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Research Article

Chitinase Producing Bacterial Dynamics among Different Chitin Exposed Sampling Sites

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ABSTRACT

Background: Chitinase is used in pest management, as a biopesticide to protect plants from fungal infestations. The purpose of this study was to identify, isolate, and screen for bacteria that produce chitinase from soil surrounding a chitin-enriched location. **Materials and Methods:** 7 samples were taken from various geographical sites to get chitinase-producing bacteria with strong chitinolytic activity at temperatures fluctuation. Dilution plate was performed and zones of hydrolysis followed by incubation at 37ºC were used to select active strain. Submerged fermentation with different substrates was tested to sort out best isolates. The effect of temperature on enzyme production was applied for selection of most potent strain. All the data evaluated statistically using student t-test. **Results:** 7 promising chitinase producers isolated from 7 samples. 28.57% were gram negative and rest are gram positive isolates. Three isolates S3A, S4A and S4B found to produce chitinase under crabshell, chitin, colloidal chitin with significant shell degradation than other strains. Among the three isolated strains S3A found to be effective showed maximum enzyme activity at 15 and 55ºC in addition to 37ºC. The total protein was 2.834, 0.343, 0.182 mg/mL with chitnolytic activity of 27.0, 9.0, 6.4 U respectively at 37, 15 and 55ºC. **Conclusion:** Soil samples exposed to chitin have effective chitinase producer at 15 to 55ºC. The active isolates identified as genera of Citrobacter sp. (S3A) and *Bacillus* sp. (S4A and S4B) are potentially used in bio control of harmful fungi and insects.

Keywords: Enzyme, Anti-fungal, Chitinase, Biopest, Chitin.

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INTRODUCTION

Chitin is a renewable biopolymer that is commonly present in molluscan shells, arthropod exoskeletons, and fungal cell walls. Every year, more than one billion tons of chitin are accumulated in the environment.[1] According to Souza *et al.*,[2] large amounts are commonly found in the excrement of aquatic animals, such as crabs, shrimp, and mollusk shells. A large variety of species share a similar chitin structure, except for the protein-calcium carbonate bond, which is primarily seen in shrimp shells. Twenty to fifty-eight percent of the dry weight of shellfish waste-roughly 75% of the total mass of shellfish such as shrimp, crabs, etc.-is composed of chitin.[3] It is crucial to identify new methods for using and disposing of the 60-80,000 tons of chitinous waste that are produced in India alone.[4] From the remains of crustaceans, nanofibers of chitin and chitosan can be extracted and utilized in biomedical and healing purposes. The cell walls of most fungi and certain algae contain chitin, which is the next most prevalent carbohydrate. A long, linear chain of carbohydrates is formed by N-acetyl-D-glucosamine monomers joined by β-1.4 linkage.[5] Chitin and cellulose are chemically similar, except for one glucoside residue that has an acetylated or deacetylated amino group in place of one of the hydroxyl groups. The growing number of chitinous wastes makes proper treatment of these wastes necessary. One potential method for managing chitinous waste is to employ them as a source of chitinase synthesis.[6] According to Saima *et al.*, bacteria from the following genera have been shown to degrade chitin in marine environments: Aeromonas, Enterobacter, Chromobacterium, Arthrobacter, Flavobacterium, Serratia, *Bacillus*, Erwinia, and Vibrio. Bacteria, fungi, action, actinomycetes, and higher plants all produce a class of enzymes called chitinases (EC 3.2.1.14).

Chitinases help to break down the chitinous waste from the seafood industry and preserve the carbon-nitrogen equilibrium in the ecosystem by exploiting contaminants from crustaceans.[7] Antimicrobial chitinases have antibacterial in nature, antifungal, insecticidal, and nematicidal properties because they weaken and break down the cell walls of a variety of diseases and pests.[8] Chitinolytic microbes therefore hold potential as alternatives to the more hazardous methods of using pesticides that kill fungi and insects.[9] The existence of chitinolytic bacteria indicates that

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chitin is present in the environment. For the successful breakdown of chitin, it is still thought that more potent chitinolytic microbes must be developed. Finding more potent chitinolytic microbes that can degrade chitin into substances is therefore necessary.[10] The significance of uncharted areas as a source of prospective microbes with economic uses is reinforced by this research.

MATERIALS AND METHODS

Sample collection

A total number of 6 different soil samples like soil from shrimp pond, Graden soil, Crab export industry site, Fish market, Beach soil, crab shell dumped soil were collected from 6 different region of Tamil Nadu, India. 10 g of soil sample was collected from each selected site carefully in an aseptic condition and the samples were placed in a sterile container and kept on ice until returned to the laboratory.

Preparation of colloidal Chitin

About 5 g of chitin powder (SRL, India) was added slowly to 50 mL of concentrated HCl (35%) and kept overnight at refrigerator at 10°C. The mixture was added to 50 mL of ice-cold 50% ethanol with vigorous stirring at 25°C and kept in the rotary shaker at 200 rpm overnight. The precipitate was collected by centrifugation at 10,000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was freeze dried to powder and stored at 37°C until further use.

Preliminary screening

Nutrient agar amended with 1% colloidal chitin was prepared by mixing 1 g of colloidal chitin with 0.3 g yeast extract, peptone, 0.55 NaCl and 2 g agar in 100 mL water.[11] the medium is sterilized by autoclaving at 121°C for 15 min. Meanwhile 1 g soil sample mixed with 10 mL normal saline. 1 mL of stock solution was mixed with 9 mL sterile water and serially diluted to get 10^{-7}. One mL of 10^{-7} dilution was transferred to sterile petridish and then chitin agar was poured over the sample. The medium is allowed to solidify and incubated at 37°C for 72 hr. All the samples were kept in triplicates and the zone of clearance due to chitin hydrolysis was recorded for up to 3 days.

