Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(3): 89 – 108 (2024)
www.ejabf.journals.ekb.eg

Red Sea Chitinase-Producing *Enterococcus* sp.: Isolation, Characterization, and Application

Mahmoud kelany*, Mohamed Abdrabo, Yasser Geneid, Mohamed Fathi

National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt
***Corresponding Author: mahmoudscience88@yahoo.com, ms.aal@niof.sci.eg**

ARTICLE INFO

Article History:
Received: April 1, 2024
Accepted: April 25, 2024
Online: May 12, 2024

Keywords:
Chitinase enzyme,
Enterobacter,
DEAE-cellulose,
Shrimp waste

ABSTRACT

This study aimed to isolate and comprehensively characterize chitinase-producing bacterial isolates collected from diverse aquatic stations in the Red Sea. Out of twenty nine isolates, chitinase-producing marine *Enterococcus hirae* (OR064172) and *Enterococcus faecalis* (OR053873) were biochemically and genetically identified. The two isolates have primary specific activity of 10.7 and 11.5U/ mg for *E. hirae* and *E. faecalis*, respectively. To enhance the purity of the chitinase enzymes, ammonium sulfate precipitation was employed, yielding 12.9 and 13.47U/ mg for 70% fractions of *E. hirae* and *E. faecalis*, respectively. Afterward, DEAE-cellulose chromatography was utilized for the purification process, resulting in two peaks for *E. hirae* and one peak for *E. faecalis*. The results of chitinase characterization indicated optimal conditions with a temperature of 37°C, a pH of 6.5, and a salt tolerance of up to 1% for both strains. The in-depth kinetic chitinase analysis unveiled the optimum K_m (0.03 and 0.07) and V_{max} (0.124 and 0.0003) conditions for chitinase activity for both *E. hirae* and *E. faecalis*, respectively. The application of the previous strains at the economic level involves the production of chitinase using marine shrimp waste in an attempt to extract beneficial products. The solid state fermentation process of this waste for both isolates was also tested, with results indicating that the specific activity of the produced enzyme was 8.48 and 11.38U/ mg, respectively, achieving the highest production after 48 hours. This study adeptly accomplished the isolation and identification of chitinase-producing marine bacterial isolates. The rigorous purification and comprehensive characterization of chitinase enzymes offer valuable insights into their production, refinement, and utilization across diverse industries, particularly within aquaculture and waste management.

INTRODUCTION

Chitin, a linear polysaccharide composed of N-acetylglucosamine units, represents one of the most abundant biopolymers on Earth, constituting a major structural component of fungal cell walls, crustacean exoskeletons, and insect cuticles **(Kobayashi *et al*., 2006; Yeul & Rayalu, 2013; Abo Elsoud & El Kady, 2019; Hisham *et al*., 2023; Utama *et al*., 2023)**. The enzymatic breakdown of chitin by chitinase is of a paramount importance for nutrient recycling and ecosystem dynamics. In natural environments, chitinase-producing microorganisms, such as bacteria, fungi, and marine organisms,

contribute to the decomposition of chitin-containing materials. This process not only releases essential nutrients like nitrogen and carbon back into the ecosystem but also influences the structure and composition of microbial communities in aquatic and terrestrial environments **(Zhang *et al.*, 2023)**. Among the myriad of organisms capable of chitin degradation, chitinase-producing bacteria stand out as key players in chitin utilization and mineralization processes **(Dwyer *et al.*, 2021; Pal *et al.*, 2021)**. Harnessing the potential of chitinase-producing bacteria holds promise for diverse applications, ranging from aquaculture to waste management.

Chitinase-producing bacteria have garnered significant attention due to their potential applications across several industries **(Golgeri *et al.*, 2022)**. In agriculture, chitinase-producing microorganisms have been explored for their role in pest control, as chitin is a major component of insect exoskeletons **(Banerjee & Mandal, 2019)**. In aquaculture, chitinase enzymes have been investigated for their ability to control fish diseases caused by chitin-containing pathogens **(Hirano *et al.*, 2019)**. Additionally, chitinase has applications in biotechnology, including bioconversion processes and the production of chitin-derived products such as chitosan, with numerous industrial uses **(Kumari *et al.*, 2023)**. By reducing the need for chemical inputs and promoting eco-friendly approaches, they align with the principles of sustainable development.

Ammonium sulfate precipitation serves as a crucial technique in the field of biochemical purification, particularly in the isolation and partial purification of chitinase enzymes from bacterial sources. This method capitalizes on the selective salting-out properties of ammonium sulfate, effectively separating proteins based on their solubility characteristics **(Dikbaş *et al.*, 2021)**. The choice of the appropriate ammonium sulfate concentration is critical in achieving the desired level of purification. Typically, a saturation level between 40 and 80% ammonium sulfate is used, depending on the specific requirements of the purification process. Chitinase enzymes exhibit varying solubility characteristics, and the optimal ammonium sulfate concentration for their precipitation may differ from one bacterial isolate to another **(Ullah *et al.*, 2022)**.

Chitinase purification using DEAE-cellulose chromatography represents a critical step in the quest to isolate and refine chitinase enzymes, which play a fundamental role in breaking down chitin, a complex polysaccharide found in the exoskeletons of arthropods and fungal cell walls. This purification method leverages the principles of ion exchange chromatography to selectively separate proteins based on their charge properties **(Singh *et al.*, 2021)**. DEAE-cellulose, or diethylaminoethyl cellulose, is a chromatography resin with positively charged diethylaminoethyl (DEAE) groups. These positively charged groups can interact with negatively charged proteins through ionic interactions. In the context of chitinase purification, DEAE-cellulose provides an ideal medium for separating chitinase enzymes from other proteins present in the sample **(Sheehan & FitzGerald, 1996)**.

The utilization of shrimp peels as a common marine waste product for chitinase production employing *Enterobacter* spp. have a promising idea for waste treatment. Shrimp peels are a readily available marine waste product. Their conversion into valuable enzymes like chitinase contributes to the sustainable utilization of marine resources and reduces waste. This can reduce the environmental impact with industrial chitinase applications and achieving economic feasibility. *Enterobacter* sp. has been identified as a promising microorganism for chitinase production due to its enzymatic capabilities and efficiency in utilizing chitin-rich substrates **(Taokaew & Kriangkrai, 2023)**.

This study consisted of several key steps. Initially, bacterial isolates were collected from different stations, with specific geographical coordinates from the Red Sea. Among these isolates, the two most potent ones were identified using API 20 kits and through genetic analysis. Subsequently, ammonium sulfate precipitation was employed to partially purify the chitinase enzymes produced by these isolates. The purification process was further refined using DEAE-cellulose chromatography. Finally, the study involved various characterizations of the purified chitinase enzymes, providing a comprehensive analysis of their properties and potential applications.

MATERIALS AND METHODS

1. Isolation of bacterial isolates with screening for chitinase production

Nine samples were collected from various locations in the Red Sea, with their longitude and latitude coordinates listed in Table (1). To screen for chitinase-producing bacteria, we utilized luria broth containing colloidal chitin, focusing on colonies exhibiting a clear zone. These selected colonies were then subjected to further detailed study. The preparation of colloidal chitin involved purchasing powdered chitin from Sigma-Aldrich and making slight modifications to the method described by **Shahbaz and Yu (2020)**. Chitin was prepared using the HCl method described by **Beltagy *et al*. (2018)**.

