

## DETERMINATION OF OPTIMUM CONDITIONS AND STABILITY STUDY OF BIOSURFACTANT PRODUCED BY *Bacillus subtilis* UKMP-4M5

(Penentuan Keadaan Optimum dan Kajian Kestabilan Biosurfaktan yang Dihasilkan oleh *Bacillus subtilis* UKMP-4M5)

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### Abstract

*Bacillus subtilis* UKMP-4M5 was isolated from a hydrocarbon contaminated site which was found to be the most potential biosurfactant producer. Isolates were grown in a mineral salt medium (MSM) supplemented with sunflower oil (1% v/v) as the main carbon source. Screening was based on both qualitative (oil spreading technique) and quantitative (emulsification index and surface tension measurement) methods. *B. subtilis* UKMP-4M5 produced the highest oil displacement activity with an emulsification index of 17%. It also reduced surface tension of culture medium from 58.95 to 41.75 mN/m. *B. subtilis* UKMP-4M5 produced biosurfactant with surface tension measurement of  $32.7 \pm 0.66$  mN/m using 2% (v/v) palm oil and 0.5% (w/v) yeast extract as carbon and nitrogen sources respectively. The tested biosurfactant exhibited excellent thermal (up to 120 °C) and pH (6.0 – 8.0) stability as well as high tolerance for varying salt concentrations (1 – 5% w/v) in terms of surface tension reducing ability. This suggests potential applications in fields such as enhanced oil recovery, bioremediation, and the food industry.

**Keywords:** *Bacillus subtilis*, biosurfactant, palm oil, surface tension

### Abstrak

*Bacillus subtilis* UKMP-4M5 yang dipencilkan dari kawasan yang tercemar dengan hidrokarbon didapati paling berpotensi sebagai penghasil biosurfaktan. Pencilan dihidupkan di dalam medium garam mineral (MSM) yang ditambahkan dengan 1% (i/i) minyak bunga matahari sebagai sumber karbon utama. Penyaringan adalah berdasarkan kepada kedua-dua kaedah kualitatif (teknik penyebaran minyak) dan kuantitatif (indeks emulsifikasi dan ukuran ketegangan permukaan). *B. subtilis* UKMP-4M5 merekodkan aktiviti sesaran minyak yang tertinggi dengan indeks emulsifikasi sebanyak 17%. Ia juga merendahkan ketegangan permukaan kultur medium dari 58.95 mN/m ke 41.75 mN/m. *B. subtilis* UKMP-4M5 menghasilkan biosurfaktan dengan ukuran ketegangan permukaan  $32.7 \pm 0.66$  mN/m menggunakan 2% (i/i) minyak kelapa sawit dan 0.5% (b/i) ekstrak yis masing-masing sebagai sumber karbon dan nitrogen. Biosurfaktan yang diuji menunjukkan kestabilan yang amat baik pada suhu (sehingga 120 °C) dan pH (6.0 – 8.0) serta toleransi yang tinggi pada kepekatan garam yang berbeza (1 – 5% b/i) dari segi keupayaannya merendahkan ketegangan permukaan. Ini mencadangkan potensi aplikasinya dalam industri seperti peningkatan perolehan minyak, bioremediasi dan industri makanan.

**Kata kunci:** *Bacillus subtilis*, biosurfaktan, minyak kelapa sawit, ketegangan permukaan

### Introduction

Biosurfactants are amphiphilic molecules containing hydrophobic and hydrophilic moieties. They are produced by a variety of microorganisms such as yeasts, fungi, and bacteria. A majority of these molecules are produced by

bacteria which are able to interact between two immiscible interfaces, such as water-oil or water-air phases. *Bacillus subtilis*, for example, produces lipopeptide surfactin, a powerful biosurfactant that can reduce surface tension of water from 72 mN/m to 27 mN/m at a concentration of 0.05% [1]. Other lipopeptide biosurfactants synthesised by *Bacillus sp.* include iturin [2] and fengycin [3].

Many organisms produced biosurfactants which metabolise water-immiscible substrates such as hydrocarbons and vegetable oil, thus increasing bioavailability and allowing for their adsorption, emulsification, and dispersion. Furthermore, the ability to reduce surface tension is a major characteristic of a surfactant which has potential applications in many consumer and industrial products such as shampoos, detergents, and toothpaste. Other potential applications include enhanced oil recovery, bioremediation, pharmaceuticals, cosmetics, and food-related industries. In comparison with the current widely used chemical surfactants, biosurfactants offer several advantages such as lower toxicity, higher biodegradability, and effectiveness at extreme temperatures and pH values [1].

Although promising, biosurfactants in general cannot compete with their chemically synthesised counterparts in the surfactant market mainly due to their high production costs. Thus the attention is now geared to the need for cost-effective processes and the use of inexpensive substrates, as these can reduce overall production costs up to 30% [1]. Optimisation of culture conditions for maximum biosurfactant production would also be among the strategies employed by researchers for the purpose of cost reduction. Studies on the biosurfactant production by *Bacillus sp.* have been performed using water-soluble substrates such as glucose [4] and glycerol [5] as well as water immiscible substrates such as diesel oil [6]. Other substrates such as molasses were also used for the production of biosurfactants by *Bacillus subtilis* [7]. Others have reported the optimization of culture conditions such as pH and temperature on bacterial cell growth which in turn affecting biosurfactant production [8].

Identification of a newly-found bacterium strain capable of producing a biosurfactant combined with use of cheap substrates for growth medium could give a promising future to biosurfactant to become economically competitive in the surfactant industry. Therefore, the objectives of this study were to screen and identify potential biosurfactant-producing bacteria, determine the best culture conditions for increased production of biosurfactants, and carry out a stability study of the biosurfactant produced.

## Materials and Methods

### Bacterial culture

Twenty five bacterial isolates were obtained from culture collection in Microbiology laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

### Preparation of standard inoculum

The amount of bacteria was standardized as described by Hamzah et al. [9] in order to obtain the same number of bacteria for all the tests.

