

# Delving of a Promising Bioemulsifier Producing Bacterium from an Oil Contaminated Coastal Site and its Enhanced Production

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Chemical surfactants are non-biodegradable and harmful, thus researchers are looking for better alternatives. The present study aimed to isolate bioemulsifier producing bacteria from oil-contaminated sediments. Nearly, 19 morphologically distinct bacteria were isolated and screened for bioemulsifier producing potential. Based on the screening, one efficient isolate PHCS 7 was selected and further subjected to molecular identification. After characterization, the isolate was identified as *Acinetobacter beijerinckii* PHCS 7 and further employed for growth kinetic profiling and optimization of physical factors for bioemulsifier production. During 48hrs incubation, *A. beijerinckii* PHCS 7 showed 64.6% emulsification activity with 8.69g/L of cell biomass. Similarly, during the optimization study pH, 8 and temperature of 35°C favored 67.9% and 69.7% emulsification activity, respectively. The current research establishes a foundation for future research on cost-effective large-scale production.

**Keywords:** Bioemulsifier; Growth kinetics profile; Oil-contaminated coast; Marine *Acinetobacter beijerinckii*; Optimization.

Microbes account for more than 90% of ocean biomass and make up the hidden bulk of life that thrives in the water. Marine microorganisms, whether as single strains or as part of microbial consortia, have distinct metabolic and physiological activity that has new industrial and environmental uses. The diversity of marine microorganisms, including bacteria, protozoa, algae, and fungi, has been documented. Bacteria and Archaea in marine waters and sediments account for a significant portion of global microbial biomass, with an estimated total population of  $6.6 \times 10^{29}$  cells<sup>1</sup>. The majority of marine bacterial communities are extremely diverse, with individual samples containing over 20,000 different species<sup>2</sup>. The

pigments produced by marine bacteria as a result of quorum sensing are of current interest due to their photoprotective, anti-cancer, anti-microbial, anti-parasitic, and immunosuppressive activities. Currently, marine bacteria are being discovered for their production of clinically and industrially significant secondary metabolites<sup>3</sup>.

Heavy metal poisoning of ecosystems as a result of anthropological action is a serious problem. Environmental sustainability principles should be followed when developing remediation methods. Metals present several unique clean-up issues since they cannot be reduced into harmless compounds<sup>4</sup>. Diverse bioremediation techniques have been suggested to eliminate or neutralise

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the toxic effects of metal contamination in the environment; nevertheless, removal effectiveness may be constrained due to the poor bioavailability of such metals in soils<sup>5</sup>. As a result, the use of biosurfactants and bioemulsifiers has been suggested as an alternative<sup>6, 7, 8</sup>. A unique class of compound called microbial emulsifiers has a wide range of potential uses in industrial development and the remediation of organic and metal-contaminated environments<sup>9, 10, 11</sup>.

Due to their distinct advantages over chemical surfactants, such as biodegradability, biocompatibility, foaming, effectiveness at low concentrations, non-toxicity and high selectivity in a range of temperatures, pH and salinities, bioemulsifiers are referred to as surface-active biomolecule materials. Bioemulsifiers have a higher molecular weight than biosurfactants because they are complex mixtures of proteins, lipoproteins, heteropolysaccharides, and lipopolysaccharides<sup>6, 12, 13</sup>.

Oil contamination has increased the number of microorganisms that may produce biosurfactants that can solubilize and utilize petroleum hydrocarbons as carbon sources, opening the way for the isolation of novel bioemulsifier-producing strains from oil-contaminated locations<sup>14, 15</sup>. Several microorganisms have been identified as having the potential to create biosurfactants in the medium. *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhodococcus* and *Halomonas* are examples of these bacteria<sup>16, 17, 18</sup>. So far, few microorganisms including *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., fungi, and yeasts have all been isolated from oil/hydrocarbon-contaminated environments, according to several publications<sup>19, 20, 21, 22</sup>.

There are many industrial and environmental uses for stable water-in-oil or oil-in-water emulsions, including in the food, detergent, pharmaceutical, agricultural, and cosmetic industries<sup>6, 23, 24</sup>. Despite their wide range of applications, they have received less research than biosurfactants. Alasan, emulsan, lipomannan, liposan, and mannoprotein, which are produced by *Acinetobacter calcoaceticus* RAG-I, *Acinetobacter radioresistens*, *Candida lipolytica*, *Candida tropicalis*, and *Saccharomyces cerevisiae*, respectively, have been studied as high molecular

weight polymeric biosurfactants or bioemulsifiers<sup>16, 25</sup>.

