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Isolation and Molecular Identification of Indegenous Bacteria in Shell Waste of Blood Cockle from Kenjeran Beach, Surabaya

Isolasi dan Identifikasi Molekuler Bakteri Indegenous Limbah Cangkang Kerang Darah di Pantai Kenjeran, Surabaya

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Abstract. Chitinase is one of the existing compounds that is quite abundant in nature. Source of chitinase can be found in several species of bacteria with chitinolytic activity. Bacterial isolation was obtained from the shell of blood cockle (*Tegillarca granosa*) collected from around Kenjeran Beach, Surabaya, Indonesia. This study aims to identify superior chitinolytic bacteria in shell waste using a molecular identification method based on the 16S rRNA gene. Bacteria were isolated by bacterial culture on selective chitin media and a clear zone around the colony as an indicator of chitinolytic activity was measured. The molecular identification process was carried out using PCR (Polymerase Chain Reaction) and sequencing techniques. The sequencing results were analyzed in silico by phylogenetic tree reconstructions through the MEGA 11. The result were then analyzed by comparing similarity values and genetic distance between sequences. Eight chitinolytic bacteria were successfully obtained, of which two isolates from the coastal area were indicated to have the highest chitinolytic index of 0.50 and 0.71. Based on the results of the observation of colony morphology, molecular analysis using Basic Local Alignment Search Tools (BLAST), and phylogenetic tree reconstruction using the maximum likelihood (ML) method. The two isolates were identified as belonging to the genus *Bacillus* and *Stenotrophomonas*.
Keywords: 16S rRNA gene; chitinolytics; coastal ecosystems; identification; shell waste

Abstrak. *Kitinase merupakan salah satu senyawa yang keberadaannya cukup melimpah di alam. Sumber kitinase dapat ditemukan pada beberapa spesies bakteri dengan aktivitas kitinolitik. Bakteri yang diisolasi diperoleh dari cangkang kerang darah (Anadara granosa) yang dikumpulkan dari sekitar Pantai Kenjeran, Surabaya, Indonesia. Penelitian ini bertujuan untuk mengidentifikasi bakteri kitinolitik unggul pada limbah cangkang kerang dengan menggunakan metode identifikasi molekuler berdasarkan gen 16S rRNA. Bakteri diisolasi dengan kultur bakteri pada media selektif kitin dan dilakukan seleksi untuk menentukan isolat yang memiliki aktivitas kitinolitik tertinggi dengan melakukan pengamatan dan mengukur perbandingan zona bening (clear zone) yang terbentuk. Proses identifikasi secara molekuler dilakukan menggunakan teknik PCR (Polymerase Chain Reaction) dan sekuensing. Hasil sekuensing dianalisis secara in silico dengan membuat rekonstruksi pohon filogenetik melalui aplikasi MEGA 11. Data yang didapatkan kemudian dianalisis dengan membandingkan nilai similaritas dan jarak genetik antar sekuens. Delapan bakteri kitinolitik berhasil diperoleh, dimana dua isolat yang berasal dari daerah pesisir terindikasi memiliki indeks kitinolitik tertinggi yaitu 0,50 dan 0,71. Berdasarkan hasil pengamatan morfologi koloni, analisis molekuler menggunakan Basic Local Alignment Search Tools (BLAST), dan rekonstruksi pohon filogenetik menggunakan metode maximum likelihood (ML), kedua isolat tersebut teridentifikasi masuk dalam genus Bacillus dan Stenotrophomonas.*
Kata kunci: *Gen 16S rRNA; kitinolitik; ekosistem pesisir pantai; identifikasi; limbah cangkang kerang*

INTRODUCTION

Chitinase is an enzyme produced by organism with chitinolytic activity used for the chitin degradation process. The availability of chitinase is quite abundant in nature, it can be found in various organism includimg bacteria, insects, fungi, plants, and animals (Pratiwi *et al*., 2016). Currently, chitinase is widely used in several fields, one of which is agriculture as a biocontrol agent for pathogenic fungi in chili and tomato plants (Buwono *et al*., 2013). Furthermore, chitinase is able to dissolve pathogenic cell walls and decompose nutrients bound in the soil (Rizal *et al*., 2019). In

addition, in the health field, the results of hydrolysis of chitinase can be used as an antitumor, anti-inflammatory, supplement, control blood sugar levels, and basic ingredients for making surgical threads (Pratiwi *et al*., 2016). The potential for the utilization of chitinase enzymes in various fields can be increased through endeavors to explore the existence of chitinase enzymes in bacteria that are indicated to have chitinolytic activity.

