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Isolation and Identification of Protease Producing Bacteria *Kocuria*sp. *Strain HR12*coexistence of *Avicennia Marina* from West Coastal Area of Qeshm Island (KonarSiah)

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ABSTRACT

Microbial proteases are an environmentally friendly indicator that has many applications in various industries including textile, detergent, leather, food industry, dairy and others. Due to the economic importance as well as the fact that microbial proteases are stable at the desired pH and tolerate harsh conditions, this study was conducted to isolate and identify bacteria that produce protease enzyme symbiosis in mangrove trees. In this study, protease-producing bacteria coexisting with the roots and leaves of mangrove trees were collected from the west coast of Qeshm Island (KonarSiah) and cultured them at 30 °C for 72 h on nutrient agar medium. Protease-producing bacteria were then screened by specific culture medium containing Skim milk agar and protease enzyme activity of the isolated bacteria was measured using 1% casein substrate. Finally, the bacterium with the highest enzymatic activity was selected and identified by 16SrRNA gene sequence analysis. The results of the analysis showed that 31 of the 46 bacteria isolated from mangrove leaves and roots were capable of producing protease enzymes. Among the six strong bacteria, one with the highest enzymatic activity was selected, which after molecular processes and sequencing led to the identification of the bacterium *Kocuria* sp. strain HR12. Kinetic studies revealed that the protease enzyme isolated from kocuriasp. HR12, exhibited its maximum activity at 50 °C and pH 9. The findings of this study provide a platform for the mass production of temperature-resistant protease enzymes from the coexisting bacteria of the Persian Gulf mangrove trees, which offers a wide range of potential uses for practical enzyme production.

Keywords: Isolation, Identification, Protease producing bacteria, Mangroves, Optimal stability

INTRODUCTION

Oceans and aquatic ecosystems are one of Earth's richest and most diverse habitats, which means that they hold a large collection of unique natural products, enzymes, and bioactive compounds 1,2]. Marine microorganisms have extraordinary advantages over their terrestrial counterparts due to their physiological compatibility, genetic structures, and metabolic characteristics [3,4]. There are in fact various communities within the marine environment, including several species of plants, animals, and microorganisms, such as bacteria, fungi, viruses, and others [5]. Microorganisms are able to produce enzymes that are very useful for a wide range of industries. The popularity of microbial enzymes over animal and plant resources is owing to their lower costs of production and ease of cultivation, which are the key reasons why they are widely used in industrial applications rather than chemical enzymes [6]. Proteases are one of the most important enzymes that rank first on the global enzyme market [7]. A protease's properties vary depending on its substrate specificity, active site, catalytic mechanism, pH and optimum temperature [8]. Among protease enzyme types, alkaline proteases alone account for more than 89% of the total annual sales of protease enzymes [9]. Bacteria are an excellent candidate for the production of industrial enzymes due to their small cultivation space requirements, rapid growth, and a low-cost production process [10,11]. Protease enzymes have many applications in various industries, such as in crumbling meat, related derivatives, producing proteins, making cheese in the dairy industry,

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food industry, use of plants as a source of perfumes and colorants, for the production of animal milk with higher levels of protein, and in the production of cosmetics and hygiene [12,13]. In view of the serious problems of chemical catalysts, their high production costs, as well as the harmful effects they can have on human health, marine enzyme technology has become one of the most important sources in this area over the past few years [14]. The bacteria that live in the ocean have excellent biological conditions, since they are tolerant of different environmental conditions, so specific metabolic pathways within these microorganisms are activated, leading to the development of active metabolites [15,16]. Mangroves are evergreen woody plants that play a vital role in protecting the saline coastal environment and food chain [17]. Mangroves have high biological productivity and are the second most abundant and rich marine ecosystem after coral reefs due to tidal influx, salinity, pH, temperature, light availability, soil oxygen content and availability of organic and inorganic nutrients. Provide an excellent environment for a wide range of living organisms, including bacteria [18, 19]. It has been discovered that mangroves are halophytes capable of producing a variety of active metabolites with diverse bioactivities, which is why more than 200 active metabolites have been isolated so far from mangroves and their associated organisms [20]. This has led to the separation of biologically active molecules from marine ecosystems, which in turn can introduce marine microbiology as one of the most valuable and interesting areas of recent research [21]. Mangrove roots release nutrients and oxygen into the soil, creating unique and diverse habitats for microorganisms and the growth of various bacterial communities [22-24]. Rhizosphere soils benefit greatly from aerial roots since their activity affects enzymatic, microbial, and bacterial activities [25].

