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Biosynthesis of silver nanoparticles and their evaluation of antifungal activity against *Magnaporthe oryzae*

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Abstract
Rice (*Oryza sativa* L) is the most important cereal grain and food stuff which forms an important part of the diet of more than three billion people around the world and popularly called as "Global grain". Blast of rice caused by *Pyricularia oryzae Cav*. (*Magnaporthe grisea* Sacc.) is one of the most destructive disease and it accounts for 30 to 100% yield losses in all rice growing areas of the country. The blast of rice causes huge losses of quality and quantity of harvest. However the fungus became resistant to chemical fungicides and these causes a global pollution so an alternate method is needed to control the blast disease so we go for biologically synthesized silver nanoparticles to control the disease. Isolation of *M.oryzae* from the infected necrotic patches of diseased leaves in rice plant. After a week to prepare a spore suspension in 3 ml of water. And then prepare a seeded media of PDA using the *M.oryzae* and carry out of the Agar well method at a different concentration by using synthesized silver nanoparticles by *B.vallismortis* BVM4, *L.macroides* LBM7 and Tricyclozole (a chemical to control blast disease) at different concentrations. By this the maximum zone of inhibition was recorded at 3.0µg/ml (30mm) of *B.vallismortis* BVM4. The minimum zone of inhibition was observed of 0.1µg/ml (20mm) of tricyclozole

Keywords: *Pyricularia oryzae, B. vallismortis* BVM4, *L. macroides* LBM7, antifungal activity

Introduction
Rice is the second most important staple cereal crop in the world. Among the rice growing countries in the world, India has the second largest area and ranks second in production next to China on global basis, it is planted on an area of 163 million hectares with a production of728.7 million tonnes (Rahman *et al.*, 2016) [21]. In India, rice was cultivated in an area of 30.68million hectares with a production of 25.03 million tonnes and productivity of 816 kg/ha during the first five year plan (1951- 52 to 1955 - 56). The area under rice cultivation in India was 43.5 million hectares with a production of 103 million tonnes during 2015-2016 (USDA Grain Report, 2016). In Tamil Nadu, rice is cultivated in an area of 1.75 million hectares with a production of 4.74 million tonnes and productivity of 2702 kg/ha during the plan period (2002-2003to 2006 - 2007) (Anon, 2009) [3].

In recent years, resistance to commercially available fungicides by phytopathogenic fungi has been increasing and has become a serious problem (Brent and Hollomon, 1995; Brent *et al*., 1998; Dekker and Georgopoulos, 1982; Goffeau, 2008) [5, 6, 8, 10], So, the search for new fungicides and alternatives is of paramount importance to combat newly emerging resistant strains of fungal pathogens (Kanhed *et al*., 2014) [15]. One solution would be nanotechnology which enhances antimicrobial activity of materials by converting them to nanoparticles. The improved antimicrobial activity of nanoparticles compared to their salts is due to their unique properties i.e. large surface area to volume ratio (Kanhed *et al*., 2014) [15]. Nanoparticles (NPs), which are 100 to 10,000 times smaller than human cells, offer unprecedented interactions with bimolecular on both the surface and inside of the cells. AgNPs have been used for numerous physical, biological, and pharmaceutical applications because their small size and similarity to cellular components enables them to enter living cells using cellular endocytosis mechanisms, especially pinocytosis. Interestingly, AgNPs have been reported to exhibit antibacterial (Kora *et al.*, 2015) [16] antimicrobial (Lemire *et al.*, 2013) [17] anti-inflammatory and anti-oxidant activities. Recent studies revealed that silver nanoparticles also possess the anti-fungal properties.

(Bahrami-Teimoori *et al.*, 2017) [4] stated that the *Amarathus retroflexus* may be a green tool for synthesizing the AgNPs with efficient anti-fungal activity, particularly against fungi: *Macrophomina phaseolina* and *Fusarium oxysporum*. The area of the biosurfactant mediated process of nanoparticle synthesis is emerging as part of green chemistry and they act

as a potent stabilizer in the silver nanoaprticles synthesis. Although the chemical and physical processes are potentially useful in the synthesis of nanoparticles, the size-controlled synthesis is still remaining as a challenge in material science (Farias *et al*., 2014) [9]. Many techniques have been devoted to synthesizing nanosize silver particles, such as chemical reduction, photochemical reduction, reverse micelle based and lamellar liquid crystals approaches, aerosol techniques and an electrostatic spraying technique. Since reverse micelles system was applied to form metal nanoparticles (Huang *et al*., 2010) [13]. In the current study an attempt is made to isolate efficient bacterial isolates from various ecosystems of Cuddalore district of Tamilnadu and evaluate their suitability to produce biosurfactant. The biosurfactant, thus obtained are studied for the synthesis and stabilisation of silver nanoparticles in water-in-oil microemulsion and further studied the feasibilities of silver nanoparticles against rice blast disease.