Table 1. Collection sites with longitude and latitude degrees

No.	Station	Longitude	Latitude	Time of sample collection
1	OyounMoussa	29°46'19.1"N	32°39'43.6"E	12:37Pm
2	RasSedr	29°35'20.9"N	32°42'41.2"E	2:00Pm
3	Pharaonic Baths	29°11'56.3"N	32°57'17.3"E	3:47Pm
4	Abo-Zenema	29°01'22.1"N	33°09'14.4"E	5:08PM
5	Belayim Petroleum	28°48'15.9"N	33°12'76.6"E	6:02PM
6	El-tour	28°12'24.8"N	33°37'34.9"E	1:58Pm
7	El-tour-desalination	28°14'12.7"N	33°36'03.9"E	3:39Pm
8	Al-Kanayes	27°55'56.7"N	33°53'36.8"E	8:55Am
9	El-tour reef	27°55'27.9"N	33°53'55.1"E	10:12Am

For bacterial screening, LB (Luria broth) served as the standard medium with some modification for screening chitinase-producing bacteria. The selective medium composition included KH_2PO_4 (14g/ L), K_2HPO_4 (6g/ L), $(NH_4)_2SO_4$ (2g/ L), $Na_3C_6H_5O_7$

(1g/ L), $MgSO_4$ (0.12g/ L), Agar (15g/ L), and 1% (w/v) colloidal chitin, adjusted to pH 7. Incubation occurred at temperatures ranging from 37°C, and the formation of clearance zones and specific activity resulting from chitin hydrolysis were recorded for up to 8 days. Based on these records, two of the most promising samples were selected for further analysis. The specific activity of all isolates was determined using the method of **Lowry (1951)** for protein measurement and **Miller (1951)** for activity assay, following the methods outlined by **Al-Agamy *et al.* (2021)**.

2. Biochemical identification using API 20E kits

Trypticase soy broth was employed to enrich the selected isolates, and the cultures were incubated for 18 hours, allowing them to reach a concentration of 0.5 McFarland units. Using either a sterile inoculation loop or swab, we selected purified colonies and transferred them into tubes containing sterile saline solution, adjusting the turbidity to a concentration of 0.5 McFarland units. Inoculation the API kit well with the standardized bacterial suspension was performed meticulously to prevent cross-contamination, followed by incubation at 37°C for 18- 24 hours. Following this incubation period, results were assessed by observing color changes in each well. These results were then compared to the reference chart or database provided by the API kit manufacturer to identify the bacterial isolate and obtain numerical codes **(Türe & Alp, 2016)**.

3. Genetic identification

The potent isolates were characterized through the analysis of the 16S rRNA (16S rDNA) gene sequences. To amplify nearly the entire length of the 16S rRNA gene from each strain, we employed primer PS3 - 353F and 809R (Forward: 'GCAGTGGGGAATATTGCA' – Reverse: 'AAGGGCACAACCTCCAA‘), as outlined in the work of **Resendiz-Nava *et al*. (2021)**. The experiment began with the preparation of bacterial samples. Colonies from each strain were cultured on TSA medium and then lysed using a mixture of NaOH and SDS, followed by boiling. After centrifugation, a portion of the lysate was used for PCR amplification. PCR reactions included various components detailed in a previous study. Amplification was conducted using specific equipment. The resulting products were purified through gel electrophoresis and extraction. Nucleotide sequences were determined using a specific sequencing kit, and analysis was carried out using various software programs, including BLASTN and FASTA for sequence identity and Clustal X for alignment **(Sint *et al*., 2011)**.

4- Partial purification using ammonium sulfate and DEAE- cellulose

Chitinase was purified from *E. hirae* OR064172 and *E. faecalis* OR053873 through a series of steps. The initial purification involved protein precipitation using ammonium sulfate at various saturation percentages: 25, 50, 75, and 90%. Subsequently, the precipitated sample was dialyzed overnight against water and barium chloride, as described by **Senol *et al*. (2014)**. The final purification step was conducted using DEAE-cellulose column chromatography with dimensions of 2:6×20cm. To determine the molecular weight of the purified enzyme, sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) was employed, following the method outlined by **Loni *et al*. (2014)**.

5. Characterization of purified chitinase

Effects of temperature, pH, salt concentration (NaCl), and reaction time on enzyme activity

The impact of pH on enzyme activity was assessed by conducting activity assays **(Miller, 1951)** at various pH levels, utilizing different buffer compounds. This examination aimed to evaluate chitinase activity across a pH range spanning from 3 to 10. To investigate the influence of temperature on chitinase activity, enzyme assays were carried out over a temperature range extending from 25 to 49°C. The assessment of thermal stability with respect to chitinase activity was designed to expose the enzyme to temperatures of 55, 65, and 70°C for durations ranging from 0 to 100 minutes. To explore the effect of salinity on enzyme activity, assays were conducted under varying saline conditions, ranging from 0.2 to 1.8%. Chitinase activity was quantified and expressed as units per milliliter (unit/ ml).

Determination of K_m and V_{max}

The K_m and V_{max} for the purified chitinase were calculated using colloidal chitin as a substrate. The substrate with a final concentration ranging from 0.1 to 1.2% was employed. Subsequently, the residual activity was determined using the standard protocol. The values of Km were obtained graphically with the assistance of the software "Sigma Plot version 10" **(Rajendran *et al*., 2024)** using the following equation:

$$\mathbf{v = V_{max} / (1 + (K_m/[S]))}$$

6. Application using shrimp waste

In the preceding phases of the research, chitin sourced from Sigma-Aldrich was employed to generate chitinase enzymes using *Enterobacter hirae* and *Enterobacter faecalis* strains. To harness the chitinase enzyme's potential in marine waste treatment, shrimp shells were utilized as a chitin source, which was processed and purified as an alternative to the previously employed chitin, following the approach outlined by **Wang *et al*. (2023)**. Additionally, a solid-state fermentation technique was employed as another practical method to assess the ability of the extracted enzymes to degrade the chitin present in shrimp shells. The experiments were conducted in 250-mL Erlenmeyer flasks containing 50g of shrimp shell powder mixed with 10mL of basal medium (pH 7.0). The basal medium consisted of 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) yeast extract, 0.028% (w/v) KH_2PO_4, 0.025% (w/v) $MgSO_4.7H_2O$, and 0.007% (w/v) $CaCl_2.2H_2O$.

Following sterilization, the solid medium had a moisture content of 70% and was inoculated with 300µL of a bacterial suspension (10^8CFU/ mL). The inoculated medium was then incubated at 37°C in a rotary shaker at 150rpm for duration of 4 days. Subsequently, the culture was extracted using 100mL of 0.1M phosphate buffer (pH 7.0) by stirring for 1 hour in an ice bath, followed by centrifugation at 8,000x g (4°C) for 15 minutes. Samples were collected at 12-hour intervals from the fermentation flasks. The

supernatant was filtered through a 0.25µM filter paper to eliminate bacterial cells, and it was utilized for enzyme activity determination, as described by **Baldoni *et al*. (2020)**.

RESULTS

1. Isolation of bacterial isolates with screening for chitinase production

The study focused on bacterial isolates collected from different Red Sea sites and tested for their activities on chitin agar plates. Moreover, various bacterial samples were analyzed for their chitinase production activity, protein concentration, and specific activity (SA). Among these isolates, two bacterial strains stood out as the most potent based on their chitinase production capabilities. Isolate 15 and 28, both identified as Gram-positive bacilli, exhibited the highest chitinase activity among all the tested isolates. Both isolates demonstrated their potential as robust chitinase producers, with isolate 28 showing slightly higher specific activity by 11.5U/ mg (Table 2).

