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### Research Article

## Optimization, Stability assay and Application of Rhamnolipid biosurfactant produced by crude oil-degrading bacterium, *Enterococcus hirae*, isolated from diesel-contaminated soil

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### ABSTRACT

Crude oil spillage causes lots of disastrous environmental pollution. The employment of biosurfactant-producing and hydrocarbon-utilizing microorganisms increase the bio-availability of petroleum hydrocarbons, thus enhancing the effectiveness of bioremediation. This study was aimed at optimizing the biosurfactant-production capability of *Enterococcus hirae*, testing the stability of the biosurfactant produced and applying the biosurfactant on emulsification of some hydrocarbons. The growth and biosurfactant production of the bacterium was optimized using one factor at a time on incubation temperature, incubation time, pH, additional carbon sources and nitrogen source. The biosurfactant was subjected to stability test on pH, temperature and salinity as well as being applied on different hydrocarbons to determine its emulsifying capability. The optimized conditions were determined as 30°C, pH 7.5, 120hours, Glycerol and Ammonium sulphate. The stability test showed that the biosurfactant retained its highest emulsifying activity of 57.58% at 40°C, 52.94% at pH 8 and 53.76% at 8% salinity. The biosurfactant showed the highest emulsifying activity against diesel (59%) and the lowest against petrol (50%). This study is the first to demonstrate *E. hirae* as an efficient biosurfactant producer with a production of 10.2g/L of biosurfactant using the optimized conditions. Furthermore, the biosurfactant produced was stable at different ranges of temperatures, pH and salinity as well as being effective in emulsifying some hydrocarbons. The stability of the biosurfactant approves its usage in bioremediation of oil spills and in microbial enhanced oil recovery (MEOR) as well as in other industri

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## INTRODUCTION

The rapid growth in industrial, municipal and agricultural activities, together with the unprecedented global population growth rate, has led to a high demand for petroleum production [1]. Petroleum industrial activities, such as exploration, drilling, refining, processing, transportation, and storage are major sources of petroleum hydrocarbon spillage, which led to terrestrial oil pollution that generally contains hydrophobic compounds [2]. The spillage of petroleum hydrocarbons (PHCs) into the environment is a critical hazard to ecosystems, and can pose serious human health and environmental problems [3]. Therefore, the remediation of PHC-contaminated soils is presently a significant task, and it is vital to develop eco-friendly and effective approaches to uphold soil ecosystems [4].

Various remediation technologies have been proven and deployed to treat petroleum-polluted soils. Recent PHC remediation methods include physical, chemical, and biological approaches [5]. Physical and chemical pollutant detoxification technologies are often expensive and pose the risk of extra air or water pollution. Biological approaches, owing to the metabolic capabilities of microorganisms and plants, are progressing techniques for the degradation of various environmental pollutants such as PHCs [1].

Bioremediation is an effective, economical, and eco-friendly approach that has gradually become popular for mineralizing organic contaminants in wastewater [6] and soil environments [7]. The most common bacterial and fungal genera known to play vital roles in PHCs degradation include *Pseudomonas*, *Nocardia*, *Vibrio*, *Corynebacterium*, *Candida*, *Arthrobacter*, *Rhodotorula*, *Brevibacterium*, *Flavobacterium*, *Sporobolomyces*, *Achromobacter*, *Bacillus*, *Aeromonas*, *Thiobacillus*, *Acinetobacter*, *Lactobacter*, *Staphylococcus*, *Articulosporium*, *Halomonas*, *Klebsiella*, *Proteus*, *Aspergillus*, *Micrococcus*, *Neurospora*, *Rhizopus*, *Penicillium*, *Mucor* and *Trichoderma* [8-9]. However, the biological activity of microorganisms is influenced by environmental factors, including energy sources, bioavailability, bioactivity, biochemistry, oxygen and nutrient availability, pH, temperature, and metabolite inhibition [10]. One of the suitable methods that can be adopted to speed up the bioremediation of sites contaminated with hydrocarbons is the involvement of biosurfactant-producing and hydrocarbon-degrading

microorganisms due to the role of biosurfactant molecules in enhancing the solubility of these hydrophobic pollutants through emulsification, thereby leading to their better bioavailability for enhanced microbial degradation [11].

