

## Article

# Dissolution of Biofilm Secreted by Three Different Strains of *Pseudomonas aeruginosa* with Bromelain, N-Acetylcysteine, and Their Combinations

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**Featured Application:** BromAc a combination of bromelain and NAC, can eradicate mesh biofilms produced by three strains of *Pseudomonas aeruginosa* in vitro, with biofilm dissolution efficacy rates of 80% and above.



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**Abstract:** Bacterial infection of hernia mesh with the formation of biofilms presents a barrier to antibiotic treatment with subsequent surgical intervention and hospitalization. Hence, in the current study, we examined the effect of BromAc, a mucolytic agent, on the dissolution of biofilm formed by three different strains of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* was carefully grown on hernia mesh and treated with various concentrations of bromelain, NAC, and their combinations at 37 °C over 4 h in vitro. Then, the biofilm dissolution activities of the agents were evaluated. Moreover, the combination index (CI) was analyzed to determine the synergy of the bromelain and NAC combination. The results indicated that biofilms were more susceptible to degradation by bromelain, whilst NAC showed growth enhancement in two of the strains. However, in combination (BromAc), the three strains were dramatically affected by the agents, with more than 80% debridement for a suitable combination of bromelain and NAC that was also strain-specific. Hence, the current study shows that the biofilms formed by these three strains of *Pseudomonas aeruginosa* were adversely affected by a single treatment of BromAc, with more than 80% debridement, indicating that subsequent treatment may abolish the biofilm completely.

**Keywords:** acetylcysteine; antibiotic resistance; biofilm; bromelain; *Pseudomonas aeruginosa*

## 1. Introduction

With the advent of modern medicine, many implantable medical devices such as breast, dental, and gluteal implants; orthopedic devices; heart valves; stents; pacemakers; hernia mesh; and a host of others have been introduced with either cosmetic or curative intent. Although these implants are successful in treating several diseases, as well as enhancing cosmetic appearance, they are prone to bacterial colonization and infection, which are often treated with antibiotics [1–3]; however, chronic recurrence often leads to hospitalization with surgical removal or debridement [4,5].

Various bacteria are known to colonize these implants, while the type of bacteria infecting certain types of implants seems to be specific [6–8] and treatment response may be organism-specific. The results for hernia repair treatments have been improved with the insertion of surgical mesh [9]. Infections in approximately 1% of the groin or up to 8% of

ventral hernia mesh repairs are seen [10]. Although different individual bacteria can invade these implants, sometimes two or three different strains of the same genus may exist in a colony [11,12], with subsequent treatment failure owing to susceptibility differences to bactericides [13]. Further, treatment can be difficult because the invading bacterial colony exudes a slimy secretion (biofilm) over the implanted mesh [14].

The biofilm is a complex material that provides residence for the bacteria, as well as protection against the external environment and bactericides. Although there are some differences in the compositions of biofilms secreted by various bacteria [15], they are similar in their basic architecture and composition. A biofilm is composed of a hydrophobic outer layer of complex carbohydrate with an interior backbone (rigid structure) composed of protein [16]. Whilst the main component of the biofilm is the polysaccharide, a hexosamine comprising mainly N-acetyl-D-glucosamine residues with  $\beta$  (1,6)-linkages, the secreted protein within the matrix with amyloid-type behavior forms protein fibrils that inter-link themselves to form the rigid backbone of most biofilms [17]. The extracellular polymeric substances (EPS) secreted by the bacteria contain micro-channels for the inflow of water and nutrients, whilst at the same time providing a barrier from the external environment [18,19]. Additionally, other vital protein molecules that provide adhesion of bacteria to the biofilm and the host tissue, known as cell wall adhesins (CWA) [20], provide an immunomodulatory function for evasion from the host's innate immune system [21,22]. Overall, the biofilm provides a safe residence for the colonizing bacteria. Hence, the prevention of the formation of biofilms will prevent the colonization of these pathogens whilst improving the safety of implants.

Treatment of bacterial infection is often difficult, since several mechanisms exist to preserve or protect bacterial colonies in biofilms. Indeed, the surface charges on the EPS may either repel or attract antibiotics with subsequent repulsion or penetration through the biofilm, resulting in the observed differential antibacterial action [23,24]. Differential antibacterial action amongst antibiotics may also be related to their mode of action, whilst at the same time residual exposure may increase resistance owing to the development of endogenous mechanisms within the bacteria against antibiotics [25]. Bacteria can also enter a resting state or sporulate with the development of very high resistance to current antibiotic therapy [26]. Further, the size of the micro-channels may regulate the passage of antibiotics [27]. Therefore, resistance to antibiotics may be due to exogenous or endogenous mechanisms. Hence, in light of the current information on the structure of biofilms, agents that disintegrate biofilms may act as an effective therapy to destroy the matrix within which bacteria reside, thereby exposing them to the innate immune system [28], with further full exposure to bactericides.

