Original Article

Diversity of Chitinolytic Bacteria from Shrimp Farms and Their Antifungal Activity

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Abstract

Background: The chitinase enzyme has received increased attention due to its wide range of biotechnological applications, especially in agriculture for the biocontrol of fungi. In this study, chitinolytic bacteria from shrimp farms (East Java, Indonesia) were isolated and evaluated for their antifungal activity. **Material and methods:** Screening of chitinolytic bacteria in the media containing colloidal chitin was determined colorimetric activity using a UV-Vis spectrophotometer. Isolates with the highest activity are determined species based on physiology, morphology, and the 16S-rRNA gene. Chitinase enzymes from selected isolates are tested as antifungals of chili plants by measuring their taste ability through the formed clear zones. **Result:** There were 24, 40, 54, and 78 isolates of bacteria from shrimp farms in Tuban (TB), Situbondo (ST), Lamongan (LA), and Sidoarjo (SD) in Indonesia respectively. Based on enzyme production, four isolates namely LA21 (0.73 U / mL), SD54 (0.663 U / mL), TB11 (1.160 U / mL), and ST7 (0.405 U / mL) were selected for further analyzed and evaluated. Phylogenetic analysis revealed that the LA21 isolates have homologous with *Bacillus cereus*, morphological and biochemical tests showed that isolate TB11 is thought to be a group of *Vibrio alginolyticus* while isolating ST 13 belongs to *Bacillus licheniformis.* The LA21 isolate displayed antifungal activity against *Fusarium oxysporum* f.sp. capsici and *Colletotrichum capsici* during the trial period. **Conclusion:** This study demonstrated that chitinolytic bacterial isolates from various shrimp farms have great potential for chitinase production and antifungal applications.

Keywords: antifungal, chitinolytic, chitinase activity, shrimp farm

Introduction

Chitinolytic bacteria are widely distributed in various types of environments, including soil, water, and living organs. In aquatic environments, chitinolytic bacteria can be found in oceans[1] brackish water lakes[2], waterways such as the Suez Canal[3], and shrimp-farm wastewater.[4] Based on their location, chitinolytic bacteria have different characteristics and properties.[5] It has been reported that some bacteria from the aquatic environment have been identified as having chitinolytic activity such as those from species *Aeromonas, Enterobacter, Chromobacterium, Arthrobacter, Flavobacterium, Serratia, Bacillus, Erwinia, and Vibrio*. Bacterial isolates from whales have also revealed some species, including *Eubacterium, Streptococcus,* and *Clostridium*. Moreover, *Bacillus licheniformis* has been found in the liquid food industry.

Other species such as *Serratia* and *Streptomyces* isolated from crustacean residues, and the *Acinetobacter johnsonii* and *Bacillus amyloliquefaciens* species isolated from shrimp residues have chitinolytic activity.[6] Chitinolytic activity has been developed recently to use it as a biocontrol agent as a substitute for chemical pesticides that harm the environment.[7] Chitinolytic bacteria can be used as a biocontrol agent against pathogenic fungi[8,9] and increase plant fertility.[10] Chitinase enzymes produced by chitinolytic bacteria have various inhibitory capabilities. The amount of chitin in the fungus cell walls varies significantly, so that

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chitinase's susceptibility also varies. The difference in inhibitory activity was affected by the amount of chitin in the fungal cell and chitinase bonds in the chitin.[11] Isolation and identification of chitinolytic bacteria can be done in several ways, including culturing bacteria on media containing colloidal chitin.[1] Chitinolytic bacteria were isolated from crab shell waste by growing the bacteria on colloidal chitin-containing media. Identification of chitinolytic bacteria can be determined by observing the formation of clear zones in the media containing chitin or by determining its activity in a colorimetric manner, assay spectrophotometer, formation of fluorescence, or zymogram.[12-15] Chitinolytic bacteria can produce chitinase enzymes that are present as intracellular or extracellular enzymes and play a role in the degradation of chitin to N-acetyl glucosamine (GlcNAc). Chitinase enzymes (EC 3.2.1.14) are a group of enzymes that can degrade chitin. Chitin is a long straight chain polymer of GlcNAc. Chitinase works by hydrolyzing β-1,4-glycosidic bonds in chitin.[16]

Chitin derivatives are beneficial in various fields such as in medical, agricultural, and industrial. It is used in food quality enhancers and act as antibacterial, antifungal, hypocholesterolemic, and antihypertensive agents.[17] Chitin and its derivatives have been widely reported for their application in the medical field. In particular, they are used as wound healers in the form of sprays, gels, and gauze. They are hydrophilic and has gel-forming properties and have high affinity for proteins.[18]