Isolation of chitinase producing bacteria

About 1 g of soil sample was serially diluted and plated on Chitin Minimal Agar (CMA) plates (Colloidal chitin, 1%; K_2HPO_4, 0.3 g; NaCl, 4 g; KH_2PO_4, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract 0.5 g and agar, 1.7% per L). The plates were incubated at 30°C for 3 days. The bacterial colony showing the zone of clearance was selected, purified by streaking on fresh CMA. Chitin solublization index was calculated as follows:

CSI= diameter of zone/ colony diameter

Effect of substrate on production of chitinase

A 50 mL of Chitin Minimal Medium (CMM) containing 0.7% (w/v); KH_2PO_4, 0.3% (w/v); K_2HPO_4, 4% (w/v); NaCl, 0.5% (w/v); $MgSO_4.7H_2O$ and 0.5% (w/v) yeast extract and pH 7.2 was prepared in a 250 mL flask.[12] The medium amended with 0.5% of inducer such as colloidal chitin, chitin and crab shell powder and then autoclaved. The sterile broth was inoculated with 5 mL of young seed culture (OD 1 at 600 nm). The flask was incubated at 37°C for 3 days. Further to incubation, the supernatant was separated via centrifugation of culture broth at 8000 g for 10 min for every 24 hr and was used as the crude enzyme. The percentage of crab shell degradation was recorded by gravimetric analysis. The dry weight of residual crab shell after 72 hr was taken followed by filtration and drying oven at 65°C.

Percentage of wt loss= Initial weight of crab shell-final weight/ In wt×100

Determination of Effect temperature on active isolates

The production medium nutrient broth amended with 0.15 colloidal chitin was prepared, sterilized and inoculated with 10% inoculums of active strains and incubated at different three (15±1, 37±1 and 55±1°C) different temperatures for 48 hr at 100 rpm. The growth rate was recorded at 600nm. The protein was isolated by methanol- chloroform extraction by mixing 1 part of cell free filtrate to 4-part methanol and vortex well. To this mixture 2 part of chloroform was added and stirrer well. The mixture was centrifuged under 10,000 rpm for 15 min at 10°C and the protein middle layer was aspirated and dissolved in TE buffer (pH 6). Sepharose column was used to purify the protein. The dialysed enzyme solution was then purified with Sephadex 75 column (20 cm×1.5 cm) equilibrated with 20 mM Tris-HCl buffer with the pH 8.0. The flow rate of 1 mL/5 min was used to elute the enzyme. The eluted fractions were estimated for the chitinase activity. The fractions showing chitinase activity were mixed together and concentrated by using a fast flow DEAE-Sepharose column (1.6 cm×20 cm) pre-equilibrated with 20 mM Tris-HCl of pH 8 (10 mmol/L). The bounded chitinase was eluted at a flow rate of 400 µL/min using different gradients of NaCl buffer (0-0.5 mol/L) at 4°C. The collected chitinase fractions were used as an enzyme source for chitinase assays and protein quantification. Lowry method used for total protein estimation.

Chitinase assay

The chitinase activity was determined by DNS colorimetry (3,5-dinitrosalicylic acid), using colloidal chitin as substrate, and the amount of reducing sugar was determined.[13] 0.25 mL sodium phosphate buffer (0.05 M, pH 7.0), 0.5 mL 1% colloidal chitin, and 0.25 mL Cell free filtrate solution were mixed and put into a water bath at 37°C for 30 min, after boiling for 5 min, the reaction was terminated. 2 mL DNS reagent was added and boiled for 5

min. The supernatant was cooled to room temperature. After centrifugation, the absorbance of the supernatant was measured at 540 nm, and the inactivated enzyme was used as blank control. The enzyme activity was defined as the amount of enzyme needed to convert the substrate colloidal chitin to produce 1 µmol reducing sugar per minute at 37°C, which was defined as one activity Unit (U). N-acetyl glucosamine as standard.

U of enzyme=Amount of sugar/221.2×4

RESULTS

Preliminary screening of isolates

Conventional Chitinase producing bacteria was isolated from six different samples by dilution plate method and the positive strains were selected for screening of enzyme production under submerged fermentation with different substrates. Based on Effect of substrate and degradation of crab shell active strain's are subjected to evaluate at different temperature (15, 37 and 55°C). Growth rate was recorded at 600 nm. Total protein was isolated by methanol-chloroform extraction (4:1:2). The total protein of crude and purified protein was estimated by Lowry' method. The chitinase assay was performed by the amount of sugar released by enzyme followed by breakdown of colloidal chitin under 37°C was calculated by DNS method. All the tests were triplicate, and the data was analyzed by student t-test method. Results: Out of 7 samples, seven chitinase positive isolates were recovered. The growth of positive strains is luxuriant on colloidal chitin enriched broth than chitin and crab shell amended broth. Extracellular crude chitinase was higher among three isolates estimate 22, 20 and 10 units on colloidal chitin followed by chitin. Further these three isolates showed degradation of crab shell during submerged state and the percentage of degradation was 24, 22 and 10%. Fish market soil, Sea shore soil and Shell dumped soil showed greater than 250 CFU respectively with five chitinase producers. Other Samples have less than 200 CFU with. Except Garden soil other samples showed one chitinase positive strains. The strains are designated as S1, S3A, S3B, S4A, S4B, S5 and S6 (n=7). Chitinase positive S1 was small spindle shape colonies with 2.8±0.002 mm citine clearance zone. Isolate from sample 3 such as S3A and S3 B where punctiform and circular opaque and the zone around the colonies were 2.4±0.002 and 3.2±0.002 mm. Sample 4 have small circular colonies with 9.8±0.002 mm zon e(S4A) and the large irregular colonies shown maximum zone 11.6±0.002 mm (S4B). Another irregular opaque colony of isolate s5 from sample 5 zone is 10.4±0.002 mm. Isolates of sample 7 was Circular, white rhizoidal with 7.8±0.002 mm zone of clearance (Table 1). The colony morphology was given in Table 2. The biochemical characters of positive isolates given in (Table 3) reveal that about 71% were gram positive in nature. Based on biochemical (Table 3) and cell wall type 4 were identified as *Bacillus* sp., and each one identified as *Citrobacter* sp. (s3b), *Pseudomonas* sp. (S5) and *Lactobacillus* sp. (S6). The growth pattern of all the isolates under minimal medium with different inducer was studied and the growth rate was represented on Figure 1. The maximum growth rate was found in mm with colloidal chitin, moderately at chitin and significant in crab shell amended broth. Isolate S3A and S4A gave maximum turbidity of growth on cc and chitin inducer