### Screening methodology for biosurfactant production

Approximately 10% (v/v) of standard inoculum from each isolate were grown in a 250 mL Erlenmeyer flask containing 50 mL of mineral salt medium (MSM) [10] supplemented with 1% (v/v) sunflower oil as a carbon source. The pH value of the medium was adjusted to 7.0 and the cultures were incubated in shaking condition at 150 rpm, 37 °C for 5 days. Biosurfactant production was tested from the cell-free culture supernatant obtained via centrifugation at 15000 rpm for 20 mins at 4 °C (RC5C Sorvall Centrifuge Instrument, USA).

### Screening of biosurfactant producer

Potential biosurfactant producing bacteria were screened via both qualitative (oil spreading technique) and quantitative (emulsification index and surface tension measurement) methods. In the oil spreading technique, the test method was carried out according to Yousseff et al. [11]. Tapis crude oil (15 µL) was added to a Petri dish containing 50 mL of distilled water. Then, 10 µL of cell-free culture supernatant was dropped onto the surface of oil. Formation of a clear zone indicates the presence of biosurfactant. The diameter of oil displaced was measured in cm.

The emulsification index was determined according to Cooper and Goldenberg [12]. Cell-free culture supernatant and Tapis crude oil (1:1, v/v) were mixed in a test tube and vortexed at high speed for 2 min. After 24 h of incubation at room temperature, the emulsification index (% E<sub>24</sub>) was determined. Isolates that give high emulsification index indicates high emulsification activity of the biosurfactant produced.

The surface tension of cell-free culture supernatant was measured using a tensiometer (Surface Tensiomat, Fisher Scientific, USA) following the Du Nouy ring method [13]. An isolate that is able to reduce surface tension < 40 mN/m is regarded as the potential biosurfactant producer [14]. Sodium dodecyl sulphate (SDS) and MSM only were used as positive and negative controls, respectively. The selected potential biosurfactant producers will be used for identification.

#### **Identification of biosurfactant-producing bacteria**

Potential biosurfactant-producing bacteria were identified based on morphological (including Gram reaction) and biochemical characteristics. Isolates were further identified by 16S rDNA analysis. Bacterial DNA was extracted via a nucleic acid extraction kit (Macherey-Nagel, Germany) according to manufacturer's instruction and amplified by polymerase chain reaction (PCR) using universal primers (p16Sf: 5'-AGAGTTTGATCCTGGCTCAG-3'; p16Sr: 5'-GGTACCTTGTTACGACTT-3'). The PCR products were purified with a PCR purification kit (Macherey-Nagel, Germany) and sent for sequencing analysis to First Base Laboratories Sdn. Bhd. The resulting sequences were compared with sequences in the GenBank database of NCBI using the BLAST network service.

#### **Determination of the best carbon and nitrogen sources for biosurfactant production**

The best carbon and nitrogen sources for increased biosurfactant production by the test bacteria was determined by the lowest surface tension value of the cell-free culture supernatant. Different types of carbon and nitrogen sources were evaluated for production of biosurfactants, followed by determining the optimum concentration of each source. For the following experiments, 10% (v/v) of standard inoculum was inoculated in a 250 mL Erlenmeyer flask containing 50 mL MSM. pH of the medium was adjusted to (7.0). The broth cultures were incubated with shaking condition at 150 rpm, 37 °C for 5 days. Production of biosurfactant was monitored by determining the surface tension value of the cell-free culture supernatant, via du Nouy ring method using a tensiometer. Measurements were performed in triplicate.

Five different carbon sources, namely crude oil, glycerol, molasses, palm oil, and palm oil mill effluent (POME) were used at 1% (v/v) added to MSM for for increased production of biosurfactant. The best carbon source will be selected based on the lowest surface tension value and used for determining optimum concentration, which may range from 0.5 to 3% (v/v).

Four nitrogen sources (ammonium chloride (NH<sub>4</sub>Cl), ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>), peptone and yeast extract) were evaluated for increased production of biosurfactant, maintaining equal amount of total nitrogen in each media at 24.21%. For the evaluation of the most optimum nitrogen concentration, the selected nitrogen source was varied from 0.1 to 0.8% (w/v). The selected carbon and nitrogen sources at their optimum concentration will be used in the subsequent experiments.

#### **Stability studies**

Stability studies were carried out using extracted biosurfactant which was carried out via acid precipitation followed by solvent extraction method [7].

To study the effects of pH on biosurfactant activity, equal volumes of extracted biosurfactant (1% w/v in distilled water) and buffer solution were mixed and adjusted to pH 2 – 5 using citrate-phosphate buffer, pH 6 – 8 using phosphate buffer, and pH 9 – 12 using carbonate-bicarbonate buffer solutions and incubated for 30 min. at room temperature before measuring surface tension using a tensiometer.

For the thermal stability study, extracted biosurfactant was used to prepare biosurfactant solutions (1% w/v) in 0.1 M phosphate buffer solution (pH 7). Test tubes containing biosurfactant solutions were incubated at different

temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 °C) for 1 hour, then autoclaved at 121 °C for 15 min. Test solutions were allowed to cool to room temperature before measuring surface tension.

A stability study at different salinity levels was performed by adding extracted biosurfactant (1% w/v) into sodium chloride (NaCl) solution at different concentrations (2 – 10% w/v). Surface tension was determined after incubation for 1 hour at room temperature.

### Statistical analysis

The results are represented as the mean  $\pm$  standard deviation from at least three replicates. One-way analysis of variance was performed on surface tension data to determine the significant differences in the values obtained. All statistical analyses were performed using SPSS (version 14.0).

