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New Chitinolytic *Alcaligenes* Species Strains Isolated from Shrimp Shells

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ABSTRACT

Chitinases have received attention in the last decades because of their wide applications in medicine, biotechnology, agriculture, waste management, and industrial applications such as food quality enhancers and biopesticides. The objectives of this research were to isolate and identify potentially chitinolytic bacterial species from shrimp shells and analyze their potency for chitinase production. The research consisted of a screening of chitinolytic bacteria based on the zone of clearance on a colored agar plate and enzyme activity assay using the dinitrosalicylic acid (DNS) method. Five bacterial isolates of chitinolytic bacteria showed high chitinolytic activity. Molecular identification of bacterial isolates was based on polymerase chain reaction (PCR) of 16S rRNA universal primers and sequences analysis. The isolates identified as chi1 and chi2 were *Alcaligenes faecalis*, while chi3, chi4, and chi5 were *Alcaligenes ammonioxydans*, *Alcaligenes aquatilis* and *E. coli*, respectively, with 99% similarity. Two of them are the high producers of chitinase, chi4 *Alcaligenes aquatilis* (4.5 U/ml) andchi3 *Alcaligenes ammonioxydans* (4.2 U/ml). A novel bacterial isolate (*Alcaligenes ammonioxydans*) that generates chitinase was identified for the first time in Egypt, and it is regarded as one of the most productive isolates in this study.

INTRODUCTION

Chitin is in the second place among the most abundant organic compounds in nature as a rich source of carbon and nitrogen that bacterial communities can transform. Bacteria can gradually hydrolyze chitin into N-acetylglucosamine monomers. Shrimp waste, constituting the primary structural component of shrimp exoskeletons and those of other crustaceans, mollusks, insects and arthropods, contains chitin in quantities ranging from 20% to 60%. Chitin is water-insoluble and hydrolyzed in nature by chitinolytic bacteria using chitinase as a hydrolytic enzyme **(Shanmugaiah *et al.*, 2008; Setia, 2015)**. Chitinase is produced in the outer cells of bacteria and attaches to chitin to break it into N-acetylglucosamine, which has a significant role in many medical applications

(Frändberg, 1997;Sato *et al.*, 2010). These substances can be employed as antibacterial agents, immunostimulants, host defense system activators, drug delivery transporters, antioxidants, hemostasis agents, as well as serving for wound healing **(Karthik *et al.*, 2017)**. Recent studies observed that chitinase selectively lysed the tumor cells *in vitro* and *in vivo* **(Pan *et al.*, 2005)**. However, various organisms including bacteria, fungi, insects, plants, animals and humans possess this enzyme for various functions, which are: 1) reshaping their matrices at different developmental stages, 2) hydrolysis of the chitin to be used as a source of energy, 3) and as a defense mechanism against microorganisms coated with chitin **(Adrangi & Faramarzi 2013; Bai *et al.*, 2016)**. Chitinolytic bacteria are found in a variety of locations, including soil, lakes, the ocean and industrial shrimp waste (**Herdyastuti *et al.*, 2021**). Bacteria are one of the main mediators of chitin in the environment and the most investigated source since they could be cultivated in a short time, and the production could be in a large-scale manner **(Vahed *et al.*, 2013)**. The aim of the present study was to isolate and identify potential chitinolytic bacterial strains from shrimp shells based on 16S rRNA sequencing and enzyme assays of chitinase.

MATERIALS AND METHODS

Collection of shrimp shells

Shrimps weighing 250g were collected from Suez (Red Sea), Egypt, and placed in a sterile zip-lock bag with ice packs for transportation to the laboratory. Upon arrival, shrimp shells were separated and frozen until the inoculation process.

Preparation of colloidal chitin

Colloidal chitin was prepared from purified chitin (Loba chemie, Mumbai, Maharashtra, India) by the modified method of Hsu and Lockwood **(Bailly & Ollis, 1986)**. In brief, chitin powder (40g) was slowly added to 600ml of concentrated HCl and kept for 60 minutes at 30°C with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 2 liters of water at 4- 10°C. The suspension was collected by filtration with a coarse filter paper and washed by suspending it in about 5 liters of DW. Washing was repeated three times until the pH of the suspension was 7. After the above treatment, the loose colloidal chitin was used as a substrate **(Jabeen & Qazi, 2014)**.