Thus, marine endophytes provide an advanced region with a great deal of biological diversity for the discovery, identification, and production of new compounds and enzymes. The use of marine resources has resulted in thousands of products, but only a small portion of its biological and chemical potential has been discovered, and many of the valuable marine resources remain intact and unknown, and their identification and extraction could have significant impacts [26]. In this study, for the first time, bacteria producing industrial protease enzyme had been isolated and identified from a mangrove tree on the western coast of Qeshm Island (KonarSiah) and their biochemical properties were analyzed.

MATERIALS AND METHODS

Sampling

In order to obtain protease-producing bacteria, leaf and root samples of mangrove trees were collected in three stages and on different days from the west coast of Qeshm Island (KonarSiah), located in Hormozgan province (Fig. 1).

The samples were collected in sterile plastic bags and stored in ice flasks at 4-10 °C before they were transferred to the laboratory. After washing, the samples were immediately sterilized and then a bacterial isolation procedure was conducted19.

Isolation of Symbiotic Bacteria from Leaves and Roots of Mangrove Trees

In order to clean the leaf and root samples collected from sand particles, the samples were first washed with sterile sea water. 2 g of each sample was weighed, rinsed with 70% alcohol for 1 min, 96% methanol for 30 s, 2.5% sodium hypochlorite for 4 min, and then sterile distilled water for each step of the procedure. Finally, the samples were homogenized with 6 ml of normal saline (physiological serum).

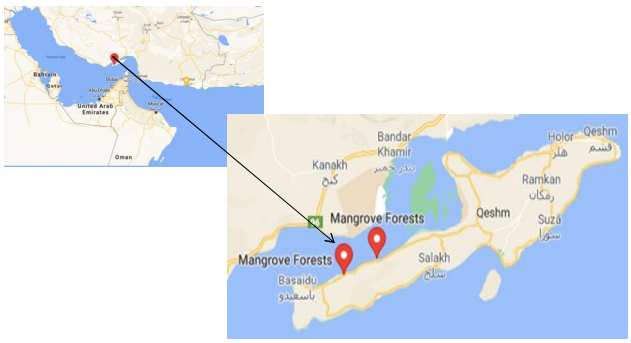

Fig. 1. Geographical position of the study location at coastal mangrove located in KonarSiah village in Persian Gulf.

The homogenized samples were serially diluted after incubation for 3 h. A 10 µl of each dilution was then spread on nutrient agar medium containing a 50% seawater solution and left at 30 °C for one week [27,28].

Primary Screening of Protease-producing Bacteria

Bacteria isolated from the leaves and roots of mangrove trees were cultured on a specific culture medium containing skim Milk agar and incubated at 30 °C for 24-72 h. Finally, the bacterial strain with the largest halo around the culture line was selected and stored on nutrient broth containing 30% glycerol and stored at -20 °C for further screening [29].

Secondary Screening and Measurement of Enzymatic Activity of Protease-producing Bacteria

Secondary screening and measurement of enzyme activity were performed based on the following methods.

Preparation of Crude Enzyme Extract

Bacteria selected for primary screening were cultured in neutral broth medium containing 50% sea water. After incubation, 80 µl of neutral broth medium with an OD 600 nm = 0.8 in 8 ml of sterile broth neutrino medium was cultured. Finally, the protease-producing strains were selected in three consecutive times (24, 48, 72 h) in a shaker incubator (WiseCube) at 200 rpm and 30 °C were grown. Then under the hood and under completely sterile conditions, 1.5 ml of each culture medium containing the selected strain was poured into the microtube and centrifuged at 12000 rpm for 20 min. Finally, the supernatant was used as a crude extract of the enzyme for the study process and to measure the enzymatic activity related to this study [30].