## Results and Discussion

### Screening of biosurfactant production

Three analytical methods were selected for screening of biosurfactant production due to their advantages in terms of simplicity, low cost, and quick implementation. Of 25 isolates screened, five isolates (3M17, 6M1, 10M2, 1M13 & 4M5) showed occurrence of clearing zone by the oil spreading technique indicating potential biosurfactant production (Table 1). Isolate 4M5 scored the most positives results by producing the largest diameter of clearing zone of > 1.5 cm, emulsification index of 17% and the lowest surface tension value at  $41.75 \pm 0.54$  mN/m.

The oil spreading technique is one of the common method used for detecting biosurfactant production [11]. This technique is more sensitive for biosurfactant detection in the cell-free culture supernatant than other screening methods such as drop collapse [15] and blood haemolysis [16]. The oil spreading technique depends on the decrease in water-oil interfacial tension due to the presence of biosurfactant regardless of its structures [17]. Of five isolates positive for oil spreading technique, it was found that isolate 4M5 produced the largest diameter of clearing zone of more than 1.5 cm, suggesting high biosurfactant concentration in the culture medium. Thavasi et al. [18] showed potential biosurfactant producers produced diameter of clearing zone of  $\geq 0.5$  cm. Karthik et al. [19], employing oil spreading technique as one of their screening methods, reported a potential biosurfactant-producing microorganism produced diameter of clearing zone in the range of 0.9 – 2.0 cm, whereas poor biosurfactant producers produced 0.1 – 0.2 cm.

Emulsification index ( $E_{24}$ ) was employed to investigate the emulsifying properties of biosurfactant. Crude oil was used as the hydrophobic substrate, for isolates capable of releasing surfactant and emulsifying crude oil may have potential application in bioremediation processes of contaminated areas with crude oil. Table 1 showed no emulsification ( $E_{24} = 0$ ) when distilled water (negative control) was mixed with crude oil, indicating no surfactant or surfactant-like molecules that may influence the results present in the system. The assumption is that if the cell-free culture supernatant contains biosurfactant, it will then emulsify hydrophobic substrates and maintain stable emulsions for at least 24 hours [20], consequently this method gives just an indication of the presence of biosurfactant. Of 25 isolates screened, only isolates 4M5 and Sf-S5-3 were able to form and maintain stable emulsions with  $E_{24}$  of 17% and 8% respectively, while all other isolates that showed negative in oil spreading technique had  $E_{24} = 0$  (Table 1).

Emulsification index (17%) by isolate 4M5 was found to be lower by 30% compared to *Pseudomonas aeruginosa* [16] and 36% compared to *Serratia sp* [21] used for selection of biosurfactant producers. Karthik et al. [19] reported that an emulsification index ranging from 5.0 – 75% was indicative of biosurfactant producers by marine actinobacteria such as *Streptomyces*, *Nocardioopsis* and *Rhodococcus sp*. Isolate Sf-S5-3 on the other hand, may produce an extracellular compound, also known as a bioemulsifier that has emulsifying activity, but it could not effectively reduce the surface tension of culture media, as the diameter of the clearing zone was negative and surface tension value of isolate Sf-S5-3 was 55.5 mN/m.

Table 1. Comparison of three methods for biosurfactant producer

Bacterial isolates	Oil spreading technique	Surface tension (mN/m)	Emulsification index (% E <sub>24</sub> )
SDS (1% w/v)	+++	32.078 ± 0.95	81.67
Distilled water	-	72.0 ± 0.21	0
MSM only	-	58.95 ± 0.3	0
10M2	+	50.18 ± 0.62	0
3M16	-	52.84 ± 0.55	0
3M17	+	51.08 ± 0.53	0
6M1	+	50.98 ± 0.42	0
4M5	+++	41.75 ± 0.54	17.04
5M7	-	57.8 ± 0.3	0
7M4	-	55.23 ± 0.87	0
1M13	+	49.78 ± 1.70	0
7M22	-	53.70 ± 1.01	0
SF-S11-47	-	55.17 ± 1.11	0
SF-S11-26	-	49.49 ± 0.86	0
SF-S5-9	-	56.60 ± 1.35	0
SF-S5-30	-	54.05 ± 0.43	0
SF-S5-3	-	55.50 ± 0.84	8.33
SF-S10-12	-	48.99 ± 0.42	0
SF-S8-13	-	57.33 ± 1.35	0
SF-S2-5	-	57.95 ± 0.05	0
LF-S11-9	-	53.18 ± 1.24	0
60	-	55.63 ± 0.51	0
19	-	55.27 ± 1.12	0
8	-	53.00 ± 0.61	0
7	-	53.60 ± 0.72	0
6	-	50.00 ± 0.2	0
5	-	54.37 ± 0.72	0
4	-	56.00 ± 1.98	0

Oil spreading technique '-' clear zone diameter <0.5cm '+' clear zone diameter 0.5-0.9cm; '++' clear zone diameter 1.0-1.5 cm; '+++' clear zone diameter >1.5cm

Quantitative analysis using surface tension measurement additionally supported the biosurfactant production potential of isolate 4M5. It was able to reduce the surface tension of culture medium from 58.95 to 41.75 mN/m. This value is much closer to the surface tension value of 40 mN/m, which was recommended for selection of biosurfactant producer [14]. All other isolates showed surface tension measurements of > 45 mN/m.

A lot of studies related to screening of potential biosurfactant producers rely on surface tension measurement due to its reliability and accuracy in quantifying soluble biosurfactant in the culture medium [15]. Thavasi et al. [18] obtained surface tension values ranging between 30.8 - 38.75 mN/m for potential biosurfactant producers among *Bacillus* species. Surface tension value above 45 mN/m is regarded as negative for biosurfactant production [22].

Since the cell-free culture supernatant of isolate 4M5 was the only isolate that satisfied all three screening methods, it was then selected for identification, determination of the best carbon and nitrogen sources for biosurfactant production and stability studies. Production of extracellular biosurfactant is advantageous from an industrial point of view because product recovery process can be simplified, therefore becoming more economical [23].

