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Research Article

Screening of Bacterial Isolates from Seafood-Wastes for Chitin Degrading Enzyme Activity

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Abstract

The safer removal of chitin is important for controlling accumulation of chitin in environment and agriculture. Chitin a long-chain polymer is a glucose-derivative and a primary component of fungal cell-wall, the exoskeleton of molluscs, fish, and insects. Chitinase enzyme (E.C. 3.2.1.14) is able to digest chitin, hydrolyzing the 1-4 linkages between the N-acetylglucosamine molecules. Present work was undertaken for the isolation and characterisation of chitinolytic bacteria from waste-seafood. Seventy-one indigenous strains were isolated from a variety of marine-sources, including scales of sea-fish, shrimps, prawns and crabs; of which 4 isolates were identified as *Alcaligenes* spp., 2 as *Kurthiaspp.*, 1 as *Acinetobacter* and 1 as *Bacillus* spp. These were higher chitinase producers with enzyme units ranging from 12.5 to 35.41 micromoles/min/ml. These chitinase producers may prove to be industrially beneficial for their enzyme production for degradation of chitin in environment clean-up, polluted with decaying sea-food and wastes originated from their processing.

INTRODUCTION

Chitin, a β -1,4-linked polymer of N-acetyl- β -D-glucosamine (NAG), is the second most abundant polysaccharide in nature after cellulose. Chitin is produced by many aquatic and terrestrial organisms in different forms [1]. It is a major cell wall constituent of higher fungi (chitridiomycetes, ascomycetes, basidiomycetes and deuteromycetes), insect exoskeletons and crustacean shells [2]. About 1011 tonnes of chitin is produced annually in the aquatic biosphere alone [3,4], arising from processing of seafood - crab, shrimp and krill shells [5]. Its complex structure chitin is resistance to natural process of degradation, creating disposal problems in environment due to its presence in sea-food wastes. Second major issue is experienced in agriculture industry, where chitin in pests is a protective-barrier against action of pesticides [6]. Despite being a potential renewable resource, most of the chitinous wastes are disposed through ocean dumping, incineration and land filling; owing to lack of cheap and commercially feasible methods for its processing leading to wastage of natural resource, economic loss and environmental pollution [7]. In India alone 60-80,000 tonnes of chitinous wastes are generated, making it necessary to design alternative methods for its disposal and utilization [8]. Chitin and chitosan nanofibers could be extracted from crustacean wastes for the development of products for medicine, and tissue engineering [9].

Chitin is insoluble in water, dilute and concentrated alkalis, alcohol and other organic solvents. These major issues have led to increased interest in chitin-hydrolyzing enzymes: chitinases.

(E.C. 3.2.1.14) hydrolyzing the 1-4 linkages between the NAG molecules [10]. These glycosyl hydrolases (GHs) are about 20-90 kDa in size and are present in various organisms [11,12]. In chitin-containing organisms, chitinases play an important role in normal life cycle functions such as morphogenesis and cell division, whereas plants produce chitinases as part of their defence against fungal pathogens. Chitinases derived from a variety of prokaryotic and eukaryotic organisms constitute the family 18 of GHs, whereas those derived from only higher plants and Gram-positive bacterium *Streptomyces* constitute the family-19. Family-20 includes N-acetylglucosaminidase from *Vibrio harveyi* and N-acetylhexosaminidase from *Dictyostelium discoideum* and human [13]. Endo-chitinases cleave randomly in the chitin chain and exo-chitinases cleave off chitobiose or chitotriose from the reducing or the non-reducing ends of the chitin chain. Microbial degradation of chitin not only leads to recycling of nutrients in the environment but also facilitates the treatment of chitinous wastes, mycolytic enzyme preparation along with generation of useful products viz. chito-oligosaccharides, N-acetylglucosamine and single cell proteins [14-16]. Chitinases have found major applications in the field of agriculture, medicine, biotechnology, food technology, waste management and industry. They act as potential biocontrol agents to fungal phytopathogens [10], mosquitoes and harmful insects [17] as well as aid in the development of fungal resistant plant/crops that carry a chitinase transgene [18-20]. Detection of chitinolytic microorganisms from natural sources is vital in the isolation of chitinase producing strains. The aim of this study was

to isolate the most prominent chitinolytic microorganisms from different marine wastes and water effluent as well as analyze the hydrolysis products formed as a result of the fermentation by these isolates.