Biosurfactants are amphiphilic molecules that contain hydrophobic and hydrophilic parts that promote the presence of interfaces between fluids with different polarities. The hydrophilic part generally consists of one of the following structures: amino acids, anionic or cationic peptides, and carbohydrates. The hydrophobic tail is generally composed of peptides, proteins, or fatty acids that can be saturated or unsaturated [12-13].

Biosurfactants have wide applications in several industries such as medicine and pharmaceutical [14], petroleum [15], food and agriculture [16] due to their wetting, spreading, stabilizing and emulsifying properties [17], foaming and cleaning properties [18], anti-bacterial, anti-fungal and antiviral activities [19]. They can be produced extracellularly by a wide range of microorganisms including bacteria, fungi, and yeasts such as *Bacillus salmalaya*, *Candida lipolytica*, *Pseudomonas aeruginosa* and *Trichosporon ashii* [20-21].

Biosurfactant can be classified into two principal groups based on their molecular weight: low molecular weight (glycolipids and lipopeptides) and high molecular weight (polysaccharides, lipopolysaccharides, proteins, and lipoproteins). Low-molecular-weight biosurfactants are more effective in reducing surface tension, while high-molecular-weight biosurfactants are better for stabilizing oil-water emulsions [22-25]. Compared to synthetic surfactants with the same functionality, biosurfactants have advantages such as being able to work in extreme temperature, pH and salinity conditions, being non-toxic or very low toxicity, and being biodegradable [26-27].

Although promising, biosurfactants in general cannot compete with their chemically synthesized counterparts in the surfactant market mainly due to their low yield and high production costs. Thus, the attention is now geared to the need for cost-effective processes such as the optimization of physical and cultural conditions to increase bacterial growth and the yield of biosurfactant produced [28]. Identification of a new bacterial strain capable of degrading hydrocarbons along with the ability to produce a high yield and stable biosurfactant could give a promising future to

biosurfactant in becoming economically competitive in the surfactant industry. Therefore, the objectives of this research were to enhance the growth and biosurfactant production capability of *E. hirae* by optimizing the physical and cultural conditions, determine the stability of the biosurfactant produced under different conditions, and determine the emulsification activity of the biosurfactant on different hydrocarbons.

## **MATERIALS AND METHODS**

### **Optimization of Physical and Cultural conditions for Enhanced Growth and Biosurfactant Production**

The effect of different physical conditions (incubation time, pH, temperature) and cultural conditions (nitrogen source and carbon source) on the growth of *E. hirae* and the ability of the strain to produce biosurfactant was determined using the methods described by [29-30]. The inoculum that was used for the optimization was standardized using Mac- Farland's standard.

The optimum incubation time for growth and biosurfactant production of *E. hirae* was studied by varying the incubation time (48, 72, 96, 120 and 144hr) of the culture medium. The culture medium was inoculated with a 24 h culture broth of *E. hirae* and incubated at 36°C in a rotary shaker incubator. Biosurfactant production was measured using  $E_{24}$ , while the growth was determined using a spectrophotometer.

The bacterial isolate was incubated at different temperatures (25, 30, 35, 40, and 45°C) using the optimum incubation time, after which the biosurfactant production and growth of the strain were determined. Also, the optimum pH for growth and biosurfactant production by the bacterial isolate was studied by varying the pH (6.0, 6.5, 7.0, 7.5 and 8.0) of the culture medium. After the optimum incubation, biosurfactant production and growth were determined using  $E_{24}$  index and spectrophotometer respectively.

The bacterial isolate was incubated with different carbon sources (glucose, glycerol and sucrose) for 48 h, after which biosurfactant production and growth were determined using  $E_{24}$  index and spectrophotometer respectively. The bacterial isolate was also incubated with different nitrogen sources (Ammonium nitrate, Ammonium Chloride and Ammonium sulphate) for 48 h, after which biosurfactant production and growth were determined using  $E_{24}$  index and spectrophotometer respectively.