Several studies have shown that the use of vegetable oils as the only source of carbon and energy stimulate biosurfactant production by microorganisms. *Pseudomonas* spp. were reported to produce higher amount of biosurfactant when using sunflower oil compared to other vegetable oil such as olive and corn oil [24], with some strains recorded surface tension value of < 40 mN/m [25]. Ferraz et al. [26] found that *Serratia marcescens* produced biosurfactant with surface tension value of 29.75 mN/m, while biosurfactant from *Enterococcus* strains recorded 68% reduction in surface tension of culture media [27] when using sunflower oil as the carbon substrate. Vegetable oil contains fatty acids that undergo modification for incorporation into surface active products [28]. Main fatty acid content in sunflower oil is linoleic acid (60%) [26], thus suggesting that this fatty acid was responsible for stimulating biosurfactant production by isolate 4M5.

#### Identification of biosurfactant-producing bacteria

Gram staining of isolate 4M5 showed that it is a Gram positive, rod-shaped bacterium. The colonies on nutrient agar were cream-coloured, flat, and circular with diameter between 5 – 6 mm. Biochemical tests revealed that isolate 4M5 is motile, hydrolysed starch and able to break down hydrogen peroxide suggesting the presence of the enzyme catalase. Isolate 4M5 fermented glucose but not sucrose or lactose and able to utilise citrate as its sole carbon source for growth. Biochemical test results for isolate 4M5 were negative for oxidase enzyme, indole, and H<sub>2</sub>S production (Table 2). All of these features strongly indicate that isolate 4M5 is *Bacillus subtilis*. This was further confirmed by analysis of 16S rRNA gene sequences (1212 bp) by BLAST database. The BLAST analysis showed 99% similar identity with *B. subtilis*. Isolate 4M5 is now designated *Bacillus subtilis* UKMP-4M5.

Table 2. Characteristics of isolate 4M5

Test	Results	Test	Results
Gram reaction	+	Shape	rod
Motility	+	Catalase	+
Growth at		Starch hydrolysis	+
20 °C	–	Methyl red	+
30 °C	+	Vogues-Proskauer	+
50 °C	+	H <sub>2</sub> S production	–
60 °C	–	Indole	–
Triple Sugar Iron	K/A	Simmon's citrate	+

+ = positive, – = negative, K/A = alkaline slant/acidic butt

The majority of Gram-positive bacteria capable of producing biosurfactant are from the *Bacillus* sp. often which was isolated from oil reservoir [29] or soil sample contaminated with petroleum [30]. The genus is reported to produce different types of lipopeptide biosurfactants, such as surfactin, lichenysin, gramicidins and polymyxins. *Bacillus subtilis*, for example, has been reported to produce three types of lipopeptides, namely surfactin, iturin and fengycin [1, 2].

#### Determination of the best carbon and nitrogen source for biosurfactant production

Production of biosurfactant by *B. subtilis* UKMP-4M5 has been found to be induced by carbon sources. All five types of carbon sources tested showed a reduction in surface tension of a culture medium (Figure 1). Surface tension values obtained were ranged from 39.7 – 51.7 mN/m indicating production of extracellular surface active

compounds by *B. subtilis* UKMP-4M5 using different carbon sources. There is no significant difference in surface tension value between palm oil and POME. Palm oil was chosen as the best carbon source for biosurfactant production based on the lowest surface tension value at  $39.7 \pm 1.23$  mN/m, compared to POME which recorded a higher surface tension value at  $43.0 \pm 0.84$  mN/m.

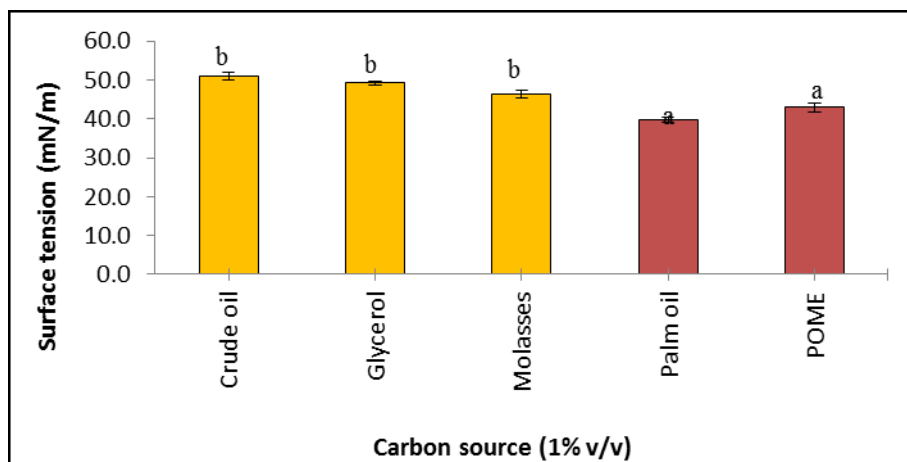


Figure 1. Effect of different carbon sources on the production of biosurfactant by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD. The letters a and b indicate significant differences ( $p < 0.05$ ) among different carbon sources.