Bacteria that have chitinolytic activity can be present in several animals in seawater areas, one of which is shellfish. A number of studies prove that shellfish are one of the marine animals of the phylum mollusca that contain chitin. Shells can be one of the sources of chitosan which is the main product of chitin derivatives (Ahmad, 2017). The shell waste can be found in several seawater locations, for instance Kenjeran Beach, one of the most popular tourist destinations in the city of Surabaya. The shell waste is generated from industrial activities, including shellfish cultivation and seafood processing. In addition, it also comes from the naturally occuring accumulation of dead clam population in the beach vicinity. This clam shell waste has the potential to be a source of bacteria with chitinolytic activity. Currently, the existence of chitinolytic bacteria in Kenjeran Beach is still underexplored. Therefore, the identification of a superior chitinolytic bacteria that can produce chitinase enzymes needs to be expanded in line with their demands in various fields. One method that can be used to identify bacteria is through a molecular-based identification process.

The molecular identification method can be supported by bioinformatics analysis based on the 16S rRNA gene using Basic Local Alignment Search Tools (BLAST) to determine the homology level between species based on their nucleotide bases and phylogenetic tree reconstruction to visualize the results of molecular data that have been obtained previously. Genotype-based identification using phylogenetic tree analysis based on the 16S rRNA gene can provide higher accuracy (Kasi *et al*., 2019). Some prior study have found that there is a relationship between bacterial species that can be determined by comparing specific regions of genetic markers, including the 16S rRNA gene (Noer, 2021). The 16S rRNA gene is commonly used because it is universally present in all bacteria and has a slow evolutionary rate (Liu, 2011). Based on the aspect of speed analysis and practicality, currently the 16S rRNA gene is widely used in various fields of bacteriological research, especially for identification purposes (Akihary and Kolondam, 2020). Based on the excess of chitinase enzyme, this study aims to identify superior bacterial isolates that have the highest chitinolytic activity isolated from shell waste on the coast of Kenjeran Beach by using a molecular analysis method based on the 16S rRNA gene.

MATERIAL AND METHODS

Sample collection was conducted in Kenjeran Beach which is located in Kenjeran, Bulak District, Surabaya. The coordinates of the beach location (latitude 7°14'16.8"S and longitude 112°47'42.6"E). The samples were taken from shell waste of blood cockle (*Tegillarca granosa*) collected in two different area of the beach including coastal and rocky areas. The sampling technique uses random purposive sampling. The collected samples are first cleaned by soaking in NaCl solution for 6 hours, then cleaned of contaminants on the surface of the shell and rinsed with aquadest and then dried. Sample testing was carried out at the Microbiology Laboratory, FMIPA, State University of Surabaya for the process of isolation, purification and selection of bacteria and the Griya Science Laboratory, Malang to conduct DNA isolation, qualitative tests and DNA amplification using PCR. The product from PCR amplification of bacterial samples was sent to 1stBASE Laboratories Sdn Bhd, Malaysia. to carry out the sequencing proces.

Selective media containing chitin consisted of chitin powder, nutrient agar (NA), and aquadest. The media was sterilized using an autoclave at 121°C for 15 minutes and poured into a sterile erlenmeyer until it solidifies and than stored. Approximately 1 gram of sample is ground until smooth, then a 10^{-1} to 10^{-6} tiered dilution is carried out on each test tube containing 9 ml of sterile aquadest that vortexed for 5 seconds. The last three dilutions (10^{-4}, 10^{-5} and 10^{-6}) were inoculated with 1 ml each into chitin media using the spread plate method (Fitriani *et al*., 2018). The samples were incubated at 37°C for 1 x 24 hours.

