Chlorpyrifos pesticide-degrading bacteria isolated from bivalves of Surabaya coastal waters, East Java, Indonesia

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Abstract. Waste from inhabited regions, factories and maricultural practices enter coastal waters through movement and penetration. A study was undertaken to determine the diversity of chlorpyrifos-degrading bacteria isolated from bivalves of Surabaya coastal waters, Indonesia. 14 of 127 bacterial strains isolated from 18 bivalves were selected due to their capability of degrading chlorpyrifos herbicide. These isolates were different in the ability of chlorpyrifos degradation. They utilize chlorpyrifos as the carbon and energy for growth. Their initial degradation at 50 mg L^{-1} concentration within the first 4 days ranged between 7.43 and 68.14%. The 16S rRNA gene sequence analyses indicated that the majority of the isolates belonged to *Vibrio* genera (10 strains), followed by *Virgibacillus* spp. (three strains) *and Bacillus* (1 strain). *Virgibacillus salarius* strain KS4.3 was selected for further study on kinetic growth and chlorpyrifos utilization. These bacterial strains have a great potential utility for the bioremediation of Surabaya coastal waters and for decreasing levels of toxic chlorpyrifos pesticide residues in bivalves.
Key Words: Bacillus, biodegradation, herbicide, Java Sea, *Vibrio*, *Virgibacillus*.

Introduction. Beginning in the mid-1960s, Indonesia introduced the Green Revolution "packages" of high-yielding varieties (HYV), chemical fertilizers, insecticides, improved irrigation, and agricultural extensions, to increase the yield and quality of crop farming (Thorburn 2015). As a result, more Indonesian farmers are using more pesticides than ever before. It is well-known that pesticides can damage human health and cause even death in humans and animals. Sabarwal et al (2018) stated that approximately 300000 deaths occur worldwide every year due to poisoning from pesticides. Many chemical residues show dangerous bioaccumulation levels in the human body and the environment (Puckowski et al 2016).

Surabaya, the second-largest city in the country, is the capital city of East Java province. The city has a high population of over 3 million people living in coastal areas (Mahriyar & Rho 2014). Located on northeastern Java, it mostly has lowlands and river estuaries. Surabaya coastal waters, located in the eastern part of the city, have been polluted by pesticides (Suryono et al 2019). The presence of pollutants in the waters will increase and affect bivalves and other marine living organisms. Humans are the final link in the food chain that will be affected. Accordingly, people become increasingly worried, as they know that they consume the polluted marine biota.

Chlorpyrifos pesticides are the most widely used pest controls in Indonesia (Joko et al 2017). This pesticide compound has the chemical structure [O, O-diethyl O- (3,5,6-trichloro-2-pyridyl) phosphorothioate] and is extensively applied in rice farming, plantations, and in others areas (Wang et al 2013; Gouma et al 2019). The persistence of these pesticide compounds in the soil is usually 9 weeks (Putnam et al 2003). This pesticide is degraded in nature to 3,5,6-trichloro-2-pyridinol (TCP). One characteristic of these pesticides is the bonding relationship between the P-O-C elements, as well as other organophosphate groups, such as diazinon, malathion, and parathion. To date, there are few reports of the degradation of chlorpyrifos compounds in shallow groundwater

(Lapworth et al 2018). TCP has antimicrobial properties that can prevent the proliferation of microorganisms and can reduce chlorpyrifos in the soil (Racke et al 1990).

Currently, many chlorpyrifos products are sold freely in the traditional market. The widespread use of this pesticide has led to wide environmental pollution, like in coastal marine environments. Several previous studies reported that organophosphate residues were detected in sediments and marine organisms. Sabdono et al (2007) reported that small amounts of organophosphate residues were detected in coral tissues from the Java coast, while Suryono et al (2019) demonstrated that organophosphates were found in bivalves from Java coastal waters.

