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Surfactant production by *Bacillus subtilis* using very low grade and cheap substrate

Niranjan Koirala[1,2ǂ*], Sareeta Khanal[1,3ǂ], Sujan Chaudhary[3,4], Sagar Gautam[5], Shiv Nandan Sah[3], Prince Subba[3], Najat Marraiki[6], Gaber El-Saber Batiha[7]

[1]Department of Natural Products Research, Dr. Koirala Research Institute for Biotechnology and Biodiversity, Kathmandu 44600, Nepal.

[2]Laboratory of Biotechnology, Faculty of Science and Technology, University of Macau, Macau SAR 999078, China.

[3]Department of Microbiology, Central Campus Technology, Dharan 56700, Tribhuvan University, Nepal.

[4]Department of Botany, Amrit Science Campus, Kathmandu 44600, Tribhuvan University, Nepal.

[5]Department of Microbiology. Trichandra Multiple Campus, Ghantaghar, Kathmandu 44600, Tribhuvan University, Nepal.

[6]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia.

[7]Department of Pharmacology and Therapeutics, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, AlBeheira, Egypt.

*Corresponding author email: Niranjan Koirala; koirala.biochem@gmail.com koirala.biotech@gmail.com

ǂ Both the authors contributed equally to this work and share the first authorship

Abstract

Bio-surfactants are surface-active molecules which are produced by the wide range of microbes including bacteria, fungi, and yeast. This study was conducted to identify bio-surfactants by *Bacillus subtilis* combined with use of cheap substrates and industrial wastes (Mustard cake, Whey and Soya cake) which are found locally in Nepal. *Bacillus subtilis*, one of the most potential bio-surfactants producer; was isolated from soil sample of hydrocarbon contaminated site. Isolates were grown in a Minimal Salt Media (MSM) with 10% (v/v) mustard oil cake, whey and soya cake separately. The presence and potential of surfactant was determined by the oil spreading technique, emulsification index (%E_{24}) and surface tension measurement. It was revealed that the surface tensions of cell free extract were 54.41, 60.02 and 56.64 mN/m for from mustard cake, whey and soya cake respectively as compared to distilled water (72.09) at 25°C. The emulsification index values are was found to be highest in engine oil from the bio-surfactant extracted from mustard cake, soya cake and whey respectively. Similarly, mustard oil showed the lowest value

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of emulsification index. The highest emulsification activity was shown in mustard oil i.e. 1.13 from the cell free extract from mustard oil and lowest in engine oil i.e., 0.07, by the extract from soya cake medium, when measured in spectrophotometer at 540 nm. In conclusion, strain of *Bacillus subtilis* was found to be the potential surface active agent producers on the mustard oil cake, which can be useful medium for various environmental, food and industrial processes.

Keywords: *Bacillus subtilis*; Bio-surfactants; emulsification index; Hydrocarbons; surface tension.

1. Introduction

Surfactants are the compounds capable of reducing surface tension between any two substances of same or different phase. Surfactants can be either chemically synthesized or obtained biologically. When surfactants are produced on living surfaces, mostly microbial cell surfaces or excreted extracellularly, they are termed as bio-surfactants. Such bio-surfactants generally contain hydrophobic and hydrophilic moieties [1]. Surfactants are characterized by their capacity to alter the surface and interfacial properties of a liquid, which allows the formation of micro emulsions, which further allows oils and related substances to become solubilized in water or vice-versa [2]. Such properties of surfactant enable a wide range of industrial applications including emulsification, detergency, foaming capacity, lubrication, moisture retention, solubilization, and phase dispersion [3].