Since the main components of biofilms, such as polysaccharides, glycoproteins, and proteins, are good substrates for enzymes, several studies have been undertaken [29,30], with the development of new therapies. Proteins are composed of amino acid residues linked together by peptide bonds, with the protein geometry being dictated by the interlinking disulfide bridges [29]; hence, the destruction of these vital linkages would effectively destroy their physical and biochemical properties [30]. In the case of EPS, which is composed mainly of polysaccharides and glycoproteins with peptide and glycosidic (-O- and -N-) linkages [31], they may serve as ideal substrates for suitable enzymes.

Bromelain, an enzymic extract from pineapple (*Ananas comosus*) fruit or stem containing proteases, esterase, cellulases, and peroxidases [32], is capable of hydrolyzing complex carbohydrates and proteins with affinity for both peptide and glycosidic linkages [33]. N-acetylcysteine (NAC) is an amino-thiol and is an antioxidant that is widely used for detoxifying acetaminophen (paracetamol) poisoning and as a mucolytic [34,35], since it is an effective reducing agent that can act on disulfide linkages in complex polymeric proteins. Additionally, both these agents have also been shown to have antimicrobial properties [36,37]. Currently, the combination of these two agents (BromAc) has been used to depolymerize the mucinous mass secreted in a rare cancer, pseudomyxoma peritonei (PMP), and is undergoing successful clinical evaluation [38]. These mucinous masses are

essentially proteinaceous in a structure that contains glycoprotein (-O- and -N-) linkages with inter-crossing disulfide bridges [39]. The similarity between PMP mucins and biofilm is that they are suitable substrates incorporating the enzymic action of bromelain and reducing potential of NAC. Hence, in light of the successful treatment of the mucinous mass in PMP, we undertook an initial investigation to determine the effects of BromAc on biofilm formed by *Pseudomonas aeruginosa* [40].

## 2. Materials and Methods

### 2.1. Microorganisms and Medium

*Pseudomonas aeruginosa* (ATCC 27853, PA01 3981, ATCC 31461) strains were screened and selected for their biofilm-forming abilities. Bacteriological agar was obtained from AMRESCO (Cat# J637-500G, Radnor, PA, USA).

### 2.2. Materials

Tissue reinforcement mesh was obtained from GORE® BIO-A® (Cat# FS2030, Newark, DE, USA) and was stored at room temperature and cut suitably in a sterile biosafety cabinet. Michel metal clips measuring 7.5 × 1.75 mm were autoclaved before use.

### 2.3. Treatments

*N*-acetylcysteine was obtained from Link Pharma (Cat# AUST R 170803; Warriewood, NSW, Australia) and stored at 4 °C at a concentration of 200 mg/mL. Bromelain was provided by Mucpharm Pty Ltd. (Batch #103-05, Sydney, NSW, Australia) as a sterile powder. The treatment solution (5.0 mg/mL) was prepared in Milli-Q water and the solution was vortexed to ensure all products were completely dissolved before filtration through a 40 µm sterile strainer (Greiner bio-one, Cat# 542 040, Kremsmünster, Austria). The solution was sterile-filtered through a 0.2 µm non-pyrogenic filter (Sartorius, Cat# ST16534-K, Göttingen, Germany). The final product was aliquoted into 1.5 mL vortex tubes and frozen at −20 °C.

### 2.4. Biofilm Formation

The bacterial strains were removed from the −80 °C freezer and incubated overnight in tryptic soy broth (TSB) at 37 °C with agitation at 110 rpm. After overnight incubation, the absorbance was measured and adjusted to an optical density (OD<sub>600</sub>) of 0.5 McFarland Standard with TSB. The suspension was aliquot into 24-well polystyrene plates either with or without mesh or metal clips according to experimental requirements. Negative control wells were included for each plate. The plates were then incubated for 24 h at 37 °C, after which the supernatant was decanted, and the preformed biofilms were washed gently with phosphate-buffered saline (PBS) to remove the planktonic bacteria whilst preserving the biofilm.

### 2.5. Treatment of Preformed Biofilms

After washing the preformed biofilms, each well was treated in replicates with either bromelain or *N*-acetylcysteine in 5% dextrose as single agents or in combination. The concentrations were adjusted according to the experiment's requirements. Bromelain and NAC concentrations were chosen based on initial work with biofilms formed by the different strains to determine minimal and maximum concentrations required for effective debridement of the biofilms. The selected bromelain and NAC concentrations also provided a large experimental range with a two-fold increase per treatment. The lowest examined concentrations provided no eradication effects in the laboratory. The highest examined concentrations provided 50–70% eradication, as complete eradication was not the aim of this study—the aim was to determine whether the combination of NAC and bromelain produced better results than for the single agents. The plates were incubated for 4 h at 37 °C. Following treatment, the supernatant was removed carefully without disrupting the biofilm architecture prior to crystal violet assays.