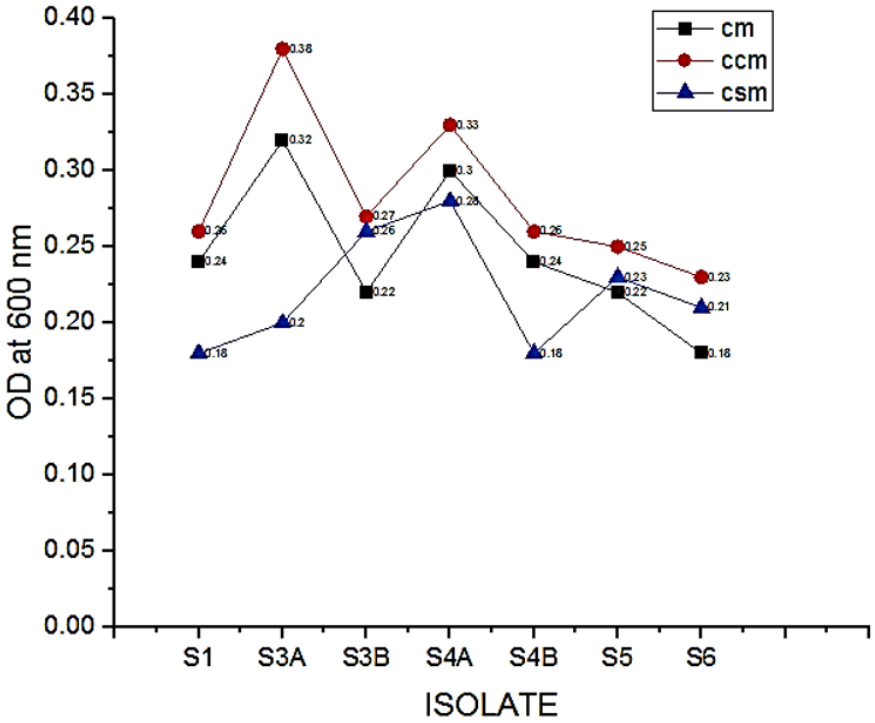

Figure 1: Growth rate of chitinase positive strains on different medium used.

enriched medium. Isolate s3b and s4a Showed higher growth on crab shell added medium and showed significant loss of crab shell (Figure 2). Further these three isolates showed degradation of crab shell during submerged state and the percentage of degradation was 24, 22,16 and 10% among S3A, S4A, S1 and S4B (Table 4).

Effect of substrate on Chitinase

The enzyme activity of extracellular chitinase among isolates were estimated in triplicate and the data is given in Table 5. Colloidal chitin medium gave maximum enzyme activity. Except isolate S1 (4.19±0 U) all other strains showed good response on cc medium. Maximum unit of enzyme assay was 22.79±4.35 U by S3A followed by 21.40±0 U by S4A and 10.22±0 U in S4b. Isolate S3B activity recorded as 9.02±0 U where as S5 and S6 activity was 8.1±0 and 6.7±0 Units. Similarly, chitin inducer expressed moderate enzyme activity among S3A, S4A, S4B and the activity of chitinase were 8.8 39±6.54>8.583±6.36>5.659±4.27>5.64±4.25U. The effect of crab shell medium showed significant enzyme activity on two strains

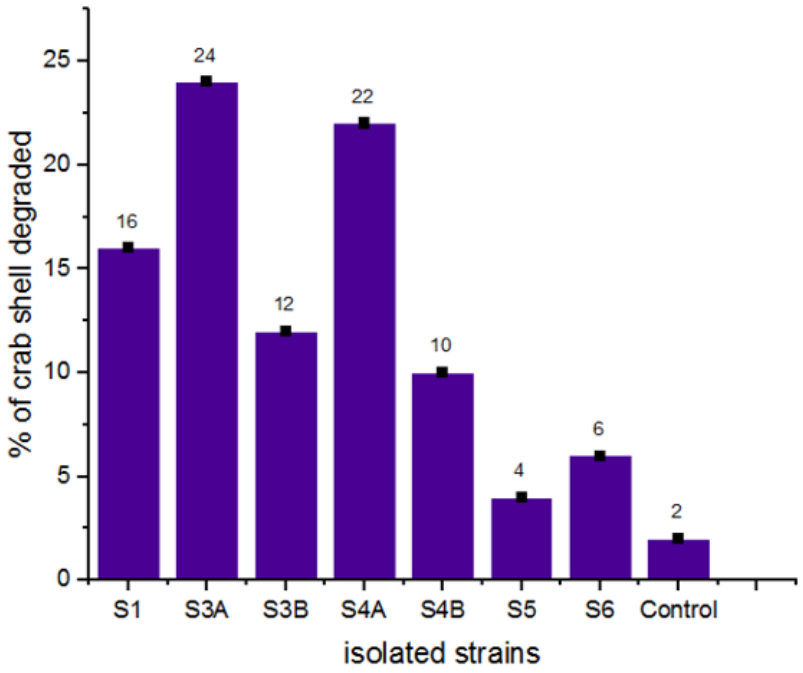

Figure 2: Crab shell degradation by isolates after 72 hr.