Due to high production costs and limited yield, research into bioemulsifier development has intensified, but industrial-scale manufacturing has not yet been achieved. This induces a necessity for exploring novel, high-yielding bioemulsifier-producing microorganisms. In association with this, the present study aimed to screen and characterize bacteria with elevated bioemulsifier production from oil-contaminated sediment. Growth kinetics and physical factor optimization for the production of bioemulsifier were also reported.

## MATERIALS AND METHODS

### Sampling

Sediment samples contaminated with petroleum hydrocarbons were aseptically collected from the coastal areas of Karaikal, Puducherry, India, and transferred to the lab in 250ml pre-sterilized bottle containers. For the serial dilution, one gram of silt from the center of the collections was weighed. Serial dilutions were performed using pre-sterilized synthetic seawater (Himedia, India), which was made at a salinity of 34ppt (parts per thousand) and a pH of 8.2.

### Isolation of marine bacteria

On Zobell marine agar (Himedia, India) plates, the spread plate method was used to isolate marine bacteria from the serial dilutions. On freshly made Zobell marine agar plates, after two days of incubation at 37°C, morphologically distinct bacteria were chosen and purely cultured. The validation of axenic cultures was accomplished by visual examination, followed by microscopic observation after the Gram staining method. For later usage, all of the axenic cultures were kept in lyophilized conditions.

### Extracellular or intracellular bioemulsifier production

Every isolate used in this investigation was cultivated separately in 30ml screw-cap tubes with 10ml of Zobell marine broth, which is identical to Zobell marine agar in all but the amount of agar. All of the cultured broths were independently centrifuged at 3000rpm for 15min after 96hrs of incubation at 37°C; the cell pellet and cell-free supernatant were collected

separately. Using an ultrasonicator, the cell pellet was dissolved in 50ml of phosphate buffer (pH 7) and sonicated for 45 seconds at 20KHz (Hielscher, USA). While the cell-free supernatant from the grown broth was immediately used for the research of bioemulsifier generation, the cell debris created during the sonication process was removed using the same applied centrifugal conditions as indicated before. This study provides evidence for either extracellular or intracellular bioemulsifier production by the test bacterial strains, or for their presence at both sites.

#### **Emulsification assay**

The emulsification assay as described in Camacho-Chab *et al.*<sup>26</sup> was used to gauge each marine isolate's percentage emulsifying activity. After the vortex, the assay was run at room temperature against crude oil as a hydrocarbon substrate. Equivalent volumes of the hydrophobic substrates and the emulsification sample were combined in the measuring centrifugation tubes, swirled on a vortex for two min., and then allowed to stand for 48hrs to determine the emulsifying activity. The percentage of the overall height that the emulsion eventually occupied after some time was judged to be the emulsifying activity.

$$\text{Emulsification activity (\%)} = \left[ \frac{\text{Total height of the emulsified layer}}{\text{Total height of the liquid layer}} \right] \times 100$$

#### **Molecular identification of potential strain**

The Roche Kit (Germany) was used to extract DNA from a 2ml bacterial strain that had been collected during the mid-exponential growth phase following the manufacturer's instructions. Universal primers<sup>27</sup> 27F-52 -AGAG TTTG ATCMTGGCTCAG-32 and 1492R-52 -TACG GYTACCTTGTTACGAC TT-32 was used. The PCR was analyzed on a thermal cycler (Eppendorf) with a 50 $\mu$ l reaction mix. The reaction mix contained 10 $\times$ amplification buffer (5 $\mu$ l), 1.5mM MgCl<sub>2</sub> (5 $\mu$ l), 1 $\mu$ l of each forward and reverse primer, 1 $\mu$ l dNTP and 0.25 $\mu$ l Taq polymerase. Amplification was performed with 35 cycles of 35s at 94 $^{\circ}$ C, the 40s at 55  $^{\circ}$ C, 2min at 72 $^{\circ}$ C, and a final extension for 8min at 72 $^{\circ}$ C following a preliminary denaturation at 95 $^{\circ}$ C for 1min. The PCR results were examined using 1.2 percent agarose gel electrophoresis (Genei).

Using the Qiagen PCR purification kit, the PCR product was improved before being sequenced on an ABI Prism 377 automated sequencer (Applied Biosystems, CA, USA). The maximum Neighbor-Joining method, maximum parsimony analysis, and maximum-composite-likelihood approach were used to compute the evolutionary distances<sup>28, 29, 30</sup>. The evolutionary analysis was directed using MEGA7 software<sup>31</sup> and the tree topologies were assessed by bootstrap method based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method.