Isolation of chitinolytic bacteria

Chitinolytic bacteria from the collected sample shrimp shells were isolated by the quadrant streaking method for getting single colony. Liquid medium was composed of nutrient broth (Oxoid) supplemented with 1% colloidal chitin; the pH of the solution was adjusted to 8 and sterilized by autoclave. Liquid medium was used to maintain the bacteria and produce the enzyme. A similar medium formula was followed for solid medium. Shrimp shells were inoculated in liquid medium and incubated at 37^{o}C for 2–7 days, and the isolation of bacteria was carried out daily. The cultured solid media were incubated at 37^{o}C for 24- 48hr for getting potential chitinolytic single colony. The chitinase producers were selected based on the clear zone or the heaviest growth on the

colloidal chitin-incorporated solid medium **(Ali *et al.*, 2020)**. Colonies obtained from plates were taken for further screening.

Screening of chitinase producing bacteria by staining plates and morphological identification

All potential chitinolytic isolates were further spotted on the chitin agar plates (nutrient agar + 1% of colloidal chitin) then incubated for 72hr at 37^{o}C, flooded with 0.1% Congo red stain and allowed to stand for 15- 20 minutes with gentle shaking. Afterwards, it was washed off with a 1.0 M NaCl solution for 10 minutes. Then, plates were observed for clear zone formation **(Krithika & Chellaram, 2016)**. This step was daily carried out; each suspected colony was inoculated onto three different plates and cultivated for three days. The plates have been divided into four quarters for four single colonies inoculated in the center of the quarter.

Extracellular enzyme activity on the chitin agar plate was measured by the method given by **Bradner *et al.* (1999)**. On each plate, the diameter of the colony and the total diameter of the zone of clearance were measured in two dimensions at 90° to each other, and the average values were noted. An index of relative enzyme activity value of 1.0 or greater was classified as having significant enzyme activity **(Duncan *et al.*, 2008)**, and isolates with such activity were taken as chitinase-positive**.**

The index of relative enzyme activity (RA) was calculated by the following equation:

$$RA = \frac{\text{Total diameter of clear zone} - \text{diameter of colony}}{\text{Diameter of colony}}$$

The screened pure isolates were stored in nutrient agar slants added with 1% colloidal chitin at 4°C to maintain the viability of chitinase producers.

Morphological identification is carried out by gram stain, selective medium (MacConkey agar), and biochemical tests.

Chitinase enzyme assay

Chitinolytic bacteria were inoculated in nutrient broth with 1% colloidal chitin for three days at 37°C in a shaking incubator at 150rpm, then tested every day for enzyme activity. Chitinase activity was determined by incubating 1.0ml of colloidal chitin (1%) and 1ml of enzyme solution at 37°C for one hour. Enzyme solution was obtained after centrifugation of the inoculated medium at 13226xg for 20min at 4^{o}C. The mixture was centrifuged at 5,000×*g* for 5min, and the reducing sugar in the supernatant formed during hydrolysis of colloidal chitin substrate was estimated by the 3,5-dinitrosalicylic acid (DNS) method **(Miller, 1959)** through adding an equal volume of DNS reagent to the mixture, then boiling it for 10min in a boiling water bath, and rapidly cooling the mixture in cold water. One unit of chitinase activity is defined as the amount of enzyme required to release 1μmol of N-acetylglucosamine (GlcNAc) in 1 minute under the above-described assay conditions **(Miller, 1959)**.

Chitinase activity was determined using a colorimetric method **(Ilyina *et al.*, 2001; Tsujibo *et al.*, 2003; Duncan *et al.*, 2008)**. The absorbance was recorded with the spectrophotometer at 540nm. The standard curve and the equation of straight line used are illustrated in Fig. (1).