Microorganisms have a crucial role in degrading organic pollutants. It has been widely reported that some bacteria can degrade chemical contaminants and their degradation pathways have been investigated in depth (Hegedus et al 2018; Huang et al 2021), but evidence about microbial degradation of organophosphates in bivalves is still very limited. Until now, little has been reported about the chlorpyrifos degrading bacteria of bivalves. Most of the research on chlorpyrifos pesticides regards mainly soil bacteria, such as *Alcaligenes faecalis* (Yang et al 2005), *Bacillus fumilis* (Li et al 2008), and *Pseudomonas aeruginosa* (Laksmi et al 2009).

Several previous studies have reported bacteria that degrade certain organophosphate pesticide groups that are identified molecularly (Sing 2009; Kumar et al 2018). Some of the chlorpyrifos pesticide degrading genes (mpd and opd genes) were found in several bacteria (Yang et al 2006). Most of these degradation genes are encoded in their plasmids in the same DNA sequence. In contrast to the results of other studies, Horne et al (2002) reported that the chlorpyrifos pesticide degrading gene in *Agrobacterium radiobacter* was encoded in its chromosomes, but retained a DNA sequence similar to the opd gene in other bacterial species. This research was carried out to isolate bacterial strains from bivalves able to efficiently degrade chlorpyrifos. The isolation of indigenous bacterial strains capable of metabolizing chlorpyrifos is favorable for *in situ* bioremediation, given that they would be well adapted to the Surabaya coastal waters.

Material and Method

Sampling and isolation of bacteria associated bivalves. The bivalves, short-necked clam *Paphia undulata* (Born, 1780), arc clams *Anadara inaequivalvis* (Bruiere 1792), and green mussel *Perna viridis* (Linnaeus, 1758), were collected from Surabaya coastal waters at 3 sampling sites, Gresik, Surabaya and Sidoarjo (07°13'26.5"S; 112°46'22.9"E) in June 2018 (Figures 1 and 2). Upon collection, samples were placed into sterile plastic envelopes, placed in a cool-box and transported from the sampling sites to the Tropical Marine Biotechnology Laboratory, Marine Science Department, Diponegoro University, Semarang, Indonesia. The samples were grounded, homogenized, and successively diluted, spread on a half strength agar medium, and incubated at room temperature for 48 hours. Colonies were randomly selected and purified by making streak plates based on morphological features such as color, shape, and size colonies (Madigan et al 2000).

Screening of chlorpyrifos-degrading bacteria. The study of chlorpyrifos degradation was carried out according to the method of Rochaddi et al (2019), with slight modifications. Approximately 0.25 µg bacterial isolate was grown in an Erlenmeyer glass containing agar and 10 mL of 50 mg L^{-1} chlorpyrifos. Furthermore, the bacterial culture was incubated in a 120-rpm rotary shaker at room temperature for 4 days. 1 mL of each culture sample was collected and centrifuged in a microcentrifuge (Microfuge 11; Beckman Instruments Inc., Fullerton, California) at 12000 rpm for 2 min. The supernatant was transferred to an Eppendorf and the chlorpyrifos concentration was measured with a spectrophotometer (Shimadzu UV-Vis double beam model 1700; ג=289 nm). The results were calibrated on a standard curve (Figure 3). A fluorometer (Turner Model III) with a 10-mm square cuvet was used to measure the growth of bacterial isolates.