These enzymes break down the 1→4 β-glycoside bond of *N*-acetyl d-glucosamine in chitin to produce mono- and oligomers. The inducible nature of chitinases, low activity of synthesized enzymes, and inertia of the substrate are only a few of the problems that need to be solved by biotechnology. Our project aims to screen bacteria capable of growing in Chitin containing medium and selection of strains which are effective producer of chitinolytic enzymes, with objective to promote their use for environment and in agriculture.

MATERIALS AND METHODS

Sample preparation for isolation of chitinolytic bacteria

Chitinase producing bacterial strains were isolated from marine sources. Several samples were collected from different regions (Alibag, Vashi and Belapur of Mumbai city) in west coastal state of India. Sources for samples included wastewater effluent from an industry in Taloja, white prawn shells (*Fenneropenaeus indicus*, formerly *Penaeus indicus*), Tiger-prawn shells (*Penaeus monodon*), sea-fish scales and sea crab shells (*Charybdis cruciata*). Each sample was separately crushed in a sterilized mortar-pestle under aseptic conditions using sterile water. Filtrates collected were serially diluted in sterile saline solution, 200 μl of 10⁻⁸, 10⁻¹² and 10⁻¹⁵ dilution was inoculated on nutrient agar plates and incubated at 37°C for 24 hours. Colonies obtained from the master plates were isolated and taken for further studies.

Cells from purified colonies of Isolates were spot-inoculated on sterile minimal M9 medium [21], with minor modification containing colloidal chitin pH 6.5 [18] and incubated at 37°C for 24-48 hours. Isolates were maintained by sub-culturing after every two weeks. These were screened for secretion of chitinase on containing chitin medium.

Identification of chitinolytic microorganisms

Isolates screened positive for chitinase production were tested using Gram's staining method [22], and identified through 16S rDNA sequencing carried out at the sequencing facility of Microbial Culture Collection at National Centre for Cell Sciences (NCCS), Pune, India. Sequences of 16S rDNA obtained were analyzed for the identification and phylogenetic relationships were studied using <http://blast.ncbi.nlm.nih.gov/Blast.cgi>; www.cme.msu.edu/RDP/html/index.html and www.ebi.ac.uk/Tools/msa/clustalw2/.

Chitinase Production

Microorganisms screened positive for chitinase production were selected for making starter inoculum culture by inoculating loop-full of colonies in 25 ml of M9 minimal medium containing 0.5% chitin flakes (pH 7) and incubated at 37°C at 100rpm for 24 h. Minimal medium containing chitin as the sole carbon source was used as enzyme production medium in fermentation process. Medium consisted of (g L⁻¹): K₂HPO₄, 0.7; KH₂PO₄, 0.3; sodium citrate, 0.05; MgSO₄·7H₂O, 0.01; ammonium sulphate, 0.1; acid

swollen chitin, 1% (w/v) [23] and colloidal chitin, 1% (w/v) [21]. 2.0 ml of fresh starter culture was inoculated to 50 ml of medium and incubated at 37°C with continuous agitation at 100 rpm for 6 days. Samples were collected at regular intervals, centrifuged at 8000 rpm for 10 min at 4°C and the clear supernatant was used for the assay of enzyme activity.

Chitinase Enzyme Assay

Chitinase activity was determined by measuring the amount of the reducing end groups, liberated in reaction mixture after degradation from acid-swollen and colloidal chitin, in a colorimetric assay using 3,5-dinitrosalicylic acid (DNS) reagent [24]. The chitinase activity was determined by mixing 1 ml crude enzyme solution with 1 ml of acid-swollen chitin (1%, w/v) in 50 mM sodium acetate buffer (pH 5) at 37°C for 1 hour. *N*-acetyl-D-glucosamine (1 mg/ml) was used as the standard. Chitinase activity was expressed as μmoles of product formed per ml of enzyme solution per minute (μmoles ml⁻¹ min⁻¹).