## 2.6. Biofilm Assays

Crystal violet (CV) staining obtained from plating was used to measure the susceptibility of preformed biofilms to each treatment. For CV staining, the biofilms were washed with PBS thrice then fixed with 99% methanol for 15 min and air-dried. The fixed biofilms were then stained with 0.5% solution of crystal violet for 10 min and excess dye was removed by washing with PBS. The plates were allowed to dry at room temperature. The biofilms were then quantified by dissolving the CV dye within with 33% acetic acid and measuring the absorbance values at 650 nm in a microplate reader. All experiments were replicated to ensure validity.

The percentage biofilm dissolution (BD) was calculated as follows:

$$\% \text{ BD} = [\text{OD}_{\text{Control}} - \text{OD}_{\text{Treated}} / \text{OD}_{\text{Control}}] \times 100$$

## 2.7. Statistical Analysis

Statistical analysis was performed on all results using GraphPad Prism version 9 for Windows, (GraphPad Software, San Diego, CA, USA). One-way and two-way analysis of variance (ANOVA) and Dunnett's test were used to statistically compare treated and control group means. Here, *p*-values of <0.05 were considered statistically significant. The combination index (CI) was analyzed using CompuSyn software (ComboSyn Incorporated., Paramus, NJ, USA). CI values were assessed according to the Chou and Talalay method, where values below 0.95 were classified as synergistic, >0.95–1.5 were additive, and above 1.5 were sub-additive [41].

## 3. Results

Three different strains of *Pseudomonas aeruginosa* (ATCC 27853, PA01 3981, and ATCC 31461) were treated with either bromelain, *N*-acetylcysteine, or their combinations for 4 h. The maximum biofilm dissolution (BD MAX) was assessed for each agent as well as their combinations.

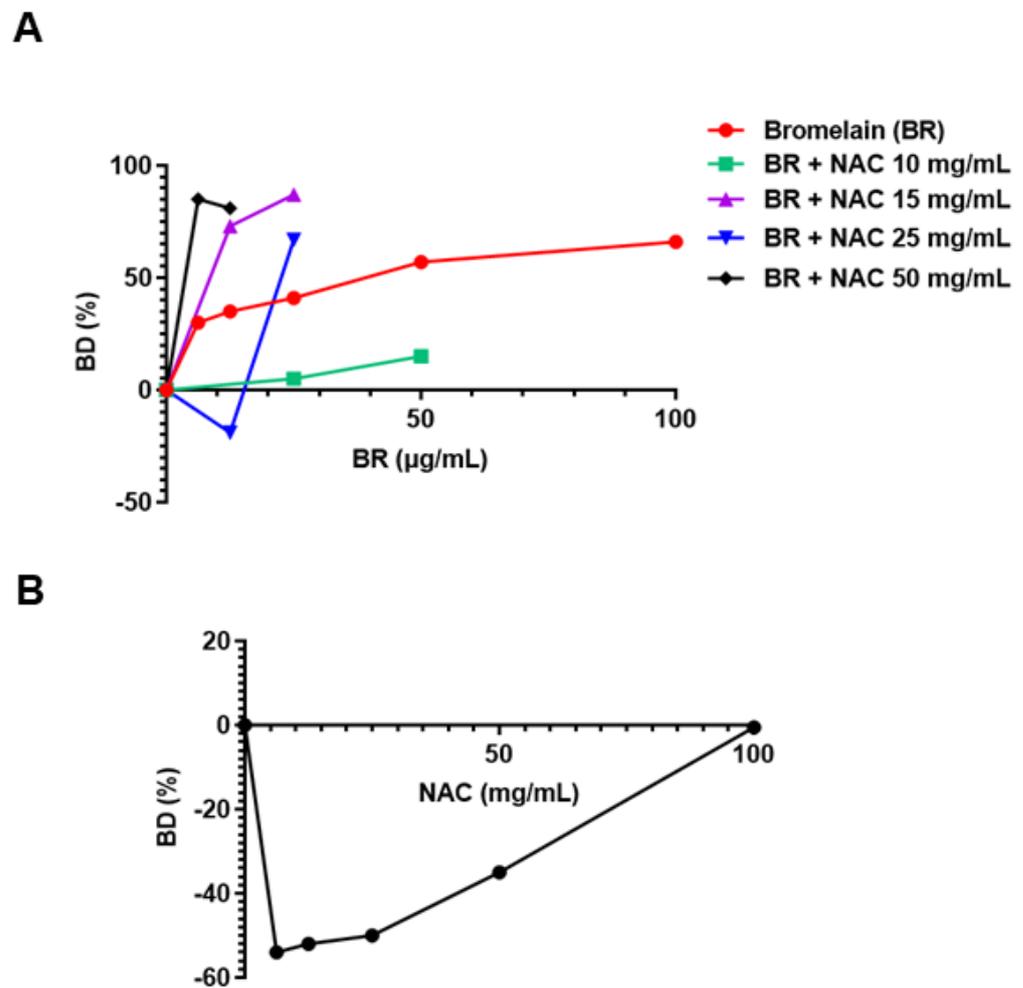
### 3.1. *Pseudomonas aeruginosa* ATCC 27853

#### 3.1.1. As Single Agents

Treatment with bromelain as a single agent (6.25, 12.5, 25, 50, 100 µg/mL) resulted in the highest dissolution of the biofilm at 100 µg/mL with 66% (BD MAX) as compared to control (Figure 1A and Table 1), while bromelain at 3.125 µg/mL showed 0% biofilm eradication. NAC as a single agent enhanced the growth of the biofilm (−54%) (Figure 1B and Table 1).

