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Novel application of *Nerium* leaf and Image J software in drop collapse assay for rapid screening of biosurfactant producing microorganisms

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Biosurfactants are attractive molecules with varied applicationsmainly oil degradation, emulsification, bioremediation, therapeutics and conjugation of nanoparticles. The existing screening methods for biosurfactants are inappropriate and too tedious. Here, we have explored a novel approach with drop collapse assay wherein we replaced the microtiter well plate with the naturally hydrophobic *Nerium* (*Nerium oleander* L.) leaf. The stability of beaded drops on the leaf indicates negative phenomenon, and spreading of drop indicates positive phenomenon for surfactant property, as confirmed by the measuring drop diameter using Image J software. Fifty five bacterial cultures isolated from oil contaminated site were screened through this novel approach which revealed that the isolates DNM49 (6.75±0.29 mm), DNM50 (7.45±0.19 mm) and DNM51 (6.14±0.82 mm) were the best in terms of surface tension reduction, although thirty other isolates were also found to be positive. A gradation of activity in terms of surface tension reduction was also established based on drop diameter. The results demonstrated promising application of *Nerium* leaf with Image J software in drop collapse assay as an eco-friendly and cost-effective and technically authenticated alternative to the existing assays.

Keywords: Cutin layer, Contact angle, Drop diameter, Critical micellar concentration

Surfactants are chemical surface-active agents with hydrophilic head and hydrophobic tail. They form micelles, reduce surface and interfacial tensions, increase miscibility and bioavailability of water-insoluble materials. They are classified as cationic, anionic and non-ionic based on their charge[1-3]. Microbial surfactants are the surface-active agents produced during their growth. They are preferred over chemical surfactants because they are less toxic, highly biodegradable and stable at extreme pH, temperature and salt concentration[4,5]. They can be produced from various sources with inexpensive, simple and inexpensive procedures and raw materials. Biosurfactants have a multitude of applications in different fields such as cosmetic, laundry, textile, therapeutics and bioremediation[6-9]. Since there are significantly fewer producers with high productivity, it leads to increased production cost and lower yield. Hence, there is a need to search for more potent biosurfactants producing microbes. Screening of microorganisms for production of biosurfactants is in great demand because of unique properties and varied applications of biosurfactants[10].

Biosurfactant molecules are structurally diverse, such as glycolipids, lipopeptides, lipopolysaccharides or phospholipids. *Pseudomonas*, *Bacillus*, *Rhodococcus and Candida* are the most common organisms known to produce different types of biosurfactants[11-13]. Therefore, several methods v*iz*., Lipase assay, Hemolytic assay, Emulsification index, CTAB assay, Drop collapse assay, Oil displacement method, Cell surface hydrophobicity and Surface tension reduction are are in practice for screening of various biosurfactants producing microorganisms[14]. However, all these methods have one or other limitations, and thus are unreliable. Therefore, a combination of three to four different methods is followed for effective screening of biosurfactants producing microorganisms[15-20]. Twigg *et al*.[21] emphasized on the utilization of multiple screening assays for confirmation of surface-active compounds as none of the prevailing assays gives complete information about its quantification and structural properties.

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The drop collapse assay though considered as a quick and easy primary protocol to screen biosurfactant producing microorganisms[22], it generates microtiter plate waste, and also consumes more time for equilibration. The automated systems for rapid and high throughput screening of biosurfactant producers incur high costs[23-25]. Lotus leaf was used by some researchers in drop collapse assay as eco-friendly and hydrophobic material. Ghasemi *et al.*[18] used Image J software for measuring the contact angle of drop (cell-free broth) for characterizing biosurfactant.

Here, we report a cost-effective modified drop collapse assay with an innovative approach by employing the common *Nerium* (*Nerium oleander* L.) leaf and Image J software, for screening of biosurfactants more rapidly.

