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# Improved Production and Physicochemical Stability of a Bioemulsifier from a Marine *Acinetobacter beijerinckii* PHCS 7

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

The commercial use of bioemulsifiers on various fields still faces the hindrance mainly because of low productivity. Hence, the current study inclines to improve the synthesis of a bioemulsifier from a marine *Acinetobacter beijerinckii* PHCS 7, which was previously isolated from a sediment sample polluted with petroleum hydrocarbons and this study also reports on the stability of the extracted emulsifier. During the optimization of biotic and abiotic factors, the use of 1% of trehalose, 0.5% yeast extract, 0.5% coconut oil, 200 rpm agitation, 30 ppt salinity and 2% of inoculum size evidenced improved production of bioemulsifier. Large-scale emulsifier synthesis was carried out

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based on the optimised conditions, followed by diethyl ether extraction and the stability of the extracted bioemulsifier was characterized. The findings demonstrated that the extracted bioemulsifier was stable under wide range of extreme situations, including those involving temperature, pH, and salinity. The improved synthesis and physicochemical stability of a bioemulsifier from a marine *A. beijerinckii* PHCS 7 reveal the wide spectrum of its uses in both bioindustrial and environmental domains.

*Keywords: Acinetobacter beijerinckii; bioemusifier; emulsification; production; optimization; stability*.

## 1. INTRODUCTION

Oil spills are one of the major threats to the ecosystem, over millions of tons of oil have been discharged into the environment and its contaminants both offshore and marine environment [1,2]. During the conjunction of oil spills into the ecosystem, hydrocarbons with low molecular weight were volatilized whereas hydrophilic components were suspended in water [3]. In addition, due to their low solubility, the majority of the oil hydrocarbons are retained on the surface of water bodies or attached to soil particles [4]. Thus, alternative solutions must be found to eliminate the negative impact of oil spills. Surfactants were frequently utilised to spread oil and hasten its mineralization. It increases the surface area of non-polar pollutants in environments that speedup microbial breakdown [4].

The utilization of chemical surfactants to promote pollutant solubility may frequently be dangerous and act as an additional source of pollution. These chemical surfactants are hazardous and non-biodegradable, which has motivated researchers to look for more effective substitutes [5]. Microorganisms can produce bioemulsifiers with similar properties, but they are less dangerous, biodegradable, and can be synthesized on-site. Hydrophobic-degrading strains synthesize emulsifiers to help the assimilation of this difficult substrates [6]. Microbial-based emulsifiers are superior to chemical surfactants because of their biodegradability, biocompatibility, low toxicity, and great substrate specificity [7]. There are various microbes have been identified based on their potential to create bioemulsifers including *Bacillus*, *Pseudomonas*, *Halomonas*, *Rhodococcus*, *Arthrobacter* and some strains of Yeast [8,9]. Numerous publications have described the isolation of *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Candida* sp., and fungal strains from zones contaminated with oily substrates and petroleum hydrocarbons [8-11].

Apart from the bioremediation process, emulsifiers from microbes can be used to improve the extraction of petroleum oils from wells, lessen the viscosity of heavy oils from storage tanks and cleaning of oily and greasy sites, boost pipeline flow and enhance fuel-water emulsions [12]. They could find usage in the food, agriculture, pharmaceutical, cosmetic industries and environmental management because they are less harmful and biodegradable than synthetic surfactants [4]. Due to a lack of efficient production methods, only a small amount of biosurfactants and emulsifiers from microbes are currently used on an industrial basis [12]. As a result, efforts to find microorganisms that may produce bioemulsifiers and be economically produced on an industrial scale are still ongoing. Alasan, Emulsan, lipomannan, mannoprotein and liposan are some examples of bioemulsifiers and they have been synthesized from *Acinetobacter radioresistens*, *A. calcoaceticus*, *Saccharomyces cerevisiae Candida tropicalis* and *C. lipolytica* [13].

Because of higher cost and limited production, although focus into the production of bioemulsifiers has risen, industrial scale production has not yet been realised [9]. This makes the screening of novel and highly productive microorganisms necessary for bioemulsifier studies. Costs associated with large-scale production can be decreased by process improvement. The types and yields of bioemulsifiers generated can be influenced by the carbon and nitrogen supply of the culture medium, and growth circumstances pH, temperature, limiting nutrients, and trace elements. It has been noted that adding immiscible substrates such as oils and fatty acids can stimulate the bioemulsifier synthesis [12].

