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An Approach for Developing a Method for Biosurfactant Activity Detection.

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Abstract

Detection of biosurfactant activity have been proposed Previously by several studies either quantitatively or qualitatively. Quantitative methods give precise measurement of the surface tension using accurate devices while qualitative measurements are considered screening methods for biosurfactant activity detection with lower precision. Due to the high cost of the direct measurement devices and in some cases the unavailability of devices, qualitative methods remain the most common used tests for screening of the surface-active properties. The need for an accurate, cheap method was the reason for this trial of developing a photometric method for a rapid, accurate biosurfactant activity detection. "α-naphthol-butanol" method is based on measuring the photometric activity of a sparingly soluble compound "α-naphthol" at its λ_{max}. Formed emulsion of oily phase "containing α-naphthol" and aqueous phase is based on the surfactant concentration and the surface tension of the solution being tested. This method passed the first and second confirmatory tests but, in the third trial it failed. Knowing the drawbacks of this method we can manage to develop a promising modification.

Keywords: Null hypothsesis, surface activity detection, α-naphthol butanol, new method development.

1. Introduction:

Biosurfactants are surface active agents of bio-origin that are produced by microorganisms whether bacteria, fungi or yeasts (**Shoeb et al., 2012)**. Detection of new biosurfactants are a matter of concern Because of the importance of biosurfactants in many life aspects; food, agricultural, pharmaceutical, cosmetic, medical, laundry industry, petroleum industry, and environmental applications (**Mukherjee and Das, 2010, Saravanan and vijayakumar, 2012 and Shoeb et al., 2015)**. However, biosurfactants are not common commercially due to the low production yield and high cost **(Joshi and Desai, 2010 , Shoeb et al., 2012 and El azzazy et al., 2015)**. Many quantitative and qualitative methods have been used by researchers for screening of biosurfactants' activity in an attempt to obtain a high yield potent biosurfactant strain.

Quantitative methods using direct surface tension measuring devices are used that gave a precise, accurate measurements of the surface tension which gave us an image of the biosurfactant type and potency. Those devices are based on functions related to the surface tension property as The DuNouyRing method, Stalagmometric Method, Pendant Drop Shape Technique and Axisymmetric Drop Shape Analysis by Profile (**Walter et al., 2010)**. Majority of surface tension measuring devices are based on DuNouyRing method which depend on measuring the detachment force of a platinum ring or plate from an interface or surface that is directly proportional to the surface tension. Most automated devices that have been reported by studies are based on this method (**Ahmad et al.,**