Materials and Methods

Screening of biosurfactants producing microorganisms

All the prominent bacterial cultures isolated from oil contaminated sites were screened for biosurfactants production as per the standard protocol prescribed by Carillo *et al.*[26] with incubation temperature of 37°C and period for three days. The centrifuged culture broth was filtered through Millipore filter (0.45 µm), and the filtrate was used for surface tension measurement, drop collapse assay, oil displacement method and emulsification index. Cultures were directly used for hemolytic, lipase and CTAB assay, mentioned in brief as under.

Lipase assay

Test isolates are spot inoculated on tributyrin agar and incubated at 37°C for 48-72 h[27]. The positive isolates are identified by a zone of hydrolysis.

Hemolytic assay

Spot inoculation is made on blood agar and incubated at 37°C for 48-72 h[28]. The isolates showing a zone of hemolysis are considered as positive for biosurfactants production.

Emulsification index

The emulsification index (E_{24}) is evaluated by a modified method of Cooper & Goldenberg[29]. Olive oil, engine oil, hexane and toluene were employed to assess emulsification index.

CTAB assay

CTAB assay is performed as described by Siegmund & Wagner[30]. Spot inoculation is done on CTAB agar plates. Dark blue halo on CTAB plate is indicative of anionic biosurfactant producing isolates.

Drop collapse assay

About 2 µL of mineral oil is equilibrated in a microtiter well plate for 1.0 h at room temperature (37°C) and 5 µL of cell free broth was added[31]**.** Drop appearance is observed after 1.0 min and the absence of biosurfactants is noticed when the drop of cell free broth remains beaded. Presence of biosurfactant is indicated when the drop becomes flat. Positive and negative controls are sodium dodecyl sulphate and uninoculated broth, respectively.

Surface tension measurement

Surface tension of cell free broth was measured (mean value of three measures) using stalagmometer by drop count as per the method described by Chakraborty *et al.*[32]

Oil displacement assay

This assay is performed in 12 well tissue culture plate instead of Petri plate. Controls employed here are similar to those used in drop collapse assay[33].

Application of *Nerium* leaf and Image J software

In this study, we used modified drop collapse assay, with *Nerium* leaf as the hydrophobic surface, instead of microtiter well plate. Further, Image J software[34] was employed to measure drop diameter on *Nerium* leaf in place of dissecting microscope with a micrometer.Leaves of *Nerium* plant were collected from the garden of the Department of Botany, Gulbarga University, Kalaburagi, India. It was authenticated and identified as *Nerium oleander* L. (Fig. 1A and B), and the specimen was deposited in Herbarium (No. HGUK-211) at the department. The mature thick, leathery and dark green coloured leaves were chosen for the study. The length, width and thickness of the leaf were 150-200 mm, 20-30 mm and 1.0 mm, respectively. The leaves collected were washed, wiped gently with tissue paper and fixed on a plane surface. Similarly Lotus leaves were also chosen for comparison of two natural hydrophobic

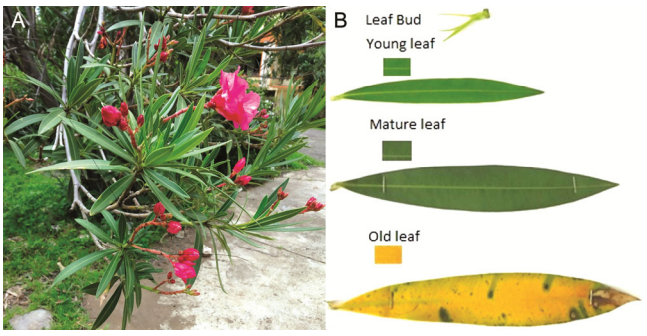

Fig. 1 — *Nerium oleander* L. (A) Plant; and (B) Leaf at different developmental stages

surfaces.10 µL of cell free broth was placed on *Nerium* and Lotus leaf at a distance of approximately 1.0 cm. A digital camera was used to capture image for measurement of drop diameter. All the measurements were set to cm or mm as a scale, rather than pixel which is common in Image J software. Diameter of each drop was measured using the short key Ctr + M.