In recent years, there has been a steady increase in the interest in bio-surfactants as they have numerous advantages over the chemical surfactants. Few of such advantages include lower toxicity, higher biodegradability, higher foaming, better environmental compatibility and effective properties even at extreme condition of temperature, pH and salinity [4]. In light of such advantages, bio-surfactants can be used for oil recovery, treatment of oil spillage and bioremediation, in foods, cosmetics and pharmaceuticals. Moreover, due to their biodegradability they can safely be used in the environment without the risks observed of some of their chemically synthesized counterparts. As a result, bio-surfactant occupies an advantageous position both for research and industrial production [5].The most prevalent bacterial species capable of producing surfactant belong to the genera are *Pseudomonas*, , *Micrococcus Flavobacterium*, , *Bacillus, Acinetobacter Achromobacter Arthrobacter, Klebsiella, Aeromonas, Alkaligenes, Streptococcus sp, Corynebacteriumsp, Moraxella sp,* and *proteobacteria* [6]. Microorganism utilize the substrate i.e. hydrocarbons to produce bio-surfactant and often minimalize them and produce ecologically harmless products [7]. *Bacillus* is one of such genus of bacteria associated with the production of vital bio-surfactant. They are gram positive rod shaped aerobic spore former bacterium commonly found in soil. Members of the genus are considered as a suitable group for research as well as synthesis of bio-surfactants industrially mainly due to their capacity to produce various metabolites including surface active ones [8]. They not only produce good bio-surfactants, but are also capable of growing under facultative or anaerobic conditions, and have also been reported to be non-pathogenic, which permits their use in wide range industries, apart from environmental applications [8]. Among the many species, *Bacillus subtilis* is one of the most potential species capable of producing a wide range of extracellular metabolites including surfactant. Surfactin, a lipopeptide bio-surfactant produced

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from *B. subtilis*, is able to reduce the surface tension of water to 25 mN/m and interfacial tension of water/hexadecane up to <1 mN/m which is among the best result given by any class of bio-surfactants. Most bio-surfactant can lower surface tension of water from 72 to35 mN/m and the interfacial tension of water/ hexadecane from 40 to 1 mN/m [9]. The cosmopolitan distribution of *B. subtilis*, ability to form resistant spores and extreme tolerance ability of the bacteria makes it a suitable candidate for further research and industrial application for the purpose.

Hydrocarbons are commonly used as the substrate for the production of bio-surfactants. It has been postulated that the biological function of surface-active compounds is related to hydrocarbon uptake, and therefore a spontaneous release occurs with these substrates [10, 11]. A wide range of hydrocarbon rich substrate has been experimented and studied for the commercial application for bio-surfactant production. However, there have been difficulties in industrial production and commercialization of the product due to the higher manufacturing cost. The cost of substrate account for 10-30 % of total production cost of bio-surfactant which makes the overall production cost higher that chemical surfactants [12].This is mainly due the use of different chemically synthesized media for production. .Consequently, chemical surfactant holds a higher position in the market. Thus, the practical and possible way to win over the market of chemical surfactant is to reduce the manufacturing cost of bio-surfactant which can then be made available in the market at lower cost. Reduction in cost of substrate by using lower grade and cheap substrate and using more efficient microbes could significantly reduce the manufacturing cost [13].

Present study aims for the production of bio-surfactant by *B. subtilis* on the industrial wastes, cheaper and low grade substrates (mustard oil cake, soya cake and whey) which are found locally and abundantly in different areas of Nepal.

2. Materials and Methods

This work was conducted at the Central Campus of Technology, Hattisar, Dharan. The eight soil samples from auto-mobile workshop (garage) were collected form Dharan-8 by using simple random sampling method. All samples were collected in aseptically dried and clean plastic bags and were transported to laboratory as soon as possible.

2.1 Bacterial isolation

The test organism (*Bacillus subtilis)* was isolated form a soil sample, by heating test samples at 80^{o}C/ 10 min by using Water bath to destroy all the vegetative cells. Then 1g of soil was weighed and was suspended in 9ml sterile distilled water. Serial dilution of soil sample was done up to10^{4}. Nutrient agar with 1% soluble starch was prepared onto which an aliquot (0.1ml) of 10^{2} to 10^{4} dilutions were inoculated and spread plate method was followed. The plates were incubated aerobically at 37^{o}C for 48 hours [14].

Colonies that showed big, creamy, wrinkle, and spreading colonies were picked and sub cultured on nutrient agar broth and on nutrient agar slant. Gram staining and endo-spore staining were carried out for the presumptive identification of the isolates [14].

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2.2 Identification

The test isolate was identified by using standard microbiological techniques as described in Bergey's Manual of Systematic Bacteriology (1986). Different biochemical tests viz; catalase, citrate, urease, indole, starch hydrolysis, methyl red voges-proskauer, sugar fermentation test (Lactose, Mannitol, Sucrose), Triple Sugar Iron (TSI) and SIM were carried out to identify the isolates.

