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Immobilization of cells using Sodium alginate for enhanced productivity of antimicrobial compound

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ABSTRACT

Immobilization of the whole cell offers high metabolic activity and Productivity. It is one of the key methods for the improvement of microbial strains producing bioactive compounds. In the present study, 18 actinomycete isolates were isolated from garden soil and screened regard with their potential against both gram-positive and negative bacteria including MRSA. Among the 18 strains, KUA06 showed good activity and they were characterized by macroscopically and microscopically. The promising isolate KUA06 were cultivated in liquid culture medium and immobilized with sodium alginate. The antibacterial compound was extracted by solvent extraction technique. Comparative studies on the total antibiotic sensitivity of the free cells and immobilized cells showed that the immobilized cells were effective against test pathogens; 25 mm against medically important organisms. Further, we concluded that the isolate immobilized KUA06 are more efficient for antibiotic synthesis against multidrug pathogens.

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INTRODUCTION

The diversity of Actinomycetes secondary metabolites is influenced and unmatched in medical significance. The rise in the number of drug-resistant pathogens and the limited successes of strategies such as combinational chemistry in providing new agents indicate an uncertain forecast for future antimicrobial therapy (Projan, 2003 and Projan and Youngman, 2002). Pharmaceutically important antimicrobial agent generated by Streptomyces can be produced by submerged fermentation in bioreactors (Vandamme, 1983) which act as biological factories for the synthesis of bioactive compounds at high concentrations (Su, 2006). Entrapment has been mainly used for the living cells and successively achieved by using natural and synthetic polymers. Entrapment of living cells which possess natural polymers like as agarose and alginate is carried out by ionotropic to increase the yield of antibiotics. Few research has demonstrated the advantages of using immobilized cells for antibiotic production. Immobilization of whole cells for extra-cellular enzyme production offers a number of advantages. They include ease of separating cell mass from the bulk liquid for possible reuse, enhancing continuous operation over a prolonged period, enhancing reactor productivity and ensuring higher efficiency of catalysis (Kar and Ray, 2008). The objective of the present study was to assess the ability of immobilized actinomycetes for the secondary metabolite production and the pathogen inhibition property.

MATERIALS AND METHODS

Sample processing and isolation of actinomycetes

1 g of soil sample was air-dried, sieved and then placed inside a small crucible. Those samples were subjected to drying and heat treatment inside an

oven at 45°C for 12 hrs. Enriched soils inside crucibles were incubated in the water bath at 27°C for 7 days before isolation (Tahtamoun *et al*., 2006). 0.1ml of diluted aliquots; 10^{-2}- 10^{-5} were spread on Petri plates containing media supplemented with Amoxicillin and cycloheximide to inhibit bacterial and fungal growth and the plates kept at room temperature for 8days. Pure culture strains of the isolates were preserved in glycerol (v/v) at 4°C.

Screening of potential antagonistic isolate

Antimicrobial activity of the isolates was tested preliminarily by using cross streak method (Chemos *et al*., 1985). Actinomycetes isolates were streaked across the diameter on Starch-Casein agar (SCA) plates. After incubation at 28°C for 24 days, the 24-hour culture of *Staphylococcus aureus*, *Streptococcus aureus,* and *Bacillus* for gram-positive organisms. *Escherichia coli, Proteus*, and *Klebsiella* for gram-negative organisms were streaked perpendicular to the Actinomycetes culture. The plates were then incubated at 30°C for 24 hours and the zone of inhibition was determined.

Characterization of potent actinomycetes isolate

The most potent Actinomycete isolate was characterized by both macroscopic, microscopic and also by morphological methods. The macroscopic method was done through colony characterization of Yeast Mannitol Agar (YMA). Colony colour and presence of pigmentation were noted. Microscopic characterization was done by coverslip culture technique and observed after 3 days. The presence or absence of aerial and substrate mycelium, spore formation, and fragmentation of the vegetative or substrate mycelium was observed.