The isolated colonies are purified using the streak method and divided into four quadrants then incubated at at 37°C for 1 x 24 hours. Bacterial isolates were then observed for macroscopic characterization of colony morphology which included color, shape, size, margin and elevation. In addition, chitinolytic activity tests were carried out on each colony by taking one ose pure bacterial then inoculated into a petri dish containing a chitin media that had solidified and incubated at a

temperature of 37°C for 48 hours. The chitinolytic index (CI) was observed and calculated. using formula by Halimahtussadiyah *et al*. (2017).

$$CI = \frac{clear\ zone\ diameter - \ colony\ diameter}{colony\ diameter}$$

The qualitative test of chitinolytic activity of isolates indicated to have the highest chitinolytic index were carried out with used a 0.1% *congo red* reagent, which is 0.1 g dissolved in 100 ml aquadest (Waling *et al*., 2021). *Congo red* is dripped to cover the entire surface of the media, then left to sit for one minute then rinsed with NaCl 1M and then observed for the color change that occurred.

Genetic identification was performed using the 16S rRNA gene. Bacteria with the highest chitinolytic activity were cultured in LB media and the DNA was isolated with ZymoBIOMICS Miniprep Kit. Amplification of the target gene was performed by PCR (Polymerase Chain Reaction) technique in a solution volume of 30 µl (Table 1) using a universal primer for the 16S rRNA gene. Forward primer used 27F (5' AGAGTTTGATC{AC)TGGCTCAG 3') and Primer reverse: 1492R (5' TACGG(CT)TACCTTGTTACGACTT 3'). The temperature setting is made as follows, pre-denaturation at 95 ^{o}C for 2 minutes, then continued with 30 cycles consisting of denaturation at 95^{o}C for 2 minutes, annealing at 50^{o}C for 30 seconds, and extension at 72 °C for 2 minutes. Next, the post elongation process is carried out at a temperature of 72 °C for 7 minutes. The PCR results were then electrophoresis on 2% agarose. The results of electrophoresis are visualized using a UV trnsluminator. After the electrophoresis process, the PCR results are then sent to First BASE, Laboratories, Malaysia for a sequencing process.

Table 1. Composition of materials in PCR reactions

Materials	Volume
PCR Master Mix Nexpro	15 µl
Sampel *DNA template* (100 ng/µl)	1.5 µl
ddH_2O	10.5 µl
Primer forward: 27F (5' AGAGTTTGATC(AC)TGGCTCAG 3')	1.5 µl
Primer reverse: 1492R (5' TACGG(CT)TACCTTGTTACGACTT 3')	1.5 µl
Total volume	30 µl

Colony morphological data and qualitative test results using *congo red* reagent were analyzed descriptively. The DNA sequencing results of each isolate obtained in the FASTA format, then compared with the database available on *GenBank* NCBI using the Basic Local Alignment Search Tools (BLAST) program to determine the level of sequence homology. The data from BLAST analysis was then used in phylogenetic tree reconstruction to determine the kinship between sequences.

RESULTS

Chitinolytic bacteria were isolated from shell waste of blood cockle (*Anadara granosa)* collected from the area of Kenjeran Beach. The first group of sample was taken around the coastal area (label A), while the second group of sample was taken from the rocky area around the beach (label B). A total of eight chitinolytic bacterial isolates found had different morphological characteristics including color, shape, size, margin, and elevation (Figure 1).

Macroscopic characteristics results shown that both sample A and sample B tend to have morphological characteristics of white color, circular shape with entire margin (Table 2). In addition, there are variations in colony size that are small, moderate and big. The last morphological characteristic that can be observed macroscopic is the elevation of the colony, which consists of elevations that are raised, flat and convex.

Based on the clear zone measurement, two isolates were found to have the highest chitinolytic activity were determined, namely isolates K.A3 and K.A4 with a chitinolytic index value of 0.50 and 0.71. These clear zones diameter are equivalent to the value of chitinolytic index in bacterial colonies. Interestingly, sample A taken from the coastal area has higher chitinolytic activity than the sample B collected from coastal rock areas. This statement supported by the results of measuring the chitinolytic index, in which K.A3 and K.A4 isolates derived from shells taken from coastal area become the two isolates that have the highest chitinolytic index (Table 3).