**Table 1.** The biofilm dissolution action of individual agents on the biofilm secreted by *Pseudomonas aeruginosa* ATCC 31461, PA01 3981, and ATCC 27853. Only values with *p* < 0.05 are shown in the table. Note: 176 BD MAX (%): Maximum Biofilm Dissolution (%).

<i>P. aeruginosa</i>	AGENT	Concentration	BD MAX (%)
ATCC 27853	Bromelain	6.25, 12.5, 25, 50, 100 µg/mL	66
	<i>N</i> -acetylcysteine	6.25, 12.5, 25, 50, 100 mg/mL	−54
PA01 3981	Bromelain	3.125, 6.25, 12.5, 25, 50, 100 µg/mL	45
	<i>N</i> -acetylcysteine	3.125, 6.25, 12.5, 25, 50, 100 mg/mL	3.0
ATCC 31461	Bromelain	3.125, 6.25, 12.5, 25, 50, 100 µg/mL	75
	<i>N</i> -acetylcysteine	6.25, 12.5, 25, 50, 100 mg/mL	57



**Figure 1.** (A) Dissolution of biofilm formed by *Pseudomonas aeruginosa* ATCC 27853 by bromelain and *N*-acetylcysteine. The graph shows biofilm dissolution (BD) > 80% of biofilm when bromelain was combined with 15, 25, or 50 mg/mL *N*-acetylcysteine (NAC). Bromelain at 100 µg/mL only gave a BD of 66%; however, in suitable combinations, the BD was above 80%, indicating very good efficacy. Further, NAC 10 mg/mL + bromelain 25 or 50 µg/mL gave very low BD values. (B) Dissolution effect of *N*-acetylcysteine on biofilm secreted by *Pseudomonas aeruginosa* ATCC 27853. The graph indicates negative dissolution activity (biofilm growth) with all additions of NAC, which returns to 0% BD at NAC 100 mg/mL.

### 3.1.2. Combination of Agents

*N*-acetylcysteine 15.0 mg/mL in combination with 25.0 µg/mL bromelain displayed the highest biofilm dissolution (87%) amongst all combinations. The combination of NAC 50 mg/mL with either 6.25 or 12.5 µg/mL bromelain also displayed rather good dissolution values (85% and 80.5%, respectively), with CI values indicating good synergy (Figure 1A and Table 2).

For this particular strain of bacteria, the combination of bromelain with NAC performed very well at three different concentrations, as shown in Table 2 (highlighted in bold font).

**Table 2.** The biofilm dissolution actions of individual agents and their combinations on the biofilm secreted by *Pseudomonas aeruginosa* ATCC 31461, PA01 3981, and ATCC 27853. Only values with  $p < 0.05$  are shown in the tables. Additions of agents with significant effects are highlighted in bold font. Note: BD MAX (%): Maximum Biofilm Dissolution (%); Antagonistic: CI value  $> 2.0$ .

<i>Pseudomonas aeruginosa</i>	N-Acetylcysteine (mg/mL)	Bromelain ( $\mu$ g/mL)	BD MAX (%)	Combination Index (CI)
ATCC 27853	15.0	12.5	73	0.063
		25.0	<b>87</b>	<b>0.025</b>
	25.0	12.5	−19	antagonistic
		25	67	0.209
	50	6.25	<b>85</b>	<b>0.008</b>
		12.5	<b>80.5</b>	<b>0.03</b>
PA01 3981	10.0	20	33	0.672
		40	50	0.268
	15.0	12.5	66	0.019
		25	71	0.022
	25	12.5	<b>95.5</b>	<b>0.001</b>
		25	<b>97</b>	<b>0.001</b>
50	6.25	<b>88</b>	<b>0.009</b>	
	12.5	<b>95.3</b>	<b>0.003</b>	
ATCC 31461	10.0	20,0	13	7.34
		40.0	27	5.76
	15.0	12.5	50	0.819
		25	<b>81</b>	<b>0.403</b>
	25.0	12.5	<b>80</b>	<b>0.357</b>
25.0		<b>85</b>	<b>0.395</b>	
50	6.25	21	2.195	
	12.5	<b>79</b>	<b>0.581</b>	

