# Investigating the Synergistic Antibacterial Activity of Epiphytic Bacterial Polyketides and Biopolymer Alginates from Marine Microalgae

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**ABSTRACT**

The objective was framed to analyse the synergistic antibacterial activity and wound-healing ability of the developed polyketide-alginate polymers. Alginates were extracted from a brown seaweed *Padina tetrastromatica* and used as a synergistic compound along with bacterial polyketides. Polyketides and alginate polymer combinations were used against test bacteria to determine the synergistic antibacterial activity. A novel wound-healing film was developed using polyketide and alginates with synergistic concentrations and its degradability and wound-healing ability was investigated. The findings in the present research showed most significantly that, *Staphylococcus aureus* showed complete synergy with the mean MIC value of 0.03 µg/ml and with best FIC value of 0.24 ($p<0.5$). Degradation of developed films revealed that more moisture leads to more release of antibacterial alginate content at the wound site and hence more degradation. This was evident from the FESEM analysis. In vitro wound-healing assay revealed that the developed polyketide-alginate polymers exhibited cell migration and proliferation after $24^{th}$ hour of incubation at $37^0$C indicating the wound-healing abilities. Hence, it can be concluded that the biochemical compounds present in the developed polyketide-alginate polymers are considered highly significant in treating any types of wounds.

**Keywords:** Alginate, *Padina tetrastromatica*, Polyketide, Synergism, Wound-healing.

## INTRODUCTION

Marine bacterial polyketides are rich class of natural products with diverse structures. Huang et al (2018) reported that the bacterial polyketides exhibits antimicrobial, antitumor, antiparasitic, antiviral, and other activities. In another study, Kajal Chakraborty et al., (2017) studied the antibacterial efficiency of polyketides from *Bacillus amyloliquefaciens* associated with edible red seaweed *Laurenciae papillosa.* Alginates are linear copolymers extracted from different types of green, brown and red seaweed in marine sources. The salt forms of alginates with several cations ($Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$) emphasize its antibacterial potential (Maria Jose Perez et al., 2016). The antibacterial potential of these marine compounds are combined together as a novel antimicrobial peptides and its synergistic antibacterial activity was evaluated against the wound pathogens. This is selected as a primary objective of the present research.

The disruption of physiological arrangement in the skin or tissues due to any accidental reasons or burns and ulcers is defined clinically as wounds (Boateng et al., 2008). Wound-healing is a complex biological process that depends on the wound condition, the patient's health, and the physical-chemical support given through external materials (Sabine et al., 2014). The development of bioactive molecules and skin substitutes for the enhancement of wound-healing process are studied widely in wound-care managements. Identifying the natural products that contain significant molecules for wound-healing process shall be explored from the marine environment. Diverse marine living beings are considered to be a great source for the search of wound-healing products (Pathum Chandika *et al*., 2015). Delay of wound-healing in the patients occurs due to different factors, like multiplication of bacteria including *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Klebsiella* sp (Richard *et al*., 2012).

Using the marine bioproducts one such wound-healing process shall be studied against the pyogenic bacterial species. It was reported that bacterial polyketides extracted from the bacteria associated with seaweeds act as a potent inhibitor of bacterial pathogens causing pyogenic infections (De Rosa *et al.*, 2002). Also, the alginates from marine seaweed reported to contain different antimicrobial compounds against bacteria associated with wounds and ulcers (Janarthanan and Senthil Kumar, 2018).

The basis of investigating the synergistic activity of polyketide and alginate was reported from the concept described by Saginur *et al.*, (2006). The researchers described the advantage of using combination therapy blending two group of drugs at different concentrations. Gorman and Jones (2002) also explained the advantage of synergism from their standard clinical practice, where a broader spectrum of antimicrobial activity would be achieved at lower concentrations of drugs. Based on the antibacterial potential of the compounds from these marine sources, the synergistic antibacterial activity of polyketide and alginates in combinations were evaluated against different test bacteria in the present research. Extraction of polyketides from bacteria associated with brown seaweed (*Padina tetrastromatica*) was reported in our previous research work (Prasad and Latha, 2019). Alginates extracted from the brown seaweed (*Padina tetrastromatica*) were analysed in the present study.