Even though there has been more research on creating bioemulsifiers, it is not practical to do so on an industrial scale due to the high cost and low yield of production. This necessitates investigating novel, extremely productive bioemulsifier producing microorganisms. Since

bacteria were isolated from oil-contaminated sediment, the current study goals included improving the synthesis of bioemulsifiers and analysing their physicochemical stabilities.

## 2. MATERIALS AND METHODS

### 2.1 Microorganisms and Culture Maintenance

A marine *Acinetobacter beijerinckii* PHCS 7 used in this study for the enhanced production of bioemulsifier and studied for its physicochemical stabilities. This bacterium was previously isolated from a petroleum hydrocarbon contaminated sediment sample collected from the coastal locations of Karaikal, Puducherry, India and characterized for potential bioemulsifier production. In our earlier study, the strain was molecularly identified and the accession number, MZ190471.1 was provided by NCBI GenBank based on the results of 16S rRNA partial sequence [14]. Further, this strain was maintained in our laboratory as axenic culture in the seawater prepared nutrient agar slants under refrigerated conditions and periodic subcultures were performed every three months.

### 2.2 Optimization of Cultural Conditions for Maximizing Bioemulsifier Production

#### 2.2.1 Basic fermentation conditions

Optimization of various cultural conditions was performed using one parameter at a time and the confirming parameters were used for the next estimations. The fermentation was performed in 500ml conical flasks containing 200ml volume of broth whose composition has the basal media conditions of 0.5% peptone, 1%glucose, pH of 8, 35ºC temperature, 150rpm agitation, 34ppt salinity and 1ml inoculum at $1.5\times10^8$ CFU/ml cell density. After 48hrs incubation, emulsification assay was performed in cell free supernatant for the quantitative estimations of bioemulsifier production [15]. Triplicate evaluations were made in all the experiments which were expressed in terms of mean ± standard deviation.

#### 2.2.2 Optimization of various growth factors

Marine *A. beijerinckii* PHCS 7 was standardized for enhanced bioemulsifier production using various growth medium components viz., carbon, nitrogen and oil sources as well as agitation, salinity and inoculum size. Regarding carbon substrates, six different sources such as glucose, galactose, sucrose, trehalose, cellulose and glycogen were individually studied for the enhanced production of bioemulsifier, similarly, six various nitrogen sources such as soya peptone, meat extract, casein, yeast extract, ammonium nitrate and ammonium phosphate were also tried. In the oil substrates, coconut, gingelly, sunflower, mustard, olive and almond oils were utilized in this study for the bioemulsifier enhanced production. The influence of different agitation conditions from 0 to 500 rpm with an interval of 100 rpm was studied for the enhanced production of bioemulsifier, similarly, various salinity parameters from 0 to 50 ppt with an interval of 10 ppt were investigated. The inoculum was prepared at the logarithmic growth phase of the marine *A. beijerinckii* PHCS 7 with the viable cell density of $1.5\times10^8$ CFU/ml, using different concentrations from 1 to 5% inoculum with an interval of 1% were evaluated for the maximizing production of bioemulsifier. Entire determinations of mean ± standard deviation was evaluated after three consecutive analysis.

### 2.3 Lab Scale Production and Extraction of Bioemulsifier

Under the standardized maximum production media conditions, bioemulsifier fermentation was performed in a 1000ml conical flask with 400ml broth volume. After 48hrs incubation, the fermented broth was centrifuged at 1000×g for 15mins and cell free supernatant containing the bioemulsifier was extracted with an equal volume of diethyl ether. After an overnight kept, the solvent phase containing the bioemulsifier was separated and dried in a rotatory vacuum evaporator. The resulting crude bioemulsifier was further lyophilised and preserved for further use.

### 2.4 Physicochemical Stability of Bioemulsifier

The extracted bioemulsifier was used for the evaluations of physicochemical stabilities under various pH, temperature and salinity conditions. The bioemulsifier dissolved in distilled water at 4mg/ml concentration was used in this evaluation and emulsification assay was used for the assessment of quantitative estimations. The pH parameters range within 2 to 12 with an interval of 1, temperature between 20 to 75ºC with an interval of 5ºC and salinity ranges between 0 to 100ppt with an interval of 10ppt were assessed for determining the physicochemical stabilities of this bioemulsifier. Triplicate evaluations were

made in all the experiments which were expressed in terms of mean ± standard deviation.