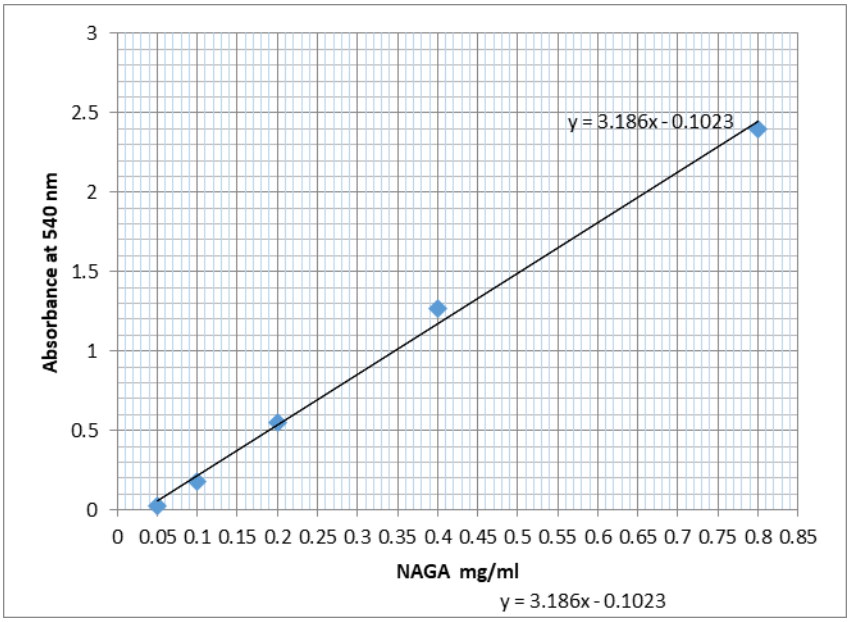

Fig. 1. N- acetylglucosamine (NAGA) standard curve for chitinase activity

DNA extraction of chitinolytic bacteria

Two loopfuls of chitinolytic bacteria were inoculated in 5ml nutrient broth and incubated for 24 hours at 120rpm at 37°C. The bacterial precipitate was suspended in 200µL of TE buffer. The suspension was incubated for 10min at 56°C and then for 10min at 95°C before being spun at 16000×*g* for 2min. After centrifugation, 5µl of the supernatant was used as templates in a 50µl PCR reaction **(Hamza *et al.*, 2020)**.

Amplification of 16S rRNA by PCR

Sequences of 16S rRNA were amplified with universal primers, Univerbac1 (5' AGGAGGTGATCCAACCGCA 3') as reverse primer, and (5' AACTGGAGGAAGGTGGGGAT 3') as forward primer **(Greisen *et al.*, 1994; Pantopikou & Papasotiriou, 2017)**. The PCR mix solution was prepared as Master Mix 25µl (Dream Taq), DNA template 5µl, primer pair 2µl, and nuclease-free water up to 50µl. The PCR reaction conditions were the initial denaturation at 94°C for 5min, denaturation at 94°C for 1min, annealing at 52°C for 1min, extension at 72°C for 1min, and final extension at 72°C for 5min. Amplicons of 16S rRNA were separated by 1% electrophoresis on agarose gel.

Sequencing and phylogeny tree construction

Positive DNA bands with targeted sizes were purified using a gel extraction kit (QIAquick, Qiagen), according to manufacturer's instructions. The BigDye Terminator 3.1 Cycle Sequencing Kit was used to sequence the purified products according to manufacturer's recommendations (Applied Biosystems, USA). Sequencing reactions (two directions) were further purified by the Centri-Sep Purification Kit according to manufacturer's instructions (Applied Biosystems) and then decoded using the 3500 genetic analyzer (Applied Biosystems). The BioEdit Sequence Alignment Editor 7.2.5 **(Hall, 1999)** was used to prepare sequence alignments. To ascertain their phylogenetic relationship, the partial sequences obtained from chitinolytic isolates were compared with reference sequences using BLAST (National Centre for Biotechnology Information, www.ncbi.nlm.gov/BLAST). Additionally, GenBank sequences for a few key groups of bacterial division were gathered. MEGA11 software was used to create an unrooted maximum likelihood phylogenetic tree with 1000 bootstrap replicates to verify the tree **(Kumar *et al.*, 2016)**. The comparative analysis of sequences and similarity matrix were performed using the CLUSTALW multiple sequence alignment program, version 7.1 of the MegAlign suite of Lasergene DNASTAR software **(Thompson *et al.*, 1994)** to determine nucleotide and amino acid sequence similarities and relationships. All obtained sequences of chitinolytic bacterial isolates were identified, assigned and uploaded to the National Center for Biotechnology Information (NCBI) with accession numbers, as rendered in Table (1).

Table 1. GenBank Accession No. of chitinolytic bacterial isolates

Code	Name	Accession No.
Chi 1	*Alcaligenes faecalis*	OM472564
Chi 2	*Alcaligenes faecalis*	OM472565
Chi 3	*Alcaligenes ammonioxydans*	OM472566
Chi 4	*Alcaligenes aquatilis*	OM472567
Chi 5	*E coli*	OM472568

RESULTS

1. Isolation and detection of chitinolytic bacteria

In the present work, chitinolytic bacterial strains were isolated from shrimp shells. Approximately, 17 pure colonies were grown on nutrient agar with 1% colloidal chitin medium, but only five isolates produced a clear zone, shown in Fig. (2), when stained with Congo red stain mainly after 24 hours. On the basis of RA index value, all 5 isolates are greater than one. All five isolates were Gram-negative non spore forming short bacilli bacteria; four isolates were non-lactose fermenters, and one isolate was only lactose

fermenter on MacConkey agar. Biochemical tests results were variable and we depended mainly on molecular identification.