Materials and Methods

Screening of Chitinolytic Bacteria From Shrimp Farms

Water samples were obtained from shrimp farms from four locations: Lamongan (LA), Sidoarjo (SD), Situbondo (ST), and Tuban (TB) in East Java, Indonesia. Samples were collected from several collecting points and were homogenized. One hundred µL of water sample was placed on solid media containing colloidal chitin.[19] Solid media consists of 1% (w/v) NaCl, 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1% (w/v) Bacto agar, and 1% colloidal chitin.[20] The culture was then incubated at 30°C for 24 hours. A clear zone around the culture indicated the presence of chitinolytic bacteria.

Preparation of Colloidal Chitin

Chitin 40 gm from shrimp shells was dissolved in 400 mL 37% HCl, then stirred for 30-50 minutes. The colloidal suspension was slowly added in cold water (5–10°C) until a volume of 2.0 liters and then filtered using filter paper no 40. The residue was washed with distilled water to a pH of 6–7.[21]

Determination of Chitinase Activity

Chitinase activity was determined using colorimetry based on methods developed by Monreal et al.[22] First, colloidal chitin substrates were dissolved in a 200 mM potassium phosphate buffer containing calcium chloride. Two test tubes were prepared, and 2 mL of chitin substrate was added to each test tube. Chitinase enzyme solution (0.5 mL) was added to the first tube, and 0.5 mL of demineralized water was added to the second test tube (blank). Test tubes were incubated in a shaker incubator at 150 rpm for 2 hours at 30°C. After incubation, test tubes were placed in boiling water at 100°C for 5 minutes and then were separated by centrifugation at a speed of 4000 rpm for 10 minutes. One mL of supernatant was added 2 mL of demineralized water and 1.5 mL of complexing reagent. The complexing reagent comprised of 96 mM of 3,5- dinitro salicylic acid solution, and sodium-potassium tartrate solution with a concentration of 5.3 M. The mixture was then heated in boiling water for 5 minutes and cooled. The absorbance was measured at a wavelength of 540 nm with a UV-Vis spectrophotometer (Shimadzu 1800). The chitinase activity was expressed as the amount of GlcNAc produced from the chitin per hour.[22-24]

Biochemical and Molecular Identification of Bacteria

The chitinolytic bacteria was identified biochemically using Microbact. The molecular method was performed by 16sRNA analysis. The DNA of the 16sRNA was amplified by polymerase chain reaction (PCR) then its nucleotide sequence was compared with similar sequences in the BLAS program database. Amplification of the 16S-rRNA gene was performed using a universal reverse primary : Uni B1 : 5'-GGTTACTTGTTACGACTT-3') and primary (Eubacterial forward primer : BactF1 : 5'-AGAGTTTGATCTGGCTCAG-3'). The genetic testing was based on 16S-rRNA through DNA isolation, amplification using PCR (ABI Prism 9700), PCR electrophoresis results, and DNA sequence determination with capillary electrophoresis genetic analysis 3130 (Applied Biosystem).[16,25]

Enzyme Chitinase as Antifungal

The culture of fungi was made from Colletotrichum capsica and Fusarium oxysporum f.sp. capsica whose optical density (OD) was measured at 625 nm. Each culture was inoculated on PDA (Potato dextrose agar) media using a paper dish. Each culture was planted on PDA solid media using a spreader and spread on the agar surface. Prepare a paper dish (6 mm in diameter) and then place it on solid media that has been planted with mushroom culture aseptically. In each paper dish, 20 µL of chitinase enzyme was added with known activity. As a control, a phosphate buffer solution of pH 6.8 was used and then incubated at room temperature for 3-4 days and the inhibition of the chitinase enzyme was observed for each fungus.[26]

Results

Isolation and Identification of Chitinolytic Bacteria From Shrimp Farms

In this study, chitinolytic bacterial isolates were successfully isolated from a shrimp farm in four different areas and there were 24 isolates from Tuban (TB), 40 isolates from Situbondo (ST), 54 isolates from Lamongan (LA), and 78 isolates from Sidoarjo (SD) and many separate colonies were found in the selected media [Figure 1a). These isolates have identified their chitinolytic abilities through chitinase activity and the formation of clear zones The chitinolytic activities of these isolates were identified by the formation of clear zones [Figure 1b]. Some isolates showed clear zones on solid media containing colloidal chitin.