Production of biosurfactant by *B. subtilis* had been reported utilizing mainly water-soluble substrates such as glucose [4], sucrose [31] and glycerol [5]. This is probably due to single-phase fermentation pathway using water-soluble substrate is simpler and thus preferred by microbes than biphasic fermentation [32]. The present study however, showed the ability of *B. subtilis* UKMP-4M5 to produce biosurfactant using various types of carbon sources. Although no experiments on cell growth towards different carbon substrates were conducted, *B. subtilis* strains were reported to be able to grow and subsequently produce biosurfactants on various carbon substrates tested such as POME [33], glycerol [5], palm oil [34], molasses [7] and crude oil [35]. In the present study, when palm oil was used as a carbon substrate, *B. subtilis* UKMP-4M5 produced biosurfactant with the lowest surface tension value ( $39.7$  mN/m) compared to the other carbon substrates used. This suggests that *B. subtilis* UKMP-4M5 is a lipase positive. This enzyme might be responsible for hydrolysing vegetable oils to produce long chain fatty acids, the important precursors for biosurfactant synthesis [36]. Furthermore, at the same concentrations, vegetable oil can generate more energy than glucose. Other studies have also shown that the use of palm oil as carbon source induced biosurfactant production by *B. subtilis* PT2 [34] and *P. aeruginosa* FR [37]. A study by Li et al. [36] demonstrated that palm oil was the best carbon source for *P. aeruginosa* GIM32 produced the highest yield of rhamnolipid ( $30.4$  g/L). The use of palm oil as carbon source is advantageous from industrial point of view since it is inexpensive substrates and abundantly available so it can drastically decrease the biosurfactant production cost. Alternative carbon sources from industrial byproducts such as POME can also be effectively used for biosurfactant production by *B. subtilis* UKMP-4M5 since it produces low surface tension values with no significant difference with palm oil. Nevertheless, palm oil produced the lowest surface tension value ( $39.7$  mN/m) compared to POME ( $43.0$  mN/m). Crude oil was found to be the least preferred carbon source for biosurfactant production. Crude oil in general contains various components such as saturated hydrocarbons, aromatic compounds, and heavy metals [38] that probably not suitable for enzyme synthesis necessary for biosurfactant production. Haddad et al. [32] reported that crude oil did not support the growth of *B. subtilis* HOB2, thus resulting in a high surface tension value for the culture medium, while Jazeh et al. [35] reported the use of crude oil as carbon substrate supported the growth of *B. subtilis* and subsequently production of biosurfactant.

Palm oil was then used to study the biosurfactant production by *B. subtilis* UKMP-4M5 at various concentrations (0.5 – 3% v/v). Figure 2 displayed that the highest biosurfactant activity was at 2% (v/v) palm oil concentration, with the lowest surface tension value of  $35.89 \pm 0.71$  mN/m. Further increases in the concentration of palm oil resulted in a significant increase in surface tension measurements.

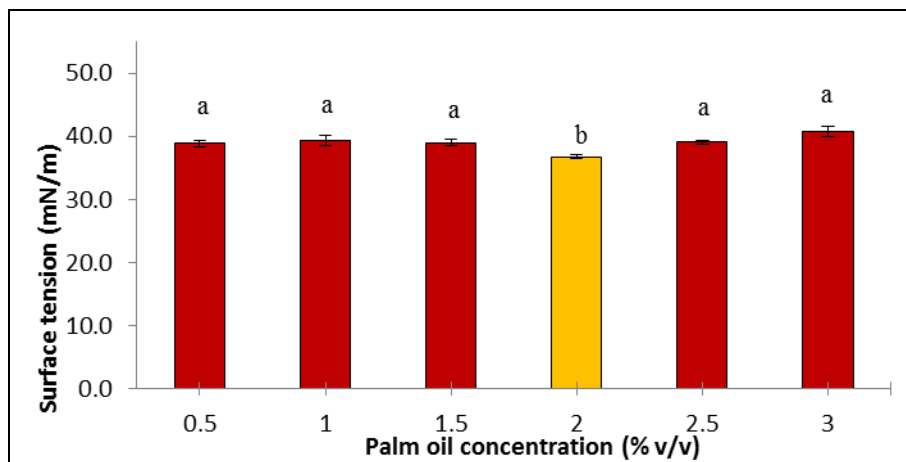


Figure 2. Effect of different concentrations of palm oil on the production of biosurfactant by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD. The letters a and b indicate significant differences ( $p < 0.05$ ) among different concentration of palm oil.

Pornsunthorntawee et al. [34] also reported in their study that optimum concentration of palm oil for biosurfactant production by *B. subtilis* PT2 was 2% (v/v) with maximum surface tension reduction of about 43%. This value is comparable to the percentage obtained in the present study, which was a 40% surface tension reduction from negative control (surface tension value of 58.89 mN/m). *P. aeruginosa* was also found to produce biosurfactant when using 2% (v/v) palm oil as carbon source with surface tension value of 30 mN/m [39]. Other researchers demonstrated the use of 5% (v/v) palm oil as an optimum concentration for maximum production of biosurfactant (30.4 g/L) by *P. aeruginosa* [37].

Medium constituents such as nitrogen source also affect the production of biosurfactants by *B. subtilis* UKMP-4M5. In this study, there was no significant difference between yeast extract and ammonium chloride in terms of surface tension reduction (Figure 3). Nevertheless, yeast extract was chosen to be the best nitrogen source as it produced the lowest surface tension value ( $35.02 \pm 0.25$  mN/m) compared to ammonium chloride ( $36.77 \pm 0.71$  mN/m).

A study by Pereira et al. [31] also showed yeast extract was found to be the best nitrogen source for optimum biosurfactant production by *B. subtilis* #309 with surface tension value of  $28.73 \pm 0.3$  mN/m. A study by Fontes et al. [40] found that the use of yeast extract in combination with ammonium sulphate were the best nitrogen sources for optimum production of biosurfactant by *Yarrowia lipolytica* (surface tension value of 21.1 mN/m and  $E_{24}=60.4\%$ ). For inorganic nitrogen, ammonium chloride was shown to be a better source of nitrogen as opposed to ammonium nitrate for biosurfactant production by *B. subtilis* UKMP-4M5, though a study by Fonseca et al. [41] showed that ammonium nitrate was the best inorganic nitrogen source for biosurfactant production by *B. subtilis* YRE207. Besides growth, nitrogen plays an important role in biosurfactant production, especially in the lipopeptide biosurfactant as a peptide structure [42].