Enzyme Assay

After enzyme extraction from the strains which formed the largest halo, 200 µ l of 50 µM phosphate buffer were added to 200 µl of enzyme extract and allowed to stand at room temperature for 1 min. Then 400 µl of 1% casein (Merck) was added as a substrate and placed at room temperature for 10 min. Afterwards 800 µl of 10% Trichloroacetic acid (TCA) (Merck) was added to stop the enzymatic reaction. The resulting solution was centrifuged at 12000 rpm for 20 min after incubating for 20 min at room temperature, and finally the supernatant was read and recorded by a spectrophotometer (Cecil Model 9000) at a wavelength of 280 nm [31].

Preliminary and Complementary Identification of Bacterial Strains

The selected protease-producing bacterial strain was characterized based on morphological characteristics, Gram staining (staining kit of the test technology company) and biochemical characteristics according to the bergey's systematic guideline [29]. The strains were then identified by amplifying 16SrRNA gene sequences with forward and reverse general primers and sequencing the resulting gene fragment.

DNA Extraction and Amplification of Bacterial 16SrRNA Fragment Using PCR

DNA extraction of the selected bacteria in this study was performed by boiling method [32,33]. Selected bacteria were re-cultured in nutrient broth medium. After 24 h, 3 ml of cultured bacteria was poured into 1.5 ml microtubes and centrifuged at 12000 g for 5 min. The supernatant was discarded and 1.5 ml of distilled water was added to the pellets and the vortex was applied for 10 s. The bacterial sample was then placed in Ben Marie (Daihan Scientific Company, model WB-11) at 100 °C for 15 min. The sample was centrifuged for 10 min at 12000 g (Sigma's model 2-16 p.) Finally, the supernatant of the selected bacterium, which contained pure DNA, was stored in a 1.5 microtube for further studies in the freezer [32,34]. PCR reaction in a final volume of 50 µl, including 0.5 µl of primer, 0.5 µl of reverse primer, 22 µl of deionized distilled water, 25 µl of mastermix (DNA polymerase, $MgCl_2$, dNTP, PCR buffer and taq enzyme) and 2 µl of bacterial DNA were extracted using general primers Fd1 (5′AGAGTTTGATCCTGGCTCAG3′) and reverse Rd1 (5′AAGGAGGTGATCCAGCC3′) in a thermocycler (BIO RAD model T100) at 95 °C for 1 min at initial denaturation at 95 °C. The 30 cycles include denaturation at 95 °C for 20 s, annealing at 63 °C for 30 s, extension at 72 °C for 1 min, and finally extension at 72 °C for 5 min. PCR product after electrophoresis on 1% agarose gel with a length of 1500 bp after purification was sequentially determined by FazaPajooh Company. Sequences were compared using the blast tool in the NCBI data base (https://blast.ncbi.nlm.nih.gov) with valid sequences

registered in the NCBI database, and the closest strain was selected based on the 16 S rRNA gene sequence and biochemical test results. In order to investigate the phylogenetic relationship between the isolated strains and draw phylogenetic tree, the 16SrRNA sequence alignment of *Kocuria* sp. strain HR12 was performed with species belonging to the genera *Kocuria*, *Bacillus*, *Mycoplasma*, *Pseudomonas* and Moorea (Moorea is a genus of cyanobacteria in the family Oscillatoriaceae) in the NCBI gene database with Clustal W software. Then the phylogenetic tree was drawn with MEGA4 software andNeibour- Joining (NJ) algorithm with bootstrap 1000 replications [35].

Investigation of the Effect of pH and Optimum Temperature on Protease Activity Extracted from Mangrove Root Symbiotic Bacteria

For the evaluation of pH's effect on enzyme activity, sodium acetate buffers were used for pH 3-6, sodium phosphate buffers for pH 7, Tris-HCl buffers for pHs 8 and 9 and sodium carbonate buffer for pH 10. Temperature-dependent enzyme activity testing was conducted at 20 to 100 °C with a 50 mM phosphate buffer, pH 7.5. It should be noted that in order to measure enzymatic activity at any temperature, At that temperature, both the substrate (casein 1%) and the enzyme solution (buffer + enzyme) must equilibrate before the enzymatic activity can be determined[5]. Protease activity was measured at the pH and temperatures with three replications [36].

RESULTS

Isolation of Protease-producing Bacteria

From the total samples collected from the leaves and roots of *A. marina* mangrove trees, 46 bacteria were isolated and their pure culture was performed on nutrient agar medium (Fig. 2). Detailed information on sampling locations and sampling dates is given in Table 1.