2.3 Substrate Preparation

The medium used for the experiment was a Minimal Salt Medium supplemented with 10% substrate (Mustard oil cake, Soya cake and Whey). It was prepared by dissolving 1.73g dipotassium phosphate, 0.68g potassium dihydrogen phosphate, 0.1g magnesium sulphate hepta hydrade, 0.33 g ferrus sulphate, 4g sodium chloride, 1g ammonium nitrate, 0.02g calcium chloride and 5g glucose [15].

2.4 Inoculation and incubation

One ml of 24 hours broth culture of the isolate was pipetted into each flask. All six flasks were plugged with cotton and allowed to stand in water bath shaker for 5 days. Temperature of the water bath shaker was maintained at 37^{o}C. After 5 days all flask were taken out from shaker and centrifugation was done to obtain cell free supernatant [15].

2.5 Oil spreading technique

The oil displacement test was performed according to the protocol described by Walter (2010). A Petri-dish (150 mm diameter) was filled with 40 ml of distilled water. 15 ml of weathered crude oil was added. The crude oil will form a thin oil layer on the water surface. Then, 10 ml of free cell culture supernatant was carefully placed on the center of the oil film. If there were microbial surfactants present in the supernatant, the oil was displaced and a clearing zone was formed [16].

2.6 Determination of emulsification activity

0.5 ml of the extracted supernatant was added to 7.5 ml of 1M tris-HCl buffer and 0.1 ml of oil (kerosene, mustard oil, sunflower oil and engine oil). The mixture was vigorously vortexed and allowed to stand for 1 hour. Absorbance was measured at 540nm 5 times at an interval of 1 hour. After obtaining the absorbance emulsification activity was calculated by calibrated graph [17].

2.7 Measurement of Emulsification Index (E_{24})

The bacterial broth was centrifuged and was studied for its emulsifying ability by a modified method of ([18]. Two ml Cell-free broth was pippeted into the screw cap test tube, and 3 ml of oil (Kerosene, Mustad, Engine and Sunflower) was then added. The mixture was vortexed at high speed for 2 min and left at room temperature. The result was observed after 24 h for the stability of emulsion. **Photograph 4** shows the test tubes left for the calculation of emulsification index. The total volume of the mixture, volume of emulsified, and volume of non-emulsified phase was observed [3]. The emulsification index (E_{24}) was calculated by the equation:

$$E_{24} = \frac{\text{Heightofemulsionlayer}}{\text{TotalHeight}} \times 100$$

The method was followed at the end of each day to obtain reading for 5 days.

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2.8 Surface tension measurement

Surface Tension was determined at room temperature i.e. 30°C by Drop Number Method by using Stalagmometer [19]. Basically, the weight of a drop of a liquid falling after passing through a capillary tube of uniform radius is approximately proportional to the surface tension of the liquid. For two liquids of surface tension Υ_1 and Υ_2, d_1 and d_2 are the densities and n_1 and n_2 are the number of drops made by the same volume of liquids then the surface tension is calculated as

$$\frac{\Upsilon 1}{\Upsilon 2} = \frac{d1Xn2}{n1Xd2}$$

Pyknometer was used to determine the density of a liquid and Stalagmometer was used to determine the number of complete drops made by a liquid [20].

3. Results

3.1 Bacterial Isolation and identification

The test organism (*Bacillus subtilis)* was isolated form a soil sample of automobile workshop. Presence of *Bacillus subtilis* was confirmed based on the macroscopic examination of colonies, microscopic examination and the different chemical tests which were performed as given in **Table 1**. The microscopic examination of the isolates after Gram staining is shown in **Photograph 1** and the biochemical test for the conformation of the isolates is shown in **Photograph 2.**

Table 1: Biochemical tests for confirmation of *Bacillus subtilis*

Biochemical test	***Bacillus subtilis* properties**
Catalase	Positive
Oxidase	Positive
Citrate	Positive
Indole	Negative
MR (Methyl Red)	Negative
VP (Voges Proskauer)	Positive
Urease	Negative

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Starch hydrolysis	Positive
Gelatin hydrolysis	Positive
Sucrose	Acid production
Lactose	Negative
Gram staining	Positive
Endospore staining	Positive (Central spore)
Nitrate reduction	Positive
Arabinose	Positive
Arabitol	Positive
Glucose	Positive
Glycerol	Positive

