Novel Mutagenesis Protocol for Isolation of Mutants Capable of Producing Enhanced Biosurfactant from No-cost Industrial Waste

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Abstract:

Low yields biosurfactant are major factor for its popularity. Therefore, strain improvement by extensive application of mutation and selection through exposure of organism to mutagen for high yield is an appropriate measure. In the present study, mutation screening technique was developed as an important tool for screening of biosurfactant hyper-producing mutants. UV- irradiation was selected as a mutagen, the dose of UV irradiation was standardized and the process of mutant screening technique was developed using curd whey as a principal media component replacing the costly synthetic medium. *Pseudomonas aeruginosa* strain PP2 was exposed at to different doses of UV irradiation for standardization of dose and irradiated cells were plated on whey amended Cetyltrimethylammonium bromide *(*CTAB*)* + Methylene blue for the characterization of mutant with enhanced biosurfactant productivities. This screening technique is applicable for both standardization of mutagen dose and isolation of biosurfactant hyper- producing mutants on the basis of zone size of the halos that developed around the colonydue to the complexation ofanionic rhamnolipid with cationic Methylene blue and CTAB. The yields of biosurfactant produced by mutant strains were improved in the range of 22-60% as compared to wild strain.

Keywords:
Biosurfactant; Curd Whey; (CTAB); *Pseudomonas aeruginosa*; Rhamnolipid.

Introduction:

Biosurfactants are promising substitutes for synthetic surfactants due to their low toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extremes of temperature, pH, and salinity an the ability to be synthesized from renewable resources (Velikonja and Kosaric 1993). Successful use of biosurfactants have been demonstrated in facilitating the degradation of organic pollutants in soil, dispersion of oil from oil spills, enhanced oil recovery, cleaning oil spills, oil emulsification and in breaking industrially derived oil in water and water in oil emulsions (Lin *et al.* 1990) . However, potential of biosurfactants is dependent on their cost and properties in relation to synthetic compounds.In recent years, the development of rapid and simple screening methods for biosurfactant-producing microorganisms has had a remarkable impact on biochemical and genetic information related to the degradation of hydrophobic compounds. Genes responsible for the production of biosurfactants have also been isolated, characterized, and cloned in heterogeneous hosts (Siegmund and Wagner 1991). They have also resulted in a several fold increase in the

yield of biosurfactants and a change in the raw-material requirement for production (Pinzon and Ju 2009). It is therefore, conceivable that super active microbial strains can be constructed through strain improvement to produce potent biosurfactants in quantity with a variety of wastewaters as substrate.

Thus, enhancing biosurfactant yield is very important. The methods used to increase metabolite production consist of strain selection, mutagenesis, manipulation of environmental and nutritional factors, and genetic manipulation. The present studies comprised of enhancing biosurfactant production through UV radiation mutagenesis of *Pseudomonas aeruginosa* strain PP2. The process of high yielding mutant screening technique was developed which emphasized on the use of whey as a principal media component replacing the costly synthetic medium reported earlier(Siegmundand Wagner 1991, Pinzon andJu 2009).

Material and methods:

Bacteria and Culture conditions: *Pseudomonas aeruginosa* that used as a parent strain was obtained from the soil sample contaminated with distillery spent wash. The identification of the strain was confirmed by Microbial Type culture collection and Gene bank (MTCC), Chandigarh, India.

Collection and processing of whey waste for preparation of CTAB-Methylene blue containing whey waste agar: Fresh curd whey waste (WW) was collected from local dairy and was processed to remove casein before use by earlier described method (Dubey and Juwarkar, 2001).

Preparation of CTAB-methylene blue containing whey waste agar for mutant screening: One liter of processed whey waste was amended with 0.005 g methylene blue, 0.2g cetyltrimethyl-ammonium bromide (CTAB) and 15 gm agar. Constituents were mixed and heated to dissolve the contents and autoclaved at 121°C for 15 minutes and poured into sterile petriplates.

UV-Mutagenesis:

Processed curd whey (100 ml) was sterilized at 121°C for 20 minutes and was inoculated with *Pseudomonas aeruginosa* strain- PP2. After the incubation period of 48 h, cells were separated by centrifugation at 8000 rpm. Supernatant which was obtained after centrifugation was further incubated for biosurfactant production to recover biosurfactant. Pellet which was obtained was washed two times with sterile water to remove toxic metabolites and was dispersed in sterile curd whey adjusted to pH 7. 2. The optical density of the culture suspension was adjusted to 1.0 by UV-VIS spectrophotometer. Cells of *Pseudomonas aeruginosa* strain- PP2 were exposed to UV- irradiation and exposure time period was increased from 10, 20, up to 150 sec. UV irradiated cell were plated on whey agar medium amended with CTAB + Methylene blue after serial dilution. Plates of whey agar mended with CTAB + Methylene blue were incubated for 72 hours and observed for sea green

coloured halos around the colony. Percentage survival of cells were recorded and at the same time the zone size of halos around the mutants of *Pseudomonas aeruginosa* strain- PP2 were measured for biosurfactant production capacities.

