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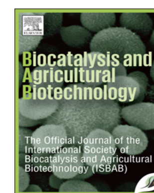

Potential biofilm dispersal by a partially purified keratinase produced by *Stenotrophomonas maltophilia* strain Kb2

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ABSTRACT

Biofilm dispersal and antibiofilm activity of the keratinase produced by *Stenotrophomonas maltophilia* Kb2 was studied against pathogenic bacteria *Staphylococcus aureus* MTCC-96 and *Escherichia coli* MTCC-739. The quantitative estimation of biofilm dispersal showed a positive correlation with the concentration of keratinase and biofilm dispersal in both the strains. The dispersal was further confirmed by the microscopic observation of keratinase treated biofilm. Among the two isolates, biofilm dispersal activity of keratinase was more pronounced in *S. aureus* as compared to *E. coli*. Inhibition of biofilm formation was observed in both the strains in presence of keratinase and a dose dependent relationship between keratinase concentration and antibiofilm activity was observed. Time course study revealed that with the increasing time duration antibiofilm and biofilm dispersal activity of keratinase decreased. The study would open new avenues in medical field for the removal of biofilms from obstructing indwelling catheters and other medical devices.

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1. Introduction

Several pathogenic bacterial species reside as complex multicellular structures in adherent surface of extrapolymeric substances known as biofilms (Abee et al., 2011, Mirani et al., 2013). Bacteria can form biofilms in wide variety of environments such as natural (sea, rivers, sub-merged rocks etc.), medical (plaque and medical implants) and engineered systems such as pipelines of waters, sewage, offshore oil and gas industry (Kavita et al., 2014). Biofilms provide protection and stability to bacteria living under adverse conditions such as nutrient limitations, desiccation, pH and temperature fluxes and complex host immune responses etc. (Hall-Stoodley and Stoodly, 2005, Kumar et al., 2013).

Staphylococcus aureus and *Escherichia coli* are the well known biofilm forming pathogenic bacteria responsible for multiple diseases in humans such as septic shock syndrome, nosocomial and community acquired bloodstream infection worldwide (Kaasch et al., 2011). According to National Institute of Health (NIH, USA) about 80% of all microbial infections are caused by biofilms, which consecutively leads to problems for treatment of human diseases (Yakandawala et al., 2006, Crossman and Dow, 2004). A variety of antimicrobial agents have been used to control biofilms but because of lower efficacy and increased resistance of the biofilm towards these antimicrobial agents, limit their effective applications (Dusane et al., 2013). Even some chemical surfactants such as cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) which are used to removal of biofilm is discouraged due to their inherent toxicity (Dusane et al., 2012). The present scenario demands attention towards developing potential biofilm dispersal agents.

Keratinases are alkaline serine and/or metalloprotease capable of degrading keratins (Radha and Gunasekaran, 2009). Due to their robust activity, microbial keratinases have gained tremendous impetus in various commercial fields such as dehairing in leather industry, slow release nitrogen fertilizers, cosmetics and detergent industry (Tiwary and Gupta, 2010). However, studies are underway to elucidate novel applications of keratinase so that these valuable enzymes can be fully exploited. In the present study, keratinase of *Stenotrophomonas maltophilia* Kb2 was used to study the biofilm dispersal and inhibition activity of *S. aureus* MTCC-96 and *E. coli* MTCC-739 biofilms.

2. Material and methods

2.1. Keratinase enzyme

A strain of *S. maltophilia* Kb2 (accession number KC878468.1) isolated from feather disposal site of Bilaspur, Chhattisgarh was

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selected as a source of keratinase production. The production and estimation of protease activity was carried out according to the method of Chaturvedi et al. (2014). The crude enzyme obtained after growth of strain Kb2 was concentrated to 60% by ammonium sulphate precipitation followed by dialysis. Concentration of protein was estimated according to the method of Bradford (Bradford, 1976)

2.2. Biofilm dispersal activity of keratinase

Biofilm dispersal activity of keratinase was determined according to the method of Kavita et al. (2014) with slight modifications. The pathogenic strains of *S. aureus* MTCC-96 and *E. coli* MTCC-739 procured from MTCC Chandigarh, India were grown under laboratory conditions at 37 °C in LB broth. The overnight grown culture was diluted to 0.1 OD_{600} with LB broth and distributed equally in 96 well plate. Biofilms formed after 48 h of incubation were treated with different dilutions of keratinase (200–1000 μg/ml) and proteinase K (positive control, 200–1000 μg/ml dissolved in 100 mM Tris–HCl buffer, pH 8.0). Well with negative control contained 100 mM Tris–HCl buffer (pH 8.0) instead of enzyme. Both sets were kept at 50 °C temperature with constant shaking at 90 rpm for 2 h for appropriate interaction of enzyme with biofilm. Wells were washed, dried and stained with 1% (*w*/*v*) crystal violet, and excess dye was washed off. Thereafter, 1 ml of 96% ethanol was added and the absorbance was measured at 590 nm using spectrophotometer. The experiment was repeated in 4 replicates. Percent biofilm dispersal was calculated by the following formula:

$$\text{Percent biofilm dispersal} = \frac{\text{absorbance of control} - \text{absorbance of treated biofilm}}{\text{absorbance of control}} \times 100$$