### **Production of Biosurfactant**

The optimized parameters were used in setting up the biosurfactant production media in a 500 mL Erlenmeyer flask containing 200 mL of the production [30].

### **Recovery / Extraction of Biosurfactant**

For the extraction of biosurfactant, cell-free supernatant was obtained through centrifugation of culture broth for 20 minutes at 10,000 rpm and this served as the source of crude biosurfactant. The supernatant was then acidified to pH 2 using 6M HCl and then stored at 4°C overnight. Biosurfactant was extracted from the refrigerated supernatant with ethyl acetate at room temperature continuously. A 1:1 mixer of ethyl acetate and supernatant were agitated vigorously and left stationary for phase separation. The organic phase was collected and then transferred to a rotary evaporator and a dark honey-colored viscous product was recovered after solvent evaporation at 40°C under reduced pressure [31].

### **Biosurfactant Stability Test**

The surface-active stability of the cell free supernatant was determined by estimating the emulsification index ( $E_{24}$ ) at different conditions such as pH, temperature and salinity using the method described by [32]. The cell-free broth was obtained by centrifuging the cultures at 10,000rpm for 20 mins. The pH of the biosurfactant (4 ml) was adjusted to 2, 4, 6, 8, 10 and 12 using NaOH and HCl after which  $E_{24}$  was determined. To test the heat stability of the biosurfactant, the broth was heated at 20, 40, 60, 80 and 100°C for 15 min, cooled to room temperature and  $E_{24}$  will be determined. The effect of NaCl on the biosurfactant was also assayed at concentrations of 2, 4, 6, 8 and 10% of NaCl.

### **Application of the Biosurfactant on Hydrocarbon Emulsification**

The biosurfactant produced was applied, using the process employed by [30], on different hydrocarbons (crude oil, diesel oil, petrol and kerosene) and the ability to emulsify these hydrocarbons was determined using  $E_{24}$ -index. Two millilitres (2 ml) of each hydrocarbon was added to the same amount of biosurfactant in three different 15 ml test tubes. The mixtures were vortexed at a high speed for 2 mins and the test tubes were placed vertically at room temperature without disturbance for 24 h. After 24 h, the height of the stable emulsion layer was measured and  $E_{24}$  index was calculated in percentages as follows:

$$E_{24}(\%) = \frac{\text{total height of the emulsified layer} \times 100}{\text{height of the liquid layer}}$$

### STATISTICAL ANALYSIS

All the parallel experiments were carried out in triplicates. The results were represented as the mean  $\pm$  standard deviation. Analysis of variance (ANOVA) was used to compare different variables at 95% probability level. All statistical analyses were done using Statistical package for social sciences (SPSS) softwares.

### RESULT

#### Optimization of Physical and cultural conditions for enhanced growth and biosurfactant production

From the result obtained, the optimum incubation time for both growth and biosurfactant production was 120hrs with the optical density (OD) reading of  $2.1536 \pm 0.1589$  and  $E_{24}$  value of  $56.00 \pm 1.0801$ . The result of the effect of incubation time on growth and biosurfactant production is presented in figure 4.1. The effect of different

incubation temperature on growth and biosurfactant production showed the optimum incubation temperature as  $35^\circ\text{C}$  by the bacterium (as shown in figure 4.2) with OD reading of  $2.2361 \pm 0.2756$  and  $E_{24}$  value of  $56.44 \pm 1.3103$ . The effect of different pH values on growth and biosurfactant production showed the optimum pH as 7.5 with an OD reading of  $2.2738 \pm 0.1612$  and  $E_{24}$  value of  $56.20 \pm 3.3921$  (figure 4.3).

Figure 4.4 shows the effect of different additional carbon source on the growth of the bacterial isolate and ability to produce biosurfactant. The result obtained shows that glycerol had the highest effect on both growth and biosurfactant production with an OD reading of  $2.4178 \pm 0.0902$  and  $E_{24}$  value of  $50.00 \pm 1.9645$ . Figure 4.5 shows that Ammonium sulphate as a nitrogen source had the best effect on the growth and biosurfactant production by the bacterium with an OD reading of  $2.2800 \pm 0.2919$  and  $E_{24}$  value of  $54.20 \pm 1.8219$ .