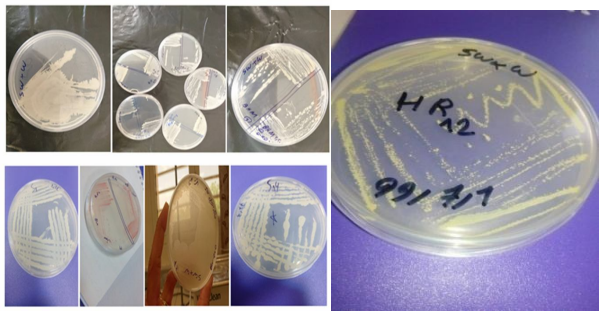

Fig. 2. Growth of *Kocuria* sp. strain HR12 on a nutrient agar culture.

Screening of Bacteria Producing Proteases

Bacteria isolated from the leaves and roots of *Avecina* marina mangrove trees were tested on a specific culture medium containing skim milk agar. The formation of a clear halo around the culture line indicated the production of protease enzyme by bacteria. The results of the initial screening showed that out of 46 isolated bacteria, 31 bacteria were able to produce enzymes and hydrolyze proteins into smaller chains of peptides and amino acids, and 15 bacteria lacked enzymes. Following the initial screening on a specific culture medium containing skim Milk agar, 9 bacteria produced a strong halo and the rest of the bacteria produced a relatively strong to weak halo diameter. Finally, the six bacteria with the highest halo were selected and screened for Protease enzyme activity. Clear zones around bacterial colonies indicated proteolysis positive results on skim milkagar.

Table 1. List of Sampling Location with Information on Sampling Time, Isolation Source and Number of Strains Isolated

No.	Sampling information	Location	Sampling time	Isolation source	Number of strains isolated
1	Sampling from 2 points intertidal zone and subtidalzonefrom different parts of mangrove randomly	The west coast of Qeshm Island, KonarSiah	2019-5-11	Root	28
2			2019-5-14	Leaves	18

Secondary Screening and Measurement of Enzyme Activity among Bacteria Producing Proteases

Among the 6 strong bacterial strains selected for enzyme activity, the HR12 bacterial strain had the highest enzyme activity, as shown in Table 2. As shown in the Table 2, the HR12 strain had a similar absorption at 280 nm and at the time of the enzyme-substrate reaction. Measurement of protease activity over a period of 24-72 h, each with three replications, also showed high absorption.Then, the bacterial strain of HR12 was morphologically characterized by measuring the diameter of the protease clear zone size (Fig. 3) and Gram staining.Results are shown in Table 3.

Molecular Identification of Protease Producing Strain Kocuria sp. HR12

After electrophoresis of PCR product on 1% agarose gel, it was found that the desired fragment had 1500 bp, which was registered in the gene bank with accession number MN988924 (Fig. 4). The blast results of the nucleotide fragment in the NCBI database showed that *Kocuria*sp.strain HR12 has the highest similarity (more than

Fig. 3. Proteolyticactivityproducing by HR12 strain on skim milk agar plates showing zone of proteolysis.

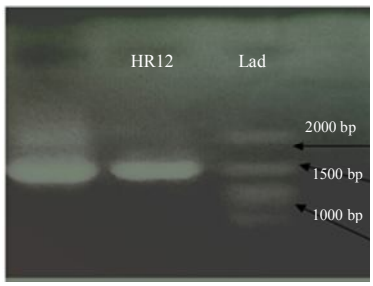

Fig. 4. Agarose gel electrophoresis of showing the PCR products amplified from 16S rRNA gene (1500 bp) of *Kocuria* sp. strain HR12; lane Lad: ladder.

Table 2. Results Obtained from Secondary Screening and Measurement of Protease Enzyme Activity by HR12 Strain

Strain code	Absorption of bacterial strains (280 nm)	Absorption of enzyme-substrate reaction[a] (Reaction time: 10 min)	Absorption at the time of enzyme assay[b]		
			24 h	48 h	72 h
HR12	0.413	0.413	0.49	0.50	0.49
			0.45	0.48	0.55
			0.48	0.55	0.53

[a]Enzyme absorptionafter casein addition and reaction time 10 min. [b]Absorptionat the time of enzyme assay after TCA addition in three replications.