Based on the results of the observations, the two isolates that have the highest chitinolytic activity are continued to the qualitative test process by adding *congo red* to detect the activity of the chitinase enzyme (Figure 2) and the results were obtained that after being dripped with *congo red* dye, the clear zone area around the bacterial colony tends to be clear or colorless, while the area outside the clear zone shows a red color indicating the presence of chitin in the media.

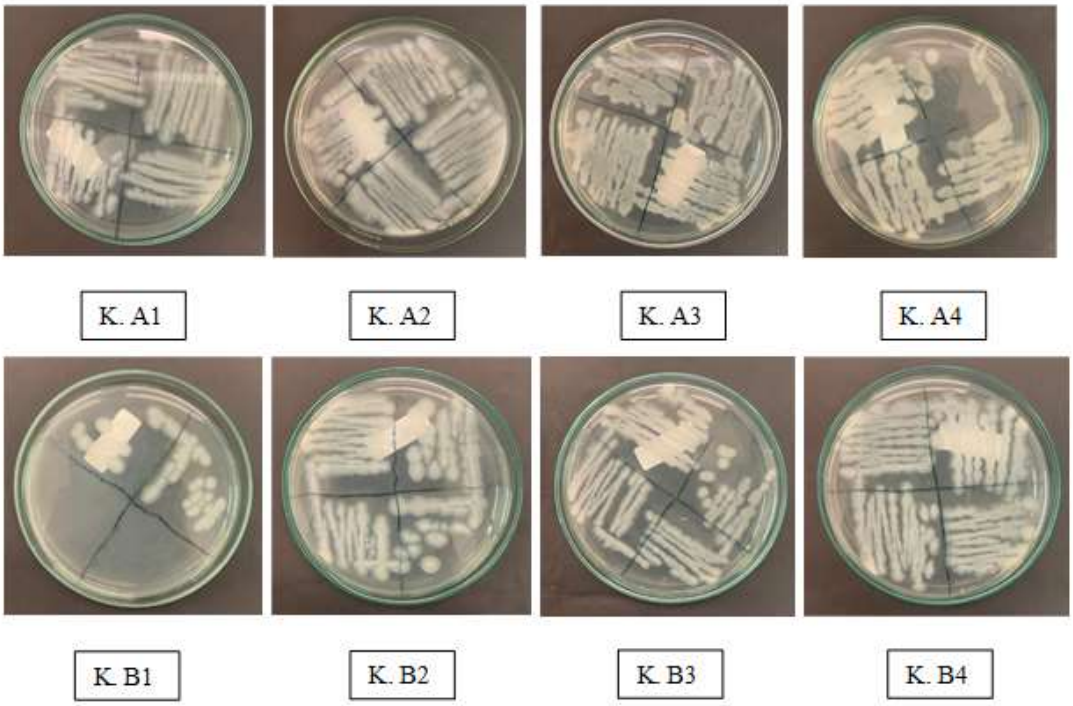


Figure 1. Bacterial isolates of shell waste of blood cockle (*Anadara granosa*)

Table 2. Observation of macroscopic characteristics of chitinolytic bacterial isolates

No	Isolate Code	Characteristics of Bacterial				
		Color	Shape	Size	Margin	Elevation
1.	K. A1	Turbid white	Irregular	Small	Undulate	Raised
2.	K. A2	White	Circular	Moderate	Entire	Flat
3.	K. A3	White	Irregular	Moderate	Curled	Flat
4.	K. A4	Cream	Circular	Moderate	Entire	Raised
5.	K. B1	White	Circular	Big	Entire	Raised
6,	K. B2	White	Circular	Big	Entire	Convex
7.	K. B3	Turbid White	Circular	Small	Entire	Convex
8.	K. B4	White	Irregular	Moderate	Lobate	Flat

Table 3. Test of chitinolytic activity of K.A3 and K.A4 bacterial isolates on chitin media based on the chitinous index (CI).