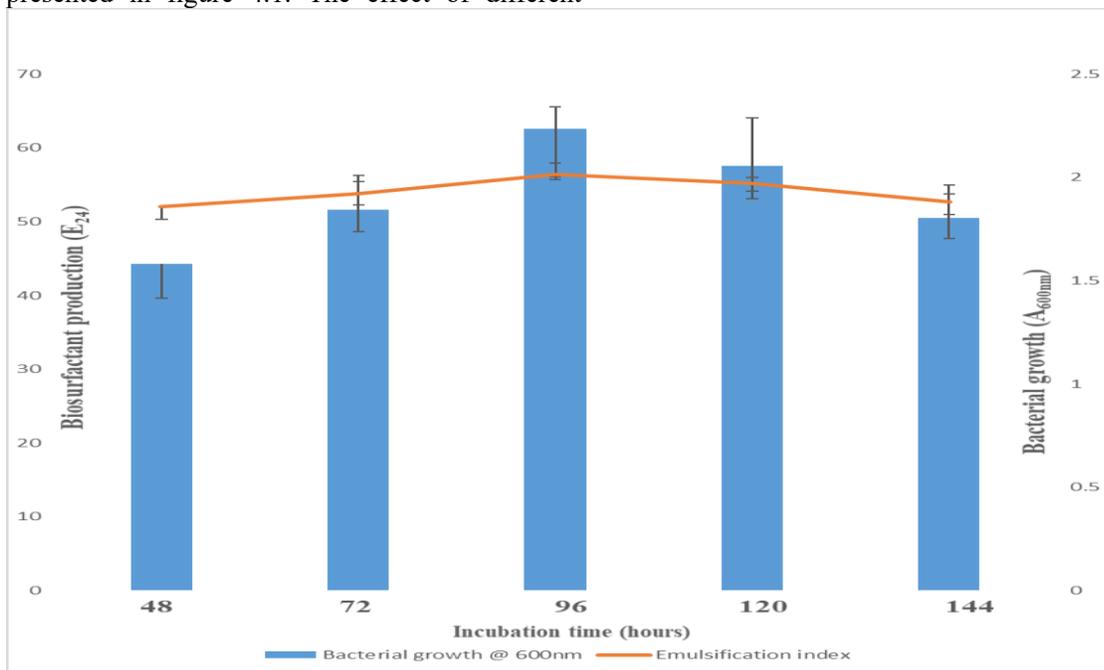


Figure 4.1: Effect of incubation time on bacteria growth and biosurfactant production