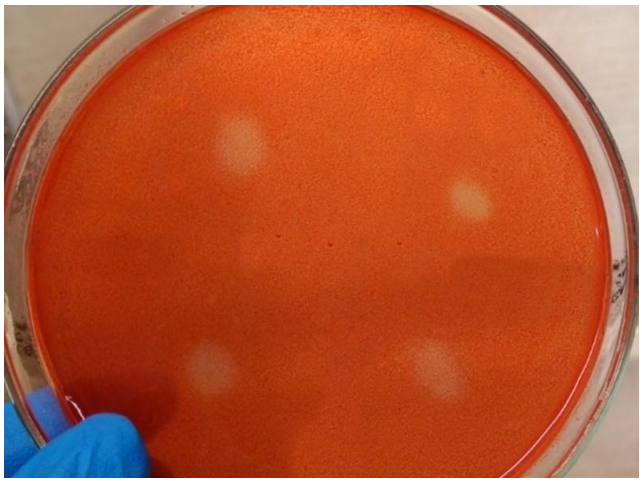

Fig. 2. Clearance zones of colloidal chitin formed by chitinases production by *E. coli, Alcaligenes faecalis, Alcaligenes ammoinoxydans* and *Alcaligenes aquatilis*

2. Chitinase enzyme assays

The five isolates selected for secondary screening test were assayed for enzyme activity using the DNS method (Table 2).

Table 2. Spectrophotometric determination of Chitinolytic activity of *Alcaligenes* sp. & *E. coli* isolates in media supplemented with colloidal chitin enzyme activity (unit / ml)

Isolates	***Alcaligenes faecalis***	***Alcaligenes faecalis***	***Alcaligenes ammonioxydans***	***Alcaligenes aquatilis***	***E coli***
Code	**Chi1**	**Chi2**	**Chi3**	**Chi4**	**Chi5**
Chitinase Activity	**4**	**3.7**	**4.2**	**4.5**	**4**

3. Molecular identification of chitinolytic isolates and phylogenetic tree construction:

According to PCR with 16s rRNA universal primer, the amplicon size was 370bp (Fig. 3).

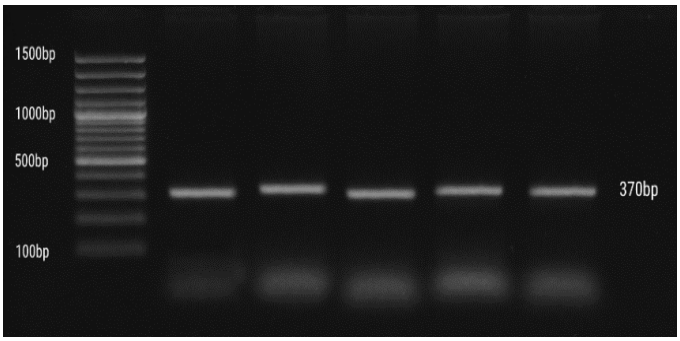

Fig. (3). Electrophoretic DNA profile of 16r RNA PCR products (370bp) of tested chitinolytic bacterial isolates, Lane 1 DNA marker (100bp), Ladder Plus. Lane 2-6 testes strains

Sequence analysis of the chitinolytic bacteria strains partial 16S rRNA, uploaded to the National Center for Biotechnology Information (NCBI) with accession numbers, as illustrated in Table (1) provided additional support for their identification. The selected strains for tree construction are listed in Table (3).

Table 3. Tested and selected strains from GenBank Accession number, source and country for phylogenetic tree construction

Bacterium	Accession no.	Source	Country
Alcaligenes faecalis	OM472564	Shrimp shells	Egypt
Alcaligenes faecalis	OM472565	Cairo, Giza markets	This Study
Alcaligenes	OM472566		
ammonioxydans	OM472567		
Alcaligenes aquatilis	OM472568		
E coli			
Alcaligenes faecalis	HM145896.1	Sea food industrial effluent	India
***Stenotrophomonas* sp.**	KR919607.2	Rhizosphere soil	India
Serratia marcescens	FJ530951.1	Fishing field	China
Acinetobacter	GQ503319.1	Marine wastes	Oman
Alcaligenes faecalis	AY959943.2	Swine wastewater sludge	Taiwan
Alcaligenes equalities	MT572474.1	Estuarine sediments of the Elizabeth River	USA
Alcaligenes ammonioxydans	CP049362.1	Wastewater from piggery husbandry	China
Alcaligenes faecalis	MW558266		Indonesia
Alcaligenes aquatilis	OM232488	sediments of potato starch wastewater	China
Alcaligenes latus	D88007	Type strain of *Azohydromonas* lat	Japan
Alcaligenes defragrans	AJ005447	Type strain *of Castellaniella defragrans*	Germany
Streptomyces sp. MT7	JQ322818	Genomic DNA	India
***Bacillus* sp.**	JN635497	Genomic DNA	China
Pseudomonas aeruginosa	M34133		USA
Aeromonas veronii	NR_044845	Type strain of *Aeromonas*	UK

		ichthiosmia	
Aeromonas hydrophila	NR_042518	Type strain of *Aeromonas hydrophila*	Switzerland
Stenotrophomonas maltophilia	EU239142		Korea
Serratia marcescens strain	ON005000	chronic ulcer tissues	China
Escherichia coli	CP048609		China
Escherichia coli	CP069624		China

Phylogenetic tree was constructed to observe the relationship between the five chitinolytic bacterial isolates in the present study and the referenced chitinolytic strains in the GenBank database, in which the evolution of the bacterial isolates was illustrated, as shown in Fig. (4).