The concentrations of polyketides and alginates selected from the synergistic studies that significantly inhibited the growth of bacteria were used to develop a novel antibacterial wound-healing films. The developed polyketide-alginate films ($PA_f$) were evaluated to determine the wound-healing efficiency using a standard in vitro wound scratch assay. The wound-healing films were developed on the basis of concept reported by Mariana *et al*., (2019). They reported that in order to reduce the bacterial colonisation at the wound site and also to increase the rate

of wound-healing, antimicrobial loaded films or equivalent dressing materials needed to be developed. Lei *et al*., (2013) described that the antimicrobial wound dressings can fasten the wound-healing process by retarding the growth of bacteria at the wound site.

## MATERIALS AND METHODS

Microorganisms (*Staphylococcus aureus, Enterobacter* sp, *Staphylococcus epidermidis, Escherichia coli* and *Klebsiella pneumoniae*) used in this study were procured from Division of Microbiology, School of Biological Sciences, CMS College of Science and College, Tamilnadu, India. The entire work was carried out in Division of Microbiology, CMS College of Science and College, Coimbatore from February 2019 to March 2020.

### Collection of brown seaweed *Padina tetrastromatica*

The seaweeds were collected fresh from Tuticorin with the help of local divers (South east coast of India – directions - Lat 8 45’ N; Long 78 10’E). The seaweed samples were transported to the laboratory in sterile polythene bags immediately after collection for further analysis.

### Extraction of biopolymer alginates from different microalgae (Torres *et al.*, 2007)

The sodium alginate was extracted from the brown seaweed *Padina tetrastromatica* by the modified method of Torres *et al.,* (2007). About 100g of milled seaweed sample was weighed and soaked in 2% formaldehyde taken in air tight conical flask for 24h. After 24h, the formaldehyde solution was filtered out and the residue was washed with distilled water for 2 to 3 times. Then 0.2 M HCl solution was added to the residue and kept at room temperature for 24h. After 24h, the solution was removed and the residue was washed with distilled water for 2 to 3 times. The residue was extracted with 2% sodium carbonate for overnight. The extract was filtered through muslin cloth bag and the filtrate was bleached with 2.5% sodium hypo chloride.

Then the solution was evaporated and dried at 60ºC in a hot air oven. The final product was scraped from the beaker and made into powder. The powdered product was weighed to calculate the sodium alginate yield.

**Antibacterial activity of Polyketides and Alginates against test bacteria**

The antibacterial activity of Polyketide and Alginates was evaluated against the test organisms by well diffusion method separately. Sterile Mueller-Hinton Agar (Composition g/L: Acid hydrolysate of Casein: 17.5g; Starch: 1.5g, Sodium chloride: 5.0g, Agar 17.0 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the test organisms were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 20µl of polyketide fractions were loaded into the well and the plates were incubated at 37ºC for 24h. Similarly, alginates were also tested separately against the test organisms. The antibacterial activity was evaluated in terms of zone of inhibition around the wells in all the inoculated NA plates. At the end of incubation, the zone of inhibition formed around each material was measured in millimetre. Experiments were carried out in triplicates and antibacterial activity was expressed in Standard deviation values using Statistical Package for Social Sciences (SPSS – 9 for Windows 7.0) software.

**Determining the Synergistic activity of Polyketides - Alginates against test bacteria (Bharadwaj et al., 2003)**

The extraction of microbial polyketides from brown seaweed (Angel Treasa *et al*., 2011) and its antibacterial potential were earlier described in our previous work (Prasad and Latha, 2019). Microbial polyketides from the bacteria associated with *Padina tetrastromatica* was found potential on the basis of their antibacterial activity against the test bacterial cultures. Based

on our earlier report, the polyketides of *Padina tetrastromatica* was selected to determine the synergistic activity along with the extracted biopolymer alginates. The synergistic antibacterial activity of polyketides and alginates against different test bacteria were described below. The synergistic activity of Polyketides - alginates was determined by the standard checkerboard titration method.

