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COMPARATIVE ANTIMICROBIAL ANALYSIS OF BACTERIAL AND FUNGAL AMYLASE

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ABSTRACT

Amylases are highly valuable in biotechnology for starch-related industrial activities and are arguably the most studied glycoside hydrolases. Currently, several amylases derived from bacteria and fungus are in use. In the food business, fungal amylases are preferred because they are safe, despite not having the same stability and pH tolerance as their bacterial counterparts. In this context, the objective of the current study was to analyse and compare the antibacterial and biofilm activity of bacterial and fungal amylase commercially produced from *Bacillus cereus* and *Aspergillus oryzae* respectively against biofilm producers *Staphylococcus aureus* MTCC 740 and *Pseudomonas aeruginosa* MTCC 1688. The antibacterial activity against the test strains by well diffusion method exhibited no significant result with bacterial amylase whereas there was a significant inhibition against *P. aeruginosa* by fungal amylase with an MIC of 70 µg/ml. There was also significant reduction in biofilm by both bacterial and fungal amylase with IC 50 37.696 mg/ml and 52.654 mg/ml against *S. aureus* respectively whereas the observed IC 50 against *P. aeruginosa* by bacterial and fungal amylase was 37.431 mg/ml and 54.32 mg/ml respectively. The decrease in the virulence factors of *P. aeruginosa* of both the microbial derived amylases resulted in 70.73% pyocyanin reduction by fungal amylase and 96.45% pigment reduction by bacterial amylase; 1.69% elastase inhibition with fungal amylase and 11.549% elastase inhibition by bacterial amylase was also recorded with the decrease in swarming motility as well.

Keywords: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, α-amylase, Las B elastase, pyocyanin, IC 50

INTRODUCTION

In the realms of medicine and the environment, biofilm removal is crucial. It can therefore be crucial to disclose the new enzymes and their combinations for the spread of pathogenic biofilms. For the first time, bacteria are able to infect humans in this way and cause acute infections; they spread quickly and multiply in both planktonic and individual forms. However, when an infection progresses to a chronic or persistent stage, it mostly colonizes tissues and other body surfaces through the formation of highly structured multicellular aggregates known as biofilms. It is difficult to eradicate multispecies biofilm using antibiotic therapy or the host defensive mechanisms. As a result, it is now crucial to develop new treatment strategies for the removal of biofilms.

The Gram-positive bacterium *Staphylococcus aureus*, and the Gram-negative bacterium *Pseudomonas aeruginosa* is the cause of an increasing amount of nosocomial and community-acquired illnesses. Many chronic infections, including bacterial keratitis, burn wound infections, urinary tract infections, and peritoneal dialysis catheter infections, are caused by these two opportunistic pathogens. The increasing incidence of antibiotic resistance of *S. aureus* and *P. aeruginosa* is due to its ability to produce biofilm and survive in extreme conditions **[1]**.

Amylases are highly valuable in biotechnology for starch-related industrial activities and are arguably the most studied glycoside hydrolases. Currently, several amylases derived from bacteria and fungus are in use. In the starch processing industry, amylases are used to hydrolyze polysaccharides like starch into their constituent simple sugars. The scope of uses for amylase has broadened into numerous new domains, including clinical, pharmaceutical, and analytical chemistry, with the emergence of new biotechnology frontiers **[2]**. Different kinds of microbes can manufacture α-Amylase, however for commercial applications Bacillus is the primary source of α-amylase. *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* produce α-amylases that have potential uses in several industrial processes, including the food, fermentation, textile, and paper sectors.

The present study aims to compare the anti-microbial activity of bacterial and fungal amylase against *S. aureus* and *P. aeruginosa* pathogens. Though the antibacterial activity of amylase is researched, there has been no research carried out in inhibiting the virulence of these pathogens. The comparative activity of bacterial and fungal amylase also will help to shed light on the mechanism of action and properties of these enzymes in antibacterial activity.

METHODOLOGY

Antibacterial activity of bacterial and fungal amylase

The fungal amylase purchased from Shughengheng Jiagnsu chemical company, Delhi, India and bacterial amylase from Shriji Exibiz Pvt Ltd., Gujarat, India were assessed for its antibacterial activity against *Staphylococcus aureus* MTCC 740 and *Pseudomonas aeruginosa* MTCC 1688 by well diffusion method **[3]**; the concentrations tested being 500 mg/ml, 250 mg/mL,125 mg/ml and 62.5 mg/mL each for bacterial and fungal amylase. The screening test was then followed by Minimum Inhibitory Concentration test wherein the concentrations of the test samples ranged from m 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.3902 mg/ml **[4]**.