Exposing of predominant actinomycetes to UV and EMS for mutation

This study deals with determining the survival ability of actinomycetes spores against UV irradiation and ethyl methanesulfonate (EMS) mutagenesis choosing mutants on the basis of random survivors by morphology and high antibiotic production. Actinomycetes were grown in ISP2 media and incubated for 10 days under room temperature at 37°C the strains used namely KUA 06. To generate mutants, the spores harvested were subjected to irradiation using a 254 nm UV source to achieve a 99% kill rate. The ISP2 plates were exposed to UV at different time intervals from 0-75 minutes. KUA06 strain was exposed to 45 minutes and the same strain was again next day exposed to 30 minutes. Also, this Actinomycetes strain was exposed to chemical mutation namely EMS at different concentration (Veerapagu *et al*., 2008). The mutated strain is stored for future use.

Fermentation process

For enhancement of the production of Actinomycetes isolates, the cultures were kept at 37°C for 4 days in a rotary shaker incubator at 200 rpm containing the medium with Glucose-0.1g; Potassium monohydrogen phosphate (K_2HPO_4)-0.1g; Yeast extract-0.5g; Tryptone-0.5g; Distilled water-100ml and kept for 8 days. After 8 days of incubation, the culture filtrate was prepared by centrifugation at 10,000 rpm for 20 minutes (Peterson and Ciegler, 1969).

Immobilization of cells

For enrichment of secondary metabolites; immobilization of cells was performed by using Sodium alginate using the ionotropic technique. At each fermentation cycle, sodium alginate beads were prepared and added to the production medium at the end of the process. After 8 days, sodium alginate beads were aseptically separated from the fermentation medium through filtration using a sterile Bucher funnel (Manjula *et al*., 2009). This is mainly done for wild-type strain and also for mutated type strain of isolated Actinomycetes.

Solvent extraction of secondary metabolites

The antibacterial compound was extracted from the filtrate through a solvent extraction method as described by Westley et al., (1979). Ethyl acetate and butanol were added to the filtrate in the ratio of 1:1. The mixture was shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase containing the antibiotic substance was separated from the aqueous phase, evaporated and dried in a water bath and the residue obtained weighed. The obtained compound was used to determine the antibacterial activity.

Determination of antibacterial activity of the metabolite

Antibacterial activity was tested *in-vitro* against pathogenic bacteria like *Proteus mirabilis, Staphylococcus aureus, E.coil, Streptococcus pyogens, Bacillus, Pseudomonas*. Antibacterial activity was performed by Disc-diffusion assay (Barry and Thornsberry, 1985) and effectiveness measured by zones of inhibition on culture plates.

RESULT AND DISCUSSION

Isolation of actinomycetes

Actinomycetes which were isolated from garden soil in Karpagam Academy of Higher Education, Coimbatore, showed a remarkable amount of colonies in that rhizospheric soil. A majority of the isolates tend to grow on fertile soils; an important characteristic feature of Actinomycetes (Stankebrandt *et al.,* 1981). Based on the colony morphology and colour of the colony 18 different strains of

Table 1: Showing the morphological and characteristic of Actinomycetes

Culture / morphological Characteristics	Observation
Temperature	28- 37°C
pH	7.7
Colour of colony	Grey then white
Form of colony	Compact, folded, lichenoid then leathery, raised
Pigmentation in medium	Present dark yellow
Aerial mycelium	Present
Substrate mycelium	Present
Spore formation	Present Spirals type of spore formation

Table 2: Table showing the OD value of fermented and immobilized wild-type strain and a mutated strain of KUA06 of Actinomycetes

Actinomycetes Strain	Free cells		Immobilized cells	
	OD value of wild-type (µg/ml)	OD value of mutated-type (µg/ml)	OD value of wild-type (µg/ml)	OD value of mutated strain (µg/ml)
KUA 06	0.45	0.57	1.23	1.67

actinomycetes were isolated. Microbes were isolated in mixed culture by spread plate method. Some of the actinomycetes were able to produce different pigments on natural or synthetic media (Dhanajeyan *et al*., 2010).

Screening of antagonistic actinomycetes

In the cross streak method, the zone of inhibition of 5 strains showed good activity towards Gram-positive bacteria, 3 strains showed good activity towards Gram-negative bacteria and 2 strains show remarkable activity towards both Gram-positive and negative bacteria. The remarkable activity was seen in KUA 06 (Figure 1 and Figure 2).