2016 and Thavasi et al., 2011). However, most microbiology & biotechnology laboratories are not equipped by those automated devices because of the high cost and the uncommon need for it. So, qualitative methods are mainly used by most studies. But, they lack the accuracy and precision however, they give general indication of the biosurfactant type and its potency (**Elazzazy et al., 2015, Nordin et al., 2013 , Shoeb et al., 2012 and , Shamsudin and Isa, 2015, Kamal et al., 2017)**. No single method of qualitative detection is sufficient and can be used alone for proving the biosurfactant activity so, many methods have been reported for proving the biosurfactant/ bio emulsifier activity as hemolysis assay, drop collapse test, microplate assay, solubilization of Crystalline Anthracene, penetration assay, oil spreading assay, emulsification capacity assay and for detection of the hydrophobicity of bacterial cells as cell surface hydrophobicity, bacterial adhesion to hydrocarbons assay, hydrophobic interaction chromatography, replica plate assay and salt aggregation assay which are not effective in testing the surface activity**(Walter et al, 2010, Meenakshisundaram et al., 2016, Nwaguma et al., 2016 and Rehman et al., 2014).** One of the methods that has been proposed by "**Willumsen and Karlson., 1997**" for detection of bio surface activity is "Solubilization of crystalline anthracene", which is a photometric detection method that works by solubilization of crystalline anthracene in the culture supernatant over 24 hr. Then, the concentration of the dissolved hydrophobic anthracene is measured photometrically at 354 nm which correlates to the production of biosurfactant. Our method may be considered a development of this method however instead of using the solubilization technique in which surface tension detection is depending on the solubilization of B-anthracene by a surfactant, we used a colloidal system of "an oily phase containing a hydrophobic photometric substance and an aqueous phase of solution containing a surfactant". In solubilization of B-anthracene method, solubilization of the hydrophobic compound "B-anthracene" is approximately started after cmc and before cmc the solubilization is negligible. On the other side, Colloidal system stabilization is increased with the increase of surfactant concentration until CMC is reached then destabilization starts **(Hussien et al., 2019, Zhang et al., 2021and Pool and Bolhuis, 2010)**. Those two functions of solubilization and colloidal system stabilization are shown clearly in the figures (1,2). In our method, we utilized the colloidal system profile using a surfactant (SDS) of gradient increasing concentrations in screening for the surface active property by photometric detection of α-naphthol compound in the aqueous phase which correlates with the formation and stability of emulsion system (Figure 2). Our system was a trial to reach a new simple, accurate and precise laboratory method and to overcome the drawbacks of the previously mentioned quantitative or qualitative methods (Walter et al., 2010). Which would have ease the screening of new biosurfactants and open the field for us for detection of high yield producing microorganisms which in turn will lead to a decrease in the production cost. In this article, however we reached a null hypothesis point of this trial but we offer a new concept of surface activity screening method in which we faced drawbacks that if they have been solved then we would reach a precise, accurate measuring method.

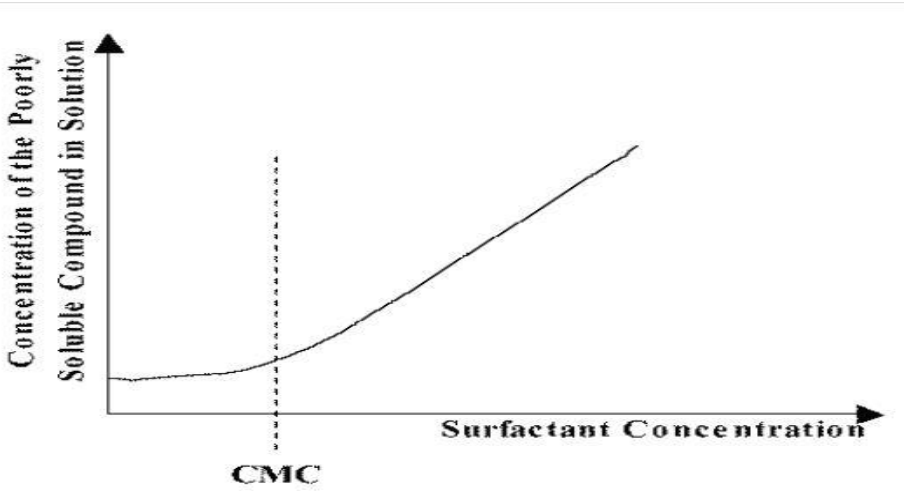

Figure (1): Solubilization of the poorly soluble compound against surfactant concentration (al kurashy et al., 2019).