Effect of different yeast extract concentration (0.1 – 0.8% w/v) for biosurfactant production by *B. subtilis* UKMP-4M5 was investigated. An increase in the concentration of yeast extract increases the production of biosurfactant as

evidenced from reduction in surface tension of culture medium (Figure 4). Although there was no significant difference in the surface tension values for yeast extract concentration between 0.3 – 0.6% (w/v); the lowest surface tension value at  $33.6 \pm 0.33$  mN/m was recorded when 0.5% (w/v) yeast extract was used. Therefore 0.5% (w/v) of yeast extract was chosen to be the best concentration for biosurfactant production rather than 0.3% (w/v) of yeast extract (higher surface tension value recorded at  $35.9 \pm 1.1$  mN/m). An increase in the concentration of yeast extract beyond 0.6% (w/v) resulted in the increase of surface tension values significantly, indicating less biosurfactant production by the test bacterium.

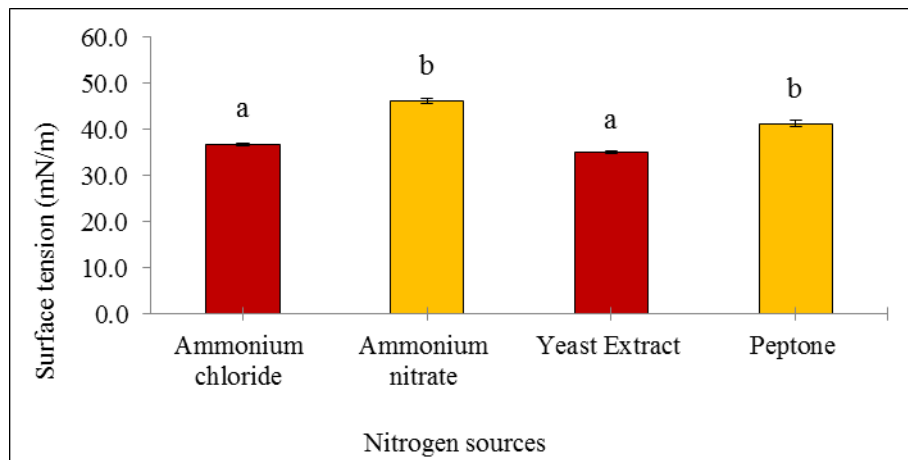


Figure 3. Effect of different nitrogen sources on the production of biosurfactant by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD. The letters a and b indicate significant differences ( $p < 0.05$ ) among different nitrogen sources.

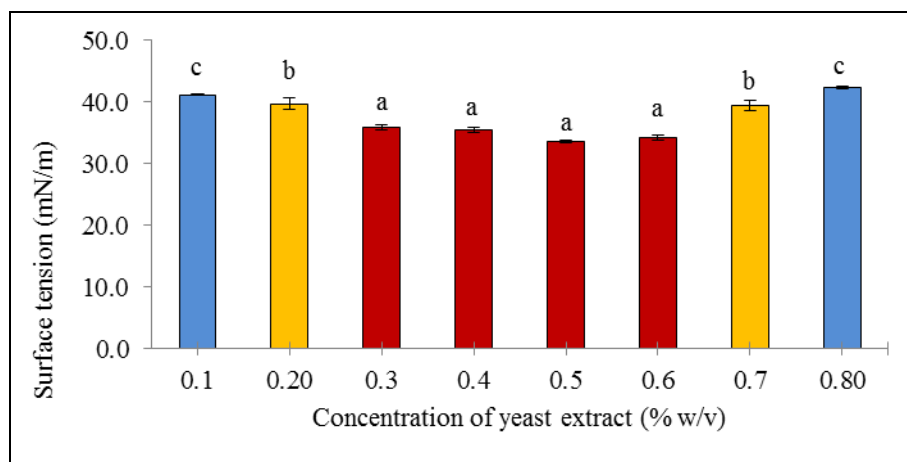


Figure 4. Effect of different concentrations of yeast extract on the production of biosurfactant by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD. The letters a, b, and c indicate significant differences ( $p < 0.05$ ) among concentrations of yeast extract.

*Bacillus subtilis* UKMP-4M5 was found to produce biosurfactant with the lowest surface tension value of  $35.02 \pm 0.25$  mN/m when using 0.5% (w/v) yeast extract. Similar results were shown by Vedaraman & Venkatesh [43] whereby the use of 0.5% (w/v) yeast extract was optimum for surfactin production (983 mg/L) by *B. subtilis* MTC2423. Rashedi et al. [44] reported in their study that the use 0.5% (w/v) yeast extract in combination with 0.5% (w/v) soy flour were the best nitrogen sources for optimal production of rhamnolipid (3.7 g/L) by *P. aeruginosa*. A study by Nalini et al. [6] reported the use of 0.3% (w/v) yeast extract was optimal for biosurfactant production by *Bacillus sp* grown in a glucose-containing medium, while Pereira et al. [31] used 0.2% (w/v) yeast extract for biosurfactant production by *B. subtilis* #309 when grown in sucrose-containing medium. Fontes et al. [32] on the other hand, used a combination of 0.05% (w/v) yeast extract and 10 g/L ammonium sulphate to obtain optimum production of biosurfactant by *Y. lipolytica*, with surface tension value of 20.9 mN/m and emulsification index of 73.1%. The present study showed 0.3% (w/v) yeast extract was enough to produce biosurfactant with significant reduction in the surface tension value; however, 0.5% (w/v) produced the lowest surface tension value. The increase in surface tension value with further increase in yeast extract concentration (beyond 0.6% w/v) may be due to nitrogen-limiting conditions no longer being a factor in biosurfactant biosynthesis. Some studies reported that biosurfactant production is more efficient under nitrogen-limiting conditions [36].

The best conditions for biosurfactant production by *B. subtilis* UKMP-4M5 were using 2% (v/v) palm oil and 0.5% (w/v) yeast extract as carbon and nitrogen sources respectively as it produced the lowest surface tension value at  $33.6 \pm 0.33$  mN/m.

