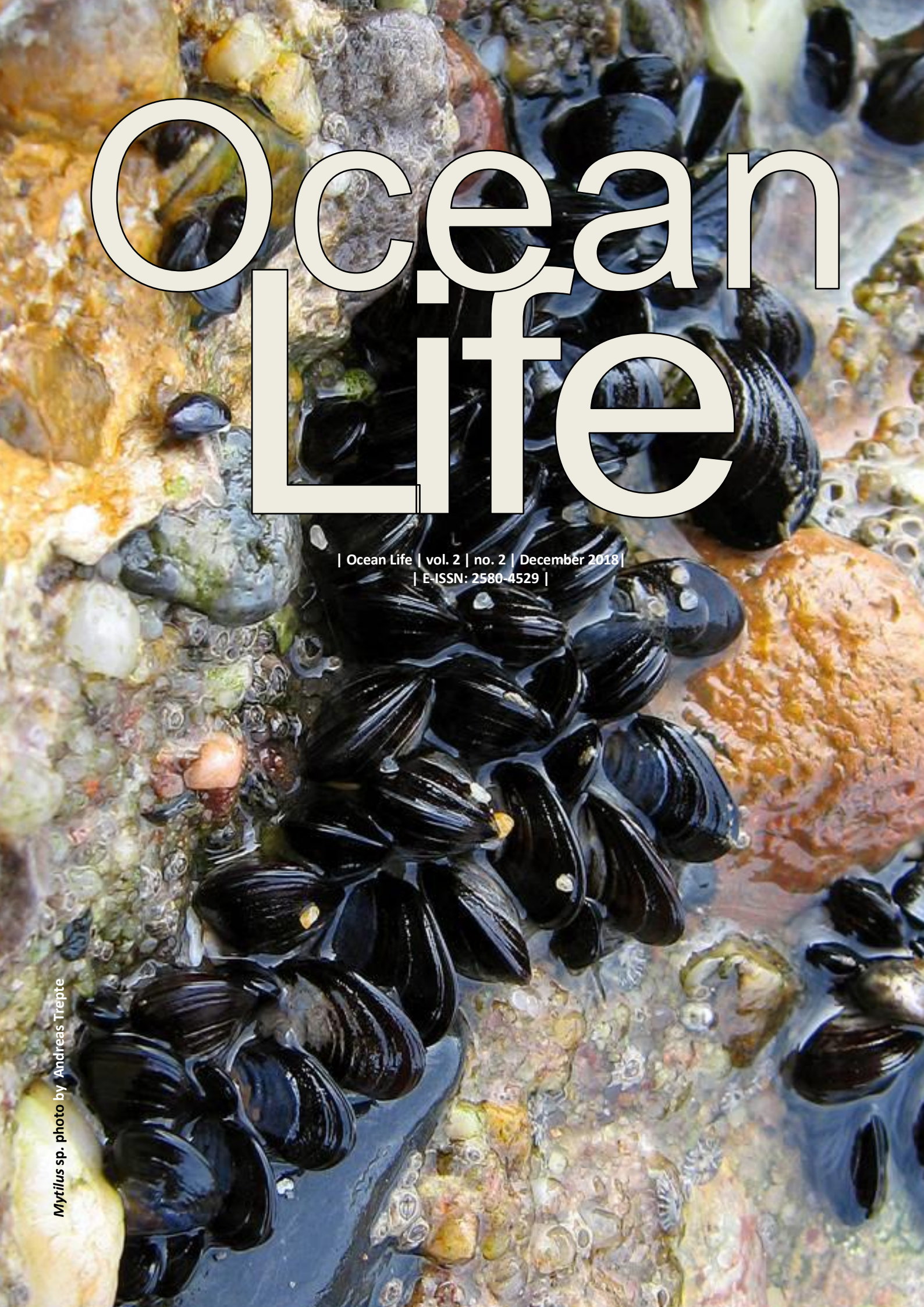

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Mytilus sp. photo by Andreas Trepte

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Tel./Fax.: +62-986-212156/211455, email: ol@smujo.id, joceanlife@unipa.ac.id, joceanlife@gmail.com

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Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

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Fisher perceptions of threats and fisheries decline in the heart of the Coral Triangle

S. NEIL LARSEN[1], CRAIG LEISHER[2], SANGEETA MANGUBHAI[3,4,♥], ANDREAS MULJADI[3], RICARDO F. TAPILATU[5]

[1]1407 6th Avenue, San Francisco, CA 94122, United States
[2]The Nature Conservancy, 4245 N. Fairfax Drive, Arlington, VA, 22203, United States
[3]The Nature Conservancy, Indonesia Marine Program, Jalan Rawa Indah Km. 9, Klawuyuk, Sorong Utara, Sorong 98412, West Papua, Indonesia
[4]Wildlife Conservation Society, Fiji Country Program, 11 Ma'afu Street, Suva, Fiji. Tel: +679 331 5174, Fax: +679 331 0178. ♥email: smangubhai@gmail.com
[5]Research Center for Pacific Marine Resources and Department of Marine Science, Universitas Negeri Papua. Jl. Gunung Salju, Amban, Manokwari, Papua Barat Province 98314, Indonesia

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Abstract. *Larsen SN, Leisher C, Mangubhai S, Muljadi A, Tapilatu RF. 2018. Fisher perceptions of threats and fisheries decline in the heart of the Coral Triangle. Ocean Life 2: 41-46.* The Coral Triangle contains the most species-diverse coral reefs in the world, and at its center is the Raja Ampat archipelago in West Papua, Indonesia. The marine resources of Raja Ampat are an important source of food and livelihood for thousands of people, but overfishing and destructive fishing practices threaten its coral reefs and fisheries. To better understand the threats, we surveyed the 'most knowledgeable fishers' in all 88 Raja Ampat's coastal villages (n = 495) from 2003-2005. We analyzed the links between declines in fish catch and threats to marine resources as perceived by fishers. Blast fishing, cyanide fishing, and 'outsiders' were perceived to be the causes of the fish declines and the greatest ongoing threats to fisheries' resources. We also found evidence of inter-generational differences in perceptions of the health of local fisheries. For fishers with over 15 years of fishing experience, 77% reported a decrease in fish catch. For fishers with less than 5 years of experience, only 41% reported a decrease in the catch. Education and outreach on illegal fishing practices and the benefits of healthy coral reef ecosystems are likely ongoing needs in communities in Raja Ampat.

Keywords: Coral reefs, destructive fishing, marine conservation, shifting baselines, Raja Ampat

INTRODUCTION

Coral reef ecosystems are threatened worldwide by several anthropogenic factors and are a high priority for conservation (Hughes et al., 2003; Bellwood et al., 2004; Brooks et al., 2006). The most species-diverse coral reefs in the Coral Triangle span Indonesia, Malaysia, Papua New Guinea, Philippines, Solomon Islands, and Timor-Leste (Veron et al. 2009). More than 100 million coastal inhabitants in this region rely directly and indirectly on coral reefs for their livelihoods (Hoegh-Guldberg et al., 2009).

In the center of the Coral Triangle is the Raja Ampat archipelago in West Papua, off the northwest tip of the Bird's Head Peninsula, Indonesia. The archipelago consists of the four main islands of Waigeo, Batanta, Salawati, and Misool, along with hundreds of smaller islands encompassing roughly 43,000 km^2 (McKenna et al. 2002). Raja Ampat was declared a maritime regency in 2003, giving it greater autonomy in adopting policies to improve fisheries management for the benefit of local livelihoods and coral reefs. Raja Ampat contains the greatest known concentrations of hard corals and reef fish species on the planet (Allen 2008; Allen and Erdmann 2009; Veron et al. 2009) and is a hotspot for cetaceans (Ender et al. 2014). Subsistence fishing predominantly using handlines from small canoes was the only form of fishing in the region before the 1960s and is still extensively practiced in Papua. However, the introduction of commercial fisheries in the 1960s resulted in a rapid decline in fishery resources due to over-exploitation (Palomares et al., 2007). Overfishing, destructive fishing practices, poorly planned coastal development, and climate change threaten the unique biodiversity and livelihoods of people in this area (Bailey et al., 2008; Mangubhai et al., 2012).

Given its global significance and the threats faced, Raja Ampat is the focus of major ongoing marine conservation efforts by government, communities, and international organizations (Coral Triangle Initiative 2009; Mangubhai et al. 2012). To support establishing a network of marine protected areas (MPAs) in Raja Ampat, The Nature Conservancy conducted a rural coastal appraisal to gather data on marine resource use and threats. This data was collected and analyzed to inform an integrated approach to marine conservation in Raja Ampat that would ensure the protection of local livelihoods and conserve the region's unique biodiversity. In addition, these data were an essential component of a marine conservation planning process to identify and later zone the MPAs in the network (Grantham et al., 2013; Halpern et al., 2013; Mangubhai et al., 2015) and provide a baseline for subsequent adaptive management and impact assessment (Lincoln Smith et al. 2000; Mascia 2003).

The rural coastal appraisal aimed to answer the research questions: (i) what are the demographic characteristics of fishers in Raja Ampat, (ii) how do they perceive fish catches have changed over time; and (iii) if a decline in catch is perceived, what is driving the decline?

MATERIALS AND METHODS

From November 2003 to March 2005, a socio-economic survey was conducted across the Raja Ampat Regency that targeted individuals who were engaged in fishing activities (Figure 1). The survey was designed by The Nature Conservancy and was pre-tested with trained enumerators at Kofiau and Misool islands before implementation. The aim of the survey was to compile fishing data that could inform marine conservation planning processes and future conservation activities. Hence, it used a purposive sampling technique (Shadish et al. 2002) rather than a random sample of the population. The survey was a structured interview with the 'most knowledgeable' fishers to understand better the factors that were perceived to affect marine resources.

When the survey team entered a village, the enumerators consulted with the village head or another community leader who identified the most knowledgeable fishers to be interviewed. Occasionally other individuals in a community would approach the survey team and ask to be interviewed. If the individuals purported to be knowledgeable about fisheries, they were added.

The survey gathered information on respondents' demographic characteristics, use of marine resources, and perceived threats to marine resources. A total of 88 out of the 89 villages in Raja Ampat were surveyed (Figure 1). The omitted village was located inland, and the community did not directly depend on fishing. All interviews were in Indonesian with enumerators from the region who had been trained in socio-economic survey techniques. Before conducting interviews, we obtained participants' free, prior, and informed consent. During the verbal consent process, participants were informed about the survey, its purpose, how the data would be utilized, that participation was voluntary, and that their responses would be kept confidential.

Interview data were entered into a pre-structured Excel database using data validation functions to minimize entry errors. Then, the data were analyzed in Excel and Sigmaplot 11.2.

RESULTS AND DISCUSSION

Results

A total of 495 people were surveyed. Most respondents were male; the average respondent was 36 years old and had completed primary school. Interestingly, 9% of respondents were women, suggesting that fishing is not a male-only activity in Raja Ampat. Less than half of the respondents (48%) identified their livelihoods as exclusively fishing. Slightly fewer (41%) indicated that they both fished and farmed, while the remainder identified themselves as farmers (6%) or had other primary occupations (4%). The majority of respondents (79%) had spent over 15 years fishing, and almost all respondents owned a boat, though only 16% said they had a boat with an engine (Table 1).

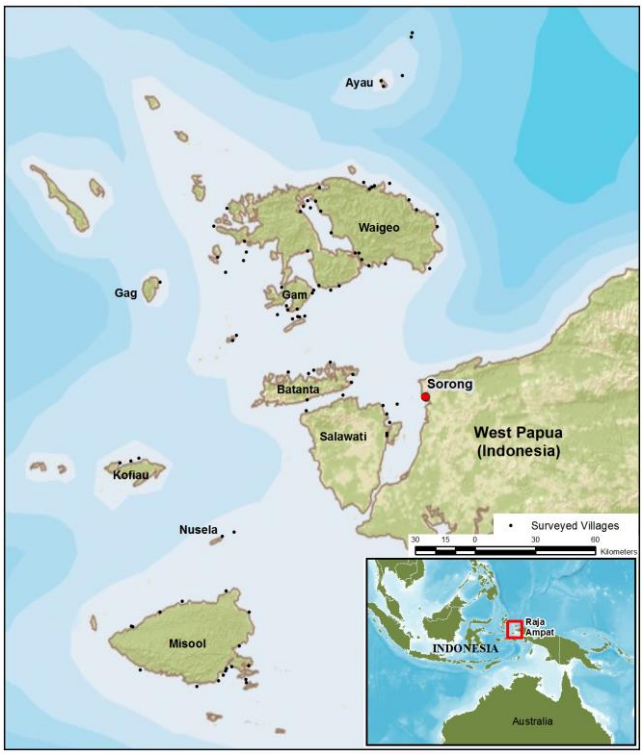

Figure 1. Villages surveyed in the Raja Ampat coastal rural appraisal 2003-2005.

Table 1. Demographic characteristics of survey respondents

Demographics	***N***	**%**
Gender		
Male	450	90.9
Female	45	9.1
Age (mean and stdev)	36.4	10.8
Education		
None	11	2.2
Primary	326	65.9
Junior	95	19.2
Senior or higher	63	12.7
Occupation		
Fisher	239	48.3
Both fisher and farmer	203	41.0
Farmer	31	6.3
Other	22	4.4
Years fishing		
<5	19	3.8
5-15	87	17.6
>15	389	78.6
Boat type		
Boat without engine	406	82.0
Boat with engine	78	15.8
No boat	11	2.2

When asked how to fish catch 'now' compared with 'before' (when the person first started fishing), 74% of respondents said that catch was less, 23.6% said it was the same, and 2% said it was more. Respondents who said that catch was less (n = 367) were asked to name the causes in an open-ended question. More than one cause could be given. The top three causes for catching fewer fish were blast fishing (62%), cyanide fishing (41%), and 'outsiders' (17%) (Figure 2).

All respondents were also presented with a list of possible threats to marine resources and asked whether the threat was ongoing or not. One-third of respondents said there were no ongoing threats on the list. In the districts of Samate, Misool, West Waigeo, and Kofiau, however, nearly 90% of respondents reported ongoing threats. In contrast, in the district of Teluk Mayalibit, only 6% of respondents listed an ongoing threat. Teluk Mayalibit is located along a nearly enclosed bay with coral reefs restricted to the mouth. This unique geographic characteristic may explain the low level of perceived ongoing threats. The ongoing threats chosen (n = 330) were largely the same as the perceived causes of a decline in fish catches: blast fishing, cyanide fishing, and outsiders. Only 4% chose overfishing as an ongoing threat (Figure 3).

In case to examine the degree to which 'outside fishers' are perceived to drive the threats, respondents were asked if they have seen or heard outside fishers in their area. Seventy-eight percent of respondents said yes (n = 388). While 'outside fishers' may be an ambiguous term that could mean people from the next village or people from outside Raja Ampat, it usually means people from the latter (A. Muljadi and S. Mangubhai, personal observations). Its utility comes in distinguishing locally based threats from external threats. When respondents who said they had seen or heard outside fishers were asked the open-ended question, "what sort of activities do outside fishers do in your area," 65% said blast fishing (Figure 4). It suggests that people from outside Raja Ampat who were engaged in blast fishing disproportionately impact the perceived ongoing threats.

When the experience levels of fishers were compared with the perceptions of fish catch, respondents who had fished longer were more likely to perceive that catch had decreased, with the opposite being true for respondents who had spent less time fishing (Figure 5). It suggests that the baseline for fishers with less than 5 years of experience is lower than more experienced fishers, that 5 years is too short a period to notice a decline in fisheries, or that the decline perceived by longer practicing fishers occurred more than five years before the survey. We found a strong correlation (chi-square 13.66, p=0.001) between years of fishing and a perceived decline in fish catch and a weaker correlation between the age of a fisher and a perceived decline in the fish catch (chi-square 4.467, p=0.011), confirming Papworth et al. (2009) supposition that a shifting baseline for fish catches should be present at different levels of experience instead of simply different ages of fishers.

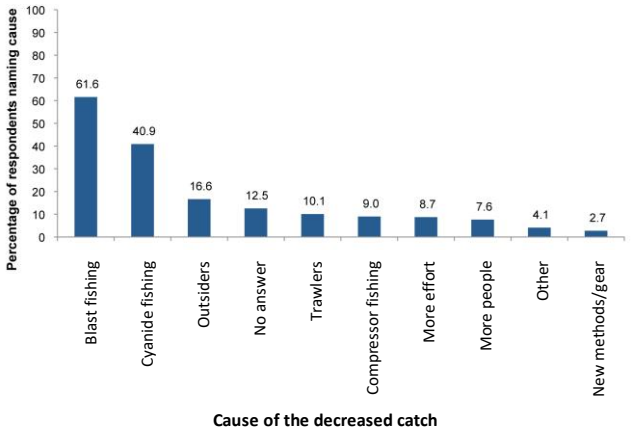

Figure 2. Respondents who said that catch had decreased responded to the question: "What are the causes for the decrease in a catch?" That was an open question where respondents could name multiple causes (*n*=367)

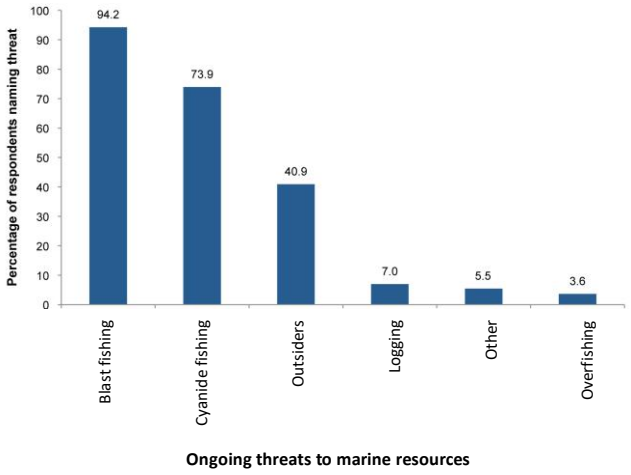

Figure 3. Ongoing threats to marine resources. Respondents could choose more than one threat (*n*=330)

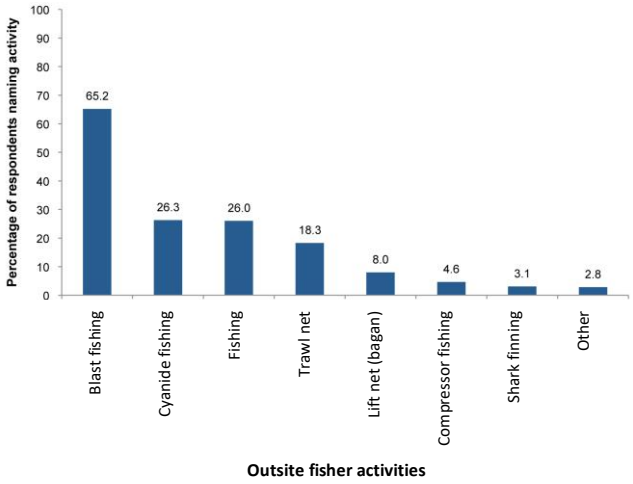

Figure 4. Activities of outside fishers according to respondents. Respondents could name more than one activity (*n*=388)

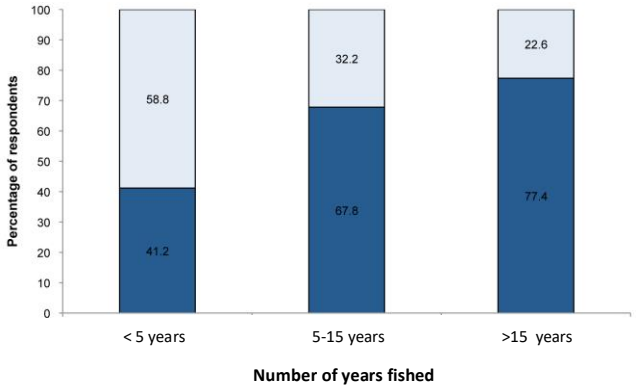

Figure 5. The relationship between the number of years a respondent had fished and their perception of how to fish catch has changed (*n*=495). The dark color represents the percentage of respondents in each age group who reported a decline in a fish caught, while the light color represents the percentage of respondents in each age group who reported that fish catch was the same or more

Discussion

We found evidence of a 'shifting baseline syndrome in the survey data, where successive generations of fishers accept a lower standard of marine resources as normal (Pauly 1995; Knowlton and Jackson 2008). It is important to note the period "before" in the survey was anchored temporally by the respondents to the time when they began fishing. Our results confirm the findings of other studies reporting evidence of shifting baselines among younger fishers in Raja Ampat and that illegal, unreported and unregulated fisheries are critical issues for marine resource management in Raja Ampat (Ainsworth et al. 2008; Varkey et al. 2010; Mangubhai et al. 2012). While it is acknowledged that perception surveys may not reflect what is actually happening in the water, fisher perceptions echoed a study of historical trends in marine resource abundance in Raja Ampat, which shows a 50% decline in fish and invertebrates since the late 1800s (Palomares et al. 2007). Similar trends have been reported from other locations. Evidence from the Gulf of California has shown that there can be a rapid shift in fishers' perception of the size and abundance of marine resources due to resource depletion (Saenz-Arroyo et al., 2005). A rapidly shifting baseline in Raja Ampat disadvantages local people and marine life. If, for example, minimal apex predator populations such as shark and grouper were to become accepted as 'normal,' the fisheries productivity of the Raja Ampat reefs is likely to be lower than if apex predators were present. Evidence from Palmyra, Christmas, and Fanning Islands suggests that reefs where apex predators are protected have 300 to 400% greater biomass than comparable reefs where apex predators have been largely extirpated by fishing (Stevenson et al. 2007).