Materials and Methods

Isolation of biosurfactant producing bacteria - Glass plate assay (Jain *et al.*, 1991) [14]

Rapid screening test for biosurfactant production is tested based on Glass plate assay described by Jain *et al*. (1991) [14]. A grease free glass plate was taken cm) wiped with alcohol to give a uniform coating of coconut oil using a absorbent cotton wool and air dried. Small drop of culture filtrate was placed on the glass plate using a sterile 2 ml syringe. If the glass plate was large enough, 15 - 20 drops of culture filtrate could be placed. A drop of distilled water served as a blank. The drops were observed carefully. Culture filtrates of the isolates which were capable of producing biosurfactant, would collapse and spread on the glass plate. The larger the collapse area, greater is the activity of biosurfactant.

Production of silver nanoparicles from biosurfactant (Xie *et al.*, 2006) [26]

The synthesis of silver nanoparticles *in situ* in the water-in-oil microemulsion was performed by the addition of synthesized two reverse micelles in the presence $NaBH_4$ as reducing agent. The synthesis involves mixing up to 0.5 ml of 0.05 Mol/I aqueous $AgNO_3$ solution, 3.0 g biosurfactant, 1.5 g n-butanol and 0.5 g n-heptane together and stirred vigorously at room temperature until homogeneous reverse micelles formed. Next, reverse micelles were also synthesized using 0.5 ml of 0.1 mol/l aqueous $NaBH_4$ solution instead of aqueous $AgNO_3$ solution. Then, the two types of reverse micelles were mixed and stirred for 60 min at 10,000 x g. Further, the reverse micelles were broken by adding ethanol (0.5 ml ethanol for 1 ml reverse micelles). When it was broken, a particle tends to precipitate from the solution. The precipitated silver particles were isolated by centrifugation at 15,000 x g. This procedure results in the production of silver nano sized particles. The particles were then sonicated in10 ml n-heptane solution and stored for further studies.

Isolation of pathogen (*Magnaporthe oryzae*)

The necrotic patches of diseased leaves were cut into small pieces. These pieces were surface sterilized by dipping in mercuric chloride solution (1:1000) for one minute and were washed by sterilized water for several times. The cut pieces were inoculated in sterilized Petri dish containing potato dextrose agar medium amended with streptomycin sulphate under aseptic condition and kept in BOD incubator at 25±10ºC for development of fungal growth. The fungus cultures were also maintained in culture tube to avoid contamination.

Agar Well method

Spore suspension of the fungus prepared with sterile distilled water from 7 days old culture. Desired concentrations of silver nanoparticles such as 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 µg/ml were prepared. Twenty ml of PDA medium was seeded with three ml of spore suspension (1×10^6 spores/ml) and allowed to solidify. Wells were cut equidistantly with the aid of sterile cork borer and test extracts were pipeted out separately and poured into each well (1.0 ml). Tricyclazole 0.2% was used for comparison. Three replications were maintained for each treatment. The plates were incubated at 28±2ºC for 48 h. The inhibition zone of the fungal growth around each well was measured and recorded.

Statistical analysis

The experimental result were statistically analyzed using Completely Randomized Design (CRD), followed by Duncan's Multiple Range at (DMRT) according to Gomez and Gonez (1984) [11].

Results

Glass plate assay - A rapid method for the detection of biosurfactantproducing bacteria (Table-1)

The glass plate assay is a rapid screening method which was performed for the detection of biosurfactant production. Among the 10 soil samples, the high microbial population was observed with Panruti soil samples. The total heterotrophic population was recorded as 16.00×10^6 CFU/g of soil followed by C. Mutlur (Chidambaram) soil sample with the total heterotrophic population of 15.33×10^6 CFU/g of soil. The percentage of total biosurfactant producers was found to be 85.74 per cent for LBM followed by 45.35 per cent for BVM. Out of 25 isolates, 10 bacterial isolates were positive for the glass plate assay method and are represented in (Table 1).