Table 2. Chitinase activity and specific activity of different marine bacterial isolates

S. No.	Gram stain	A (U/l)	P(mg/l)	SP(U/mg)
1	(G+vecocci)	114.3	90.5	1.3
2	(G+ve bacilli)	0	0	0
3	(G+vecocci)	103.6	84.32	1.23
4	(G+vecocci)	0	0	0
5	(G+vecocci)	0	0	0
6	(G+vecocci)	145.4	96.25	1.5
7	(G-vecocci)	135.9	92.14	1.48
8	(G+vecocci)	167.5	103.47	1.62
9	(G-vecocci)	0	0	0
10	(G+ve bacilli)	0	0	0
11	(G-ve bacilli)	0	0	0
12	(G-ve bacilli)	0	0	0
13	(G+ve bacilli)	225.7	109.45	2.1
14	(G-vecocci)	278	121.32	2.3
15	**(G+ve bacilli)**	1198.4	112.35	10.7
16	(G+vebacilli)	165.3	112.98	1.5
17	(G-vecocci)	193.6	111.26	1.8
18	(G-vecocci)	211.7	98.65	2.15
19	(G+vecocci)	0	0	0
20	(G+ve bacilli)	0	0	0
21	(G+vecocci)	0	0	0
22	(G+ve bacilli)	359.7	122.54	3.94
23	(G+ve bacilli)	0	0	0
24	(G+ve bacilli)	65.75	89.2	0.74
25	(G+vecocci)	258.6	96.34	2.7
26	(G-vecocci)	344.4	110.5	3.12
27	(G+ve bacilli)	918.8	107.6	8.5
28	(G+ve bacilli)	1264	110.04	11.5
29	(G+vecocci)	634.8	110.48	5.75

Note: A (U/l): Activity (Unit per liter); **P (mg/l):** Protein (milligram per liter); **SP(U/mg):** Specific activity (Unit per milligram).

2- Biochemical identification of most potent isolate using API 20E kits

The biochemical identification of the most potent isolate was performed using API 20 Strep kits. Based on the results, both No. 15 and No. 28 isolates were identified as belonging to the *Enterococcus* genus. Specific API 20 Strep codes for both strains were 20100 and 20160, respectively.

3- Genetic identification of most potent isolate

In the pursuit of a comprehensive characterization of the most potent isolate, genetic identification was conducted. The following results were obtained: Isolate No. 15: *Enterococcus hirae* with Accession Number: OR064172. While Isolate No. 28: *Enterococcus faecalis* with Accession Number: OR053873

4- Partial purification using ammonium sulphate

The chitinase activity, protein concentration, and specific activity (sp) at various ammonium sulfate precipitation levels for two isolates, *E. hirae* OR064172 and *E. faecalis* OR053873 are shown in Table (3). Both isolates displayed chitinase activity, with variations in specific activity at different levels of ammonium sulfate precipitationchitinase fractions. The specific activity provides insights into the enzyme's efficiency at each fraction, with the 70% fraction demonstrating the highest specific activity for both isolates (Table 3).

Table 3. Chitinase fractions of ammonium sulphate for *E. hirae* OR064172 and *E. faecalis* OR053873

	***E. hirae* OR064172**			***E. faecalis* OR053873**		
	Activity (U/l)	**Protein (mg/l)**	**SP (U/mg)**	**Activity (U/l)**	**Protein (U/mg)**	**SP (U/mg)**
Crude E.	1198.4	112.35	10.7	1264	110.04	11.49
20%	780.35	138.54	5.63	963.63	107.85	8.93
35%	1325.65	152.36	8.7	1450.36	156.65	9.26
50%	2021.75	192.3	10.51	2146.54	198.74	10.8
70%	2650.2	205.36	12.9	2860.6	212.32	13.47
90%	1720.65	215.36	7.98	1964.82	228.87	8.58

The ammonium sulfate fractions of chitinase precipitation by *E. hirae* and *E. faecalis* show that the specific activity of chitinase was the highest in the 70% ammonium sulfate fraction for both *E. hirae* and *E. faecalis*. The specific activity decreased in the 50 and 35% ammonium sulfate fractions, and was the at its lowest level in the 20% ammonium sulfate fraction. The protein concentration was at its lowest in the 20% ammonium sulfate fraction for both strains. The protein concentration increased in the 35, 50, and 70% ammonium sulfate fractions (Fig. 1).

5. Chitinase purification using DEAE-cellulose

Fig. (2A) includes purification of chitinase using DEAE-cellulose data for *E. hirae*. Two lines in the chart "Activity" and "Specific activity" show the activity of chitinase in each of these fractions, varying across the different fractions with ranges from as low as 52.63 to as high as 279.85U/ l. The specific activity values in the column purification vary from 5 to 30U/ mg. Higher specific activities indicate that more of the total protein in the fraction is chitinase, suggesting greater purification. For *E. hirae*, fraction 17 has the highest chitinase activity, but its specific activity is relatively low. Moreover, fraction 17 has the highest specific activity, suggesting that it is the most purified fraction.

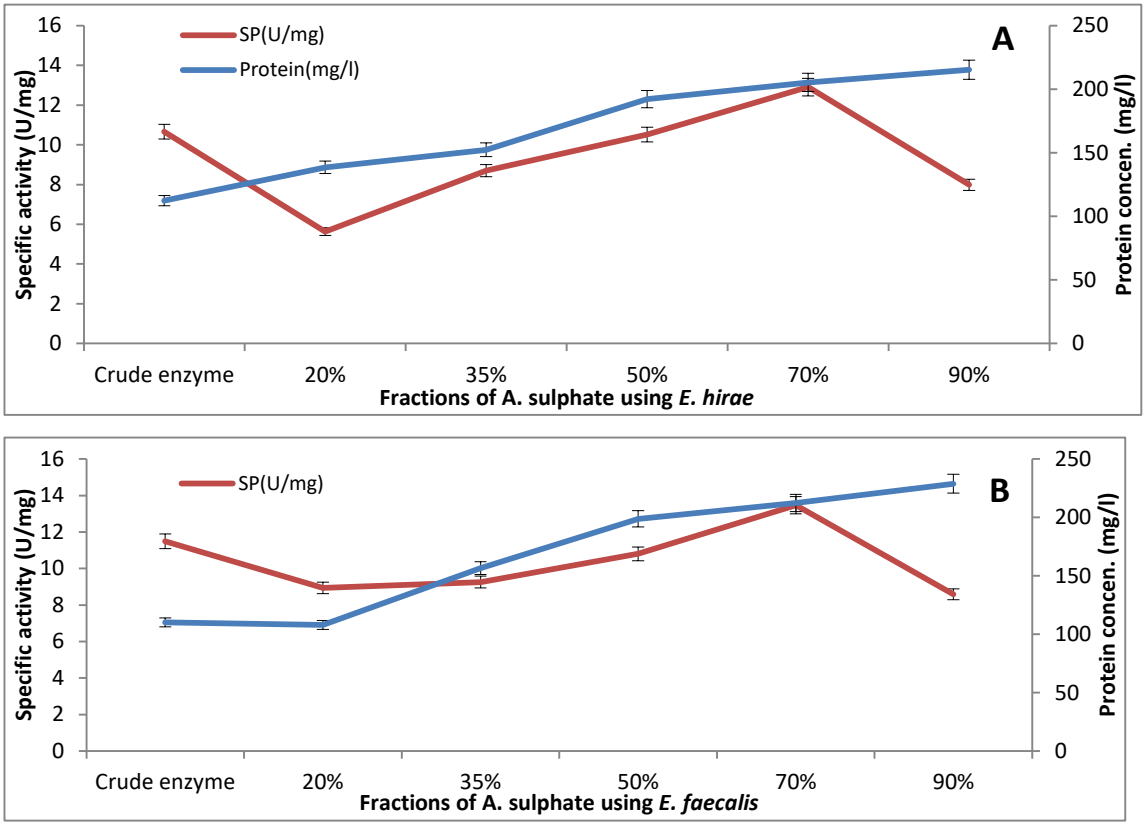


Fig. 1. Specific activity (U/ mg) and protein conc. (mg/ l) of amm. sulphate fraction of chitinase produced by **(A)** *E. hirae* and **(B)** *E. faecalis*

On the other hand, DEAE purification for *E. faecalis* shows variations in fractions' activities. Fractions 1 to 8 have relatively low specific activities. The specific activity starts at 7.44U/ mg and gradually increases to 16.53U/ mg. Fractions 9 to 13 show an increase in specific activity, indicating that the purification process is becoming more effective. This suggests that chitinase is becoming more concentrated in these fractions. Fractions 14 to 21 exhibit a decline in specific activity. The highest activity and specific activity is observed in fraction 12 by 252.05 and 25.56U/ mg, respectively (Fig. 2B).