No	Isolate Code	D. Clear Zone (mm)	D. Colony (mm)	Chitinolytic Index
1.	K. A3	10.5	7	**0.50**
2.	K. A4	12	7	**0.71**

The visualization of the DNA isolation process showed that the DNA band was clearly visible, moreover absence of smearing indicating that the DNA was intact and not fragmented (Figure 3a). Both of these characteristics indicate that the DNA has been well isolated so that it can be continued for the PCR amplification process. The results of the amplification process were then qualitatively tested using 2% agarose gel and showed the presence of clearly visible DNA bands with sizes that matched the expected target. The DNA bands of the two isolates had a length of approximately 1,300 bp (Figure 3b). This indicates whole DNA was successfully isolated and sufficient to be used for identify the bacterial species. The visualization results that were appropriate then continued to the sequencing process.

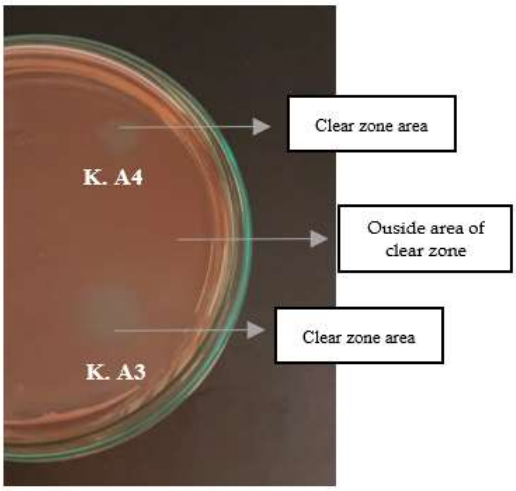
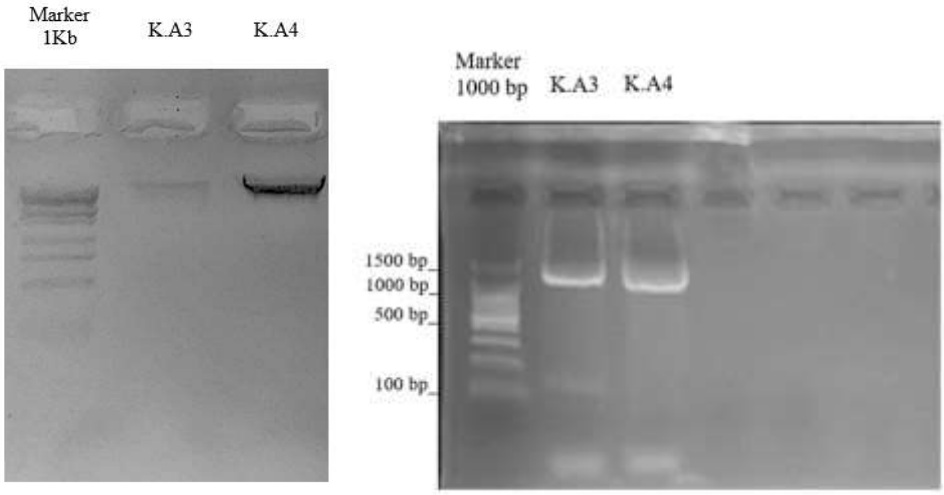


Figure 2. Qualitative test of bacterial isolates K A3 and K.A4 with *congo red* reagent



(a) **(b)**

Figure 3. Results of visualization of agarose gel electrophoresis; (a) DNA isolation results, (b) PCR amplification results

The sequencing results were then further analyzed using Basic Local Aligment Search Tools (BLAST) which was accessed through the National Center for Biotechnology Information (NCBI) website. Based on the BLAST analysis process, it was found that K.A3 isolate had the highest query coverage value with the species *Bacillus cereus* strain S19/A with a value of 98% and a percent identity of 96.05%, while the K.A4 isolate had the highest query coverage value with the species *Stenotrophomonas maltophilia* strain R1076 with a value of 96% and a percent identity of 94.60%. Results of BLAST analysis on K.A3 and K.A4 isolates (Table 4).