Table 1: List of bacterial populations from different soil samples

Soil samples	Total heterotrophic bacterial population*+	Bacterial population after addition of crude oil*+	Biosurfactant producing bacterial isolates*+	Percentage of total biosurfactant producers
Thalanguda	10.33	06.66	0.53	15.34
Devanampattinam	13.66	10.33	4.66	45.21
Panruti	14.00	20.66	10.33	85.74
Cuddalore	10.66	05.33	2.00	20.00
Samiyarpettai	15.33	10.00	2.33	35.00
Pudhukuppam	15.33	05.33	3.33	35.80
Parangipettai	12.33	10.00	5.33	45.30
Killai	12.66	11.33	5.00	40.83
C.Mutlur	10.66	05.33	3.66	45.38
Kollidam	05.66	04.66	1.00	25.24

*Values are a mean of five replicates ± SD + Population expressed in CFU x 10^6/g of soil.

Evaluation of silver nanoparticles against *Magnaporthe oryzae* by agar well method using biosurfactant from *Lysinibacillus macroides* LMB7 and *Bacillus vallismortis* BVM4

Synthesized silver nanoparticles were tested for their antifungal activity of *Magnaporthe oryzae* at different concentration (0.1, 0.5,1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 µg/ml) by agar well method. In the BVM4 synthesized silver nanoparticles showed maximum zone of inhibition (30.50mm) at 3.0µg concentration. The minimum zone of inhibition was recorded at 0.1µg concentration (20.00 mm). (Figure -1). Silver nanoparticles were synthesized by using LBM7 were showed the maximum zone of inhibition at 3.0µg (30.50 mm) concentration and the minimum zone of inhibition was recorded at 0.1 µg (20.00 mm) (Table-2). The inhibition zone formed by the silver nanoparticles performed better at 3.0 µg/ml over the commercial fungicide Tricyclazole @ 0.2%.

Table 2: Evalution of silver nanoparticles against *M. oryzae* by agar well method

S.no	Concentration µg /ml	Inhibition zone (mm)		Tricyclazole 2%	Control
		BVM4	LBM7		
1.	0.1 µg	20.00	20.00	19.50	0.00
2.	0.5 µg	25.50	23.40	27.00	0.00
3.	1.0 µg	24.10	20.80	26.20	0.00
4.	1.5 µg	22.00	21.20	24.00	0.00
5.	2.0 µg	21.00	24.00	26.00	0.00
6.	2.5 µg	23.10	22.50	23.30	0.00
7.	3.0 µg	30.50	29.10	21.30	0.00
8.	3.5 µg	28.20	26.00	22.00	0.00
9.	4.0 µg	27.50	24.00	25.10	0.00
10.	4.5 µg	29.50	25.20	29.30	0.00
11.	5.0 µg	28.10	27.50	25.10	0.00

µg=microgram, mm=millimetre, %=percentage, BVM4= *Bacillus vallismortis*, LBM7= *Lysinibacillus macroides* Antimicrobial activity of silver nanoparticles against *Magnaporthae oryzae.*

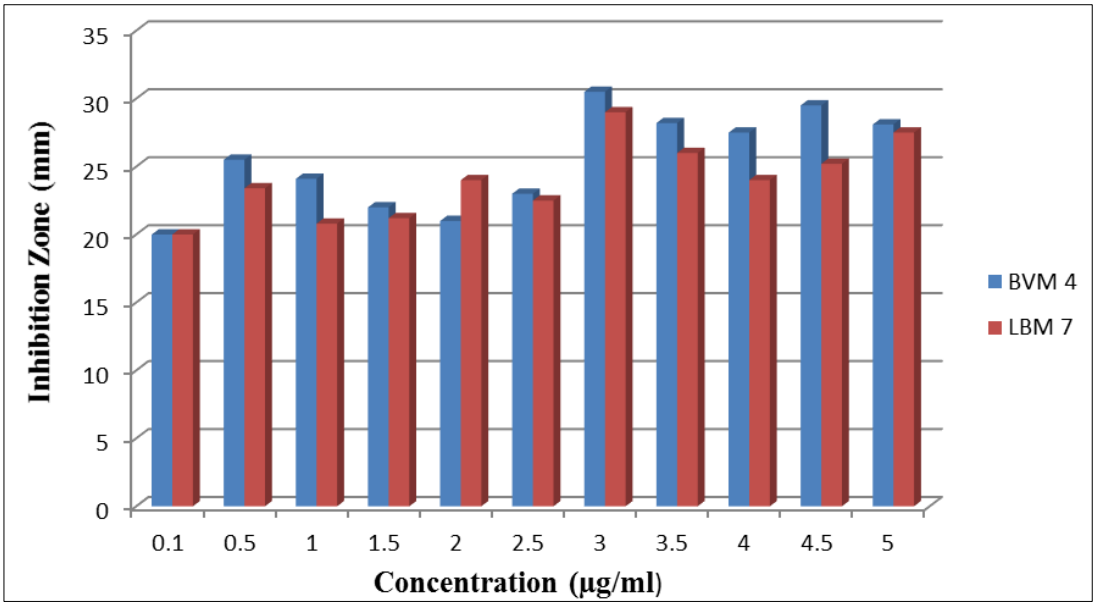

Fig 1: Evalution of Biosynthesis of silver nanoparticles against *M.oryzae* by agar well method**.**

