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**Original Article**

# Evaluation of Anti-Biofilm Activity of Bacterial Amylase Against Human Pathogens

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## ABSTRACT

Global healthcare crisis has a significant impact on human health and economy because of antimicrobial resistance. Emergence of antibiotic resistance due to excessive antibiotic use results in resistant microbes. Biofilm shows integral resistance to antimicrobial agents and the host defense system. **Objective:** To test the efficacy of bacterial amylase against biofilm formation of pathogenic microbes. **Methods:** Amylase producing bacterial isolates (isolated from a variety of sources, such as waste, rhizosphere of vegetable soil, rice field, potato, and sugarcane field soils sources (ArPs, ArDs, ArSs, ArVs and ArRs) were utilized to reduce the microbial biofilm development against pathogenic isolates of Staphylococcus aureus and Pseudomonas aeruginosa. Six bacterial isolates (SB, SW, SU, PB, PW, and PU) obtained from cancer patients of tertiary care hospitals. **Results:** Amylase enzyme extracted from ArPs showed maximum growth inhibitory effect on SW pathogen, while ArRs showed minimum growth effect upon SB pathogens. However, amylase showed highest anti-biofilm activity against SW pathogen. In general, the highest biofilm inhibition was recorded at pH7 at 37°C as compared to rest of pHs and temperatures. **Conclusions:** The efficacy of bacterial amylase in biofilm inhibition of human pathogens, seem promising and having significant potential in health care systems.

## INTRODUCTION

Microbial infections persist to pose a serious hazard to human health and a significant economic concern for society. Massive efforts are necessary to enhance and produce antibacterial macromolecules in order to overcome this situation. Antimicrobial macromolecules must be improved and developed in large quantities to report this crisis. Polysaccharides, proteins, amylase, fatty acids, and other natural antibacterial macromolecules comprise a high number of molecules that conduct good antimicrobial activity due to their remarkable biochemical features. The practice of these biological macromolecules and their composites is a possible strategy to diminish disease resistance in materials [1]. The philosophy of science is to advance the worth of human life, and for many years, a large number of people have focused on increasing this quality. Of the previous attempts, using amylase enzyme as an antibiofilm activity is a particularly promising strategy. These materials, have medicinal, cosmetic, and even nutritional properties [2]. Enzymes have a high selectivity, allowing them to be used effectively in the removal of organic adhesive residues from paper. A-Amylase enzyme was utilized in the conservation, particularly in the eradication of starch paste. The amylase enzyme was chosen for its capacity to hydrolyze starch and remove it from paper documents [3]. Biofilm production is one of the many antimicrobial resistance (AMR) tactics, and it's linked to a lot of death and morbidity. Targeting critical proteins/targets that are important for biofilm development and control might lead to novel treatment techniques for battling biofilm, thereby lowering the resistant infections and morbidity associated with A. baumannii biofilm [4]. As a whole, the formation of biofilms can be categorized roughly in a few stages; adhesion, conidial germination and development of hyphae, biofilm maturation and cell dispersion. Fungi in biofilms can adjust to the in-host environment [5]. Global healthcare disaster has a significant influence on human health and economy because of antimicrobial resistance. The lack of novel

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antimicrobials, particularly against Gram-negative bacteria, and the rise of high-risk multidrug-resistant (MDR) clones are exacerbating the situation. The rise of MDR Klebsiella pneumoniae strains resistant to carbapenems, the final class of antibiotics used to treat severe Gram-negative infections, is one of the most serious concerns [6]. The course, duration and cost of hospitalizations and mortality are the factors affected by the spread of resistance and the emergence of nosocomial infections caused by resistant bacteria. Major human pathogens such as Staphylococcus aureus and Staphylococcus epidermidis have high resistance to antibiotic treatment. The biofilm-related biofouling of modern surfaces, especially layers, stays a genuine concern, which moves specialists to create functional answers for the decrease of their effect [7]. Enzymes play substantial role in the elimination of biofilm in natural environments and may be promising agents for this reason. A proteolytic enzyme, bromelain was found good to have preventive and biofilm degradative ability and considering the proteolytic ability defined for pepsin and the biopolymer matrix structure of bacterial biofilms [8]. Microbes are huge number of enzymes that can help in industrials than other conventional methods. These microbes are worthy catalysts. Reaction rate can be increased through it. It can work in every condition and production process can be control by genetic manipulation. Amylases, which hydrolyze the polysaccharide backbone of EPS, might help with biofilm control [9]. Biofilms are a microbial community made up of one or more microorganisms that may develop on a variety of surfaces. Bacteria, fungus, and protists are examples of microorganisms that create biofilms. Dental plaque, a slimy build-up of bacteria that occurs on the surfaces of teeth, is an example of a biofilm. Biofilm method of development gives various favorable circumstances to indwelling microbes. They quickly create obstruction towards any AMR as well as anti-toxins are inadequate to enter thick biofilm and neglected to annihilate the biofilms earlier [10]. Amylases are also called as industrial enzymes. These enzymes are used in starch handling industries, syrup production companies and starch hydrolysis [11]. Considering the efficacy of bacterial enzymes as a strategy for removal of bacterial growth and biofilms, present study was hypothesized on the fact that the activity of amylases extracted from indigenous bacterial isolates from environmental sources might have antibiotic and anti-biofilm potential against pathogenic microbes of clinical significance.