**Checkerboard titration method (Qaziasgar and Kermanshahi, 2009 and Odds, 2003)**

To determine the inhibitory concentrations of each polymer separately and in combinations, the minimal inhibitory concentrations (MIC) was simultaneously identified in this method. The fractional inhibitory concentrations (FIC) of the polymers were calculated from MIC values to determine the synergism between the two polymer combinations (Polyketides - alginates). To assess antimicrobial combinations, the checkerboard method was selected. In this technique by using agar dilution method, the concentrations tested for each antimicrobial agent were typically ranged from four or five below the expected MIC to twice the anticipated MIC as in the 45-degree line in Fig.1 (each square represents one plate). The predetermined concentrations (µg/ml) used for this method were 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1.0 and 2.0. According to Figure-1, the plates in the left-hand column were used for the predetermined concentrations of Polymer-A (Polyketide), the plates in bottom row were used for Polymer-B (Alginate) and the plates in the 45-degree line were used for mixed polymer combinations. In all the arranged plates, 1ml of predetermined dilutions of the antimicrobial agents was added with sterile and molten Muller-Hinton agar. Then the surface of each plate was inoculated with 1 X $10^4$ CFU/spot of bacteria. After 16-20 hours incubation at 37 ºC, the plates were examined for evidence of visible growth. The experimental set up was made for all the polymer combinations

in triplicate. Fractional inhibitory concentration index (FICI) was calculated using the following equation.

*Formula to determine synergy*

FIC *index* = $FIC_A + FIC_B$

$$FIC_A = \frac{MIC_A \text{ in combination}}{MIC_A}$$

$$FIC_B = \frac{MIC_B \text{ in combination}}{MIC_B}$$

where, A was the minimal inhibitory concentration (MIC) of Polymer-A in a plate that was the lowest inhibitory concentration in its row, and B was the MIC of Polymer-B in a plate that was the lowest inhibitory concentration in its column. $MIC_{AB}$ was the lowest inhibitory concentration of Polymer A and B in combination in the 45-degree line. According to Odds (2003), the synergy between two drugs was indexed and presented below.

| **Mean FICI value (≤ 0.5)** | **Index** |
|---|---|
| Mean FICI < 0.5 | Synergy |
| Mean FICI > 0.5 | Partial synergy |
| Mean FICI ≥ 2.0 | Antogonism |

**Developing Polyketide-Alginate films ($PA_f$) as wound dressing material (Mohamed Fertah *et al*. 2017)**

About 2.5ml of extracted alginate and 2.5ml of polyketides were mixed in a sterile glass test tube. Equal volumes of the polymer extracts were taken based on their synergism. The synergistic polymers were mixed with equal volumes of poly vinyl alcohol (1% wt./wt. in water) in the ratio of 1:1. The polymer solutions were mixed in a sterile beaker and stirred for 1h at 60-ºC to obtain a homogeneous solution. To this solution, 1ml of 2% glutaraldehyde solution in

water was added under stirring conditions in a magnetic stirrer (120rpm) at room temperature (25ºC). The entire content was slowly added as a thin layer on a sterile petri dish and incubated in the incubator at 37° C for 18h to form a thin polyketide-alginate film ($PA_f$). The developed $PA_f$ was slowly allowed to dry and smoothly scraped off from the petri dish without damaging the inner and outer edges of the film. The scrapped film was measured for its diameter and stored in a sterile zip lock covers at 4°C for further analysis.

**Degradation studies of the developed $PA_f$ (Ana R Costa-Pinto *et al*., 2014)**

Degradation studies were performed in triplicate by incubating $PA_f$ in 10ml of phosphate-buffered saline (pH 7.0), with lysozyme from chicken egg white (Sigma, USA) (3mg/L) at 37°C under agitation (60rpm) for 8 weeks. The enzymatic concentrations used were similar to those found in human blood serum. Before and at the end of each degradation period, the samples were removed and its topographical changes were determined using FESEM analysis.

**In Vitro Wound-healing studies (Liang et al., 2007)**

The migration rates of fibroblast cells ($L_{929}$) were assessed by the scratch assay method. The cell density of $2 \times 10^5$ cells was seeded into each well of a 24-well plate and incubated with complete medium at 37°C and 5% $CO_2$. After 24h of incubation, the monolayer confluent cells were scrapped horizontally with a sterile $P_{200}$ pipette tips. The debris was removed by washing with PBS. The cells were treated with polyketide-alginates with the selected concentration (100µg/ml) by diluting with serum-free Dulbecco's modified eagle medium (DMEM). The cells treated with allantoin (Sigma Aldrich, Germany) were used as positive control. The scratch induced as the represented wound, was photographed at 0h using phase-contrast microscopy at 40X magnification. After 12h and 24h of incubation, the second set of images was photographed.