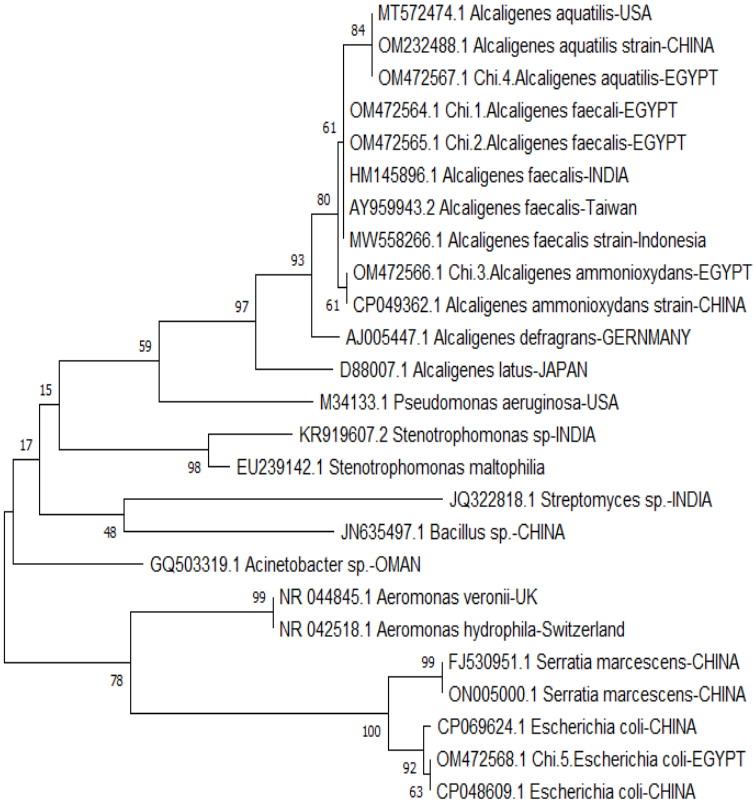

Fig. 4. Phylogenetic analysis of the present chitinolytic isolates sequences. The unrooted maximum likelihood tree shows the relationship between the present study isolated and other chitinolytic strains referenced in the GenBank database, in which related taxa and closed branches are clustered together, illustrating the evolution.

Additionally, the identity matrix of partial sequences of 16S RNA of *Alcaligenes* species and *E. coli* with other bacterial species illustrated similarities and variations percentage, as listed in Table (4).

Table 4. Distance matrix of partial sequences of 16S RNA of *Alcaligenes* species and *E. coli* with that of other species obtained by Megalign (Lasergene DNASTAR Inc., Madison, WI) revealing the Percentage of similarity and divergence