Screening of hyper-productive mutants: Relationship between concentration of biosurfactant produced by *Pseudomonas aeruginosa* Strain - PP2 and the zone size of halos developed on whey agar medium amended with CTAB + Methylene blue was determined. Single colonies obtained after mutagenesis were compared for the zone size of halos that developed due to the complexation of anionic rhamnolipid with cationic Methylene blue and CTAB after incubation as compared to wild strain.

Result and discussion:

UV-mutagenesis and mutant screening technique for enhanced production of biosurfactant:

UV irradiation was selected as a mutagen and the dose of UV irradiation was standardized to be 120 seconds with 98%killing**.**UV- irradiation was selected as a mutagen, the dose of UV irradiation was standardized and the process of mutant screening technique was developed using curd whey as a principal media component replacing the costly synthetic medium. *Pseudomonas aeruginosa* strain PP2 was exposed at to different doses of UV irradiation for standardization of dose and irradiated cells were plated on whey amended Cetyltrimethylammonium bromide *(*CTAB*)* + Methylene blue for the characterization of mutant with enhanced biosurfactant productivities. Biosurfactant hyper- producing mutants were screened on the basis of zone size of the halos that developed around the colonydue to the complexation of anionic rhamnolipidwith cationic Methylene blue and CTAB. Biosurfactant production of the parent as well as mutant strains was calculated by measuring area of clear zone formed by different concentration of biosurfactant were measured. When area of clear zone was plotted against the different biosurfactant concentration a direct relationship was obtained which can be used as a measure of biosurfactant quantity. Colour of halos developed around the colony were not blue as reported by Seigmund and Wagner (1991) however, being of sea green colour indicated complexation between anionic rhamnolipid and cationic Methylene blue and CTAB.

Evaluation of mutants of *Pseudomonas aeruginosa*strain- PP2 for biosurfactant production capacities:

Resultpresented inFigure 1 shows the direct relationship between the zone size and the biosurfactant concentration. As the concentration of biosurfactant increases the zone size of halos also increases. Strain improvement of the *Pseudomonas aeruginosa*was carried out and was

compared with the wild strain. Figure 2 shows that the mutant 5 (M5) resulted in the highest zone size and also gives the highest biosurfactant yield as compared to wild strain and other mutants. As the time period increases the ability to produce the biosurfactant by mutants also increases. Ability to produce the higher amount of biosurfactant by mutant strain of *Pseudomonasaeruginosa* was increase by 60% as compare to wild strain (Figure 3).

The CTAB agar plate method is a semi-quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner, 1991. The microbes of interest are cultivated on a light blue mineral salts agar plate containing the cationic surfactant cetyl- trimethylammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyl-trimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos. To strengthen the visual effect of this method, small wells can be melted into the agar surface with the heated point of a glass stick or pipette. The cultures are placed and incubated in the wells. Even hydrophobic substrates like plant oils can be included in this test. Therefore, the oil droplets are stabilized with Gum Arabicum. Oil, agar and 1 g/L Gum Arabicum are mixed separately with ultrasound in a small volume of water. The homogenous mixture is added to the medium before sterilization.

The CTAB agar assay is a comfortable screening method, but it is specific for anionic biosurfactants. It has been applied in several screenings. Different culture conditions can be applied directly on the agar plates, e.g., different substrates or temperature. Furthermore, it could be transferred to liquid culture conditions. Interest in biosurfactants has led to the development of a multitude of methods for the screening of biosurfactant producing strains. As every method has its advantages and disadvantages, a combination of different methods is appropriate for a successful screening. Mutant screening technique developed and presented herewith will be simultaneously helpful for both standardization of mutagen dose and isolation of biosurfactant hyper-producing mutants on the basis of the size of the halo developed around the colony.Exposure of organism to mutagen gives high yield. Therefore, mutation technique was adopted as an important method for over production of biosurfactants in the present study.