Analysis of chitin hydrolysis products

Samples showing maximum activity as crude enzymes were further analyzed through high performance liquid chromatography. 1 ml of crude enzyme was incubated with 1 ml of acid swollen chitin (1%, w/v) prepared in 0.1 M sodium acetate buffer (pH 5) at 37°C for 1 hour. After centrifugation, the reaction mixture was filtered using syringe filter (0.2 μm), and samples were analyzed through dual λ Absorbance detector HPLC (Waters 2487, India). *N*-acetyl glucosamine (1 mg/ml, 1000 ppm) was taken as standard and samples (20 μl) were analyzed at 210 nm, on C-18 column using acetonitrile: water (70:30) as mobile phase with flow rate of 0.8 ml min⁻¹ according to the method described by [25].

RESULTS

A large number of bacterial strains were isolated on nutrient agar plates from samples collected from a variety of seafood wastes and wastewater effluent. The isolates were categorized in five groups based on their source of isolation. Morphological features of the chitinolytic organisms were studied by Gram staining method; microscopic studies confirmed majority of isolates were bacilli or cocci, detailed results for five groups are summarised in (Tables 1-5).

These isolates were subjected to a process for screening of chitinolytic strains by spot-inoculating on plates of a minimal medium containing colloidal chitin (CCM). In this repeated screening method using 71 different bacterial isolates, only 32 were found to be chitinase positive. Pictures of CCM plates showing chitinase positive bacterial colonies are presented in (Figure 1 A-C).

All 32 chitinase positive isolates were further taken for a secondary screening in production medium for enzyme production and samples at regular interval were assayed for chitinase units produced by isolates, the results for are presented in (Figure 2) and (Table-6).

The higher enzyme producers strains (PASB-11, 12, 14, 51, 54 and 55) were isolated from samples of Shrimps (large king

Table 1: Characterization of strains isolated from samples collected from wastewater.

Isolate No.	Nutrient Agar *	Colloidal-chitin M9 Medium*	Morphology of isolates	Gramstain Test (+/-)
PASB 1	+	-	White, raised, opaque, rough	-
PASB 2	+	+	White, raised, opaque, rough	+ (bacilli)
PASB 3	+	+	White, raised, translucent, smooth	+ (cocci)
PASB 4	+	-	White, raised, opaque, rough	-
PASB 5	+	+	Orange, raised, opaque, smooth	+ (cocci)
PASB 6	+	+	Yellow, raised, opaque, smooth	+ (cocci)
PASB 7	+	-	Tan, raised, opaque, rough	-
PASB 8	+	-	White, raised, opaque, smooth	-

*+ indicates growth; - indicates no growth

Table 2: Characterization of strains isolated from samples collected from Squid (Decapodiformes){9, 10}; and Shrimps(large king prawns) {11-16, 50-55}.

Isolate No.	Nutrient Agar *	Colloidal-chitin M9 Medium*	Morphology of isolates	Gramstain Test (+/-)
PASB 9	+	+	White, raised, translucent, rough	+ (cocci)
PASB 10	+	-	White, raised, translucent, rough	-
PASB 11	+	+	Yellow, raised, translucent, smooth	+ (bacilli)
PASB 12	+	+	White, raised, opaque, smooth	+ (bacilli)
PASB 13	+	+	Yellow, raised, opaque, rough	+ (bacilli)
PASB 14	+	+	Brown, raised, opaque, rough	+ (cocci)
PASB 15	+	-	Orange, raised, opaque, smooth	-
PASB 16	+	-	White, raised, opaque, smooth	-
PASB 50	+	+	Orange, raised, opaque, rough	+ (bacilli)
PASB 51	+	+	Transparent, raised, smooth	+ (bacilli)
PASB 52	+	+	White, raised, opaque, smooth	+ (bacilli)
PASB 53	+	+	Transparent, raised, smooth	+ (bacilli)
PASB 54	+	+	White, raised, opaque, smooth	+ (bacilli)
PASB 55	+	+	Brown, raised, opaque, smooth	+ (bacilli)

*+ indicates growth; - indicates no growth

Table 3: Characterization of strains isolated from samples collected from Sea Crab (*C. cruciata*)/