Table 3. The Morphological Characteristics and Gram Staining ofHR12 Strain Isolated from Mangrove

Morphological characteristicsand Gram staining	Gram stain	Form of bacterial cells	Colony color	Size	Form and shape of bacterial colony	Average clear zone size (Time: diameter in mm)
Results	+	Cocci	Yellow	Small	dry form	24 h: 3 mm 48 h: 7 mm 72 h: 12 mm

Table 4. Nucleotide Sequence Results of Kocuria sp. Strain HR12 at NCBI Site

Description	Scientific name	Per. Identity (%)	Accession
Kocuria sp. Strain HR12 16S rRNA gene	*Kocuria* sp.	100	This study
Kocuria sp. Strain CW544 16S rRNA gene	*Kocuria* sp.	99.91	MK034270.1
Kocuria sp. Strain SJU14 16S rRNA gene	*Kocuria* sp.	99.91	MN511781.1
Kocuriaplustris 16S rRNA gene	*Kocuriaplustris*	99.91	LR215141.1
Kocuriaassamensis strain ADL041 16S rRNA gene	*Kocuriaassamensis*	99.91	MK818797.1
Kocuriaassamensis strain SCSIO-43735 16S rRNA gene	*Kocuriaassamensis*	99.91	MH283819.1
Kocuria sp. strain InS-100 16S rRNA gene	*Kocuria* sp.	99.91	KY964250.1

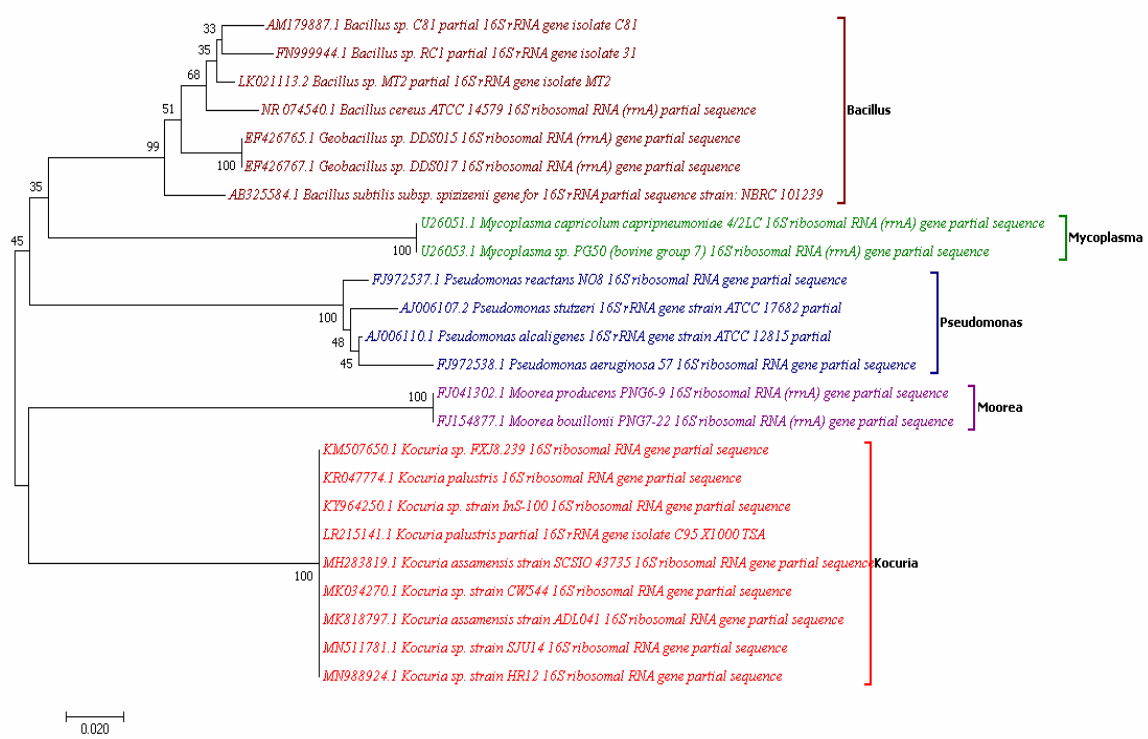

Fig. 5. Phylogenetic tree drawn from the nucleotide sequence of the 16SrRNA gene of the identified bacterium *Kocuria* sp. HR12 strain with bacterial species belonging to the genera Kocuria, Bacillus, Mycoplasma, Pseudomonas and Moorea.