#### Stability studies

Biosurfactant activity was tested over a range of pH values (2 – 12). Results showed the activity of biosurfactant was greatly affected by changes in pH. Low biosurfactant activity was detected at extreme pH; 2 to 4 and above 10 (Figure 5). Surface tension values remained relatively stable between pH 6 to 8 with no significant difference; however the lowest surface tension value was achieved at pH 6. The result also showed that highly acidic pH caused a greater reduction in surface activity than highly alkaline pH (surface tension value of 38.77 mN/m at pH 2 as opposed to 36.9 mN/m at pH 12).

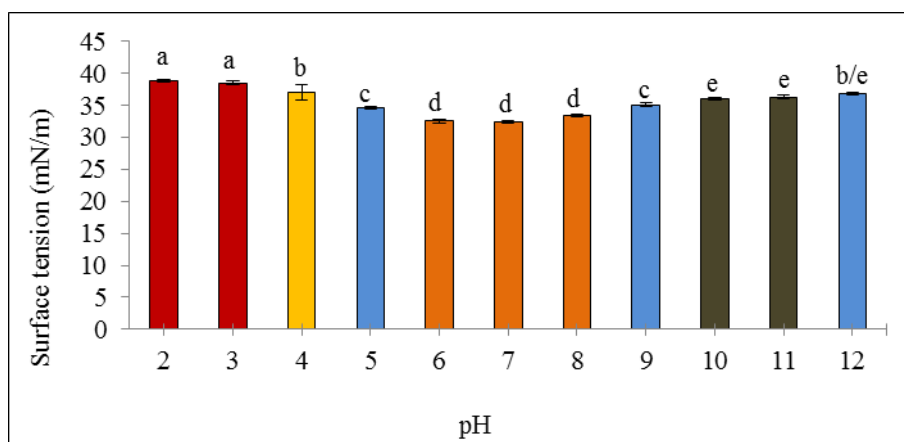


Figure 5. Effect of pH on biosurfactant activity produced by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD. The letters a, b, c, d, and e indicate significant differences ( $p < 0.05$ ) among different pH.

pH values between 2 to 4 showed a significant decrease in biosurfactant activity as shown by the increase in surface tension values. These results are in accordance with those reported by Vaz et al. [45], whereby biosurfactant stability from *B. subtilis* EG1 was found to be unstable by acidic pH. Abdel-Mawgoud et al. [7] also reported similar findings in their study in which surfactin activity started to decrease at  $pH < 5$ , samples become turbid due partial precipitation of biosurfactant in acidic conditions. The test biosurfactant showed better stability at alkaline

than acidic conditions, as shown by lower surface tension values when incubated in alkaline conditions as compared to acidic conditions. Similar results reported by El-Sersy [8] indicated that biosurfactant from *B. subtilis* N10 was found to have high surface activity predominantly in the alkaline condition, pH between 6.0-12. Abdel-Mawgoud et al. [7] reported that surfactin from *B. subtilis* was soluble in aqueous solutions at pH values higher than 5.0 with optimum solubility at pH 8.0-8.5. This could be due to the acidic nature of surfactin, as the presence of two carboxylic groups from glutamic and aspartic acid residues confers an anionic nature [7]. Similar results had been reported for biosurfactant production from other microorganisms such as *P. aeruginosa* [14], which was stable at a wide range of pH between 6.0-10.0, whereas stability of biosurfactant from *Nocardiopsis* sp. was found to be stable between pH 8 – 12 [46].

Thermal stability of biosurfactant was investigated over a wide temperature range (10 – 100 °C) for 1 hour and cooled to room temperature. Results showed that the biosurfactant from *B. subtilis* UKMP-4M5 exhibited excellent surface activity with no significant difference ( $p > 0.05$ ) of the temperature tested (Figure 6). Surface activity remained relatively stable with surface tension values between 32.47 – 33.10 mN/m even after exposure to high temperature (100 °C). Interestingly, the test biosurfactant retained its surface activity even after autoclaving, with a negligible increase in surface tension value (0.67 mN/m) before and after autoclaving (Figure 7), suggesting that no degradation occurred.

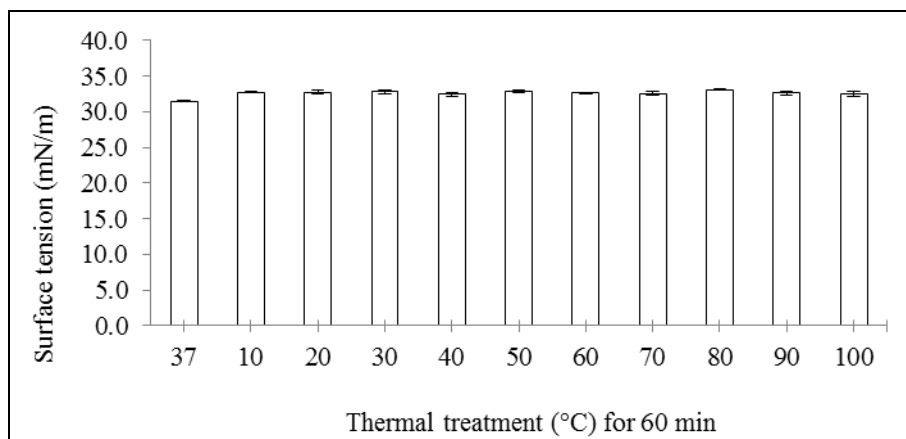


Figure 6. Effect of temperature on biosurfactant activity produced by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD.