## METHODS

Sample collection bacterial isolation and purification: Soil samples (n=10) for amylase producing bacterial isolates from the sources dump soil, garden soils, Rhizosphere soil in vicinity of plants of Tomato, Rice, Potato plant, Sugarcane were collected. All samples were collected in sterile zipper bags and transported in research laboratory, Lahore Garrison University for processing. All soil samples dilution was plated from each serially diluted sample into their respective Nutrient Agar plates. Plates were incubated at 37°C aerobically for 24hours.These isolates were further purified by quadrant streaking methods on same plates [12]. The isolated colonies were further characterized using cultural characteristics, staining behavior and biochemical tests. Isolation of pathogenic bacteria: The isolation of infective bacteria was also done from infected wounds, blood and urine samples of cancer patients of Tertiary care hospital Lahore. Selected colonies were purified by streaking. The selected colonies were named as SB, SU, SW, PB, PU, PW. Staphylococcus Aureus and Pseudomonas aeruginosa were selected. The isolated colonies were further characterized using cultural characteristics, staining behavior and biochemical tests. Enzyme Extraction and Quantification of Bacterial Amylase production: For quantification of amylase production, bacterial isolate screened from starch agar plates were inoculated in Luria-Bertani medium (LB) and incubated at 37 °C for 24 hours. Fresh bacterial cultures were used as inoculum (with cell densities adjusted to OD 0.5 600nm in LB media for 4 gram of autoclaved wheat bran (WB) available from local market. The media was kept at 37°C temperature for 48 hours for fermentation period. After 48 hours of fermentation the fermented media were taken out and supplemented with 50ml of 20 m M phosphate buffer (pH=7.0) for 30 minutes at 4°C in a rotary shaker at 250rpm.The content was filtered through muslin cloth, filtrate was centrifuged at 8325 × g for 10 min and it was centrifuged (HERMLE) at 8000 rpm for15 minutes at 4°C. After this the supernatant has been collected which is enzyme extract. Clear brown supernatant was used as the enzyme source of alpha amylase activity of the extract was measured by DNS method following the procedure of [13]. The reaction was stopped by adding 1 mL of 3, 5-dinitrosalicylic acid then followed by boiling for 10 min. The final volume was made up to 12 mL with distilled water and the reducing sugar released will be measured at 540nm.The quantity of reducing sugar freed throughout the experiment was calculated using a UV-VIS spectrophotometer to measure color development at 540 nm (Spec ORD 200plus). Biofilm development of pathogenic bacteria: Modified methods of Qurashi and Sabri 2012 were followed for pathogenic bacterial biofilm quantification [14]. Biofilm formation was checked in the presence and absence of bacterial amylase. To determine

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the anti- biofilm activity of amylase, biofilm formation of pathogenic bacteria was also determined in the presence of enzyme in similar method. Statistical analysis: Statistical analysis was done by pair-wise comparisons of means using one way analysis of variance (Fishers least significant difference (LSD) method at $p<0.05$) level of significance. All experiments were carried out in triplicates, average values were expressed.