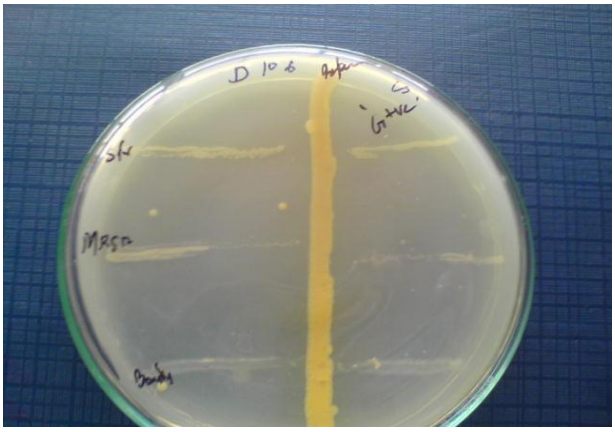

Figure 1: Antagonistic effect of KUA06 against Gram-positive bacteria

Pathogens used are *Bacillus subtilis (*MTCC 441)*, Staphylococcus aureus* (MTCC 737) and *MRSA* Pathogens used are *Proteus vulgaris*, *Escherichia coli (ATCC 25922)* and *Pseudomonas* aeroginosa (MTCC 424).

The putative isolates, when subjected to submerged culture, showed different activity from that of primary screening. Some isolates did not show the activity in the liquid medium. During the screening of the novel secondary metabolite, Actinomycete isolates are often encountered which show antibiotic activity on agar but not in liquid culture (Shantikumar Singh *et al.,* 2006).

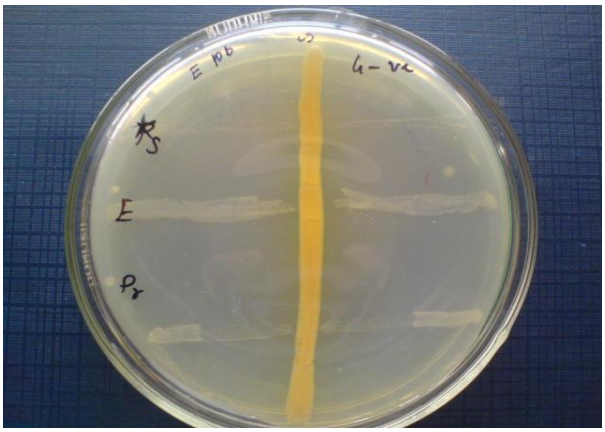

Figure 2: Antagonistic effect of KUA06 against Gram-negative bacteria

Characterization of the most potent actinomycetes isolate

Both the macroscopic and microscopic methods were used to describe KUA 06 according to Waksman (1961), such as colour and form (Table 1) was exhibited by colonies of Actinomycetes. However, the colour and growth form of the colony could not serve as bases for pointing out the genus to which Actinomycetes belong. Hence, morphological properties must include as the primary bases of characterization. A distinguishing morphological property of the isolate KUA06 is the presence of aerial mycelium, a characteristic possessed by all members of Actinomycetes Lechevalier *et al.,* (1994). As they showed good sporulation with compact, chalk-like dry colonies variation of different colony variation from yellow to white colour. The isolates were found to be Gram-positive organisms with branched mycelium in their cell morphology similar to fungal characteristics (Holt, 1989).

After growing from the broth, KUA 06 was inoculated on ISP2 medium and was seen to exhibit a

white colony. However, the subsequent transfer of the isolate showed a white colony, the form of the colony was described as compact, folded, raised and lichenoid to leathery. It was also observed that it produced dark yellow pigmentation on the medium, only a substrate mycelium can be observed from the colony. Microscopic observations showed that KUA 06 forms spores on the tips. A well-developed substrate mycelium which partly penetrates the medium and the formation of a single spore are the two characteristics that may well qualify it as a species of Actinomycetes. Waksman (1961) has also mentioned the formation of dark- yellow pigment that dissolves into the medium, to be a possible characteristic of the genus, even though not exclusively.