Isolate No.	Nutrient Agar *	Colloidal-chitin M9 Medium*	Morphology of isolates	Gramstain Test (+/-)
PASB 17	+	-	White, raised, opaque, smooth	-
PASB 18	+	-	Yellow, raised, opaque, smooth	-
PASB 19	+	-	White, raised, opaque, smooth	-
PASB 20	+	+	Raised, transparent, smooth	+ (bacilli)
PASB 29	+	+	Yellow, raised, opaque, smooth	+ (bacilli)
PASB 30	+	+	Red, raised, translucent, smooth	+ (bacilli)
PASB 31	+	-	White, raised, opaque, rough	-
PASB 32	+	-	White, raised, translucent, smooth	-
PASB 47	+	-	White, raised, opaque, smooth	-
PASB 48	+	-	White, raised, opaque, smooth	-
PASB 49	+	-	Red, raised, opaque, smooth	-
PASB 56	+	-	Yellow, raised, opaque, smooth	-
PASB 57	+	+	Yellow, raised, opaque, smooth	+ (bacilli)
PASB 58	+	+	White, raised, opaque, smooth	+ (bacilli)

PASB 59	+	-	Yellow, raised, opaque, smooth	-
PASB 60	+	+	Transparent, raised, smooth	+ (bacilli)
PASB 61	+	+	Yellow, raised, opaque, smooth	+ (bacilli)
PASB 62	+	+	White, raised, opaque, smooth	+ (bacilli)
PASB 63	+	-	Red, raised, opaque, smooth	-
PASB 64	+	-	Transparent, raised, smooth	-
PASB 65	+	+	Transparent, raised, smooth	+ (bacilli)
PASB 66	+	-	Translucent, raised, rough	-
PASB 67	+	-	Transparent, raised, smooth	-
PASB 68	+	+	Yellow, raised, opaque, smooth	+ bacilli
PASB 69	+	+	White, raised, opaque, rough	+ bacilli
PASB 70	+	+	Yellow, raised, opaque, smooth	+ bacilli
PASB 71	+	-	Transparent, raised, smooth	-

*+ indicates growth; - indicates no growth

Table 4: Characterization of strains isolated from samples collected from a variety of Fish Scales.

Isolate No.	Nutrient Agar *	Colloidal-chitin M9 Medium*	Morphology of isolates	Gramstain Test (+/-)
PASB 21	+	-	White, raised, opaque, rough	-
PASB 22	+	-	Brown, raised, opaque, rough	-
PASB 23	+	-	White, raised, opaque, rough	-
PASB 24	+	-	White, raised, opaque, smooth	-
PASB 25	+	+	White, raised, opaque, rough	+ (bacilli)
PASB 26	+	-	Cream, raised, opaque, smooth	-
PASB 27	+	-	Transparent, raised, rough	-
PASB 28	+	-	Yellow, raised, translucent, smooth	-

*+ indicates growth; - indicates no growth

Table 5: Characterization of strains isolated from samples collected from Prawn shells.

Isolate No.	Nutrient Agar *	Colloidal-chitin M9 Medium*	Morphology of isolates	Gramstain Test (+/-)
PASB 33	+	+	Red, raised, opaque, smooth	+ (bacilli)
PASB 34	+	-	White, raised, opaque, smooth	-
PASB 35	+	-	Orange, raised, opaque, smooth	-
PASB 36	+	+	Cream, raised, opaque, smooth	+ (bacilli)
PASB 37	+	-	Transparent, raised, smooth	-
PASB 38	+	+	Orange, raised, opaque, smooth	+ (cocci)
PASB 39	+	-	Yellow, raised, opaque, smooth	-
PASB 40	+	-	Red, raised, opaque, smooth	-
PASB 41	+	-	White, raised, opaque, rough	-
PASB 42	+	-	White, raised, opaque, smooth	-
PASB 43	+	-	Cream, raised, opaque, rough	-
PASB 44	+	+	Yellow, raised, opaque, smooth	+ (bacilli)
PASB 45	+	-	Transparent, raised, smooth	-
PASB 46	+	-	White, raised, opaque, smooth	-

*+ indicates growth; - indicates no growth

prawns), PASB-38 was obtained from prawns; their morphology results are presented in tables 2 and 4. The 8th good chitinase producer designated as PASB-57 was isolated from sea crab shells (*Charybdis cruciate*), morphology results are presented in table-3.