### 3.2. *Pseudomonas aeruginosa* PA01 3981

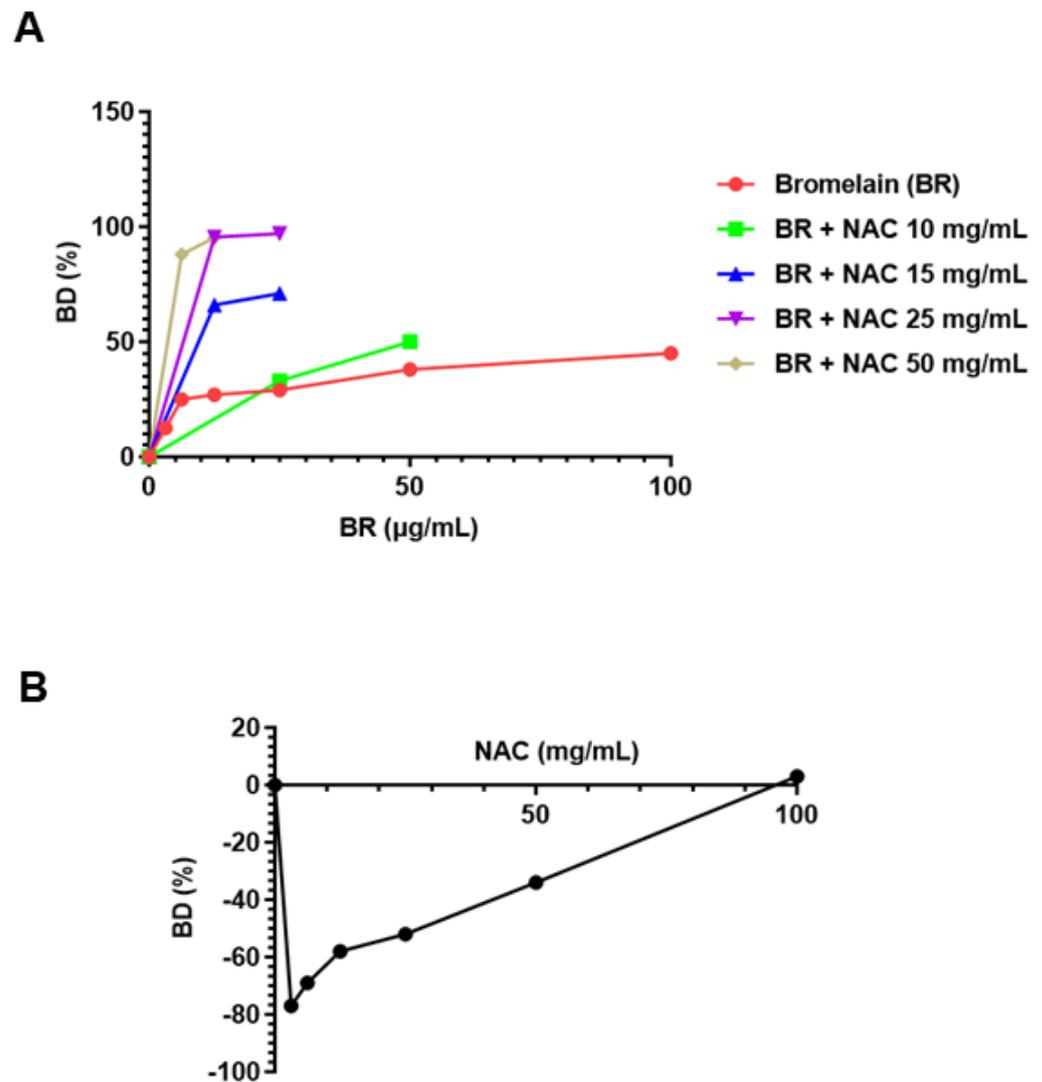
#### 3.2.1. As Single Agents

Treatment with bromelain as a single agent (3.125, 6.25, 12.5, 25, 50, 100  $\mu$ g/mL) resulted in the highest dissolution of the biofilm at 100  $\mu$ g/mL with a value of 45% (BD MAX) as compared to control (Figure 2A and Table 1), whilst NAC as a single agent affected the biofilm by only 3.0% (Figure 2B and Table 1).

#### 3.2.2. Combination of Agents

N-acetylcysteine 25.0 mg/mL in combination with either 12.5 or 25  $\mu$ g/mL bromelain displayed the most efficient biofilm dissolution values (BD MAX = 95.5 and 97%, respectively) amongst all combinations in a synergistic manner. The combination of NAC 50 mg/mL with either 6.25 or 12.5  $\mu$ g/mL bromelain also displayed good dissolution values (BD MAX = 88% and 95.3,) respectively), with CI values indicating good synergy (Figure 2A and Table 2).

Hence, in this particular strain of bacteria, the combination of bromelain with NAC performed very well at four different combinations (BD MAX 88–97%), as shown in Table 2 (highlighted).

