DROP COLLAPSE ASSAY ON LOTUS LEAF (Nelumbo nucifera): A SIMPLE AND COST EFFECTIVE METHOD FOR RAPID DETECTION OF BIOSURFACTANTS

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ABSTRACT

An alternative technique to perform drop collapse assay for rapid detection of surface active agents produced by microorganisms is described. The method is rapid, simple, economical and sensitive as it can detect the biosurfactant even in low concentrations. The limit of detection for Triton-X 100:0.01 mM (6.25 µg mL⁻¹), CTAB: 0.1 mM (36.44 µg mL⁻¹) and SDS: 0.001 mM (0.288 µg mL⁻¹) using lotus leaf assay. This method uses Lotus (Nelumbo nucifera) leaf as the surface for performing drop collapse studies and can be useful as an initial step for screening microorganisms for biosurfactant production and for detection of surfactant activity. The underline principle was that the leaves of lotus are super hydrophobic, i.e. drops of water roll off free of residue. The lotus leaf has surface roughness and posses water-repellent wax crystals which attribute towards super hydrophobic properties. Hence, if surface active agent capable of reducing surface and interfacial tension acting as wetting agent is produced by the Microbispora sp. V2 even in low concentrations, the drop collapse will occur on lotus leaf and wetting of leaf can occur. Lotus leaf can be a better alternative to microtitre plates as due to its rapidity, sensitivity, simplicity, ease, cost effectiveness and reproducibility. The simplicity of this technique makes it suitable in rapid screening of large number of surfactant producing microbes without the need of expensive high throughput systems.

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1 Introduction

Biosurfactants or bioemulsifiers have tremendous applications in petrochemical, cosmetic and pharmaceutical industries making them one of the favorite candidates for commercialization and environment cleanup (Shoeb et al., 2013). Biosurfactants of microbial origin exhibit versatility in structure and function with variation in genus and ecological origin. Biosurfactant producing microorganisms are collected from natural environments using various established methods such as drop collapse assay, microplate assay, penetration assay, oil spread method, solubilization of crystalline anthracene, etc. (Van Hamme & Ward, 2001; Youssef et al., 2004; Walter et al., 2010; Viramontes-Ramos et al., 2010). In present study drop collapse assay was modified with an intention to make it rapid and cost effective to overcome some limitations in the existing assay (Jain et al., 1991).

The original drop collapse method makes use of microtiter plate lid where 2.0 μL of mineral oil is equilibrated for 1 hr at room temperature, to which 5.0 μL of cell free broth is added. The collapse of the drop on the surface of oil is inspected after 1 min. The diameter of drop should be at least 0.5 mm larger than that of negative control to be scored positive (Jain et al., 1991; Bodour & Miller-Maier, 1998). As such the assay has been extensively used for the screening of biosurfactant producers, but it does not give quantitative data on the extent of drop collapse, cannot measure minute concentrations of the surfactant and also generates microtiter plate waste, hence is not ecofriendly or economical, and is not rapid as the equilibration time itself is 1 h. Although automated systems have emerged for the rapid and high throughput screening of biosurfactant producers, but incur high costs (Maczek et al., 2007). Keeping in view the limitations of the existing method and the cost economics, the paper describes a simple, rapid and economical method where lotus leaf is used as base for screening instead of microtiter plates.

2 Materials and Methods

2.1 Drop collapse assay on lotus leaf

The 20 μL cell free broth (or suspension to be screened for biosurfactant activity) was placed on a clean lotus leaf (N. nucifera) and size of the drop was measured using Pro Logger software 3.8.6. The lotus leaf serves as base instead of microtiter plates and several samples can be simultaneously screened on one leaf. Here, mineral oil is not used unlike in drop collapse assay, where it is used to provide the required hydrophobicity to the base. The lotus leaf is naturally hydrophobic therefore can be directly used for the assay, which will save mineral oil and time of equilibration. The surface roughness and water-repellent wax crystals confer hydrophobic properties to the leaf (Ensikat et al., 2011) and a surface active agent would reduce the surface and interfacial tension leading to the collapse of the drop. This method also overcomes the inconvenience of drop size measurement on microtitre plate. Gel documentation software (e.g. Pro Logger) were used to measure the size of drop. Here, the drops on lotus leaf can be easily imaged and their diameter easily measured by the imaging software. Hence, wetness can be recorded as drop collapse even if the surfactant is in low concentrations. On lotus leaf, the drop size of liquids having surfactant activity is greater than those lacking the activity. This increase in drop size is due to the collapse of drop which in turn is due to the wetting ability of the surfactant. The diameter of drop should be at least 0.5 mm larger than that of negative control to be scored positive (Nasr et al., 2009). The use of lotus leaf for the drop collapse assay is shown in Figure 1.