Mutation and fermentation processes

Mutation a key method for the improvement of microbial strains producing bioactive compounds. Mutagenesis can be induced by exposure to UV light which can result in a 10- to 1000- fold increase in metabolite production (Adrio & Demain, 2006). In an ISP_2 medium, KUA 06 was exposed to Ultraviolet radiation (UV) at different intervals. The colour change was observed. There was a change of colour in KUA 06 from yellow to pale yellow colour (Figure 3 and Figure 4). The inoculation into fermentation medium, after 4 days the centrifugation was done and the supernatant was collected. It was filtered to remove impurities.

Figure 3: Wild-type strain of KUA06

Figure 4: Mutant strain of KUA06

In this present study, the spores of Actinomycetes were irradiated with physical mutagens; UV at 254 wavelength and chemical mutagen EMS, Physical and chemical mutagens were enhancing the production of secondary metabolites. Maximum sporulation control it is 55 CFU survive and the percentage of killing is at 92%. Spores of Actinomycetes treated with UV and EMS only four colonies survived when compared to the control it is 66 and the percentage of killing is 94% (Figure 5). Morphology mutated colonies were observed and isolated (Figure 6).

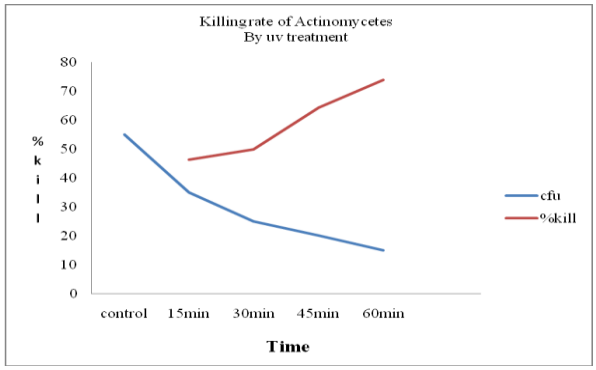

Figure 5: Showing the effect of Actinomycetes by UV treatment

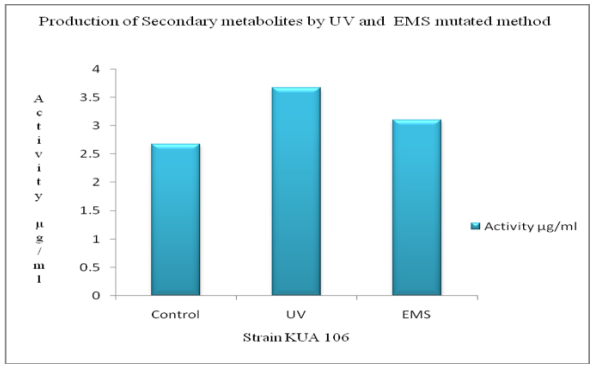

Figure 6: Production of secondary metabolites by UV and EMS mutant strain

A comparative profile of the total antibacterial sensitivity of free cells and mutant cells indicated that the immobilized cells are found to be more effective against test pathogens than free cells. Similarly, Manjula et al., (2009) reported that the promising immobilized isolates showed good activity against tested organisms. As compared to the control UV mutated strain showed an increased trend in the inhibition zone against test pathogens.

Ellaiah et al., have reported that the 156% fold increases in lipase yield from *Aspergillus niger* by UV mutagenic treatment whereas the present study, the lipase yield of UV mutant BTUV3 was 164% higher than the parent strains (BTUV12) and wild strains (BTS-24). Caob and Zhang reported an increase in lipase production of 3.25 fold by using a Pseudomonas mutant with UV and NTG. Philips reported that UV-mutated actinomycetes increase antibiotics production than non-mutated actinomycetes. He also reported that the Penicillium chrysogenum after mutation produced antibiotics 10,000 times higher than the non-mutated strains

Immobilization of cells

The strains namely KUA 06 is taken and the extract is treated with sodium alginate to form beads. After air-dried, the beads were added to the ISP_2 medium. It was then centrifuged to obtain cell-free extract and estimation of protein is performed and optical density (OD) value is noted. The best result is observed in KUA 06 mutated strain (Figure 7) the results of the OD value for KUA06 were 1.67µg/ml (Table 3).