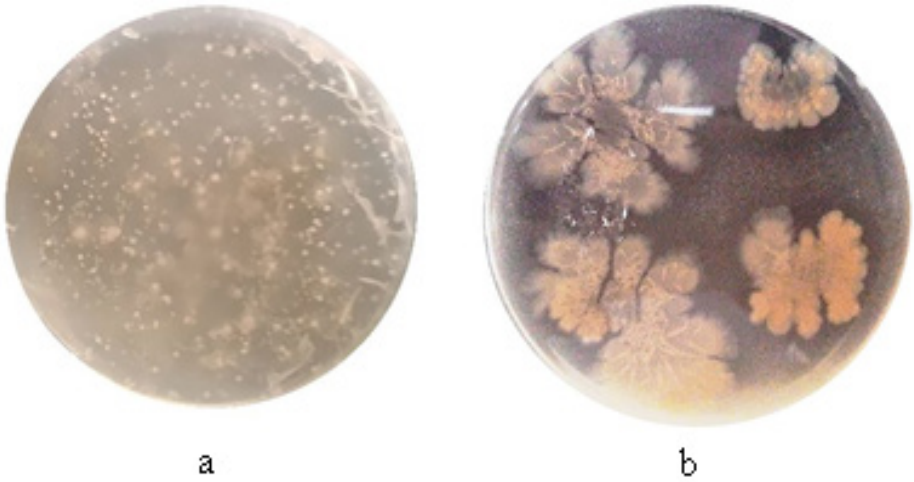

Figure 1: Mixed cultures from shrimp farms (a), and the formation of a clear zone in media containing colloidal chitin (b)

From each location, isolates with high chitinase activity have been determined by comparing the results of the Monreal and Reese methods. [Figure 2].

The chitinase activity obtained at each place shows varying activity values. In each place there are isolates that have the highest activity indicated by isolates LA21, isolates SD54, isolates TB11 and ST7 with activity 0.730; 0.663 ; 1.160 and 0.405 U/mL.

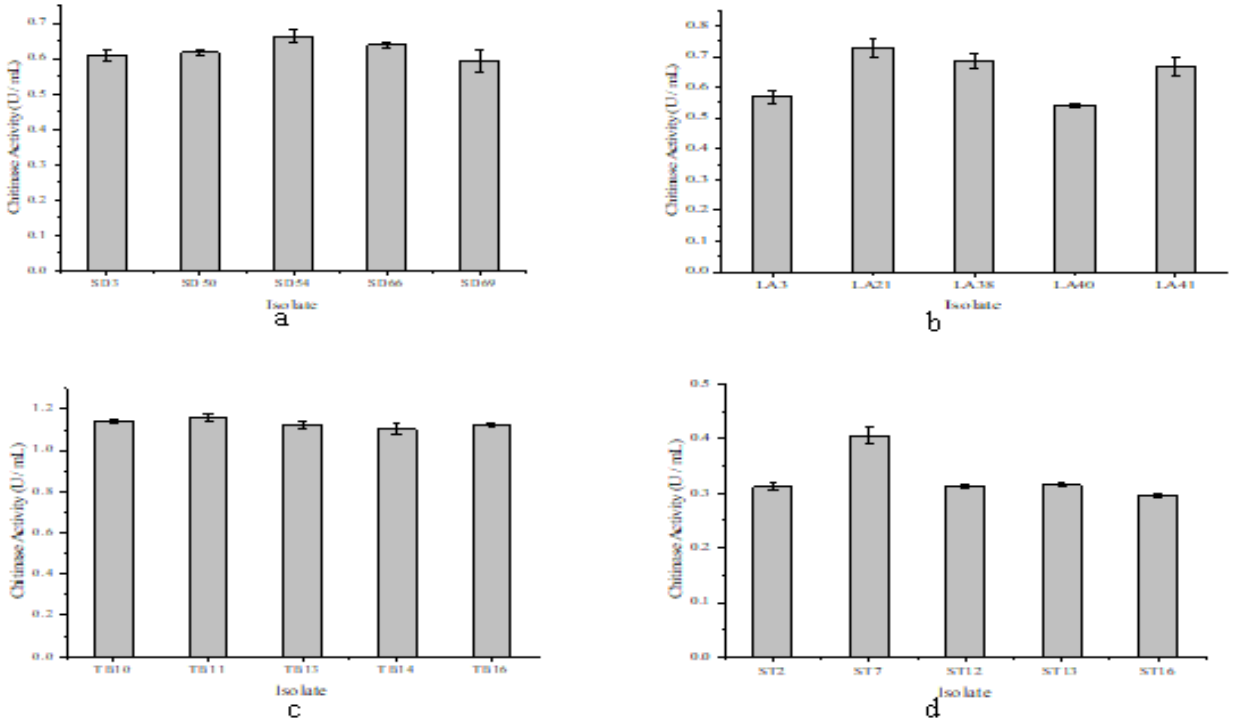

Figure 2: The chitinase activity of SD (a), LA (b), TB (c) and ST (d) isolates obtained from different shrimp farm locations.