Higher Chitinase enzyme producers PASB-11, 12, 14, 38, 51, 54, 55, 57 with enzyme units 25.61, 31.64, 35.41, 26.34, 30.5, 19.9, 12.5 and 25.6 IU, respectively were studied for their HPLC profile (Figure 3). These higher chitinase producers were shortlisted for identification through 16s rDNA sequencing followed by analysis through BLASTn (NCBI), CLUSTAL W (EBI) and SEQMATCH (RDP database). The results suggested the strains as *Acinetobacter spp.* (PASB 14), *Bacillus spp.* (PASB-51), *Kurthiagibsonii* (PASB-38; 54) and *Alcaligenes spp.* (PASB 11; 12; 55 and 57).

DISCUSSIONS

Microbial production of chitinase has captured the worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method. A wide range of

microbes capable of producing chitinases have been isolated from different sources such as soil, peanut hulls, marine waste, etc. In the present study chitinase producing microorganisms were isolated from seafood wastes shrimp shells, prawn shells, crab shells and other marine samples. Around 32 chitin degrading organisms were isolated, out of which four potential chitinolytic bacteria were selected for further studies based on their high extracellular chitinase production. Their identification and characterization was done through 16S rDNA sequencing followed by analysis using various bioinformatics tools. The four isolates were identified as *Bacillus spp.*, *Kurthiagibsonii* and *Alcaligenes spp.* Earlier studies have reported the chitinase production by *Alcaligenes faecalis* AU02 to be maximum (258 U/mL) after 48 h at 37°C in a medium containing 1% shrimp and crab shell powder in basal medium (pH 8.0) [26]. *Bacillus sp.* TKU006 has been reported to produce protease and chitinase on shrimp shell powder and crab shell powder at 25°C for 2 days [27]. The chitinolytic activity in the culture supernatant of *Bacillus pumilus* reached a maximum of 79.8 U/100 ml after 8 days of incubation using basal medium [28]. In comparison to these findings, our results show an increased enzyme activity of

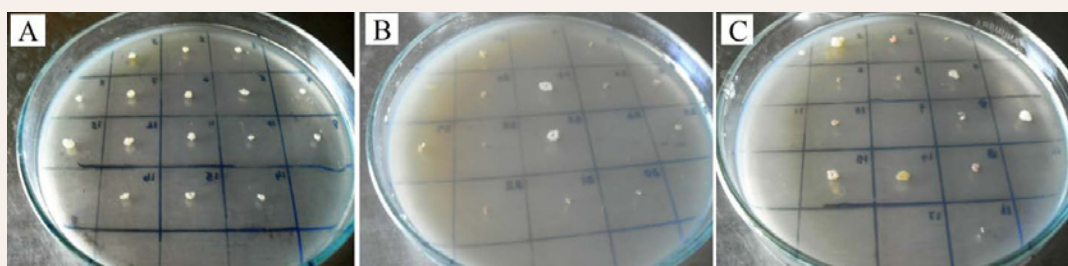


Figure 1 Pictures of CCM plates showing chitinase positive bacterial colonies.