Table 1: The number of positive isolates recovered from soil samples.

Sample	CFU x10^{-5}	positive strain	Strain code
Sea food industry soil	184	1	S1
Garden soil	182	0	0
Sea shore soil	282	2	S3A S3B
Shell dumped soil	270	2	S4A S4B
Shrimp pond soil	168	1	S5
Fish market soil	254	1	S6

Table 2: Size of zone on secondary screening by positive isolates.

S. code	Chitinase positive	Colony nature	Zone diameter mm
S1	1	Small spindle shape white opaque	2.8±0.002
S2	0	--	--
S3	2	Puncti form, white opaque	2.4±0.002
		Circular opaque raised	3.2±0.002
S4	2	Small circular white flat opaque's	9.8±0.002
		Large irregular white opaque	11.6±0.002
S5	1	Large irregular white opaque	10.4±0.002
S6	1	Circular, white rhizoidal	7.8±0.002

Table 3: Biochemical characters of chitinase positive strains.

S. code	Gram stain	Indole	MR	VP	Citrate	Catalase	Oxidase	Genera
S1	Positive Rod	-	+	-	+	-	-	*Bacillus* sp.
S3a	Negative rod	-	+	-	+	+	-	*Citrobacter* sp.
S3b	Positive slender rod	+	++	-	+	-	+	*Bacillus* sp.
S4a	Positive cocco bacilli	+	++	-	+	-	-	*Bacillus* sp.
S4b	Positive rod	+	+	+	+	+	-	*Bacillus* sp.
S5	Negative rod	-	++	+	+	+	+	*Pseudomonas* sp.
S6	Positive rod	-	++	-	+	+	+	*Lactobacillus* sp.

+Weakly positive, ++ positive, - negative.

Table 4: Crab shell degradation after 72 hr incubation.

S. Code	Initial. weight	residual weight	percentage
S1	0.5	0.42	16
S3A	0.5	0.38	24
S3B	0.5	0.44	12
S4A	0.5	0.39	22
S4B	0.5	0.4	10
S5	0.5	0.48	4
S6	0.5	0.47	6
Control	0.5	0.49	2

Table 5: Crude Chitinase activity among isolates on different inducer.

strain		crab					cm			CC		
	sugar	SD	units	SD	sugar	SD	units	SD	sugar	SD	units	SD
S1	0.175	0.07	3.16	1.36	0.152	0.12	2.749	2.19	0.013	0.03	4.19	0
S3A	0.441	0.07	7.98	1.30	0.488	0.36	8.839	6.54	1.260	0	22.79	4.35
S3B	0.419	0.32	7.59	5.84	0.253	0.19	4.581	3.49	0.498	6.8	9.02	0
S4A	0.353	0.03	13.13	9.69	0.474	0.35	8.583	6.36	1.183	0	21.40	0
S4B	0.258	0.20	4.66	3.66	0.312	0.23	5.659	4.27	0.565	0	10.22	0
S5	0.317	0.24	5.74	4.51	0.239	0.18	4.323	3.36	0.451	6.8	8.159	0
S6	0.236	0.19	4.28	3.47	0.186	0.14	3.368	2.64	0.375	6.8	6.7822	0

Table 6: Concentration of protein (mg/mL) among active chitinase producers.

Protein	S3A	SD (±)	S4A	SD (±)	S4B	SD (±)
crude 55°C	0.220	0.010	0.285	0.005	0.245	0.003
pure 55°C	0.182	0.006	0.214	0.008	0.202	0.006
crude 37°C	3.005	0.019	2.527	0.019	2.390	0.039
pure 37°C	2.834	0.078	2.390	0.052	2.299	0.071
crude 15°C	0.434	0.034	0.571	0.034	0.434	0.059
pure 15°C	0.343	0.019	0.434	0.034	0.320	0.052

S3A and S4A with 7.98±1.30 and 13.13±9.69U respectively. It was noted that isolated those produced low level of chitinase in chitin and crab shell medium expressed greater enzyme activity in cc medium (Figure 3). The effect of temperature on active strain reveals that the maximum enzyme and total protein was recorded at 37°C followed by 15 and less significantly at 55°C. Total protein of isolate S3A, S4A and S4B grown at 15, 37 and 55°C was given in Table 6 and concentration of crude and pure represented on Figure 4. Among the temperature cells grown at 37°C shows maximum crude extracellular protein on minimal medium and was estimated as 3.005±0.019, 2.527±0.019, 2.390±0.039 whereas the gel permeation purification shows reduced protein content estimated as 2.834±0.078, 2.390±0.052 and 2.299±0.071 mg/mL. Growth at 15°C showed 5-8-fold decreased crude protein content compared to 37°C. The concentration of crude at 15°C were 0.434±0.034, 0.571±0.034 and 0.434±0.059 and the pure protein was 0.343±0.019, 0.434±0.034, 0.320±0.052 mg/mL. It was noted there was 10-fold decrease in protein estimation among isolates grown in 55°C (Figure 4a). The variation among protein content is directly proportional to the growth of cells on the medium at given temperature which influences the growth. The total protein of crude was higher than purified protein (Figure 4b) but the enzyme activity was indirectly proportional.