## 3. RESULTS AND DISCUSSION

According to the previous finding, most of the bioemulsifier synthesising bacteria have been isolated and identified in the moderated environmental condition whereas bioemulsifier producing bacteria of this study was isolated from an oil contaminated coastal site [14]. Makkar and Cameotra [12] and Plaza et al. [16] also attempted to isolate emulsifying bacteria from extreme conditions.

### 3.1 Optimization of Culture Condition for Bioemusifier Production

Yields of bioemulsifiers were greatly influenced by the composition of culture medium especially carbon, nitrogen and oil substrates as well as abiotic conditions [12,17].

#### 3.1.1 Carbon

The majority of microorganisms choose sugars including glucose, galactose, lactose, and sucrose as carbon substrate for emulsifier synthesis. In the present study, the influence of different carbon sources such as glucose, galactose, sucrose, trehalose, cellulose and glycogen on enhancing maximum emulsification activity was tested. The results showed that trehalose favoured high yield with 72.3±3.1% of activity. Followed that, glucose (64.5±2.4%), galactose (56.7±2.2%) and sucrose (54.9±2.3%). The least emulsification activity of 42.3±1.8% was observed in glycogen (Fig. 1). According to Ashtaputre and Shah [18], *Sphingomonas paucimobiliz* GS1 uses sucrose as a substrate and creates a highly viscous emulsifier made up of glucose, acetate, galacturonic acid and glucuronic acid. Whereas, in an another study polysaccharide carbon sources including glucose, galactose, fructose, mannose and sucrose were failed to induce bioemulsification activity and maximum production was noted in benzoate.

#### 3.1.2 Nitrogen

Nitrogen sources are another significant factor in the performance of bioemulsifier production [12]. To achieve the maximum yield of emulsification activity, various nitrogen sources (0.5% concentration) including soya peptone, meat extract, casein, yeast extract, ammonium nitrate and ammonium phosphate were tested in the present investigation. Fig. 2 showed that yeast extract enhanced maximum emulsification activity (75.6±3.2%) and noted activity was also recorded in ammonium nitrate (71.2±2.9%) and ammonium phosphate (66.7±3.1%). Whereas, meat extract favoured the least emulsification activity of 43.6±1.9%. In contrast to the present study, urea was noted as the best nitrogen source for the production of emulsifier using *Athrobacter paraffineus* [19]. Mahdi et al. [20] emphasized that ammonium ions are considered the optimum nitrogen sources for maximum bioemulsification activity.