Figure 1 Drop collapse assay on Lotus leaf (Nelumbo nucifera) here Collapse of 20 μL of drop of cell free supernatant of various fermented media is compared with respective sterile media compared with water as negative control and 10 mM each of SDS, CTAB and Triton X-100 as positive control. I- water. II- ISP2 medium a- sterile control . b- cell free supernatant. III- ISP3 medium a- sterile control. b- cell free supernatant. IV- ISP4 medium a- sterile control. b- cell free supernatant. V- Madhuca latifolia L flower extract medium a- sterile control . b- cell free supernatant. VI- Madhuca latifolia L flower extract medium + anthracene a- sterile control . b- cell free supernatant, VII- partially purified biosurfactant (6 μg 20μL-1), VIII- 10 mM SDS, IX- 10 mM Triton X-100 and X- 10 mM CTAB

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2.2 Viability and efficiency of the lotus based drop collapse assay

In order to assess the viability of the lotus based drop collapse assay, drop collapse of representative anionic, cationic and non-ionic detergents in their critical micelle concentration (CMC) range as well as outside the range were determined in present study. Sodium dodecyl sulphate (SDS), Cetyl trimethylammonium bromide (CTAB) and Triton X-100 were used as standard chemical surfactants, whereas distilled water served as negative control. 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 Tensiometer (Dataphysics DCAT 11) (Pornsunthornrattawee et al., 2008). The surface activity of the produced biosurfactant is expressed as the percentage reduction of surface tension by the following equation and table 1 represented the concentration of surfactant, values of drop size and surface tension of the standard chemical surfactants.

\[
\text{% surface tension reduction} = \left( \frac{\gamma_m - \gamma_c}{\gamma_m} \right) \times 100
\]

where, \(\gamma_m\) is the surface tension of the sterile uninoculated medium and \(\gamma_c\) is the surface tension of the centrifuged cell free broth.

In order to assess the efficiency and sensitivity of the lotus based drop collapse assay towards biosurfactants, screening of purified biosurfactant based drop collapse assay towards biosurfactants, screening of

Table 1 Concentration of surfactant, values of drop size and surface tension of the standard chemical surfactants.

| Sample                          | Wetting ability (drop size in mm) on Lotus leaf | Surface tension reduction (%) |
|--------------------------------|-----------------------------------------------|-----------------------------|
|                                 | Wetting ability (drop size in mm)              |                             |
|                                 | X co-ordinate | Y co-ordinate |                             |
| Sterile distilled water (negative control) | 3.88 ± 0.01 | 2.50 ± 0.01 | 0.00                         |
| 10 mM SDS (positive control) | 10.85 ± 0.02 | 8.78 ± 0.02 | 56.49                       |
| 10 mM TritonX-100 (positive control) | 9.33 ± 0.02 | 7.94 ± 0.02 | 56.28                       |
| 10 mM CTAB (positive control) | 8.29 ± 0.03 | 6.94 ± 0.03 | 66.53                       |
| Partially purified biosurfactant | 8.63 ± 0.01 | 8.28 ± 0.01 | 45.11                       |

Wetting ability of 20 µL of water (negative control), 10 mM each of SDS, TritonX-100 and CTAB (positive control) and partially purified biosurfactant

Table 2 Drop collapse assay on Lotus leaf (N. nucifera).