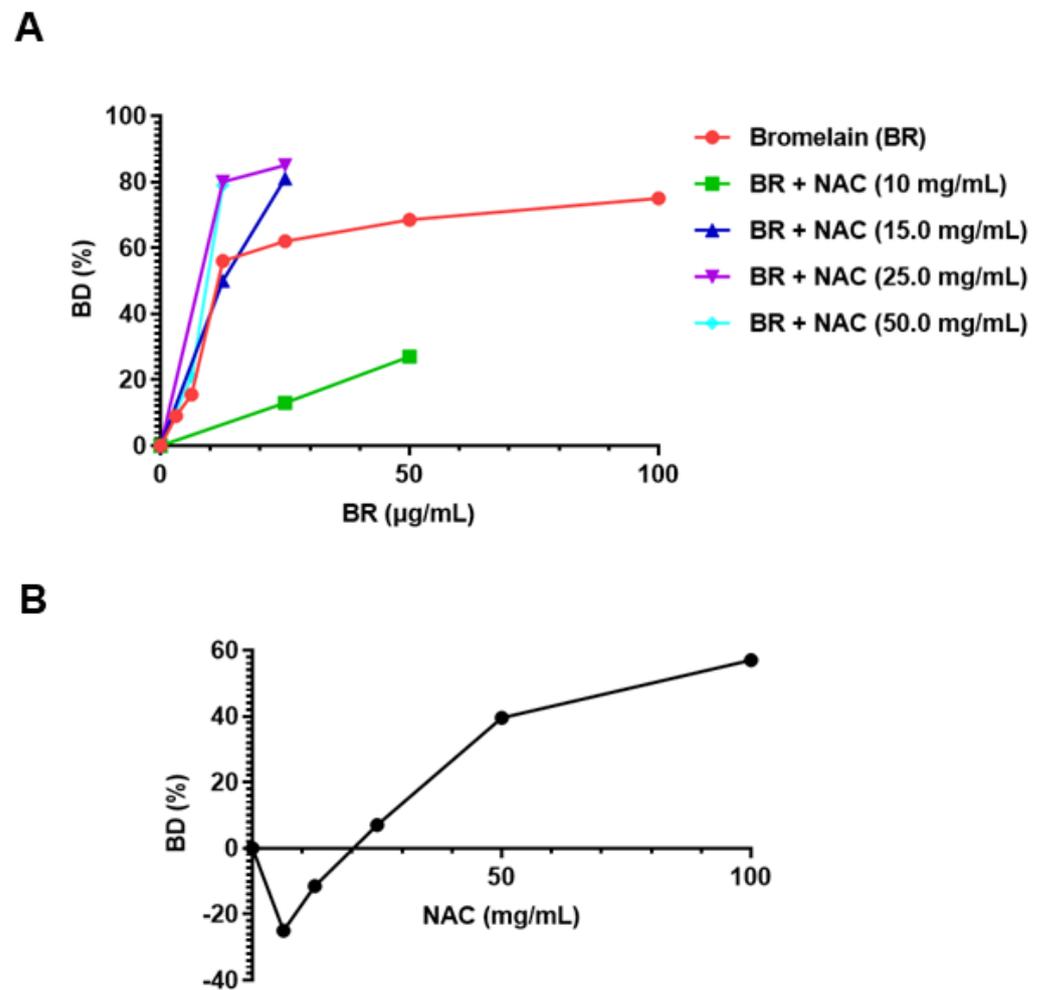


**Figure 2.** (A) Dissolution of biofilm formed by *Pseudomonas aeruginosa* PA01 3981 by bromelain and *N*-acetylcysteine. The graph shows that the addition of *N*-acetylcysteine (NAC) at 15, 25, or 50 mg/mL to bromelain increased the biofilm dissolution (BD) substantially (well above 80%). Bromelain (3.125–100 µg/mL) alone did not even produce 50% BD. (B) Dissolution effect of *N*-acetylcysteine on biofilm secreted by *Pseudomonas aeruginosa* PA01 3981. The graph shows a negative effect (enhancement of biofilm growth) with the initial small addition of NAC, which reduced its negative activity with an increase in NAC concentration.

### 3.3. *Pseudomonas aeruginosa* ATCC 31461

#### 3.3.1. As Single Agents

Treatment with bromelain as a single agent (3.125, 6.25, 12.5, 25, 50, 100 µg/mL) displayed the highest biofilm dissolution at 100 µg/mL with a value of 75% (BD MAX) as compared to control (Figure 3A and Table 1), whilst NAC as a single agent resulted in dissolution of the biofilm (57%), indicating that the two agents affected the biofilm to varying degrees, with bromelain being more effective than NAC (Figure 3B and Table 1).



**Figure 3.** (A) Dissolution of biofilm formed by *Pseudomonas aeruginosa* ATCC 31461 by bromelain and *N*-acetylcysteine. The graph shows that with the addition of 15, 25, and 50 mg/mL *N*-acetylcysteine (NAC) to bromelain, the biofilm dissolution (BD) values were above 80%, indicating good dissolution of biofilm. Bromelain alone showed a maximum biofilm dissolution (BD MAX) of 75% at 100 µg/mL. (B) Dissolution effect of *N*-acetylcysteine on biofilm secreted by *Pseudomonas aeruginosa* ATCC 31461. The graph indicates that NAC below 25 mg/mL had a negative effect (enhancement) on biofilm growth, while at 50 and 100 mg/mL NAC showed about 40–57% BD MAX values, indicating that it had a dissolution effect on biofilm.