## RESULTS

Five bacterial colonies which were isolated named as ArDs, ArVs, ArRs, ArPs and ArSs. All isolates showed the ability to hydrolyze starch on agar plates. Cultures were characterized on the basis of staining, morphology and biochemical tests and results were obtained. The isolates from different sources showed different results. All bacterial isolates showing amylase activity shared gram positive behavior and showed spore formation. Biochemical behavior of bacterial isolates showed that all isolates gives negative results for coagulase, and urease. Bacterial isolates showed positive results for catalase assay and in some cases oxidase test. Isolation of pathogenic bacteria from blood culture, wound swab and urine samples were done by direct swabbing from the samples to N agar plates. Bacterial isolates shared gram staining behavior. Isolates SU, SB and SW showed gram positive staining. Isolates PU, PW and PB shared gram-negative behavior. Biochemical behavior of bacterial isolates showed positive results for catalase and coagulase test (SU, UW and UB). While other bacterial isolates PW, PB and PU showed positive results for oxidase test and negative results for indole test and urease test. A starch–iodine test was used to assess the total enzyme activity of the crude, purified, and amylase enzymes. Highest amylase production was showed by sources ArDs and ArVs as compared to rest of sources as shown in figure 1.

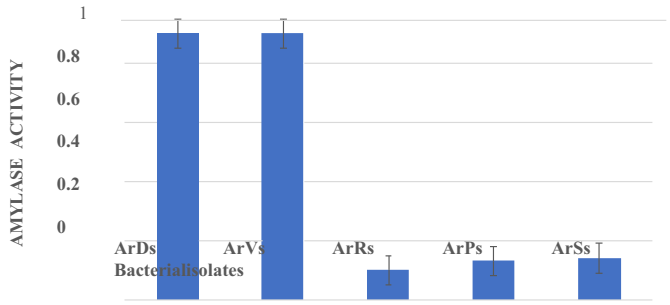


**Figure 1:** Amylase activity of bacterial isolates

Formation of biofilm of pathogenic isolates Staphylococcus aureus and Pseudomonas aeruginosa (SB, SU, SW, PB, PU and PW) respectively was determined by using microtiter plate assay. There was a general trend of increased biofilm formation in all bacterial isolates at different temperatures and ph. When the activity of amylase was tested for biofilm formation by these pathogenic isolates the highest biofilm formation was shown by PW as compared to rest to isolates as shown in figure 2.