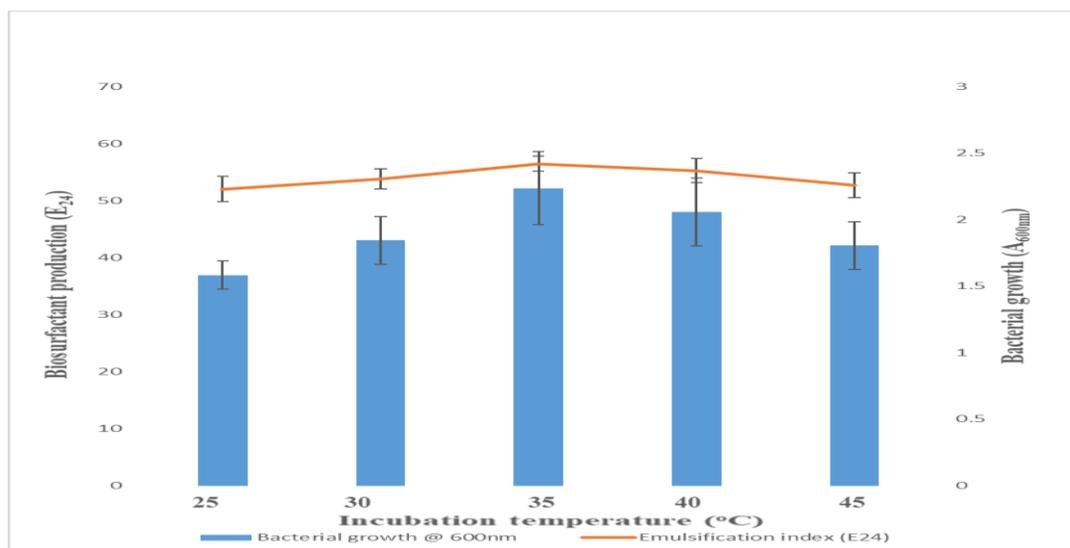


Figure 4.2: Effect of incubation temperature on bacteria growth and biosurfactant production

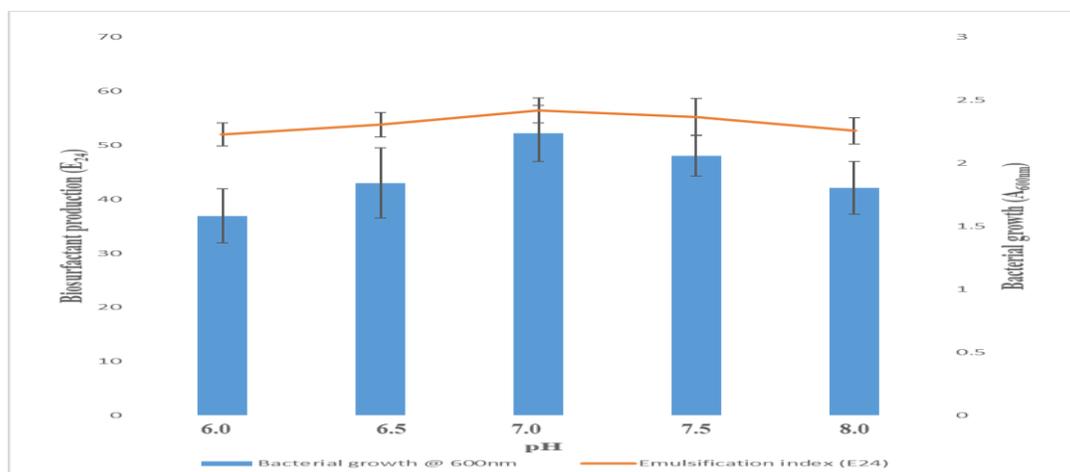


Figure 4.3: Effect of pH on bacteria growth and biosurfactant production

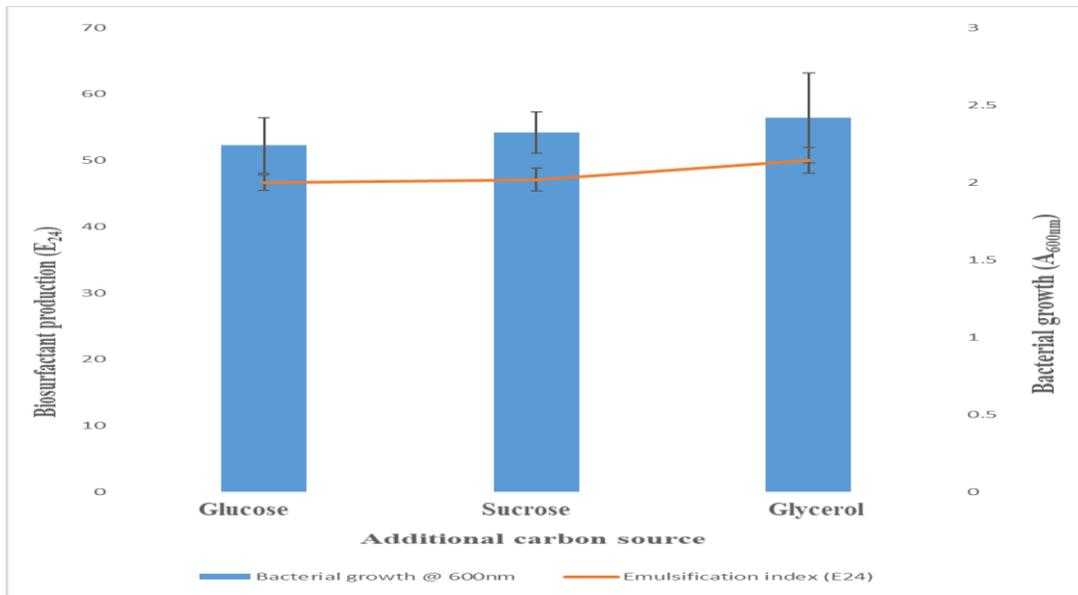


Figure 4.4: Effect of Additional carbon source on bacteria growth and biosurfactant production