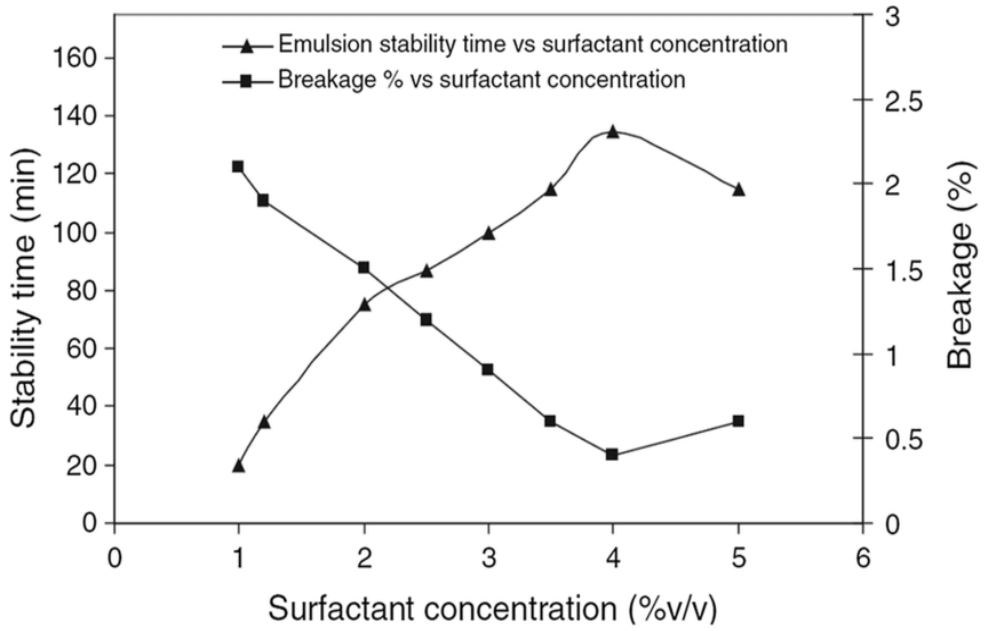

Figure (2): Colloidal system stability in presence of different surfactant concentrations (Hussein et al., 2019).

2. Methods:

For proving of application of our method in detection of susceptible biosurfactant activity. Our Principle should be proved first on a definite surface active agent with certain surfactatnt activity and known critical micelle concentration. We choosed in our method sodium dodecyle sulphate as the most common surface active agent that have been used in many life aspects. Principle of our method is based on that the colloidal system is composed of oily phase that contains soluble α-naphthol and aqueous phase of water that contains different gradient sodium dodecyle sulphate "SDS" concentrations. After vortex of the system, emulsion is formed of the two phases which its stability is directly corresponding to the surfactant concentration. If the water phase contains a surfactant, the emulsion formed of the two phases will be formed with stability that is increased to reach a maximum at critical micelle concentration. SDS will be the surfactant to be used for verification and calibration of this method with increasing concentrations around the critical micelle concentration (CMC= 2g/l). After complete stabilization of the colloidal system, volume of the aqueous phase that contains the formed stabilized emulsion is withdrawn and tested for presence of α-naphthol at its "λ_{max}= 340 nm". The intensity of absorbance is directly corresponding with the presence of α-naphthol which is directly corresponding to the emulsion stability that depends on the surfactant's concentration. For verification and calibration of this method each step should be proved, verified and calibrated individually then, combination of those steps will give us the complete method.

A) Absorbance of α-naphthol at 340 nm:

At a concentration of 5 g α-naphthol /100 ml butanol), absorbance should be taken at λ_{max} =340 against a blank of n-butanol.

B) Absorbance of (α-naphthol) in the aqueous phase, of the colloidal system and at gradient concentrations of SDS:

We dissolved α-naphthol in butanol with sufficient concentration "5g /100ml" which give deep colored solution with no saturation "oily phase" while the aqueous phase is formed from gradient increasing concentrations of SDS. Then, vortex of the two phases to form emulsion and testing of the aqueous phase for α-naphthol at λ_{max} 340 nm.

Knowing that critical micelle concentration of sodium dodecyl sulphate is 2 g/1l (**Moroi et al., 1974).** So, gradient increasing concentrations of SDS should be prepared (0, 0.25, 0.5 , 0.75 , 1 , 1.25 , 1.5 , 1.75, 2 , 2.25, 2.5, 2.75 ,3, 3.25, 3.5 ,3.75,4). Each 3 ml of the solution shout be vortexed for 3 min with 0.2 ml 5 g α-naphthol butanol to form emulsion. Then, to be leaved for 24 hr. Then, 1 ml of the lower aqueous phase are drawn gently with Pasteur pipette, measured at 340 nm using spectrophotometer.