Biofilm inhibition of the test sample

Anti-biofilm activity by Crystal violet assay: Bacterial and fungal amylase were dissolved in distilled water at a concentration of 500 mg/mL. This was further diluted with distilled water to obtain test concentrations of 6.25, 12.5, 25, 50, 100, 125, & 250 mg/mL. Similar dilutions were prepared for standard Tetracycline. Biofilm quantification was performed following the method of Alva *et al*, 2019 **[5]**. A 100 µl sample of the diluted culture of *S. aureus* and *P. aeruginosa* strains were placed in a microtiter plate and incubated for 24 hours at 37°C. The attached cells were washed three times with PBS at pH 7.4. Then, 125 µl of 0.1% freshly prepared crystal violet solution was added to the dried pellet and incubated for 10 minutes. After staining and washing, 200 µl of 30% acetic acid was added to the pellet and incubated for 15 minutes to dissolve the stain. A 100 µl aliquot was transferred to a new plate, and the optical density was measured at 600 nm using an ELISA reader (Biorad, USA). The reduction in biofilm formation in the presence of plant extracts was calculated as percent inhibition using the formula: [(OD of control – OD of treated) / OD of control] x 100.

Las B Elastolytic assay: The Elastin Congo Red assay was conducted following the protocol of Alva *et al*, 2019 **[5]**. The cells were cultured at 37°C for 14 hours in LB broth, then centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant (50 µl) was mixed with 2 ml of 10 mM Na_2HPO_4 containing 30 mg of Elastin Congo Red. The mixture was incubated at 37°C for 14 hours, followed by centrifugation. The released Congo Red was then measured at 495 nm using a spectrophotometer.

Pyocyanin Quantification Assay: The assay was carried out following the method of Alva *et al*, 2019 **[5]**. Cells were cultured in LB broth at 37°C for 24-48 hours and then centrifuged. A 5 ml sample of the culture supernatant was mixed with 3 ml of chloroform and vortexed. The separated

lower organic phase was collected, and 2 ml of 0.2 M HCl was added. The absorbance of the resulting lower organic phase was measured at 520 nm using a spectrophotometer. The pyocyanin concentration was expressed in µg/mL of culture supernatant and calculated by multiplying the optical density by 17.072.

Swarming Motility Assay: A 5 µL sample of the overnight culture was inoculated onto 0.4% nutrient agar and incubated for 24 hours. The swarm diameter on the agar plates was then measured in millimeters **[5]**.

STATISTICAL ANALYSIS

All the experiments were performed in triplicates and the results are expressed as Means ± SD (n=3). The results were analysed for statistical significance using the unpaired Students T-test, One- way ANOVA, and Dunnets test (SPSS Inc. 20. 0 version). Probability values (P) ≤ 0.05 were statistically significant.

RESULTS AND DISCUSSION

Antibacterial activity of bacterial and fungal amylase

The antimicrobial activity of fungal and bacterial amylase was assessed against two organisms wherein bacterial amylase shown maximum activity found with test organism *Pseudomonas aeruginosa* and fungal amylase did not show any activity for *S. aureus* and *P. aeruginosa*. The results are tabulated in **Table 1**.

Minimum Inhibitory Concentration (MIC) of bacterial amylase was performed against *P. aeruginosa* at concentrations ranging from 0.45 mg/ml to 0.0035 mg/ml. The lowest concentration which inhibited the growth of the bacterial strain was taken as the MIC value. The MIC of bacterial amylase against *P. aeruginosa* was 0.007 mg/ml. The results are tabulated in **Table 2**.

Antibiofilm Activity by Crystal Violet Assay Method

The **IC 50** values of the bacterial amylase against *S. aureus and P. aeruginosa* were 37.696 & 37.431 mg/ml whereas for the fungal amylase was recorded to be 52.654 mg/ml & 54.32 mg/ml respectively. The Percentage of biofilm inhibition is depicted in **Figure 1A & 1B**.