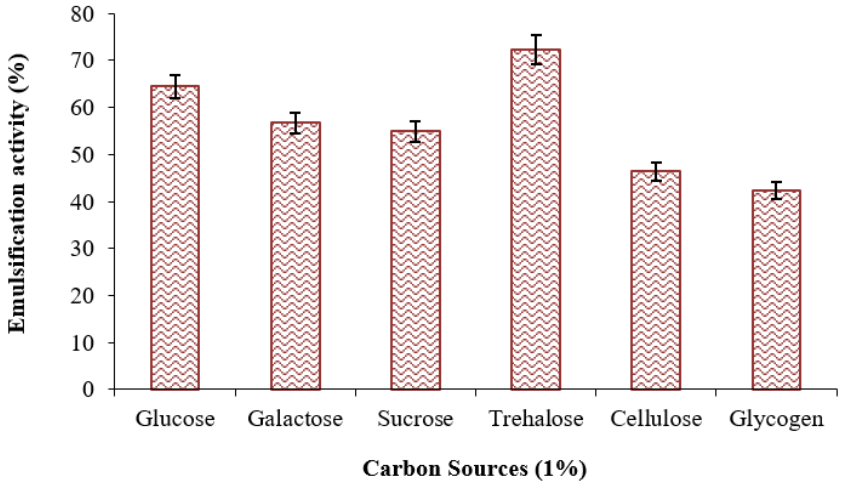


**Fig. 1. Influence of different carbon sources on the bioemulsifier synthesis**

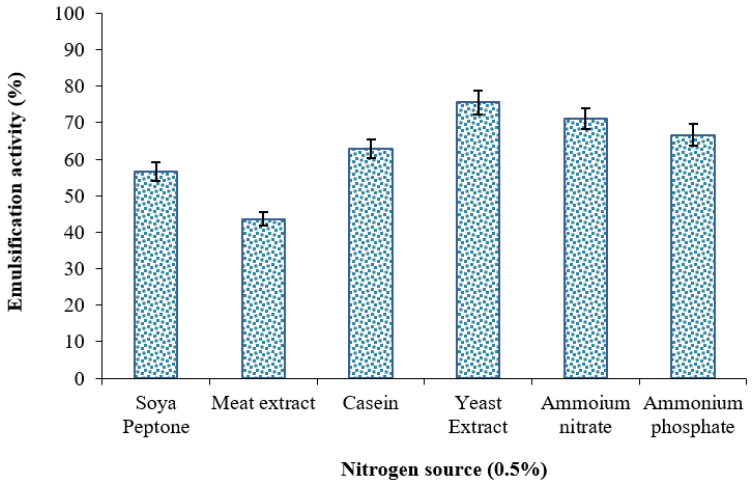


**Fig. 2. Influence of various nitrogen sources on the bioemulsifier synthesis**

### 3.1.3 Hydrophobic substrate

The effect of 0.5% of hydrophobic substrates was tested to enhance the maximum emulsification activity and the selected hydrophobic substrates including coconut oil, gingerly oil, sunflower oil, mustard oil, olive oil and almond oil (Fig. 3). Among all the oils tested, coconut oil favoured maximum bioemulsifier synthesis with 78.1±3.3% emulsification activity. Kazim et al. [21] studied *B. elkanii* SEMIA 587 for the production of bioemulsifiers with different oils and hydrocarbons. The results of Lopes et al. [22] shows that emulsifier activity was observed from wild-type strain, SEMIA 587 when used sunflower oil as its carbon source. Using sesame oil the production of bioemulsifier from *S. marcescens* S10 gave the maximum emulsifier synthesis [23]. In this investigation were able to stabilise emulsions in the presence of edible oils, indicating the possibility of their use in the food sector as cleaning and emulsifying agents as well as to treat lipid-rich wastewater.

### 3.1.4 Agitation

Synthesis of bioemulsifier was optimized using different agitation ranging from 0 to 500 rpm. The results showed that 200 rpm favoured maximum emulsification activity with 87.5±4.1%. Similarly, 300 rpm also favoured 87.5±4.1% emulsification activity and the least activity of 81.2%±2.2 was noted at 500 rpm (Fig. 4). Moussa et al. [24] stated that increased rate of agitation greatly influence emulsifier activity and maximum activity was noted at 150 rpm.