3.2 Emulsification activity (EA)

This study recorded the emulsification activity was observed highest in mustard oil with the cell free extract from mustard oil cake whereas engine oil showed the lowest **(Fig: 1).** Emulsification activity in mustard oil from extract from mustard oil cake was found to be 1.13, 0.99, 0.81, 0.95 and 0.76 at the time interval of 1, 2, 3, 4 and 5 hour respectively. However, EA of engine oil was found to be 0.13, 0.06, 0.08, 0.07 and 0.07 at the time span of 1, 2, 3, 4 and 5 hour respectively **(Fig: 1).** Similarly, EA by extract from whey was also found to be higher of mustard oil. Emulsification value of mustard oil was found to be 0.69, 0.58, 0.46, 0.53 and 0.42 at the time span of 1, 2, 3, 4 and 5 hour respectively **(Fig: 2).** The EA by the extract from whey was followed by sunflower oil, kerosene oil and engine oil with the lowest emulsification activity **(Fig: 2).** EA in mustard oil was again found dominating in the extract from the soya cake followed EA in sunflower oil, kerosene oil and engine oil **(Fig: 3)** respectively. The emulsification activity of cell free broth extracted from mustard cake, Whey and Soya cake are shown in figure 1, 2 and 3 respectively.

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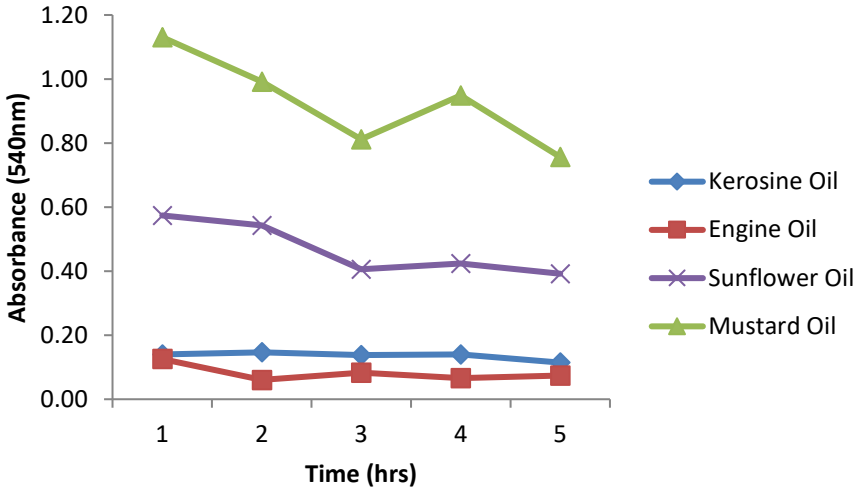

Figure. 1: The emulsification activity of cell free broth extracted from mustard cake

