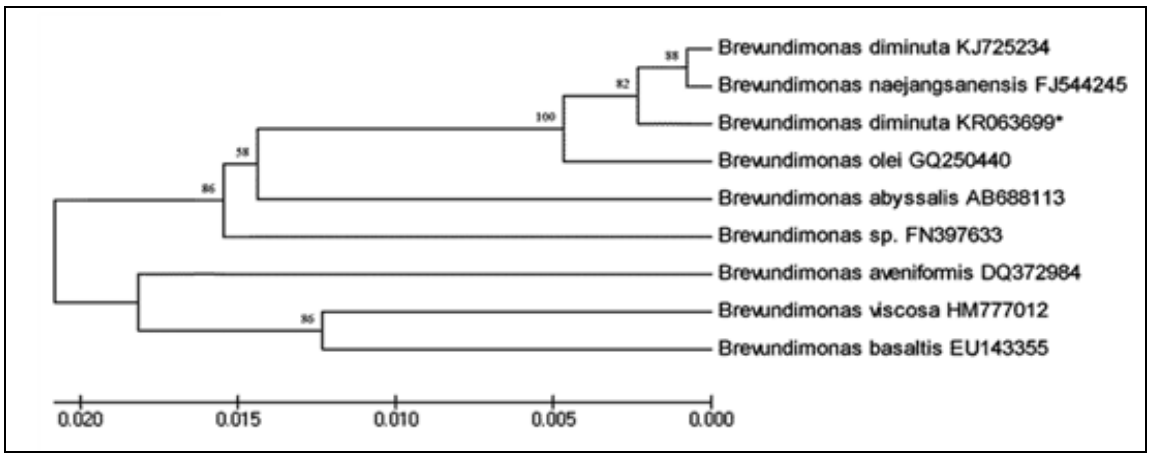

FIG. 2: THE PHYLOGENETIC TREE OF 16S RDNA OF ISOLATE *BREVIDOMONAS DIMINUTA*

The base pairs of 1305 (B44) and 1271 (B44a) were aligned. The 16S rDNA sequence of strain B44 was highly homologous to many strains of *Brevidomonas diminuta* (B44) and *Pseudomonas aeruginosa* (B44a). The phylogenetic tree was constructed **Fig. 2** by the neighbor joining method [34], with results showing the B44 strain belongs to *Brevidomonas diminuta* (accession number KR063699) and strain B44a belongs to *Pseudomonas aeruginosa* (accession number KR873425).

Optimization of Chitosanase Production: The strain B44a was renamed as B46. On the 3rd day of incubation, strain B44 showed high activity, and the activity was seen to be higher if the concentration of the substrate, chitosan, was increased to 1.5%.

The B44 strain showed a higher activity of 2.103 mg/mL with 1.5% and B46 with 2.01 mg/mL on the 5th day **Fig. 3.**

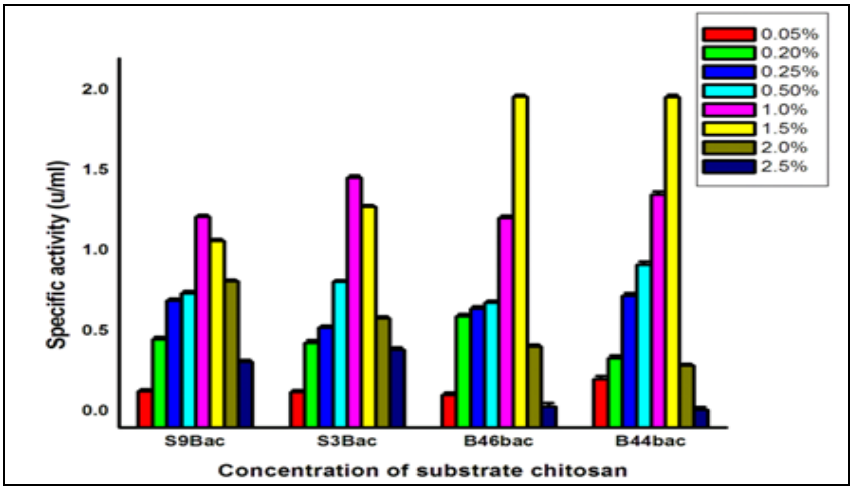

FIG. 3: THE OPTIMIZATION OF SUBSTRATE CHITOSAN FOR THE PRODUCTION MEDIA

The organism showed the highest enzyme activity at pH 6.5. The strain B44 enzyme was sensitive, as minimum activity was observed below pH 5 and above pH 7 due to the instability of the proteins **Fig. 4.**