To assess the viability of the *Nerium* based drop collapse assay, drop collapse of representative anionic, cationic and non-ionic detergents in their critical micelle concentration (CMC) range as well as beyond the range were determined. Sodium dodecyl sulphate (SDS), Cetyl trimethylammonium bromide (CTAB) and Triton X-100 were used as standard chemical surfactants, whereas distilled water and sterile nutrient broth served as negative controls. A correlation between drop size, concentration of the surfactant and surface tension was established after measuring the surface tension of the above surfactants using a Stalagmometer[35].

Statistical assessment and graphical representation of data for drop diameter was calculated using IBM SPSS statistics 25 and Microsoft excel 2007. All the assays were performed in triplicates and results were represented as mean± standard error (SE). Further, statistical correlation of surface tension and drop size were evaluated using Pearson's correlation coefficient ($p = 0.01$, two tailed).

Results and Discussion

Screening of Biosurfactants producing microorganisms

Table 1 illustrates the evaluation of seven different methods for screening of surfactant producing bacteria. Among the examined 55 bacterial isolates

Table 1 — Evaluation of screening methods for the production of biosurfactants by bacterial isolates

Isolates	Hemolytic assay[a]	CTAB assay[b]	Drop collapse assay[c]	Oil spreading assay[d]	Lipase assay[e]	Emulsification index[f]				Surface tension (ST) (mNm^{-1})[g]
						Olive oil	Engine oil	Hexane	Toluene	
DNM1	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM2	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM3	++	nil	+	+	+++	48	nil	nil	nil	59.06±0.02
DNM4	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM5	nil	++	+	+	nil	50	20	10	10	58.33±0.08
DNM6	++	++	++	++	nil	51	nil	nil	nil	59.93±0.01
DNM7	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM8	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM9	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM10	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM11	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM12	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM13	nil	+	+	+	nil	nil	35	nil	nil	60.78±0.006
DNM14	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM15	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM16	+++	nil	++	++	nil	45	15	20	17	56.40±0.004
DNM17	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM18	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM19	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM20	nil	+	+	+	nil	nil	nil	nil	nil	57.23±0.01
DNM21	nil	+	+	+	nil	nil	nil	nil	nil	60.00±0.02
DNM22	++	nil	++	++	nil	47	51	nil	nil	54.20±0.01
DNM23	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM24	nil	+	+	+	nil					54.06±0.004
DNM25	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM26	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM27	nil	+	+	+	++	nil	nil	nil	nil	59.98±0.02
DNM28	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM29	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM30	nil	+++	+	+	+++	30	nil	nil	nil	54.06±0.005
DNM31	nil	+++	+	+	nil	25	nil	nil	nil	54.10±0.003
DNM32	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM33	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM34	++	nil	+	+	nil	nil	40	nil	nil	58.10±0.01
DNM35	++	nil	+	+	nil	nil	42	nil	nil	60.20±0.01

(*contd.*)

Table 1 — Evaluation of screening methods for the production of biosurfactants by bacterial isolates (*contd.*)