A 2011 survey of resource use in Raja Ampat's Kofiau and Boo Islands MPA found that 15% of the fishers in the area were not community residents, and these outsiders were responsible for 70% of the catch volume (Muhajir et al. 2012a). Thus, outsiders may be responsible for a disproportionate share of the blast fishing and a disproportionate share of the fish catch. That suggests that enforcing the laws on destructive fishing practices and empowering local communities to exclude outsiders from area fishing grounds may benefit Raja Ampat's people and reefs. Long-term monitoring suggests "hard coral cover, the populations of key fisheries species and fish functional groups are being maintained or improving within the Bird's Head MPA network" (Glew et al. 2015). One study comparing co-managed marine reserves, national parks, and traditional fisheries management approaches found that effective conservation approaches almost always include the ability to exclude outsiders (McClanahan et al. 2006). Since 2012, monitoring efforts have been modified in Raja Ampat to take a more impact evaluation approach which may provide insights into temporal and spatial patterns of MPA impacts once analyzed (Ahmadia et al., 2015; Fox et al., 2017).

While the survey respondents perceived destructive fishing practices and outsiders as the primary causes for a decline in the catch in Raja Ampat, some respondents named additional factors including greater fishing effort, increases in the number of fishers, and the introduction of modern gear. All of these factors may be contributing to declining fish catches, but understanding the magnitude of each factor was beyond the data in this study. For local fisheries management and coral reef conservation efforts addressing the primary perceived causes may provide greater near-term benefits.

A 2010 survey of fishers in northern and western Raja Ampat found that blast fishing was perceived to have decreased compared with five years prior. However, there were still reports of illegal blast fishing perpetrated by outsiders (Sala et al., 2011). In southern Raja Ampat, resource use surveys from 2006-2011 confirmed a decline in the use of destructive fishing gear and destructive methods such as blast fishing within MPAs (Muhajir et al. 2012a, 2012b). While these surveys suggest that destructive fishing practices are declining, blast fishing occasionally occurs within MPAs, showing the need for continuing enforcement and outreach initiatives to protect marine resources in Raja Ampat.

Along with ecological data from Raja Ampat, the results of the rural coastal appraisal were used in the designation of a Raja Ampat MPA network that covers 1,185,940 ha (Mangubhai et al. 2012) and its subsequent zonation (Grantham et al. 2013). The results also helped to guide the development of socio-economic criteria for the zoning of the Raja Ampat MPA network (Table 1 in Mangubhai et al. 2015). Once the MPAs were declared in 2007, the data from the appraisal was used to guide initial conservation efforts in Raja Ampat and to build support for conservation by engaging with local communities (Leisher et al., 2012). A tourism entrance fee system in Raja Ampat has generated revenues for local government priorities, community conservation initiatives, and community well-being programs, as well as helped build support for conservation action by incentivizing compliance with fishing regulations (Mangubhai et al. 2011).

Given the large area of the Raja Ampat Regency and the finite budget for enforcing fishing regulations, local communities may benefit from having MPAs that are large enough to support apex predators and maintain ecosystem processes (Salm et al. 2000; McLeod et al. 2008; Jaiteh et al. 2016) but not so large that enforcement costs become prohibitive. A study from Indonesia and Papua New Guinea found that MPAs within the community's line of sight had greater success with excluding outsiders and curtailing illegal fishing activities than those not fully visible to local communities (McClanahan et al. 2006). While the proximity of MPAs to communities is advantageous for self-policing, communities still need adequate resources to conduct patrols and communicate infractions in real time to authorities (Sala et al., 2011).

Education and outreach on legal and illegal fishing practices and the benefits a restored coral reef ecosystem can provide are likely ongoing needs in Raja Ampat. From 2005 to 2010, the knowledge and attitudes in MPAs in southern Raja Ampat shifted towards more sustainable use of marine resources (Leisher et al. 2012), but continued efforts are needed, especially among younger fishers who may have baselines that do not fully reflect an understanding of the benefits that an intact coral reef ecosystem can provide. In addition, a much broader outreach strategy is required that extends to communities outside of MPAs to ensure other reefs in the regency are not being degraded and continue providing food and livelihoods for local communities.

To conclude, most fishers interviewed perceived that the marine resources they depend on for their livelihoods were declining. The primary cause of this decline was illegal, destructive fishing methods that many associated with the presence of outsiders. Subsequent studies have shown that while there has been progressing in addressing these threats, they still exist and pose an ongoing threat to people and nature in Raja Ampat. Our results have since guided engagement with local communities and informed the development and implementation of an MPA network to address these threats. In addition, this survey is unique in that it provides a valuable baseline of how fishers from all of the coastal villages in Raja Ampat perceived the status of and major threats to local marine resources at the time when major national and international conservation initiatives were beginning in the region. Thus additional research examining how fisher perceptions of marine resource abundance and threats in Raja Ampat have changed since the 2003 survey would allow a valuable assessment of the effectiveness of ongoing conservation, education, and enforcement initiatives in the region.

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Recent coral reef conditions in Weh Island, Aceh Province, Indonesia

RIZKIE SATRIYA UTAMA♥, TRI ARYONO HADI
Research Centre for Oceanography, Indonesian Institute of Science Jl. Pasir Putih I, East Ancol, North Jakarta 14430, Jakarta, Indonesia.
Tel.: +62-21- 64713850, Fax.: +62-21-64711948, ♥email: rizkie.s.u.biogama@gmail.com

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Abstract. *Utama RS, Hadi TA. 2018. Recent coral reef conditions in Weh Island, Aceh Province, Indonesia. Ocean Life 2: 47-53.* Over the past several decades, coral reef conditions have declined globally due to human activities and natural disturbances. In the last decade, several natural phenomena, such as a 2010 tsunami and 2016 coral bleaching event, have been recorded on Weh Island and resulted in coral cover decline. The aims of this study are to observe the current status of coral diversity and reef conditions at Weh Island. The study was carried out in February 2017 at ten study sites. The methods used were Underwater Photo Transect (UPT) analyzed with CPCe 4.1. software. Live coral coverage ranges from 10% to 57,33%, with average live coral cover at Weh island at 28.48% ± 5.334 (moderate condition). About 82 species, 31 genera, and 13 families of coral were recorded in this study, with four species found at all sites. There are 'no take and no anchor zones' from Panglima Laot regulation in the Iboih areas, which positively impact coral existence.

Keywords: coral cover, coral reef conditions, Weh Island

INTRODUCTION

Coral reefs are important ecosystems that provide many benefits (food and services) to many coastal societies (Moberg and Folke 1999). Weh Island water is influenced by three different ecoregions (Andaman Sea, Indian Ocean, and Malacca Strait). These thus affect the area's marine biodiversity, especially on the reef. Unfortunately, Weh Island is far from the coral triangle area and the national capital, making it difficult to obtain coral reel data from this area (Gibson et al. 2007). However, based on records from the nearest locations of Thailand and West Sumatra, there are 339 corals from the Andaman Sea to West Sumatra and around 59 species of which are *Acropora* (Veron et al. 2009; Wallace et al. 2012).

Over the past several decades, coral reef conditions have declined globally due to human activities and natural disturbances. Human impacts contribute to a huge portion of the decline in developing worlds through factors such as sedimentation, nutrient enrichment, and habitat loss by destructive fishing (Bruno et al. 2003; Erftemeijer et al. 2012; Nyström et al. 2000) and are the main cause of the decline of coral communities in these areas. Furthermore, climate change accelerates natural influences such as coral disease prevalence, coral bleaching events, and frequent cyclone hurricanes (Hoegh-Guldberg 2011). In addition, prolonged habitat degradation could affect coral community structure and diversity. In the last few decades, several natural phenomena such as tsunami (2004) and coral bleaching in 2010 and 2016 have been recorded in Weh Island and resulted in coral cover decline (Ampou et al. 2017; Rudi et al. 2012). Therefore, obtaining information on the current status of coral diversity and reef conditions at Weh Island is important.

MATERIALS AND METHODS

Study site

This research was conducted at 10 sites from the west to the east coast of Weh island (

Figure **1**). In the west part of Weh Island, Aceh Province, Indonesia, the reefs are short with strong current and wave action, and the slope is steep, which creates a wall-like structure and high water visibility. In the eastern part, the reefs consist of a long flat area with moderate current and wave action, and the slope inclines less than 30^o. In the bay areas, the reefs are short with low current and wave action, and the slope inclines between 30-45^o. Of these three areas, only the west portion was less populated. The eastern and southern areas were moderately populated with many tourism activities.

Coral survey method

In this study, we used a modified Underwater photo transect (UPT) method (Giyanto et al. 2010). Three replicates of 10-meter-long transects were laid parallel to the coastline on the reef slope at approximately 5-7 meters depth. A 20-meter distance separated each replicate transect. A total of 30-meter transect lines were used in this study, with a photo of the quadrat frame (44 x 58 cm^2) for each meter. An underwater camera (Canon Power Shoot G1X, 14 Megapixel image resolution) protected with a waterproof casing (Ikelite 6146.01) was used to take images of the benthic substrate communities along the transect lines. Additional close-up images of corallites were taken to help with the coral identifications.

Image processing and data analysis

For assessment of benthic community compositions and substrate compositions, photos were analyzed using CPCe

4.1 software (Kohler and Gill 2006). A total of 50 random points were placed on each photo, and each point was assigned to one of the categories (in Giyanto et al. 2010), and the species of live coral was identified. To avoid clumped points, we used stratified random as the spreading method with 5 columns and 5 rows with 2 points on each box. Corals were identified following the Indo-Pacific Coral Corals of the World (Veron and Smith 2000) and Revision and Catalogue of Worldwide Staghorn Corals Acropora and Isopora (Scleractina: Acroporidae) in the Museum of Tropical Queensland for determined *Acropora* (Wallace et al. 2012). Each Overall total live coral cover was calculated. In addition, corals were further separated into morphological categories to assess coral morphology composition. The percent cover of living corals was categorized into 4 ranks, i.e., poor (0-24,9% live cover), moderate (25-49,9% live cover), good (50-74,9% live cover), and excellent (75--100% live cover).

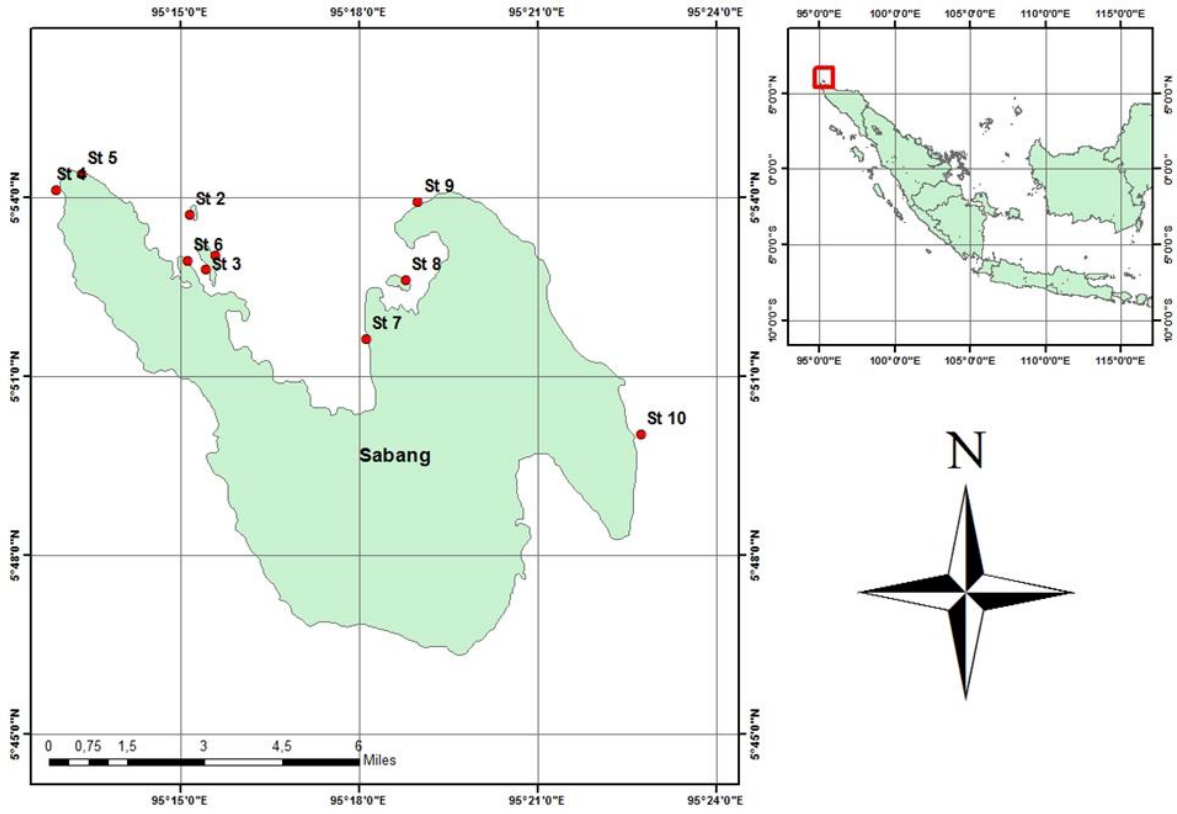

Figure 1. Location of study sites in Sabang waters

Table 1. Code for each benthic category (biota and substrate)

Morphology/Categories	Code	Description
Acropora	AC	Acropora branching, tabulate, digitate and submassive
Branching Coral	CB	Branching non-Acropora corals, especially Porites cylindrica, some other spp.
Encrusting Coral	CE	Low relief, often small colonies, Tabular non-Acropora
Massive Coral	CM	Massive or dome-like corals of all sizes.
Foliose Coral	CF	Foliose, either horizontal or vertical, non-Acropora, especially Montipora, Echinopora
Submassive Coral	CS	Multilobate or "lumpy" corals, sometimes columnar or mixed massive- columnar, especially Goniopora, Galaxea
Mushroom Coral	CMR	Free-living fungiid corals
Millepora	CME	Various species of Millepora. (hidrocoral) Blue coral (hidrocoral)
Heliopora	CHL	Free-living fungiid corals
Dead coral with algae	DCA	Recent death coral and dead coral covered by algae
Soft coral	SC	Alcyoniidae and gorgonians
Sponge	SP	All sponge
Fleshy seaweed	FS	Macroalgae and turf algae
Other fauna	OT	Other benthic fauna
Rubble	R	Broken dead coral (Substrate)
Sand	S	Sand and silt (Substrate)
Rock	RCK	Natural rock (Substrate)

Data analyses

A multivariate analysis of benthic categories and substrate types was evaluated using multidimensional scale (MDS) ordination. The data were transformed by using log (x+1) to improve the spread of the data. The statistic test was performed using Primer software.

RESULTS AND DISCUSSION

Coral condition and reef classification

Based on UPT data, the live coral coverage ranged from 10% to 57,33% (Figure 2). The average live coral cover at Weh island was 28.48% ± 5.334 (SE). The highest live coral cover was found at site 02, and the lowest coral cover was at site 01. These two sites had different substrates, and site 02 is mostly DCA substrate; site 01 is mostly sand substrate. The coral larva that attaches to stable substrates like DCA or rock had a more significant chance to grow than unstable substrates like sand and rubble. Therefore, at site 01, coral stations grew in clusters with rock substrate or DCA. Dead coral dominated abiotic categories with algae (DCA) with an average coverage of 35,92%, followed by sand at 18,78%. The highest DCA coverage was recorded at station site 06, with coverage of 61,44%. At station site 04, another biota was recorded at high coverage of 10.51%. Encrusting sponges and zoanthid dominated this site. At this station, current and waves can be too strong for juvenile coral to attach to the substrate.

Coral diversity and distribution

Based on image processing data analysis, 82 species, 31 genera, and 13 families of coral were recorded in this study, with four species found at all sites (Table S1). Site 5 had the largest number of species (37), followed by site 2 (31). On the other hand, the smallest number of species were observed at site 10 (7 species). Acroporidae and Faviidae were the most diverse families at Weh Island. There were 21 species of Acroporidae and 31 species of Faviidae. Though less diverse, corals can still be found at 25 meters in depth.

Poritidae was dominant with an average cover of 17,05%, followed by Acroporidae, Helioporidae, and Faviidae with coverages of 3.61%, 3.50%, and 1.99%, respectively (Table 2). *Porites* were the most widely spread coral and were dominant among other genera, with an average coverage of 17,05%. Other genera had average coverage of < 5%. *Porites* were found well distributed and abundant at bay areas and along the east coast of Weh Island. On the west part of Weh Island (site 4 and site 5), *Porites* coverage was low and was dominated by *Pocillopora* and *Montipora*.

The multivariate analysis shows the characteristics of the study sites concerning the benthic category and substrate type (Figure 3). The results show that there are 4 clusters of study sites. The first cluster was comprised of site 1, site 3, site 5, site 7, site 8, site 9, and site 10. All sites in this cluster were dominated by *Porites* but had a great diversity of corals. However, another cluster consisted of one site (site 2, site 4, and site 6) for each cluster, which means each site has unique characteristics. Site 2 was placed near the center of the vector and was the most diverse among all sites. Site 4 was dominated by *Pocillopora*, and DCA dominated the substrate. Finally, site 6 was dominated by *Heliopora*.