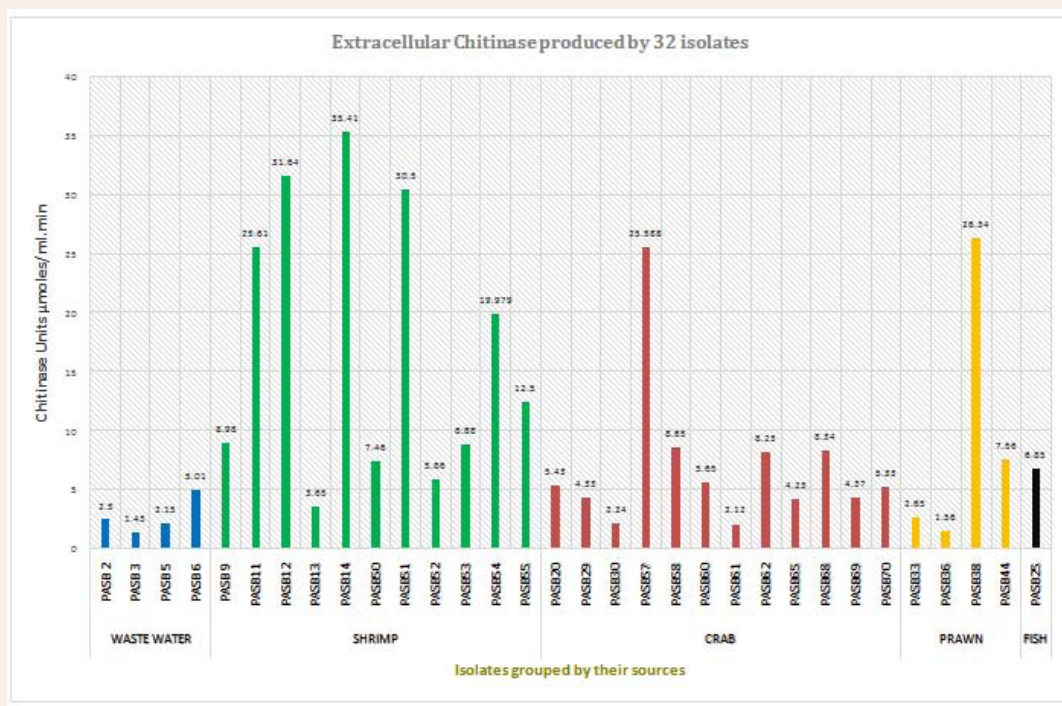


Figure 2 Enzyme production and samples at regular interval.