99%) to *Kocuria*sp.strain CW544 (Table 4). Phylogenetic tree results between *Kocuria* sp. strain HR12 with bacterial species belonging to the genera *Kocuria*, *Bacillus,Mycoplasma*, *Pseudomonas* and Moorea showed that *Kocuria* sp. strain HR12 was supported with 100% identity in the *Kocuria*clade (Fig. 5).

pH and Temperature Effects on the Activity of Protease Enzyme Extracted from *Kocuria*sp Strain HR12

Figure 6 shows the result of pH and temperature effects on the activity Kocuria sp. strain HR12 protease enzyme. The results showed that the bacterial enzyme has the

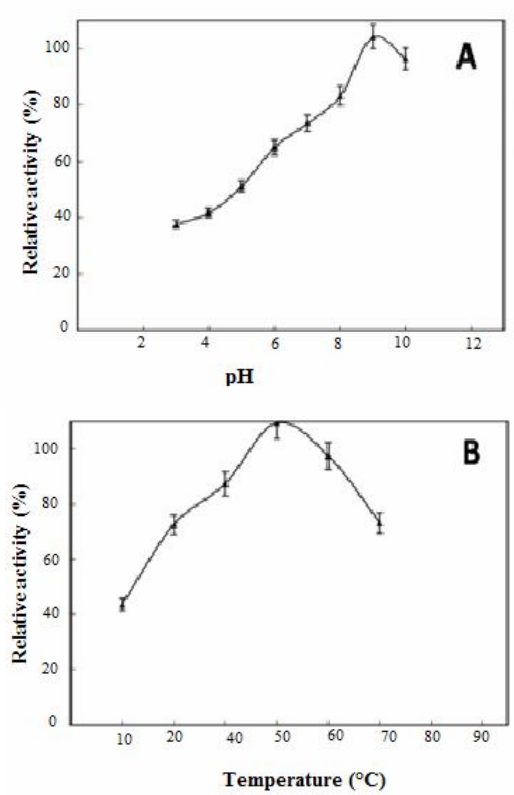

Fig. 6. (A) Effect of pH on protease activity. (B) Effect of Temperature on enzyme activity. Enzyme activity was measured and the results were presented on graph. The optimum temperature was determined for the protease at pH 9 and the optimum temperature of 50 °C. Bars represent means ± standard deviations for 3 replicates.

maximum enzyme activity at pH 9 and optimum temperature of 50 °C (Fig. 6A, B). Also, this enzyme maintained more than 80% of its activity at pH 10 and temperature of 50 to 70 °C. As a result, it can be concluded that the protease enzyme extracted from Kocuria sp. strain HR12 coexisting with mangrove roots at alkaline pH and high temperatures has the highest enzyme activity, so this bacterium has the ability to produce industrial protease enzyme at high temperatures.