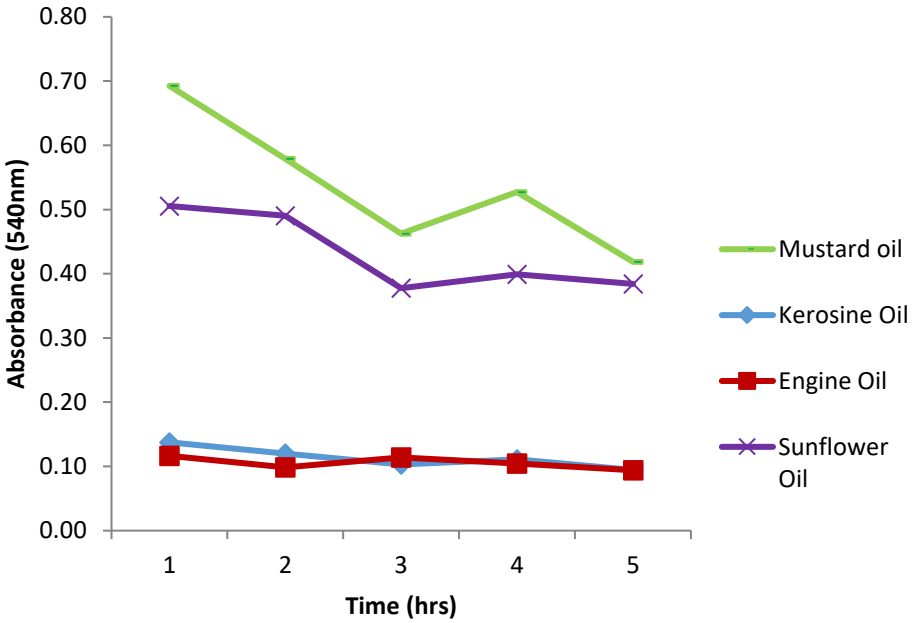

Figure. 2: The emulsification activity of cell free broth extracted from whey

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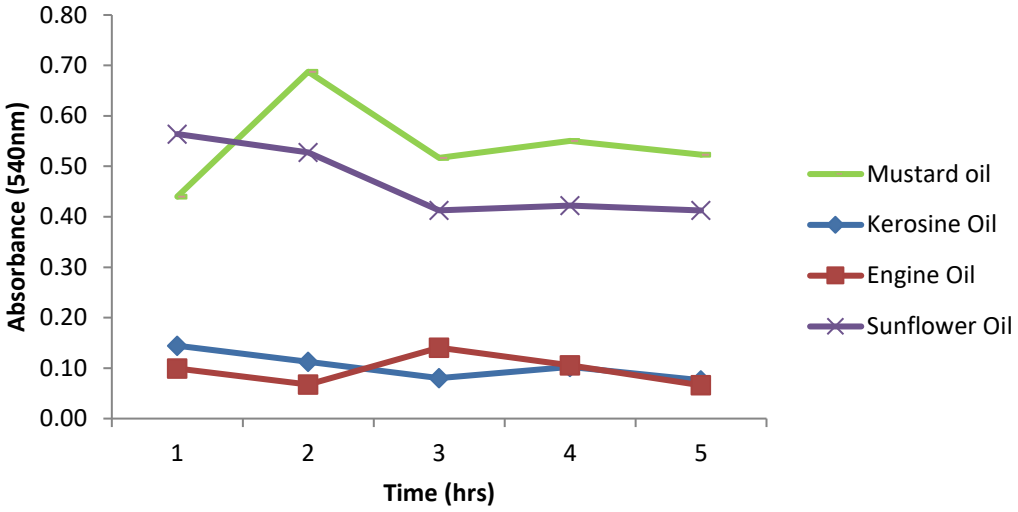

Figure. 3: The emulsification activity of cell free broth extracted from soya cake

3.3 Emulsification index (E_{24})

The emulsification index revealed highest in engine oil by the extract from mustard oil cake whereas mustard oil showed the lowest values **(Table: 1).** Corresponding E_{24} in Engine oil by extract form mustard oil cake substrate was found to be 22.09, 28.55, 29.96, 32.85 and 38.82 in the time span of 1, 2, 3, 4, and 5 day respectively while E_{24} in mustard oil was 1.43, 5.57, 7.27, 9.32 and 12.21 in 1-5 day respectively. Similarly, E_{24} in engine oil with extract from whey was found to be highest with 11.40, 14.48, 23.60, 33.45 and 36.30 in the time span of 1, 2, 3, 4 and 5 day respectively. Emulsification index of extract from the whey in different medium was followed by sunflower, kerosene and mustard oil. Similar result was found with the extract from soya cake. Emulsification index of bio-surfactants obtained from Mustard oil, Whey and Soya cake are listed in table 1, 2 and 3 respectively.

Table 1: Emulsification Index of bio-surfactants obtained from Mustard oil cake

Time (Day)	Kerosene oil	Sunflower oil	Mustard oil	Engine oil
1	4.44	19.28	1.43	22.09
2	5.80	20.01	5.57	28.55
3	9.26	20.81	7.27	29.96
4	12.50	22.74	9.32	32.85
5	17.86	23.26	12.21	38.82

Table 2: Emulsification Index of bio-surfactants obtained from Whey.

Time (Day)	Kerosene oil	Sunflower oil	Mustard oil	Engine oil
1	0.85	16.40	2.79	11.40
2	3.27	18.38	5.43	14.48

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3	6.69	19.62	6.63	23.60
4	9.36	20.08	8.48	33.45
5	11.61	21.29	10.33	36.30

Table 3: Emulsification Index of bio-surfactants obtained from Soya cake.