Thermal stability studies of biosurfactant from *B. subtilis* UKMP-4M5 indicated that the surfactant was thermostable, as heating up to 100 °C caused no significant effect on the biosurfactant performances. The high thermal stability of the test biosurfactant is on par with other synthetic surfactants such as linear alkylbenzene sulfonate (LAS) and alkylpolyglucoside [45], but superior to sodium dodecyl sulphate, which exhibits a significant loss of activity at temperatures above 70 °C [46]. Interestingly, the test biosurfactant retained its surface activity even after autoclaving at 121 °C for 20 min, suggesting its potential application in food, pharmaceutical and cosmetic industries where heating to achieve sterility are important. Biosurfactants from *B. subtilis* have been reported to exhibit excellent thermal stability. Surfactin for example showed no change in surface tension value before and after heating treatment at 100 °C, though slight increase was observed at 121 °C for 10 min. This may be due to precipitation of heat-dependent substances such as proteins contaminating the surfactin solution which might have been co-extracted during the extraction steps [7]. Haddad et al. [32] investigated the stability of biosurfactant from a *B. subtilis* strain at 100 °C at different exposure time. The authors reported that no interference on the surface activity could be found, thus corroborating the results obtained in the current study. Results from the current study have thus illustrated the stability of biosurfactant produced by *B. subtilis* UKMP-4M5 at high temperatures which may be encountered in oil fields.

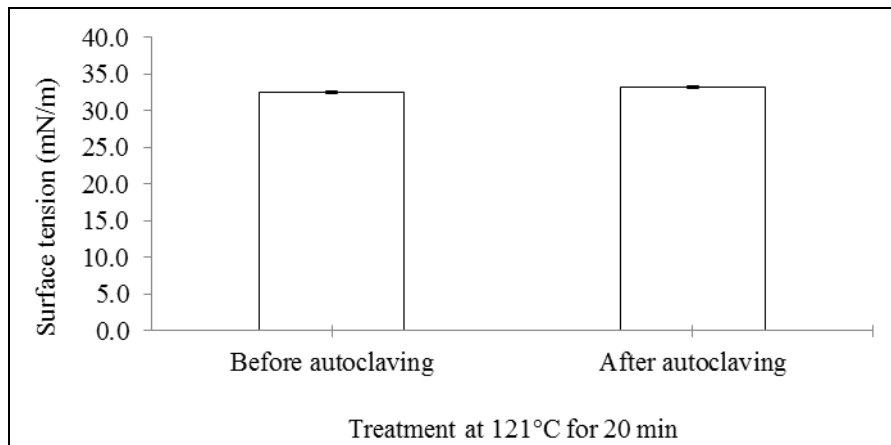


Figure 7. Effect of autoclaving on biosurfactant activity produced by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD.

The effect of different NaCl concentrations (1 – 15% w/v) on biosurfactant activity from *B. subtilis* UKMP-4M5 was studied to evaluate its potential application in bioremediation of contaminated marines. Figure 8 shows that the changed in surface tension value of the tested biosurfactant was not significant between 1 – 5% (w/v) NaCl concentrations. Optimum stability of biosurfactant was observed at 3% (w/v) NaCl with the lowest surface tension value at 33.18 mN/m.

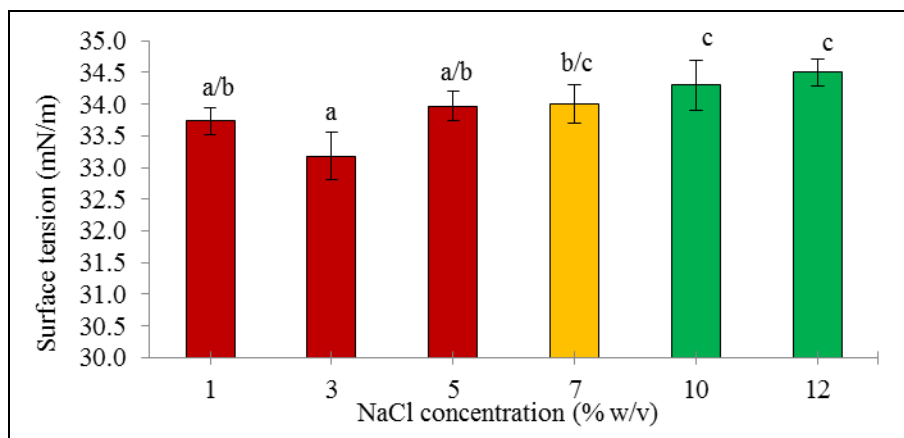


Figure 8. Effect of NaCl concentration on biosurfactant activity produced by *B. subtilis* UKMP-4M5. All values are mean of three replicates  $\pm$  SD. The letters a, b, and c indicate significant differences ( $p < 0.05$ ) among NaCl concentrations

The addition of NaCl in the range tested had a weak effect on the surface tension of biosurfactant produced by *B. subtilis* UKMP-4M5. Stability of biosurfactant from *B. subtilis* had been reported to be excellent in the presence of NaCl up to 20% (w/v) [8]. Ghojavand et al. [29], who reported that NaCl concentration, did not affect the surface activity of biosurfactant from *B. subtilis* PTCC 1696, the highest surface tension value recorded at 34 mN/m at 20% (w/v) NaCl. Similarly, Haddad et al. [32] reported that 25% (w/v) NaCl concentration showed only slight variation in the surface tension value (4 mN/m) of biosurfactant from *B. subtilis* HOB2. The stability of the biosurfactant

obtained from the present study is shown to be superior to other strain of *B. subtilis* which was reported to be losing its surface activity (surface tension value at 45 mN/m) when level of NaCl reached 11% (w/v) [5]. These findings have revealed that the tested biosurfactant had a high level of tolerance to ionic strength, and is thus an excellent candidate if compared with chemical surfactants for use in bioremediation of contaminated marine environments. Chemical surfactants, however, are deactivated by 2 – 3% salt concentrations [47].

### Conclusion

In the present study, a bacterial isolate identified as *B. subtilis* UKMP-4M5 was found to be the most potential biosurfactant producer. This isolate can produce biosurfactant using 2% (v/v) palm oil with addition of yeast extract with low surface tension value. The semi purified biosurfactant is thermostable to extreme temperatures. Furthermore, the test biosurfactant has proved to be robust and stable over huge ranges of pH and salinity. Thus, the biosurfactant has great potential for commercial use in industries such as enhanced oil recovery, bioremediation, and food-related industries as an alternative to the synthetic surfactants. Further work is required to fully characterise the biosurfactant to better predict its potential applications.

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