DISCUSSION

Based on the results of this study, the optimum temperature for protease activity isolated from Kocuria sp. strain HR12 was 50 °C. These results are in agreement with Nawab et al. regarding the optimal temperature of protease enzyme by *B. subtilis*, which produces protease industrial enzyme [37]. According to BadoeiDelfard study, pH 9 is the optimal pH for the maximum activity of the temperature-resistant bacterial protease enzyme of B. *Tequilensis* [38]. Verma and Pandey (2019) reported that the gram-positive bacterium Citrococcus sp belongs to the family Micrococcuaceae, which was identified based on the Bergey bacteriological manual. The bacterium identified in this study as *Kocuria* sp. strain HR12 belonging to the family Micrococcuaceae were reported [39]. Karn *et al.* (2011), conducted a study on the degradation of pentachlorophenol (PcP), a highly toxic environmental pollutant, at the paper mill treatment plant in Haryana, India, by *Kocuria* sp. CL2 and reported the performance of this bacterium in various industrial applications [40]. Sarafin *et al.* (2014), reported the isolation and identification of *Kocuriamarina* BS-15 from halophilic bacteria isolated from India, indicating the high application potential of this bacterium for industrial uses 41. According to Mukhtar *et al.* (2019), bacteria producing hydrolytic enzymes from the saline rhizospheres of both salsolastochsii and atriplexamnicola led to the identification of Kocuria spp.42. Feng *et al.* (2012), reported that alkaline serine protease belonging to *Kocuriakristinae* F7 has maximum enzymatic activity at pH 9 and 60 °C [43]. Studies have shown that the optimum temperatures for some marine microbial protease strains, such as AP-4 and F1 strains of *Bacillus stearothermophilus*, are 55 °C and 60 °C, respectively [44,45], *penicillumgriseofulvin* at 28 °C [46], *B.licheniformis* at 39.5 °C [47], *Bacillus* sp. Y at 35 °C [48] and *Bacillus* sp. B21-2 has been reported to be 30 °C [49]. Kathiresan and Manivannan (2007) isolated and characterized protease-producing *Streptomyces* sp. from Coastal mangrove sediment. They reported that high level of protease activity was detected at pH 8.5 and was stable for 144 h at 30 °C. The pH of the medium affects the stability of bacterial growth, which has been determined by research on bacterial growth [50]. The pH optimum for alkaline protease are usually above 7.5 [51] and temperature optimum for thermophilic microbes was found to be at a range of 70-80 °C [52,53] as in the present study with *Kocuria* sp. strain HR12 displays high enzyme activity

at pH 9 and temperature at 50 °C. Mamangkey *et al*., 2021; isolated the protease-producing bacteria from the *Bruguiera cylindrical* a mangrove plant and identified them based on the 16S rDNA sequencing. They reported that *Vibrio alginolyticus*as a newly proteolytic agent with protease activity at 0.5% (w/v) sucrose and soy peptone in the fermentation medium[54].Khiftiyah *et al.* (2019) isolated potential microbes withproteolytic activity from mangrove soil. Their results showed that bacterial strains NA1 and PDA2013 are able to produce proteolytic enzymes 55 .Ntabo *et al.* (2021) isolated forty-two bacterial from the leaves and roots of six mangroves and analyzed them based on 16S rRNA. Their studies showed that 95% of the strains had protease activity [56]. Utomo *et al*. (2019) reported that *Enterobacteragglomerans* and *Yersiniaenterocolotica* in mangrove sediment are proteolytic bacteria with optimum pH and temperature of 7 and 33 °C, respectively [57]. Based on studies on proteolytic microbes, it has been reported that the optimum temperature for the production of protease enzyme in *Micrococcus* sp. (50 °C), *Bacillus clausii* I-52 (60 °C), *Pseudomonas aeruginosa* MN1 (60 °C) (Sharma *et al*., 2019), the strain used in this study was 50 °C [58]. The optimum pH for alkaline proteases is in the range of 9-11 [59]. For example, the results show that the optimal pH for *Bacillus firmus* 7728 (pH 9), *Bacillus* sp. JB-99 (pH 11), *Bacillus* sp.SSR1 (pH 10), Micrococcus sp. (pH 11), *Bacillus pumilus* MK 6-5 (pH 11.5) And the optimal PH of Kocuria sp. HR12 from the present study was 958 [60]. Luang-In *et al*. (2019) reported that the highest protease activity was found in Bacillus thuringiensis at pH 8 and 65 °C [61]. Based on the results of this study, the bacterium *Kocuria* sp. strain HR12 appears to be a desirable strain capable of producing thermostable proteases with maximum enzymatic activity at 50 °C.

CONCLUSIONS

This investigation was conducted to isolate protease-producing bacteria from symbiotic bacteria associated with mangrove leave and roots on the west coast of Qeshm Island (KonarSiah), which were characterized based on morphological characteristics, Gramstaining and molecular identification, and finally identified as *Kocuria* sp. This study concluded that the isolated strain is capable of producing alkaline protease. As a result, the highest activity was detected at pH 9 and 50 °C, which suggests that this organism is active in alkaline conditions and produce industrial proteases at high temperatures. This development may provide a viable method for the production of industrial enzymes and widely used proteases from marine bacteria coexisting in mangrove roots. The present study is to draw more researchers' attention to the technology of marine industrial enzymes.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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