To determine the migration rate, the images were analysed using “image-J” software, and the closed area was compared with the value obtained at 0h.

## RESULTS AND DISCUSSION

### Antibacterial activity of Polyketides and Alginates against test bacteria

Antibacterial activity of polyketide and alginate was tested separately before investigating its synergistic behaviour against the test organism. Maximum inhibitory zones of 16.6 ± 0.76mm and 16.6 ± 1.25mm were evident for polyketide and alginate against *Staphylococcus aureus.* Followed by the inhibitory zones of 16.3 ± 0.57mm and 16.6 ± 1.04mm was found evident for *Escherichia coli*. Other significant test organism also exhibited inhibitory zones against the marine peptides. *Enterobacter* sp exhibited inhibitory zones of 15.6 ± 1.25mm and 15.3 ± 0.76mm when tested against polyketide and alginate respectively. *Staphylococcus epidermidis* and *Klebsiella pneumoniae* showed inhibitory zones range between 13.3 ± 0.57mm and 14.6 ± 1.04mm. All the values were presented in Table-1.

### Synergistic activity of Polyketide-Alginate against test bacteria

Synergistic activity of polyketide-alginate combinations was determined against the test bacteria. In Table-2 all the five test organisms showed complete synergistic effect for polyketides - alginates combination. Most significantly, *Staphylococcus aureus* showed complete synergy with the mean MIC value 0.03 µg/ml (Fig.2) and with best FIC value 0.24 ($p<0.5$). Similar MIC and FIC values were observed for *Enterobacter sp* and *Escherichia coli*. Other significant organism *Klebsiella pneumoniae and Staphylococcus epidermidis* also showed complete synergy with the mean MIC value 0.12 µg/ml and with best FICI value 0.72 ($p<0.5$).

The MIC of polymer combination obtained for all the test organisms was very low in comparison with the individual effect of each polymer. It showed that greater antibacterial effect was achieved with lower concentration of polymers. This assumption agreed with that of the following factors described by Saginur *et al.,* (2006). They reported that the accepted clinical practice to treat biofilm-associated infections was the use of combination therapy in which two

or more antimicrobials are blended at different combinations. This approach comes from standard clinical practice, such that a broader spectrum of activity is achieved and lower concentrations of the antimicrobial are required, resulting in more effective therapy and decreased resistance (Gorman and Jones, 2002).

**Effect of the developed PAf on degradation studies**

Topographical analysis of developed wound dressing films using polyketide and alginate was investigated by FESEM at three different stages viz., before degradation, during degradation analysis and after degradation. The images revealing the three different stages were illustrated in Fig. 3, 4 and 5.

In Fig.3, it was evident that the surface of the film was clear and did not show any cracks or equivalent. In Fig.4, initial level cracks were observed on the developed films indicating that the films are in the degradation stages due to hydrophilic properties of the alginate. The moisture content in the wound emphasize this hydrophilic properties. So, the developed film at the wound site undergoes degradation releasing the antibacterial alginate and polyketides. The release concentrations of the synergistic polymers influences qualitative wound-healing by reducing the bacterial numbers and infection caused by them. The moisture content varies from one condition to another condition depending on the types and impact of wounds. These factors influences the degradation rate of the developed films. If the moisture content is more at the wound site, then the degradation of developed alginate films will be higher; and hence more antibacterial content will be released. In Fig.5, more degradation of the film was observed which substantially supports the above conditions. The alginate films were broken and more cracks were observed in PA films. From the FESEM analysis it was evident that the films are highly biodegradable with simultaneous expectation of wound-healing. The wound-healing properties of the developed

films were significantly due to their antibacterial compounds present in it natively. This was proved in the in vitro wound-healing assay method of the developed wound closure PA films.