Bacterial species were identified based on their physiological and morphological characteristics and the 16S-rRNA gene. Based on the morphological and biochemical characteristics, TB11 isolate was identified as Vibrio alginolyticus, while ST7 isolate was identified as Bacillus licheniformis. LA21 isolate

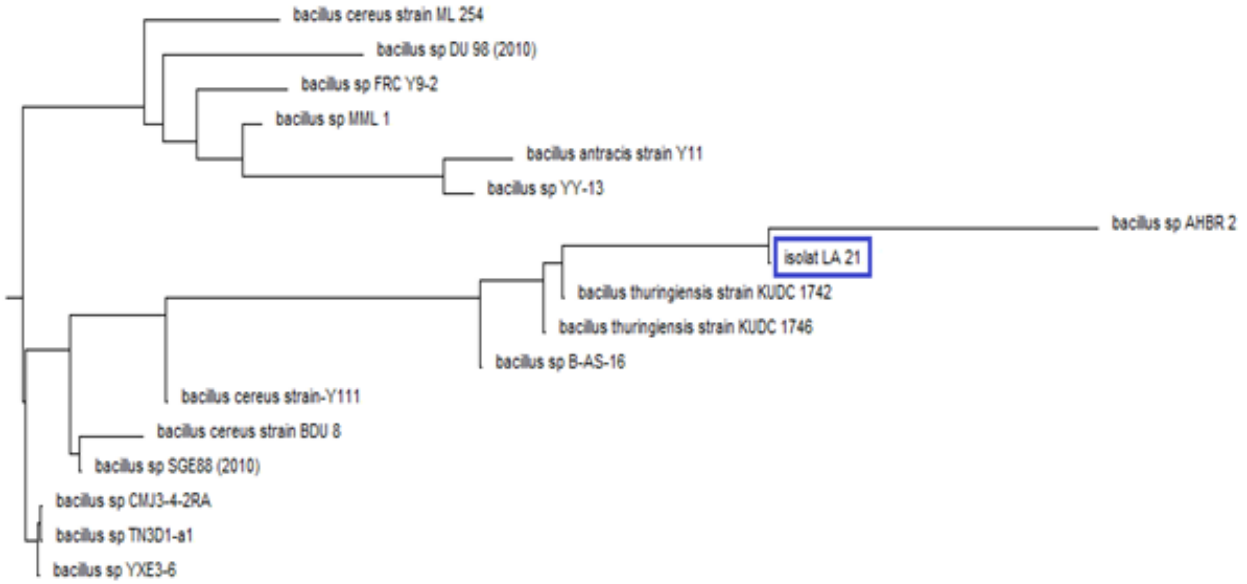

Figure 3: Phylogenetic tree analysis of LA21 isolate based on 16S-rRNA analysiswas identified as Bacillus cereus group based on the the16S-rRNA analysis [Figure 3].

Anti-Fungal Potential in Chili Plants

The ability of Bacillus sp 21 as an antifungal against *Fusarium*. *oxysporum f. sp.* capsica and *Colletotrichum capsici* showed significant results [Figure 4 and Table 1]. *Fusarium oxysporum f. sp. capsici* and *Colletotrichum capsici* are pathogenic fungi in red chili (*Capsicum annuum L.*) and cause wilt and anthracnose, respectively.