Table 4. Results of the highest query coverage of K.A3 and K.A4 isolates using the BLAST analysis

Isolate code	Description	Max score	Total score	Query cover	E-value	Identity	Accession
Isolate K. A3	*Bacillus cereus* strain S19/A	2054	2054	98%	0.0	96.05%	OR349523.1
Isolate K. A4	*Stenotrophomonas maltophilia* strain R1076	1786	1786	96%	0.0	94.60%	OP861522.1

The reconstruction of the phylogenetic tree reveals the formation of four main clades formed (Figure 4). K.A3 isolate was in the first clade with the bacterial sequences of *Bacilus cereus* strain G34 and *Bacilus cereus* strain SI9/A with a bootstrap value of 95%, while K.A4 isolate was in the fourth clade with the bacterial sequences *Stretophomonas maltophilia* strain R1076 and *Stenotrophomonas pavanii* strain AS1F with a bootstrap value of 76%.

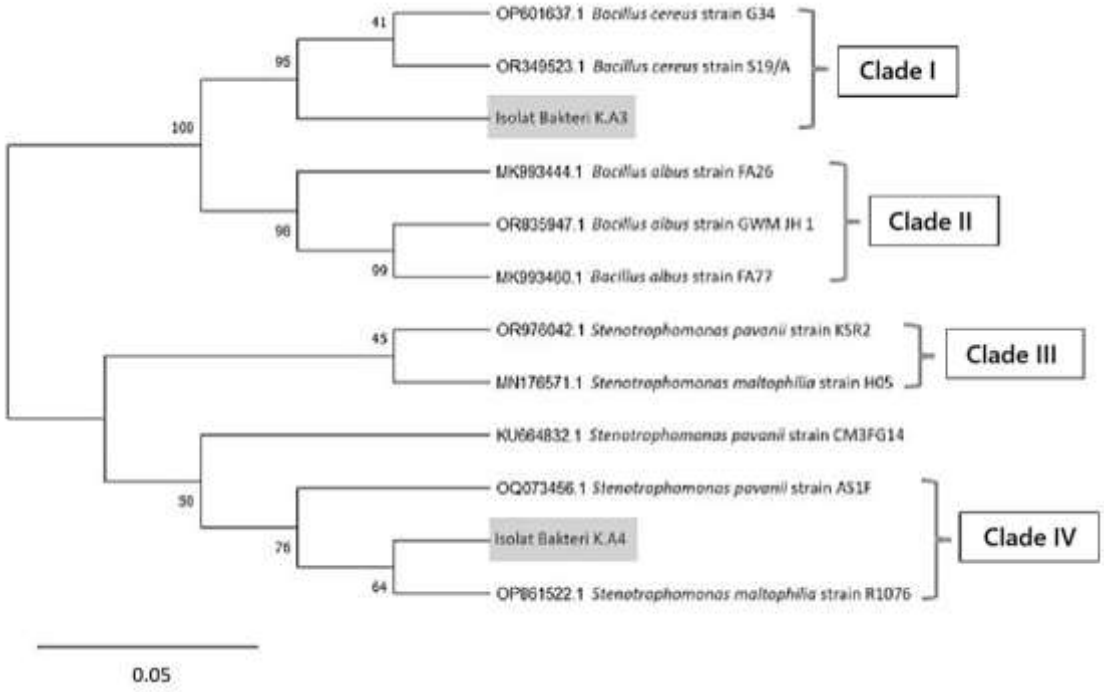


Figure 4. Results of visualization of phylogenetic trees of K.A3 and K.A4 isolates

DISCUSSION

Morphological observation results shown that isolated bacteria tend to have morphological characteristics of white color, circular shape with entire margin (Table 2). This finding is in agreement with previous study about the chitinolytic bacteria that were found in the marine area of Banda Aceh and Aceh Besar, which are the majority of these bacteria have white and milky white colors and circular shapes with entire margins (Fitri and Yasmin, 2011). In addition, chitinolytic bacteria isolated from vannamei shrimp waste also have turbid white colors and circular shapes (Maria *et al*., 2020).