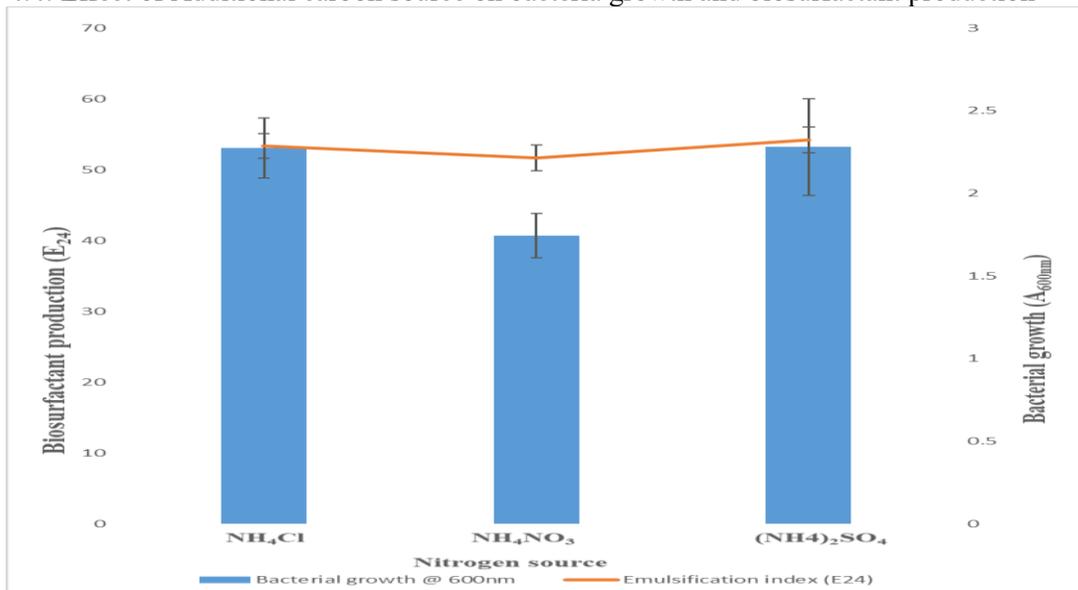


Figure 4.5: Effect of Nitrogen source on bacteria growth and biosurfactant production

#### Stability of the biosurfactant produced

The emulsification index value obtained from stability studies of the biosurfactant are presented in table 4.1. Effect of temperature on biosurfactant activity revealed that the biosurfactant retained its activity at the temperature range of 20 – 100°C with a slightly higher activity at 40°C ( $E_{24} = 57.58\%$ ).

Biosurfactant activity was also observed at pH range of 2 – 12, with higher activity at pH 8 ( $E_{24} = 52.94$ ). Emulsification index of the biosurfactant at different concentrations of sodium chloride (NaCl) (2 – 10%) revealed activity over the wide range of concentrations studied. However, the best activity was observed at 8% NaCl with  $E_{24}$  value of 53.7

Table 4.1: Biosurfactant stability test

TEMP (°C)	Mean E <sub>24</sub> (%)	%NaCl	Mean E <sub>24</sub> (%)	pH	Mean E <sub>24</sub> (%)
20	56.77	0	56.47	2	44.74
40	57.58	2	42.68	4	45.45
60	53.33	4	45.12	6	50.00
80	52.63	6	50.00	8	52.94
100	51.52	8	53.76	10	48.57
		10	48.39	12	45.28

#### Application of the biosurfactant on hydrocarbon emulsification

When the biosurfactant produced was applied on four different hydrocarbons, it showed varying degrees of emulsification. The highest emulsification was observed with diesel ( $E_{24} = 58.54 \pm 0.38$ ) while the least emulsification was observed in petrol ( $E_{24} = 50.50 \pm 0.41$ ) as shown in Figure 4.6.