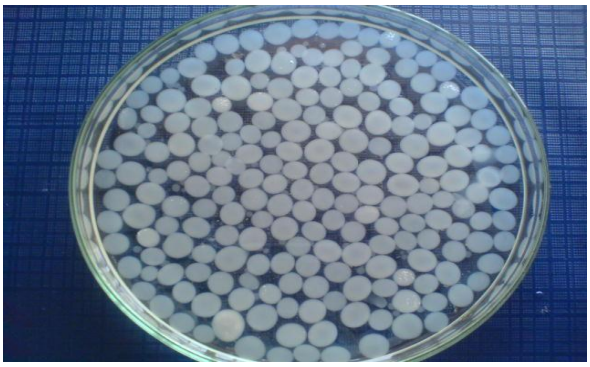

Figure 7: Immobilized Sodium alginate beads

Mahmoud *et al.* demonstrated the entrapment of *S. rimosus* in sodium alginate and used in bubble column reactors. Ogaki *et al.,* demonstrated use of immobilized cells of *S. rimosus* for the continuous production of oxytetracycline. Farid *et al.,* (2002) attempted entrapment of *S. rimosus* in calcium alginate gels and reported a good level of antibiotic production in repeated batch fermentation for 28 days. Previous studies indicated that the sodium alginate concentration for the immobilization of cells plays a prominent role in the production of antibiotics due to the porosity of the beads which limiting the nutrient supply and oxygen diffusion (Chun and Agathos, 1991). Sallam *et al.* studied the application of immobilization technique using alginate for the production of cyclosporine A by a local strain of *Aspergillus terreus.* Alginate at 2% wt/vol was found to be an optimum concentration for the formulation of spherical and stable beads with better antibiotic production (Srinivasulu *et al.,* 2005 and Ellaiah, *et al.,* 2000). The above concentration is used for our study.

Comparative data on total OD value for free cells and immobilized cells as shown in table 3. The enhancement of secondary metabolites was done by fermentation (i.e.) ISP_2 media is prepared and both wild-type and mutated strain were subjected for fermentation OD (optical density) value is taken in that KUA 06 showed an increased amount of OD value namely 1.67µg/ml when compared with other strains. It was discovered that the immobilized cells in sodium alginate were efficient for the production of antibiotics which was confirmed by further study.

Antibacterial activity of the crude extract

The solvent-treated extract of mutated KUA 06 is added to the already inoculated bacterial plates. The extract added to the disc in different concentrations. At 25µg/ml concentration, a remarkable zone of inhibition against *Proteus* was observed (Figure 8). Strain improvement is one of the main factors involved in the achievement of higher titers of industrial metabolites.

At a different concentration of KUA06 extract (25 µg) showing good activity against Proteus sp.

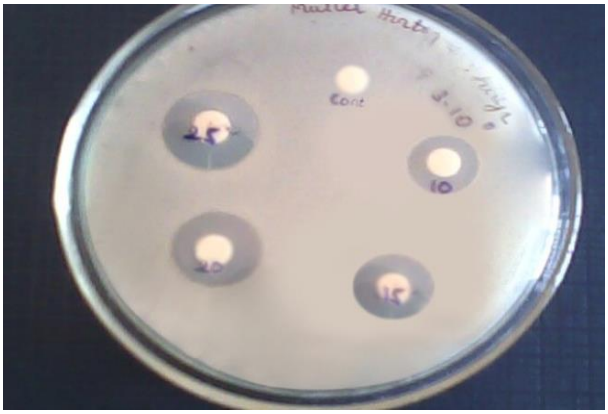

Figure 8: Antibacterial activity of KUA06 against Proteus sp.

The solvent extraction is further undergone by the antibacterial activity of KUA 06 which showed the best activity towards *Proteus* sp. The zone of inhibition against the medically important organisms was found to be 25 mm in diameter.

CONCLUSION

Due to mutation, the partial active gene of these strains that were responsible for the production of antibiotics could have been activated. Random mutagenesis and fermentation screening have been reported as effective ways to improve the productivity of industrial microbial cultures.

From our study, we conclude that the application of immobilization technique by using sodium alginate was more efficient for the production of a secondary metabolite from KUA06. The possible route of antibiotic synthesis enhancement and its biological efficacy require further investigations.

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