### 3.3.2. Combination of Agents

*N*-acetylcysteine 25.0 mg/mL in combination with 25.0 µg/mL bromelain displayed the highest biofilm dissolution value (85%) amongst all combinations. The combinations of NAC 15 mg/mL + 25 µg/mL bromelain, 25.0 mg/mL NAC + 12.5 µg/mL Bromelain, and 50 mg/mL NAC + 25 µg/mL bromelain showed good biofilm dissolution rates of about 80% in a synergistic manner, as indicated by the CI values below 0.5 (Figure 3A and Table 2).

Hence, in this particular strain of bacteria, the combination of bromelain with NAC performs very well at four different concentrations, as shown in Table 2 (with highlight).

### 3.4. Comparison of Maximum Biofilm Dissolution (BD MAX) Values of the Two Different Agents on Different Strains of *Pseudomonas aeruginosa*

Amongst the three strains of *Pseudomonas aeruginosa*, bromelain at 100 µg/mL was most active in the dissolution of biofilm in ATCC 31461, whilst being slightly less active in ATC 27853 (75% vs. 66%, respectively). Bromelain was least active in PA01 3981, with BE MAX value of 45% (Table 3).

**Table 3.** Comparison of maximum biofilm dissolution (BD MAX) activity levels of bromelain (BR) and *N*-acetylcysteine (NAC). OA = order of activity. Note that negative values indicate the synthesis or growth of biofilm.

<i>Pseudomonas aeruginosa</i>	BR 100 µg/mL	OA	NAC 100 mg/mL	OA
ATCC 27853	66	2	−54	3
PA01 3981	45	3	3	2
ATCC 31461	75	1	57	1

NAC at 100 mg/mL resulted in negative dissolution (enhancement of biofilm formation) in ATCC 27853, whilst it had a very minor effect (3%) in PA01 3981. On the contrary, it was comparatively very active with 57% dissolution in ATCC 31461 (Table 3).

The biofilms generated by all three strains of *Pseudomonas aeruginosa* reacted very well to the dissolution treatment of a combination of bromelain and *N*-acetylcysteine (BromAc), with BD MAX values ranging from 79 to 97%, indicating good efficacy (Table 4).

**Table 4.** Comparison of maximum biofilm dissolution (BD MAX 80% and above) using a combination of agents in the three strains of *Pseudomonas aeruginosa*. The sensitivity to *N*-acetylcysteine with 50 mg/mL + bromelain 12.5 µg/mL indicates that strain PA01 3981 was the most sensitive, whilst the other two strains were almost equally sensitive. CI = combination Index.

		ATCC 27853		PA01 3981		ATCC 31461	
NAC (mg/mL)	BR (µg/mL)	BD MAX (%)	CI	BD MAX (%)	CI	BD MAX (%)	CI
15	25	87	0.025	-	-	81	0.403
25	12.5	-	-	95.5	0.001	80	0.357
25	25	-	-	97	0.001	85	0.395
50	6.25	85	0.008	88	0.009	-	-
50	12.5	80.5	0.03	95.3	0.003	79	0.581

There was a slight individual variation between the biofilms generated by the strains, with PA01 3981 being slightly more receptive and with the highest BD MAX value range of 88–97% depending on the concentrations of the two agents used. A combination of 25 mg/mL NAC with 25 µg/mL bromelain produced the highest % of biofilm dissolution (BD MAX) in PA01 3981. The biofilms formed by the other two strains ATCC 27853 and ATCC 31461 appeared to be almost equally affected by the treatment at equivalent combinations, i.e., 15.0 mg/mL NAC + 25 µg/mL bromelain had BD MAX values of 87% vs. 81%. Similarly, with a combination of 50 mg/mL NAC with 12.5 µg/mL bromelain, the BD MAX values were 80.5% vs. 79%. Overall, the combination of bromelain and NAC (BromAc) gave dissolution values (BD MAX) of 80% and above in the three strains of *Pseudomonas aeruginosa*, indicating the efficacy of BromAc (Table 4).

#### 4. Discussion

Bacterial colonization on surgical implants may be treated with bactericides; however, the presence of biofilms presents a barrier to the treatment, mainly because they impede drug penetration and act as reservoirs for bacterial colony formation with protection from the host's innate immune system. Hence, the removal of the biofilm alone may eradicate the bacterial colonies, since they will become more susceptible to the immune system, while subsequent treatment with bactericides may result in complete eradication [42].