Table 1 : Chitinase inhibition of Bacillus sp 21

Fungi	Inhibition Diameter (cm) in day -				
	1	3	5	7	11
Colletotrichum capsici	0.70	1.83	2.14	2.16	2.16
Fusarium oxysporum f.sp. capsici	0.80	0.84	1.64	1.89	1.89

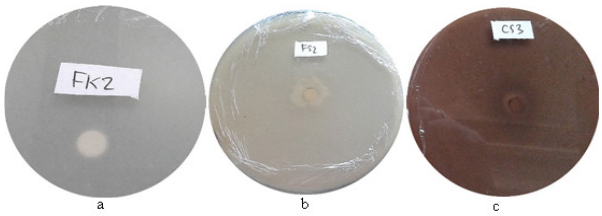

Figure 4: Results of chitinase inhibition in control (a), *F. oxysporum f.sp.* capsica (b), and *C. capsica* (c)

Discussion

Shrimp farms are excellent substrates for the growth of chitinolytic bacteria. Chitin is a very abundant natural biopolymer as a polysaccharide with β-1,4-N-acetylglucosamine bonds. It is found in many fungal cell walls and crustacean's exoskeleton such as shrimp.[27] The density chitinolytic bacteria collected from shrimp farms in this study was very high, so it specific media for culturing these bacteria. The use of a specific growth media is convenient for growing certain microorganism species and preventing other undesirable strains of microorganisms. An enrichment growth media can be used to obtain the desired species as this is a selective and fast way to grow bacteria with high growth yields that can be adapted for cultivation.[28] Some species have been reported to possess chitinolytic activity, but it is still unknown what group of microorganisms dominates in the decomposition of chitin compounds in aquatic environments. Although some researchers have mentioned that thse marine ecosystems as the main source of chitinase producers. Some studies mention that only 4% represent chitinolytic bacteria.[6]

Chitinolytic bacteria can be identified in several methods, such as the formation of clear zones around isolated cultures on growth media containing colloidal chitin.[29-32] The clear zones occurred due to the degradation process of the chitin into monomers. According to Agrawal *et al.*[33] and Vaidya et al.[34] visualizing the clear zone is difficult and the possibility of the emerging clear zone is very small.[33,34] The clear zone formation generally takes quite a long time that is 3 – 7 days of incubation. Another method to determine chitinolytic colonies is using a bromocresol purple (BCP) indicator, namely

by the formation of the purple zones around the colony. The purple complex was formed by the two molecules of GlcNAc and BCP through hydroxyl, carboxyl, and amino groups. The color binding mechanism occurs through the adsorption process and is strongly affected by pH.[35]

The chitinase activity showed varying values when compared to activities obtained from chitinolytic bacteria isolated from other shrimp pond areas.[2,36] Saules et al.[37] obtained 52 chitinolytic isolates with the highest activity of 51.2 U/mL with an incubation time of 72 hours.[37] Chitinase activity could be influenced by several factors, such as the lack of suitable nutritional conditions during chitinase induction and the cell walls on the surface, which are blocked by proteins bound to chitin molecules thus inhibiting enzymes – substrates interaction.[38] Chitin hydrolysis by chitinase can occur by involving two types of enzymes and requires other enzymes to release the GlcNAc [Figure 5].[39] Endochitinase cannot cut the inner chain of chitin into shorter fragments, but exochitinase can hydrolyze chitin at the end terminal by producing chitobiose.[40]

LA21 isolate was considered as Bacillus cereus group, and based on the the16S-rRNA analysis [Figure 3], it has 97% similarity to Bacillus specie AHBR.[41] The diversity of chitinolytic bacteria from shrimp ponds was also shown in the study, as Ray *et al.*[2] obtained a new actinobacterial strain called Streptomyces chilikensis RC1830, which was isolated from the sediments from Chilika lake in Indian.[2] Twenty-seven isolates from the Aspergillus flavus group (AUMC 13576) obtained from marine waste El-Sokhna, Egypt, have - shown to have 620.54 U/I chitinase activity (3). The chitinolytic bacteria Paenibacillus specie D10-2 were isolated from shrimp farms in Yogyakarta, Indonesia (4).Ali et al.[32] obtained 20 bacteria with two isolates namely Streptomyces laurentii SN5 and Cellulosimicrobium funkei SN20[32], which are new species which demonstrated chitinolytic activity. The diversity of chitinolytic bacterial groups obtained from various locations is influenced by chitin in] the associated environment and the dominant bacterial.[27