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
1		100.0	100.0	100.0	100.0	98.8	98.8	98.8	99.4	99.4	93.6	97.7	77.8	81.3	83.0	88.3	84.2	84.8	81.3	81.3	84.8	77.2	77.2	77.2	77.2	77.2	1	OM472564.1
2	0.0		100.0	100.0	100.0	98.8	98.8	98.8	99.4	99.4	93.6	97.7	77.8	81.3	83.0	88.3	84.2	84.8	81.3	81.3	84.8	77.2	77.2	77.2	77.2	77.2	2	OM472565.1
3	0.0	0.0		100.0	100.0	98.8	98.8	98.8	99.4	99.4	93.6	97.7	77.8	81.3	83.0	88.3	84.2	84.8	81.3	81.3	84.8	77.2	77.2	77.2	77.2	77.2	3	MW558266.1
4	0.0	0.0	0.0		100.0	98.8	98.8	98.8	99.4	99.4	93.6	97.7	77.8	81.3	83.0	88.3	84.2	84.8	81.3	81.3	84.8	77.2	77.2	77.2	77.2	77.2	4	HM145896.1
5	0.0	0.0	0.0	0.0		98.8	98.8	98.8	99.4	99.4	93.6	97.7	77.8	81.3	83.0	88.3	84.2	84.8	81.3	81.3	84.8	77.2	77.2	77.2	77.2	77.2	5	AY959943.2
6	1.2	1.2	1.2	1.2	1.2		100.0	100.0	98.2	98.2	92.4	96.5	76.6	80.1	81.9	87.1	83.0	83.6	80.1	80.1	83.6	76.0	76.0	76.0	76.0	76.0	6	OM472567.1
7	1.2	1.2	1.2	1.2	1.2	0.0		100.0	98.2	98.2	92.4	96.5	76.6	80.1	81.9	87.1	83.0	83.6	80.1	80.1	83.6	76.0	76.0	76.0	76.0	76.0	7	MT572474.1
8	1.2	1.2	1.2	1.2	1.2	0.0	0.0		98.2	98.2	92.4	96.5	76.6	80.1	81.9	87.1	83.0	83.6	80.1	80.1	83.6	76.0	76.0	76.0	76.0	76.0	8	OM232488.1
9	0.6	0.6	0.6	0.6	0.6	1.8	1.8	1.8		100.0	94.2	97.1	78.4	81.9	83.6	87.7	84.8	85.4	80.7	80.7	85.4	77.8	77.8	77.8	77.8	77.8	9	OM472566.1
10	0.6	0.6	0.6	0.6	0.6	1.8	1.8	1.8	0.0		94.2	97.1	78.4	81.9	83.6	87.7	84.8	85.4	80.7	80.7	85.4	77.8	77.8	77.8	77.8	77.8	10	CP049362.1
11	6.7	6.7	6.7	6.7	6.7	8.0	8.0	8.0	6.1	6.1		93.6	80.7	83.6	83.6	87.7	83.0	83.6	83.6	83.6	85.4	78.4	78.4	78.4	78.4	78.4	11	D88007.1
12	2.4	2.4	2.4	2.4	2.4	3.6	3.6	3.6	3.0	3.0	6.7		78.9	81.9	83.0	88.9	83.0	84.8	80.1	80.1	84.8	75.4	75.4	75.4	75.4	75.4	12	AJ005447.1
13	25.8	25.8	25.8	25.8	25.8	27.5	27.5	27.5	25.0	25.0	21.8	24.3		82.5	80.7	79.5	81.3	83.6	79.5	79.5	82.5	79.5	79.5	78.4	78.4	78.9	13	JQ322818.1
14	21.6	21.6	21.6	21.6	21.6	23.2	23.2	23.2	20.8	20.8	18.5	20.9	19.5		80.1	84.2	80.1	81.9	78.4	78.4	83.6	75.4	75.4	77.8	77.8	77.8	14	JN635497.1
15	18.7	18.7	18.7	18.7	18.7	20.2	20.2	20.2	17.9	17.9	17.9	18.6	22.6	22.6		80.7	84.2	84.8	84.8	84.8	95.3	78.9	78.9	77.8	77.8	77.8	15	KR919607.2
16	12.7	12.7	12.7	12.7	12.7	14.1	14.1	14.1	13.4	13.4	13.5	12.1	23.9	18.0	21.8		87.1	89.5	84.8	84.8	83.6	80.1	80.1	82.5	82.5	82.5	16	M34133.1
17	17.8	17.8	17.8	17.8	17.8	19.3	19.3	19.3	17.1	17.1	19.4	19.3	21.2	23.1	17.2	14.2		97.1	87.1	87.1	86.5	84.8	84.8	87.1	87.1	86.5	17	Acinetobacter
18	17.0	17.0	17.0	17.0	17.0	18.5	18.5	18.5	16.3	16.3	18.6	17.0	18.0	20.8	16.4	11.4	3.0		85.4	85.4	86.0	81.9	81.9	84.2	84.2	83.6	18	GQ503319.1
19	21.6	21.6	21.6	21.6	21.6	23.2	23.2	23.2	22.4	22.4	18.5	23.1	23.5	25.6	16.6	17.1	14.2	16.4		100.0	84.8	84.2	84.2	85.4	85.4	85.4	19	NR_044845.1
20	21.6	21.6	21.6	21.6	21.6	23.2	23.2	23.2	22.4	22.4	18.5	23.1	23.5	25.6	16.6	17.1	14.2	16.4	0.0		84.8	84.2	84.2	85.4	85.4	85.4	20	NR_042518.1
21	17.1	17.1	17.1	17.1	17.1	18.6	18.6	18.6	16.3	16.3	16.3	17.0	19.5	18.7	4.2	18.7	14.9	15.6	17.3	17.3		81.9	81.9	81.9	81.9	81.9	21	EU239142.1
22	27.4	27.4	27.4	27.4	27.4	29.2	29.2	29.2	26.6	26.6	25.7	30.0	23.4	30.1	24.5	23.4	17.0	20.8	18.2	18.2	21.1		100.0	96.5	96.5	95.9	22	FJ530951.1
23	27.4	27.4	27.4	27.4	27.4	29.2	29.2	29.2	26.6	26.6	25.7	30.0	23.4	30.1	24.5	23.4	17.0	20.8	18.2	18.2	21.1	0.0		96.5	96.5	95.9	23	ON005000.1
24	27.4	27.4	27.4	27.4	27.4	29.1	29.1	29.1	26.5	26.5	25.7	29.9	25.2	26.6	26.2	20.2	14.1	17.8	16.7	16.7	21.2	3.6	3.6		100.0	99.4	24	OM472568.1
25	27.4	27.4	27.4	27.4	27.4	29.1	29.1	29.1	26.5	26.5	25.7	29.9	25.2	26.6	26.2	20.2	14.1	17.8	16.7	16.7	21.2	3.6	3.6	0.0		99.4	25	CP048609.1
26	27.4	27.4	27.4	27.4	27.4	29.1	29.1	29.1	26.5	26.5	25.7	29.9	24.3	26.6	26.2	20.2	14.8	18.5	16.7	16.7	21.2	4.2	4.2	0.6	0.6		26	CP069624.1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		