6. Characterizations of purified chitinase by *E. hirae* and *E. faecalis*

In a series of experiments, the chitinase production capabilities of two bacterial strains, *E. faecalis* and *E. hirae*, were investigated under varying conditions (Fig. 3A). The first set of experiments explored the influence of pH levels on chitinase production. Notably, *E. hirae* consistently outperformed *E. faecalis* in chitinase production across all pH levels, with its highest activity observed at pH 6. This suggests that *E. faecalis* is an inferior chitinase producer compared to *E. hirae*, which is a valuable information for biotechnological applications.

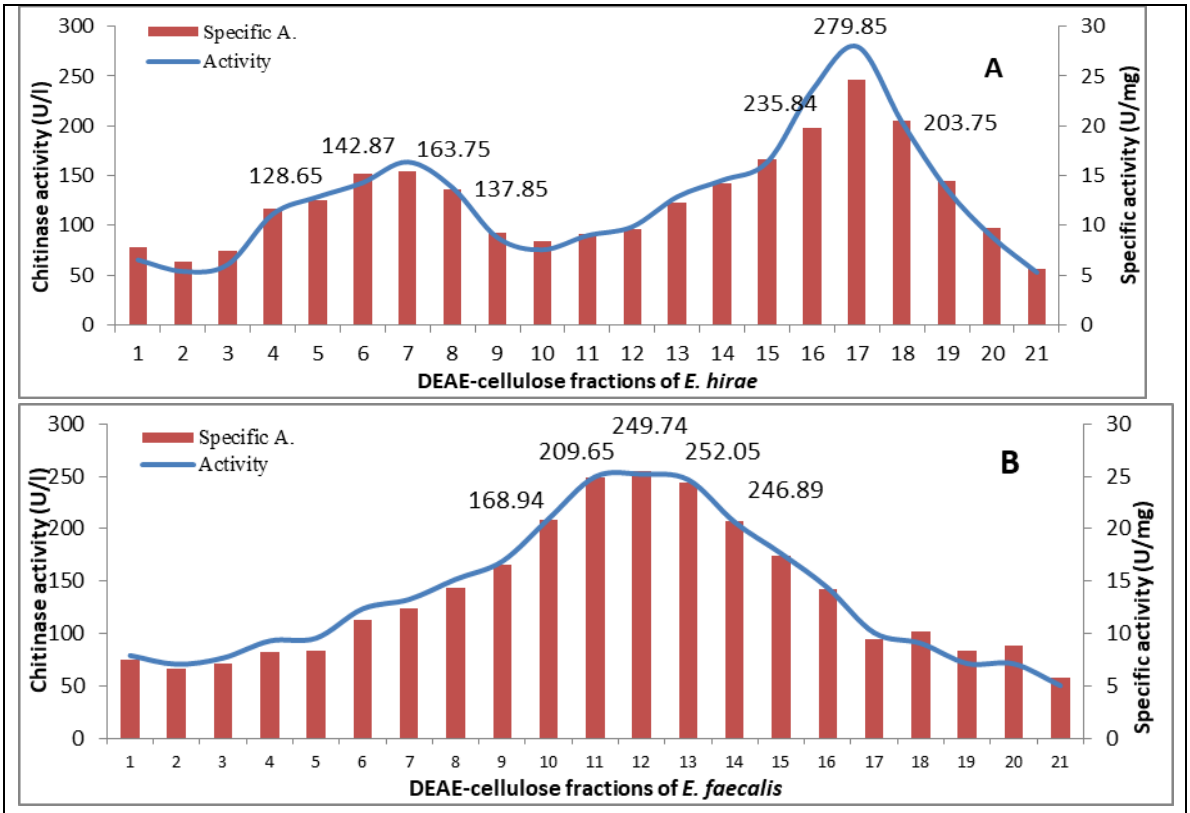


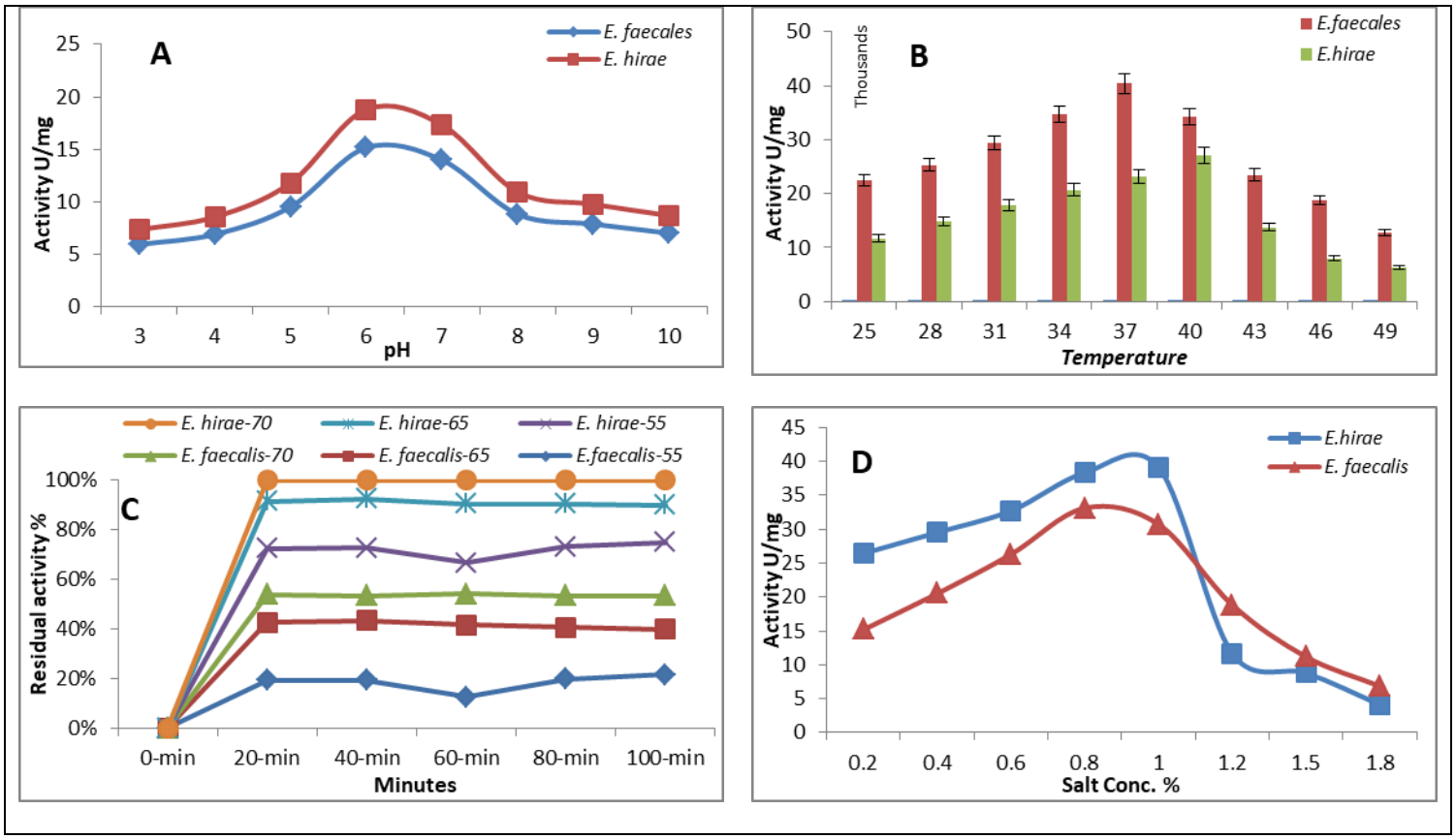
Fig. 2. Chitinase activity (U/ mg) and specific activity (mg/ l) of chitinase purified fractions produced by **(A)** *E. hirae* and **(B)** *E. faecalis*

The second set of experiments examined the effect of temperature on chitinase production. *E. faecalis* exhibited higher chitinase activity than *E. hirae* across all temperature ranges. The peak activity for *E. faecalis* occurred at 37°C (Fig. 3B). These findings reinforce the notion that *E. faecalis* is a more effective chitinase producer than *E. hirae*, providing crucial insights for biotechnological applications.