**In vitro wound healing potential of Polyketide-Alginate films**

In this study, the polyketide-alginate concentrate used for the cell adhesion studies was determined for its ability to improve wound-healing by acting directly on $L_{929}$ mouse fibroblast cells. After creating a scratch on $L_{929}$ mouse fibroblast cell lines, the cell migration, cell proliferation and wound closure were measured for a known concentration (100µg) of polyketide-alginate at three different time periods ($0^{th}$ hour, $12^{th}$ hour and $24^{th}$ hour). The wound-healing ability of the developed polyketide-alginate films showed that (Fig.6), at $0^{th}$ hour, no cell migration and proliferation was observed for the known concentrate (100µg) including control (Distilled water). At $12^{th}$ hour, the concentrates showed positive cell migration and cell proliferation when compared to the control sample. After 24hours, more cell proliferation was evidence indicating that the known concentrates of polymers have assisted in wound closure. In vitro scratch assay could be noted as an appropriate and inexpensive method and was used in the present research to identify the wound healing potential of the polymer.

According to the finding above, it can be concluded that the developed PAf could be used to treat in different types of wounds. The images of migration and proliferation of fibroblast cells after treated with developed PAf could able to increasing the levels of cytokines, growth factors and keratinocytes at the wound area which it could be useful for effective wound healing in diabetic wounds.

**CONCLUSION**

Alginates were extracted from a brown seaweed *Padina tetrastromatica* and used as a synergistic compound along with bacterial polyketides. Polyketides and alginate polymer combinations were

used against test bacteria to determine the synergistic antibacterial activity. *Staphylococcus aureus* showed complete synergy with the mean MIC value of 0.03 µg/ml and with best FIC value of 0.24 ($p<0.5$). A novel wound-healing film was developed using polyketide and alginates and its degradability revealed that more moisture leads to more release of antibacterial alginate content at the wound site and hence more degradation. In vitro wound-healing assay revealed that the developed polyketide-alginate polymers exhibited cell migration and proliferation after 24 hour of incubation Hence, it can be concluded that the biochemical compounds present in the developed polyketide-alginate polymers are considered highly significant in treating any types of wounds. The wound-healing quantitatively controlled in terms of development before the application of the polymers shall be studied in future.

**Conflict of Interest**

Authors declare no conflict of interest in the research

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**Table-1: Antibacterial activity of Polyketides and Alginates against test bacteria**

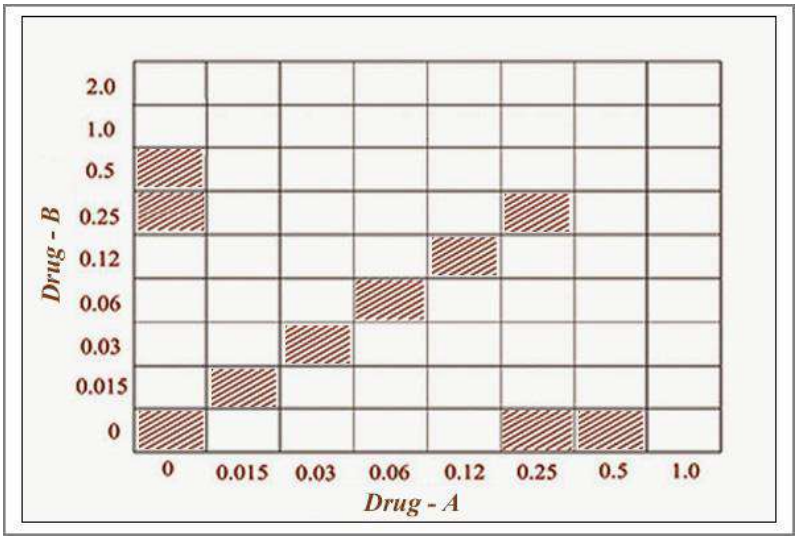
| S. No. | Test Bacteria | Zone of Inhibition (mm) | |
|---|---|---|---|
| | | **Polyketides** | **Alginates** |
| 1 | *Staphylococcus aureus* | 16.6 ± 0.76 | 16.6 ± 1.25 |
| 2 | *Enterobacter* sp | 15.6 ± 1.25 | 15.3 ± 0.76 |
| 3 | *Staphylococcus epidermidis* | 14.6 ± 1.04 | 13.3 ± 0.57 |
| 4 | *Escherichia coli* | 16.3 ± 0.57 | 16.6 ± 1.04 |
| 5 | *Klebsiella pneumoniae* | 13.6 ± 0.76 | 14.3 ± 1.25 |