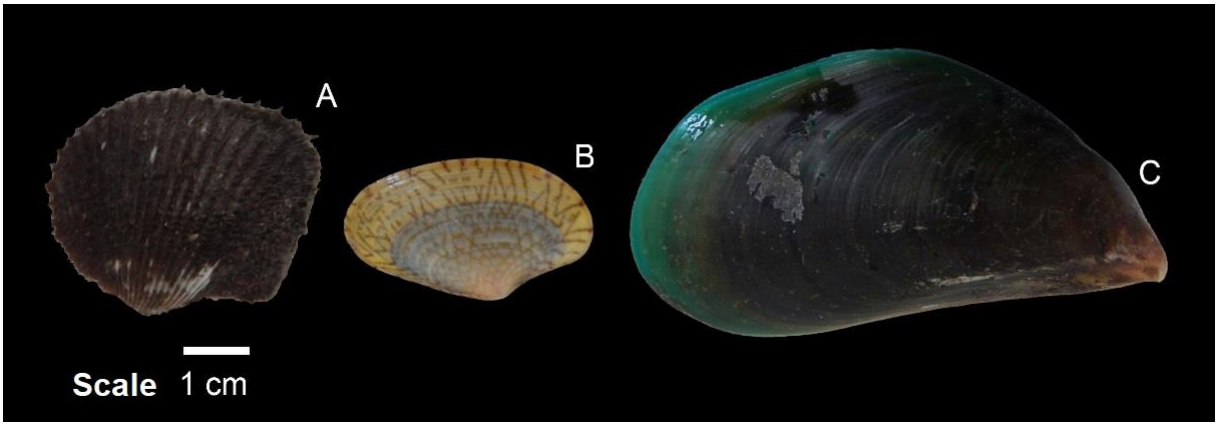

Figure 1. Samples of bivalves: A - short-necked clam *Paphia undulata* (Born, 1780); B - arc clam *Anadara inaequivalvis* (Bruiere, 1792); C - green mussel *Perna viridis* (Linnaeus, 1958).