2.3. Microscopic analysis of biofilm dispersal

The biofilm dispersal activity of keratinase was also performed in glass slides. Partially purified keratinase and proteinase K was diluted upto approximately 500 μg/ml in Tris–HCl buffer (pH 8.0) was used to treat preformed biofilm of both the selected strains. After 1 h of incubation the glass slides were examined microscopically using inverted microscope (Leica DMIL LED, Germany) at 10 × magnification.

2.4. Effect of temperature on biofilm dispersal

In order to determine the effect of temperature in biofilm dispersal the preformed biofilm of *S. aureus* was treated with 500 μg/ml keratinase and proteinase K under temperature ranging from 25–60 °C.

2.5. Antibiofilm activity of keratinase

In order to study antibiofilm activity of keratinase and proteinase K, the overnight grown bacterial culture was diluted to 0.1 OD_{600} with LB broth and distributed equally in 96 well plate. The culture was incubated with 200–1000 μg/ml of keratinase and proteinase K for 48 h. Untreated bacterial culture was considered as control. The culture was discarded after incubation and antibiofilm activity of keratinase and proteinase K was calculated as:

$$\text{Percent antibiofilm activity} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

2.6. Time course study of biofilm development in presence of keratinase

To determine the effect of keratinase on biofilm formation the overnight grown bacterial culture was diluted to 0.1 OD_{600} with LB broth and distributed equally in 96 well plate. The culture was incubated for different time period (24–96 h) with 500 μg/ml of keratinase and proteinase K. Untreated bacterial culture was considered as control. The culture was discarded after incubation and inhibition of biofilm formation was calculated as:

$$\text{Percent inhibition of biofilm formation} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

3. Results and discussion

3.1. Effect of keratinase in biofilm dispersal

In the present investigation, dispersal of preformed biofilms of *S. aureus* MTCC-96 and *E. coli* MTCC-739 by a partially purified keratinase from *S. maltophilia* Kb2 and proteinase K has been studied. A varying concentration of keratinases and proteinase K (200–1000 μg) were tested to evaluate biofilm dispersal. Biofilm dispersal increased to a significant extent ($p < 0.05$) and reached to a maximum of 75.5 ± 0.13% and 79.8 ± 0.027% respectively with the highest concentration of keratinase and proteinase K in *S. aureus* while the dispersal was found to be 63.02 ± 0.55% and 68.17 ± 0.11% in *E. coli* (Table 1). The observation clearly indicated that the keratinase and proteinase K could efficiently disperse biofilm formed by *S. aureus*, as compared to *E. coli* biofilm. This finding is consistent with previous reports which show that in gram positive bacteria such as *S. aureus*, the biofilm matrix is made up of glycocalyx which is composed of teichoic acids and proteins (Archer et al., 2011) whereas the matrix of *E. coli* biofilm is mainly composed of exopolysaccharides such as cellulose, and colanic acid (Beloin et al., 2008). Therefore, the matrix of Staphylococcal biofilms is easy to break with the help of proteases.

The result was corroborated with microscopic examination of biofilms formed on glass slides, which also showed significant reduction by the action of keratinase and proteinase K respectively. Fig. 1A clearly depicts the visual observation of biofilm formation by *S. aureus* (lane 1) and biofilm dispersal by keratinase (lane 2) and proteinase K (lane 3). Fig. 1C and D indicates the dispersal of biofilm formed by *S. aureus* by keratinase and proteinase K when compared with intact biofilm (Fig. 1B). Similar result was observed in *E. coli* biofilm. The role of proteolytic enzymes in biofilm dispersal has already been reported but this is the first report on biofilm dispersal activity of keratinase. Several native proteases of bacteria involved in biofilm dispersal have already been reported in literature such as Spl proteases of *S. aureus* (Kaplan, 2010) and cystein proteases, Spe B of *Streptococcus* (Conolly et al., 2011).

3.2. Effect of temperature on biofilm dispersal

The optimium activity of partially purified keratinase from *S. maltophilia* Kb2 was found to be 50 °C. So, biofilm dispersal assay was performed at this temperature with keratinase and proteinase K respectively. To evaluate activity of keratinase and proteinase K at other temperatures, biofilm dispersal assay was performed in different temperature ranging from 25–60 °C. The results showed that biofilm dispersal gradually increased starting from 25 to 50 °C (Fig. 2). Further increase in temperature from 50 °C caused

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