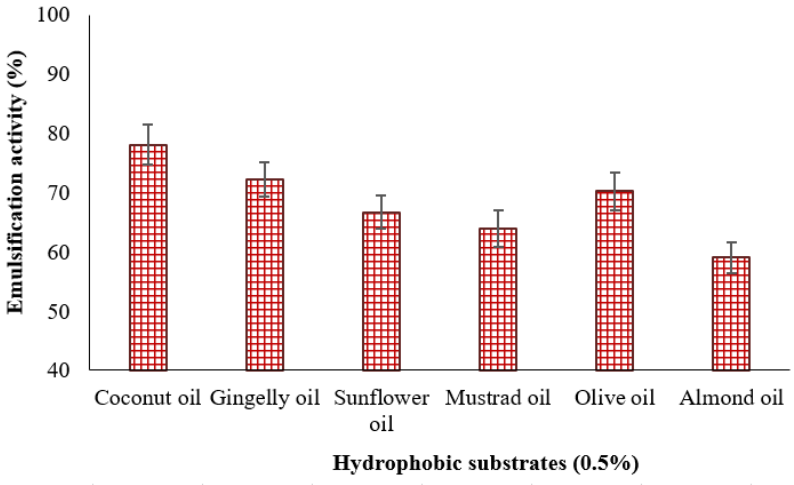


**Fig. 3. Influence of various hydrophobic substrate on the bioemulsifier synthesis**

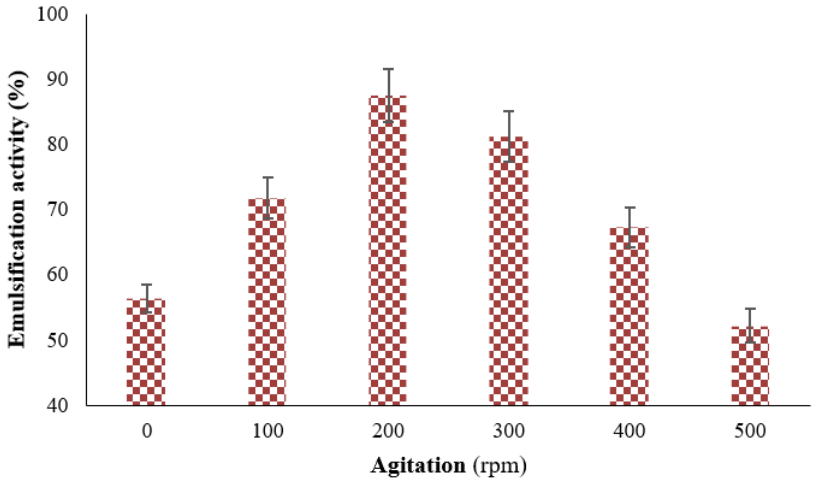


**Fig. 4. Effect of agitation parameters on the bioemulsifier synthesis**

### 3.1.5 Salinity

The influence of salinity against bioemulsifier production was tested with different salinity concentrations ranging from 0 to 50 ppt. The results showed that 30 ppt favoured maximum emulsification activity with 89.4±4.2% followed that 40 ppt also showed maximum 85.2±4.1% emulsification activity. The least bioemulsifier production with 27.2±1.3 emulsification activity was observed in 0 ppt (Fig. 5). Adetunji and Olaniran [25] reported that 3% of NaCl favoured maximum emulsifying activity using *Acinetobacter* sp. They also emphasized that away from optimum salinity declined emulsification activity was observed.

### 3.1.6 Inoculum size

Changes in inoculum size greatly influence bioemulsifier production and in this study effect of inoculum size ($1.5\times10^8$ CFU/ml) on the production of bioemulsifier was tested. Among different inoculum sizes tested (1.00 to 5.00%), 2.00% inoculum size enhanced maximum emulsification activity (92.3±4.4%). Notable bioemulsifier synthesis (89.3±4.3% and 70.8±3.3%) was also recorded in 1.00% and 3.00% of inoculum size, respectively. Increased inoculum size decreased emulsification activity (Fig. 6). Ferreira et al. [26] reported that 5% inoculum in 150 rpm agitation enhances maximum emulsification activity.