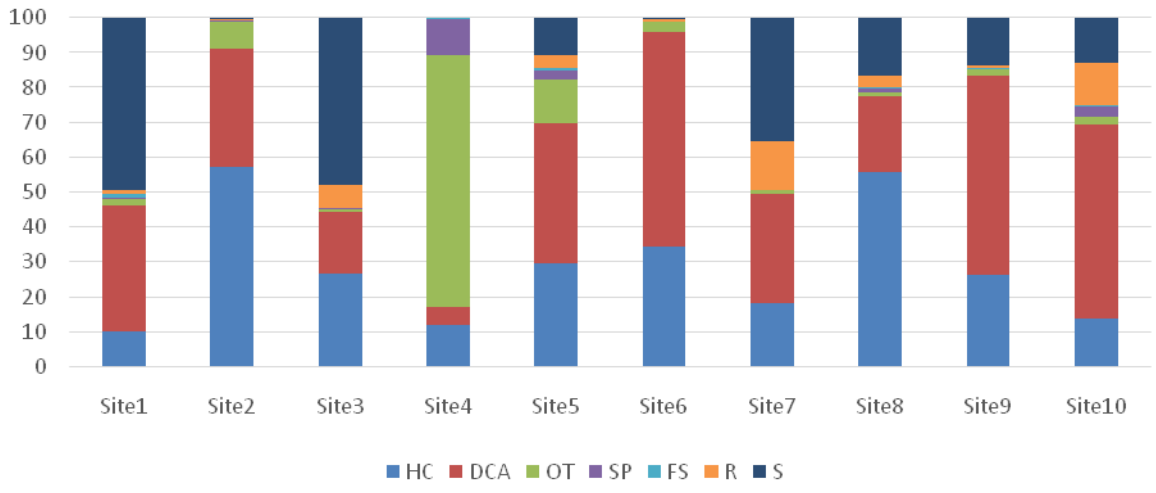

Figure 2. Percentage coverage of benthic categories and substrate. HC: Hard coral cover; DCA: dead coral with algae; OT: other biotas; SP: sponge; R: rubble; S: sand

Table 2. Percentage coverage of coral families

Categories	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10
Acroporidae	1.22	1.56	0.44	4.11	17.67	0.44	3.78	4.33	1.78	0.78
Faviidae	1.44	5.78	1.00	0.67	5.00	0.00	2.67	2.11	0.78	0.44
Poritidae	6.00	46.78	24.67	0.89	4.78	0.89	5.89	46.78	22.89	11.22
Helioporidae	0.44	0.56	0.00	0.00	0.00	32.00	0.00	0.33	0.44	1.22
Pocilloporidae	0.00	0.11	0.00	5.78	1.11	0.78	4.22	0.11	0.11	0.00
Milleporidae	0.33	0.56	0.00	0.22	0.33	0.00	0.22	0.11	0.00	0.11
OHC	0.56	2.00	0.55	0.33	0.89	0.44	1.44	2.11	0.44	0.11

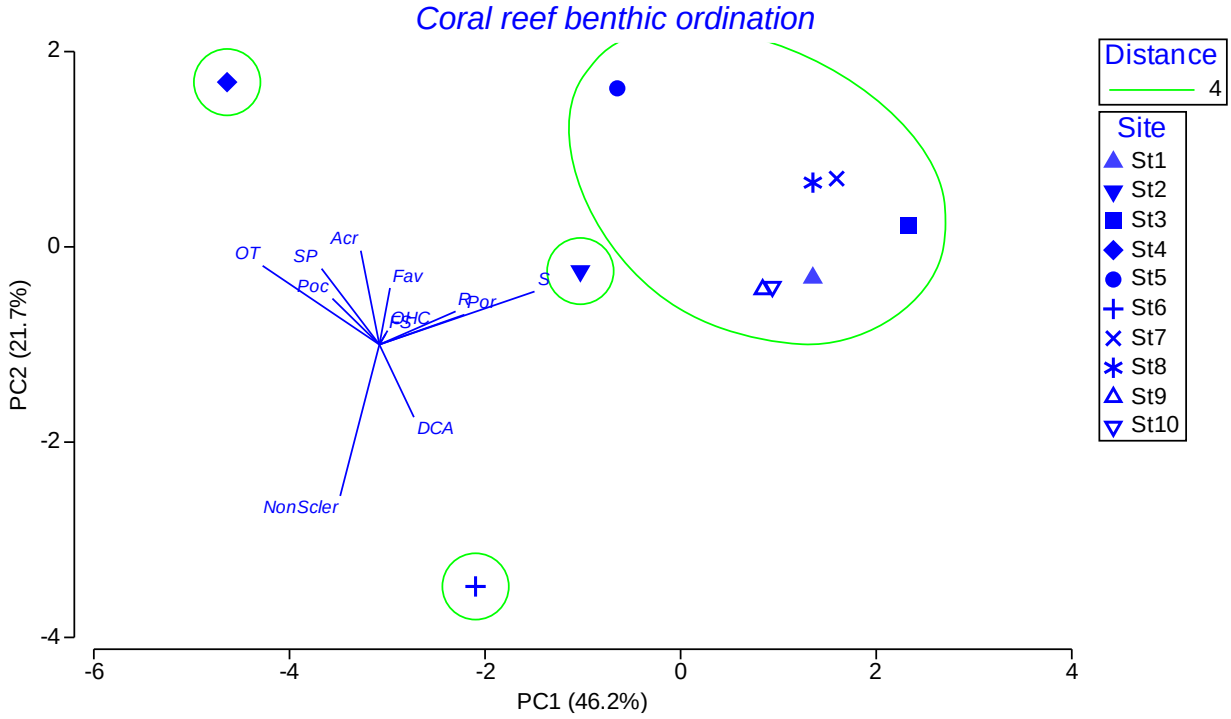

Figure 3. Multivariate analysis of benthic and substrate categories. Benthic and substrate categories: Acr (Acroporidae), Fav (Faviidae), Por (Poritidae), Poc (Pocilloporidae), OHC (Other Hard Coral), Non-Scler (Milleporidar and Helioporidae), SP: Sponge; FS: Fleshy Seaweed; OT: Others; R: Rubble; S: Sand; DCA: Dead Coral with Algae) (DC and RCK category merged with DCA; Si merged with S

Discussion

Coral conditions at Weh island were generally at moderate condition. There is no difference in terms of coral condition with previous studies conducted by Rudi (2010) (LIT method) and Hastuty and Adrianto (2014) (PIT Method). Most reef sites in developed areas, with many resorts and residential sites, have been a focal point for tourists to venture into water sports activities such as snorkeling, diving, and boating, and these activities may be harmful to coral reef health (Erftemeijer et al. 2012; Roche et al. 2016). However, a proper management system can reduce the risk of damage due to human impacts. Weh Island has two MPAs: Weh Island Marine Recreational Park (WMRP) and Weh Island Marine Protected Area (WMPA), which are managed by government enforcement (Natural Resources Conservation Agency and Marine Affairs and Fisheries Agency), NGO's and the local community (Panglima Laot). In this area, they have a no take zone policy and restricted activities (only recreational, research, restoration, and fishing with limited gears). According to (Hughes et al. 2007) the existence of managed areas and the prohibition of fishing is an important way to ensure the occurrence of the food chain, the existence of a good ecosystem function, and the resilience of a coral reef. Good cooperation between government and the local community in the development of rules and regulations, enforcement, and monitoring is a major factor for successful management of marine protected areas in Sabang (Kusumawati and Huang 2015)

Based on multivariate analysis, there were 4 cluster groups by living coral and substrate (Figure 3). Site 4 was different from other sites with corals from Acroporidae and Pocilloporidae families, growing fast and dominant among other stony corals. This site was located northwest of Weh

island, with good water visibility, good water circulation, low sedimentation, and good conditions for *Acropora* and *Pocilloporat*o to grow quickly (Wallace 2011). Site 5 was dominated by Poritidae, but this site was clustered differently from the other sites. We can find all the corals families observed on this research site. Finally, another site (site 6) was dominated by stress-tolerant corals like massive coral and non-scleractinian.

We found 82 hard coral species from 31 genera and 13 families based on data. This number was high for an area far from the heart of the Coral Triangle. *Porites*, *Heliopora*, and *Montipora* were the highest coral genera recorded in this study. Previous studies reported that Weh Island was dominated by *Porites* and *Heliopora* (west) and *Acropora* (north and east) (Baird et al., 2012; Rudi, 2010). *Porites* and *Heliopora* are stress-tolerant corals (Edinger and Risk 2000; Loya et al. 2001). *Porite* tissue is seated deeper in the skeleton; thus, it is better shaded from high irradiance. Porites are less prone to bleaching than *Acropora* (Hoegh-Guldberg and Salvat 1995). Porites colonies can survive at high suspended sediment by removing sediment with their own cleaning mechanism (Pichon 2011). Therefore, it can be assumed that the dominance and abundance of *Porites* in Weh island are caused by the changing of the environment due to global warming and the increase of human activities in that area

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Table S1. List of coral species at Weh Island, Aceh Province, Indonesia

Species	Site									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Acroporaclathrata	-	-	-	+	-	-	-	-	-	-
Acroporacytherea	-	-	-	-	+	-	-	-	-	-
Acroporaflorida	-	-	-	-	+	-	-	-	-	-
Acroporagemmifera	-	-	+	-	+	-	-	-	-	-
Acroporamonticulosa	-	-	-	-	+	-	-	-	-	-
Acroporasolitaryensis	-	-	-	+	-	-	-	-	-	-
Acropora sp.	-	+	-	+	+	+	-	-	-	-
Astreoporamyriophthalma	+	-	-	-	+	-	+	-	-	-
Astreoporaocellata	-	-	-	-	-	-	+	-	-	-
Astreopora sp.	-	+	-	-	-	-	-	-	-	-
Barabattoiaamicorum	-	-	-	-	-	-	-	+	-	-
Coeloserismayeri	-	+	-	-	-	-	-	+	-	-
Cyphastrea japonica	+	+	-	-	+	-	-	+	+	-
Cyphastreaocellina	-	-	+	-	-	-	-	-	-	-
Cyphastreaserailia	-	-	-	-	-	-	-	+	-	-
Cyphastrea sp.	-	-	+	-	-	-	-	-	-	-
Diploastreaheliopora	-	+	-	-	-	-	+	-	-	-
Echinophylliaaspera	-	+	-	-	-	-	-	-	-	-
Echinoporagemmacea	-	-	-	-	-	-	+	+	-	-
Faviamatthaii	-	+	-	-	+	-	+	-	-	-
Favia pallida	+	-	-	-	-	-	+	-	-	-
Faviatruncatus	-	-	-	-	-	-	+	+	+	+
Favia sp.	+	-	-	-	-	-	-	-	+	-
Favitesabdita	-	-	-	-	+	-	-	-	-	-
Favitescomplanata	+	+	-	-	+	-	+	-	-	-
Faviteshalicora	-	-	-	-	+	-	-	-	-	-
Favitesmicropentagona	-	+	-	-	+	-	-	-	-	-
Favitespentagona	-	+	-	-	+	-	+	+	-	-
Favites sp.	+	-	-	-	+	-	-	-	-	-
Fungiadanai	-	+	-	-	-	-	-	-	-	-
Fungiafungites	+	-	+	-	-	-	-	-	-	-
Fungiarepanda	-	-	-	-	-	+	-	-	-	-
Galaxeafascicularis	+	-	-	-	-	-	+	+	+	-
Galaxea sp.	+	-	-	-	-	-	-	-	-	-
Gardineroserisplanulata	-	+	-	-	-	-	-	-	-	-
Goniastreaminuta	-	+	-	-	-	-	+	-	-	-
Goniastreapectinata	-	-	-	-	-	-	+	-	-	-
Goniastrearetiformis	+	+	-	-	+	-	-	+	-	-
Helioporacoerulea	+	+	-	-	-	+	-	+	+	+
Hydnophoramicroconos	+	-	-	-	-	-	-	-	-	-
Leptastreabeickensis	-	+	-	-	-	-	-	-	-	-
Leptastreabottae	-	-	-	-	-	-	-	+	-	-
Leptastreapruinosa	-	+	-	-	-	-	-	-	-	-
Leptastreapurpurea	+	+	+	-	+	-	+	+	+	-
Leptastrea sp.	-	+	-	-	-	-	-	-	-	-
Leptastreatransversa	-	-	-	+	-	-	-	-	-	-
Leptoriaphrygia	-	-	-	-	-	-	-	-	-	-
Leptoserisyabei	-	-	-	+	-	-	-	+	-	-
Millepora sp.	+	+	-	+	+	-	+	+	-	+
Montastreaannuligera	-	-	-	-	+	-	-	-	-	-
Montastreavalenciennesi	-	+	-	-	+	-	+	-	-	-
Montiporacaliculata	-	-	-	-	+	+	+	+	+	+
Montiporagrisea	+	+	-	+	+	-	+	+	-	-
Montiporainformis	+	-	-	-	+	-	-	-	-	-
Montiporaorientalis	-	-	-	-	+	-	-	-	-	-
Montiporapeltiformis	-	-	-	-	+	-	-	-	-	-
Montipora sp.	-	+	+	+	+	-	+	+	+	-
Montiporastellata	-	-	-	-	+	-	-	-	+	-
Montiporatuberculosa	-	-	-	-	+	-	+	+	-	-
Montiporaturgescens	-	-	-	-	-	-	+	-	-	-
Montiporaundata	+	-	-	-	+	-	-	+	-	-
Montiporavenosa	+	+	-	-	+	-	-	+	-	-
Oulophylliabennettae	-	+	-	-	-	-	-	-	-	-

Pachyserisgemmae	-	-	-	-	-	-	+	+	-	-
Pavonaminuta	-	+	-	-	-	-	-	-	-	-
Pavonavarians	-	+	-	-	+	-	-	+	-	-
Pavonavenosa	-	-	-	-	-	-	-	+	+	-
Platygyradaedalea	-	+	-	-	-	-	-	-	-	-
Platygyraryukyuensis	-	-	-	-	+	-	-	-	-	-
Physogyralichtensteini	-	-	-	-	-	-	-	+	-	-
Pocilloporadamicornis	-	+	-	-	-	+	+	-	-	-
Pocilloporameandrina	-	-	-	+	-	-	-	-	-	-
Pocillopora sp.	-	-	-	+	-	-	-	-	-	-
Pocilloporaverrucosa	-	-	-	+	+	+	+	+	+	-
Porites lobata	+	+	+	+	+	-	+	+	+	+
Porites lutea	+	+	+	+	+	+	+	+	+	+
Porites rus	-	-	-	-	-	+	-	+	-	-
Porites solida	-	-	-	-	+	-	-	+	+	+
Porites sp.	+	-	+	-	-	-	-	-	-	-
Porites stephensoni	-	-	-	-	+	-	+	-	-	-
Psammocora sp.	-	-	-	-	+	-	-	-	-	-
Pseudosidera streatayami	-	-	-	-	-	-	-	+	-	-
Number of species	21	31	9	13	37	7	26	30	14	7
Number of colony	31	94	28	34	108	36	65	88	51	25

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Measurement of microplastic density in the Karimunjawa National Park, Central Java, Indonesia

SULISTIYONO LIE[1,2,♥], AHMAD SUYOKO[1], AULIA ROMADHONA EFFENDI[2], BENARIFO AHMADA[2], HERDI WIRA ADITYA[2], ISTRIA RIMBA SALLIMA[2], NI PUTU AYU NITA ARISUDEWI[2], NAJLAA ILLIYYIEN HADID[1,2], NURULITA RAHMASARI[2], AKBAR REZA[3]

[1]Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sinduadi, Mlati, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-580839. ♥email: sulistiyonolie5@gmail.com
[2]Gadjah Mada Diving Society, Universitas Gadjah Mada. Gelanggang Mahasiswa UGM, Jl. Pancasila No. 1, Sleman 55281, Yogyakarta, Indonesia
[3]Laboratory of Ecology and Conservation, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sinduadi, Mlati, Sleman 55281, Yogyakarta, Indonesia

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Abstract. *Lie S, Suyoko A, Effendi AR, Ahmada B, Aditya HW, Sallima IR, Arisudewi NPAN, Hadid NI, Rahmasari N, Reza A. 2018. Measurement of microplastic density in the Karimunjawa National Park, Central Java, Indonesia. Ocean Life 2: 54-58.* Plastic debris enters the marine ecosystem in various sizes, ranging from micrometers to millimeters. Specific densities of plastic particles can vary greatly depending on the type of polymer and the manufacturing process. The highest microplastic density is usually related to the shoreline and circulation of currents in the middle of the sea. Microplastics are then degraded into fragments or particles that are very small and digested by marine biota. In recent years, there have been increasing environmental concerns about microplastics. The purpose of this study was to determine the types of microplastics and their density in the Karimunjawa Islands region and to determine the environmental impact of microplastics. The method used was sediment sampling, with sand samples taken at a depth of 2-5 cm from the sand surface in a plot 10 meters away, with another plot in a 50-meter straight line. Afterward, the laboratory separated microplastics from sand samples using a saturated saline solution. Next, the microplastic identification process was performed by differentiating based on color, size, number, and microplastic form or category. In this study, four types of microplastic were found, i.e., fiber, fragment, film, and foam. At Legon Lele Beach and Ujung Gelam, fiber was the most abundant, with 111 and 66 particles, respectively. The smallest number was film- with 6 particles in Ujung Gelam Beach and 3 in Legon Lele Beach.

Keywords: Karimunjawa, microplastics, sand samples, sediment sampling

INTRODUCTION

The worldwide production of plastics has increased considerably since the development of synthetic polymers in the middle of the 20th century (Andrady 2011). When discarded into the marine environment, plastics can be an environmental hazard (Moore 2008). Plastic debris enters the marine ecosystem in a wide range of sizes, from micrometers to millimeters (Barnes et al. 2009). In recent years, there has been increasing environmental concern about 'microplastics': tiny plastic granules used as scrubbers in cosmetics and air-blasting and small plastic fragments derived from the breakdown of macroplastics (Derraik 2002). Microplastics have been attributed to numerous size ranges, varying from study to study, with diameters of <10 mm, <5 mm, 2-6 mm, <2 mm (Ryan et al. 2009), and <1 mm (Claessens et al. 2011). This inconsistency is particularly problematic when comparing data referring to microplastics, making it increasingly important to create a scientific standard (Claessens et al., 2011, Costa et al., 2010).

Plastics manufactured to be microscopic are defined as primary microplastics. These plastics are typically used in facial cleansers and cosmetics or as air-blasting media, while their use in medicine as vectors for drugs is increasingly reported (Patel et al. 2009). Secondary microplastics, described as tiny plastic fragments, are derived from the breakdown of larger plastic debris at sea and on land (Ryan et al., 2009, Thompson et al., 2004). Over time, a culmination of physical, biological, and chemical processes can reduce the structural integrity of plastic debris, resulting in fragmentation (Browne et al. 2007).

However, plastic debris on beaches has high oxygen availability and direct exposure to sunlight, so it will degrade rapidly, turning brittle and forming cracks and "yellowing" (Moore 2008). In addition, with a loss of structural integrity, these plastics are increasingly susceptible to fragmentation resulting from abrasion, wave-action, and turbulence (Browne et al. 2007). This process is ongoing, with fragments becoming smaller over time until they become microplastic in size (Ryan et al. 2009).

While it is apparent that microplastics have become both widespread and ubiquitous, information on the biological impact of this pollutant on organisms in the marine ecosystem is only just emerging (Barnes et al., 2009). Nevertheless, the possibility that microplastics threaten biota, as their small size makes them available to a wide range of marine organisms, is of increasing scientific concern (Thompson et al. 2004). In addition to potential

adverse effects from ingesting the microplastics themselves, toxic responses could also result from (i) inherent contaminants leaching from the microplastics and (ii) extraneous pollutants adhered to the microplastics disassociating (Cole et al. 2011).

Karimunjawa Island is a coastal area with abundant fishery and potential as a tourist destination. However, microplastic marine waste may cause turmoil in the local community. Waste may pollute the coastal and marine areas. In addition, the absence of preliminary information about microplastic in this region is one of the obstacles to managing fisheries and marine potential based on environmentally-friendly technology. Based on this fact, a study needs to be done to determine the microplastic distribution on the sandy beaches in the Karimunjawa Islands region. Therefore, the purpose of this study was to determine the types of microplastic and their densities in the Karimunjawa Islands region and to determine the environmental impact of microplastics here.

MATERIALS AND METHODS

Study area

The study was carried out in the Karimunjawa Island (Karimunjawa National Park), Karimunjawa Sub-district, Jepara District, Central Java Province, Indonesia, i.e. (i) Legon Lele Beach, -5.8622312,110.4446497,17; and (ii) Ujung Gelam Beach, -5.8396515,110.4087674,17) (Figure 1).

Materials

This study used distilled water, aluminum foil, sand, saturated salt, filter paper, and paper tape.

Equipment

Tools used were twine ropes, plastic containers, small shovels, beaker glasses, tweezers, nails, Petri dishes, transects, scissors, stereo microscopes, and pencils.

Procedures and data analysis

Literature studies

A detailed literature review was conducted to identify the main methodological procedures that require standardization in sampling and extracting beach sand. We divided the findings into sampling procedures and extraction procedures. Data regarding variability in sampling procedures included: the definition of microplastic size, beach zone sample, sample size, and sample depth. Data regarding variability in extraction procedures included: sample drying temperature/duration, completion time, number of re-extraction, and quantitative units. The sampling and extraction procedures were then analyzed and compared in terms of methodological variability.

Case studies

The design of the case study depends on a literature review. Thus, the main findings were collected here, and further findings were outlined in the results section. Whether to determine how much variation was identified in this literature, in sampling and extraction procedures influenced by the study results, case studies were carried out in the Karimunjawa Islands National Park. Sand samples were taken from the high tide line at Legon Lele and Ujung Gelam beaches in the dry season April-May 2018. Sand samples were taken randomly on a straight line determined by a 50-meter transect with 5 plots separated by gaps of 10 meters.

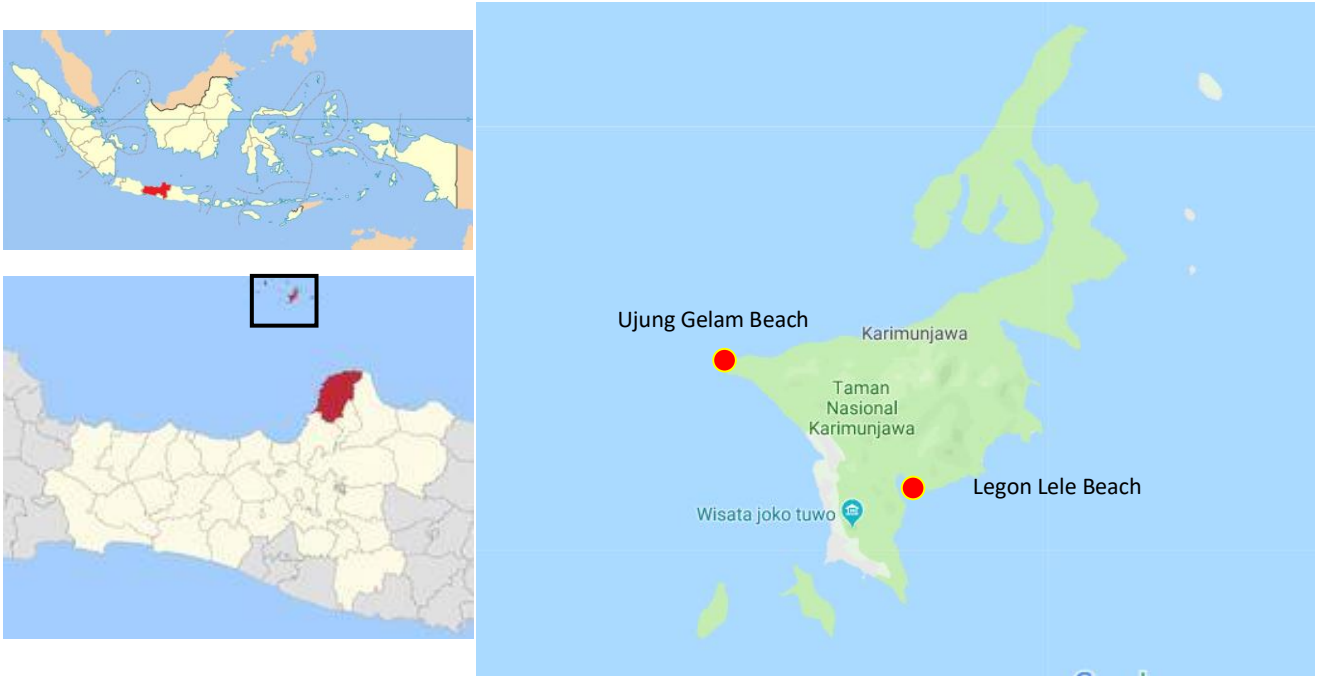

Figure 1. Study area in Karimunjawa Island, Jepara District, Central Java Province, Indonesia, i.e., Legon Lele Beach and Ujung Gelam Beach

Investigation of sampling procedures

Sampling was carried out using a 30 × 30 cm squared sampling quadrat, which was placed at the sampling location. Sand samples were taken at a depth of 2-5 cm from the sand surface with a small shovel and inserted into a plastic container that had been coated with aluminum foil on the edges or walls in a closed container. A sample of 0.2-2 kg of sand per closed container was obtained from different plot points.