After proving the concept of emulsion stabilization profile, now we have to optimize the conditions of the test through modification on :

vortex time, volume to volume ratios, α-naphthol concentrations, time gap.
All experiments are done in triplets, against blank, with critical micelle concentration of sodium dodecyl sulphate (2g/l). Spectro photometered at 340nm.All readings was taken after 24 hr and 3 series was done.
Vortex time: Different vortex time of 0.1 ml α-naphthol butanol (5g/100 ml) is done with 2 ml of critical micelle concentration of sodium dodecyl sulphate against blank of SDS

Volume to volume ratios:
- For 2 ml aqueous solution, gradient volumes of 5g/100 ml "α-naphthol butanol" will be vortexed for 2 min and spectrophotometer reading will be taken after 24 hr.
- For 3 ml aqueous solution, gradient volumes of 5 g α-naphthol / 100 ml butanol is used, vortexed and reading are taken at spectrophotometer after 24 hr .
- For 4 ml aqueous solution, series of gradient volumes of α-naphthol butanol was used, vortexed and reading with spectrophotometer was taken after 24 hr.
- For 5 ml aqueous solution, series of gradient volumes of α-naphthol butanol was used, vortexed and reading with spectrophotometer was taken after 24 hr .

α-naphthol concentrations:
For choosing a good concentration of α-naphthol butanol, we have to choose a concentration where sub-saturation occurs which gives a reasonable reading, for this 5,6,7,8,9,10 grams of α-naphthol are dissolved in 100 ml butanol.

Period gap of the test:
Different period gaps have been used to allow the emulsion formed to be stabilized to its final formation. Period gaps used were 12, 24, 36 and 48 hrs.

3. Results:

3.1. Absorbance of α-naphthol at 340 nm:
Absorbance was measured at (λ_{max} =340 nm,) against blank of butanol to be equal 1.933.
3.2. Absorbance of (α-naphthol) in the aqueous phase, of the colloidal system and at gradient concentrations of SDS are illustrated in Table 1.

Table (1): Absorbance of aqueous phase at wavelength 340 nm with gradient increasing concentrations of SDS.

Concentration	S1	S2	S3
0	0.5	0.55	0.52
0.25	0.43	0.5	0.49
0.5	0.67	0.7	0.72
0.75	0.7	0.72	0.72
1	1.37	1.4	1.5
1.25	1.65	1.67	1.55
1.5	1.81	1.85	1.6
1.75	1.9	1.92	1.65
2	1.75	1.77	1.53
2.25	1.3	1.35	1.6
2.5	1.6	1.62	1.7
2.75	1.8	1.77	1.5
3	1.77	1.8	1.4
3.25	1.9	1.8	1.43
3.5	1.84	1.81	1.53
3.75	1.81	1.8	1.6
4	1.77	1.77	1.58

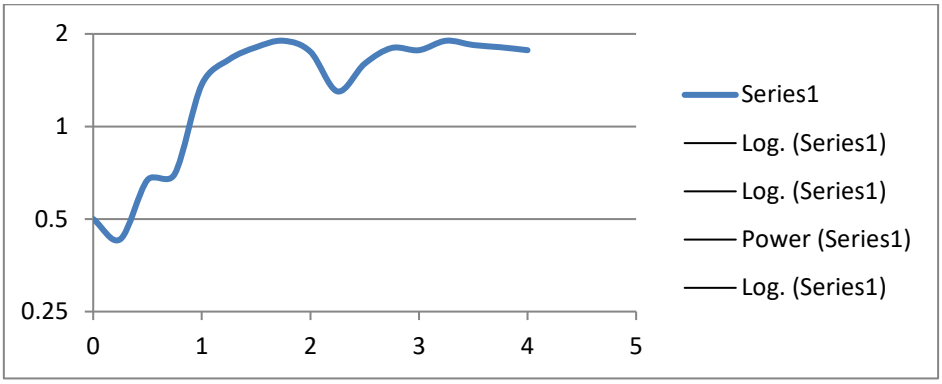

Figure (3): showing the absorbance spectrum of aqueous phase at wavelength 340 nm with gradient increasing concentrations of SDS.