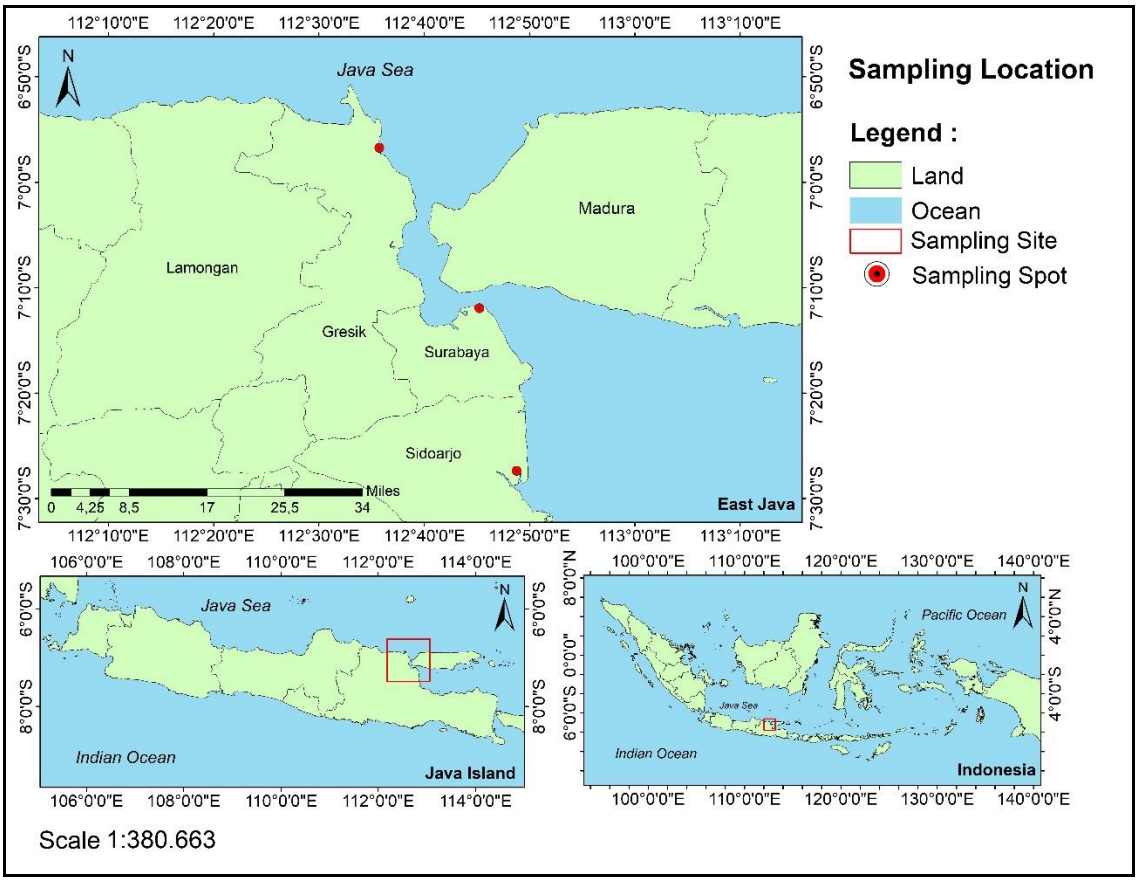

Figure 2. Sampling site locations at Surabaya coastal waters.

Kinetic growth and chlorpyrifos degradation of KS4.3 isolate. Isolate KS4.3 was inoculated in Erlenmeyer flasks (250 mL) containing the nutrient agar (NA) medium (100 mL) supplemented with 30 mg L^{-1} chlorpyrifos with three replications, and placed on a rotary shaker at 120 rpm at room temperature. The bacterial growth and remaining chlorpyrifos were measured by UV-VIS spectrophotometry after 4, 6, 8 10, 12, 16, 20, 24, 30, 36, 48, 72, and 96 hours. After centrifugation, samples were removed, and the supernatant was decanted into an Eppendorf. Spectrophotometry at 289 nm and the calibration curve were used to measure chlorpyrifos concentrations. The KS4.3 bacterial growth was measured by UV-VIS spectrophotometry at a λ of 600 nm.

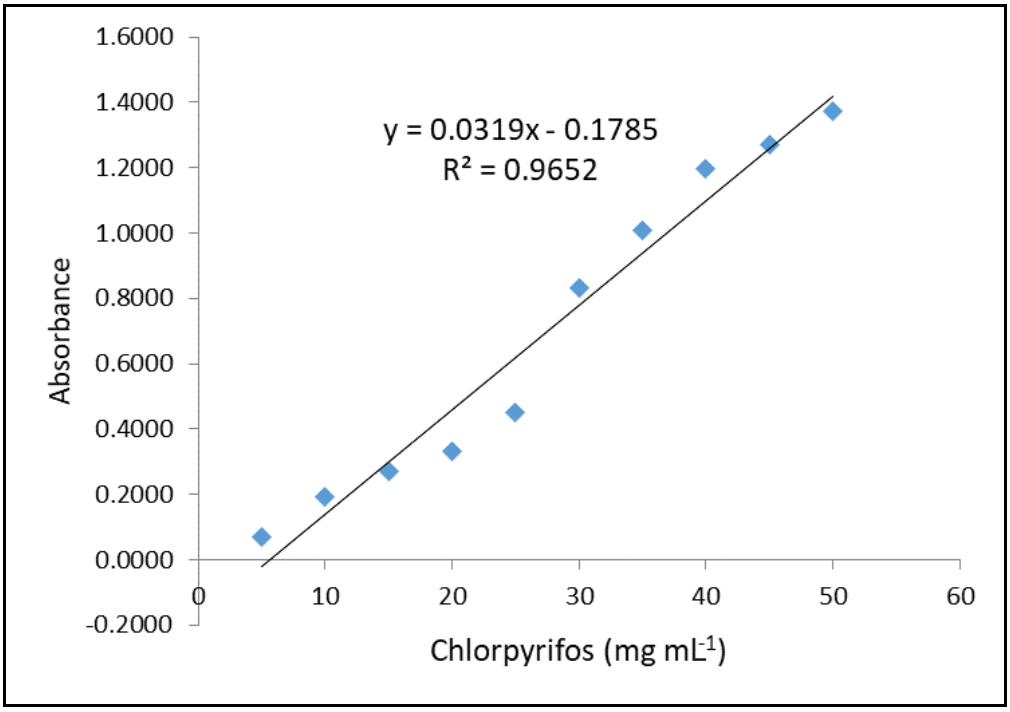

Figure 3. Chlorpyrifos absorbance curve at ʎ=289 nm.