Crude and pure protein enzyme activity

The crude enzyme activity among isolates grown at 37°C calculated from the monosaccharide produced (Table 7). The concentration of sugar was 1.298±0.05, 1.194±0.01 and0.632±0.02 mg/g respectively on S3A, S4A and S4B. Further the t-test among strains reveals that the data were statistically highly significant at $p<0.05$. Likewise, the activity of protein isolated at 15°C the estimated sugar 0.403±0.12, 0.346±0.02 and 0.46±0.07 mg but the *t*-test reveals the data is not significant among the strains ($p>0.05$) S3A, S4A and S4B (Table 8). The amount of estimated sugar by the activity of crude chitinase isolated at 55°C were given in Table 9. About 0.270±0.01, 0.213±0.016 and 0.178±0.003 mg/g of free sugar was recorded, and the data is significant at $p<0.05$. Figure 5 represents the comparative analysis of effect of enzyme produced under different temperatures. Maximum conversion of chitin into monosaccharide was found at 37°C, moderately at 15°C and less significantly on 55°C. Chitinase activity of S3A among crude isolated from different growth temperature reveals a maximum activity 23.478±1.07 at 37°C and moderate activity 7.28±2.25 U at 15°C whereas minimum of 4.87±0.30 U at 55°C. Isolate S4A at 37°C had maximum 21.57±0.3 U, 7.28±2.25 U at 15°C and 3.84±0.30 U at 55°C. Similarly, chitinase activity of S3B at 37, 15 and 55°C were 11.42±0.51, 8.324±1.36, 3.21±0.04 U (Figure 6). The independent t-test among isolates at 37°C was significant at $p<0.05$ whereas not significant at 15 and 55°C ($p>0.05$) reveals

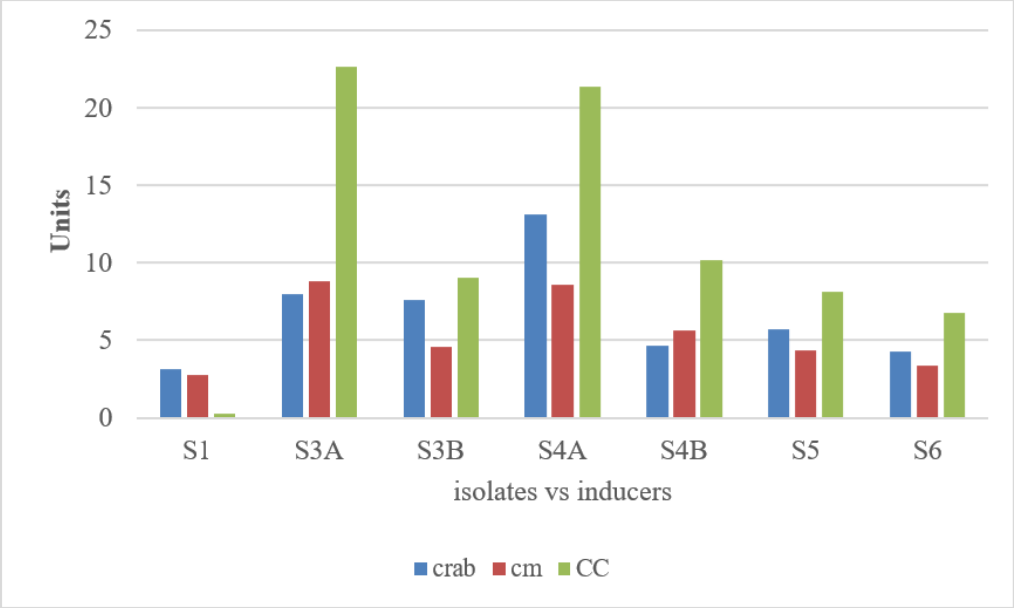

Figure 3: Amount of chitinase estimated (units/mL) among isolated positive strains.

Table 7: The amount of sugar released by crude chitinase produced under 37°C.

properties	S3A OD	Mg/mL	S4A OD	mg/mL	S4B OD	mg/mL
MEAN	0.443333	1.298	0.406667	1.194	0.21	0.632
SD	0.020817	0.05	0.005774	0.01	0.01	0.02
P	0.055351354		0.001002435		0.001927571	

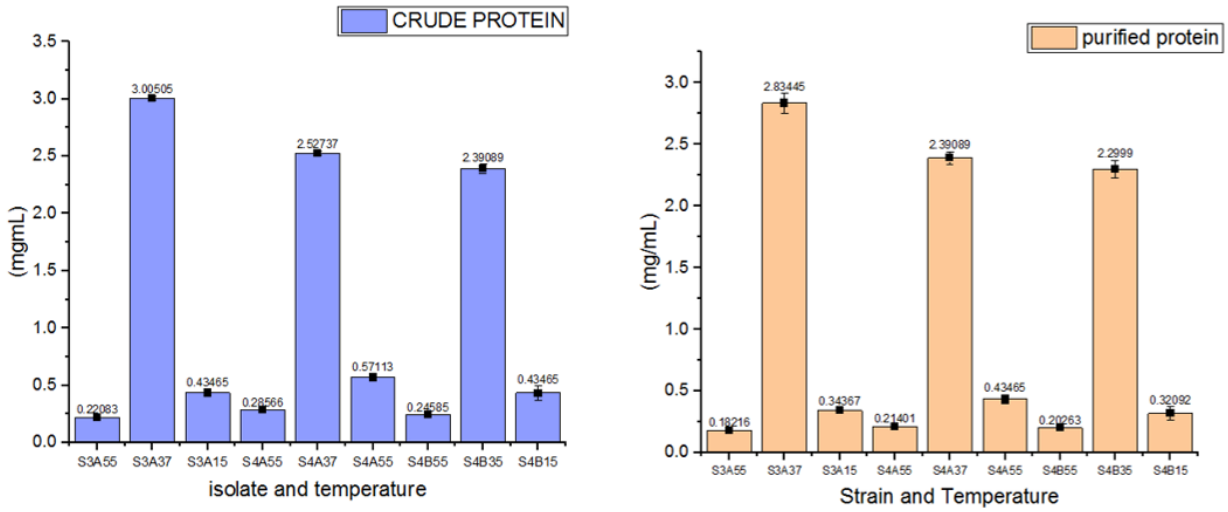

Figure 4: Effect of different temperature a) Crude total protein b) Purified protein.