3.3. Experiment optimization:

3.3.1. Vortex time:

Readings of a system of variant vortex time are presented in Table 2.

3.3.2. Volume to volume ratios:

Data are presented in Tables 3-6.

3.3.3. α-naphthol concentration:

Different α-naphthol solution concentrations readings are presented in Table 7

3.3.4. Time gap:

Different period gap readings are illustrated in Table 8.

Table (2) : Readings of a system of variant vortex time.

Time	S1	S2	S3
1	1.76	1.7	1.8
2	1.84	1.77	1.84
3	1.75	1.84	1.81
4	1.84	1.61	1.87
5	1.68	1.6	1.68

Table (3): Readings of a system of 2 ml aqueous solution is vortexed with gradient volumes of α-naphthol solution.

Volume "α-naphthol butanol" "ml"	S_1	S_2	S_3
1- 0.1	1.38	1.63	1.73
2- 0.2	1.29	1.13	1.07
3- 0.3	0.05	0.2	0.16
4- 0.4	0.41	0.49	0.63
5- 0.5	0.32	0.35	0.23
6- 1	0.43	0.47	0.32

Table (4): Readings of a system of 3 ml of SDS solution is vortexed with gradient volumes of α-naphthol solution.

Volume	S_1	S_2	S_3
1- 0.1	1.01	1.02	0.89
2- 0.2	1.49	1.42	1.49
3- 0.3	1.27	1.27	0.93
4- 0.4	0.8	0.8	0.57
5- 0.5	0.8	0.8	0.51
6- 1	1	1	0.66

Table (5): Readings of a system of 4 ml of SDS is vortexed with gradient volumes of α-naphthol solution.

Volume	S_1	S_2	S_3
1- 0.1	0.92	0.81	0.94
2- 0.2	1.73	1.78	1.65
3- 0.3	0.89	1.02	0.83
4- 0.4	0.89	1.1	0.9
5- 0.5	0.52	0.54	0.59
6- 1	0.60	0.56	0.65

Table (6): Readings of a system of 5 ml of SDS is vortexed with gradient volumes of α-naphthol solution.

Volume	S_1	S_2	S_3
1- 0.1	0.94	0.8	0.81
2- 0.2	0.87	0.95	1.03
3- 0.3	1.14	1.2	1.14
4- 0.4	1.06	0.82	0.92
5- 0.5	0.88	0.84	0.88
6- 1	0.52	0.47	0.50

Table (7): Different α-naphthol solution concentrations readings.

Concentration/100ml	S_1	S_2	S_3
5	1.34	1.38	1.36
6	1.39	1.41	1.4
7	1.4	1.41	1.31
8	1.52	1.52	1.5
9	1.69	1.69	1.67
10	1.44	1.88	1.65

Table (8): Different period gap readings.

Period	S_1	S_2	S_3
12 hr	1.84	1.83	1.82
24 hr	1.34	1.38	1.35
36 hr	1.21	1.12	1.15
48 hr	1.06	0.96	1.01
60 hr	1.06	1.05	1.06