Therefore, in the present study, we investigated the dissolution action of BromAc (bromelain and *N*-acetylcysteine) on biofilm formed by *Pseudomonas aeruginosa* using three different strains in order to study the efficacy, with variability shown within the strains. Bromelain as a single agent was very effective at certain concentrations, showing some variability within the strains. Strain ATCC 31461 was the most sensitive, with a BD MAX of 75%, whilst PA01 3981 was less sensitive, with a BD MAX of 45%. Treatment with NAC

showed that strain ATCC31461 was the most sensitive (BD MAX = 57%), whilst ATCC 27853 was not only the least sensitive but showed biofilm growth (enhancement) in the presence of NAC (BD MAX = −54%). These variations may be due to differences in the compositions of the biofilms, as well as other variables within the strain.

Bromelain may act as a proteolytic agent, hydrolyzing the peptide and glycosidic bonds found within the glycoproteins and proteins in the biofilm and simultaneously hydrolyzing complex carbohydrates [33]. These multiple enzymic actions may be due to bromelain's complex composition, having numerous enzymes [32]. Hence, it reduced the expression of the biofilm as a single agent. However, the reducing action of NAC (antioxidant) largely depends on the disulfide bridges found within the biofilm and favored in strain ATCC 31461, whilst in strain ATCC 27854, it acted in an opposite manner, providing more than 50% extra growth. This may indicate that this particular strain may be able to use the amino thiol as a source of sulfur and nitrogen for protein synthesis, as well as to enhance the formation of glycosidic linkages with subsequent bacterial multiplication and ensuing biofilm formation; however, this needs further investigation. The abundance of biofilm is directly related to the density of the invading bacteria. The destruction of the bacteria may relate directly to the abundance of biofilm. Further studies on the viability of these microbes in the presence of these agents may provide information that may clarify the distinction between microbial density and biofilm abundance.

Comparing the three agents, it is evident that bromelain is more effective as a biofilm dissolution agent in at least two strains, ATCC 27853 and ATCC 31461 (66 and 75% BD MAX, respectively) (Table 3). Since bromelain possesses antibacterial properties, the dissolution or debridement of the biofilms may also be attributed to the reduction in bacterial colonies, while the biofilm reduction may be attributed to both the chemical dissolution activity on the polysaccharide and glycoprotein matrix together with antibacterial action on the bacterial colony; however, this needs further study to determine the presence and magnitude of both these properties of bromelain.

All the three strains of *Pseudomonas aeruginosa* were well affected by a combination of bromelain and NAC (BromAc), indicating BD MAX values of 80% and above (Table 4). This indicates that a suitable combination of these two agents would provide a good therapy to eradicate biofilm. Of note, strain PA01 3981 was most sensitive, with a BD MAX value of 97% at a combination of 25 mg/mL NAC + 25 µg/mL bromelain, with a ratio of 1000:1 on a weight basis. This may also indicate that the high dose of NAC required is reflective of the number of disulfide bonds present within the biofilm. The CI values for all effective combinations indicate very good synergy, with extremely high synergy in the case of strain PA01 3981. The other two strains reacted equally well compared to each other, with BD MAX values of 85–87%, although ATCC27853 showed a BD MAX value of 87% with a combination of 15 mg/mL NAC + 25 µg/mL bromelain (ratio of 600:1 by weight) and for strain ATCC 31461 with a weight ratio of 1000:1 at a BD MAX of 85% (25 mg/mL NAC + 25 µg/mL bromelain). These variations may be related to the slight differences in percentage compositions of proteins, glycoproteins, and carbohydrates, as well as the abundance of biofilm secreted. Further, the low concentration of bromelain required with a relatively high (100-fold) NAC is indicative of the chemical reactivity of the two agents; bromelain is an enzyme that regenerates, whilst NAC, being an antioxidant, is used up during the degradation of the biofilm. The amount of NAC required may also reflect the abundance of disulfide linkages present within the biofilm.

In the clinical setting, delivery of BromAc by either intravenous or oral route would be impractical, since bromelain is readily bound to blood albumin, macroglobulin, and other blood and cellular components, such that it would be impossible to achieve the effective dose used in this study [43]. However, although a high NAC dosage may be given by IV with proven safety, as in acetaminophen toxicity [44], it is envisaged that the medicament is more suitable for local delivery at the site of the mesh or other implants. The safety of BromAc via intraperitoneal delivery has been established both in vivo [45] and in clinical trials [38].

## 5. Conclusions

Since BromAC at suitable concentrations of the two agents (bromelain and NAC) produced biofilm dissolution with a BD MAX values of 80% and above in the three strains of *Pseudomonas aeruginosa*, it appears that subsequent dissolution of the established biofilm with BromAc may enable 100% eradication of the biofilm, with a substantial effect on the bacteria. However, this requires further investigation. Although the current study shows promises for the development of BromAc as a dissolution agent for biofilm in hernial mesh implants and possibly in other implants, further studies are required to detect the antimicrobial action of both agents, particularly in these three strains of *Pseudomonas aeruginosa*.

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