Isolates	Hemolytic assay[a]	CTAB assay[b]	Drop collapse assay[c]	Oil spreading assay[d]	Lipase assay[e]	Emulsification index[f]				Surface tension (ST) (mNm^{-1})[g]
						Olive oil	Engine oil	Hexane	Toluene	
DNM36	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM37	++	++	+	+	nil		35	nil	nil	54.60±0.02
DNM38	nil	+	+	+	nil	nil	30	nil	nil	60.30 ±0.03
DNM39	nil	+	+	+	nil	nil	20	nil	nil	60.09±0.02
DNM40	++	+	++	++	nil	nil	41	nil	nil	60.98±0.01
DNM41	++	+	++	++	nil	nil	38	nil	nil	53.00±0.003
DNM42	nil	+	+	+	nil	nil	nil	nil	nil	59.81±0.01
DNM43	nil	+	+	+	++	nil	nil	nil	nil	59.93±0.02
DNM44	++	nil	+	+	nil	nil	nil	nil	nil	60.23±0.03
DNM45	++	++	++	++	nil	nil	nil	nil	nil	54.50±0.006
DNM46	++	++	++	++	nil	nil	nil	nil	nil	59.03±0.001
DNM47	++	+	+	+	nil	nil	nil	nil	nil	60.13±0.03
DNM48	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM49	++++	++++	++++	++++	+++	56	52	37	16	28.40±0.001
DNM50	++++	++++	++++	++++	++	50	51	33	5	23.23±0.002
DNM51	++++	++++	++++	++++	++	40	50	36	6	29.93±0.005
DNM52	nil	+	+	+	++	nil	nil	nil	nil	60.26±0.01
DNM53	++	++	+	+	++	nil	42	nil	nil	61.18±0.01
DNM54	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
DNM55	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
P.a.2297	+++	++	+	+	nil	38	50	nil	nil	38.53±0.01
B.s.2423	+++	++	+	+	+	30	30	nil	nil	49.93±0.01
P. otitidis	+++	++	+	+	++	20	30	nil	nil	58.00±0.01
D/w	-	-	nil	nil	-	nil	nil	nil	nil	71.20±0.02
Uninoculated broth	-	-	nil	nil	-	nil	nil	nil	nil	62.50±0.01
8 mM SDS	-	-	+++	+++	-	70.26	57.16	52.57	63.78	40.05±0.03

[[a] nil, no hemolysis; +, incomplete hemolysis; ++,complete hemolysis with a diameter of lysis <1cm; +++, complete hemolysis with a diameter of lysis between 1 and 3cm.; ++++, complete hemolysis with diameter more than 3 cm. [bcd] nil, Negative; +, Positive activity; ++, Moderate activity; +++, Good activity; ++++ Very good activity. [e] nil, negative; +, incomplete zone of hydrolysis; ++, complete hydrolysis with a diameter of lysis <1 cm; +++, complete hydrolysis with a diameter of lysis between 1 and 3 cm. [f] Data are mean of three separate experiments. [f,g] Data are mean of three separate experiments with standard error; nil, no reduction in surface tension]

(DNM1-55), 25 were completely negative for all the seven methods assessed, indicating no production of biosurfactants. However, three methods, namely oil displacement, drop collapse, and surface tension reduction, successfully exhibit positivity for biosurfactants production by more isolates (30). Further, other four methods namely CTAB, emulsification index, hemolytic and lipase were able to exhibit positivity for 24 (-6), 19 (-11), 17 (-13) and 11 (-19) bacterial isolates, respectively, for production of biosurfactants, among 30 isolates. Figure values mentioned within parenthesis indicates the number of negative isolates. DNM 49, 50 and 51 were selected as the best isolates, as they were not only positive for all seven methods, but also have shown highest activity. They were identified as *Pseudomonas* spp. by 16s rDNA sequencing (Gene bank accession Id: MK351590,MK351591, MK351592) and used for future studies (not reported here).

Screening results indicate that out of all the methods performed, drop collapse and oil displacement were more reliable, followed by surface tension measurement. This is because all the biosurfactants producing microorganisms showed positive results for oil displacement, drop collapse and surface tension reduction whereas gave negative results, with either of all other screening tests performed. But measuring surface tension is tedious task for screening multiple samples at a time. Thus, drop collapse and oil displacement can be used as primary screening methods and other methods can be used for secondary screening. These observations were in accordance with the work reported by Youssef *et al.*[22]. The recommended order for screening of biosurfactant producing microorganisms are measurement of surface tension, oil spreading or drop collapse assay followed by emulsification index. According to Plaza *et al.*[36] drop collapse assay cannot detect biosurfactant at significantly low concentration compared to oil displacement method.