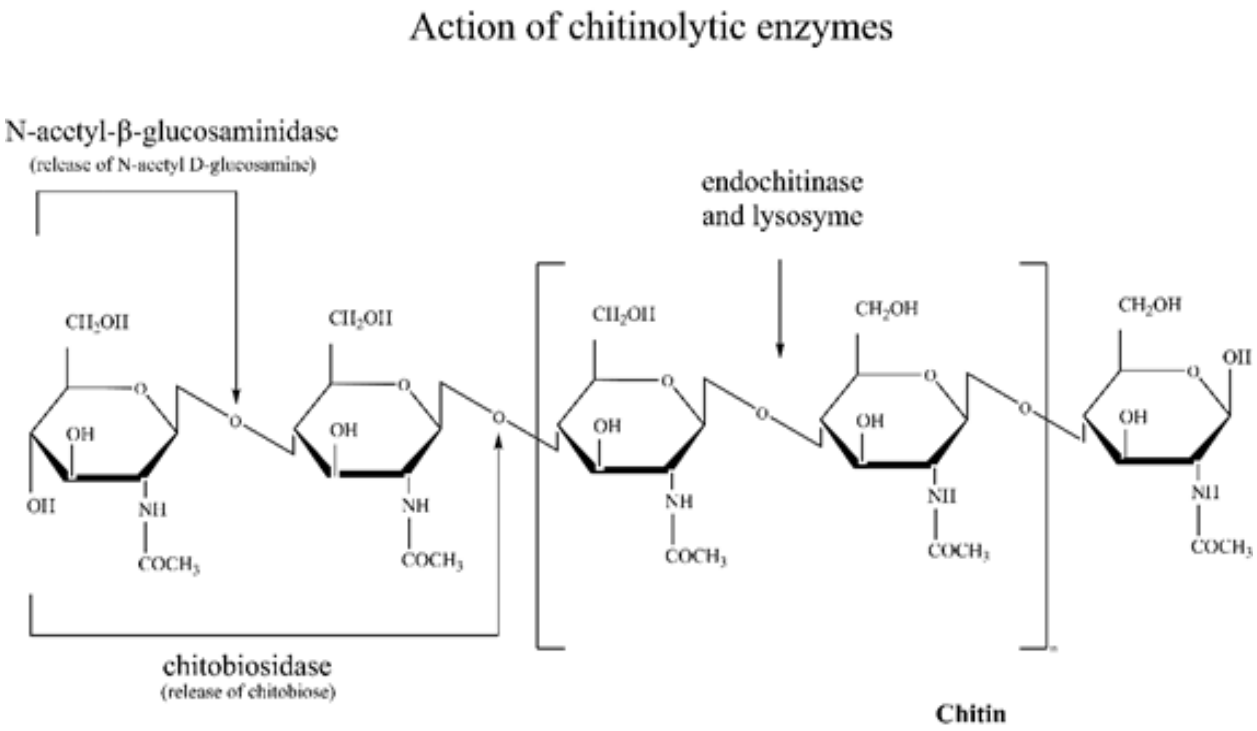

Figure 5: The action of the chitinolytic enzyme on chitin, which randomly cleaves glycosidic bonds in the chitin molecule[39]

Chitinolytic microorganisms play an important role as biocontrol agents and pathogen antagonists to control post-harvest rot.[9] Chitinolytic bacteria that are isolated from pathogenic insects or fungal cell walls act as biocontrol agents due to chitinase release, which can degrade chitin. Chitin is the main cuticle component of the peritrophic membrane, used as a protective gut lining in insects.[42] The chitinase enzyme hydrolyzes chitin into its monomers. The degradation of chitin in fungi and pathogenic insects via the chitinase enzyme is indicated by the presence of a clear zone when cultured on solid growth media. Bacteria may act as biocontrol agents through several mechanisms, including nutritional competition, direct contact with pathogenic hyphae, and the production of enzymes that can break down cell walls.[43] In this study, the diameter of inhibition

of *Bacillus specie* 21 against fungal pathogens in chili plants increased with the increasing incubation time. The diameter of inhibition against *Colletotrichum capsici* was wider than that of *Fusarium oxysporum f. sp. capsica,* but the diameter did not change even after seven days of incubation.[44] Studies on chitinase as an antifungal agent have been conducted by many researchers.[9,31,45,46,47,48,49,50,51,52] Chitinolytic bacteria use fungal hyphae as a source of chitin. The released chitinase lyses the fungal hyphae and causes fungal death. The chitinase of *T. asperellum* PQ34 can prevent anthrax disease caused by *Colletotrichum sp.* on mangoes and chilies. Fungi that grow on mangoes and chilies can be inhibited by chitinase after 96 hours of treatment. The diameter of fungal growth decreased from 1.67 to 0.88 cm in mangoes and 2.85 to 1.45 cm in chili peppers.[53]

Conclusion

Based on the results, chitinolytic isolates from aquatic sources provide different chitinolytic species. This can be influenced by the presence of chitin in the environment and the structure of the dominating bacterial community in the environment.

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Conflicts of Interest

There are no conflicts of interest.

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