Molecular identification. The method of Radjasa et al (2007) was used to perform DNA extraction, PCR amplification of partial 16S rRNA gene of the bacterial strain, purification of PCR product, and subsequent sequencing analysis. The BLAST database was used to analyze the homology of the DNA sequence of strains.

Phylogenetic-tree construction. The maximum-likelihood analysis was used to construct a phylogenetic tree. To prevent uncertain alignments within highly variable positions of the 16S rDNA, an alignment position with less than 50% of sequences of the entire data set were excluded. Multiple alignments/pairwise DNA sequence was analyzed by CLUSTAL X (Thompson et al 1997). Moreover, the PAUP*4.0 (Phylogenetic Analysis Using Parsimony) software package was used to establish the phylogenetic relationships (Swofford 1998).

Nucleotide sequence accession numbers. Partial 16S rDNA gene nucleotide sequences of the selected strains were deposited in the GenBank database under accession numbers MK774741- MK774754.

Results and Discussion

Chlorpyrifos-degrading bacteria. 127 bacterial strains were isolated from 27 bivalves from 3 sampling locations along the Surabaya coastal waters. 9 bivalves, 3 individual bivalves from each species, were sampled from each location. The bacterial isolates were different in their ability to degrade chlorpyrifos. The assay results showed that 14 (11.02%) out of 127 strains were able to degrade chlorpyrifos. These selected isolates were identified molecularly by analyzing the 16S rDNA. Several previous studies demonstrated that some bacteria were capable of degrading chlorpyrifos from land. Rayu et al (2017) found that three chlorpyrifos-degrading bacteria isolated from farm soils could mineralize chlorpyrifos completely to be used as carbon and energy. Meanwhile, other chlorpyrifos-degrading bacteria such as *Pseudomonas* sp. (Farhan et al 2012), *Klebsiella* sp. (Farhan et al 2013), and *Stenotrophomonas* sp. (Deng et al 2015) were also reported. The degrading ability of 14 strains varied from 7.43 to 68.14% (Table 1).

Only strain KS4.3 showed a very good growth and the highest degradation among these isolates. This strain was used for in-depth further study on chlorpyrifos degradation.

Table 1.Chlorpyrifos-degrading bacteria isolated from bivalves of Surabaya coastal waters

No	*Isolate*	*% degradation*
1	KS-4.3	68.14
2	KS-4.1	47.47
3	KS-3.1	39.59
4	KS-3.2	37.51
5	KS-1.1	30.52
6	KS-5.2	27.40
7	KS-5.1	26.96
8	KS-7.2	24.67
9	KS-7.3	24.62
10	KS-1.2	24.62
11	KS-2.2	21.76
12	KS-4.4	21.52
13	KS-3.3	17.76
14	KS-5.3	7.43

Growth kinetics and chlorpyrifos degradation by KS4.3 strain. The degrading ability of the KS4.3 strain was examined in culture media containing chlorpyrifos. The strain could moderately degrade 30 mg L^{-1} of chlorpyrifos during the logarithmic phase of the bacterial growth (12-30 h). The degradation rate gradually slowed down with the decreased bacterial growth rate after 36 h. 68.14% of chlorpyrifos was removed at the 96 h mark. The results showed that KS4.3 was capable of utilizing the herbicide chlorpyrifos as a source of carbon and energy (Figure 4). Compared to previous studies, the KS4.3 strain could degrade chlorpyrifos better than *Stenotrophomonas* sp. G1 (Deng et al 2015). Rayu et al (2017) reported that *Xanthomonas* sp. 4R3-M1*, Pseudomonas* sp. 4H1-M3, and *Rhizobium* sp. 4H1-M1 hydrolyzed 10 mg L^{-1} concentration of chlorpyrifos within 96 h. Rokade & Mali (2013) showed that *Pseudomonas desmolyticum* NCIM 2112 could degrade 63.52% of chlorpyrifos after 96 h of incubation. However, there are other bacteria that can degrade chlorpyrifos better than the KS4.3 isolate, such as *Bacillus megaterium* CM-Z19 and *Pseudomonas syringae* CM-Z6 (Zhu et al 2019). These strains had a 92.6% and 99.1% degradation of chlorpyrifos-methyl (100 mg L^{-1}) within 5 days of incubation, respectively.