Moving on to the third set of experiments, the study assessed the impact of thermal stability during different exposure time on chitinase production by both bacterial strains. Both *E. faecalis* and *E. hirae* showed increased chitinase production over time, with *E. faecalis* consistently surpassing *E. hirae* in chitinase activity (Fig. 3C). This

underscores the superiority of *E. faecalis* as a chitinase producer, which is an essential information for researchers interested in utilizing chitinase for biotechnological purposes. The chitinase production activity of two bacterial strains, *E. faecalis* and *E. hirae*, across varying salt concentrations was evaluated. The activity production of chitinase is at its peak at a concentration of 1g/ l. *E. hirae* shows the highest activity level. However, *E. faecalis* is low at a concentration of 0.5 g/l, and high at a concentration of 1g/l. Overall, Fig. (3D) shows that the activity of chitinase is at its highest at a salt concentration of 1g/ l.

Lastly, the study delved into the Michaelis-Menten constants (K_m) and maximum velocities (V_{max}) of chitinase production by both bacterial strains. These constants provide insights into substrate concentration and reaction rates. *E. faecalis* displayed a K_m of 0.0003mg/ ml and a V_{max} of 0.7mg/ mL (Fig. 3E), which varied among different strains. Similarly, *E. hirae* displayed a K_m of 0.124mg/ mL and a V_{max} of 34.5mg/ mL (Fig. 3F), indicating genetic variability in its chitinase production capabilities.



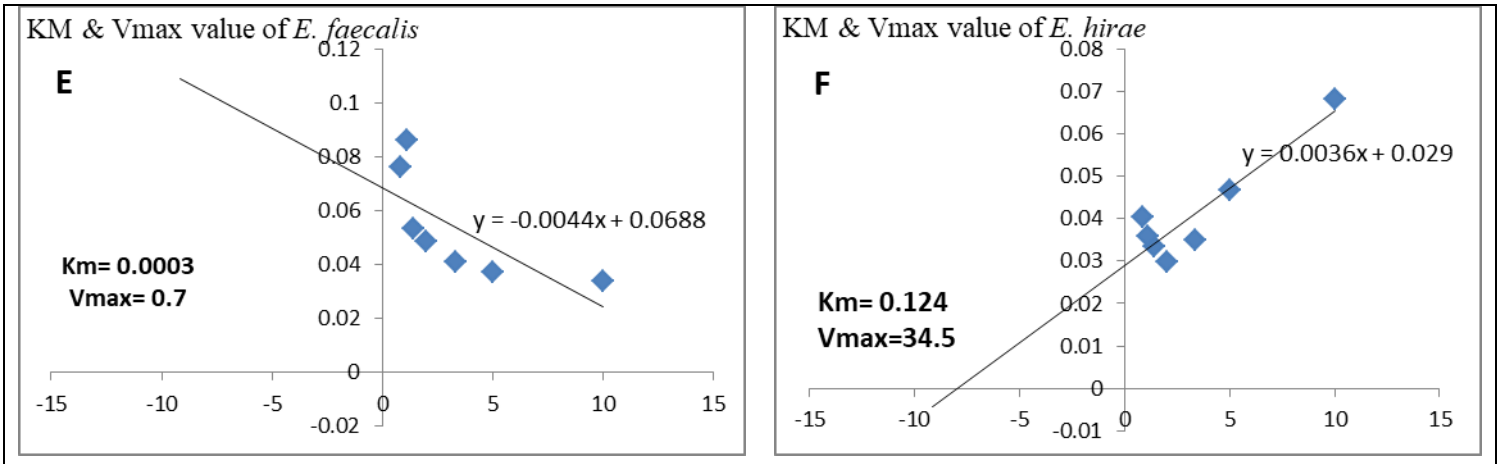


Fig. 3. Effect of some physiological characterization on chitinase production: **(A)** pH, **(B)** temperature, **(C)** Thermal stability, **(D)** Salinity, **(E)** Kinetic for *E. faecalis*, and **(F)** Kinetic for *E. hirae*

7. Chitinase application

In the study, the usage of *E. faecalis* and *E. hirae* for the degradation of shrimp peels was investigated. Table (4) presents data on the enzymatic activity, protein concentration, and specific activity of chitinase produced by *E. faecalis* and *E. hirae* when applied for the degradation of shrimp peels. These results showed that both *E. faecalis* and *E. hirae* have the potential to contribute to the degradation of shrimp peels, with *E. hirae* exhibiting a higher specific activity, indicating its greater efficiency in breaking down chitinaceous components within the shrimp peels.

Table 4. Application of *E. faecalis* and *E. hirae* for degradation of shrimp peels chitinase

Isolate	**Shrimp peels (U/l)**		
	Activity (U/l)	**Protein (mg/l)**	**Sp (U/mg)**
E. faecalis	1440.5	169.9	8.48
E. hirae	1814.5	159.47	11.38

The solid-state fermentation of shrimp peels using two different bacteria, *E. faecalis* and *E. hirae* showed that *E. hirae* produced more chitinase than *E. faecalis* during all fermentation hours tested. The highest activity of chitinase production by *E. faecalis* was at 48 hours, followed by 36, 24, and 60 hours. The lowest activity of chitinase production by *E. faecalis* was at 96 hours. While *E. hirae* produced the highest chitinase than *E. faecalis* at all fermentation hours tested, except the 96 hours. The highest activity of chitinase production by *E. hirae* was at 60 hours, followed by 48, 36, and 24 hours. The lowest activity of amylase production by *E. hirae* was at 96 hours (Fig. 4).

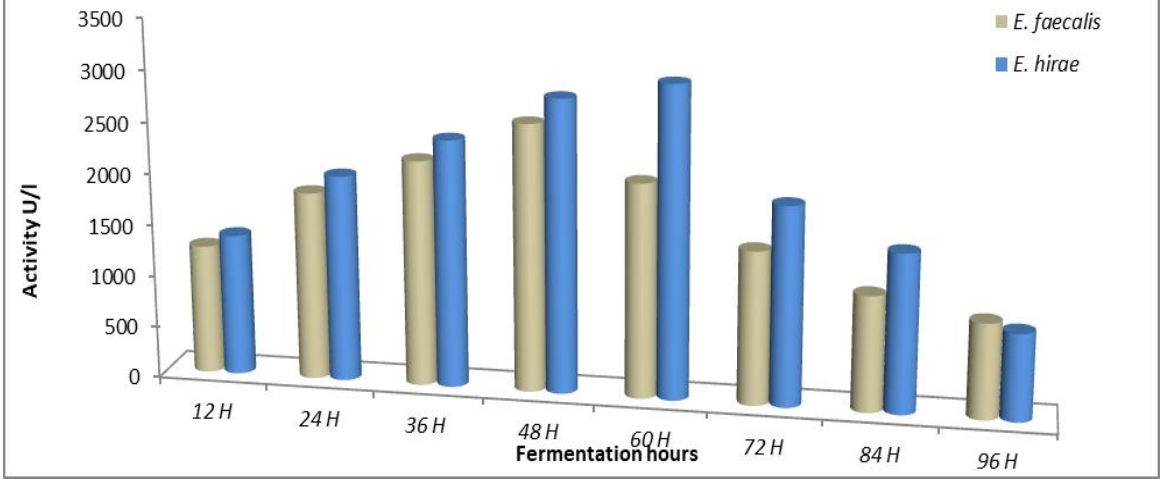


Fig. 4. Solid state fermentation of shrimp peels using *E. faecalis* and *E. hirae*

DISCUSSION

Chitinase-producing bacteria have recently gained significant attention for their role in chitin degradation and their potential applications across various industries **(Bhattacharya *et al.*, 2007; Jahromi & Barzkar 2018; Thakur *et al.*, 2023)**. In this study, we undertook the successful isolation and characterization of chitinase-producing bacterial isolates from the Red Sea, revealing *Enterococcus hirae* (OR064172) and *Enterococcus faecalis* (OR053873) as robust chitinase producers. Chitinase activity is a crucial parameter in evaluating the efficiency of these isolates in breaking down chitin, a major component of the exoskeletons of various organisms **(Kotb *et al.*, 2023)**. Our research encompassed the purification process, including ammonium sulfate precipitation and DEAE-cellulose chromatography, which facilitated the identification of optimal conditions for chitinase activity. Additionally, we delved into understanding the impact of various factors on chitinase activity, such as pH, temperature, salt concentration, and reaction time, shedding light on how these enzymes behave in diverse environmental conditions.