Table 8: The amount of sugar released by crude chitinase produced under 15°C.

properties	S3A OD	Mg/mL	S4A OD	mg/mL	S4B OD	mg/mL
MEAN	0.143333	0.403	0.11	0.346	0.15	0.46
SD	0.020817	0.12	0.01	0.02	0.026458	0.07
P	0.219024257		0.060058655		0.311017764	

Table 9: The amount of sugar released by crude chitinase produced under 55°C.

properties	S3A OD	Mg/mL	S4A OD	mg/mL	S4B OD	mg/mL
MEAN	0.083333	0.270	0.063	0.213	0.051233	0.178
SD	0.005774	0.01	0.005	0.016	0.001079	0.003
P	0.0066178		0.011706006		0.000347356	

Table 10: Chitinase assay among crude protein produce under different temperature among isolates.

strain	units	SD	P
S3A37	23.478	1.071394	0.054908899
S4A37	21.57926	0.30277	0.001008309
S4B37	11.42254	0.515397	0.001932118
S3A15	7.287523	2.254749	0.297116696
S4A15	6.799277	1.076063	0.184151465
S4B15	8.324292	1.360131	0.184151465
S3A55	4.876432	0.30277	2.17706E-18
S4A55	3.84569	0.30277	0.029059689
S4B55	3.218807	0.047844	0.004487613

Table 11: The amount of sugar released by pure chitinase isolated from S3A.

properties	37°C		15°C		55°C	
	OD	mg/mL	OD	mg/mL	OD	mg/mL
Mean	0.513333	1.49878	0.163333	0.498858	0.113333	0.356012
SD	0.025166	0.071898	0.020817	0.059472	0.011547	0.032989
P	0		F		387	

that the 37°C act as optimum temperature for three isolates (Table 10).

The amount of sugar released by the purified chitinase among isolates was represented in Figure 7. The activity of purified protein from S3A on chitin and the estimated sugar was given in Table 11. Maximum amount of sugar 1.49±0.071898 was recorded at 37°C whereas 0.49±0.059 at15°C and 0.35±0.03 mg at 55°C. The one-way ANOVA reveals the *p* value was 0 and F value was 387. The activity of S4A on chitin were 1.327±0.03, 0.41±0.01, 0.28±0.02 mg respective at 37,15 and 55°C with *p* value of 0 and F value 971 (Table 12). Isolates S4B chitinase showed 0.794±0.016, 0.517±0.04 and 0.211±0.007 mg of free sugars. The calculated *p* value is 0 and the F value was 255 (Table 13). The maximum chitinase assay was 27.09464±1.29 units in S3A at 37°C followed by S4A grown at 3°C (23.9±0.5 U). The activity of S3A at 15 and 55°C was comparatively less than 37°C growth but higher than other two Isolates. The activity of S4A at 15°C was less than S4B (9.35±0.89) but higher than S4B at 55°C (5.125±0.36) Strain S4B showed maximum of 14.35±0.29 U at 37°C growth and minimum of 3.82±0.12 U at 55°C growth whereas moderate chitinase activity 9.35±0.89U at 15°C (Figure 8). The independent t-test of S3A has F vale 356 and the *p* value<0.0001. The data of S4A was significant and *p*<0.0001 with an F value 1135.4. Isolate S3B one-way ANOVAs reveals *p* value<0.0001 and the F value 274. Whereas the temperature dependent *p* values are <0.0.000201 at 37°C, 0.124421 at 15°C (not significant *p*>0.05) and 0.006457 at 55°C (Table 14).

DISCUSSION

The isolation and identification of active bacterial strains capable of producing chitinase from various ecological conditions are reported in this study. Chitinolytic ability has been investigated from a variety of locations, including a shrimp pond, an industrial site, a crab shell site, and a fish market area. Clear zones were formed by the chitinolytic activity of established colonies on agar plates, and they were chosen for further research. The majority of samples showed the presence of chitinase producer due to the previous exposure of chitin. The unexposed soil from the garden reveals absence of chitinase producer. The hydrolytic colonies are circular, spindle shaped and large irregular opaque in nature. Large irregular colonies have maximum clearance than circular and spindle shape colonies. The investigation of seawater isolates found in this was comparable to that found by Liang *et al.*,[14] who

Table 12: The amount of sugar released by pure chitinase isolated from S4A.

properties	37°C		15°C		55°C	
	OD	mg/mL	OD	mg/mL	OD	mg/mL
Mean	0.453333	1.327365	0.133333	0.413151	0.086667	0.28346
SD	0.011547	0.032989	0.005774	0.016494	0.005774	0.020382
P	0		F		971	

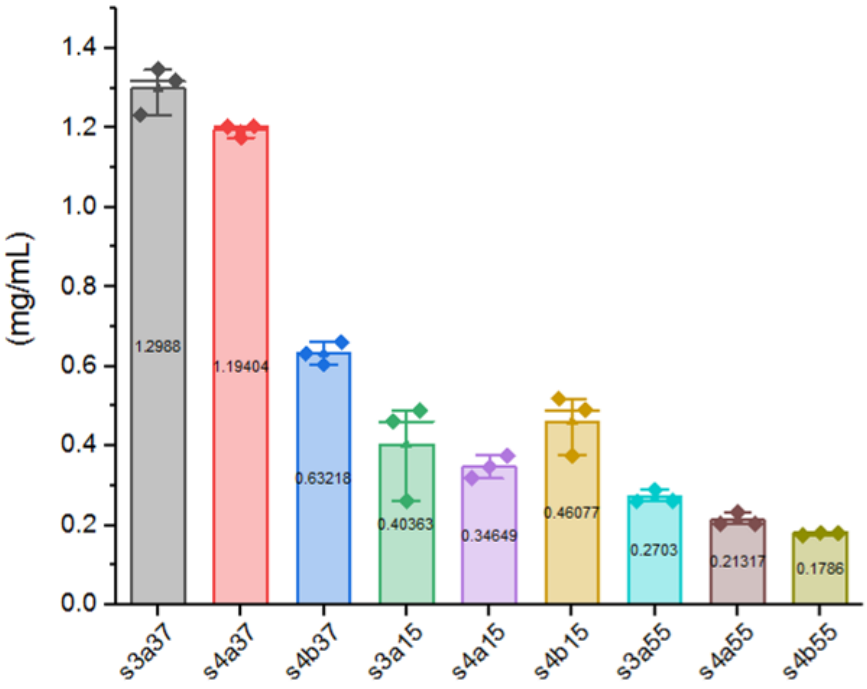

Figure 5: Estimated sugar (mg/mL) released by crude Chitinase activity.