On the contrary, Anuraj *et al.*[37] reported that drop collapse assay can detect significantly small amount

of surfactant. As mentioned in previous literature, it is understood that the remaining four methods are not reliable as hemolytic activity can be shown by other compounds as well, and not specific to any one biosurfactants[22]. CTAB assay can detect only anionic biosurfactants but not all types of biosurfactants[30]. All positive isolates do not necessarily show lipid hydrolysis. All the biosurfactants are not good emulsifiers, and hence emulsification index is also not a good criterion[38,39]. Drop collapse and oil displacement give equally good results, but measuring displaced oil is quite difficult and inaccurate. Thus, to make screening rapid, drop collapse is a better option as many samples can be screened at a time. Also, the collapse of drop indicates surface tension reduction[4] which is peculiar feature of any biosurfactant, thus it can be used as rapid screening method for biosurfactants producing microorganisms.

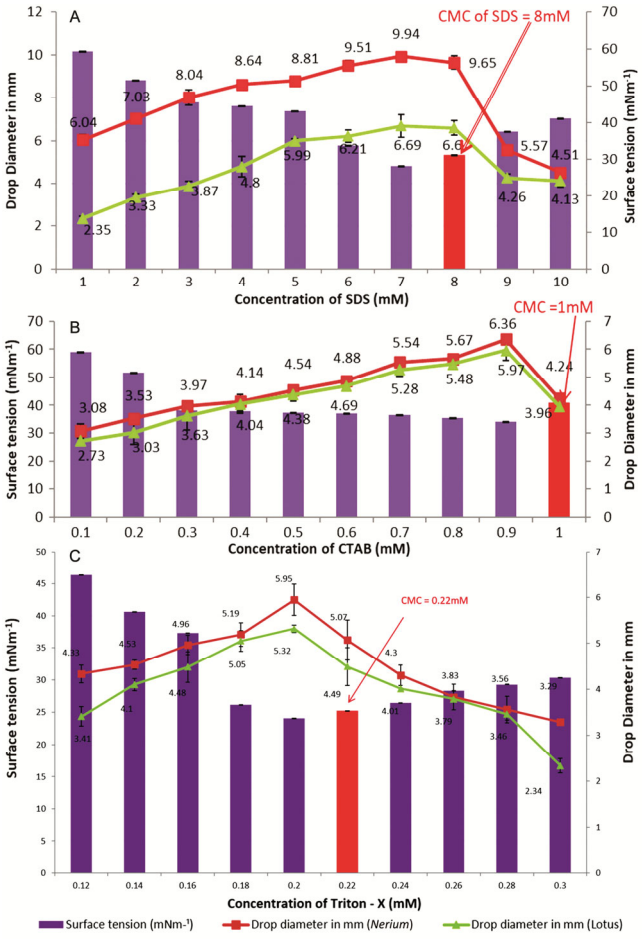

Fig. 2 — Correlation of concentration, surface tension and drop diameter of (A) anionic surfactant SDS; (B) cationic surfactant CTAB; and (C) non-ionic surfactants Triton X on *Nerium* leaf

Application of *Nerium* leaf and Image J software

Surface tension and drop collapsing ability of SDS, CTAB and Triton X

Fig. 2 (A-C), represents drop collapse assay and correlation of concentration, surface tension and drop diameter of anionic surfactant SDS, cationic surfactant CTAB and non-ionic surfactant Triton X on *Nerium* leaf in drop collapse assay. Surfactant concentration is inversely proportional to surface tension till it reaches critical micellar concentration (CMC). CMC's of SDS, CTAB and Triton X on *Nerium* leaves were found to be 8 mM, 1.0 and 0.22 mM, respectively, which is in good agreement with the report of Samsonoff[40]. A positive correlation was observed between the surface tension reduction and increase in drop size. At 8 mM of SDS, there was no reduction in surface tension, and hence the drop size was decreased which indicates that drop size is inversely proportional to surface tension as shown in Fig. 2A. Similar findings were observed with CTAB and Triton X with CMC of 1.0 and 0.22 mM, respectively (Fig. 2 B and C).