Accurate genetic identification of our isolates as *E. hirae* (OR064172) and *E. faecalis* (OR053873) was achieved through the development of PCR assays, building upon the work of **Knijff *et al.* (2001)**. This genetic data not only strengthens the credibility of our findings but also lays a solid foundation for future research aimed at unraveling the genetic mechanisms governing chitinase production in these isolates. These findings provide crucial genetic information about the isolates, enabling a deeper understanding of their taxonomic classification and potentially shedding light on their functional characteristics. The genetic identification, along with the biochemical data presented earlier, contributes to a more comprehensive profile of these isolates, which is

essential for further research and potential applications in aquaculture and related fields **(Liang, 2014)**.

Chitinase enzyme purification often requires a multi-step approach to separate it from complex cellular components. Two common purification methods are partial precipitation with ammonium sulfate and subsequent chromatography using DEAE-cellulose. Ammonium sulfate selectively precipitates proteins based on their solubility, size, and charge, concentrating the enzyme and partially removing impurities. This method efficiently concentrates chitinase and simplifies the sample. It serves as a cost-effective initial purification step, as demonstrated in this study.

After ammonium sulfate precipitation, DEAE-cellulose chromatography is employed for further purification of chitinase that separates proteins based on their charge by negatively charge resins bind with positive charge of protein containing chitinase. As mentioned in the result, the highest specific activity is mentioned in fraction 17 of *E. hirae* and fraction 12 of *E. faecalis*. The specific activity should increase indicating that the chitinase enzyme is being concentrated and purified. The fractions of purification step are important for elution profile progress. The fractions with the highest chitinase activity represent the most purified enzyme as achieved in *E. hirae* and *E. faecalis*. The high-purity and high-activity chitinase enzymes in various industrial and commercial fields have been steadily growing. While crude chitinolytic enzymes from bacteria have been used for chitin degradation **(Ali *et al*., 2020)**, purified enzymes open up broader possibilities for their application. Several notable examples of purified bacterial chitinase enzymes include those from *Serratia marcescens* **(Wang *et al*., 2014)**, *Salinivibrio* sp. **(Le & Yang, 2018)**, and *Aeromonas hydrophila* **(Stumpf *et al*., 2019)**.

Our comprehensive characterization of the purified chitinase enzymes provided valuable insights into their behavior under various conditions. Notably, *E. faecalis* and *E. hirae* recorded valuable chitinase production across different pH levels and temperatures, highlighting its potential as a robust chitinase producer. Furthermore, the enzymes exhibited impressive thermal stability, which holds significance for industrial applications requiring enzymatic processes at elevated temperatures. Our investigation into the effects of salt concentration on chitinase activity indicated that both strains exhibited increased activity with higher salt concentrations, making them adaptable for use in diverse saline environments. For instance, *Serratia marcescens* DSM 30121T displayed the highest chitinase activity (0.556U/ ml) when cultured in a medium containing 1% colloidal chitin concentration at 30°C and pH 6 **(Lamine *et al*., 2012)**. *Bacillus thuringiensis* isolate CMBL-Bt4 exhibited superior chitinase activity (0.23U/ ml) compared to 12 other *Bacillus thuringiensis* strains after 4 days of incubation at 37°C, with a pH of 7 for the culture medium **(Saleem *et al*., 2014)**. Additionally, *Streptomyces* sp. S242 demonstrated the highest chitinase activity (0.162U/ ml) after 4 days of incubation at 30°C, with a pH of 7 for the culture medium **(Saadoun *et al*., 2009)**. *Aeromonas* sp. CQNU6-2's crude chitinase activity was active over a broad pH range

from 3 to 10, with the maximum activity observed at pH 6, showing potential in the biological control of pests and diseases **(Ajayi *et al.*, 2016)**. Moreover, the enzyme exhibited chitin hydrolysis capability across a wide temperature range of 10 to 90°C. Its optimal activity, reaching 67.2%; this was noted at a low temperature of 10°C. Remarkably, even at the high temperature extreme of 90°C, it retained a substantial portion of its activity, specifically 41.9%.

The two isolated strains displayed differences in both K_m and V_{max}. In *E. faecalis*, the K_m value was low compared to *E. hirae* strain, indicating that the first isolate has a high affinity for substrate, achieving higher productivity in the presence of small quantities of substrate, unlike the other isolate which had a high K_m, meaning it produces a higher amount of the enzyme in the presence of larger quantities of substrate. As for V_{max}, which represents the maximum rates of the enzyme's ability to convert substrate into the final product which required that, the substrate concentration is much higher than K_m in order for the enzyme to be working at its maximal capacity. The V_{max} for *E. hirae* was higher than that of the other strain. The V_{max} for the chitinase enzyme extracted from *Aspergillus flavus* was higher than that mentioned in this study. Furthermore, the V_{max} for the *Streptomyces chilikensis* RC1830 falls within the range referred to in our research **(Beltagy *et al.*, 2018; Ray *et al.*, 2019)**.

The application of *E. faecalis* and *E. hirae* in the degradation of shrimp peels showcased their potential for eco-friendly waste management in the seafood industry. *E. hirae*, in particular, displayed higher specific activity in breaking down chitinaceous components within shrimp peels, indicating its superior efficiency in this process. Our solid-state fermentation results further supported the potential of these bacteria in degrading chitin-containing waste, with *E. faecalis* demonstrating a higher chitinase production.

Considering the challenges associated with chitin degradation and the substantial amount of shrimp waste generated in the shellfish industry, the utilization of chitin resources has become crucial for maintaining a proper carbon-nitrogen balance **(Bhattacharya *et al.*, 2016)**. Compared to physical and chemical methods, biological approaches offer several advantages for chitin decomposition, including higher yield and environmental friendliness **(Liu *et al.*, 2019)**. Consequently, there has been a growing focus on screening and utilizing novel chitinase-producing organisms **(Kumar *et al.*, 2017; Stumpf *et al.*, 2019; Cardozo *et al.*, 2023)**. Historically, shrimp shells and crab shells have been traditional resources for the production of commercialized chitin **(Doan *et al.*, 2019)**. Leveraging these resources not only addresses environmental pollution resulting from shrimp shell waste but also provides an effective solution for waste management.

CONCLUSION

It could be concluded that, the study of chitinase-producing bacteria has gained significant attention due to their role in chitin degradation and their potential applications in various industries. This research successfully isolated and characterized chitinase-producing bacteria from the Red Sea, identifying *E. hirae* (OR064172) and *E. faecalis* (OR053873) as robust chitinase producers.