Discussion

Rice blast disease

Blast of rice caused by *Pyricularia oryzae Cav.* (*Magnaporthe grisea* Sacc.) is one of the most destructive disease (Ou, 1985) and it accounts for 30 to 100% yield losses in all rice growing areas of the country (Padmanabhan, 1965) [19]. It has been found in over 85 countries across the world and reached the United States in 1996. Every year the amount of crops lost to rice blast could feed 60 million people. Although there are some resistant strains of rice, the disease persists wherever rice is grown. The disease has never been eradicated from a region. (Rice blast achieved 2010, Cereal knowledge Bank).

Biosurfactants

Biosurfactants are surface active molecules produced by microorganisms as metabolites. They are classified based on their chemical composition as glycolipids, lipoaminoacids, lipopeptides, polymers etc. They have numerous compared to chemically synthesized surfactants, such as low toxicity, biodegradability, possess high specificity, ease of production, ability to be synthesized from renewable substrates, high foaming, high selectivity, specific activity at extreme temperature, pH, salinity and can be reused through regeneration too as compared to synthetic surfactants.

On the other hand, they have high production costs due to low yields and fastidious purification. In the present study, an attempt was made to develop the economically attractive biosurfactant production process by using cheapest renewable substrates from agro-industrial wastes, and optimized the bio-processes for obtaining maximum productivity. An attempt was also made to synthesize silver nanoparticles in microemulsion, stabilized by low cost biosurfactant synthesized using cheapest renewable substrates. Further,

application of silver nanoparticles in the production of antimicrobial textiles was studied.

In the present study, the biosurfactant producing bacteria were isolated from the hydro carbon enriched soils collected from 5 different location of cuddalore district in Tamil Nadu. Totally 25 isolates were screened for the biosurfactant production. Among them, two promising isolates namely BVM4 and LBM7 were selected for further works. The genera of the isolated 25 were as follows: *Bacillus* (8), *Escherichia coli* (3), *Klebsiella (2), Lactobacillus* (1), *Proteus,* (3), *Pseudomonas* (6) and *Staphylococcus aureus* (2).

There are several reports on the biosurfactant producing microorganisms isolated from various eco systems in cuddalore district (Darvishi *et al*., 2011) [7].

Saimmai *et al*. (2012) [23] collected 89 sediment soil samples from mangrove environment, from the east and west coasts of southern Thailand, screened for the biosurfactant producers collected by an enrichment culture technique. They isolated 95 isolates positive for biosurfactant production according to the qualitative drop-collapsing test. The 95 isolates also showed promising biosurfactant activity by exhibiting a surface tension reduction of pure water to 20mN/m. Govindammal and Parthasarathi (2013) [12] also isolated five strains from mangrove ecosystem and selected the best biosurfactant producing organism *Pseudomonas fluorescens* MFS03 for biosurfactant production using renewable substrates.

Screening of biosurfactant producers

Satpute *et al.,* (2010) [24] reported that the single screening method was not suitable to identify all types of biosurfactants and hence recommended more than one screening methods as to identify potential biosurfactant producers. Therefore, in the present study, the selected isolates were performed with different screening test to check the biosurfactant production ability and to find the efficient biosurfactant producer by following the standard methods described by the earlier authors viz., glass-slide test (Persson and Molin, 1987) [20].

Antifungal activity of silver nanoparticles

Abbas Nasehi *et al.* (2018) [1] revealed that confirmed the beneficial effects of plant tonic in controlling rice blast disease. The antifungal efficacy of plant toxic against *M. oryzae* under *in vitro* conditions. Tapan kumar das *et al.* (2013) [25] proved that the antifungal activities of the silver nanoparticles are very significant and indicate that the synthesized silver nanoparticles may have an important advantage over conventional antifungal antibiotics. Anima nanda *et al.* (2014) [2] studied the evaluation of antimicrobial activity of biologically synthesized silver nanoparticles from filamentous fungi. These silver nanoparticles showed good antibacterial activity against various bacterial pathogens and also enhances the antibacterial activity of Amoxicillin were studied.

Conclusion

From the current study, it may be concluded that the silver nanoparticles synthesized by using biosurfactants found to be inhibiting rice blast fungus at 3.0µg/ml Concentration,

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