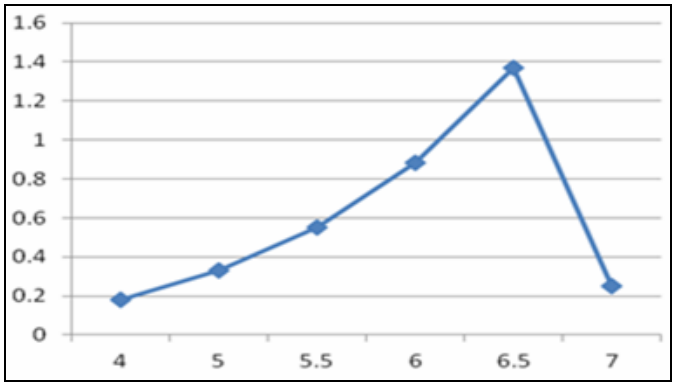

FIG. 4: EFFECT OF PH ON CHITOSANASE ACTIVITY

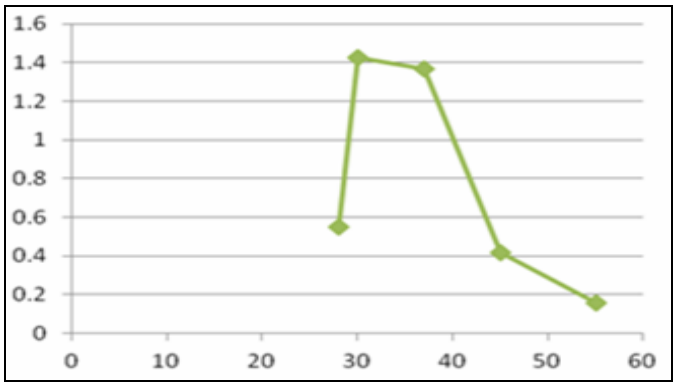

FIG. 5: EFFECT OF TEMPERATURE ON CHITOSANASE ACTIVITY

In the temperature optimization studies, the crude enzyme showed higher activity at 30°C and 37°C but decreased at 45°C and 55°C **Fig. 5**. Our study found that crude enzyme activity was highest at pH 6.5, 30°C, with 1.5% chitosan. The chitosan activity was higher in glucose and starch [35, 36]. The higher enzyme activity of bacterial colonies showed the utilization of 1% glucose and 1% fructose as major carbon sources **Fig. 6.** It has been known that glucose will catabolically repress chitosanase production [37], but from our results, xylose at 1% has influenced the production of all the bacterial isolates. The isolates B44 and B46 have shown maximal enzyme production of 4.562 U/L and 2.263 U/L, respectively, with xylose concentrations at 1%. Sucrose at 1% enhanced the

maximum chitosanase production in B46 by 1.963 U/L. The concentration of starch at 1% also influenced the bacterial isolates B44 (3.769 U/L) and B46 (1.314 U/L) for the maximum production of chitosanase.

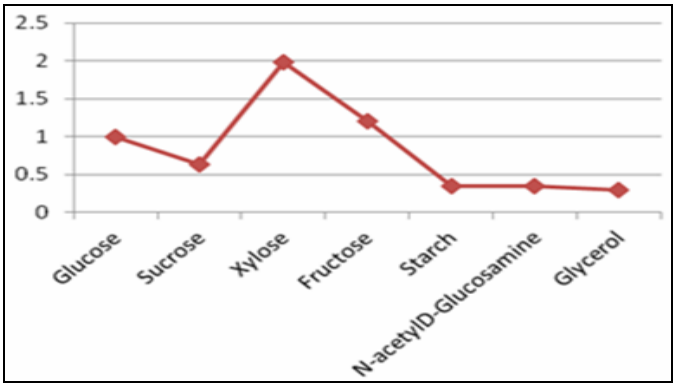

FIG. 6: EFFECT OF CARBON SOURCES ON CHITOSANASE ACTIVITY

TABLE 4: EFFECT OF NITROGEN SOURCES ON CHITOSANASE PRODUCTION

	B46bac	B44bac
AC	1.199±0.002[a]	1.390±0.021[a]
AS	0.701±0.011[b]	0.692±0.042[b]
PN	0.462±0.002[c]	0.319±0.002[c]
U	0.316±0.004[d]	0.094±0.005[d]
YE	0.833±0.004[e]	1.580±0.076[e]
PEP	1.291±0.027[f]	1.406±0.005[a]

Keynote: Values were given as mean ± S.D; Each column with different superscript letters Indicates are significantly different determined by one-Way ANOVA Post-hoc Tukey's test ($p \leq 0.05$).