Chitinase enzyme is a type of enzyme that has the ability to hydrolyze chitin macromolecules in the vicinity of the chitinolytic microbial environment (Purkan *et al*., 2014). Therefore, measurements of the clear zone formed around the colony and qualitative test analysis using *congo red* dye were then carried out to determine the indication of the presence of chitinase enzyme around the bacterial colony. Based on the results of observation and measurement of the clear zone, two isolates with the highest chitinolytic index values were obtained (Table 3). This indicates that the chitinolytic activity and ability to secrete the chitinase enzyme in both isolates were detected to be superior to other isolates. Moreover, *congo red* staining process was showed the clear zone area around the bacterial colony tends to be clear and colorless, while the outer area of the clear zone shows a red color (Figure 2). The chitin contained in the medium will be degraded by the enzyme chitinase. Consequently, when the staining process is carried out using the *congo red* reagent, the area around the colony indicated to have chitinolytic activity will show a clear color, this occured because there is no bond between chitin and the *congo red*. Meanwhile, in areas where chitin is still present, it will bind with the *congo red* reagent, causing the media to show a red color (Alamsjah, 2014).

Based on the results of qualitative tests, it can be found that isolates derived from samples taken around coastal areas and potentially higher exposure to seawater have chitinolytic activity that tends to be higher than isolates derived from samples collected from coastal rock areas. This can occur due to several factors, such as the pH level of seawater in the Kenjeran Beach area which shows a value of 7.8, where this value is a pH that is quite optimal for chitinase enzyme activity (Pamungkas *et al*., 2022; Afiuddin and Novitrie, 2017). In addition, the optimum salinity level of beach water in the range of 25-29 ppt is the optimal salinity value to support the activity of the chitinase enzyme (Chen *et*

al., 2019; Wulansari and Kuntjoro, 2018). These two factors are indications of the cause of samples that come into direct contact with seawater can have appropriate pH and salinity levels to support the productivity of chitinase enzymes.

The molecular identification process was then carried out on the two isolates that were identified to have the highest chitinolytic activity through the PCR (Polymerase Chain Reaction) method based on the 16S rRNA gene which was then visualized with a UV transluminator. The visualization results show that the DNA bands in the two isolates have a length of approximately 1,300 bp (Figure 3b). This indicates that the amplification procedure of the two isolates has been done well and the length of the target DNA has been reached and is sufficient to identify the bacterial species. This statement is reinforced by research conducted by Masri *et al*. (2021) that successfully carried out molecular identification of chitinolytic bacteria based on the 16S rRNA gene with a length of 1300 bp. The visualization results that were appropriate then continued to the sequencing process.. Nucleotide sequence data obtained in the form of FASTA was analyzed using BLAST to analyze the homological level of nucleotide base sequence between the tested isolates and the NCBI data.

The BLAST analysis showed that bacterial isolates with the code K.A3 had the highest homology level with the *Bacillus cereus* strain S19/A species with the highest query coverage value of 98% and percent identity of 96.05%. This is supported by research conducted by Maria *et al*. (2020) also succeeded in identifying two bacterial isolates that are indicated to have the highest chitinolytic activity that are able to produce active chitinase in media containing shrimp skin powder, where the two isolates were identified as *Bacillus subtilis* and *Bacillus sp*. Moreover, research exploration by Buwono and Grandiosa (2021) succeeded in identifying chitinolytic bacteria from crab waste that are identical to the species *Bacillus cereus* with a homological value of 98% and a percent identity of 96.05%, where the isolate has a white colony morphology and is indicated to have a chitinolytic index value of 1.99 mm.

Isolates with the code K.A4 has the same nucleotide base as the species *Stenotrophomonas maltophilia* strain R1076 which is shown with a query coverage value of 96% and a percent identity of 94.60%. The results of the identification are in line with the research conducted by Soeka and Sulistiani (2012) who succeeded in isolating chitinolytic bacteria from the genus *Stenotrophomonas* from groundwater samples in the Mount Bromo area, East Java using a molecular identification method with morphological characteristics cream color, circular shape with entire margin. The morphological characteristics are similar to K.A4 bacterial isolate (Table 2). Chitinolytic bacteria from the genus *Stenotrophomonas* can also be found in the waste of aquatic animals such as shrimp, this is supported by the research of Ovilla *et al*. (2019) successfully identified the species *Stenotrophomonas maltophilia* from shrimp waste collected around the Las Escolleras-Puerto Madero area, Chiapas, Mexico.