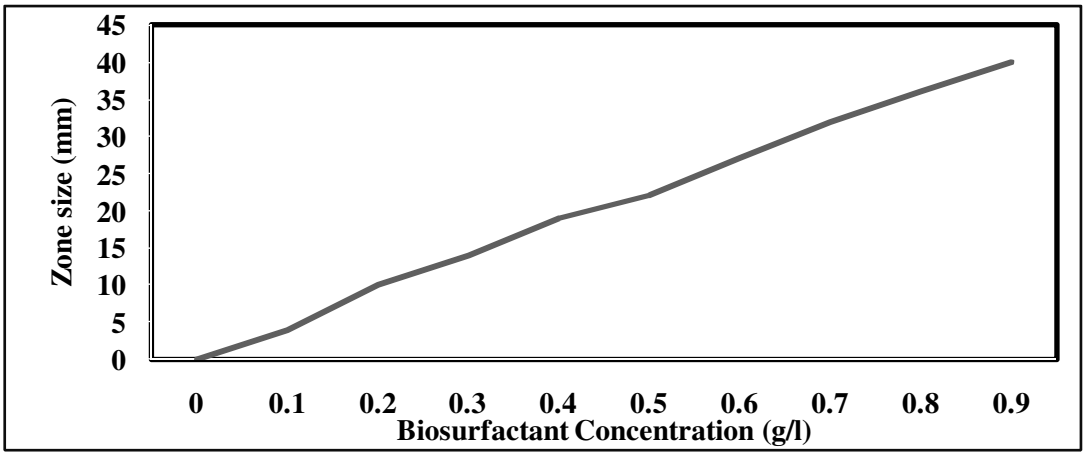

Figure1: Relationship between concentration of biosurfactant produced by *Pseudomonas aeruginosa* Strain –PP2 and zone size of halos of complexation developed on whey agar medium amended with CTAB + Methylene blue.

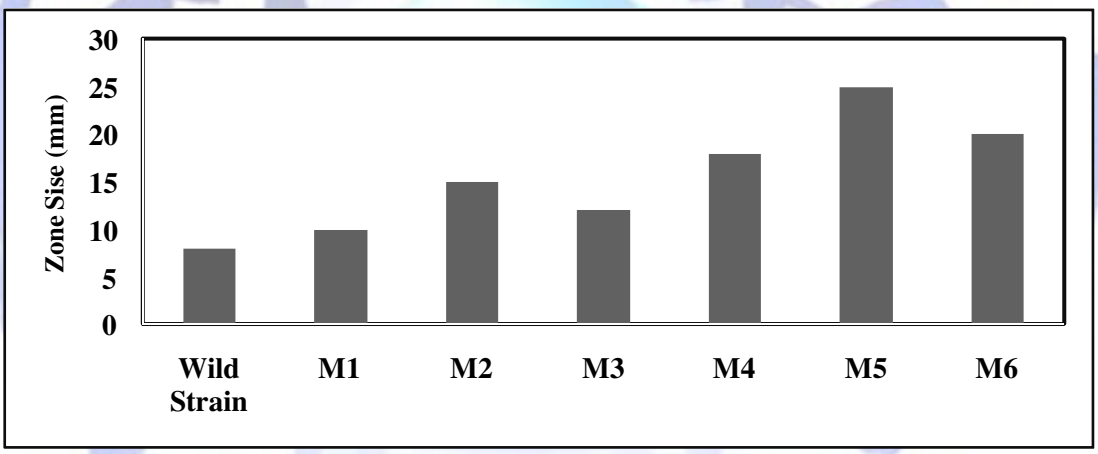

Figure 2: Relationships between the zone size of halos produced by wild and mutants strains (M1-M6) of *Pseudomonas aeruginosa*strain PP2on whey agar medium amended with CTAB + Methylene blue

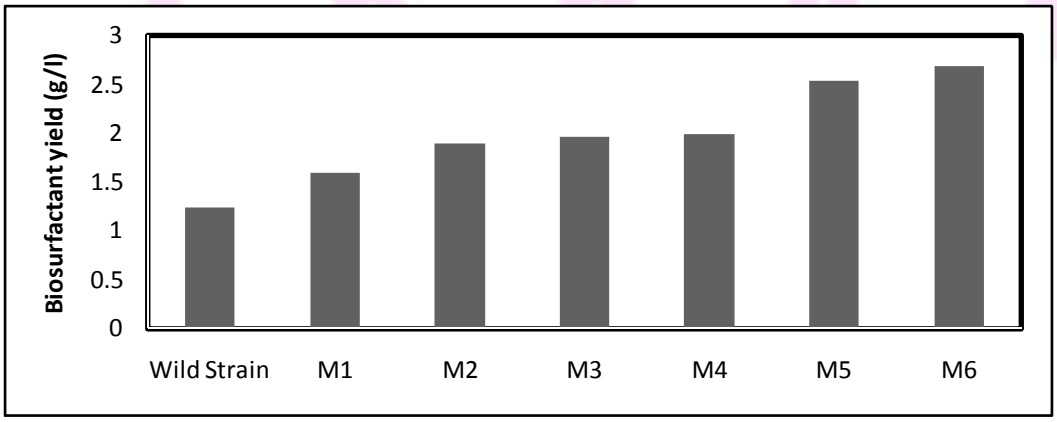

Figure 3: Biosurfactant production capabilities of wild and mutants strains (M1-M6) of *Pseudomonas aeruginosa*strain PP2

Conclusion:

Present study has shown that the process of mutation screening technique developed emphasized on the use of whey as a principal media component replacing the costly synthetic medium. Exposure of organism to UV-mutagen resulted in high yields. Mutant screening technique developed is applicable for both standardization of mutagen dose and isolation of biosurfactant hyper-producing mutants on the basis of zone size of the halos that developed around the colonydue to the complexation ofanionic rhamnolipid with cationic Methylene blue and CTAB. The yields of biosurfactant produced by mutant strains were improved by 22-60% as compared to wild strain. Utility of this technique can also be explored for screening of other types of anionic biosurfactant producing mutants.

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