Investigation of extraction procedures

The sand was transported back to the laboratory, dried, and stored at room temperature until the extraction process. Microplastic in sand samples was extracted using a saturated salt solution by mixing distilled water with NaCl. Beach sand mixed with the saturated salt solution was filtered with filter paper. Microplastic extraction was achieved by density separation. Microplastic quantities were calculated from sand samples taken from Legon Lele Beach and Ujung Gelam with 10 sand samples on filter paper. The filter paper was then examined under a stereo-microscope at up to 40-fold magnification, and microplastic was calculated systematically, allowing for microplastic quantification in the range of 0.3-5 mm (NOAA, 2015). Based on the most commonly used definitions in the literature review, microplastic was defined as a plastic material smaller than 5 mm in the largest dimension. After that, the microplastic identification process was performed by differentiating it based on color, size, number, and microplastic form or category. Finally, microplastic density data were analyzed using Microsoft Excel.

RESULTS AND DISCUSSION

Based on this research, microplastic density was obtained by presenting the following data (Figure 2-5). This study found four microplastic types: fiber, fragments, films, and foam. These four types of microplastic were the most common in Legon Lele Beach and Ujung Gelam on Karimunjawa Island. The number of microplastic particles was sorted based on size. Microplastic particles of 1-5 mm in size were the most common in both sampling locations, Legon Lele Beach and Ujung Gelam. Microplastics of 1-5 mm at Ujung Gelam Beach were found to be as many as 44 particles. Meanwhile, microplastic sizes of 1-5 mm in Legon Lele Beach were found to be as many as 62 particles.

This study sorted the number of microplastic particles based on colors. Blue microplastic was the most common in both sampling points. A total of 101 microplastic particles were found at Legon Lele Beach. Meanwhile, 51 blue microplastic particles were found at Ujung Gelam Beach.

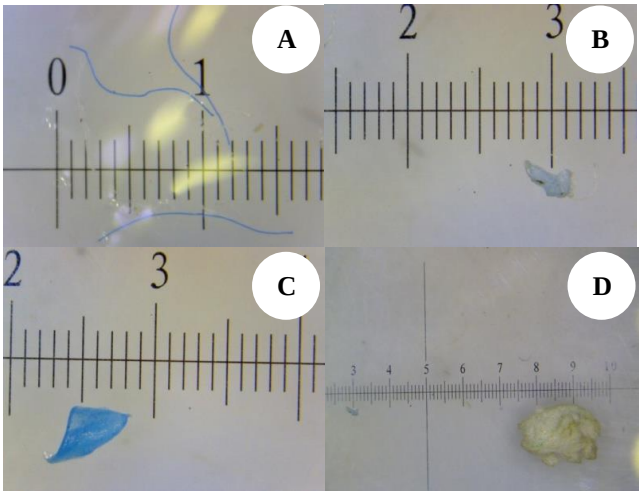

Figure 2. Microplastic types: (A) fiber, (B) fragments, (C) films, (D) foam found in Legon Lele Beach and Ujung Gelam, Karimunjawa Island, Central Java, Indonesia

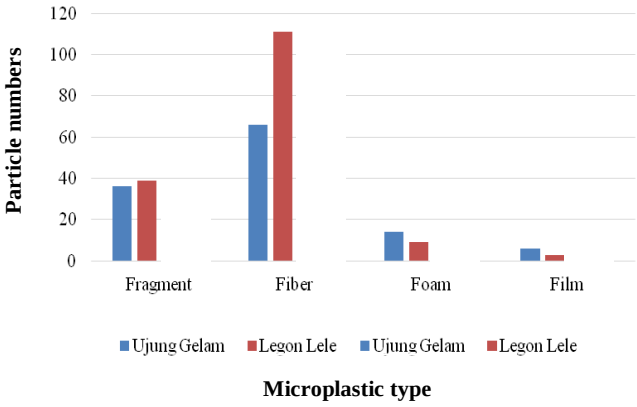

Figure 3. Types and densities of microplastics found in Karimunjawa Island, Central Java, Indonesia

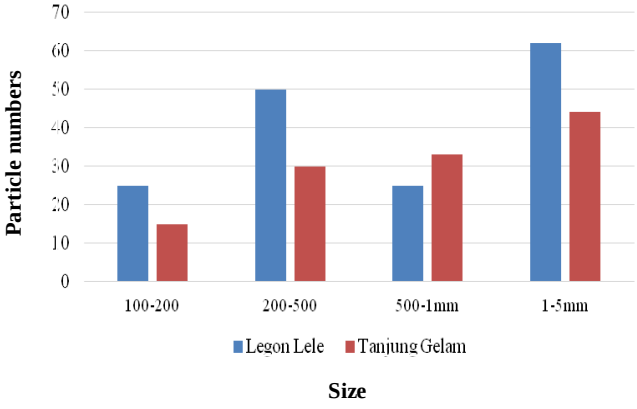

Figure 4. Sizes and particle numbers of microplastics in Karimunjawa Island, Central Java, Indonesia

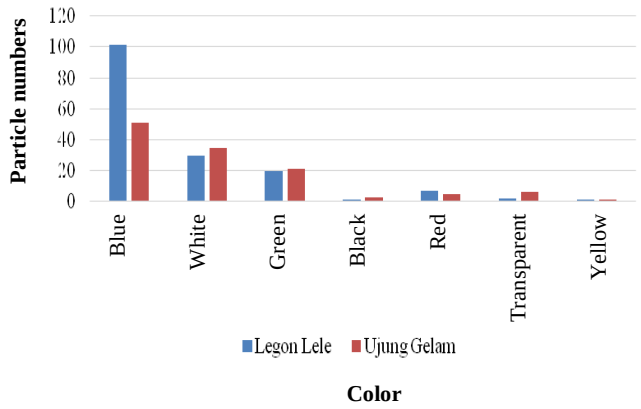

Figure 5. Colors and particle numbers of microplastics in Karimunjawa Island, Central Java, Indonesia

Discussion

This study found four microplastic types: fiber, fragments, films, and foam. A plastic factory in the vicinity of the study area was absent, so no pellets were found in this study. According to Kingfisher (2011), pellets are the primary microplastic directly produced by factories as raw material for manufacturing plastic products. The microplastic density of fiber type was in the highest order. After fiber, there were sequential types of microplastic fragments, foam, and films. Fragments result from cutting plastic products with very strong synthetic polymers (Kingfisher 2011), films have a density lower than fiber, so they are easily transported (Hastuti 2014), and fiber is derived from fishing activities. Fiber can come from high fishing activity around the area, contributing debris to seawater (Katsanevakis and Katsarou 2004). Fragments are derived from plastic consumer products. The origins of these fragments can be in the form of fishing nets, fiber lines (polypropylene strands), industrial raw materials (for example, from the ship breaking industry), and polymer fragments of plastics that can be decomposed by oxidation. Other specific microplastic sources such as small facial cleansers and microplastic polyethylene or low-density polyester fibers may eventually reach the sea. Our findings are in line with previous studies, where no clear distribution patterns of microplastics were found at different sampling sites (Besley et al., 2016).

The specific density of plastic particles can vary greatly depending on the type of polymer and the manufacturing process. The density values for plastics range from 0.8 to 1.4 g cm $^{-3}$, specifically for polypropylene are from 0.85 to 0.94 g cm $^{-3}$, polyethylene are from 0.92 to 0.97 g cm $^{-3}$, and for polystyrene are from <0.05 to 1.00 g cm $^{-3}$. These values refer to the pure resin without considering the effects of the density of various additives that may be added during the manufacture of the product. The general density for sand or other sediments is 2.65 g cm^{-3}. This difference separates plastic particles that are lighter than heavier sediment or sand grains by mixing sediment samples with saturated solutions and shaking them for a while. After mixing, the sediments are expected to settle down quickly, while low-density particles remain suspended or float to the solution's surface (Hidalgo-Ruz et al. 2012). Microplastic characteristics determine the distribution and impact on the environment. For example, solid plastic particles spend more time in contact and collide more strongly with abrasive sedimentary particles than lighter microplastic ones. This difference is important because it can affect the level of degradation, surface characteristics, and shape of microplastic particles. There is no minimum size set for microplastic. The smallest size reported is 1 µm in diameter and 20 µm in length in sediment samples. Most studies show values above 500 µm for sediment samples and 300 µm for seawater samples. This differentiation depends directly on two main factors: the tools used during the sampling and processing steps. More than 500 µm of particles are stored in filters, and standard cans, then sorted using a surgical microscope. Less than 500 µm particles are usually only obtained by research with separation of density and filtration, and particles less than 2 µm cannot be represented in a usual manner (Browne et al. 2010). Color can facilitate separation when microplastic is spread among much other debris. Eye-catching color particles have a high probability of being isolated for subsequent identification as microplastic.

In contrast, those with dull colors are easily forgotten, so they have the potential to cause bias. Color is also a photodegradation index and residence time at sea level. The discoloration process (yellowing) shows a longer exposure time to seawater, increasing the likelihood of the polymer oxidizing (Frias et al. 2010).

In general, microplastics move differently from macroplastics at sea: macroplastic distributions can often be explained by prevailing currents and winds, while mechanisms encouraging microplastic distribution are less known and may be affected by particle aggregation or animal activity. Comparative studies should be carried out to determine the dynamics of microplastic accumulation along wave exposure gradients and tidal height. Studies from subtidal zones reveal that microplastics are more abundant in subtidal sediments than on sandy beaches and estuary habitats (Browne et al., 2011).

Marine food chains generally involve these organisms (with order symbolized by the arrow): phytoplankton → zooplankton → small fishes → large fishes. Since most microscopic planktons, microplastics may be mistakenly eaten as food by larger organisms such as fishes. Sizes of particles categorized as microplastics have not yet been properly defined. Some say microplastics may be as large as 5 mm (Cole 2011). The size is larger than most plankton. Generally, the size of phytoplankton falls under 35 um, and the size of zooplankton is between 35-157 µm (nauplii and rotifers) or above 157µm (exclusively copepods and cladocerans) (Kim et al. 2000). Proof of microplastic ingestion by zooplankton has been documented. Desforges et al. (2015) reported that *Neocalanus cristatus*, a copepod consumed microplastics with an encounter rate of one particle/every 34 copepods analyzed (0.026 ± 0.005 particles/individual zooplankton) and *Euphausia pacifia*, a euphausiid, with an encounter rate

of one particle/every 17 euphausiids (0.058 ± 0.01 particles/zooplankton). Cole et al. (2013) show that the ingestion may be size-independent such as in *Centropages typicus* and *Temora longicornis* that consumed 7.3, 20.6, and 30.6 µm polystyrene beads, size-dependent such as in *Acartia clausi* that ingested 7.3 µm beads but ingested significantly fewer 20.6 and 30.6 µm beads, and *Calanus helgolandicus* that showed significantly less affinity for 30.6 µm beads than for 7.3 µm beads, life-stage selective such as in decapod Brachyurans where brachyuran zoea showed no affinity for 20.6 µm beads, while the more developed brachyuran megalopa readily ingested such beads, or individually selective such as in *Obelia* sp., Paguridae larvae, and Porcellinidae (zoea). Cole et al. (2013) also reported that the rate of algal feeding was reduced in copepods due to microplastics. That may be caused by the clogging of zooplankton's alimentary canal.

In conclusion, based on the research that has been done, it can be concluded that there are four types of microplastic found, i.e., fiber, fragment, film, and foam. In this study, the most abundant microplastic type found was fiber at both sampling points, namely Legon Lele Beach and Ujung Gelam. Microplastic particles of 1-5 mm were the most common in both sampling locations. Blue microplastic was the most common at both sampling points. Food chains of marine organisms involve several organisms. Because most planktons are microscopic, microplastic may be eaten by large organisms such as fish. The rate of algal consumption is also reduced in the microplastic copepods. The highest microplastic abundance is usually related to the coastline and circulation of currents in the middle of the sea. Microplastics are then degraded into fragments or particles that are very small and digested by marine biota.

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Potential application of biosurfactant from marine bacteria in bioremediation

MANORAMA MOHANTY, SURAJIT DAS♥
Department of Life Science, National Institute of Technology. Rourkela-769008, Odisha. Tel.: +61-661-2462684
♥email: surajit@nitrkl.ac.in.

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Abstract. *Mohanty M, Das S. 2018. Potential application of biosurfactant from marine bacteria in bioremediation. Ocean Life 2: 59-72.* Marine bacteria were screened for their potential ability to produce biosurfactants which can effectively reduce polycyclic aromatic hydrocarbons (PAHs) as the only carbon and energy source. Having many toxic effects, the PAHs are very harmful to flora and fauna as well as affecting humankind adversely. This work aimed at investigating the potential applications of biosurfactant in aerobic degradation of PAHs under stress conditions. The antimicrobial and anti-adhesive capacity of the biosurfactant were also tested against different pathogenic species. Marine bacteria were collected from sediment samples of Paradip Port, Visakhapatnam Port, Rishikulya, Bhitarakanika and screened for their biosurfactant production. Its growth was optimized in carbon and nitrogen sources for maximum biosurfactant production. Naphthalene and PAHs degrading isolates were evaluated for their biodegradative potential through UV-Vis spectroscopy and phenotypical characterization by SEM studies. Five candidate isolates, identified to be *Ochrobactrum*, *Streptococcus*, *Pseudomonas* sp., *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* showing 99.9%, 99.6%, 99%, 99.3%, 98.6% of Phenanthrene degradation (100mg/L) and 99%, 99.1%, 89.75%, 94.01%, 97.02% of Naphthalene degradation (100 mg/L), respectively.

Keywords: Bioremediation, biosurfactants, PAHs, polycyclic aromatic hydrocarbons

INTRODUCTION

Given the fact that 70% of the earth surface is covered by salt water, the marine environment becomes the most significant habitat as compared to other habitats in the biosphere. Industrialization and extraction of natural resources have caused in large-scale environmental pollution. Large amounts of toxic wastes have been scattered in thousands of contaminated sites which spread across our nation whose natural sink is the coastal marine regions. Thus, all of us is being exposed to contamination from past and present industrial practices, emissions in natural resources (water, air, and soil) even in the most isolated regions; hence, the risk to human and environmental health is rising. Our challenge is to develop innovative and cost-effective solutions to clean up polluted environments, to make them safe for human habitation and consumption, as well as to protect the functioning of the ecosystems which support life.

The pollution is mainly originated from various anthropogenic sources like an oil spill, plastic debris, pesticides, fertilizers, chemicals, radioactive substances, heavy metals, biological, solid wastes. Polycyclic aromatic hydrocarbons (PAHs) are the major constituent of oil and are abundant everywhere in nature, for example, in the soil, air, water, flora, and fauna. Most of PAHs are carcinogenic, mutagenic and teratogenic to many organisms including mammals.

About 23,000 metric tons of PAHs are discharged to the marine environment by anthropogenic sources every year (Eisler 1987). PAHs may come into the marine environment by spillage of petroleum and petroleum products, atmospheric deposition of PAHs, domestic and industrial sewage, surface land runoff, biosynthesis. PAHs incorporated with some airborne particles settle down in the bottom of the sea. The petroleum and petroleum products undergo diffusion, evaporation, some chemical changes, sunlight effect (photo-oxidation) (www.intechopen.com/download/pdf/29372).

PAHs are the most toxic pollutants in the hydrocarbon family. The toxicity of PAHs varies substantially in the marine environment. In crustaceans, the level of toxicity is even higher, and in teleosts, it is deficient. Some aquatic plants and animals' uptake the PAHs, then accumulate it. Absorption of the PAHs is highly species specific. The fish and crustaceans readily absorb pahs whereas some algae and mollusks are unable to metabolize these PAHs. Many marine organisms can eliminate these PAHs, so biomagnification is not observed in the food chain (Eisler 1987). Some PAHs are pooled in the cell membrane of the microbes due to their lipophilic nature. Exposure to PAHs, however, leads to cell damage, carcinogenesis, teratogenesis, and mutagenesis as PAHs binds covalently with the macromolecules like RNA, DNA, and protein.

Various physicochemical methods are introduced to remove these contaminations from the environment. But these are too expensive, non-specific and also they introduce the secondary contaminants to the environment. An eco-friendly, cost-effective and bio-based method is adapted to treat these contaminations, called bioremediation. Bioremediation is a way of detoxification and degradation of toxic pollutants either through intracellular accumulation

or by enzymatic transformation to less toxic or non-toxic compounds (Singh et al. 2008). Microorganisms exhibit the potential to degrade, transform or chelate the poisonous chemicals, but this transformation process is prolonged. The major components of this bioremediation are the microbes and their products. The critical factors of bioremediation are the availability of contaminants, availability of microbes and a suitable environment — also, the nutrients, pH value, oxygen influence the bioremediation of PAHs (Singh Cameotra and Makkar 2010).

Due to the low water solubility and hydrophobic characteristic of PAHs, many microorganisms have developed several mechanisms to increase the availability of such compounds to utilize them as carbon and energy source. Microorganisms produce two main types of surface-active compounds: biosurfactants and bioemulsifiers. Biosurfactants greatly reduce the air-water surface tension while bioemulsifiers do not decrease as much the surface tension but stabilize oil-in-water emulsions. Example of some biosurfactants: Rhamnolipid, Surfactants, Sophorolipid, Lipopeptide, Trehalose tetra ester.

Biosurfactants in the degradation of heavy metals, PAHs, pesticides in soil and water environment, are of great significance. The microbes generally use organic compounds as the source of carbon and energy for their growth. When a hydrocarbon (C_xH_y) or any insoluble substrate is the carbon source, microorganisms regulate their diffusion into the cell membrane by producing a variety of biosurfactants. Some bacteria and yeasts produce ionic surfactants which emulsify the C_xH_y substrate in the growth medium (Karanth et al. 1999). For example, Rhamnolipids which are excreted by different *Pseudomonas* spp. (Guerra et al. 1984), non-ionic trehalose corynomycolates are generated by many *Mycobacterium* spp. and *Arthrobacter* spp.

Biosurfactants are biodegradable, usually low toxic, biocompatible, digestible for which they are used in cosmetics, pharmaceuticals and as functional food additives. They can be produced from industrial wastes and by-products, and this is of particular interest for bulk production (e.g., for use in petroleum-related technologies). Furthermore, biosurfactants can be efficiently utilized in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in decontamination soil. They are more effective at extreme pH, temperatures, and salinity.

In the last decades, there has been a growing interest in isolating microorganisms that secrete surface active molecules with good surfactant characteristics like low critical micelle concentration (CMC) and high emulsification activity. Such microorganism is simultaneously presenting low toxicity and good biodegradable ability. The type and amount of the biosurfactants generated by microbes depend on the producer organism, carbon and nitrogen, trace elements, and temperature. Biosurfactants are categorized mainly by their microbial origin and chemical composition (a type of polar group present). Based on the structure of their hydrophilic part, biosurfactants are classified primarily into five categories: (i) Glycolipids, (ii) Lipopeptides, (iii) Fatty acids, (iv) Polymer type, (v) Particulate biosurfactants (Desai and Banat,1997; Gautam and Tiagi, 2005).