1% yeast extract proved to be the best nitrogen source for B44 [38], which exactly matched Zhang *et al*., 2022. Among the nitrogen sources, peptone, yeast extract, and ammonium chloride were shown to influence the growth of organisms, whereas the growth of the organisms ceased with urea-added production media [39]. The nitrogen sources in our present optimization study revealed that strain B44 utilized peptone (0.5%) and ammonium chloride (0.5%) for the highest activity. The results of Aktuganov *et al*. (2019), reporting that higher chitosanolytic activity was observed when optimization media were supplemented with 1% yeast extract, were similar to our present study **Table 4.** The activity was repressed when a yeast extract of 0.5% was employed in the production medium [40]. During the carbon and nitrogen combination optimization studies, B44 showed higher activity in production media containing 10% peptone with all carbon sources at 20%. The highest activity was seen only in the combination of ammonium chloride (20%) and sucrose (10%). The activity of the isolate B44 was also higher with peptone (20%) and fructose (10%). From the results, it is clear that the proportion of peptone (10%) with carbon sources induced the organisms to yield the highest enzyme production. The optimization studies on chitosanase activity in our present study revealed the maximal activity observed with culture conditions of pH 6.5, 30°C, 1.5% chitosan, 10% peptone, and 2% xylose or fructose.

Molecular mass-SDS-PAGE: The SDS-PAGE pattern of the partially purified B44 chitosan enzyme **Fig. 7** was found to be approximately 43 KDa [41], which was similar to Singh *et al.* (2021) but was higher, ranging from 41 KDa to 45 KDa [42].

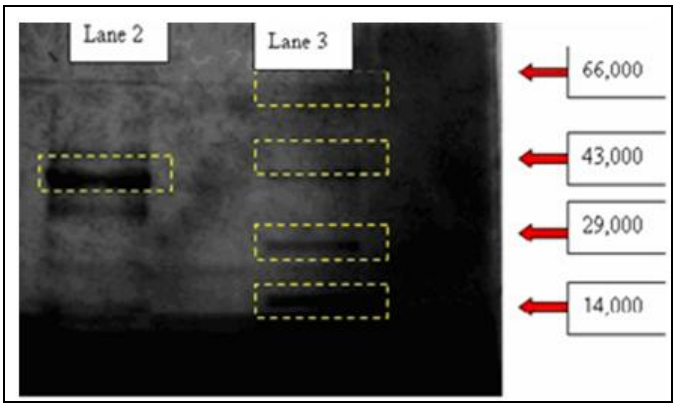

FIG. 7: SDS PAGE OF THE CHITOSANASE FROM THE ISOLATES. LANE 2: PARTIALLY PURIFIED ENZYME EXTRACT OF B44, LANE 3: MARKER MOLECULE

Application of the Chitosanase Enzyme:

Preparation of Chitooligosaccharides and their Solubility: The de-polymerization of chitosan into small oligomers indicated by the no precipitate formation of the mixture when added to alkali was similar to the result [43] observed by Tabassum *et al*. 2022.

Solubility of Chitooligosaccharides: The solubility of B44 and B46 chitooligosaccharides was investigated in various solvents, such as water, ethyl acetate, and glacial acetic acid, at room temperature. The solubility of chitooligosaccharide in water, ethanol, ethyl acetate, and glacial acetic acid except diethylether [44], whereas in the present investigation, the chitooligosaccharide not dissolved in ethanol and diethylether **Table 5**.

TABLE 5: SOLUBILITY PATTERN OF THE CHITOOLIGOSACCHARIDE

S. no.	Samples	Solvents	Soluble
1	B44	Water	Yes
		Ethanol	No
		Diethyl ether	No
		ethyl acetate	Yes
		glacial acetic acid	Yes
2	B46	Water	Yes
		Ethanol	No
		Diethyl ether	No
		ethyl acetate	Yes
		glacial acetic acid	Yes

The chitooligosaccharides showed the highest zone of inhibition at 60µl for all pathogens in the study. The MIC and MBC of the chitooligosacharides were also determined from 0.2 g to 100 g, and the present chitooligosacharide hydrolysate showed a higher zone of inhibition at 60µg/ml for the selected pathogens **Fig. 8** & **Table 6.**