**Table-2: Effect of Polyketides - alginates against test bacteria**

| Test Bacteria | $MIC_A$ | $MIC_B$ | $MIC_{AB}$ | $FIC_A$ | $FIC_B$ | $FIC_{AB}$ | Index |
|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | 0.25 | 0.25 | 0.03 | 0.12 | 0.12 | 0.24 | S |
| *Enterobacter* sp | 0.25 | 0.25 | 0.03 | 0.12 | 0.12 | 0.24 | S |
| *Staphylococcus epidermidis* | 0.25 | 0.5 | 0.12 | 0.48 | 0.24 | 0.72 | S |
| *Escherichia coli* | 0.25 | 0.25 | 0.03 | 0.12 | 0.12 | 0.24 | S |
| *Klebsiella pneumoniae* | 0.25 | 0.5 | 0.12 | 0.48 | 0.24 | 0.72 | S |

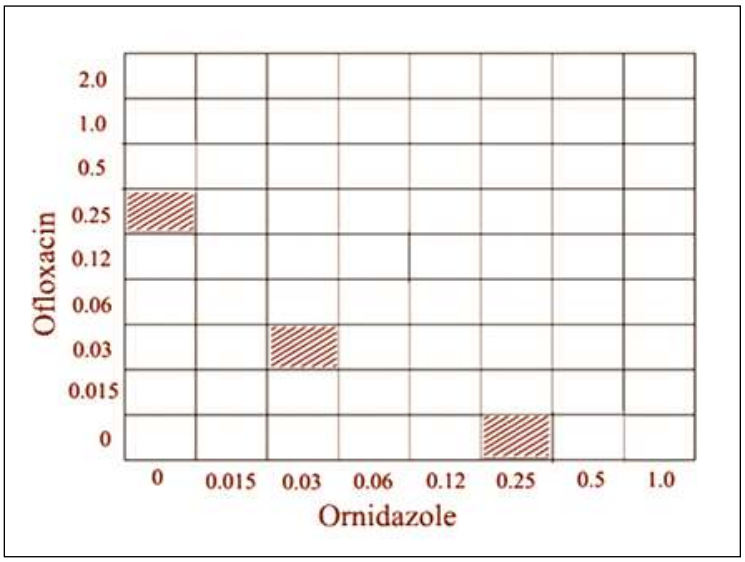
A – Polyketide, B - Alginate, AB- Combined concentration of Polyketides - alginates
S – Synergy, Units for all the presented values - µg/ml

**Fig. 1: Checkerboard model to determine synergism of two polymers**



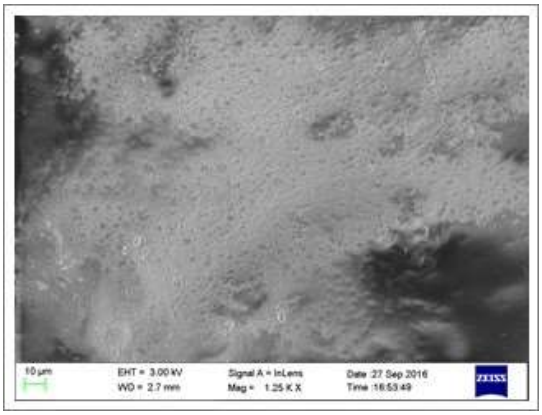

(The picture was adapted from Qaziasgar and Kermanshahi, 2008)
Bottom row: To determine MIC of Polymer-A [Polyketides]
Left column: to determine MIC of Polymer-B [Alginate]
Centre: MIC of (Polymers) - Polyketides – alginates