Time (Day)	Kerosene oil	Sunflower oil	Mustard oil	Engine oil
1	1.77	16.79	3.73	17.76
2	4.90	18.68	4.74	22.01
3	8.54	20.50	6.31	25.80
4	9.08	21.64	7.57	32.70
5	12.84	22.20	8.06	37.05

The emulsification index was found to be increasing every day and the maximum result was obtained at day 5. The comparative study of the emulsification index of three substrates with different oils is given in **Fig. 4**.

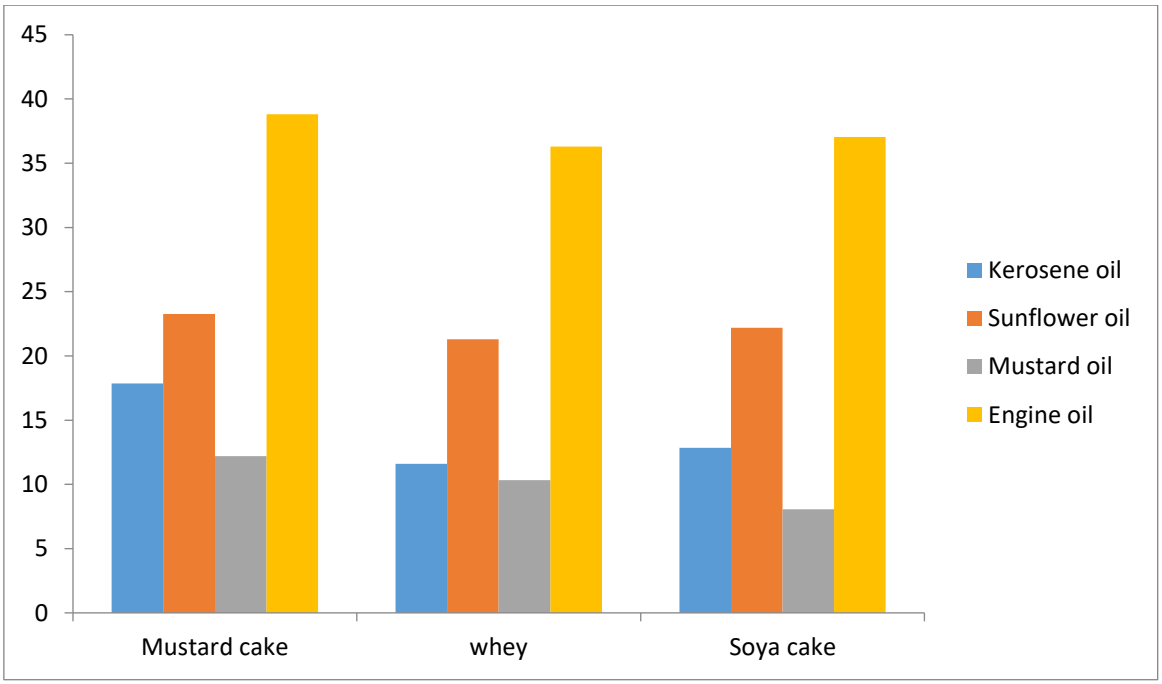

Figure. 4: Bar diagram for comparative study of emulsification index of three substrates with different oil at maximum level (5 days).

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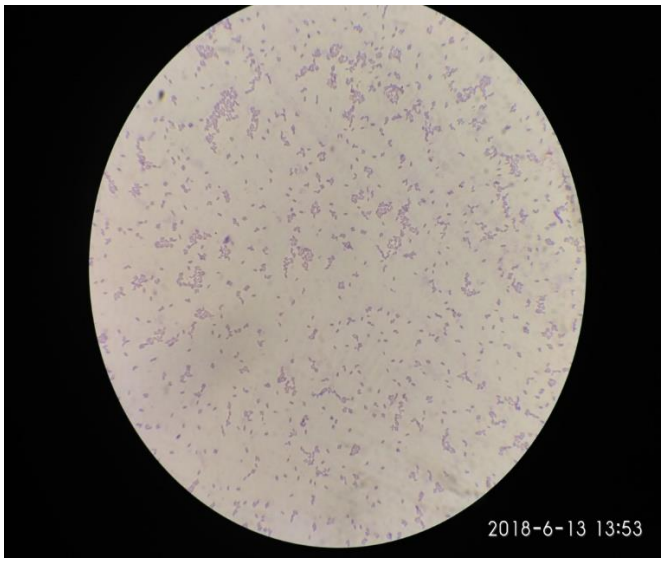
3.4 Surface tension