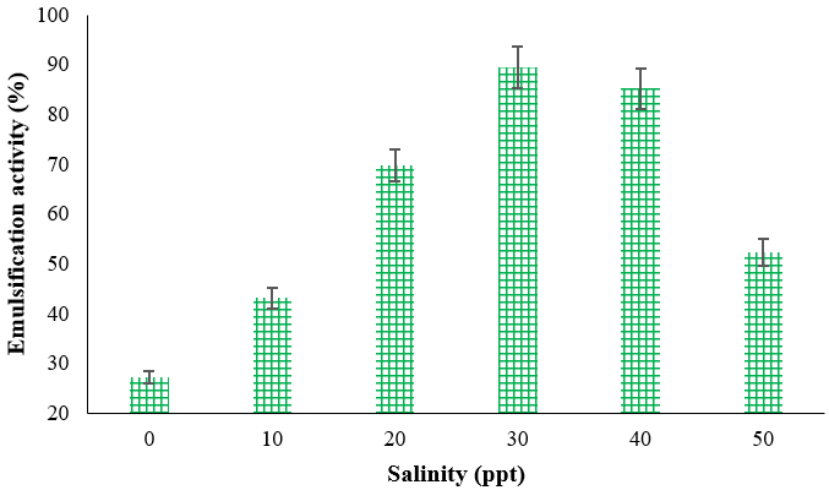


**Fig. 5. Effect of salinity parameters on the bioemulsifier synthesis**

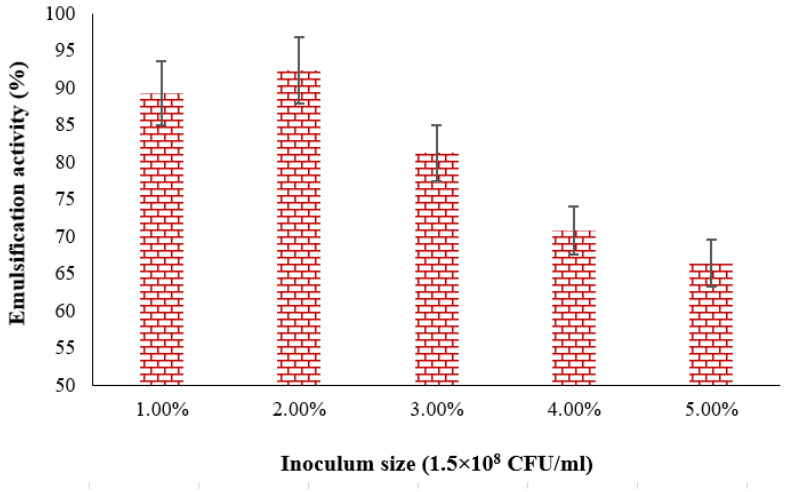


**Fig. 6. Effect of different inoculum size on the bioemulsifier synthesis**

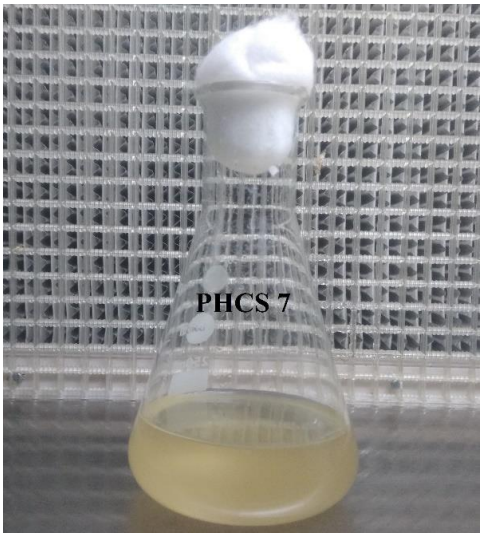


**Fig. 7. Lab scale production of bioemulsifier using *A. beijerinckii* PHCS 7 under optimized conditions**

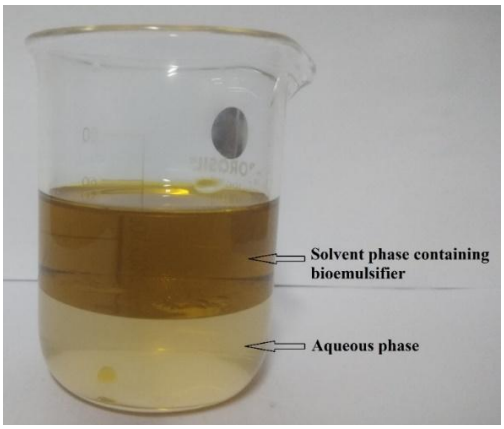


**Fig. 8. Diethyl ether extraction of bioemulsifier from *A. beijerinckii* PHCS 7 cultured broth**

### 3.2 Lab Scale Bioemulsifier Synthesis

Based on the optimized condition, lab scale bioemulsifier synthesis was carried out using *A. beijerinckii* PHCS 7 (Fig. 7). Followed that diethyl ether extraction was done to purify bioemulsifier which was synthesis by *A. beijerinckii* PHCS 7 (Fig. 8) and stability of bioemulsifier was characterized.

### 3.3 Stability of Bioemulsifier

The stability of bioemulsifier is extensively dependent on various physical and chemical properties [27]. In this study, the stability of the purified bioemulsifier was characterized and the factors tested included pH, temperature and salinity. Among different pH (2 to 12) tested, pH 8 showed maximum bioemulsifier stability and decreased stability was noted with increased and decreased pH (Fig. 9). *Bacillus subtilis* bioemulsifier is active at pH 7.0, with a marked decline in activity at pH levels above 7.0. At pH 7.0, bioemulsifier made from a new *Pseudomonas* sp. 2B also exhibited its best emulsifying activity [25]. Similarly, during different temperatures tested (20 to 75 ◦C) 30 to 40◦C showed maximum stability of bioemulsifier and declined temperature showed decreased bioemulsifier stability (Fig. 10). Stability of bioemulsifier was tested at different salinity conditions and results showed that 0 to 40 ppt showed maximum stability and least stability was noted at 100 ppt (Fig. 11).