#### **Growth standardization for maximum bioemulsifier production**

##### **Basal cultural conditions**

Among various optimization methods one parameter at a time, the potential isolation was maximized for the creation of bioemulsifiers, and the standardized conditions were fixed for further evaluations. A 250ml conical flask with a 100ml working volume of broth and the following composition of peptone (0.5%) and glucose (1%) with a final pH of 8.2 $\pm$ 0.2 was used for the growth standardization process, along with the other cultural conditions of 37 $^{\circ}$ C temperature, agitation at 150rpm and 1ml inoculum. While the inoculum was made using the exponential growth phase of potential strains in the same baseline cultural medium conditions, the OD620 nm of the inoculum was set to 0.1 as per the McFarland turbidity 0.5 standards which are equivalent to 1.5 $\times$ 10<sup>8</sup> CFU/ml bacterial cell concentration. Additionally, the emulsification assay<sup>26</sup> stated above was used to calculate the synthesis of emulsifiers. All the estimations were performed in triplicate and the results were expressed as mean  $\pm$  standard deviation.

##### **Time course on bioemulsifier production**

The prospective strain was standardized for their maximum bioemulsifier production concerning the development of cell biomass at regular intervals of 6hrs from 0 to 102hrs. A part of the cultured broth was used to monitor the tests, and then the cell biomass and cell-free supernatant were separated with the use of centrifugation at 3000rpm for 15min. Emulsifier activity was measured from the cell-free supernatant, and bacterial cell growth was quantified using the dry weight of cell biomass obtained from centrifuged cell pellets that were hot air oven-dried at 50 $^{\circ}$ C for 30min.

### Optimization of pH and temperature conditions

Optimizing parameters like pH and temperature played a key role in the enhanced production of bioemulsifier. The effect of different pH conditions between pH 5 to 10 with an interval of pH 0.5 was evaluated for the effective optimization of the medium. Similarly, different temperatures ranging from 20 to 50°C with an interval of 5°C were applied to the production medium for their determinations.

## RESULTS AND DISCUSSION

Diversified microorganisms were present in the oil-contaminated samples. In the present study, bacterial strains were isolated from petroleum hydrocarbon-contaminated sediment samples which were collected from the shoreline areas of Karaikal, Puducherry, India. Nearly 19 morphologically distinct bacterial cultures were isolated using the spread plate method and it was performed on Zobell marine agar. Further morphological characterization was done using the Gram staining method and this method confirmed the axenic culture of the isolates. Isolates were initially named by labelling PHCS 1 to PHCS 19 in which PHCS stands for Petroleum Hydrocarbon Contaminated Sediment followed by the serial numbers of the isolates. Further, cultures were maintained in the Zobell marine broth and they were screened for both intra and extracellular production of bioemulsifier producing. In the present study, among 19 isolates 12 were gram-positive and 7 were identified as gram-negative bacteria. According to Bicca *et al.*<sup>32</sup>, the microbes isolated from the oil-contaminated site showed an extensive gram-positive population and this could be due to the enhancement of these populations' survival in such hostile conditions. In another study, 192 morphologically distinct microbes have been isolated from the oil-contaminated site of which 3 fungi, 4 yeast and 185 bacteria<sup>19</sup>.

The method commonly used for screening includes oil spread, direct surface or interfacial tension measurements, emulsification index, emulsification assay, bacterial adhesion to different hydrocarbons, hemolysis, drop collapse and cetyltrimethylammonium bromide (CTAB) agar plate test<sup>33,34,35</sup>. Camacho-Chab *et al.*<sup>26</sup> reported that emulsification assay using hydrocarbon substrate

provides a fast and readily available method. Thus, in the present investigation screening was done according to the method of Camacho-Chab *et al.*<sup>26</sup>. The screening results revealed that except for PHCS 1 and PHCS 18 all the other isolates showed either extracellular or intracellular emulsification activity (Table 1). Among the different isolates tested, the following isolates showed maximum extracellular emulsification activity. PHCS 7 showed maximum of 64.2±2.5% emulsification activity and this was followed by PHCS 11 with 43.3±1.8%, PHCS 13 (38.9±1.7%) and PHCS 4 (34.5±1.3%). Similarly, intracellular emulsification activity was also recorded and results showed that PHCS 17 produced a maximum of 41.2±1.6% intracellular emulsification activity. This was followed by PHCS 3 (32.3±1.2%), PHCS 12 (27.8±1.2%) and PHCS 5 (26.5±1.1%). In an early report, 25% of bioemulsifier producers were screened in the bacteria isolated from the contaminated site when compared to uncontaminated soil (hydrocarbon-impacted soil).