Based on the results of BLAST analysis, it was found that K.A3 isolate had the highest query coverage value with the species *Bacillus cereus* strain S19/A with a value of 98% and a percent identity of 96.05%, while the K.A4 isolate had the highest query coverage value with the species *Stenotrophomonas maltophilia* strain R1076 with a value of 96% and a percent identity of 94.60%. The sequence will be similar to the database in GenBank if the query cover value is close to 100%, the E value is close to 0, and the percentage per ident is close to 100% in each database. E value percentage shows a significant level of alignment, lower E value describing the more significant of matching sequence, while per ident shows the level of sequence matching with the data in GenBank (Gaffar *et al*., 2020). The higher query cover value is equivalent with the higher level of homology (Nugraha *et al*., 2014). Query cover shows the length of the nucleotides that are aligned with the data on the GenBank.

Phylogenetic tree visualization has shown that K.A3 isolate is located in the first clade with two other sequences, namely *Bacillus cereus* strain SI9/A and *Bacillus cereus* strain G34, while K.A4 isolate is located in the fourth clade with the sequences *Stenotrophomonas malthophilia* strain R1076 and *Stenotrophomonas pavanii* strain AS1F. Sequences that are in the same clade can indicate a high degree of kinship between sequences. One of the factors that can cause kinship between sequences that are in the same clade is due to the presence of homologous nucleotide base sequences. In addition, the grouping of sequences in one clade can be influenced by genetic recombination factors (Djuita, 2012), as well as gene transfer or flow that can encourage evolution in species. The occurrence of evolution in species causes the possibility of similarity in morphological characters or biological functions so that species are in the same phylogenetic tree branch.

The genetic distance analysis showed that K.A3 bacterial isolate had the closest kinship relationship with *Bacillus cereus* G34 bacteria with a genetic distance of 0.022 and a similarity value of

97.78% and *Bacillus cereus* S19/A with a genetic distance of 0.027 and a similarity value of 97.29%, while K.A4 bacterial isolate was closely related to *Stenotrophomonas pavanii* strain AS1F with a genetic distance of 0.044 and a similarity value of 95.62% and *Stenotrophomonas pavanii* strain CM3FG14 with distance genetics 0.049 and a similarity value of 95.06%. Based on the results of the similarity analysis of the 16S rRNA gene, it shows that the similarity value of ≥99% can be expressed as one of the same species, while if the similarity value of the 16S rRNA gene shows a figure of <97%, it tends to be grouped into one genus or one taxonomic level above it (Syah, 2022).

Based on the results of phylogenetic analysis, the existence of sequences in the same branching or clade indicates the existence of a close degree of kinship between sequences, while the bootstrap value shows the validity value of the phylogenetic tree construction results. To confirm the suitability of the phylogenetic tree construction results, genetic distance and similarity between sequences were analyzed so that it could be concluded that K.A3 isolate belongs to the genus *Bacillus* while K.A4 isolate can be grouped in the genus *Stenotrophomonas* because the similarity value is below the value of 99%.

CONCLUSION

Based on this research, five of eight chitinolytic bacterial isolates obtained from the shell waste of blood cockles (*Tegillarca granosa*) around Kenjeran Beach are indicated to have chitinolytic activity, characterized by the formation of a clear zone around the colony. Qualitative testing of the chitinolytic index for all eight bacterial isolates revealed that two isolates, coded K.A3 and K.A4, exhibited the highest chitinolytic activity. Further analysis, including observation of colony morphology, molecular analysis using Basic Local Alignment Search Tools (BLAST), and phylogenetic tree reconstruction, identified the K.A3 isolate as belonging to the genus *Bacillus*, while K.A4 isolate is identified in the group of the genus *Stenotrophomonas*.

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