Surface tension and drop collapsing ability of bacterial Isolates

The correlation of surface tension and drop diameter of bacterial isolates on *Nerium* and lotus leaf in the modified drop collapse assay are depicted in Fig. 3. The bacterial isolates DNM 49, 50 and 51 have reduced the surface tension to 28.40±0.001, 23.23±0.002 and 29.93±0.005, respectively. Also, the drop size was 6.75±0.29, 7.45±0.19, 6.14±0.82 on *Nerium* and 5.38±0.33, 5.85±0.40 and 5.17±0.14 on lotus leaf, respectively. There were significant virtual differences between drop diameters on *Nerium* and lotus leaves, which depict that the interpretation is more easy and convenient on *Nerium* leaf than that of lotus for drop collapse assay (Fig. 4).

The results of statistical correlation between drop diameter on *Nerium* leaf and surface tensions was calculated using SPSS, version 25. There was a strong negative correlation between drop diameter and surface tension (Pearsons correlation coefficient; r_s = -

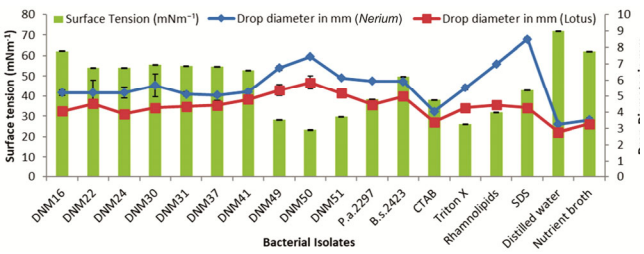

Fig. 3 — Correlation of surface tension and drop diameter of bacterial isolates on *Nerium* leaf (Drop collapse assay)