**Table 1.** Emulsification activities of axenic bacteria isolated in this present study where the abbreviation "PHCS" stand for Petroleum Hydrocarbon Contaminated Sediment

Strain No.	Emulsification activity (%)	
	Extracellular activity	Intracellular activity
PHCS 1	-	-
PHCS 2	14.7±0.5%	-
PHCS 3	-	32.3±1.2%
PHCS 4	34.5±1.3%	-
PHCS 5	-	26.5±1.1%
PHCS 6	27.8±1.1%	-
PHCS 7	64.2±2.5%	-
PHCS 8	32.4±1.2%	12.4±0.4%
PHCS 9	-	-
PHCS 10	29.3±1.3%	-
PHCS 11	43.3±1.8%	-
PHCS 12	-	27.8±1.2%
PHCS 13	38.9±1.7%	-
PHCS 14	17.5±0.6%	-
PHCS 15	-	21.6±0.7%
PHCS 16	18.6±0.7%	-
PHCS 17	-	41.2±1.6%
PHCS 18	-	-
PHCS 19	31.2±1.1%	-

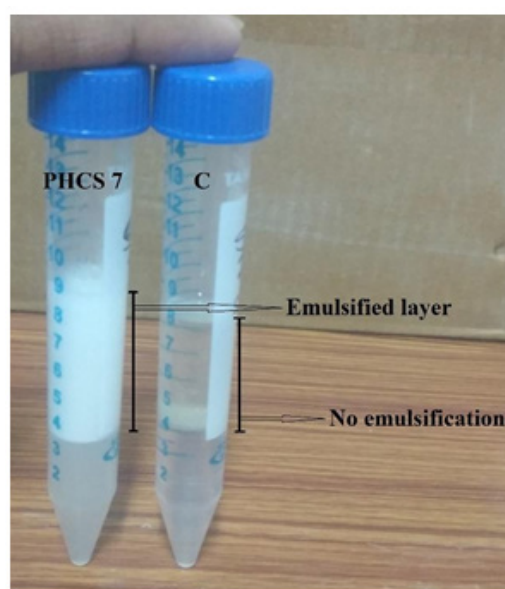
In the present study, based on the screening, PHCS 7 proved to be a promising candidate to produce bioemulsifier and Fig. 1 shows the axenic culture of potential bacterium, PHCS 7 on Zobell marine agar plate and Fig. 2 demonstrates emulsification activity of the potential bacterium, PHCS7 against coconut oil. Further, PHCS 7 was characterized by molecular profiling using the 16s rRNA gene sequencing method. The molecular characterization revealed that the selected bacterial isolate was *Acinetobacter beijerinckii* and 100% similarity was obtained in BLAST analysis when compared with already reported strains. Then, the sequence was deposited in GenBank, NCBI and acquired accession number MZ190471.1. The



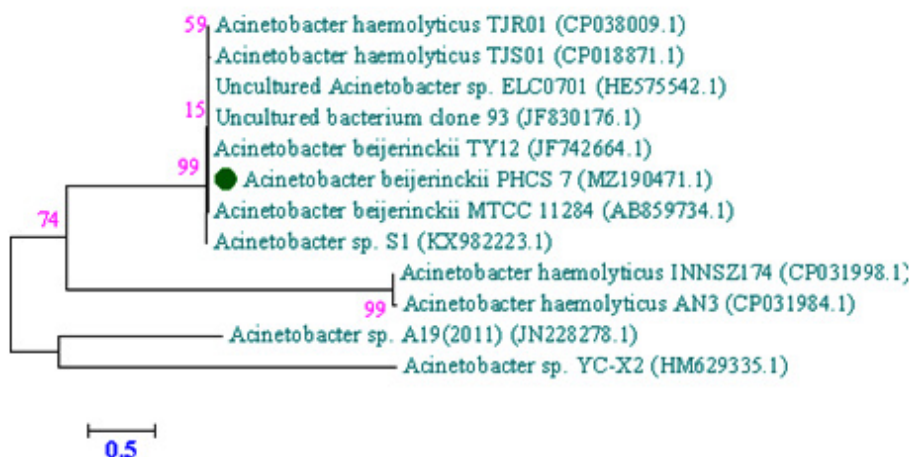
**Fig. 1.** Axenic culture of the potential bacterium, PHCS 7 on Zobell marine agar plate

phylogenetic tree of *A. beijerinckii* PHCS 7 was illustrated using the neighbor-joining method (Fig. 3). The Neighbor-Joining approach was used to infer the evolutionary history and the ideal tree is displayed, with a branch length sum of 8.06519181. The resulting dataset contained 1517 locations in total, and MEGA7 was used for the evolutionary analyses<sup>31</sup>.