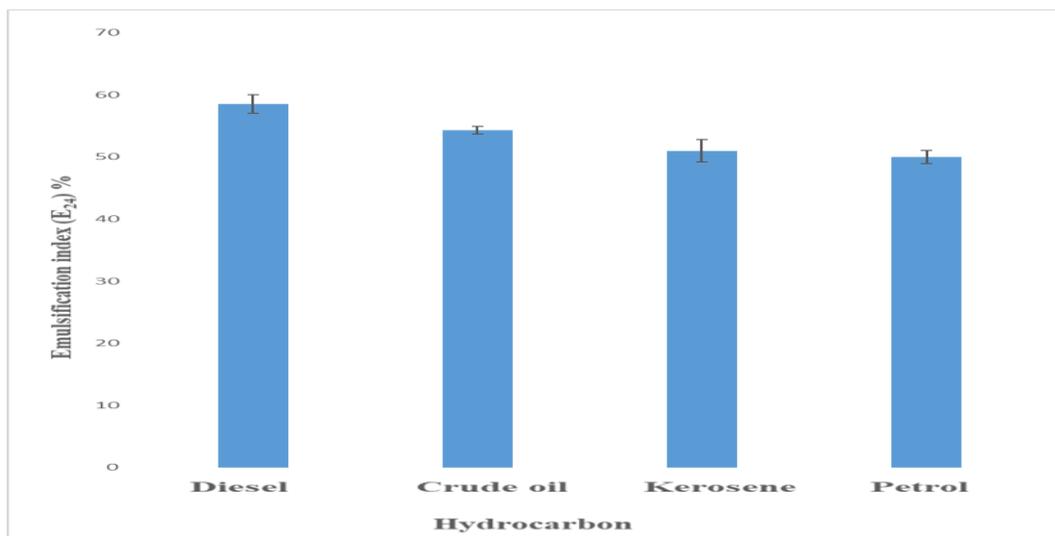


Figure 4.6: Emulsification of different hydrocarbons by the biosurfactant produced by *E. hirae*

#### DISCUSSION

This study evaluated the enhancement of biosurfactant production capability of *Enterococcus hirae*, the stability assay and applicability of the biosurfactant produced in hydrocarbon emulsification. Many studies have reported the isolation and distribution of biosurfactant producing bacteria in hydrocarbon contaminated sites [33-35]. Although biosurfactant producing bacteria are ubiquitous in nature, they are mostly found in hydrocarbon contaminated environments [30].

The effect of incubation time (48, 72, 96, 120 and 144hr) on the ability of *E. hirae* to grow well and produce biosurfactant was investigated in this study. The optimum incubation time was 120hr. This result is similar to that obtained by [30] who reported optimum growth and biosurfactant production after 120hr with *Klebsiella pneumoniae* strain IVN51.

Optimization of cultural temperature of *E. hirae* showed the highest biosurfactant production and growth at 35°C. Similar result has been reported by [36] and [30] who reported maximum biosurfactant production for *P. aeruginosa* and *K. pneumoniae* respectively. These isolates were also isolated from oil contaminated soil samples. Different bacteria produce biosurfactant at different temperature range. However, most of them produce at the range of 30-37°C [37]. [38] reported that a change in temperature can cause alteration in the composition of biosurfactant. The result of pH optimization for growth and biosurfactant production by *E. hirae* showed maximum growth and biosurfactant production occurred at neutral pH (7.5). This is consistent with several reports that have reported the effect of pH on biosurfactant production by bacteria. The effect of different additional carbon sources (glucose, sucrose and glycerol) on biosurfactant