Table 1: Antibacterial screening of bacterial amylase against *P. aeruginosa* and *S. aureus*

Organisms	Concentration of Bacterial amylase (mg/mL)	Zone of inhibition (mm)			Mean ± SD
		Trial 1	Trial 2	Trial 3	
Staphylococcus aureus	500	-	-	-	-
	250	-	-	-	
	125	-	-	-	
Pseudomonas aeruginosa	500	26	27	26	26.3 ± 0.577
	250	25	25	26	25.3 ± 0.577
	125	23	24	24	23.6 ± 0.577

Data shown are the average and standard deviation based on triplicate runs (Mean + Standard Deviation)

Table 2: Minimum Inhibitory Concentration of bacterial amylase

Organisms	Concentration of bacterial amylase (mg/mL)	Zone of inhibition (mm)			Mean ± SD
		Trial 1	Trial 2	Trial 3	
Pseudomonas aeruginosa	125	32	31	32	31.66 ± 0.577
	62.5	30	30	31	30.33 ± 0.577
	31.2	29	27	29	28.33 ± 1.154
	15.6	26	27	27	26.66 ± 0.577
	7.8	25	26	25	25.33 ± 0.577
	3.9	25	25	24	24.66 ± 0.577
	1.95	24	25	25	24.66 ± 0.577
	0.97	24	23	23	23.33 ± 0.577
	0.48	23	22	23	22.66 ± 0.577
	0.224	23	23	21	22.33 ± 1.154
	0.1125	22	21	22	21.66 ± 0.577
	0.0562	21	22	21	21.33 ± 0.577
	0.0281	21	20	20	20.33 ± 0.577
	0.014	21	21	20	20.66 ± 0.577
	0.007	18	16	16	16.66 ± 1.154
	0.0035	-	-	-	-

Data shown are the average and standard deviation based on triplicate runs (Mean + Standard Deviation)

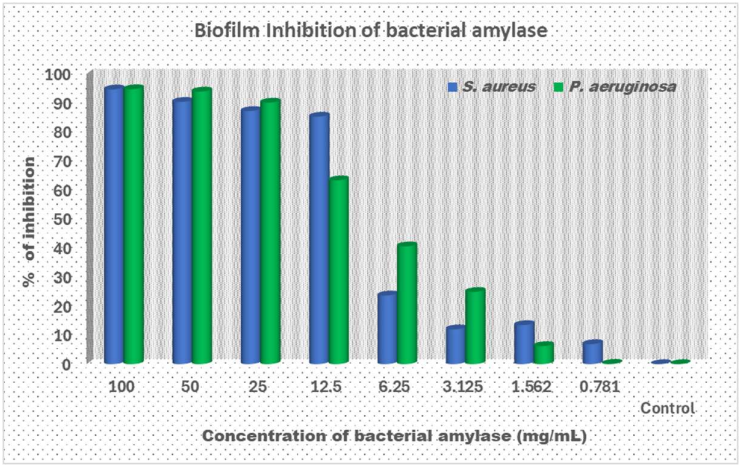

Figure 1A: Biofilm inhibition of bacterial amylase against *S. aureus* and *P. aeruginosa*

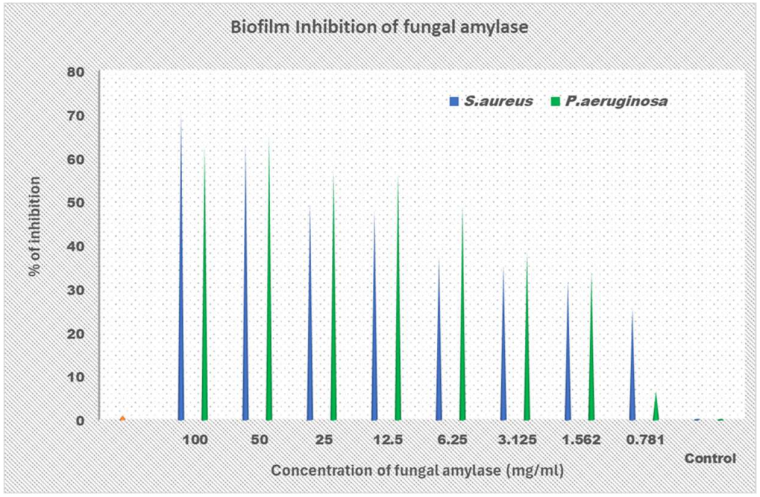

Figure 1B: Biofilm inhibition of fungal amylase against *S. aureus* and *P. aeruginosa*

Assessment of virulence factors of *Pseudomonas aeruginosa* in the presence *of fungal amylase*.

The fungal amylase and bacterial amylase were able to reduce the elastase production, pyocyanin production and swarming motility assay in *P. aeruginosa* (**Figure 2A, 2B & 2C**).