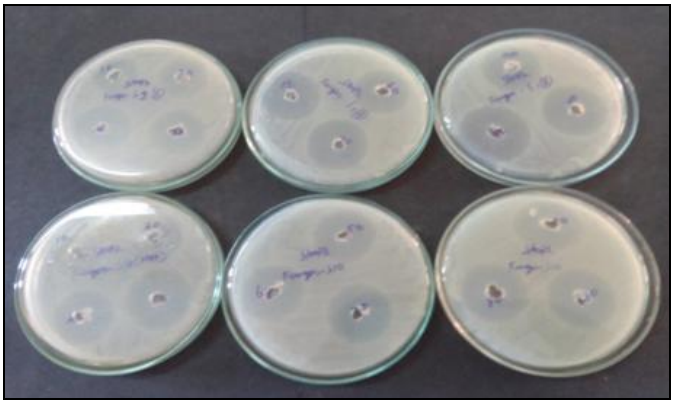

FIG. 8: ANTIBACTERIAL SCREENING ACTIVITIES OF CHITOOLIGOSACCHARIDES BY USING WELL DIFFUSION ASSAY

TABLE 6: ANTIBACTERIAL SCREENING ACTIVITIES OF CHITOOLIGOSACCHARIDES BY USING WELL DIFFUSION ASSAY

Pathogens	Zone of inhibition in mm										Crude Enzyme 100µg / well	Chitosan 100µg/well Zone in mm
	Concentration of CHOS in µg											
	10	20	30	40	50	60	70	80	90	100		
E.coli	-	2	5	7	10	11	12	13	13	15	-	11
P.aeruginosa	3	9	12	14	16	16.7	17	18.5	19	20	-	14
S. aureus	4	6.2	7	10	11	11	12	13	14	15.1	-	9

The crude enzyme showed no zone of inhibition, whereas chitosan showed an inhibition zone lower than that of chitooligosaccharides in 100µl **Table 7.** Chitosan was more effective against *E. coli* than chitoligosaccharide [45].

TABLE 7: MIC AND MBC VALUES OF CHITOSAN OLIGOSACCHARIDE SAMPLES

Organism	MIC and MBC values (mg/ml) of Oligochitosan Samples		
	Initial MIC [mg/ml]	Final MIC [mg/ml]	MBC [mg/ml]
Escherichia coli	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.01
Staphylococcus aureus	0.1 ± 0.01	0.2 ± 0.02	0.1 ± 0.02
Pseudomonas aeruginosa	0.15 ± 0.02	0.19 ± 0.01	0.15 ± 0.03

In the present study, chitooligosaccharide hydrolysate showed advantages as an antibacterial agent compared to chitosan against all pathogens taken for the study. The MIC and MBC of the chitooligosacharides were also determined from 0.2 to 100 µl, and the activity was similar against E. *coli* and *S. aureus* at 10 mg/ml [46, 47]. The antimicrobial activity of the chitosanase enzyme in

the present investigation showed no inhibitory effect against the selected pathogens. Chitooligosaccharides showed significantly stronger antimicrobial activities than chitosan and chitosanase **Fig. 9.**

FIG. 9: ANTIMICROBIAL ACTIVITY OF THE CHITOOLIGOSACCHARIDE HYDROLYSATE

The inhibitory zones of chitooligosaccharides were significantly higher than those of chitosan and chitosanase [48]. Our present investigation has controversial results compared to Li *et al.*, 2020 as the antibacterial effect of the chitooligoosaccharide was greater than that of chitosan. Further, the present result of the chitooligosaccharide hydrolysate obtained disagrees with Aranaz *et al.*, 2021 finding that the slightly hydrolysed chitosan hydrolysate was more active than chitosan and chitooligomers [49]. Further, the present result of the chitooligosaccharide hydrolysate obtained disagrees with Aranaz *et al.*, 2021 finding that the slightly hydrolyzed chitosan hydrolysate was more active than chitosan and chitooligomers [50]. As per the literature survey, chitosan showed higher activity against Gram-positive organisms than chitooligosaccharides [51], but in our study, chitooligos activity was similar to both Gram-positive and Gram-negative organisms.

CONCLUSION: The present investigation is aimed at the selection of bacteria for chitosanase synthesis and the substantial yield of bio-functional chitooligomers. We collected various marine samples from multiple locations for our inquiry. From the findings we obtained, the marine muddy soil proves to be an intriguing resource for the isolation of novel chitosanase-producing organisms for the synthesis of bio-functional chitooligosaccharides. The optimization approaches used to enhance enzyme production ranged from 1.34 to 4.94 ul/l. The chitooligosaccharide hydrolysate derived from the partly purified enzyme has shown superior antibacterial efficacy against multidrug-resistant organisms than chitosan.

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Declaration of Patient Consent: Patient's consent not required as there are no patients in this study.

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CONFLICTS OF INTEREST: There is no conflict of interest.

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