| Fermentation Medium | Wetting ability (drop size in mm)* | Surface tension reduction (%) |
|---------------------|-----------------------------------|-----------------------------|
|                     | Uninoculated broth | Cell free supernatant |                             |
|                     | X co-ordinate | Y co-ordinate | X co-ordinate | Y co-ordinate |                             |
| ISP2                | 7.24 ± 0.02 | 4.50 ± 0.02 | 7.60 ± 0.02 | 6.22 ± 0.02 | 0.00                         |
| ISP3                | 4.83 ± 0.02 | 4.14 ± 0.02 | 5.19 ± 0.02 | 4.49 ± 0.02 | 0.00                         |
| ISP4                | 7.26 ± 0.03 | 5.88 ± 0.03 | 7.94 ± 0.03 | 7.62 ± 0.03 | 0.00                         |
| M. latifolia flower extract medium | 6.57 ± 0.02 | 4.83 ± 0.02 | 8.63 ± 0.02 | 6.56 ± 0.02 | 25.00                       |
| M. latifolia flower extract medium + anthracene | 6.21 ± 0.01 | 5.87 ± 0.01 | 8.63 ± 0.01 | 6.91 ± 0.01 | 35.00                       |

Wetting ability of 20 µL of cell free supernatant of fermented media is compared with respective uninoculated media. The experiment was done in triplicate with standard error mentioned above.
3 Results and Discussion

3.1 Drop collapse assay

The drop collapse occurred with the cell free supernatants of fermented *M. latifolia* flower extract medium with anthracene. However, the drop collapse effect with other media was either very less or not seen (Table 2). This indicated that *Microbispora* sp. V2 could produce surface active agent (wetting agent) with *M. latifolia* flower extract medium with anthracene.

3.2 Viability and efficiency of the lotus based drop collapse assay

About 35% reduction in surface tension was reported with fermented *M. latifolia* flower extract medium with anthracene when measured by tensiometer which validated the results obtained by drop collapse on lotus leaf ascertaining its authenticity. The surface tension reduction and drop collapse assay on Lotus leaf (*N. nucifera*) with various concentrations of Triton-X 100 (Table 3); CTAB (Table 4) and SDS is shown in Table 5. The assay of biosurfactant produced by *Microbispora* sp. V2 extracted from *M. latifolia* flower extract medium with anthracene (20 µg mL⁻¹) by drop collapse method on Lotus leaf (*N. nucifera*) is represented in Table 6.

There is a report on drop collapse test for screening surfactant-producing microorganisms (Jain et al., 1991). Study has also been done on rhamnolipid biosurfactant production by *Pseudomonas aeruginosa* due to their potential applications in a wide variety of industries and the high levels of their production (Toribio et al., 2010). Biosurfactant production by endophytic *Nocardiopsis* sp. *mrinalini* 9 isolated from *Hibiscus rosasinensis* leaves has also been studied (Singh & Sedhuraman, 2015).
Table 4. Surface tension reduction and Drop collapse assay on Lotus leaf of various concentrations of CTAB.

| CTAB (mM) | Surface tension reduction (%) | Drop size (mm) | X-co-ordinate | Y-co-ordinate |
|-----------|-------------------------------|----------------|---------------|---------------|
| 0.001     | 51.72                         | 4.17           | 3.52          |
| 0.01      | 53.71                         | 4.80           | 3.89          |
| 0.1       | 54.28                         | 5.80           | 5.08          |
| 0.2       | 56.27                         | 5.45           | 5.10          |
| 0.3       | 57.83                         | 5.45           | 5.12          |
| 0.4       | 57.86                         | 5.77           | 5.12          |
| 0.5       | 58.24                         | 6.08           | 5.21          |
| 0.6       | 58.32                         | 6.08           | 5.44          |
| 0.7       | 58.49                         | 6.11           | 5.44          |
| 0.8       | 58.54                         | 6.40           | 5.44          |
| 0.90      | 59.39                         | 6.40           | 5.45          |
| 0.91      | 59.56                         | 6.40           | 5.76          |
| 0.92      | 59.66                         | 6.43           | 5.76          |
| 0.93      | 59.71                         | 6.43           | 5.76          |
| 0.94      | 59.73                         | 6.47           | 5.76          |
| 0.95      | 60.30                         | 6.59           | 5.77          |
| 0.96      | 60.33                         | 6.72           | 5.77          |
| 0.97      | 60.37                         | 6.73           | 5.90          |
| 0.98      | 60.55                         | 6.79           | 6.08          |
| 0.99      | 60.80                         | 6.91           | 6.08          |
| 1.0       | 60.82                         | 6.99           | 6.08          |
| 2         | 60.87                         | 7.10           | 6.09          |
| 3         | 61.0                          | 7.10           | 6.11          |
| 4         | 61.44                         | 7.19           | 6.39          |
| 5         | 62.65                         | 7.30           | 6.40          |
| 6         | 62.71                         | 7.49           | 6.40          |
| 7         | 62.76                         | 7.60           | 6.41          |
| 8         | 62.82                         | 7.60           | 6.43          |
| 9         | 63.85                         | 8.32           | 6.53          |
| 10        | 66.53                         | 8.40           | 7.42          |
| Water     | -                             | 3.88           | 2.50          |