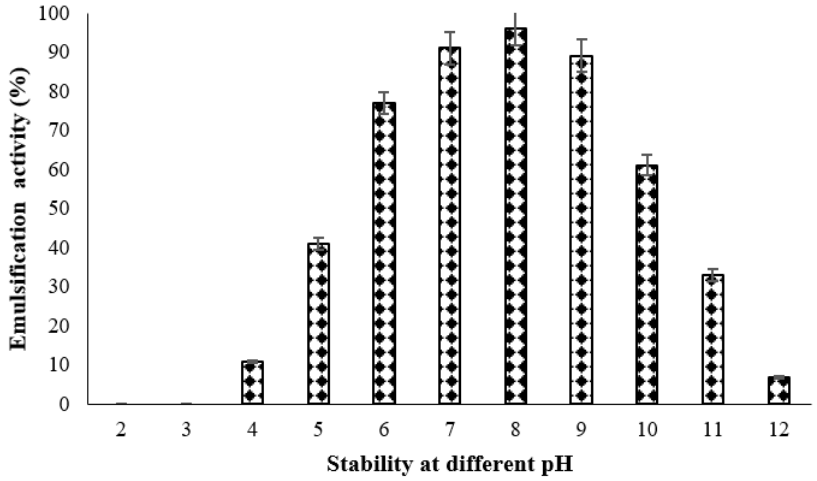


**Fig. 9. Stability of bioemulsifier at different pH conditions**

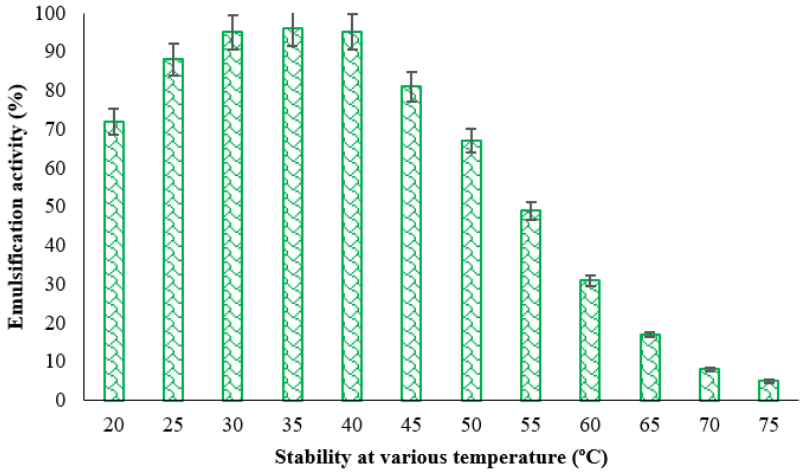


**Fig. 10. Stability of bioemulsifier at various temperature conditions**

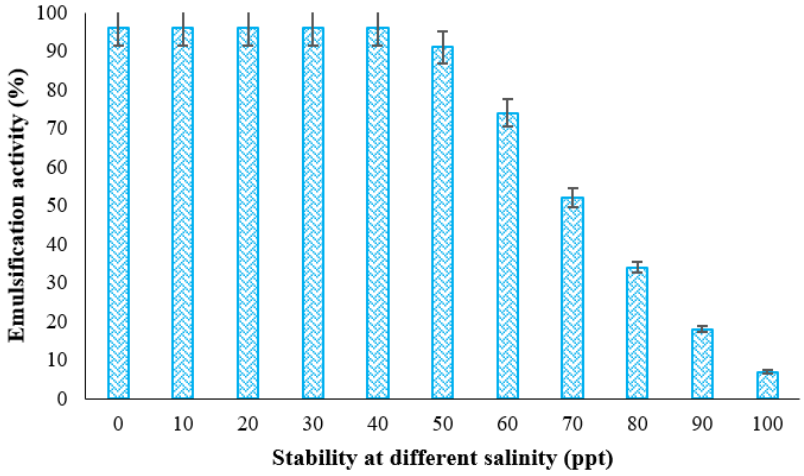


**Fig. 11. Stability of bioemulsifier at different salinity conditions**

## 4. CONCLUSION

This study proved the improved production of bioemulsifier with the easily consumable lab resources of biotic and abiotic conditions using a marine *A. beijerinckii* PHCS 7 which confirms its productivity at an affordable cost. Further, this study revealed great stability of the extracted bioemulsifier over a wide range of pH, temperature and salinity conditions which emphasize its role on the wide spectrum of uses. These features suggesting the inevitable use of this bioemulsifier in the field of many bioindustrial and environmental applications.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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