The objectives of the present study are : (i) Screening, phenotypic and biochemical characterization of biosurfactant producing marine bacteria, (ii) Growth optimization of the isolates in different carbon and nitrogen sources, (iii) Extraction and chemical characterization of a biosurfactant produced from the strains, (iv) Antioxidant, anti-adhesive and antimicrobial activity study of the extracted biosurfactant, (v) Biosurfactant based bioremediation of PAHs.

MATERIALS AND METHODS

Fourteen bacterial strains were collected from Paradeep port, Vishakhapatnam, Rishikulya, Bhitarakanika marine water. They were streaked in Luria Bertani (LB) agar plate and maintained at pure culture.

Colony morphology

Colony morphology was observed for twenty-four hours incubated cultures of the isolated bacteria.

Screening for biosurfactant activity

Drop collapsing test

Fourteen bacterial strains were maintained overnight in LB broth with ten mM $MgCl_2$. Next day these were sub-cultured into MMMF media and incubated for 24 h at 23^0C. 10 µL of the supernatant of each strain was spotted on the polystyrene coated glass plate that was covered by immersion oil (Taguchi et al. 2006).

Oil spread method

Bacterial strains were inoculated in LB broth with ten mM $MgCl_2$ for 24 h. Next day 20 µL of immersion oil was layered uniformly to a 20 mL of distilled water that was kept in the Petri plate. 20 µL of the culture was added to a different spot on the immersion oil after doing vertex for 2 minutes. After 30 sec, it was examined whether they are giving a clear zone or not. (Morikawa et al. 2000).

Emulsification test

The bacterial strains were inoculated in LB broth with ten mM $MgCl_2$ for overnight. Two mL of the bacterial culture was transferred to a test tube, and n-octane (1 mL) was added to it. The mixture was vortexed for 2 min and kept for 24 h to see the emulsification result (Cooper et al. 1987).

$$\text{Emulsification index} = (\text{Emulsification height}/\text{Total height}) \times 100$$

Blood Haemolysis Test

On the blood agar plates, the isolated new colonies were streaked and kept in an incubator for 24 hours at 37°C. The biosurfactant producing organisms was determined from the presence of the clear zones around the colonies in the plates (Satpute et al. 2008).

Physical characterization of bacterial isolates

Gram staining

First, smears of bacterial suspensions were made with one drop of distilled water on a clean and dry slide glass. These slides were fixed by heat, and the fixed smears were flooded with crystal violet solution and kept for 1 min, and were rinsed off with distilled water. These slides were then treated with Gram's iodine solution, allowed to remain for 1 min, then rinsed with distilled water. These slides were treated with 1-2 drops of Gram's decolorizer and kept for 1-5 sec. Then the slides were rinsed with water. Lastly, these were flooded with safranin and maintained for 1 min, then again rinsed with distilled water. These slides air dried and observed under the light microscope at 40X objective. If the cells retained the pink color of safranin, then these were identified to be, and if they kept the violet color of crystal violet, they were defined as Gram-positive.

Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy (SEM)

The freshly cultured strains were pooled at 8000 g, 4^0C for 5 min and the cells were washed with 0.1 mM phosphate buffer saline (PBS) 3 times. Following this step, the cells were fixed by adding 2 % glutaraldehyde prepared in 0.1 M PBS and incubated at room temperature overnight for fixation. Later, the next day the cells were washed thrice with PBS. These cells were centrifuged at 8000 g, 4˚C for 5 min; then dehydration was done of each sample by a series of ethanol concentrations ranging from 30 %, 50 %, 70 %, 90 %, 100 % and incubated for 18 hours. The fixed samples were incubated for one h with ethanol (100 %), air dried and observed at various resolutions under SEM.

Biochemical tests of bacterial isolates

Mannitol motility test

Freshly cultured bacterial strains were inoculated in mannitol motility nitrate agar and incubated for 24 h and were checked for motility. The strains are non-motile if they showed growth along the line of the inoculation. If the bacteria showed growth by spreading over the medium, then they are motile. This mannitol motility test also confirms whether bacterial strains can ferment mannitol or not. A color change from red to yellow indicates a positive result.

Nitrate reduction test

In this test, 1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl- Naphthylamine reagent were added to the kit medium to check whether these bacterial strains can convert nitrate to nitrite or not. If the color immediately changed into the pinkish red color on the addition of reagent indicates a positive reaction. If there is no change in color, it shows a negative result.

Sulphide Indole Motility (SIM) Test

Fourteen strains were inoculated in Sulphide indole motility (SIM) media and incubated these to test whether they are motile or non-motile and whether they are producing sulfide or not. If the color of the medium will change from yellow to black, then H_2S production result will be positive.

Growth curve

Bacterial strains were inoculated in LB broth, and 300 µL of the freshly inoculated bacterial cultures were taken in the microtiter plate (Tarson). Then, the O.D. was measured at 595 nm in the ELISA Reader (Perkin Elmer) at every two h interval for 24 h. The readings were plotted against time to determine the growth curve.

Growth optimization of the strains in various carbon and nitrogen sources

Growth optimization in Carbon sources

Two aliphatic carbon sources (Glycerol and Sucrose) and five aromatic carbon sources (Kerosene, Biphenyl, Naphthalene, Pyrene, Phenanthrene) were drawn to optimize the growth of these strains. Two percent of each aliphatic carbon sources were added to Bushnell Haas Broth (BHB) with NaCl supplementation (19.450 mg/L), and concentration of 100 mg/L of each aromatic source was taken, and the absorbances were monitored at 595 nm (Onwosi and Odibo 2012).

Growth optimization in Nitrogen sources

Two percent of each nitrogen sources (KNO_3, Urea and Yeast extract) was added to the BHB (supplementation with NaCl- 19.450 gm/l) with the respective optimized carbon sources for the growth optimization of these strains. Absorbance was read at 595 nm (Onwosi and Odibo 2012).

Extraction of biosurfactant

Five strains were inoculated in BHBroth as described above with respective carbon and nitrogen sources and then incubated at 25^0C for seven days with shaking conditions. Supernatants were pooled by centrifuging at 6000 rpm, 4^0C for 20 minutes. The pH of these supernatants was adjusted by adding 1M $H2SO_4$. Then an equal volume of chloroform: methanol was added to this supernatant in the ratio of 2:1. The mixture was shaken well for proper mixing and then left overnight for evaporation. The presence of biosurfactant is confirmed when white colored precipitates were seen in the interface between the two liquid (Dhouha et al. 2012).

Characterization of biosurfactant

Carbohydrate and protein estimation

Estimation of carbohydrate was done by the phenol-sulfuric acid method (Dubois et al. 1956), while the evaluation of protein was conducted by Bradford method (Bradford 1976).

Surface tension measurement

Fifty mL of the crude biosurfactant of 5 strains were taken for surface tension measurement concerning distilled water. The surface tensions of each strain were plotted by digital tensiometer. Hence, the surface tensions concerning distilled water were determined (Abu-Ruwaida et al. 1991).

Fourier Transform Infrared analysis (FTIR)

FTIR spectroscopy was performed using crude biosurfactant extract obtained from the acid precipitation of the cell-free culture supernatant to determine the chemical nature of the biosurfactants. FTIR Prestige- 21 Fourier Transform Infrared spectrophotometer (Shimadzu, Japan) was used to determine the chemical nature of the biosurfactant by the KBr pellet method (Das et al. 2008a, b; Mukherjee et al. 2009).

Antimicrobial activity test

Twenty mL Muller Hinton Agar media with supplementation of NaCl (19.450gm/l) was prepared each for Petri plates on each of which three wells were made and named as A, B, and C respectively. The plates were swabbed with *Bacillus, Streptococcus, Shigella, Escherichia coli, Proteus, Salmonella.* To the wells A, purified biosurfactants were added. To the wells, B, diluted biosurfactants (10 fold) were added. To the wells, C, only distilled water (control) was added. The plates were incubated at 37°C for 24 hours and checked for the presence of a clear zone which marked the antimicrobial activity of biosurfactant. Mean of the three readings of the clear zone diameter were taken for each well to calculate the actual zone diameter (Rodrigues et al. 2006).

Antioxidant activity test

The antioxidant potential of the biosurfactant was measured by their scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH method is the easiest method to determine the antioxidant activity of compounds and is widely used. The aliquots of the different biosurfactant level were poured to 5.0 mL of a 0.004% (w/v) solution of DPPH. Absorbance at 517 nm and IC50 were determined after 30 min of reaction. IC50 (the half maximal inhibitory concentration) value denotes the level of sample required to scavenge 50% of the DPPH free radicals. The radical scavenging activity at various biosurfactant concentration was calculated by the equation below:

Equation: $S_{DPPH} = 100 \times (1 - A_{sample}/A_{DPPH})$

Where A_{sample} indicates the absorbance of the solution in the presence of test samples, and A_{DPPH} shows the absorbance of the DPPH solution in the absence of the test samples (Yalcin and Cavusoglu 2010).

Anti-adhesive test

Two-hundred µL of the crude biosurfactants (100 mg/mL in PBS) were filled in the wells, and the control wells were filled only with PBS. The plate was later incubated at 4˚C for 18 h and washed with PBS three times. The pathogenic bacteria cultures, *Streptococcus pneumoniae,* and *Bacillus* sp. were centrifuged, and pellets were collected then resuspended in PBS and added to these wells. The plate was then kept in an incubator at 4˚C for four h. The plate was washed with PBS three times, then fixed by 200 µL methanol for 15 min. The plate was dried and then stained with 2% crystal violet (200 µL) for 5 min. The dye was re-solubilized with 200 µL of 33% (v/v) glacial acetic acid per well. Then absorbance was measured at 595 nm (Rufino et al. 2011).

% of microbial inhibition = $[1- (A_c/A_0)] \times 100$

Where:

A_C = Absorbance of the well with a biosurfactant concentration c.

A_0 = Absorbance of the control well

Biodegradation of Polycyclic Aromatic Hydrocarbon (PAH) by Biosurfactant

Phenanthrene biodegradation

First, bacterial cultures were inoculated in LB broth for 24 h. The next day, 100 µL of these bacterial cultures were subcultured in 50 mL Bushnell Haas media (with supplementation of NaCl 19.450 gm/l) with 100 mg/L of phenanthrene for seven days for enrichment culture. On the 7th day, the pellets of each bacterial culture were collected by centrifuging at 6000 rpm, 10 min at 4^0C. Then these pellets were re-suspended in B H broth (2 mL). Then the O.D. of each bacterial pellet (300 µL) was read at 595 nm in ELISA Plate Reader. As O.D. of each strain was found less than 1, then 50 µL of an enriched pellet of each strain was moved to 5 mL of BHB with Phenanthrene (100 mg/L) and kept in a shaker incubator (in the dark) at 180 rpm, 37˚C. On the Day-1, Day-3, Day-5, Day-7, extraction was done by adding the equal volume of n-Hexane. After adding n-Hexane, the sample was vortexed for 5 minutes, then centrifuged at 6000 rpm for 10 min at 4^0C to collect the Hexane layer. Then O.D. of the Hexane extract was taken at 292 nm and also scanned from 200 nm to 400 nm (Tao et al. 2007).

Naphthalene biodegradation

First bacterial cultures were inoculated in LB broth for 24 h. Next day 100 µL of these bacterial cultures were subcultured in 50 mL Bushnell Haas media (with supplementation of NaCl 19.450 gm/l) with 100 mg/L of naphthalene for seven days for enrichment culture. At 7th day the pellets of each bacterial culture were collected by centrifuging at 6000 rpm, 10 min at 4˚C. Then these pellets were re-suspended in BHB (2 mL). Then the O.D. of each bacterial pellet (300 µL) was read at 595 nm in ELISA Plate Reader. As O.D. of each strain was lower than 1, then 50 µL of an enriched pellet of each strain was moved to 5 mL of BHB (with NaCl 19.450 gm/l) with Naphthalene (100 mg/L) and run in a shaker incubator (dark) at 180 rpm, 37^0C. Then at Day-1, Day-3, extraction was done by adding the equal volume of n-hexane. After adding n-Hexane, the sample was vortexed for 5 min, then centrifuged at 6000 rpm for 10 minutes at 4^0C to collect the Hexane layer. The O.D. of the Hexane extract was measured at 254 nm and also scanned from 200 nm to 400 nm (Tao et al. 2007).

RESULTS AND DISCUSSION

Cell morphology

Cell morphologies of these five strains (JV502, JV501, JP022, JV201, and JP011) were given in Figure 1.

Screening for biosurfactant activity

Drop collapse test

Ten µL cell suspension of each strain was placed on the polystyrene coated glass plate that was covered by immersion oil. If the cell suspension contains biosurfactant, the drop will collapse or spread due to the reduction of a hydrophobic surface. On the other hand, if no biosurfactant present in the cell suspension, the drops will remain stable as the polar water molecules are repelled from the hydrophobic surface. The stability of the drop depends on the biosurfactant level. Only strains JV801 and NR802 gave negative results and rested gave positive results (Table 1).

Oil spread method

Cell-free culture broth of 14 strains was added to the plate that contained distilled water and oil. The 12 strains (JV201, JV501, JV502, JP022, JP011, NE3B01, NE3B02, NP202, NP103, ATCC, NR802, and JV801) showed the zone of displacement in oil. The zone of displacement displayed the biosurfactant production in these strains, and the results were noted down (Table 1).

Emulsification test

In the emulsification test, JP022 showed 41% of emulsification activity, and the other strains ranging between 35-40 % of emulsification activity and harmful emulsification activity was observed in NP202, NP103, and JV101 (Figure 2).

Blood hemolysis test

Fourteen strains were streaked on blood agar plates. Among the strains, JV501, JV201, JP022, JP011, and ATCC showed hemolytic activity by forming a clear zone around the colonies (Figure 3).

Among these fourteen strains, five strains gave good results in the screening of biosurfactant test, so they proceeded for further study.

Physical characterization of bacterial isolates

Gram staining

Cell morphology of these ten strains was studied by gram staining and observed under oil immersion microscope. Among these strains, JV501 was Gram-positive bacteria, and others were Gram-negative. Similarly, JV501 was coccus, and the others were rods. The results have been given below in Table 2.

Table 1. Results of drop collapse test and oil spread method

Strain name	Drop collapse test	Oil spread method
JV501	Positive	Positive
JV201	Positive	Positive
JV502	Positive	Positive
JP022	Positive	Positive
JV202	Positive	Negative
JV101	Positive	Negative
JP011	Positive	Positive
JV801	Negative	Positive
NE3B02	Positive	Positive
NE3B01	Positive	Positive
NP202	Positive	Positive
NP103	Positive	Positive
ATCC	Positive	Positive
NR802	Negative	Positive

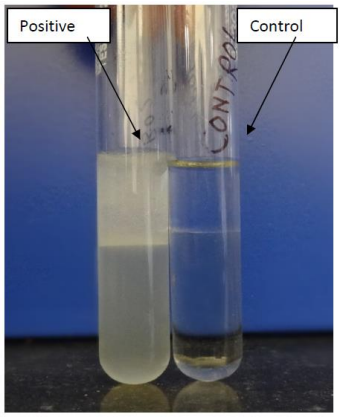


Figure 2. The tubes showing emulsification activity

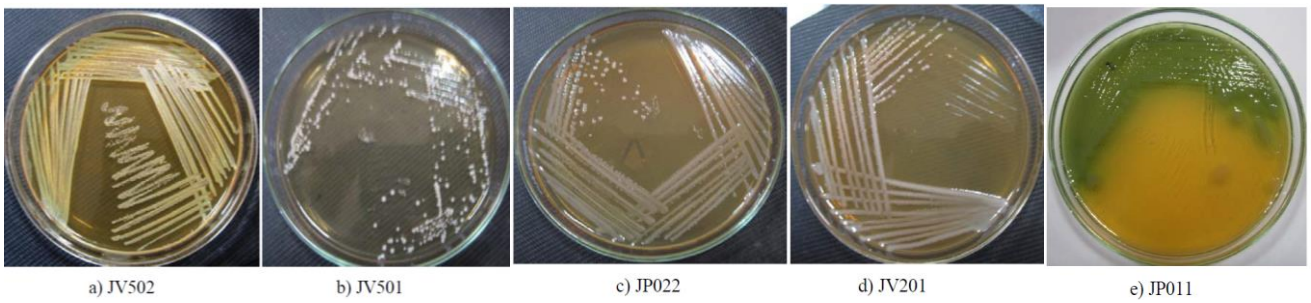


Figure 1. Cell morphology of the isolated strains

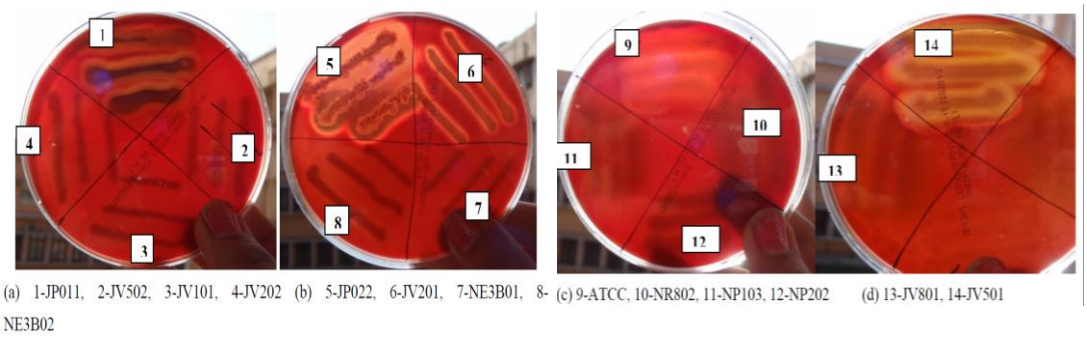


Figure 3. Hemolytic activity of the isolates, JP011- α hemolysis, JP022- β hemolysis, JV201- β hemolysis, ATCC- hemolysis, JV501- β hemolysis and others are showing a negative result.

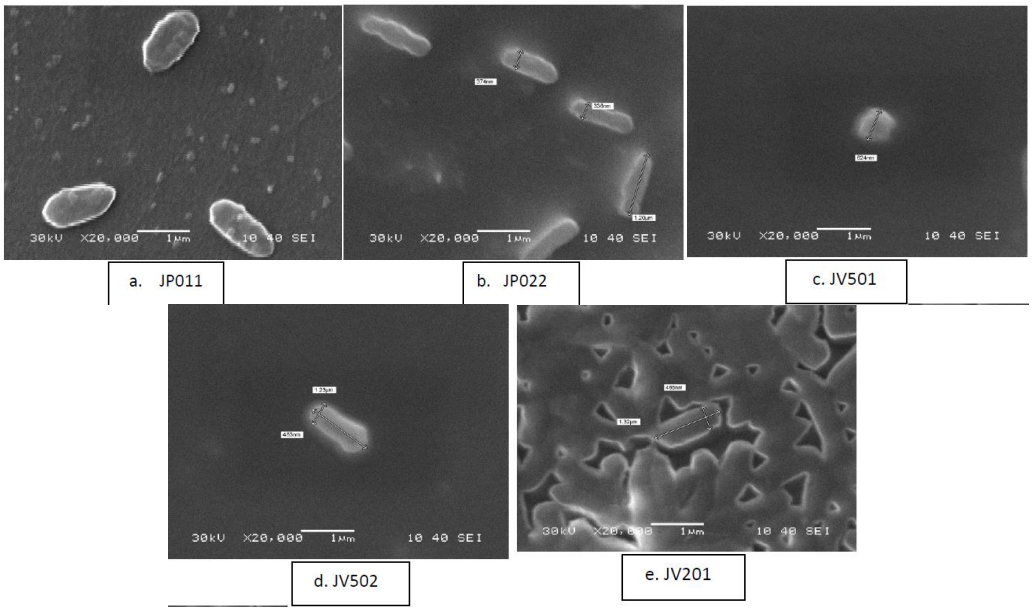


Figure 4. SEM results of the isolates

Table 2. Cell morphology of the isolates

Strain name	Colour	Gram staining	Shape
JV501	Purple	+ ve	Cocci
JV201	Pink	-ve	Rods
JV502	Pink	-ve	Rods
JP022	Pink	-ve	Rods
JP011	Pink	-ve	Rods

Table 3. Results of all biochemical tests

Strain name	Mannitol test	Motility test	Nitrate reduction test	Sulfide production test
JV501	+ ve	Motile	+ ve	- ve
JV201	+ ve	Motile	- ve	- ve
JV502	+ ve	Motile	+ ve	- ve
JP022	- ve	Non-motile	- ve	- ve
JP011	- ve	Non-motile	- ve	- ve

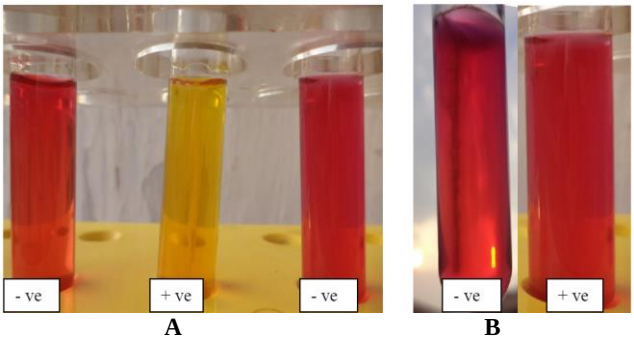


Figure 5. A. Mannitol test, + ve showing mannitol fermentation, B. Motility test, + ve showing Motile and - ve showing Non-motile

Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy (SEM)

Cell surface topography of the five strains was observed by SEM image (Figure 4).