DISCUSSION

In the present work, chitinolytic bacterial strains were isolated from shrimp shells from the Red Sea, Suez, Egypt. Out of 17 pure single colonies were grown on nutrient agar with 1% colloidal chitin medium, only five isolates produced a clear zone when stained with Congo red after 24 hours (Fig. 2). While, **Krithika and Chellaram (2016)** reported that clear zone produced by *Chitiniphilus shianonensis*, *Acinetobacter* sp., *Bacillus badius*, *Acinetobacter venetianus*, and *Brevibacillus borstelensis* after three days. It has been reported that the production of the chitinolytic enzyme by bacteria is controlled by an inducer and repressor system, with chitin acting as an inducer and glucose acting as a repressor **(Liu *et al.*, 2015)**. Thus, the colloidal chitin was used as a substrate in this study to induce the production of chitinase from bacterial isolates. Since chitinase produced by the isolates degrades colloidal chitin present in the agar medium, it leads to the hydrolysis of the polysaccharide (chitin), causing the loss of stain binding to the agar and the formation of clear zones **(Verma & Garg, 2019)**. Choosing shrimp shells as they contain chitin in their structure to facilitate the isolation of chitinolytic bacteria, which adapt to their environment through the production of chitinase. The rate of chitin waste degradation is influenced by chitinase activity, environmental factors, indigenous

microorganisms' metabolism, carbon utilization as a source of energy, protein synthesis, and releasing cell metabolism products **(Bailly & Ollis, 1986)**.

According to PCR of the 16S rRNA universal primer, the amplicon size was 370bp (Fig. 3). Four chitinolytic bacterial isolates were identified as *Alcaligenes* sp. based on 16S rRNA gene sequences. Chi1 and Chi2 were referred to *Alcaligenes faecalis*, while Chi3 and Chi4 were *Alcaligenes ammonioxydans* and *Alcaligenes aquatilis*, respectively, and Chi5 was *E. coli* (Table 2).

Alcaligenes spp. are common in soil and aquatic environments but are also found as normal inhabitants of vertebrate intestinal tracts. *Alcaligenes* spp. are a Gram-negative and non-endospore-forming bacilli that are motile, oxidase, catalase and citrate positive, while some of them are negative but nitrate reduction negative (**Klem, 1999**). This result matches the biochemical tests' findings in this study. *Alcaligenes ammonioxydans* was the first recorded in Egypt to produce chitinase enzyme, and it showed potential enzymatic activity, but *Alcaligenes faecalis* was known to be a chitinolytic bacteria (**Annamalai *et al.*, 2011**). Moreover, the bacterial isolates are variable in their chitinolytic activity based on enzymatic assay. Two isolates, *Alcaligenes ammoinoxydans* and *Alcaligenes aquatilis* (Chi3 and Chi4), revealed the highest chitinase production of 4.5 and 4.2U/ ml, respectively, after 24hr (Table 1). Similar observation was reported by **Karunya *et al.* (2011)** for *Bacillus subtilis* and *Serratia marcescens*. **Wang and Chang (1997)** reported maximum enzyme production by *Pseudomonas* sp. after 72hr, while, **Wang and Hwang (2001)** found maximum enzyme production by *Bacillus cereus, Bacillus alvei*, and *B. sphaericus* after 48hr of incubation.