According to the previous report *Acinetobacter* spp. attained distinctive attention in bioemulsifier production. Emulsan and Alasan



**Fig. 2.** Emulsification activity of the potential bacterium, PHCS7 against coconut oil



**Fig. 3.** Evolutionary relationships of *A. beijerinckii* PHCS 7 using Neighbor-Joining Method

were one of the best and most extensively utilized bioemulsifier and greatly enhance oil recovery and hazardous substance degradation<sup>37</sup>. For commercial exploration growth, the kinetic of bacterial isolates need to be studied. In the present study, the growth kinetic profile of *A. beijerinckii* PHCS 7 for maximum emulsifier production was done at a regular time intervals. The results elaborated that maximum growth and emulsifier activity was recorded at 48 to 78hrs of incubation. The emulsifier production was evidenced from the

initial incubation time of the lag phase till the end period of the decline growth phase examined (Fig. 4). Additionally, the *A. beijerinckii* PHCS 7 saw a high emulsifier production peak at the end of the exponential or beginning of the stationary growth phase (48hrs later), and it persisted until the end of the stationary or beginning of the declining growth phase (78hrs). During 48hrs of incubation, *A. beijerinckii* PHCS 7 showed 64.6% emulsification activity with 8.69 g/L of cell biomass.

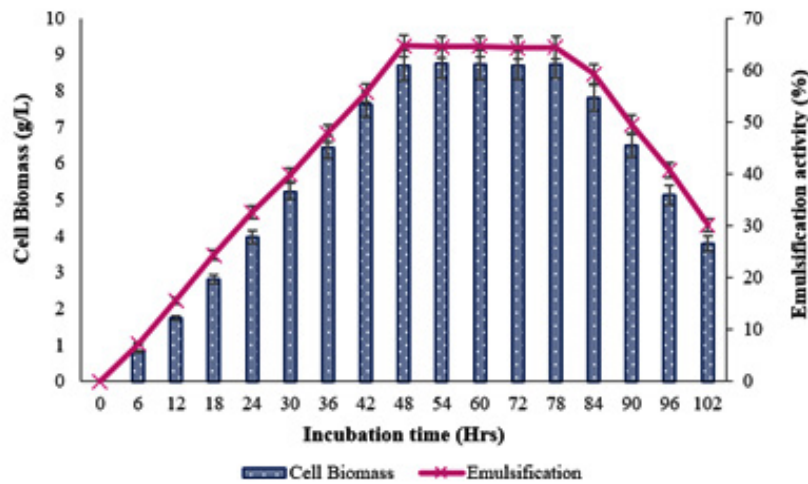


Fig. 4. Time course on bioemulsifier production using the marine strain *A. beijerinckii* PHCS7