Biochemical tests

Table 3 shows the results of all biochemical tests like Mannitol motility test, Nitrate reduction test, Sulphide indole motility test.

Mannitol motility test

In this test, JV501, JV201, JV502 shows a positive result, and others were showing the adverse effect (Figure 5). In the case of the motility test, all strains were found motile excluding JP022 and JP011.

Nitrate reduction test

Only JV501, JV502 were showing a positive result, and others were showing an adverse effect.

Sulfide indole motility (SIM) test

All strains showed a negative result.

Growth curve

Five strains were inoculated to check their growth curve. The OD was measured and plotted against time in ELISA Reader (Perkin Elmer) (Figure 6).

Growth optimization in various carbon and nitrogen sources

In different carbon sources

Two aliphatic carbon sources (glycerol and sucrose) were taken to monitor the growth of these strains.

Aliphatic carbon sources

Strain JV502, JV201, and JP011 showed robust growth in glycerol (Figure 7). Strain JP011, JV501, JV201, showed good results in sucrose as the carbon source (Figure 8).

Aromatic carbon sources

JV501, JV201 showed good growth results in Kerosene (Figure 9). JV201, JV501, JV502 showed good growth in Pyrene medium (Figure 10). JV501, JP011, and JV502 grew well in Biphenyl (Figure 11). In Naphthalene, JV501, JV201, and JP022 showed good growth (Figure 12). Meanwhile, the strains JV501, JV201, and JP011 showed the excellent result in Phenanthrene (Figure 13).

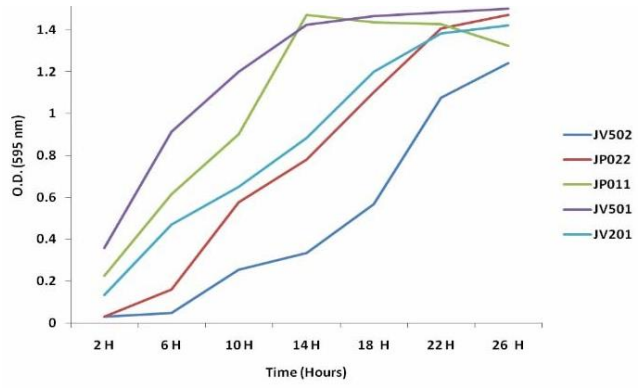


Figure 6. Growth curve of the bacterial isolates

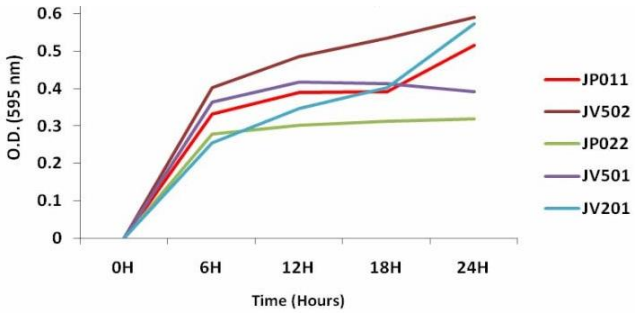


Figure 7. Growth optimization of the isolates in Glycerol (2% v/v)

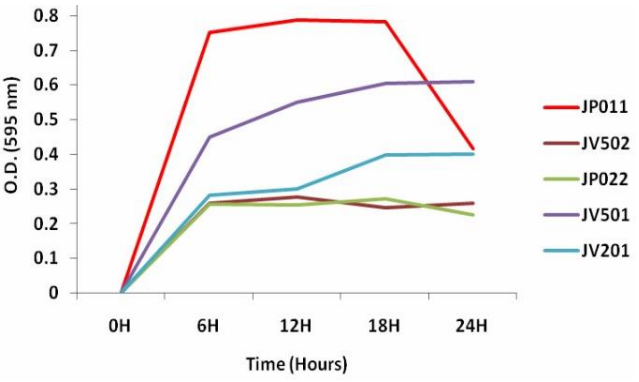


Figure 8. Growth optimization of the isolates in Sucrose (2% w/v)

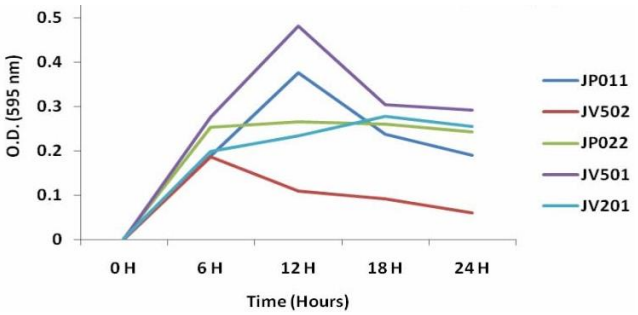


Figure 9. Growth optimization of the isolates in Kerosene (2% v/v)

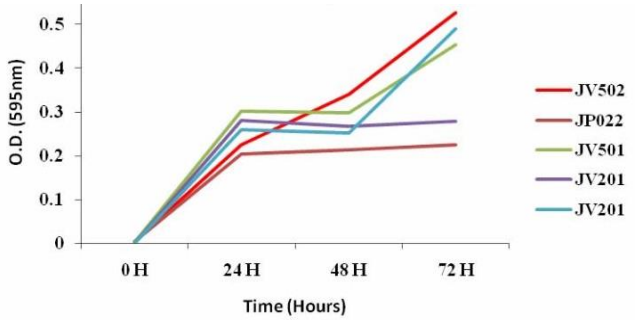


Figure 10. Growth optimization of the isolates in Pyrene (100 mg/L)

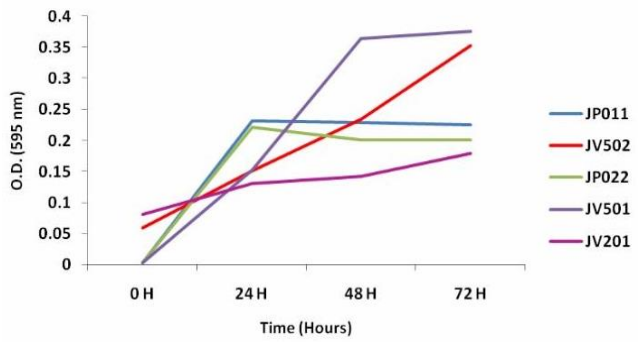


Figure 11. Growth optimization of the isolates in Biphenyl (100 mg/L)

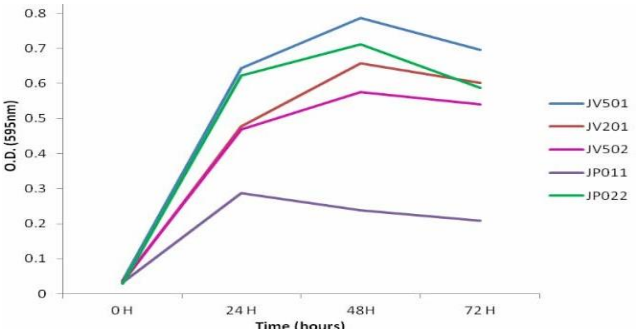


Figure 12. Growth optimization of isolates in Naphthalene (100 mg/L)

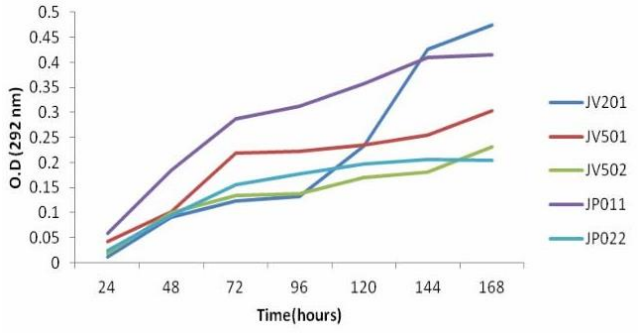


Figure 13. Growth optimization of the isolates in Phenanthrene (100 mg/L)

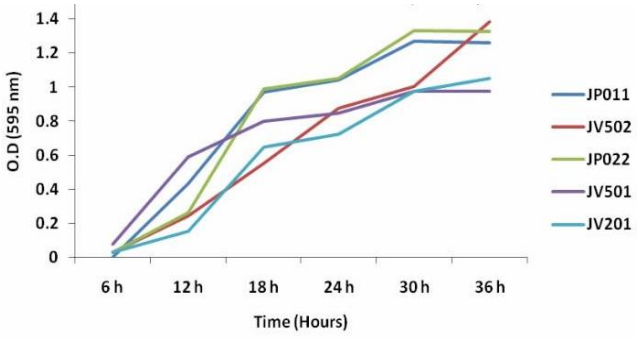


Figure 14. Growth optimization of the isolates in Yeast Extract (2% w/v)

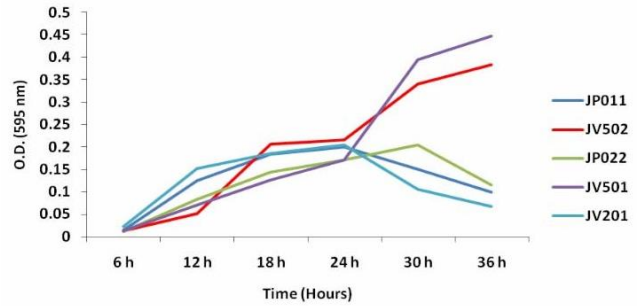


Figure 15. Growth optimization of the isolates in urea (2% w/v)

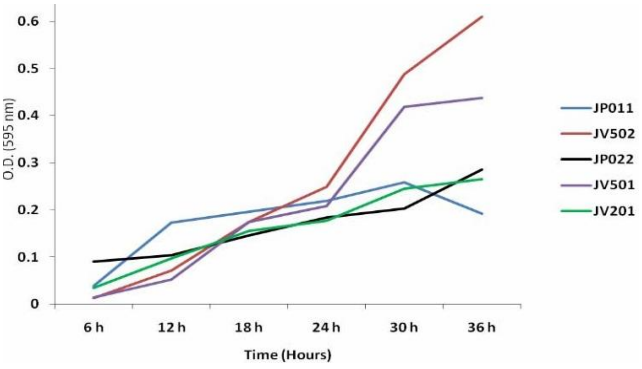


Figure 16. Growth optimization of the isolates in potassium nitrate (2% w/v)

Growth optimization of the strains in different nitrogen sources

The strains were optimized for better growths in three nitrogen sources yeast extract, urea, potassium nitrate. All the strains showed good growth in the presence of yeast extracts (Figure 14) whereas strain JV501, JV502 showed good growth in urea (Figure 15) and also in Potassium nitrate (Figure 16).

Extraction of biosurfactant

The five strains which were inoculated in BHB with respective carbon and nitrogen sources for seven days showed good results. Supernatants were obtained by centrifuging at 6000 rpm at 4^0C for 20 min and an equal volume of chloroform: methanol (ratio 2:1) was added for acid precipitation with 1M H_2SO_4. After the pH was adjusted to 2, the mixture was left overnight for evaporation, and if white colored precipitate were found in between two immiscible liquids, then biosurfactant production was observed. The biosurfactant productions of all five strains were detected (Figure 17).

Characterization of biosurfactant

Carbohydrate estimation

The carbohydrate level present in the biosurfactants was calculated from the standard curve (Figure 18). Table 4 showed that strain JV201 has the maximum carbohydrate content.

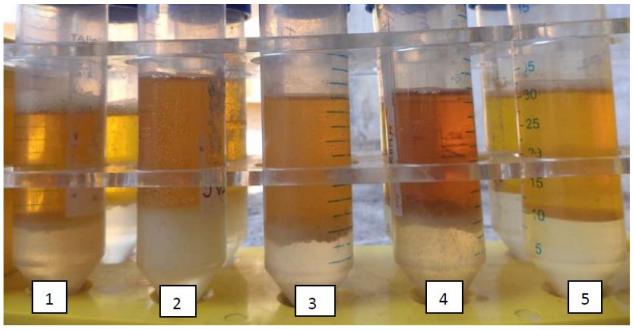


Figure 17. Biosurfactant production (1-JV501 2-JV201, 3-JV502, 4-JP022, 5-JP011. All are producing approximately 100mg/25 mL)

Protein estimation

The concentration of protein present in the biosurfactants was calculated from the standard curve (Figure 19). JV201 showed the maximum carbohydrate content (Table 5).

Surface tension measurement

A digital Tensiometer determined the surface tension of crude biosurfactant for distilled water (Table 6, Figure 20).

Fourier Transform Infrared analysis (FTIR)

The determination of the functional group present in the crude biosurfactant by using Fourier Transform Infrared Spectroscopy (Figure 21-29).

Table 4. Carbohydrate estimation of the biosurfactants

Strain name	O.D. at 490 nm	Concentration (µg/mL)
JV501	0.035	0.217979
JV201	0.172	0.709865
JV502	0.063	0.318511
JP011	0.028	0.192847
JP022	0.048	0.264655

Table 5. Protein estimation of the biosurfactants

Strain name	O.D. at 595 nm	Concentration (µg/mL)
JV501	0.022	1.122537
JV201	0.183	2.952162
JV502	0.015	1.042988
JP011	0.017	1.065717
JP022	0.019	1.088445

Table 6. Surface tensions of the isolates

Strain name	Surface tension
JV501	55.234±0.028 mN /m
JV201	55.368 ± 0.028 mN /m
JV502	51.251 ± 0.028 mN /m
JP011	43.776 ± 0.029 mN /m
JP022	52.292 ± 0.028 mN /m

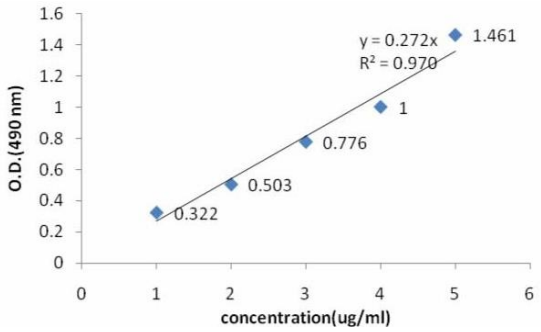


Figure 18. A standard curve of D-glucose

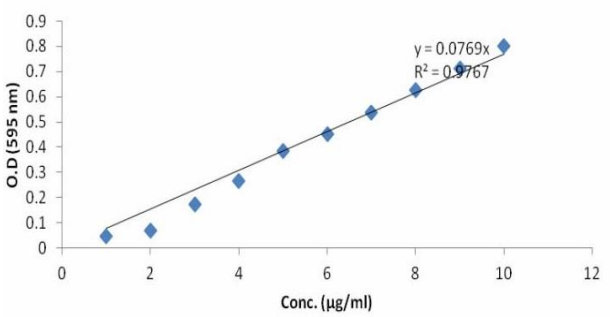


Figure 19. A standard curve of protein (BSA)

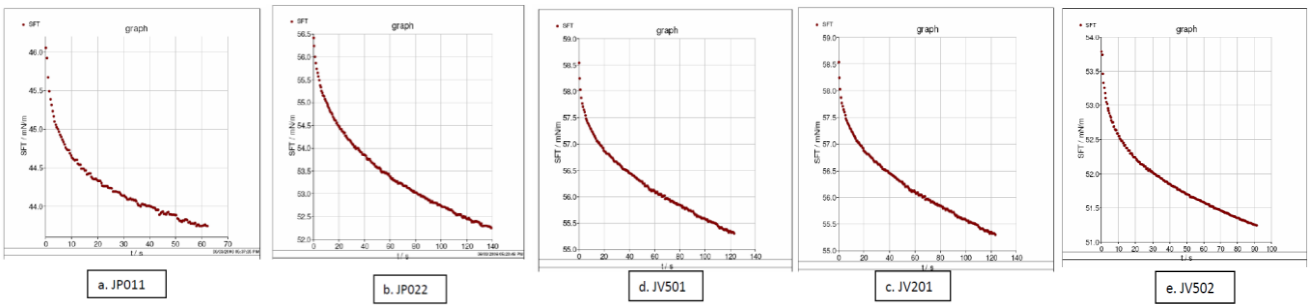


Figure 20. Surface tension measurement of the isolates

Antimicrobial activity

The antimicrobial activities of the biosurfactants found in 5 strains against six pathogenic strains. These were *Bacillus, Shigella, Streptococcus, Escherichia coli, Proteus and Salmonella)* are shown in Figure 25-32.

Antioxidant activity test

The biosurfactant extracted were checked for their antioxidant activity showing negative results (Table 7).

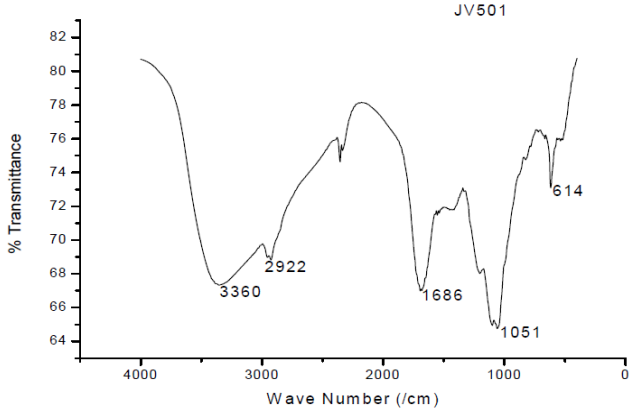


Figure 21. FTIR result of JV501

Inference

Wave number	Bonds	Functional group
3360	N-H stretch	Primary, Secondary amines, Amides
2922	C-H stretch	Alkanes
1686	C=O stretch	Carbonyls (general)
1051	C-N stretch	Aliphatic amines
614	C-Cl stretch	Aalkyl halides

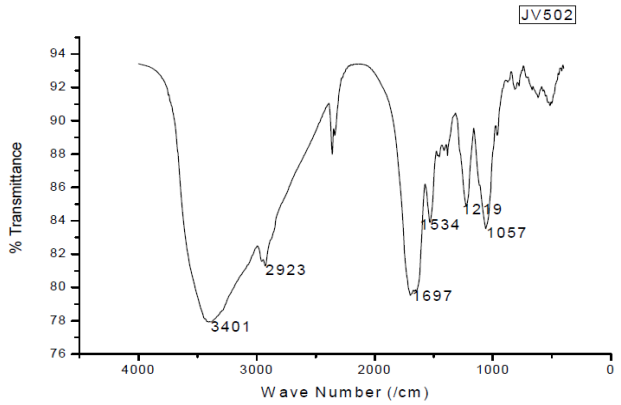


Figure 22. FTIR result for JV502

Inference

Wavenumber	Bond	Functional group
3401	O-H stretch, H-bonded	Alcohols, phenols
2923	C-H stretch	Alkanes
1697	C=O stretch	Carbonyls (general)
1534	N-O asymmetric stretch	Nitro compounds
1219	C-H wag (-CH_2X)	Alkyl halides
1057	C-N stretch	Aliphatic amines

Anti-adhesive test

Anti-adhesive test against Bacillus

Anti-adhesive property mainly depends upon the level of the biosurfactant and the microorganisms used. Here, the crude biosurfactants were extracted from five strains. *Bacillus* was taken as pathogenic strain to test the anti-adhesive property of these five biosurfactants, and PBS (phosphate buffer saline) was used as a control that contained no biosurfactant. Our findings demonstrated that biosurfactant extracted from JV201 showed anti-adhesive value 22.61% (Table 8) for the microorganisms *Bacillus* at a minor concentration (1.87mg/mL) means 22.61% adhesiveness inhibited.