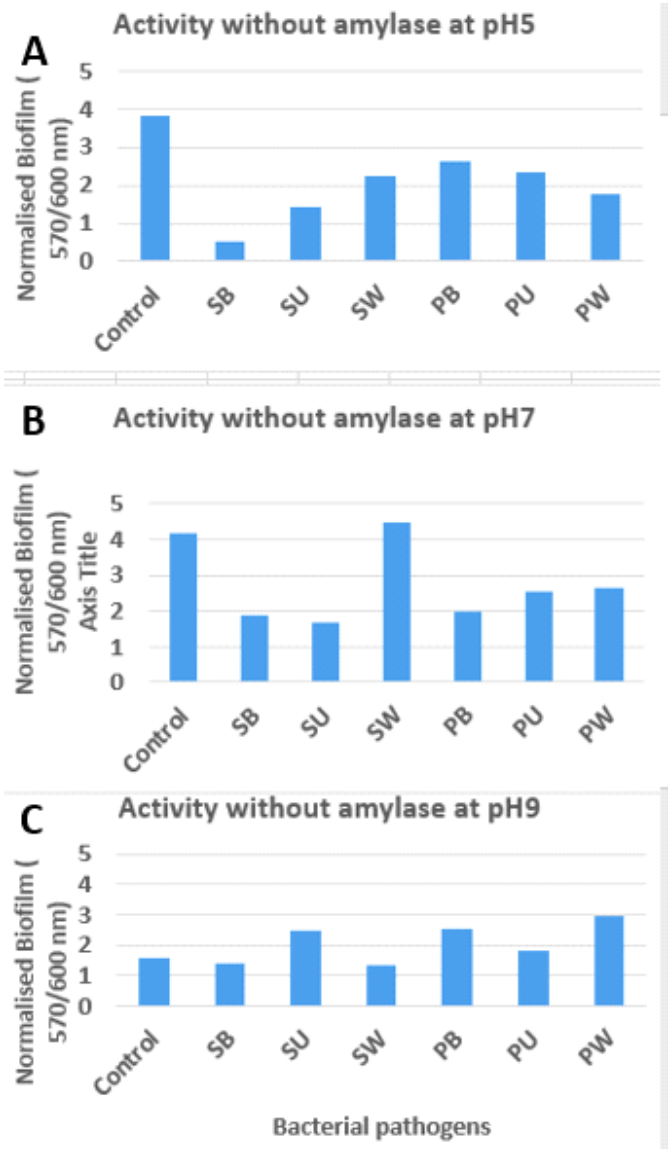


**Figure 2:** Biofilm forming assay of bacterial pathogens

Anti-biofilm activity of amylase against pathogenic isolates showed consistent results. When the anti-biofilm activity of pathogens in the presence and absence of amylase was checked at varying temperatures and pHs the efficacy of biofilm inhibition was highest for pathogen SW as compared to rest of bacterial pathogens. The most significant data was observed against SW at pH5 (Figure 3A). At pH7 (Figure 3B) significant biofilm inhibition was recorded for pathogen SU by amylase from source ArPs as compared to rest of treatments. At pH9 minimum amylase production and pathogens inhibition observed (Fig 3C).

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Lowest value of SU was observed at pH5 significant biofilm inhibition was recorded for pathogen SW by amylase from source ArVs as compared to rest of treatments (Figure 3.3A).At pH9 significant biofilm inhibition was recorded for pathogen SW by amylase from source ArPs as compared to rest of treatments at temperature58°C. In general, the highest biofilm inhibition was recorded at pH7 at 37°C as compared to rest of pHs. There was a general trend of higher biofilm inhibition by isolate SW was recorded. Results showed that isolate SW showed significant results .Biofilm formation was significantly decreased for isolates SB and SU at pH5 and pH9 respectively while SW showed more biofilm development by pathogens in the presence of amylase. Figure 4 shows growth of bacterial isolates at varying temperatures and PHz and maximum bacterial growth was observed at pH7 and temperature 37°C optimal conditions. Least growth curve was noticed at pH9 at temperature 60°C. At 30°C pathogenic bacterial isolates showed normal growth but at 60°C the growth was reduced to 0 shown in figure 4.