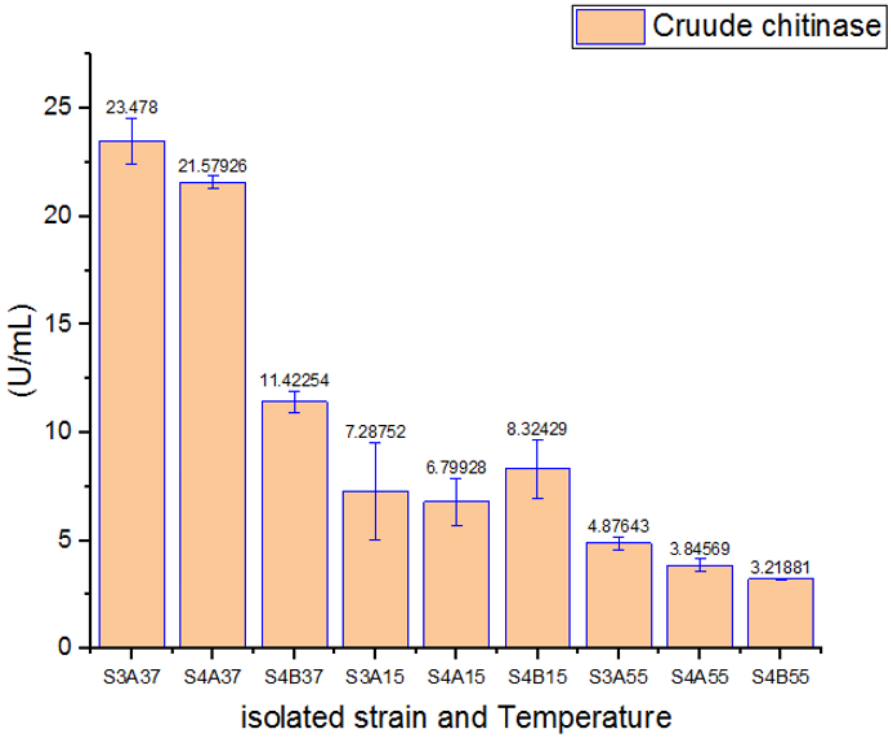

Figure 6: Estimated crude Chitinase activity (U/mL).

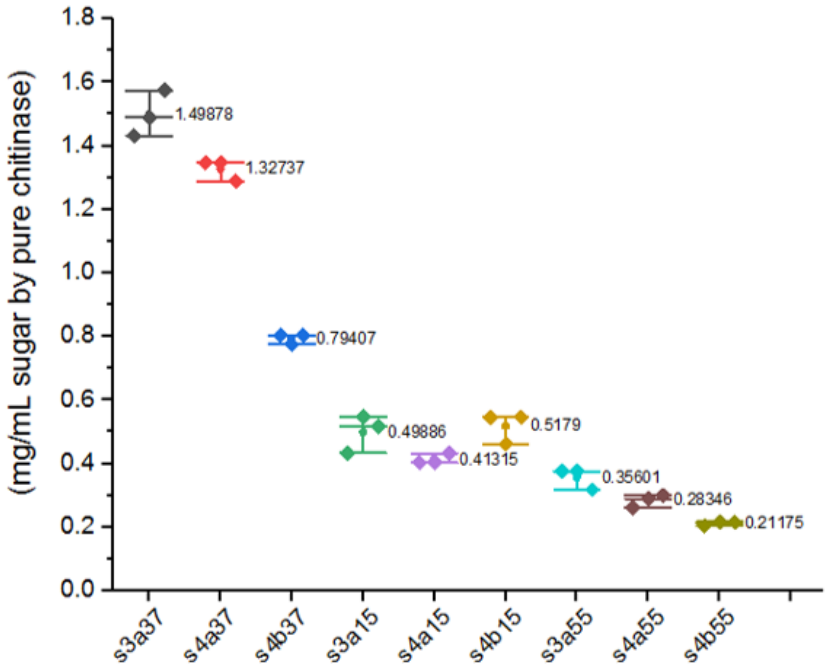

Figure 7: amount of sugar released by pure chitinase activity (mg/mL).

Table 13: The amount of sugar released by pure chitinase isolated from S4B.

properties	37°C		15°C		55°C	
	OD	mg/mL	OD	mg/mL	OD	mg/mL
Mean	0.266667	0.794073	0.17	0.517904	0.062	0.211753
SD	0.005774	0.016494	0.017321	0.049483	0.002	0.007062
P	0			F	255	

Table 14: Chitinase assay among purified protein produce under different temperature among isolates.