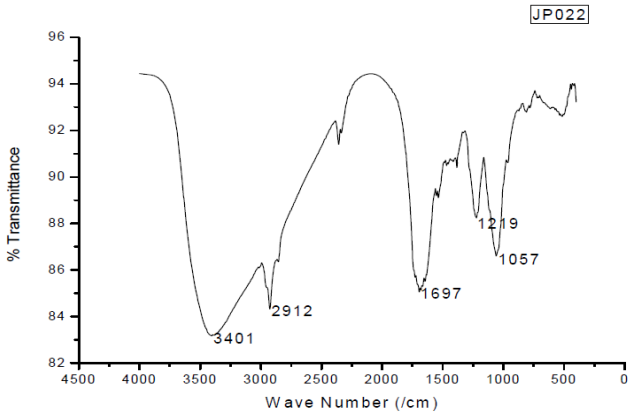


Figure 23. FTIR result of JP022

Inference

Wavenumber	Bond	Functional group
3401	O-H stretch, H-bonded	Alcohols, phenols
2912	C-H stretch	Alkanes
1697	C=O stretch	Carbonyls (general)
1219	C-O stretch	Alcohols, carboxylic acids, esters, ethers
1057	C-N stretch	Aliphatic amines

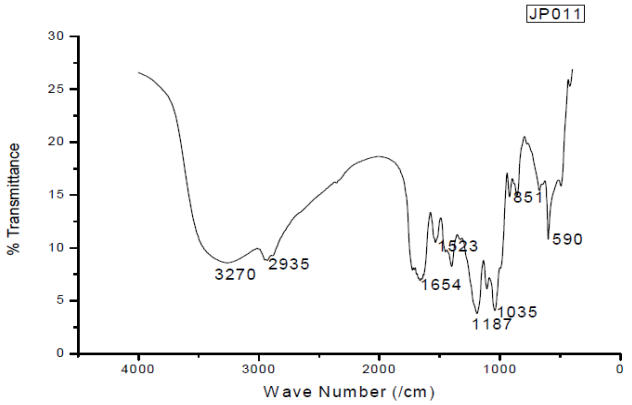


Figure 24. FTIR result of JP011

Inference

Wavenumber	Bond	Functional group
3270	N-H stretch	Primary, Secondary amines, Amides
2935	C-H stretch	Alkanes
1654	-C=C- stretch	Alkenes
1523	N-O asymmetric stretch	Nitro compounds
1187	C-H wag (-CH2X)	Alkyl halides
1035	C-O stretch	Alcohols, Carboxylic acids, Esters, Ethers
851	C-H "oop"	Aromatics

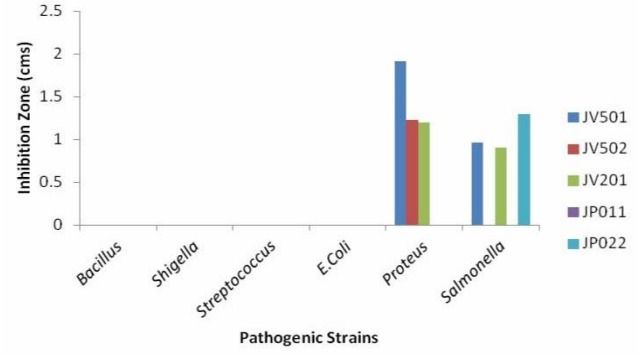


Figure 25. Antimicrobial activity of crude biosurfactant against pathogenic strains

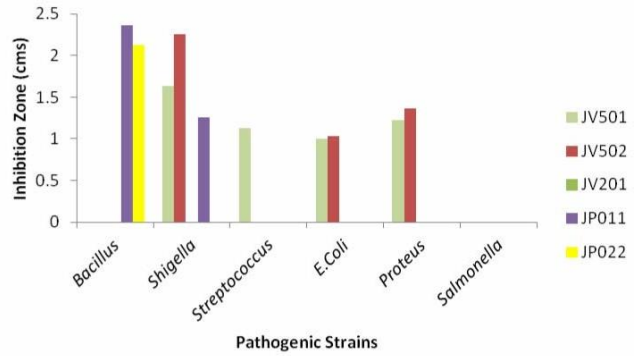
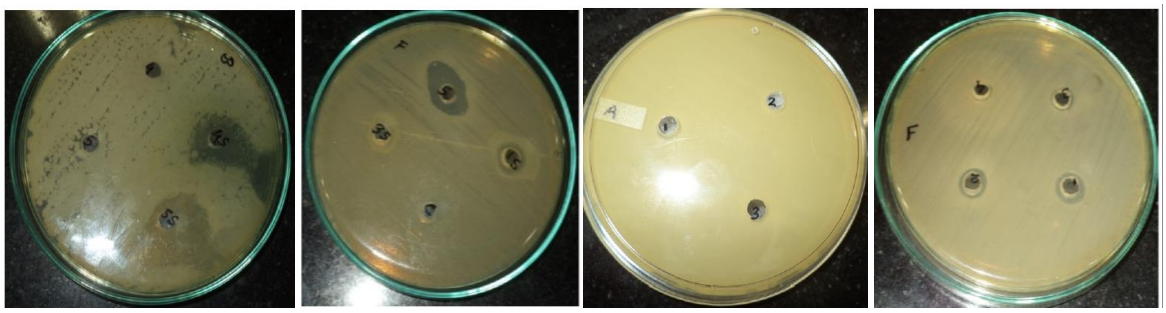


Figure 26. Antimicrobial activity of supernatant (biosurfactant) against six pathogenic strains

Table 7. The O.D. at 517 nm showing negative antioxidant activity

Strain name	Control	O.D. after 30 min	O.D. at 1 h	O.D. after 1.30 h
JV501	1.290	1.434	1.412	1.376
JV502	1.290	1.834	1.757	1.706
JV201	1.290	1.717	1.525	1.480
JP011	1.290	1.760	1.577	1.517
JP022	1.290	1.629	1.457	1.328



A. Positive B. Nebative

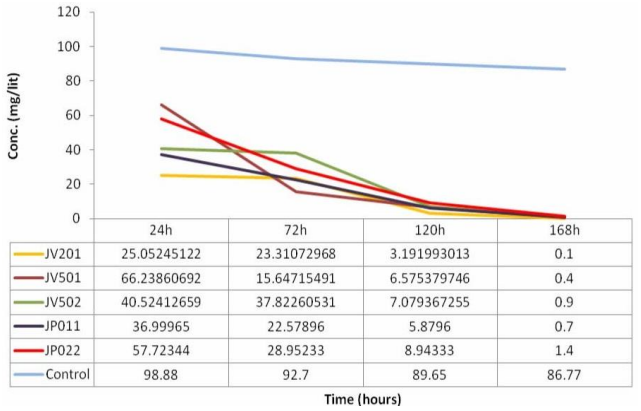
Figure 27. Plates showing Antimicrobial Activity by the biosurfactant extracted from the strains

Table 8. Percentage of Anti-adhesiveness against *Bacillus*

Strain name	Biosurfactant conc. (mg/mL)										Control
	50	25	12	7.5	3.75	1.87	0.93	0.46	0.23	0.11	
JV501	15.47	11.90	16.66	9.52	10.71	8.33	10.71	4.76	8.33	14.28	0.084
JV201	-59.52	19.04	21.42	8.33	20.23	22.61	21.42	14.28	10.71	9.52	0.084
JV502	9.52	-7.14	20.23	13.09	17.05	13.09	1.19	9.52	-2.38	-3.57	0.084
JP022	-8.33	4.76	11.9	8.33	14.28	13.09	4.76	-9.52	-17.85	-3.57	0.084
JP011	-126.19	-70.23	-48.80	-25	-22.61	-44.04	-48.80	-29.76	-21.42	4.76	0.084

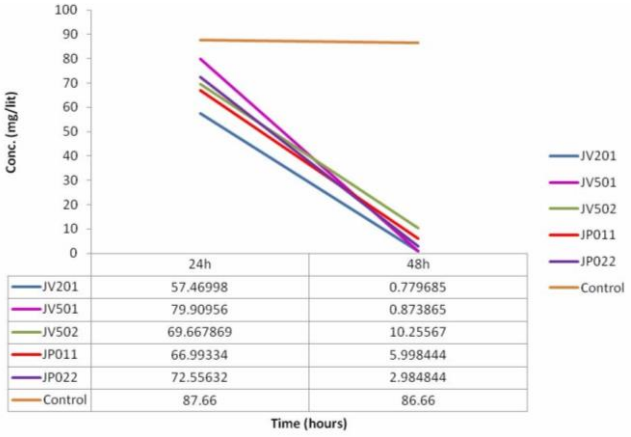
Table 9. Percentage of anti adhesiveness against *Streptococcus*

Strain name	Biosurfactant conc. (mg/mL)										Control
	50	25	12	7.5	3.75	1.87	0.93	0.46	0.23	0.11	
JV501	-16.66	8.33	7.14	3.57	2.38	-8.33	-25	-94.04	-140.4	-129.7	0.084
JV201	-85.71	20.23	21.23	22.61	22.61	22.61	23.8	2.38	-4.76	-40.47	0.084
JP022	-70.23	-26.19	1.19	2.38	2.38	-2.38	3.57	14.28	-13.09	-51.19	0.084
JP011	19.26	8.55	25.89	7.98	6.55	-7.54	-43	-94.8	-2.98	-6.89	0.084
JV502	3.12	6.77	15.28	28.94	18.78	4.89	5.99	3.14	-5.89	-3.29	0.084



	24h	72h	120h	168h
JV201	25.05245122	23.31072968	3.191993013	0.1
JV501	66.23860692	15.64715491	6.575379746	0.4
JV502	40.52412659	37.82260531	7.079367255	0.9
JP011	36.99965	22.57896	5.8796	0.7
JP022	57.72344	28.95233	8.94333	1.4
Control	98.88	92.7	89.65	86.77

Figure 28. Isolates showing phenanthrene degradation



	24h	48h
JV201	57.46998	0.779685
JV501	79.90956	0.873865
JV502	69.667869	10.25567
JP011	66.99334	5.998444
JP022	72.55632	2.984844
Control	87.66	86.66

Figure 29. Isolates showing naphthalene degradation

Anti-adhesive test against *Streptococcus*

Streptococcus was used for the anti-adhesive analysis. Like above PBS was used as a control. Here biosurfactants from JV501.JV201, JP022 were taken to test the anti-adhesive property against *Streptococcus*. It was observed that JV201 showed the anti-adhesive value for *Streptococcus* was 23.80% at a shallow concentration (0.93 mg/mL) and JP011 and JV502 showed anti-adhesive value for *Streptococcus* at 25.89% at 12 mg/mL concentration and 28.94% at 7.5 mg/mL concentration. These were given in Table 9.

Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHS) by biosurfactant

Phenanthrene biodegradation

Phenanthrene was added to the BHB in the concentration 100 mg/L for five strains (JV201, JV501, JV502, JP011, and JP022). O.D. was read at 292 nm for four days (1st, 3rd, 5th and 7th day) by extracting it with n-hexane. From five strains, JV201 and JP011 degraded phenanthrene from initial of 100 mg concentration to 3.19 and 8.94 mg within 120 hours. The other strains also displayed excellent degradation results (Figure 28).

Naphthalene biodegradation

Naphthalene was added to the BHB in the concentration 100 mg/L for five strains- JV201, JV501, JV502, JP011, and JP022. Absorbance was taken at 254 nm for four days (1st day, 3rd day, 5th day and 7th day) by extracting it with n-hexane. Among these five strains, it was observed that JV201 and JP011 had shown higher efficiency in the degradation of naphthalene and strain JV501 showed complete degradation of naphthalene very quickly (Figure 29).

Discussion

Fourteen bacterial strains were isolated from Paradeep port, Vishakhapatnam, Rishikulya, Bhitarakanika marine water and streaked in LB agar plates and maintained at pure culture. These strains were screened to check the biosurfactant production.

In the oil displacement test, twelve strains showed positive results, and two strains (JV801 and NP802) were negative. In drop collapse method, all strains showed positive results except JV202 and JV101. In emulsification assay, JP022 showed 41% of emulsification activity and the other strains ranging between 35-40%, except for NP202, NP103, and JV101, they gave negative results. In the hemolytic blood test, JP011 showed α hemolysis, JP022- β hemolysis, JV201- β hemolysis, JV501- β hemolysis, ATCC- α hemolysis and others were showing negative results.

From these biosurfactant screening assays, five strains named JV501, JV201, JV502, JP011, and JP022 were showing positive results of producing biosurfactant. Therefore, these five strains were taken for further study. SEM result showed that four strains are rod except for JV501 (cocci). Based on biochemical tests, mannitol motility test, nitrate reduction test, and sulfide indole motility tests, it was found that JV501, JV201, JV502 were showing mannitol fermentation and also motile. The remaining showed negative results in mannitol fermentation and also non-motile. Similarly, in the nitrate reduction test, JV501 and JV502 showing in the reduction of nitrate to nitrite and others displayed negative results. In sulfide production tests all were showing negative results.

Despite numerous report on the antimicrobial activities of biosurfactants, the biosurfactants are produced mostly by the micro-organisms of terrestrial origin. The number of reports on marine antimicrobial biosurfactant molecules is negligible. Therefore, their antimicrobial potentials have not been explored in details. This problem was addressed in the present work, and the biosurfactants isolated from marine bacteria as well as petrochemical wastes were tested for antimicrobial action against a battery of pathogenic test organisms. Six pathogenic strains namely *Proteus*, *Bacillus*, *Shigella*, *Escherichia coli*, *Streptococcus*, *Salmonella* were used for the antimicrobial test. Biosurfactants produced from JV501 showed antimicrobial activity against *Proteus*, *Salmonella*, JV502 against *Proteus*, JV201 against *Proteus*, *Salmonella* and JP022 against *Salmonella*. Supernatants of these five strains

proceeded to the antimicrobial test. Among these, JP011 demonstrated antimicrobial activity against *Bacillus* and *Shigella*, JP022 against Bacillus, JV501 against *Shigella*, *Streptococcus* and *Escherichia*, JV502 *Shigella*, *Escherichia coli*.

Antimicrobial activity of the tested strains is evident in this study and thus can be useful in many domestic and commercial uses. The isolated biosurfactant showed activity against both Gram-positive and Gram-negative bacterial strains. The result is quite in contrast to earlier reports on antimicrobial actions of the biosurfactants where the lipopeptide biosurfactants were reported to be active mostly against Gram-positive bacteria (Singh and Cameotra 2004).

The growth pattern of the isolates was usually between 16-20 hrs having good biosurfactant production during this period. The chemical characterization of the produced biosurfactant using FTIR demonstrated that the peak obtained through this analysis usually corresponds to primary and secondary amines functional groups, also having carboxylic acid stretch, alkane stretch as well as aromatic groups present. The carbohydrate estimation using Phenol-sulphuric acid test and protein estimation using Bradford assay showed that there were much carbohydrate and protein content in the extracted biosurfactant. Different aliphatic and aromatic carbon sources were utilized as a substrate for the growth of biosurfactant producing bacteria to have an optimization study of which references are widely utilized. All the five strains could easily use both glycerol and sucrose. The growth was also seen in different aromatic compounds like naphthalene, pyrene, phenanthrene, biphenyl, kerosene suggesting that the bacteria could efficiently utilize naphthalene and phenanthrene more readily, and then comes biphenyl. The rationale behind biosurfactant production on hydrocarbon utilization must have stimulated itself by enhancing the substrate availability. In some literature, it was mentioned that biosurfactant production in the presence of hydrocarbons showed better production of biosurfactants (Kumar et al. 2006). Here the result was the same; the bacterial strains were showing better production of biosurfactant by utilizing the PAHs as a carbon source. Biosurfactants usually lower the tensioactive force between the two phases. The surface tension of this fraction of the strains JP011, JP022, JV201, JV501, JV502 was found to be in the range of 40-55 mN/m, with the lowest (43.776 mN/m) being from the strain JP011 indicating its powerful surface tension-reducing property.

The anti-adhesive nature of biosurfactant was also tested for the five strains against *Bacillus* and *Streptococcus* of which few strains producing biosurfactant showed good anti adhesiveness. This property can be attributed for the cleaning of pathogenic organisms present in medical equipment and efficiently used in medical uses. The antioxidant potential of the biosurfactant was determined by their scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH method is widely used and the easiest way to measure the antioxidant activity of compounds. But the isolated strains did not show any positive antioxidant results.

Biosurfactant utilized in bioremediation has been harnessed relentlessly for biotechnological purposes. We have isolated and identified five potent strains having high surface tension reducing property: *Ochrobactrum*, *Streptococcus*, *Pseudomonas* sp., *Pseudomonas aeruginosa*, and *Achromobacter xylosoxidans* which have 99.9%, 99.6%, 99%, 99.3%, 98.6% of phenanthrene degradation (100mg/L) and 99%, 99.1%, 89.75%, 94.01%, 97.02% of naphthalene degradation (100 mg/L) respectively having good antimicrobial and anti-adhesive properties.

To conclude, nowadays the production of biosurfactant is increasing due to its properties like low toxicity, biodegradability, digestibility and biocompatibility and also due to its vast applications in bioremediation of various toxic substances like PAHs. It is produced on living surfaces mainly microbial cell surfaces or synthesized extracellularly amphiphilic compounds reduces the interfacial tension between the surfaces and interface respectively. When bacteria are present in stress conditions like hydrophobic environment, they utilize these hydrophobic substances like carbon and energy sources. Bacteria produce biosurfactant which helps in conversion of the hydrophobic layer into small micelles which can be easily engulfed as a carbon source which is the primary nutritional requirement. PAHs are released into the environment by various sources. These can be classified into natural sources and anthropogenic sources, but anthropogenic sources produce higher PAHs than natural sources. The anthropogenic sources are the waste products of various industries (Petroleum, Diesel), domestic sewage, the oil spill in the marine environment, smoking, by burning of coal, diesel, and petrol (fuel for energy). The hydrocarbons contaminate the subsoil and groundwater. It enters the food chain and disturbs it. Some of the light polyaromatic hydrocarbons bind to the dust particles in the atmosphere and persist for a long time. It enters into the human body through inhalation, food, skin and causes mutagenic and carcinogenic effects. These compounds are highly toxic, even a low amount of them present in the soil may cause serious problems.

Eco-friendly technologies such as degradation by microorganisms must be used to clean the environment. Bioremediation has been accepted as an essential method for the treatment of oil pollution by biosurfactant excreted by bacterial colonies. Under certain conditions, living microorganisms especially bacteria can metabolize many classes of hydrocarbons compound. Since hydrocarbons carry high organic matter, it can be assimilated by the bacteria as a carbon source. There are many ways used to clean up the organic contaminants. Some non-biological methods such as excavation and discharge of contaminated soil to landfill sites are employed. Biological methods are the processes that utilize plants (phytoremediation) or microorganisms (bioremediation) to remove these pollutants from soil. Therefore, employing biobased techniques like the production of biosurfactant in large quantities through bioreactors can be efficiently commercialized in industries and can be applied in highly

polluted areas for complete biodegradation of the toxic polycyclic aromatic hydrocarbons.

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Genetic biodiversity of spiny lobsters (*Panulirus* spp.) from coastal waters of Southern Java, Indonesia

FLORENCIUS EKO DWI HARYONO[1,♥], AMBARIYANTO[2,♥♥]

[1] Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Jenderal Soedirman. Jl. Dr. Soeparno, Kompleks GOR Susilo Sudarman Karangwangkal, Purwokerto Utara, Banyumas 53123, Central Java, Indonesia. Tel./fax.: +62-281-642360. ♥email: marine_2807@yahoo.com
[2]Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro. Jl. Prof. Sudarto S.H., Tembalang, Semarang 50275, Central Java, Indonesia. Tel.: +62-24-7474698, ♥♥email: ambariyanto@undip.ac.id

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Abstract. *Haryono FED, Ambariyanto. 2018. Genetic biodiversity of spiny lobsters (*Panulirus *spp.) from coastal waters of Southern Java, Indonesia. Ocean Life 2: 73-78.* Spiny lobsters of the genus *Panulirus* inhabit southern Java; however, species identification of early life stages based on morphological features analysis is equivocal. The condition results from differences in morphology of the larval stage and the adult, a long period of the larval life cycle. Oceanographic currents' impact inhibits and restricts the route and direction of larval dispersal that causes genetic changes. However, this area's genetic biodiversity of spiny lobsters has not been investigated. The aim of this/our study was to identify lobsters' genetic diversity in Southern Java waters. The research was conducted from January through to August 2015. A total of 1,137 lobsters were collected from seven different locations for morphological analyses, and 40 lobsters were taken randomly for genetic analysis. Observations of nuclear and mitochondrial DNA, the relationships of genetics in phylogenetic using Codon of code and MEGA 5.0, and dendrogram using Primer_e software. Morphological analysis obtained 6 species of spiny lobsters: *P. homarus, P. versicolor, P. ornatus, P. penicillatus, P. polyphagus,* and *P. longipes*. Phylogenetic analysis obtained two clades, and morphological identification results obtained *P. penicillatus* (spiny rock lobster). Still, genetically acquired identification included a group of genetic *P. homarus* (spiny sand lobster). Such discrepancies are possibly due to a long period of larval life, adults in the low light of intensity environment, and extreme oceanographic conditions with the marine oil spill.

Keywords: Biodiversity, genetic, spiny lobster, Southern Java

INTRODUCTION

As an archipelagic and tropical country, Indonesia is the center of mega-biodiversity (Brooks et al., 2006; Ambariyanto, 2010). The biological aspect of marine environment conditions shows that marine organisms are very diverse (Hutomo and Moosa 2005; Allen 2008), and most marine organisms are in high demand as commodities. As a result of the conditions, the exploitation of marine resources is very high. Especially for the resource to be consumed and traded mainly have high economic value (Berkes et al. 2006). Various types of marine organisms are important export commodities for Indonesia, which have an important role in the country's economy (Baily 2006; Lambaga 2009).