ABBREVIATIONS

DEAE-cellulose: Diethylaminoethyl cellulose; **API**:application programming interface; **Km and Vmax**: Michaelis-Menten constant (KM) and maximal velocity (Vmax); **DNA**: deoxyribonucleic acid; **sp**: Species; **K_2HPO_4**: di potassium hydrogen orthophosphate; **KH_2PO_4**: potassium dihydrogen orthophosphate; **(NH4)2SO4**: di-ammonium sulphate; **Na3C6H5O7**: tri-sodium citrate; **$MgSO_4$**: magnesium sulphate; **Pm**: post meridiem time; **Am**:ante meridiem time; **N**: Northern; **E**: Eastern; **rDNA**: Ribosomal deoxyribonucleic acid; **F**: Forward; **R**: Reverse; **SDS**: Sodium dodecyl sulphate; **M**: Molar; **NaOH**: Sodium hydroxide; **rpm**: rotation per minute: **PCR**; Polymerase chain reaction: **MgCl2**; magnesium chloride: **^{O}C**; degree celsius: **OD**: optical density; **dNTPs**: Deoxynucleotide triphosphates; **µl**: micron; **mM**: mill mole; **GCG Wisconsin Package**: genetic computer groups Wisconsin Package; **CFU/mL**: Colony foarming units per ml.

Authors' contributions

Mahmoud S. kelany (KM), Mohamed Abdel-Rahiem Ali Abdrabo (AM), Yasser A. Geneid (GY) and Mohamed Fathi (FM) have made significant contributions to the design of the practical parts, sample collection and the writing of the manuscript. KM, AM and GY provided water and sediment sample collection. KM and AM performed the bacterial isolation, identification, screening for chitinase production, and purification. KM performed characterization with application. All authors read, reviewed, and approved the final manuscript.

Acknowledgment

The authors are grateful to The National Institute of Oceanography and Fisheries Science, Egypt, for providing the space and possibilities for this research.

Availability of data and material

The raw data supporting the conclusions of this manuscript would be available by the authors, without undue reservation, to any qualified researcher.

Ethical statements

The trial was performed following the Universal Directive on the protection of animals used for scientific purposes according to ethical guidelines approved by the ethics of scientific research committee, Faculty of NIOF Committee for Ethical Care of Marine Organisms and Experimental Animals (NIOF-IACUC), Cairo, Egypt, with approved code No. NIOF- AQ5- F23-D-035. This article does not contain any studies with human participants or animals performed by any author.

REFERENCES

Abo Elsoud, M.M. and El Kady, E.M. (2019). Current trends in fungal biosynthesis of chitin and chitosan. Bulletin of the National Research Centre, 43(1): 1-12.

Ajayi, A.; Onibokun, E.A.; George, F. and Atolagbe, O.M. (2016). Isolation and characterization of chitinolytic bacteria for chitinase production from the African Catfish, Clarias gariepinus (Burchell, 1822). Research Journal of Microbiology, 11(4): 119-125.

Al-Agamy, M.H.; Alhuzani, M.R.; Kelany, M.S. and Hamed, M.M. (2021). Production and partial characterization of α-amylase enzyme from marine actinomycetes. BioMed research international, 21(1): 1-15.

Ali, M.H.; Aljadaani, S.; Khan, J.; Sindi, I.; Aboras, M. and Aly, M.M. (2020). Isolation and Molecular Identification of Two Chitinase Producing Bacteria from Marine Shrimp Shell Wastes. Pakistan journal of biological sciences, 23(2): 139-149.

Baldoni, D.B.; Antoniolli, Z.I.; Mazutti, M.A.; Jacques, R.J.S.; Dotto, A.C.; de Oliveira Silveira, A.; Ferraz, R.C.; Soares, V.B. and de Souza, A.R.C. (2020). Chitinase production by Trichoderma koningiopsis UFSMQ40 using solid state fermentation. Brazilian Journal of Microbiology, 51(1): 1897-1908.

Banerjee, S. and Mandal, N. C. (2019). Diversity of chitinase-producing bacteria and their possible role in plant pest control. Microbial Diversity in Ecosystem Sustainability and Biotechnological Applications, Volume 2. Soil & Agroecosystems: 457-491.

Beltagy, E.A.; Rawway, M.; Abdul-Raouf, U.M.; Elshenawy, M.A. and Kelany, M.S. (2018). Purification and characterization of theromohalophilic chitinase producing by halophilic Aspergillus flavus isolated from Suez Gulf. The Egyptian Journal of Aquatic Research, 44(3): 227-232.

Bhattacharya, D.; Nagpure, A. and Gupta, R.K. (2007). Bacterial chitinases: properties and potential. Critical reviews in biotechnology, 27(1): 21-28.

Bhattacharya, S.; Das, A.; Samadder, S. and Rajan, S. S. (2016). Biosynthesis and characterization of a thermostable, alkali-tolerant chitinase from Bacillus pumilus JUBCH08 displaying antagonism against phytopathogenic Fusarium oxysporum. 3 Biotech, 6(1): 1-8.

Cardozo, F.; Feitosa, V.; Pillaca-Pullo, O. and Pessoa, A. (2023). Endochitinase and Chitobiosidase Production by Marine Aeromonas caviae CHZ306: Establishment of Nitrogen Supplementation. Bioengineering, 10(4):431-446.

Dikbaş, N.; Uçar, S.; Tozlu, G.; Öznülüer Özer, T. and Kotan, R. (2021). Bacterial chitinase biochemical properties, immobilization on zinc oxide (ZnO) nanoparticle and its effect on Sitophilus zeamais as a potential insecticide. World Journal of Microbiology and Biotechnology, 37(1): 1-14.

Doan, C.T.; Tran, T.N.; Nguyen, V.B.; Nguyen, A.D. and Wang, S.L. (2019). Production of a thermostable chitosanase from shrimp heads via Paenibacillus mucilaginosus TKU032 conversion and its application in the preparation of bioactive chitosan oligosaccharides. Marine drugs, 17(4): 217-231.

Dwyer, K.; Bentley, I.S.; Tighe, E.; McGleenan, E.; Gaffney, D. and Walsh, G. (2021). Recombinant production and characterisation of two chitinases from Rasamsonia emersonii, and assessment of their potential industrial applicability. Applied Microbiology and Biotechnology, 105(20): 7769-7783.

Golgeri, M.D.B.; Mulla, S.I.; Bagewadi, Z.K.; Tyagi, S.; Hu, A.; Sharma, S. and Nadda, A. K. (2022). A systematic review on potential microbial carbohydrases: current and future perspectives. Critical Reviews in Food Science and Nutrition, 46(3): 438-455.

Hirano, T.; Okubo, M.; Tsuda, H.; Yokoyama, M.; Hakamata, W. and Nishio, T. (2019). Chitin heterodisaccharide, released from chitin by chitinase and chitin oligosaccharide deacetylase, enhances the chitin-metabolizing ability of Vibrio parahaemolyticus. Journal of Bacteriology, 201(20): 10-1128.

Hisham, F.; Akmal, M.M.; Ahmad, F.; Ahmad, K. and Samat, N. (2023). Biopolymer chitosan: Potential sources, extraction methods, and emerging applications. Ain Shams Engineering Journal, 15(2):102424-102435.

Jahromi, S.T. and Barzkar, N. (2018). Marine bacterial chitinase as sources of energy, eco-friendly agent, and industrial biocatalyst. International journal of biological macromolecules, 120(B): 2147-2154.

Keyhani, N.O. and Roseman, S. (1999). Physiological Aspect of Chitin Catabolism in Marine Bacteria. Biochimica et Biophysica Acta, 1473(1): 108-122.

Knijff, E.; Dellaglio, F.; Lombardi, A.; Andrighetto, C. and Torriani, S. (2001). Rapid identification of Enterococcus durans and Enterococcus hirae by PCR with primers targeted to the ddl genes. Journal of Microbiological Methods, 47(1): 35-40.

Kobayashi, S.; Makino, A.; Matsumoto, H.; Kunii, S.; Ohmae, M.; Kiyosada, T. and Shoda, S.I. (2006). Enzymatic polymerization to novel polysaccharides having a glucose-N-acetylglucosamine repeating unit, a cellulose− chitin hybrid polysaccharide. Biomacromolecules, 7(5): 1644-1656.