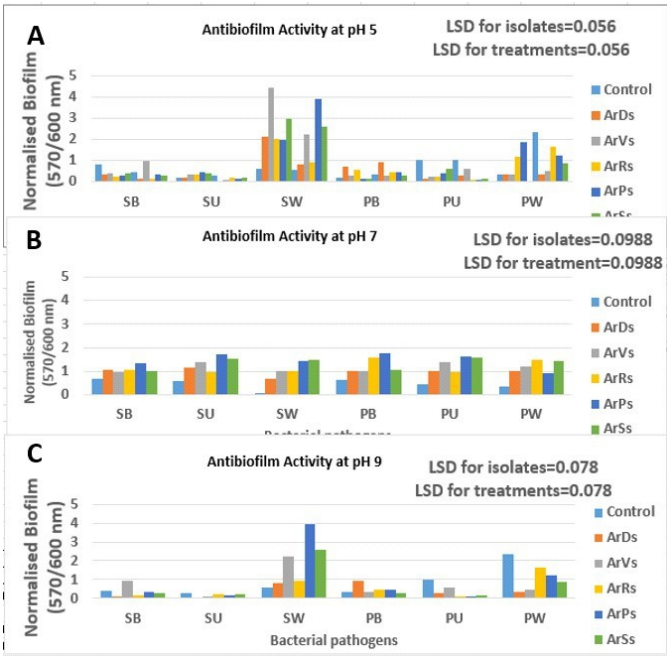


**Figure 3**: Anti-biofilm activity of amylase against pathogenic isolates at A; Ph 5, B:pH 7, pH 9.

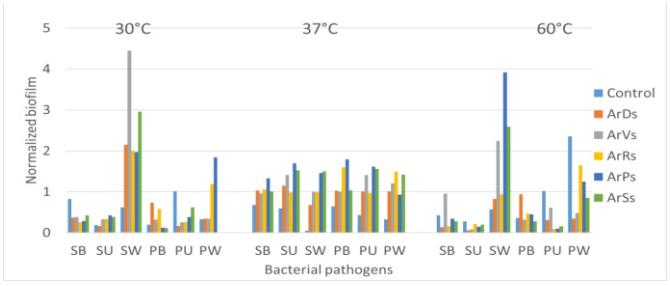


**Figure 4:** Anti-biofilm activity of amylase against pathogenic isolates at varying temperatures of 30°C, 37°C, 60°C.

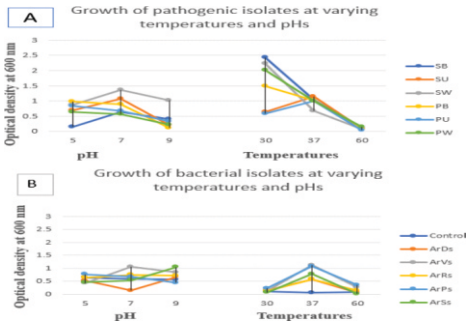


**Figure 5:** Growth of bacterial isolates at varying pHs and temperatures.

A shows growth of pathogenic bacterial isolates while B shows growth of amylase producing bacterial isolates.

## DISCUSSION

Biofilm colonies offer significant advantages to bacteria in terms of protective creation, but they are notorious for their antibiotic resistance. Biofilms are thick cell formations of bacteria encased in exopolysaccharides that help in biological conflict by enhancing surface adherence [16]. Berger et al (2018) found biofilm-forming species of Candida albicans, Candida glabrata, Enterococcus faecalis, S. mutans, V. dispar, and Fusarium nucleatum [17]. Similarly, Kalpana et al., 2012 showed the effectiveness of Amylase with substantial antibiofilm activity in S, aureus and P. aeruginosa [18]. Amylase generating bacterial strains was utilized to extract amylase enzyme from a variety of sources, such as waste soil, rhizosphere of vegetable soil, rice field soil, potato soil, and sugarcane field soil sources, in order to reduce microbial biofilm development. With this in mind, the current study examined the efficiency of amylase in preventing biofilm by using harmful bacteria from Staphylococcus aureus (blood culture, urine culture, wound swab) and Pseudomonas aeruginosa (blood culture, urine culture, and wound swab) from biofilm formation. SB, SW, SU, PB, PW, and PU are six bacterial isolates obtained from cancer patients: SB and PB from blood cultures, SU and PU from urine cultures, and SW and PW from cancer patients' wound swabs. Biofilm formation was tested in bacterial pathogens. Biofilm formation was demonstrated by all pathogens. Biofilm development is a common occurrence in bacterial infections that allows them to spread their presence. ArPs showed maximum growth effect on SW pathogen, while ArRs showed minimum growth effect upon SB pathogen [19]. However, amylase showed highest anti-biofilm activity against SW pathogen. Molecular docking interactions of amylase with P. aeruginosa and S. aureus extracellular polymeric substances (EPS) imply the potential of an energy-driven impulsive mechanism. Therefore, this work demonstrates that naturally isolate amylase from Bacillus has the ability to remove biofilms of

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hazardous bacterial isolates implicated in generating different nosocomial illnesses utilizing a mix of experimental and computational approaches [15]. To assess the variety and distinctive characteristics of bacterial isolates while generating amylase, a cultural, morphological, and biochemical profile was performed. All bacterial isolates tested positive for catalase and coagulase, according to their biochemical characteristics. Urease tests were negative for all while oxidase tests showed some positive behavior. Except for EMB, bacterial isolates had a metallic sheen and a green hue on MacConkey. In the presence of extracted amylase enzyme, biofilm development of harmful bacterial isolates was examined. In earlier studies, surface active molecules with anti-biofilm effectiveness were found in bacterial metabolites such as Exopolysaccharides. The development of biofilms by pathogenic bacterial isolates was studied. Biofilm development of isolate PW remained constant or increased, according to the findings. In general, pathogenic isolates generated less biofilm such as PW, which formed a lot of biofilms [20]. The current study demonstrates that amylase is effective against harmful bacteria that form biofilms. The crude amylase enzyme's capacity to remove biofilms and disperse them makes it particularly well suited to the treatment of invasive bacteria and illnesses. For the prevention of microbial biofilms on abiotic and living surfaces, the search for new chemicals with anti- biofilm activity is thus necessary. In the future, the findings of this study should be expanded to identify bacteria at the species level so that favorable

## CONCLUSIONS

The efficacy of bacterial amylase in biofilm inhibition of human pathogens, seem promising and having significant potential in health care systems. The findings of this research is to provide a summary of the current understanding of the amylase enzyme efficiency as an anti-biofilm agents to combat some of the important issues include food spoilage in food and beverage industry, medical devices such as urinary catheters and surgical instruments etc.

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