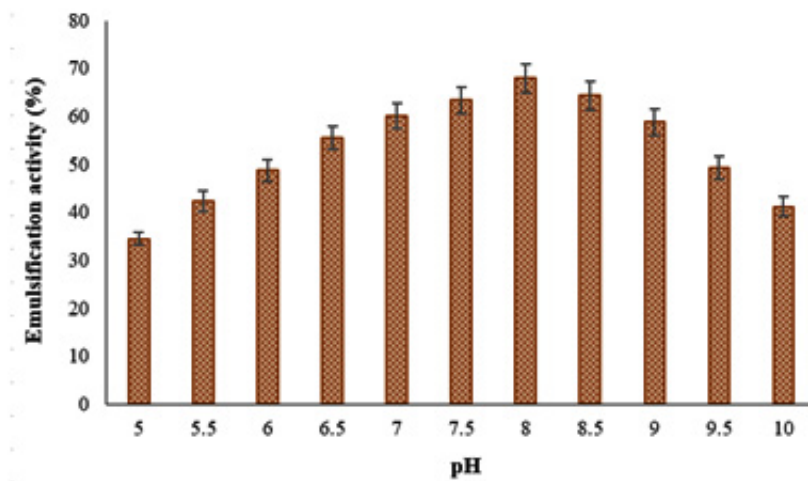
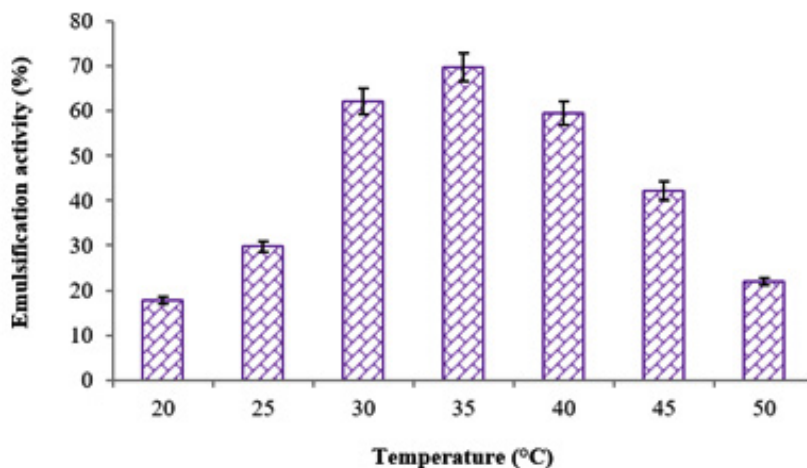


Fig. 5. Effect of pH on bioemulsifier production using the marine strain *A. beijerinckii* PHCS7



**Fig. 6.** Influence of different temperatures on bioemulsifier production using the marine strain *A. beijerinckii* PHCS7

Similarly, 64.4 and 64.5% of bioemulsifier synthesis was recorded during the 54<sup>th</sup> and 60<sup>th</sup> hrs of incubation with 8.75 and 8.72g/L of cell biomass. The least emulsification activity was observed during 6hrs of incubation and Fig. 4 shows the growth kinetics profile as a function of time on emulsification production using *A. beijerinckii* PHCS 7. The maximum emulsifier production during the stationary phase of the studied bacterial strain *Bacillus subtilis* PT2<sup>38</sup>. Different authors have also reported emulsifier production during their stationary phase<sup>19, 39</sup>. Few researchers observed that emulsifier produced from *Acinetobacter baumannii* AC5 was associated with bacterial growth<sup>40</sup>. According to the observation, the productivity increased with cell biomass up to 72hrs and maximum bioemulsifier production was gained at the end of the logarithmic phase.

The important factors which greatly influences cell growth and metabolite production in bacteria are pH and temperature. In the present study, the influence of physical factors such as pH and temperature was optimized to synthesize the maximum amount of emulsifier. The influence of different pH ranges from 5 to 10 on emulsification production was tested using *A. beijerinckii* PHCS 7 (Fig. 5). Among the tested pH conditions, pH 8 favored a high amount of bioemulsifier with 67.9% and this was followed by pH 8.5 with 64.3% and pH 7.5 with 63.4% of emulsification

activity. Further, the least emulsification activity of 34.5% was recorded at pH 5. Maximum emulsifier from *S. marcescens* was attained at pH 8 and this was closely related to the present findings<sup>41</sup>. In contrast, some studies highlighted that at pH *C. guilliermondii* S9 produced the maximum amount of bioemulsifier<sup>42</sup>.

The effect of different temperatures ranging from 20 to 50°C was tested against the production of bioemulsifier using *A. beijerinckii* PHCS 7 (Fig. 6). The results showed that enhanced emulsification production was recorded at 35°C with 69.7% of emulsification activity and then 30°C and 40°C showed 62.1% and 59.5% emulsification activity respectively and the least emulsification activity was observed at 20°C with 17.8% emulsification activity. Increased and decreased temperature showed declined emulsifier production. Zheng *et al*<sup>43</sup> reported that *Rhodococcus ruber* Z25 showed maximum emulsifier production at 34°C; while biomass and biosurfactant were completely inhibited at 50°C and this was correlated to our findings.

## CONCLUSION

Marine microbial diversity is enormous with many environmental and biotechnological applications. Microbial emulsifier producer was extensively reported from the terrestrial

microorganisms compared to the oil-contaminated marine microbes. The present study proved that oil-contaminated sediment is an excellent source for the isolation of emulsifier-producing bacteria. Thus, these kinds of sources are still to be explored to identify novel bioemulsifier producer microbes. The present study provides a baseline for future perspectives on cost-effective large-scale production.

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### Conflict of interest

The authors declare that they have no conflict of interest on publication of this article.

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