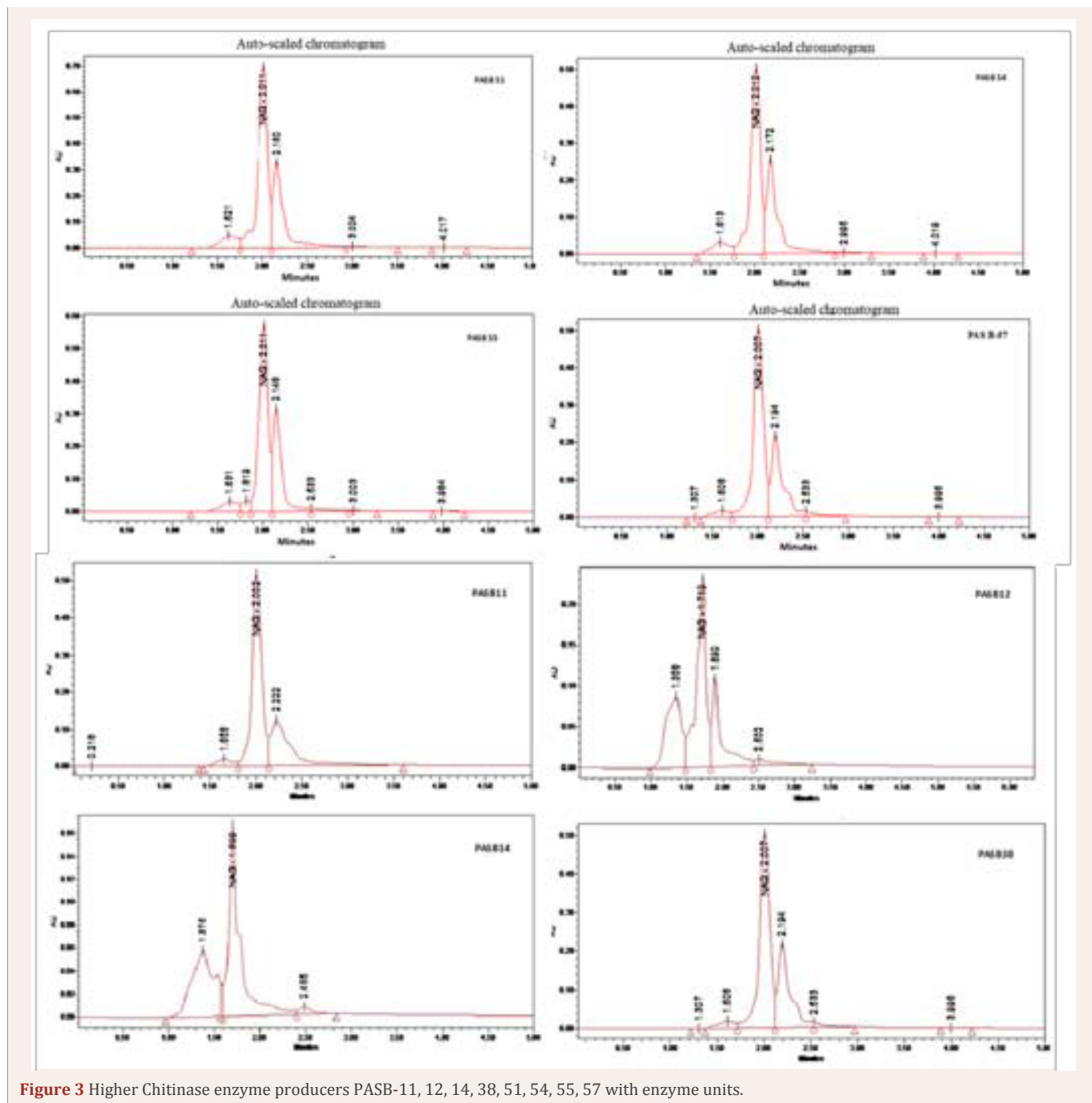


Figure 3 Higher Chitinase enzyme producers PASB-11, 12, 14, 38, 51, 54, 55, 57 with enzyme units.

30.502 $\text{mmoles min}^{-1}\text{ml}^{-1}$, 19.979 $\text{mmoles min}^{-1}\text{ml}^{-1}$, 12.5 $\text{mmoles min}^{-1}\text{ml}^{-1}$ and 25.588 $\text{mmoles min}^{-1}\text{ml}^{-1}$ for *Bacillus* spp. PASB-51, *Kurthiagibsonii* PASB-54, *Alcaligenes* spp. PASB-55 and PASB-57, respectively [29].

First reported that the chitinolytic activity of *Kurthia gibsonii* Mb126 strain isolated from prawn shell powder could be increased up to 65 U/ml under optimum conditions. They further purified the enzyme through four steps including ammonium sulphate precipitation, affinity adsorption, ion exchange chromatography and gel filtration chromatography. The chitinase was purified 16.11-fold through Sephadex G 100 gel filtration. The specific activities of the crude and purified enzyme were 0.64 U/mg and

10.31 U/mg proteins, respectively. The enzyme was most active at pH 6.5 at 40°C [30]. In our study, the chitinolytic activity of *Kurthiagibsonii* (PASB-54) has been found to be 19.979 mmoles/min/ml . A qualitative HPLC analysis successfully detected the presence of monomer NAG, a part of chitin hydrolysis product, thereby proving chitinase production in the medium by these four strains. Also, these isolates were capable of degrading chitin to its monomer unit NAG.

With respect to present results and comparison with the best knowledge about other chitinase producers, these isolates may have the capability for production of chitinase on an industrial scale. Also, many thermostable chitinases have been identified

Table 6: ChitinaseEnzyme synthesis by isolated bacterial cultures.

Isolate source/name	Extracellular Chitinase produced (micromoles/ ml.min)
Wastewater/PASB 2	2.5
Wastewater/PASB 3	1.43
Wastewater/PASB 5	2.15
Wastewater/PASB 6	5.01
Shrimp PASB 9	8.98
Shrimp PASB11	25.61
Shrimp PASB12	31.64
Shrimp PASB13	3.65
Shrimp PASB14	35.41
Shrimp PASB50	7.46
Shrimp PASB51	30.5
Shrimp PASB52	5.86
Shrimp PASB53	8.88
Shrimp PASB54	19.979
Shrimp PASB55	12.5
Prawn PASB36	1.56
Prawn PASB38	26.34
Prawn PASB44	7.56
Crab PASB29	4.33
Crab PASB30	2.24
Crab PASB57	25.588
Crab PASB58	8.65
Crab PASB60	5.65
Crab PASB61	2.12
Crab PASB62	8.23
Crab PASB65	4.23
Crab PASB68	8.34
Crab PASB69	4.37
Crab PASB70	5.33
Fish PASB25	6.85

from a variety of microorganisms. The chitinolytic microbes identified in this study can be further analyzed for their stability over high temperatures and thus could prove useful for other biotechnological applications.

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