4. Discussion:

Many methods have been proposed by researchers in the approach of biosurfactants' screening whether quantitative methods based on direct surface tension measuring or qualitative methods based on the surface tension property detection which give an indication of the biosurfactants being produced by microorganisms. While quantitative methods are costy and require advanced equipments, qualitative methods give a strong indication of biosurfactant activity however, they are not accurate nor precise (**Walter et al., 2010)**. The idea behind this approach is to develop a simple and precise method of surface tension detection with high thoroughout put. In this approach we present a method that would have been a precise qualitative test in general surface activity screening and would have been a quantitative photometric method if we have a relevant graph profile of α-naphthol photometric readings against its measured surface tension for the same surface-active agent being tested. This method may be considered a development of the method of "solubilization of crystalline anthracene" (Willumsen and Karlson., 1997) but the anthracene method has a great drawback that Solubilization below CMC is negligible and it starts after CMC depending on micelles formation (**Tehrani-Bagha and Holmberg, 2013**). On contrast for colloidal systems, their emulsion stability is increased till CMC is formed then a decrease in stability occurs. For the application of the colloidal system stabilization using a surfactant , we have used an oily phase of inert photometric solvent (butanol) that have embedded photometric colored substance (α- naphthol) with λ_{max} at 340nm and the aqueous phase was water with gradient concentrations of SDS above and below the CMC.

α-naphthol showed photometric absorbance in butanol solution with concentration of (5g/l) to be 1.933 which will give us an image of the absorbance intensity and we will use it as a background for the following designed colloidal system. Using the colloidal system mentioned above, the unique pattern profile of emulsion stability is what we exactly observed, the absorbance spectrum of "(α-naphthol) in the aqueous phase was an indication of emulsion stability that was correlating to "the gradient concentrations of SDS" in trial one and two which was considered a primary and secondary confirmatory practical application of the theoretical pattern profile of emulsion stability (**Hussien et al., 2019,Zhang et al., 2021and Pool and Bolhuis, 2010**). The reading of the absorbance at λ_{max} showed that the concentration of α-naphthol is being increased logarithmically, till approximately it faces the CMC then a slight decrease is noticed in a plateau shape that confirm the critical micelle concentration point with maximum surface tension reduction.

Based on this primary and secondary confirmation we tried to optimize this method by regulating its parameters to verify it for practical approved application in surface tension detection. Best vortex time; which

optimize the test is 2 minutes, more vortex didn't result in better values. Volume to volume ratio; different ratios of both the aqueous phase and oily phase have been used and we found that the system 0.2/3 ml (α-naphthol oily phase/ aqueous surfactant containing solution) was the best to be used as for oily phase less than 0.2 ml reults in no reservoir for the emulsion formed and so the result will not be accurate while more than 0.2 ml give low readings which may be attributed to "the increased volume results in coalescene of oily phase at the upper, while 3 ml is the best because less volume is corresponded with disturbance and incorrect readings and more volume reults in dissolving of all the oily phase and absence of the reservoir. Best α-naphthol concentration was found to be 5 g/100 ml as it is the most diluted and hence the most sensitive to surface active property. Best Time gap was found to be 48 hr as less time is accompanied by no stabilization and more time is invain.

However, this method is considered a breakthrough as a theoretical principle and a proved practical application during the primary and secondary confirmation. But, unfortunately during the triple confirmation this method failed which lead us to a null hypothesis instead of verification of this method. We rely this fail on the great drawback that the oil phase reservoir is in the upper phase so, in order to withdraw the required aqueous phase that contains the hydrophobic substance we need to indulge through the upper phase making destorion of it. So, for a reliable, trustworthy method we need to overcome this drawback first that distort our result.

5. Conclusion:

This method is a trial of developing an indirect surface tension detection/measuring test by utilizing the emulsion stability/formation. A feature which is more accurate, precise than solubilization of anthracene method as it utilizes the emulsion formation/stability feature not solubilization feature which they differ totally in their run profile. However, the null hypothesis we offer in this paper is based on drawbacks of the procedure we faced that we need to overcome either by modulation of steps or the apparatus that we deliver our experiment on. But, it stills a promising method of detection/measuring of surface active property if we fixed this drawback of the indulge of upper oil phase.

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