Lobsters can have a special ability compared to other marine species, the special ability was able to survive without water for a certain period, and this excess used to be distributed under life conditions (Landau 1991), and these advantages lead to high economic value. Spiny lobster is an important species and one of the species that have the support of global food stocks. The world stock of lobster as supporting fishery production was currently around 260,000 tons per year (de Lestang et al. 2015). The tropical lobster consists of several species, commonly known as lobster/mangkara/barong

The study of diversity lobster is not only morphological but also genetic diversity (DeBoer et al. 2008; Starger et al. 2010; Vogler et al. 2012; Barber et al. 2014). Family *Panulidae*, genus *Panulirus* consists of 19 species, all of these species live spread in the marine world environment, seven species live in Indonesia ocean, five species namely *P. homarus* Linnaeus (1758), *P. penicillatus* Olivier (1791), *P. longipes* A. Milne-Edwards (1868), *P. ornatus* Fabricus (1798) and *P. versicolor* Latreille (1804). Lobsters live in Indonesia and are found in the Pacific Ocean (Reddy 2013). Southern of Central Java lobsters life in Cilacap waters are 4 species, i.e., *P. homarus, P.ornatus. P. penicillatus, and P. ornatus* (Hartoyo et al. 2002). Busono (2008) found 5 species in southern Central Java waters. While Haryono and Tjahya (2008) state that the spiny lobster in Cilacap waters based on morphological characteristics obtained 6 species, i.e., the "sand lobster" (*P. homorus*), "pearl lobster" (*P. ornatus*), "bamboo lobster" (*P. versicolor*), "stone lobster" (*P. penicillatus*), "batik lobster" (*P. longipes*), and "brown lobster" (*P. polyphagus*).

Identification of lobster based on morphological results many discrepancies. The conditions are based on the differences in morphology of the larval stage to the adult morphology (Chow et al. 2006). As a result of a long period of the lobster larval life cycle, oceanographic currents limit the impact that inhibits the larval dispersal routes and directions, causing genetic changes in lobster in other regions (Abdullah et al. 2014). Genetic diversity and population structure of marine species due to the long life

of the larval stage (Palero et al. 2008), and the present species was the product of a long history of models of the effects of past and oceanographic processes. Another cause of the irregularity was intermarriage causes genetic diversity (Palero et al. 2009).

Liquid and solid wastes have polluted extremely in Southern Java waters and will affect the benthic organism, including spiny lobster. Biophysical conditions such as oceanographic conditions concerning the behavior of the larvae contribute significantly to population genetic differentiation for marine and pelagic phases and genetically distinct effects on the development of non-pelagic (Laurenzano et al. 2013). Some of the causes of disruption of oceanographic parameters that have occurred in the southern waters of Java, among others, are the batik industry in Yogyakarta and Surakarta, with chemical wastes in the process of dyeing fabrics leading to coastal waters. Extreme pollution also occurs with many ship incidents containing crude oil polluting the sea, among others Alisa XVII on November 1989 loading 11.000 drums of asphalt, MT King Fisher loaded full of crude oil in April 2000. Crude oil and bitumen spills are distributed toward the northwest during when highest tide; otherwise, the oil spreads toward the southeast during when lowest tide (Widhayanti et al., 2015). Banana VI and a ship were leaked with loaded 5,000 tons of airplane fuel on January 15, 2010, MT Alisa XVII 18.101 GT aground and leaked, including many fishing boats. During 2006-2008, four tanker accidents were reported as shipwrecks (Fitri 2008). MT HHC has loaded the bulk of asphalt, and MT Harmony Seven sank on October 17, 2015, with 33,000 tons of diesel.

Such conditions have implications for fisheries management related to the stock lobster for evaluation of long-term management of lobster. Species in deeper water are more sensitive to overexploitation, and the long period of overexploitation impacts genetic diversity (Palero et al. 2010). Irregularity of species organs, such as the communication organ for lobster, was the result of evolution. Based on morphological data, two adult organ morphology of organs in the same species exist. Based on that, irregularities need special lobster management. The aim of the study was identification the genetic diversity of lobster in the Southern coastal Java water, Indonesia.

MATERIALS AND METHODS

Study area

The coastal waters of Southern Java are known as an area with high lobster catches. Study area especially in Southern waters of Central Java Province and Special Region of Yogyakarta Province, Indonesia, which Southern Central Java waters consist of four districts, namely Cilacap, Kebumen, Purworejo, and Wonogiri. Special Region of Yogyakarta consist tree districts, namely Kulonprogo, Bantul, and Gunungkidul. Lobsters were captured from the southern waters of Java with lobster data collection stations covering lobsters living in Teluk Penyu waters of Cilacap where the tourism coast Teluk Penyu as Station 1, Station 2 in the outside the Cilacap Ocean Fishing Port (PPSC) waters, Station 3 and 4 in the Kebumen water with landing center at Pantai Ayah and Karang Duwur, 5th, 6th, and 7th stations are waters of Yogyakarta with the concentration of catching in Depok-Bantul, Baron and Sadeng-Gunungkidul waters.

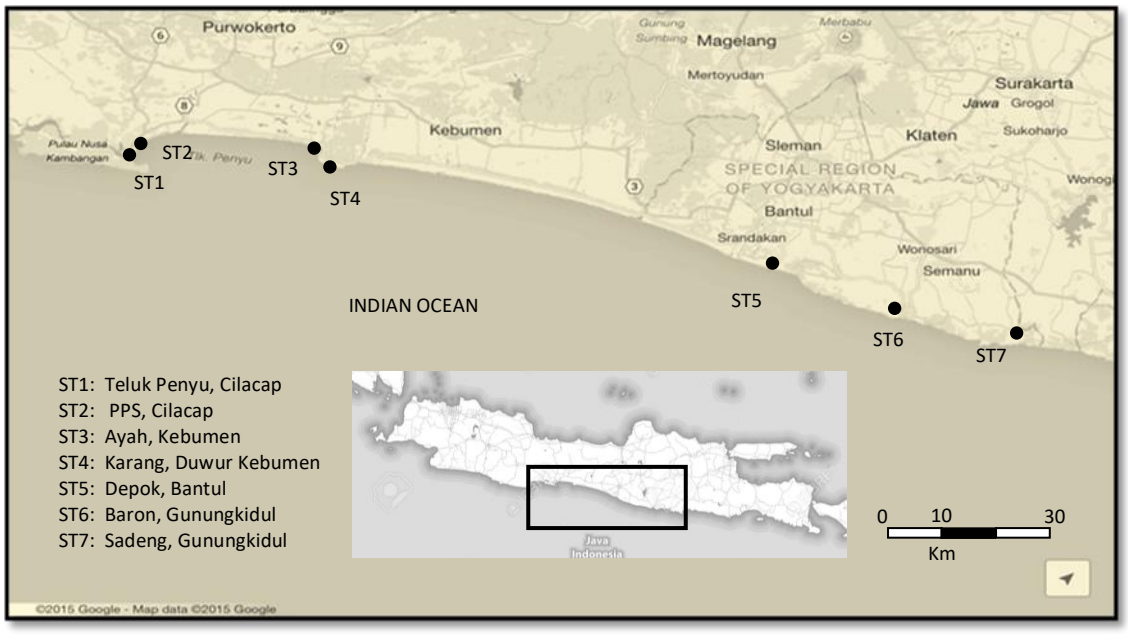

Figure 1. Station site (ST), Southern water of Java, Indonesia

Procedure

Lobster sampling was done randomly at the Southern Central Java waters (Cilacap, Kebumen, Purworejo districts) and Southern Special Region of Yogyakarta waters (Kulonprogo, Bantul, and Gunung Kidul districts). These locations are known as high lobster fishing areas. Sampling was conducted from February to August 2015 using lobster gillnet with a mesh size of ¾ inch. Sampling was performed six times at the locations. All lobsters captured were put in a cool box to be identified morphologically in situ based on Carpenter and Niem (1998). Genetic analyses of the sampled lobsters were carried out at the Integrated Laboratory of Diponegoro University, Semarang, Indonesia.

Lobster DNA was extracted in five steps. First, 1 g of leg tissue was crushed and placed in a mini tube containing a DNA extractor. The solution was then centrifuged for 10 minutes, incubated at 95^0C for 20 minutes, and centrifuged for 10 minutes. Next, extracts of each sample were transferred to a microtube. The amount of mtDNA (in base pairs, bp) was determined using a Nanodrop, and the number of its base pairs that are used as a basis of comparison with its standard lobster base pairs (bp) 566-571 (Abdullah et al. 2014). The solution was diluted sample extract DNA template if more than bp standard and vice versa. Amplification of mtDNA base pairs molding method Polymorphic Chain Reaction (PCR) based methods Lavery et al. (2014). Nucleotide base pairs multiplication process begins by mixing a lobster leg muscle extract results from a DNA template preparation by PCR solution, namely primary brand Promega releases (LCOI) and reserve (HC0I) each at a concentration of 2 uL and 2.5 uL and added solution PCR 25 uL, add 18.5 uL H2O, the overall concentration is 50 uL (DNA extraction Promega). PCR method with the first phase template DNA sample was heated 95^0C for 4 minutes, 94^0C for 10 min, 20 min 47^0C, 72^0C for 30 minutes, and 72^0C for 5 minutes. Overall, the DNA template is heated 35 times and lasts indefinitely to heat 40^0C. The separation of samples of cellular genetic molecules with the electrophoresis process is to form an mtDNA fragment. Next, soak in an ethidium bromide solution, and the solution will infiltrate into the DNA, which in turn serves to visualize as it will fluoresce when irradiated with ultraviolet light.

Data analysis

DNA sequencing was performed at the PT. Genetic Center Indonesia. Philo-genetic analysis using codons code and software MEGA5.0 and Dendrogram use Primer_e software.

RESULTS AND DISCUSSION

A total of 1137 *Panulirus* spp. individual lobsters were collected, i.e. Cilacap (243 individual lobsters), Kebumen (129), Purworejo (13), Kulonprogo (55), Bantul (275), Gunungkidul (422), and Wonogiri not obtained data. Morphological identification of spiny lobster from Southern water of Java obtained six species: *P homarus*, *P. versicolor*, *P. ornatus*, *P. polyphagus*, *P. penicillatus*, and *P. longipes*. Genetic analysis of 40 leg muscles of lobsters sample with distribution from Cilacap, Kebumen, Kulonprogo, Bantul, and Gunung Kidul districts (Table 1).

Table 1. Number of *Panulirus* species collected per district and sequencing analysis codes

Districts	Spesies	Code	Districts	Spesies	Code
Cilacap	*P. homarus* 0	CHP0	Bantul	*P. homarus* 1	BHP 1
	P. homarus 1	CHP 1		*P. homarus* 2	BHP2
	P. homarus 2	CHP 2		*P. homarus* 3	BHP3
	P. homarus 3	CHP 3		***P. homarus* 4**	**BHP4**
	P. homarus 4	CHP 4		*P. homarus* 5	BHP5
	P. homarus 5	CHP 5		*P. homarus* 6	BHP6
	P. ornatus 1	CM 1		*P. penicullatus*1	BBT1
	P. versicolor	CB		*P. penicullatus*2	BBT2
	P. polyphagus	**CP**		*P. penicillatus*3	BBT3
	***P. polyphagus* 1**	**CP 1**		*P. ornatus*	BM
Kebumen	*P. homarus*	KHP		*P. ornatus* 1	BM 1
	P. penicullatus	KBT	Sadeng	*P. homarus*	SHP
	P. ornatus	KM	(Gunung Kidul)	*P. homarus* 1	SHP1
	P. polyphagus	KP		*P. homarus* 2	SHP2
	P. versicolor	**KBB**		*P. homarus* 3	SHP3
Kulon Progo	*P. homarus* 0	KPHP0		***P. polyphagus* 1**	**SBT1**
	P. homarus 1	KPHP1		*P. polyphagus* 2	SBT2
				P. polyphagus 3	SBT3
				P. polyphagus 4	SBT4
				P. versicolor	SBB
				P. longipes	**SBS**

Mitochondrial DNA (mtDNA) lobster sample of PCR process results, optimized through the electrophoresis stage. In the electrophoresis process, the band fraction that was not visible at the base pair (bp) reading under uLtraviolet rays was 6 samples (Table 1 bold letters), i.e., CP, CP1, KBB, SBS, BHP4, and SBT1, We suspect the consequences of leg tissue lobster with less good condition.

Electrophoresis under ultraviolet light was shown in that DNA base pair lobster sample at 600 bp. Two clades were obtained out of the 34 sequenced individuals. Clad 1 consisted of ten species of lobster *P. homarus* (8) and *P. penicillatus* (2), namely CHP2, KBT, KPHP1, BHP3, SHP2, BHP1, KHP1, SBT3, SHP0, and SBT2. Clad 2 consists of 8 species, namely BBT2, BBT3, BBT1, SBB, CB, KM, BHP4, and BM1. Kinship and morphological identification of lobster in clad 1 were identified as rock lobster *P. penicillatus* (SBT and KBT2). Phylogenetic group, common ancestor by lobster *P. homarus* (even being a clad and in groups of lobster *P. homarus*). The conditions of the aquatic habitat of seabed fishing ground in Gunung Kidul district were rocks. Identification of spiny lobster morphologically was *P. penicillatus* (rock lobster), and genetic analysis resulted from *P. homarus* (sand lobster). The condition presumed the identification of spiny lobster morphologically as *P. penicilatus* and genetically identified as P. homarus. The condition was estimated to be an adaptation form in a rock environment. Laveri et al. (2014) state that diversity of geographic patterns of genetic diversity resulted in an evolution of *P homarus*. Based on the color of spiny lobsters, Tlusty and Hyland (2005) state that genetic aspects and mechanisms of diet influence the phenotype color of lobsters. Variability color is a tool to identify lobster and differences in lobster color genetically by the phenotype of lobster. Seabed conditions Gunungkidul fishing ground as rock and color of lobster darker.

Spiny lobster has a complex life cycle and bio-oceans (Diniz et al. 2010), a tropical species with a very wide distribution (Lavery et al. 2014). Aspects of evolution, distribution, ecology, and biology of lobster and how they relate to genetic aspects (Reddy. 2013), but the association between the environment and the gradient is important in identifying the impact of natural selection (Sork and Waits 2010).

Diversity genetics can occur in different individuals within the same species (intra-specific), may also occur in some species (interspecific), and also diversity genetically between genus to and between families with each other family (Hoshino et al. 2012). Genetic variation is a spatially significant contribution to the genetic mapping and mapping effects on genetic variations that can be used to identify and restrict gene flow (Sork and Waits 2010). For example, the loss of genetic diversity can contribute to the collapse of the lobster fishery and cause ecological and social impacts on ecosystems and fisheries (Worm et al. 2006).

Discussion

We identified six spiny lobster species in southern Java waters. These were the *lobster Pasir* (*P. homarus*), *lobster mutiara* (*P. ornatus*), *lobster bambu* (*P. versicolor*), *lobster batu* (*P. penicillatus*), *lobster batik* (*P. longipes*), and *lobster cokelat* (*P. polyphagus*). Lobster catches in Cilacap, Kebumen, Purworejo, Bantul, and Kulon Progo districts were dominated by *P. homarus*, whereas catches in the Gunung Kidul district were composed especially of *P. polyphagus*. Additionally, *P. homarus* was the most abundant species caught in the muddy sand substrate, while *P. homarus* was the most abundant species in the rock substrate.

Morphological identification of specimens collected in the Gunung Kidul district resulted in two different species, the spiny rock lobster and the spiny sand lobster. However, genetic analyses clustered them into the same clade. The condition is assumed to adapt to the environment and low light intensity.

Spiny lobster *Panulirus* sp. has a very complex life cycle and bio-oceans (Diniz et al. 2010), as its terms of tropical species that live with a very wide distribution (Lavery et al. 2014), aspects of evolution, distribution, ecology, and biology lobster associated with genetic aspects (Reddy 2013), but the relationship between the environment and the gradient is important because it identifies the impact of natural selection (Sork and Waits. 2010). Genetic diversity may occur in individuals who are different even though the same species (intra-specific), also can occur in several species (interspecific), and genetic diversity can also occur when there is a cross between a genus one genus to another and between the families one with another family (Hoshino et al. 2012). The loss of genetic diversity can contribute to the collapse of the lobster fishery, causing ecological and social impacts on ecosystems and fisheries. Significant genetic variations are also a result of the spatial contribution, so the genetic condition of a species is important for genetic mapping; the genetic map is important because it can be used to identify and restrict gene flow (Sork and Waits. 2010).

Species of *Panulirus* spp. in Southern Java waters were obtained of six species, namely *P. homarus*, *P. ornatus*, *P. versicolor*, *P. penicillatus*, *P. longipes*, and *P. polyphagus*. Species *P. homarus* dominant were caught in Cilacap, Kebumen, Purworejo, Bantul and Kulon Progo districts and the dominant of lobster were caught in Gunungkidul *P. penicillatus*. The dominant lobster caught in the muddy sand seabed substrate were lobster *P. homarus* and substrate rock *P. penicillatus*.

Identification morphologically obtained different species, but genetically in the same group, such as morphologically identification of the lobster that dominant life at Gunungkidul and life in rock substrate, namely *P. penicilatus.* Genetically was the same clad as *P. homarus*. The condition was assumed adaptation to the environment and low intensity. The condition is also important to review the conditions associated with the impact caused by environmental pollution on the beach as the lobster habitat. Within a few years, a ship tanker with a cargo full of raw fuel oil had to sink and pollute the Southern waters of Java. Also, some ship tanker leaks occur in shipping channels into the crude oil refinery in Cilacap.

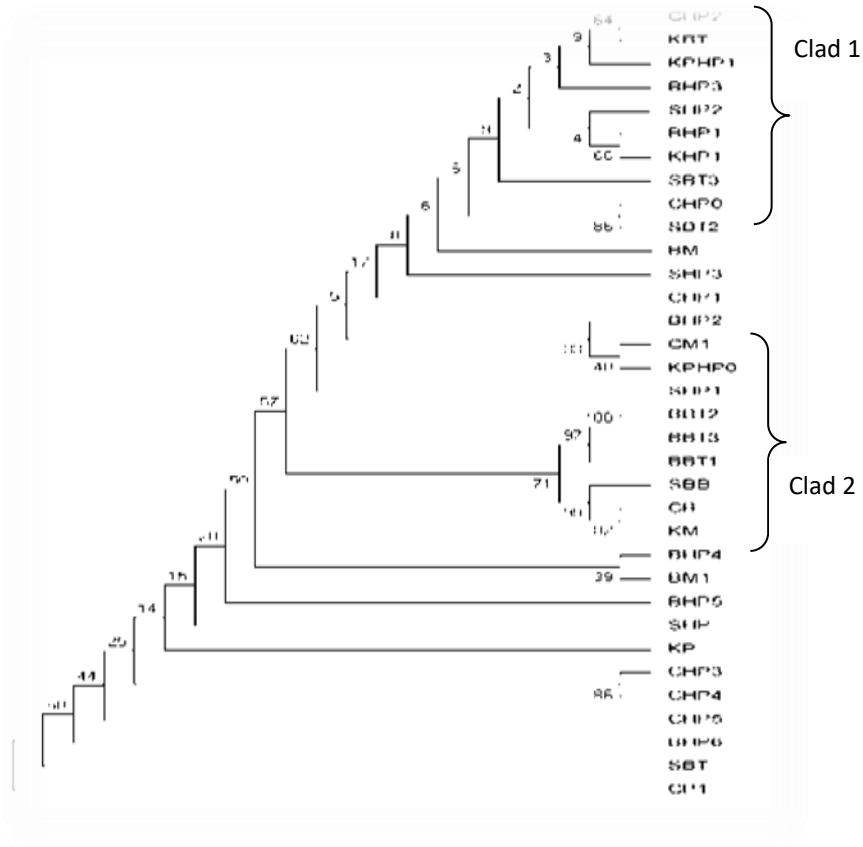

Figure 1. Phylogenetic of spiny lobster (*Panulirus* sp.) from Southern water of Java

Liquid and solid wastes have extremely polluted Southern Java waters. Risk levels of hydrocarbon in Cilacap sediment ranged from low to medium-low. They were obtained from a location that has a greater impact on the ecological risk for marine organisms (Syakti et al., 2015). Sewage disposal outlets decreased population density and changes in size spectra (de-la-Ossa-Carretero et al. 2010) that impacted from Cilacap refinery site stations obtained heavy metal Cr, Ni, and Zn concentrations in sediments. It may cause the adverse effect to occur over a wider range of organisms and can contribute to a more serious harmful effect (Syakti and Hidayati 2015). Coastal areas of Cilacap has sensitive and very sensitive to the presence of oil spills (Wibowo et al. 2008).

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