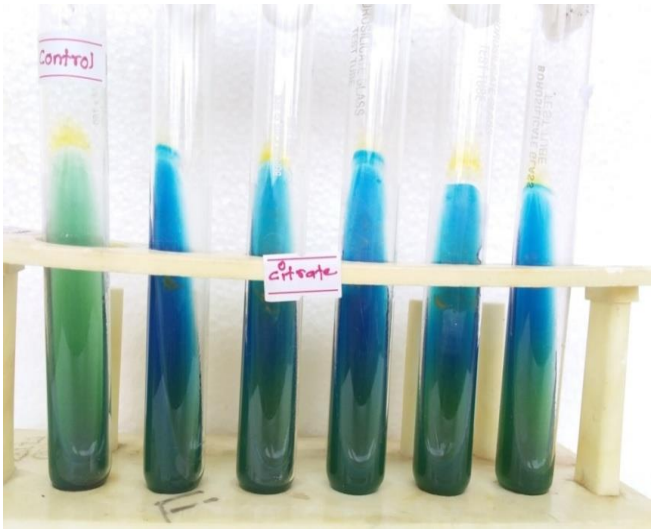
The surface tension of water at 30°C was 71.34 dyne/cm. The result calculated from cell free broth of mustard oil cake, whey and soya cake were found to be 54.41, 60.02 and 56.64 dyne/cm respectively **(Table 4). Photograph 3** shows the cell free broth extracted using different substrates.

Table 4: The surface tension of cell free broths

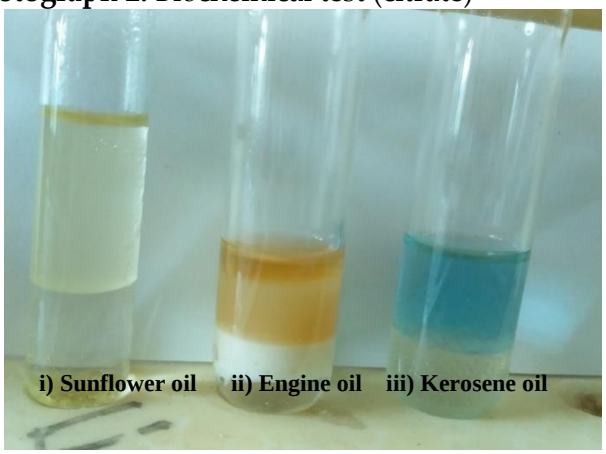
Cell free broth	Surface tension (dyne/cm)
Mustard Cake	54.41
Whey	60.02
Soya cake	56.64



Photograph 1: Gram stain of *B. subtilis*



Photograph 2: Biochemical test (citrate)



Photograph 3: Bio-surfactants extraction (cell free broth) **Photograph 4:** Emulsification index of

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Hydrocarbons.

4. Discussion

Bio-surfactants, compounds with such wonderful advantages over chemical ones, have not yet been commercialized significantly as a result of high production cost. Since the cost of substrates, chemical ones used more widely, account for 10-20% of the production cost of bio-surfactants, the production cost is higher [12]. The possible measure to minimize the cost is to reduce the cost of substrate i.e. use of cheap substrates preferably natural substrates like mustard oil cake, soya cake and whey. Present study was performed with the objective of identifying the utility of the substrates mentioned above for production of bio-surfactants using bacterial species isolated from oil contaminated soil. From the total 4 soil samples, one isolate of *Bacillus subtillis* was isolated based on its morphological and bio chemical characteristics based on the study of Banat et al. [21] and used for the production of bio-surfactant. However, the study couldn't identify the exact strain as a result of less resources for genetic analysis. The isolate was then accessed for its capacity to produce bio-surfactant in mustard oil cake, soya cake and whey. Luckily it was able to grow on all the substrate used. The oil spreading technique proved that the isolate has the ability to produce the surfactin as it spread the oil when cell free broth was poured on it. The result is comparable to the study of Banat et al. [22].