Molecular characterization of chlorpyrifos-degrading bacteria. In this research, 14 chlorpyrifos-degrading strains were obtained from bivalves in the Surabaya coastal waters. Their 16S rDNA gene was sequenced to investigate the phylogenetic and evolutionary correlations among the bacterial strains. The DNA sequences of 14 bacterial strains were successfully amplified using PCR, and identified by using BLAST nucleotides based on GenBank databases. The sequence DNA analysis showed that nucleotide identities varying from 98% to 100% are based on the consensus sequences of 7 species: *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Virgibacillus salarius*, *Virgibacillus harveyi*, *Virgibacillus marismortui,* and *Bacillus tropicus* (Table 2). The best isolate, KS4.3, was identified as *Virgibacillus salarius* and was deposited in the GenBank with Acc. No. MK774748. Phylogenetic analysis clustered the isolates into three clades, *Vibrio* sp., *Virgibacillus* sp., and *Bacillus* sp. (Figure 5). Furthermore, Figure 5 showed that KS4.3 isolate clustered into the *Virgibacillus* sp. group. Earlier studies reported the number of *Virgibacillus* sp. having variable potential for substrate degradation, such as *Virgibacillus flavescens* sp. nov., isolated from marine sediment, could degrade casein, starch, pectin, polygalacturonic acid, carboxymethyl-cellulose, alginic acid, and agar (Zhang et al 2016). Essghaier et al (2012) reported that *Virgibacillus marismortui* strain M3-23 isolated previously from shallow salt lakes in Tunisia could degrade chitin. Montriwong et al (2012) demonstrated that *Virgibacillus halodenitrificans* SK1-3-7 produced high proteinase.