Table 5. Surface tension reduction and Drop collapse assay on Lotus leaf of various concentrations of SDS.

| SDS (mM) | Surface tension reduction (%) | Drop size (mm) | X-co-ordinate | Y-co-ordinate |
|----------|-------------------------------|----------------|---------------|---------------|
| 0.0001   | 47.01                         | 5.48           | 4.60          |
| 0.001    | 47.12                         | 5.72           | 5.16          |
| 0.01     | 47.35                         | 6.00           | 5.20          |
| 0.1      | 47.57                         | 6.10           | 5.26          |
| 0.5      | 47.67                         | 6.20           | 5.30          |
| 1        | 47.95                         | 6.32           | 5.40          |
| 2        | 48.14                         | 6.36           | 5.50          |
| 3        | 50.26                         | 6.38           | 5.56          |
| 4        | 52.87                         | 6.46           | 5.58          |
| 5        | 52.9                          | 6.74           | 5.62          |
| 6        | 53.09                         | 6.74           | 5.63          |
| 7        | 54.0                          | 6.75           | 6.00          |
| 7.5      | 54.77                         | 6.75           | 6.00          |
### Table 6 Assay of biosurfactant produced by *Microbispora* sp. V2 extracted from *M. latifolia* flower extract medium with anthracene (20 µg mL⁻¹) by drop collapse method on Lotus leaf.

| Test | Drop (mm) | X- co-ordinate | Y- co-ordinate |
|------|-----------|----------------|----------------|
|      | Left side of lotus leaf | Right side of lotus leaf | Left side of lotus leaf | Right side of lotus leaf |
| I    | 8.672     | 8.630          | 8.250          | 8.285          |
| II   | 6.240     | 6.260          | 4.940          | 4.947          |
| III  | 7.250     | 7.285          | 4.940          | 5.000          |
| IV   | 7.025     | 7.028          | 6.260          | 6.240          |
| V    | 5.950     | 5.985          | 6.600          | 6.660          |
| VI   | 5.727     | 5.760          | 6.240          | 6.245          |
| VII  | 4.427     | 4.420          | 4.450          | 4.460          |
| VIII | 3.880     | 3.900          | 2.560          | 2.560          |

I: Biosurfactant produced by *Microbispora* sp. V2 in *M. latifolia* flower extract with 20 µg mL⁻¹ anthracene; II: Triton X-100(0.09 mM); III: Triton X-100 (1.1 mM); IV: SDS (7 mM); V: SDS (11 mM); VI: CTAB (0.9 mM); VII: 1.2 mM CTAB; VIII: distilled water.

### Conclusion

The results indicate lotus leaf can be a better alternative to microtitre plates because of its rapidity (no time lag), sensitivity (detection of surfactant in low concentrations), simplicity, ease, cost effectiveness and reproducibility. The simplicity of this technique makes it suitable in rapid screening of large number of surfactant producing microbes without the need of expensive high throughput systems. Cost economics, equilibration time, microtiter plate lids emergence, measurement of the drop diameter and quantitation, sensitivity.

### Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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