Las B Elastolytic Assay

The elastase inhibition with the fungal amylase and bacterial amylase were measured at 495nm. OD value of the control was found to be 0.355 nm and the fungal amylase was 0.349nm and bacterial amylase was 0.314nm. There was 1.69% elastase inhibition with fungal amylase and 11.549% elastase inhibition by bacterial amylase.

Pyocyanin Reduction Assay

The pigment yield of the control and the samples were calculated by multiplying with 17.072. The pigment yield of the control was calculated as 47.118 µg/ml and the fungal amylase was found as 13.79µg/ml and bacterial amylase was found as 1.67µg/ml. There was 70.73% pigment reduction by fungal amylase and 96.45% pigment reduction by bacterial amylase.

Swarming Motility Assay

There was a significant difference in the swarming motility exhibited by the control and fungal and bacterial amylase. The control showed 19 mm diameter zone of inhibition whereas the Fungal amylase shown 5mm diameter zone of inhibition and bacterial amylase shown 7mm zone of inhibition.

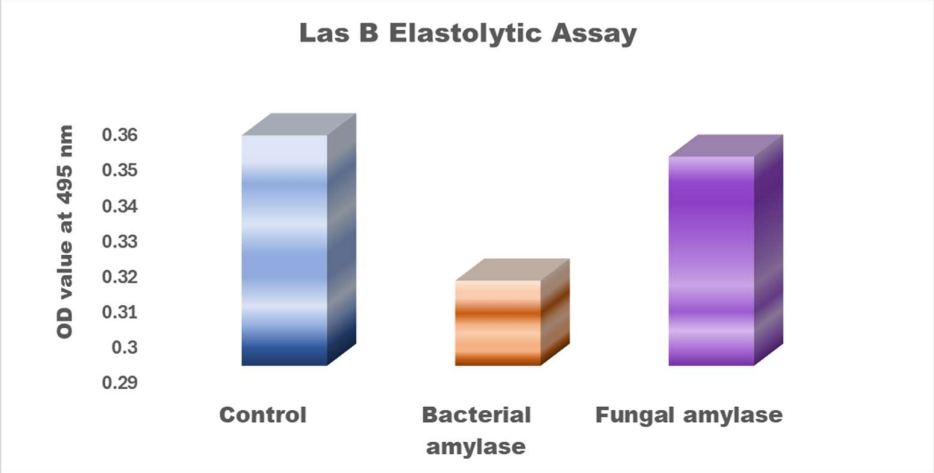

Figure 2A: Las B Elastolytic Assay of fungal and bacterial amylase against *P. aeruginosa*

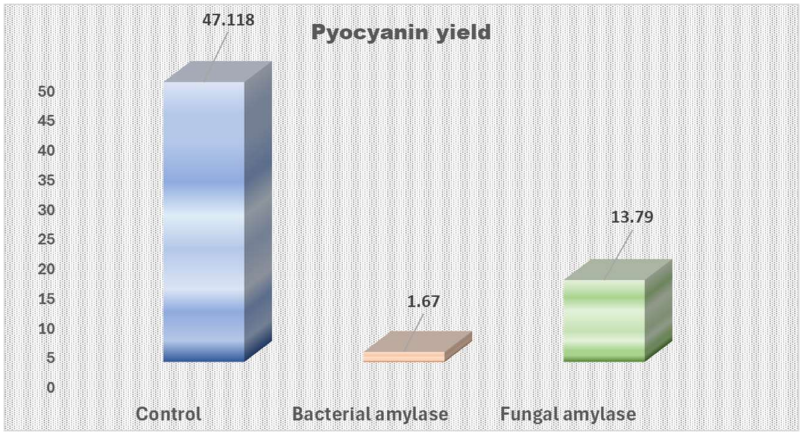

Figure 2B: Pyocyanin Reduction Assay of fungal and bacterial amylase against *P. aeruginosa*

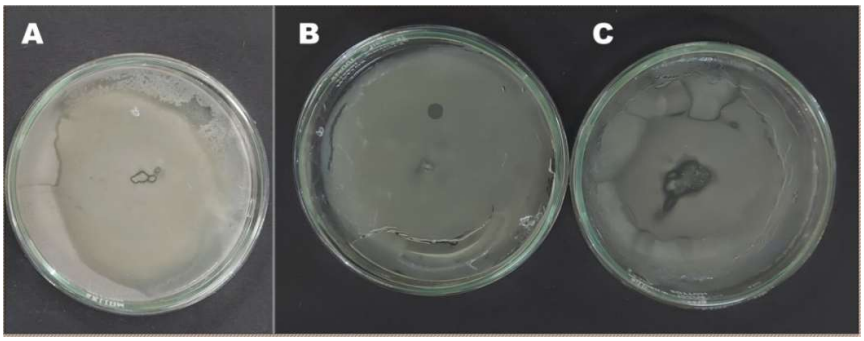

Figure 2C: Swarming motility assay against *P. aeruginosa*
A -fungal amylase (1- Control, 2- fungal amylase), bacterial amylase (1- Control, 2- Bacterial amylase)