production and growth of *E. hirae* investigated in this study revealed that the maximum biosurfactant production and growth was obtained when grown in a Mineral salt medium amended with glycerol as the additional carbon source. Although the isolate was able to grow in the presence of other carbon source, glycerol gave the highest result for biosurfactant production and growth. This result is similar to that obtained by [30]. Production of biosurfactant by microorganisms while using water-soluble substrates had been reported often probably due to single-phase fermentation pathway using water-soluble substrate is simpler and thus preferred by microorganisms than biphasic fermentation [39, 37, 40-41]. Nitrogen plays an important role in the production of biosurfactant by microorganisms. There are observations that different nitrogen sources can stimulate biosurfactant production by some microorganisms. The result of the effect of different nitrogen sources on the growth and biosurfactant production capability of *E. hirae* showed maximum growth and biosurfactant production when Ammonium sulphate was used. This is similar to the study conducted by [42] who reported maximum biosurfactant by *P. aeruginosa* UKMP14T with ammonium sulphate as the nitrogen source. The difference observed in the production of biosurfactant when *E. hirae* was grown in the presence of different nitrogen sources may be due to the preferential demand for a particular nitrogen source for growth and secondary metabolite production by the bacterium.

The production yield of *E. hirae* was 10.2g/L dry weight using all the optimized conditions. This result revealed *E. hirae* as an excellent biosurfactant producer. However, it is quite an appreciable quantity compared with that observed from previous researchers such as [43] who got 9g/L of biosurfactant produced by *S. mercensens*; [44] who got 3.4g/L of Rhodofactin after optimization. Nevertheless, differences may be attributed to the initial substrate used as carbon source since the quantity and type of biosurfactant produced vary on the substrate type. The present study used crude oil in addition to glycerol as the carbon sources while the study by [44] made use of hexadecane.

The applicability of biosurfactant in several fields depend on their stability at different temperature, salinity and pH values. Stability studies indicated that the Rhamnolipid type of biosurfactant produced by *E. hirae* retained >52% of its activity at 20 – 100°C, >45% at pH 4 – 12 and >45% at 4 – 10% salinity. In general, this biosurfactant has a good stability regardless of extreme temperature, salinity and pH conditions. At present, many saline-alkali soils are contaminated by petroleum [45], therefore, under such extreme condition, the excellent stability of the biosurfactant produced by *E. hirae* can be advantageous to remediating the oil polluted sites. The biosurfactant also exhibited potential for suitability in microbial enhanced oil recovery (MEOR) since conditions in an oil reservoir which include high temperature, salinity and pH were found to be optimal for the activity of the biosurfactant. There are reports on the stability of biosurfactants at extreme conditions [46, 20, 47, 43, 32]. The stability of the biosurfactant at high temperature, acidic or alkaline pH and high salinity posit the biosurfactant to be useful across industries such as pharmaceutical, food, cosmetics and even in bioremediation of oil spills in marine environment [32].

The application of the Rhamnolipid biosurfactant produced by *E. hirae* in this study against different hydrocarbons showed varying degrees of emulsification against the tested hydrocarbons (crude oil, diesel, kerosene and petrol). The biosurfactant showed higher emulsification activity against aromatic and cyclo-aromatic hydrocarbons than straight chain hydrocarbons. The biosurfactant demonstrated good emulsification activity against crude oil. Strong emulsification activity of a hydrocarbon degrading bacteria is always considered to be a very imperative aspect in the context of hydrocarbon degradation [48].

#### CONCLUSION

This study has shown that the bacterium, *Enterococcus hirae* isolated from diesel contaminated soil was capable of producing biosurfactant and degrading crude oil. It was observed that temperature, pH, incubation time, carbon sources and nitrogen sources all impacted on the ability of *E. hirae* to produce biosurfactant. The rhamnolipid biosurfactant

produced was found to be stable at a wide range of temperature, pH and salinity for emulsification activity thus confirming its potential use in different environmental applications under various environmental conditions. In addition, the biosurfactant produced was found to demonstrate emulsification activity against crude oil, diesel, kerosene and petrol, a feature which is attractive for application in the biodegradation of petroleum hydrocarbons and microbial enhanced oil recovery (MEOR).

#### Conflict of Interest

The authors declare no conflict of interest

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