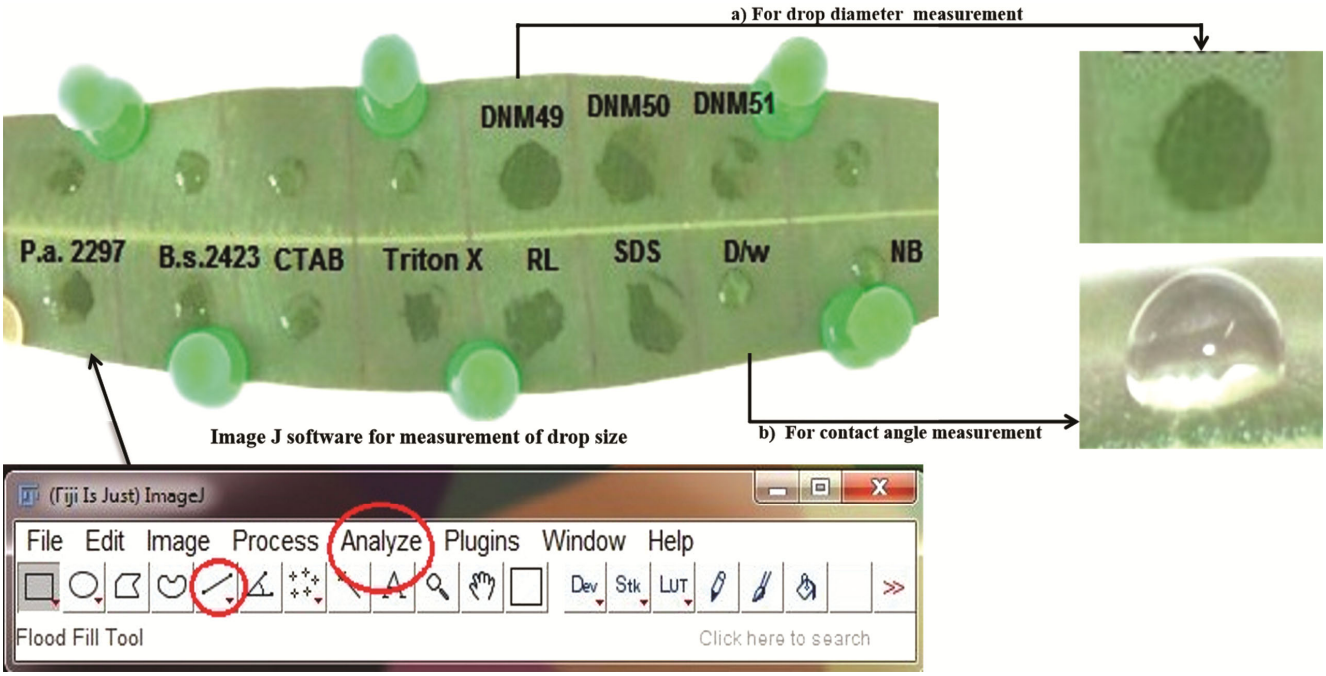

Fig. 4 — Novel application of *Nerium leaf* and Image J software for the determination of (A) drop diameter; and (B) contact angle

0.825**). ** - Correlation is significant at 0.01 levels.

To the best of our knowledge, there are no reports available on the use of *Nerium* leaf in drop collapse assay. Lotus leaf was utilized for drop collapse assay previously as it is superhydrophobic with epicuticular wax crystals showing contact angle of more than 160°[41] confirming its hydrophobicity. However, the problem associated with lotus leaf is seasonal availability, hydrophytic nature and thick venation system which hinder its use as hydrophobic surface in a typical laboratory condition. Also, its storage under water leads to removal of waxy material as observed while performing the drop collapse assay on lotus leaf. In contrary to this, *Nerium* is widespread in tropical and subtropical areas of the world. It is cultivated worldwide as an ornamental plant, naturalize very easily and it is sub spontaneous in many areas[42,43]. Branislava *et al.*[44] reported that *Nerium* leaves show large number of epidermal hairs, thick cuticle and sunken stomata that indicate their xenomorphic character. The leaves lack epicuticular wax and are naturally hydrophobic[45] which is also confirmed by measuring its contact angle which is around 110°. Cutin is one of the major components of plant cuticle a waxy polymer made up of esters of fatty acids[46]. The leaves contain small amount of latex. This surface chemistry contributes to the hydrophobicity of *Nerium* leaf and makes its use in drop collapse assay more sensitive. Often mature leaves exhibit a stable hydrophobicity and provide a larger surface area when compared to younger leaves. However, older leaves may become wettable[47]. The size of the leaf varies according to climatic conditions, thus proper selection of mature leaf should be done with more emphasis on its thickness. Since lotus is superhydrophobic, drop collapse in terms of drop diameter was lesser (approx. >1.0 mm) as compared to the hydrophobic surface of *Nerium*. As the drop diameter was large and clearly visible in *Nerium* leaf, it offers a better substitute to microtiter well plate for rapid screening of biosurfactants producing microorganisms.

Image J software is an image processing program based on JAVA (programming language) developed at the National Institute of Health (NIH) and Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin by Wayne Rasband in 1997[34]. Image J can calculate area and pixel value statistics of user defined selection, measure distance and angles, etc. Ghasemi *et al.*[18] used Image J software for measuring contact angle of drop (cell free broth) for characterizing biosurfactant but no reports are available where this software is used for screening of biosurfactants producing microorganisms by measuring drop diameter. Gel documentation software (e.g., Pro logger) was used previously for measurement of drop diameter in drop collapse assay which seems to be complicated as