DISCUSSION

Out of the major three menaces to global public health is multidrug resistance that is principally caused by over usage of drug or prescription, use of microbicidal agents inappropriately, and use of low-quality drugs. The comprehension of the mechanism of resistance of these bacteria is essential for the designing of new microbicidal agents or other alternative strategies to tackle these clinical problems **[6]**. The primary objective of the study was to evaluate the antimicrobial activity of fungal and bacterial amylase against nosocomial infections causing organisms. Both bacterial as well as fungal amylase was subjected to antibacterial activity by Kirby Bauer method followed by the Minimum Inhibitory Concentration of the bacterial amylase which has shown promising activity against *S. aureus* and *P. aeruginosa*. Though previous studies facilitated the potential of bacterial amylase as potent biofilm inhibitor **[7]**, the antimicrobial activity of the same has not been carried out against *P. aeruginosa* and *S. aureus.*

Both bacterial and fungal amylase proved to a potent biofilm inhibitor wherein different assays were carried out like Pyocyanin Inhibition, Swarming motility and elastase production.

The objective of checking the antimicrobial activity of fungal amylase was implemented for the first time though attempts were done in isolating fungal amylase from various fungi **[8]**. The comparative antimicrobial activity and biofilm inhibiting potency of bacterial and fungal amylase was researched for the first time which would really help to narrow down the isolation process of amylase for medicinal uses.

CONCLUSION

The antimicrobial activity of fungal and bacterial amylase was assessed against two organisms wherein bacterial amylase showed maximum activity with the test organism *P. aeruginosa* whereas fungal amylase did not show any activity against *S. aureus* and *P. aeruginosa*. In context to this Minimum Inhibitory Concentration (MIC) of bacterial amylase was performed against *P. aeruginosa* at concentrations ranging from 0.45 mg/ml to 0.0035 mg/ml. The lowest concentration which inhibited the growth of the bacterial strain was taken as the MIC value. The MIC of bacterial amylase against *P. aeruginosa* was 0.007 mg/ml. The secondary phase of the study focused on the biofilm inhibition of both bacterial and fungal amylase. The biofilm inhibition activity of the fungal and bacterial amylase was performed against the organisms namely, *S. aureus* and *P. aeruginosa*. The inoculum without the sample was taken as the control and Tetracycline was used as the positive control. Two test concentrations of the sample were taken from 100 – 0.781 mg/mL for both samples. The IC 50 values of the bacterial amylase against *S. aureus* and *P. aeruginosa* was 37.696 & 37.431 mg/ml whereas for the fungal amylase was recorded to be 52.654 mg/ml & 54.32 mg/ml respectively. The antibiofilm activity of both amylases were followed by the assessment of the inhibition of virulence factors of *P. aeruginosa*; resulted in the reduction of the elastase production, pyocyanin production and swarming motility assay of *P. aeruginosa*. There was 1.69% elastase inhibition with fungal amylase and 11.549% elastase inhibition by bacterial amylase. The fungal amylase and bacterial amylase were able to inhibit the production of pyocyanin wherein the OD value of the control was measured as 2.76 nm and the fungal amylase and bacterial amylase was measured as 0.808 nm and 0.098nm respectively. The pigment yield of the control was calculated as 47.118 µg/ml and the fungal amylase was found as 13.79 µg/ml and bacterial amylase 1.67 µg/ml. There was 70.73% pigment reduction by fungal amylase and 96.45% pigment reduction by bacterial amylase by Pyocyanin Reduction Assay. There was a significant difference in the swarming motility exhibited by the control and fungal and bacterial amylase thus emphasizing the

application of bacterial and fungal amylase as potent biofilm inhibitors.

DECLARATIONS

Ethics Approval and Consent to Participate

"This article does not contain any studies with human participants or animals performed by any of the authors."

Competing Interests

Visnupriya V., Kavya K and Tessy Anu Thomas "declare that they have no conflict of interest."

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There are no funding bodies in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors Contributions

The first and the second author, Vishnupriya V. and Kavya K. have carried out the present study under the supervision and guidance of the corresponding author, Dr. Tessy Anu Thomas.

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