All *Alcaligenes* isolates were clustered in the first clade, with a high homology score originating from the major ancestor *Alcaligenes latus* from the USA (D88007) for all *Alcaligenes* species with 97% frequency, as shown in Fig. (4). *Alcaligenes ammonioxydans* chi3, the potent novel chitinolytic strain in the present work, was present in the first clade, acting as a major ancestor for other *Alcaligenes* species with a sub-frequency of 80%. Other *Alcaligenes aquatilis* Chi4 revealed a close phylogenetic relationship with *Alcaligenes aquatilis* (USA and China), whereas *Alcaligenes faecalis* revealed a close phylogenetic relationship with other *Alcaligenes faecalis* (India, Taiwan, and Indonesia). While, isolate Chi5 *Escherichia coli* exhibited a close phylogenetic relationship with *Aeromonas hydrophila* (NR 042518) and *Aeromonas veronii* (NR 044845). It is worth noting that these bacteria, being Gram-negative rods, include certain species of Aeromonas that produce the chitinase enzyme. This enzymatic activity is particularly prevalent among marine bacteria, serving as an adaptation and defense mechanism **(Kuddus & Ahmad, 2013)**.

Alcaligenes is phylogenetically located among the beta subgroup of proteobacteria, which also contains the genus *Pseudomonas*. Therefore, it is not surprising that species in this genus are quite metabolically diverse, like the *Pseudomonads* **(Klem, 1999)**. The phylogenetic tree revealed that *Pseudomonas*

aeruginosa from the USA (M34133) is closely related to most potential chitinolytic strains of *Alcaligenes* species (Fig. 4). It was reported that *Stenotrophomonas maltofilia* is the most potent producer of chitinase, followed by *Pseudomonas* and *Alcaligenes* spp. **(Shaikh *et al.*, 2018)**. Genus *Acinetobacter* and *Bacillus* were dominant bacteria in industrial wastewater, along with other genera such as *Pseudomonas, Spirillum, Hyphomicrobium, Agrobacterium, Propionibacterium, Rhizobium, Corynebacterium, Cytophaga, Thiobacillus,* and *Alcaligenes* **(Bitton, 2005)**.

CONCLUSION

A significant portion of the chitinolytic bacteria recovered from shrimp shells in the present work belongs to the *Alcaligenes* species. Phylogenetic estimations using sequence data from partial 16S rRNA genes revealed highly genetic similarity between isolated chitinolytic bacteria and other chitinase producing bacteria from different countries. To the best of our knowledge, the isolation of *Alcaligenes ammonioxydans* is the first record of a chitinolytic bacterial strain in Egypt and within the *Alcaligenes* species family. Compared to other bacteria, the current bacterial isolates could be a valuable source of the chitinase enzyme for various applications, given their ability to produce a significant amount of the enzyme within a short period of time (24 hours). The development of low-cost chitinolytic enzymes is critical for the utilization of shrimp shell waste since it does not only offer environmental biocontrol but also lower the cost of bacterial chitinases. In addition, the growing interest in chitinases as anticancer and immunomodulatory drugs in cancer therapy is expected to drive up the market demand for chitinases

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Arabic summary

حظيت انزيمات الكيتينيز بالاهتمام في العقود الأخيرة، بسبب تطبيقاتها الواسعة في الطب، والتكنولوجيا الحيوية، والزراعة، وإدارة النفايات، والتطبيقات الصناعية مثل محسنات جودة الأغذية والمبيدات الحيوية. لاحظت الدراسات الحديثة أن الكيتينيز يحلل بشكل انتقائي الخلايا السرطانية في المختبر وفي الجسم الحي. كان الهدف من هذا البحث هو عزل وتحديد الأنواع البكتيرية من قشور الجمبري التي يحتمل أن تحلل الكيتين وتحليل قدرتها على إنتاج الكيتينيز. يتكون البحث من عزل البكتيريا المحللة للكيتين من قشور الجمبري، بناءً على تكسير الكيتين المعلق الموجود في الوسط الصلب الملون وظهور منطقة خالية من اللون حول المستعمرة البكتيرية وكذلك تقدير نشاط الإنزيم باستخدام طريقة DNS (حمض ثنائي نيتروساليسيليك). أظهرت خمس معزلات بكتيرية من البكتريا المحللة للكيتين نشاط عالي لإنتاج الكيتينيز. تم استخدام التعريف الجزيئي لهذه المعزلات البكتيرية بناءً على تفاعل البلمرة المتسلسل لتحليل البادئات العامة S16 rRNA و كذلك تحليل هذه التسلسلات الناتجة. أسفرت النتائج عن تصنيف أربع معزولات من *Alcaigenes* spp. و معزولة واحدة هي*E. coli* .تم إكتشاف معزولة بكتيرية جديدة تنتج الكيتينيز لأول مرة في مصر و على مستوى الدراسات السابقة و تعتبر من ضمن المعزولات الأعلى إنتاجية في هذه الدراسة و هي *Alcaligenes ammonioxydans*