Strain	S3A37	S3A15	S3A55	S4A37	S4A15	S4A55	S4B37	S4B15	S4B55
mean	27.09464	9.011453	6.431585	23.99638	7.462327	5.125861	14.35202	9.355033	3.829171
SD	1.292199	1.071394	0.605539	0.595099	0.30277	0.368571	0.292329	0.897868	0.127704
P	<0.0001			<0.0001			<0.0001		
F	356.4			1135.43			274.64		
37P	0.000201; F 139								
15P	0.124421; F 3.67								
55P	0.006457; F 22.89								

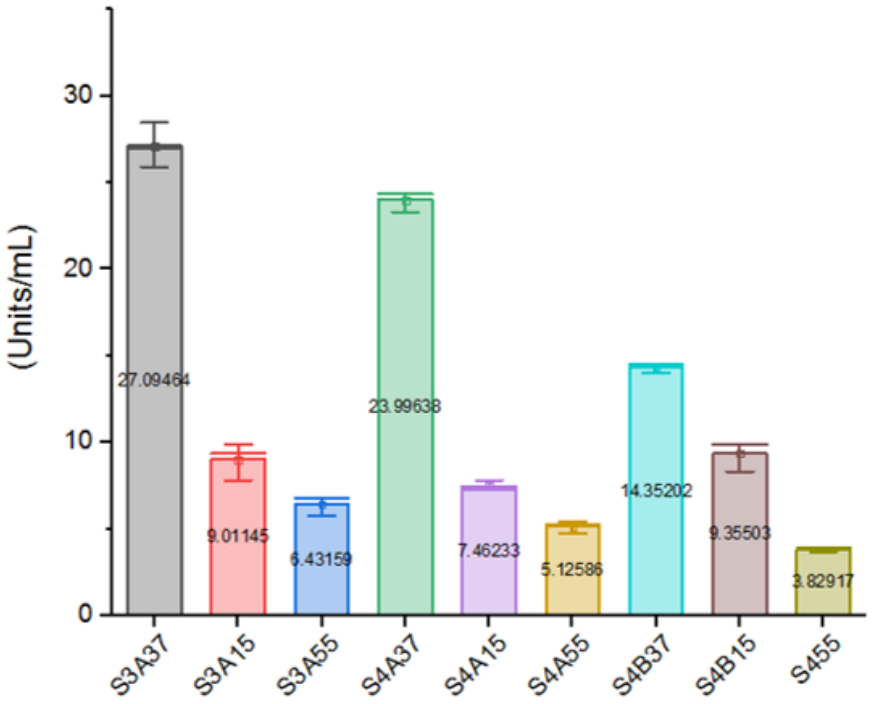

Figure 8: Chitinase activity of purified protein (U/mL).

recovered chitinolytically active organisms from marine sources. It was discovered that the chitin-amended soil outperformed garden soil in terms of results. According to several more recent research, adding chitin to soil increases the population of chitin degrading bacteria by selecting them. The Zarei *et al.*,[15] report is connected with the chitinase that was isolated from the shrimp pond. The results revealed a preponderance of G+ Bacilli isolates, which Thomas *et al.*,[16] had previously observed in 2020. The chitinase activity of *Bacillus* species from wastewater was reported by Laribi-Habchi *et al.*[17] The chitinase production with three different substrate data showed that it was the highest activity in colloidal chitin and found to be among the tested substrates. It was assumed that Colloidal chitin and its degradation products played a role as an inducer system in stimulating the production of chitinase, as reported by Jha *et al.*[18] In most cases, colloidal chitin, chitin flakes or chitin powder was utilized as a carbon source in the production of chitinase.[19,20] Though the crab shell less significantly induced chitinase production the strains showed degradation of crab shell. The reduced enzyme activity in filtrate may be due to impurities of crab shell powder. Biodegradation of lobster shells by chitinolytic microorganisms depends on the concentration of shells and load of microbial inoculums.[21] The culture conditions were optimized for maximum chitinase production at different temperature. All the three strains exhibited maximum enzyme activity at 37°C. Sephadex column chromatography purification, containing enzyme specific activity increased three-to-five-fold purification using the Sephadex column, and the protein recovered was decreased. However, the cold and hot temperature does not influence the enzyme activity, but the data confirms that these isolate capable to tolerate both extreme conditions. Adaptability of the microorganisms to grow at cold temperatures with chitnolytic activity was reported by Liu *et al.*[22] The report is in contrast to chitinolytic of *B. licheniformis*[23] demonstrated a value of 494.5 U/mg of protein, whilst the strain of *Bacillus* sp. bacteria demonstrated a production rate of 11.1 U/mg of protein in crude extract[24] Further optimization of

carbon, nitrogen, minerals are require to enhance the chitinase production.

CONCLUSION

Seven different isolates of chitinase-producing bacteria have been isolated from soil samples taken from a local site exposed to chitin. The biochemical characteristics of positive isolated reveals the presence of *Bacillus* spp., *Citrobacter* sp., *Pseudomonas* sp. and *Lactobacillus* sp. The crude extract with high extracellular chitinase induced by colloidal chitin. The crude and pure enzyme was produced at a maximum 37ºC temperature. Only one isolates *Bacills* sp. S3A found to be a more thermo tolerant chitinase producer.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

No animal studies involved and not supported by any funding grant.

ABBREVIATIONS

ANOVA: Analysis of Variance; **SRL:** Sisco Research Laboratories Pvt. Ltd.; **HCl:** Hydrochloric Acid; **K_2HPO_4:** Dipotassium Hydrogen Phosphate; **NaCl:** Sodium Chloride; **$MgSO_4 \cdot 7H_2O$:** Magnesium Sulfate Heptahydrate; **CMA:** Chitosan Minimal Agar; **DE-AE:** Diethylaminoethyl; **DNS:** 3, 5-Dinitrosalicylic Acid.

SUMMARY

Chitinase produces bacteria isolated from marine and tertiary soil. Totally seven sample were processed. Colloidal chitin agar was used for chitinase screening. Seven chitinase producers were isolated and degradation of crab shell was performed. Isolates designated as S3A, S4A, S1 were found to be maximum chitinolatic activity. Among the three-substrate used colloidal chitin was found to be an effective inducer. Out of seven three strains were selected for thermo stability. Isolates S3A, S4A, S4B highly produced chitinase at 37ºC and moderate enzyme activity was record at 15ºC. Whereas at 55ºC less significant enzyme activity (<5U) was recorded among the isolates. By the enzyme producers identified as *Bacillus* and *citrobacter* using biochemical studies.

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