The produced extracellular metabolites were then compared by calculation of emulsification activity and emulsification index on 4 different oil medium; Kerosene oil, Mustard oil, Engine oil and Sunflower oil. The emulsification activity was found to be highest for mustard oil while lowest for engine oil. This shows that the bio-surfactants extracted possessed lower ability to emulsify the mustard oil but has higher to engine oil. Since, the habitat of microbial used in the study was from automobile workshop, the organism showed higher ability to emulsify the engine oil as the organism might had adapted to the oil contaminated soil.

This study revealed that the emulsification index on all tested oils mediums was found a little higher by the bio-surfactant produced in mustard oil cake as compared to soya cake and whey. This might be due to the ability of *B. subtilis* to utilize nitrogen from various sources for cell multiplication and biosynthetic pathway [23]. This point was also supported by Patel and Desai [24] where optimum C/N ratio would be a favorable factor in mustard oil cake for the production of surfactants by *B. subtilis.*

Bio-surfactant produced from the respective substrate has the highest emulsification index value of 38.82, 37.05 and 36.05% (Table 1, 2 and 3) respectively in engine oil while they have the lowest emulsification index value of 12.21, 10.33 and 8.06% (Table 1, 2 and 3) respectively in mustard oil. Similarly, the emulsification index in sunflower was also found higher value than in the mustard oil. This difference of emulsification index in different medium can be attributed to biochemical properties of the medium. Sunflower oil contains the long chain of mono and polyunsaturated fatty acids (91.49 ± 1.91) compared to mustard oil (86.80 ± 3.07) [25]. A similar prediction based on the length of hydrocarbon chain can be made about the higher value of emulsification index in engine oil. However, any solid research is lacking regarding the topic and is open for speculations.

The Emulsification index in each of the oil medium is found to be increasing gradually with increase in time in case of each of the bio-surfactant extracted. The fifth day showed the maximum value of emulsification index for each of the cell free extracts, which is quiet obvious as the index is found to increase with time in most of the previous similar studies. With increase in time, the emulsion gradually gets back together forming a separate layer as before being vortexed. This study has also revealed that the surface tension of the extracted bio-surfactants from the substrate mustard cake, whey and soya cake was found to lie within the normal range as prescribed by [26, 27]

Many similar studies have identified the bacterial strain as well as the possible substrates of bio-surfactant production. Makkar and Cameotra [28] have reported the studies on bio-surfactant production by Bacillus strains under thermophilic conditions on sucrose and molasses as substrate. Al-Bahry *et al.* [29] has reported production of bio-surfactant by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. Nevertheless, the mustard oil cake, an agro-industrial waste, could be the potential substrate for the commercial bio-surfactant production as suggested by present study. Although the potential of the crude bio-surfactant produced is lower than expected, it cannot be denied that higher potential could have been achieved with better resources. Moreover, the extracted crude surfactant was also able to give satisfying result despite of being crude. However, the study could have been considered more achieving if the purity of the crude extract could have been increased.

5. Conclusion

The result of this study showed that the strain of *Bacillus subtilis* isolated from Mustard cake, Whey and Soya cake was found to be the potential surface active agent producers which are useful tools for various environmental, food and industrial processes. The organism isolated from oil contaminated area showed greater ability to produce bio-surfactants. Furthermore, each of the substrates obtained from industrial and household waste were found to show great potential as substrate for the production of bio-surfactant. Among them, the mustard oil cake substrate is cheap and best among the three studied substrate for bio-surfactant production.

Author's Contribution: Conception, data acquisition, analysis and drafting were done by S.K., S.C., P.S., and S.G. Experimental work was performed by S.K., S.G., S.C., and P.S. Writing and preparation of manuscript was performed by S.C., S.G, and S.K. Analysis and interpretation of data and critical revision of manuscript was done by P.S., S.N.S., G.E.-S.B and N.M. Supervision by N.K and Funding acquisition was done by G.E.-S.B, N.M and N.K. Final approval of manuscript was done by all the authors.

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Conflict of Interest: The authors declare that there is no conflict of interest with present publication.

Declaration: We hereby declare that this study is our own work and that to the best of our knowledge and belief. It contains no matter and data previously published or produced by another party.

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