**Fig. 2: Simplified Checkerboard Method of Polyketides - alginates**



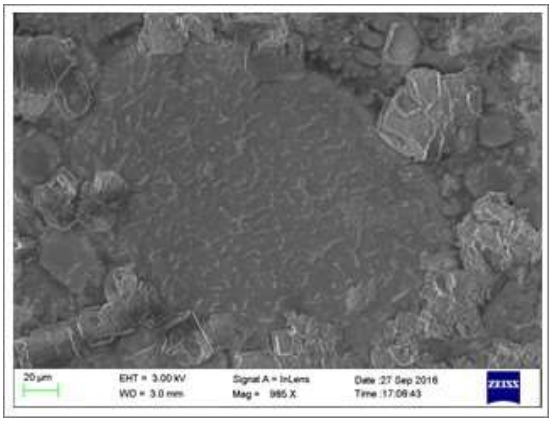

MICA – 0.25, MICB – 0.25, MICAB – 0.03
Test Bacteria: *Staphylococcus aureus*

**Fig. 3: Before degradation studies of the developed $PA_f$**



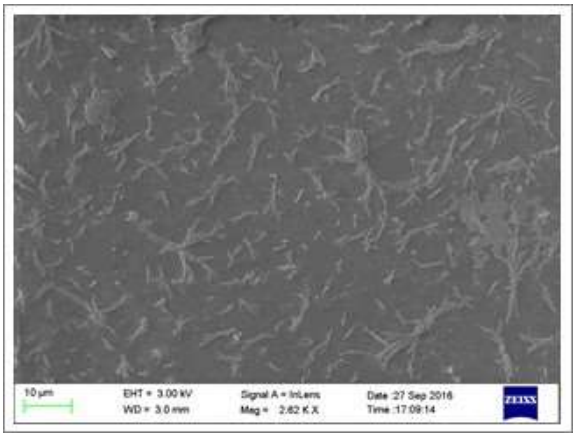
**(Before degradation)**

**Fig. 4: During degradation studies of the developed $PA_f$**



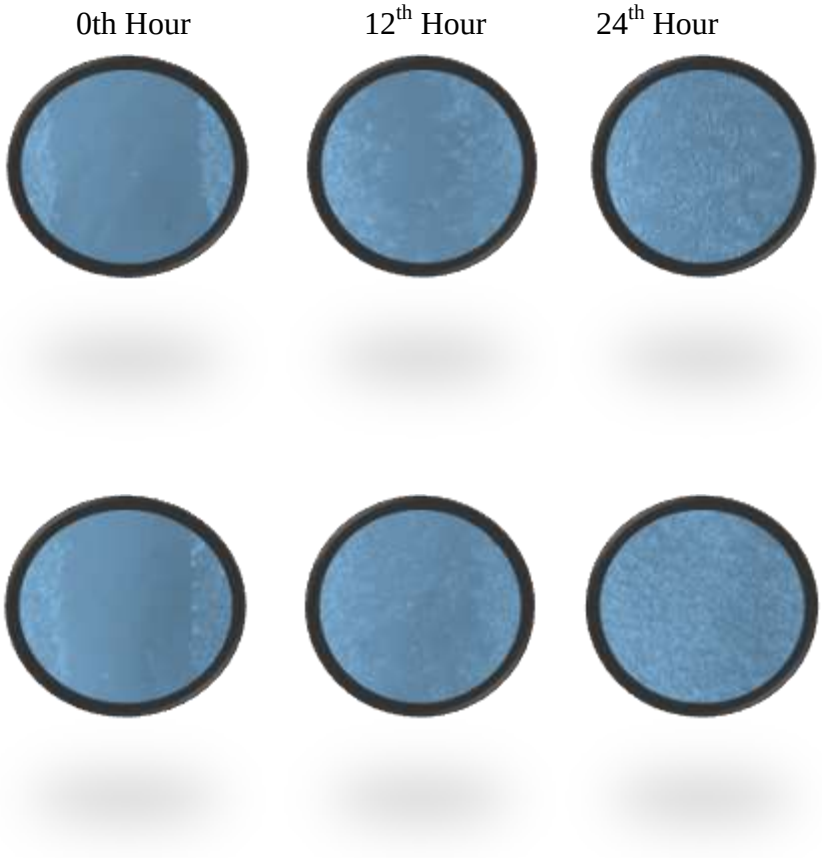
**(During degradation)**

**Fig. 5: After degradation studies of the developed $PA_f$**



**(After degradation)**

**Fig. 6: Wound-healing ability of $PA_f$**




A: Control (Distilled water) [Top three pictures]
B: Polyketide-alginate sample (100µg) [Bottom three pictures]