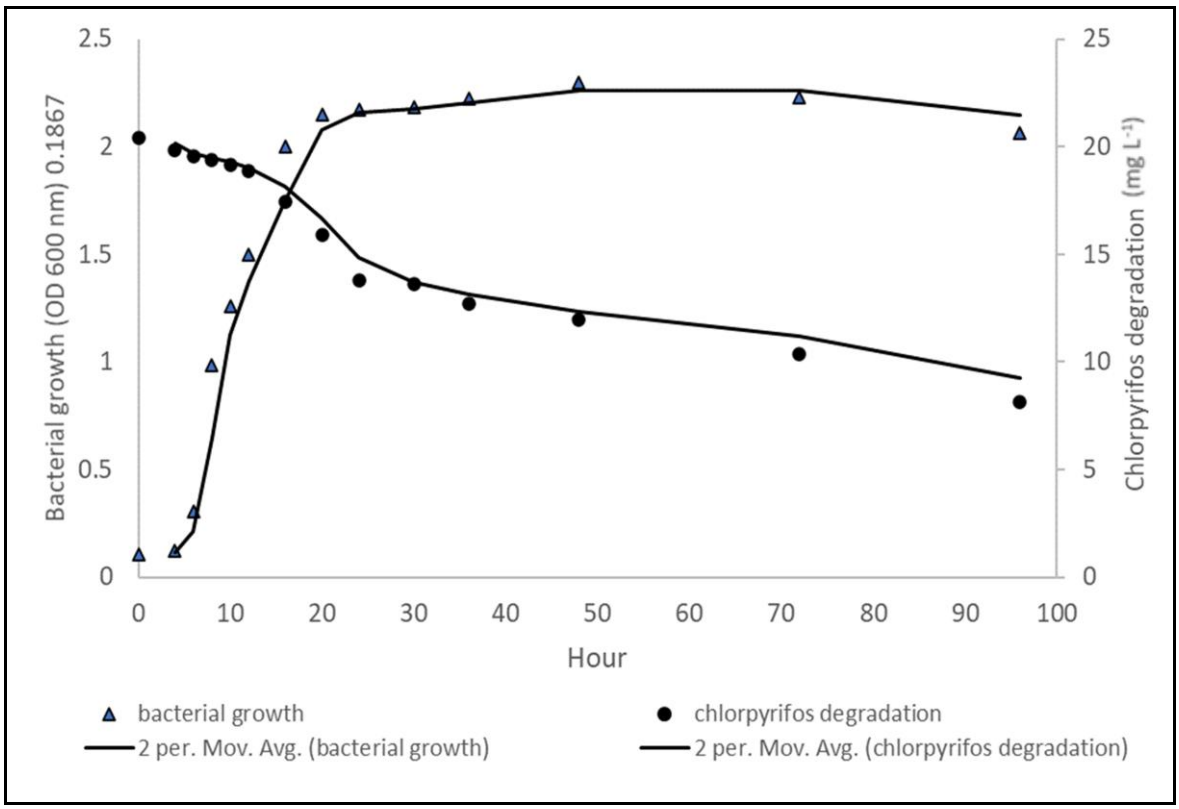

Figure 4. Bacterial growth and chlorpyrifos degradation of KS4.3 isolate; OD - optical density; Mov. Avg. - moving average.

Table 2

Homology analyses and accession no. of chlorpyrifos degrading bacteria

No	*Sequence length (bp)*	*Isolate code*	*BLAST results*	*Homology (%)*	*Accession number*
1	994	KS 1.1	*Vibrio parahaemolyticus*	98	MK774741
2	1439	KS 1.2	*Vibrio alginolyticus*	99	MK774742
3	1427	KS 2.2	*Vibrio parahaemolyticus*	99	MK774743
4	1425	KS 3.1	*Vibrio alginolyticus*	99	MK774744
5	1440	KS 3.2	*Vibrio alginolyticus*	99	MK774745
6	1417	KS 3.3	*Vibrio alginolyticus*	99	MK774746
7	1448	KS 4.1	*Virgibacillus marismortui*	100	MK774747
8	1452	KS 4.3	*Virgibacillus salarius*	99	MK774748
9	1426	KS 4.4	*Vibrio alginolyticus*	99	MK774749
10	1438	KS 5.1	*Vibrio alginolyticus*	99	MK774750
11	1438	KS 5.2	*Vibrio harveyi*	99	MK774751
12	1420	KS 5.3	*Vibrio alginolyticus*	99,58	MK774752
13	1452	KS 7.2	*Virgibacillus salarius*	99	MK774753
14	861	KS 7.3	*Bacillus tropicus*	98,26	MK774754