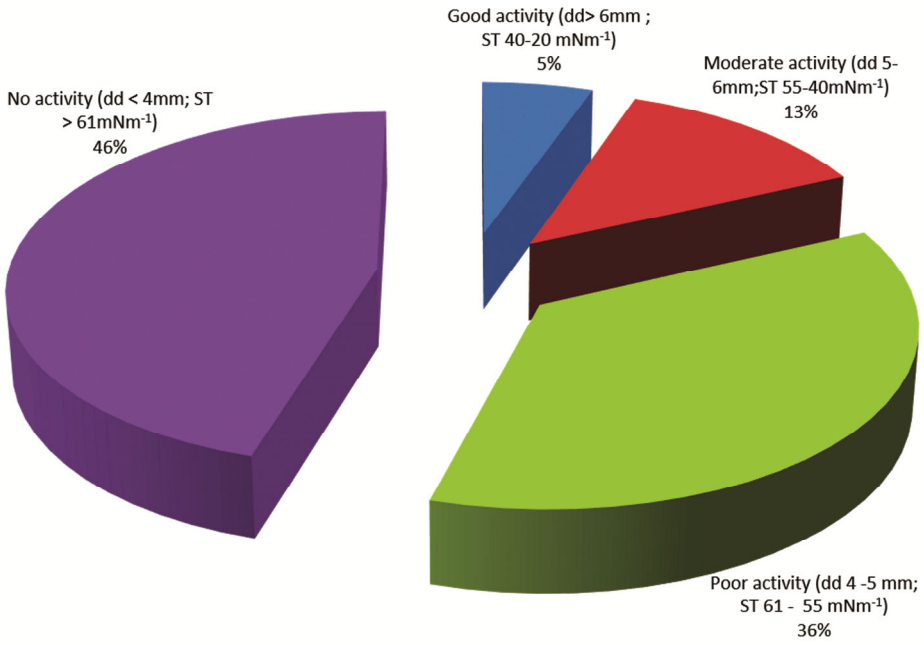

Fig. 5 — Gradation of activity of biosurfactant based on drop collapse assay on *Nerium* leaf

compared to image J software. Therefore, the novel combination of *Nerium* leaf and Image J software offers an eco-friendly and user-friendly approach to screen large number of samples at a time. This study does not determine the concentration of biosurfactants at the screening stages rather determines its activity in terms of surface tension which is inversely proportional to its concentration. This is because the variation in chemical properties of different biosurfactants affects its drop size. This limitation can be overcome in later stages after screening by using standard curve of the known biosurfactants to make this assay quantitative.

Based on the statistical comparison, it can be suggested that the drop collapse method is reliable for screening purpose and hence a graded range of drop diameter is established as Good (>6 mm), Moderate (5-6 mm), Poor (4–5 mm) and No activity (<4 mm) for screening of isolates (Fig. 5).

Conclusion

The modified drop collapse assay on hydrophobic surface of *Nerium* leaf shows substantial increase in drop diameter which is inversely proportional to the surface tension. A virtual observation of the collapse of drops, which occurs in few seconds make the assay rapid for detection of surfactant producing microorganisms. The measurement of the drop diameter using Image J software validates the drop collapse assay. The modified drop collapse assay can be more significant in search of other biosurfactants by overcoming the most of the limitations associated with other prevailing screening methods. The results indicate that *Nerium* leaf can be a natural and better alternative to microtiter plates for drop collapse assay. This is in terms of rapidity, sensitivity, at ease and economically viable. A large number of samples can be screened quickly using this novel combination of hydrophobic *Nerium* leaf and Image J software for measuring drop diameter. A gradation of biosurfactant activity based on drop size was also proposed in the present study. Due to its multiple advantages, the *Nerium* leaf is anticipated to serve as an eco-friendly tool for rapid detection of surfactant producing microorganisms.

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Conflict of interest

Authors declare no competing interests.

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