Kotb, E.; Alabdalall, A.H.; Alghamdi, A.I.; Ababutain, I.M.; Aldakeel, S.A.; Al-Zuwaid, S.K.; Algarudi, B.M.; Algarudi, S.M.; Ahmed, A.A. and Albarrag, A.M. (2023). Screening for chitin degrading bacteria in the environment of Saudi Arabia and characterization of the most potent chitinase from Streptomyces variabilis Am1. Scientific Reports 13(1):11723-11735.

Kumar, M.; Brar, A.; Vivekanand, V. and Pareek, N. (2017). Production of chitinase from thermophilic Humicola grisea and its application in production of bioactive

chitooligosaccharides. International journal of biological macromolecules, 104(B), 1641-1647.

Kumari, R.; Kumar, M.; Vivekanand, V. and Pareek, N. (2023). Chitin biorefinery: A narrative and prophecy of crustacean shell waste sustainable transformation into bioactives and renewable energy. Renewable and Sustainable Energy Reviews, 184(1): 113595-113616.

Lamine, B.M.; Lamine, B.M. and Bouziane, A. (2012). Optimization of the Chitinase Production by Serratia Marcescens DSM 30121Tand Biological Control of Locusts. Journal of Biotechnology and Biomaterials, 2(1): 13-138.

Le, B. and Yang, S.H. (2018). Characterization of a chitinase from Salinivibrio sp. BAO-1801 as an antifungal activity and a biocatalyst for producing chitobiose. Journal of basic microbiology, 58(10): 848-856.

Liang, T.W.; Chen, Y.Y.; Pan, P.S. and Wang, S.L. (2014). Purification of chitinase/chitosanase from Bacillus cereus and discovery of an enzyme inhibitor. International journal of biological macromolecules, 63(1): 8-14.

Loni, P.P.; Patil, J.U.; Phugare, S.S. and Bajekal, S.S. (2014). Purification and characterization of alkaline chitinase from Paenibacillus pasadenensis NCIM 5434. Journal of basic microbiology 54(1): 1080-1089.

Pal, K.; Rakshit, S.; Mondal, K.C. and Halder, S.K. (2021). Microbial decomposition of crustacean shell for production of bioactive metabolites and study of its fertilizing potential. Environmental Science and Pollution Research, 28(1): 58915-928.

Ray, L.; Panda, A.N.; Mishra, S.R.; Pattanaik, A.K.; Adhya, T.K.; Suar, M. and Raina, V. (2019). Purification and characterization of an extracellular thermo-alkali stable, metal tolerant chitinase from Streptomyces chilikensis RC1830 isolated from a brackish water lake sediment. Biotechnology Reports 20(1): e00311-e00318.

Resendiz-Nava, C.N.; Silva-Rojas, H.V.; Rebollar-Alviter, A.; Rivera-Pastrana, D.M.; Mercado-Silva, E.M. and Nava, G.M. (2021). A comprehensive evaluation of Enterobacteriaceae primer sets for analysis of host-associated microbiota. Pathogens 11(1): 17-28.

Saadoun, I.; Al-Omari, R.; Jadarat, Z. and Ababneh, Q. (2009). Influence of Culture Conditions of Streptomyces sp. (Strain S242) on Chitinase Production. Polish Journal of Microbiology, 58(4): 339-345.

Saleem, F.; Younas, A.; Bashir, R.; Naz, S.; Munir, N. and Shakoori, A.R. (2014). Molecular Cloning and Characterization of Exochitinase A Gene of Indigenous Bacillus thuringiensis Isolates. Pakistan Journal of Zoology, 46(1): 1491-1501.

Senol, M.; Nadaroglu, H.; Dikbas, N. and Kotan, R. (2014). Purification of Chitinase enzymes from Bacillus subtilis bacteria TV-125, investigation of kinetic

properties and antifungal activity against Fusarium culmorum. Annals of clinical microbiology and antimicrobials 13(35), 1-7.

Shahbaz, U. and Yu, X., (2020). Cloning, isolation, and characterization of novel chitinase-producing bacterial strain UM01 (*Myxococcus fulvus*). Journal of Genetic Engineering and Biotechnology 18(1): 1-11.

Sheehan, D. and FitzGerald, R. (1996). Ion-exchange chromatography. Protein Purification Protocols, 59(1): 145-150.

Silva-Castro, G.; Uad, I.; Gónzalez-López, J.; Fandiño, C.; Toledo, F. and Calvo, C. (2012). Application of selected microbial consortia combined with inorganic and oleophilic fertilizers to recuperate oil-polluted soil using land farming technology. Clean Technologies and Environmental Policy 14(1): 719-726.

Singh, R.V.; Sambyal, K.; Negi, A.; Sonwani, S. and Mahajan, R. (2021). Chitinases production: A robust enzyme and its industrial applications. Biocatalysis and Biotransformation, 39(3): 161-189.

Sint, D.; Raso, L.; Kaufmann, R. and Traugott, M. (2011). Optimizing methods for PCR-based analysis of predation. Molecular ecology resources 11(5): 795-801.

Stumpf, A.K.; Vortmann, M.; Dirks-Hofmeister, M.E.; Moerschbacher, B.M. and Philipp, B. (2019). Identification of a novel chitinase from Aeromonas hydrophila AH-1N for the degradation of chitin within fungal mycelium. FEMS microbiology letters, 366(1), fny294-303.

Taokaew, S. and Kriangkrai, W. (2023). Chitinase-assisted bioconversion of chitinous waste for development of value-added chito-oligosaccharides products. Biology 12(1): 87-107.

Thakur, D.; Chauhan, A.; Jhilta, P.; Kaushal, R. and Dipta, B. (2023). Microbial chitinases and their relevance in various industries. Folia Microbiologica, 68(1): 29-53.

Türe, M. and Alp, H. (2016). Identification of bacterial pathogens and determination of their antibacterial resistance profiles in some cultured fish in Turkey. Journal of Veterinary Research 60(1): 141-146.

Ullah, N.; Rehman, M.U.; Sarwar, A.; Nadeem, M.; Nelofer, R.; Shakir, H.A. and Alqahtani, F. (2022). Purification, characterization, and application of alkaline protease enzyme from a locally isolated Bacillus cereus strain. Fermentation, 8(11), 628-641.

Utama, G.L.; Oktaviani, L.; Balia, R.L. and Rialita, T. (2023). Potential Application of Yeast Cell Wall Biopolymers as Probiotic Encapsulants. Polymers, 15(16): 3481-3500.

Wang, J.L.; Chen, Y.C.; Deng, J.J.; Mo, Z.Q.; Zhang, M.S.; Yang, Z.D.; Zhang, J.R.; Li, Y.W.; Dan, X.M. and Luo, X.C. (2023). Synergic chitin degradation by Streptomyces sp. SCUT-3 chitinases and their applications in chitinous waste

recycling and pathogenic fungi biocontrol. International Journal of Biological Macromolecules 225(1): 987-996.

Wang, K.; Yan, P.S. and Cao, L.X. (2014). Chitinase from a novel strain of Serratia marcescens JPP1 for biocontrol of aflatoxin: molecular characterization and production optimization using response surface methodology. BioMed research international, 2014(1): 1-8.

Yeul, V.S. and Rayalu, S.S. (2013). Unprecedented chitin and chitosan: A chemical overview. Journal of Polymers and the Environment, 21(1): 606-614.

Zhang, C.; Zhao, X.; Liang, A.; Li, Y.; Song, Q.; Li, X. and Hou, N. (2023). Insight into the soil aggregate-mediated restoration mechanism of degraded black soil via biochar addition: Emphasizing the driving role of core microbial communities and nutrient cycling. Environmental Research, 228(1): 115895-115905.