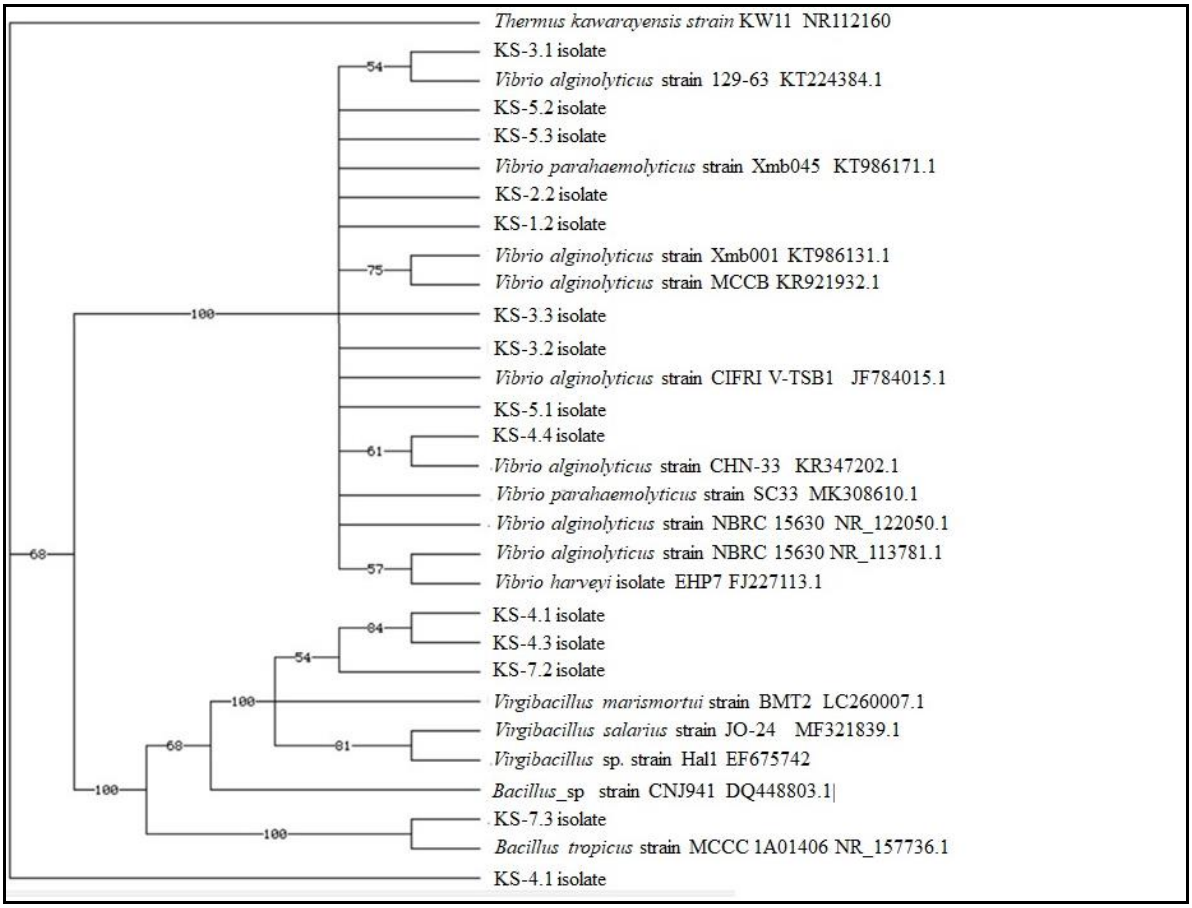

Figure 5. Phylogenetic-tree construction based on comparative 16S rRNA gene sequence analysis of chlorpyrifos-degrading bacteria associated with bivalves. Bootstrap values are expressed as randomization of 1000. *Thermus kawarayensis* strain KW11 NR112160 was used as an outgroup.

Several genera of *Bacillus* were also reported to degrade chlorpyrifos pesticide. However, most of them were isolated from soils, such as *Bacillus cereus* (Liu et al 2012; Jiang et al 2019), *Bacillus megaterium* CM-Z19 (Zhu et al 2019), and *B. subtilis* Y242 (El-Helow et al 2013).

Conclusions. Our laboratory was one of the first to take an interest in the study of bacteria associated with bivalves such as *Virgibacillus*, *Bacillus*, and *Vibrio* genera. In this study, *Virgibacillus salarius* strain KS4.3 was able to remove 68.14% of 30 mg L^{-1} chlorpyrifos in 96 h. This was an important finding since the chlorpyrifos degrading bacterial strains have the potential to develop into promising candidates for bioremediation of coastal waters.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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