

IN VITRO ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF ULTRAFILTRATED-BROMELAIN AGAINST DENTAL CARIES PATHOGENS

Johari AS¹, Tarmizi NH², Zulkepli NA², Mokhtar N³, Ya'kub MK⁴, and Sarmoko S⁵.

¹IC-Innovation in Biomedical, SIRIM Industrial Research, SIRIM Berhad, 09000 Kulim, Kedah, Malaysia

²Centre for Medical Laboratory Technology Studies, Faculty of Health Sciences, Universiti Teknologi MARA Cawangan Selangor, Kampus Puncak Alam, 42300 Bandar Puncak Alam, Selangor, Malaysia

³Dental Simulation and Virtual Learning Research Excellence Consortium, Department of Dental Science, Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200 Kepala Batas, Pulau Pinang, Malaysia

⁴SMART KJ Agro (Asia) Plt, Bandar Amanjaya, 08000 Sungai Petani, Kedah Darul Aman, Malaysia

⁵Department of Pharmacy, Sumatera Institute of Technology, Lampung, Indonesia

Correspondence:

Nur Ayunie Zulkepli,
Centre for Medical Laboratory Technology Studies,
Faculty of Health Sciences,
Universiti Teknologi MARA Cawangan Selangor,
Puncak Alam Campus,
42300 Bandar Puncak Alam,
Selangor, Malaysia
Email: nayunie@uitm.edu.my

Abstract

Dental plaque on tooth surfaces significantly contributes to dental caries leading to acidic demineralization of the tooth enamel and dentin. This is mainly caused by the formation of pathogenic bacterial biofilm. Dental caries affects approximately 3.5 million people around the world. Compared to synthetic anticariogenic agents such as chlorohexidine, plant-based compounds were found to have fewer side effects and higher economic value. Pineapple (*Ananas comosus*) is one of the largely commercialized plants in Malaysia with beneficial attributes such as its Bromelain enzyme. This study aimed to purify Bromelain enzyme from MD2 pineapple core and to evaluate their antibacterial properties against dental caries-associate pathogens *in vitro*. This study evaluated the effect of Bromelain enzyme isolated from MD2 pineapple core using 50% ammonium sulphate precipitation incorporated with centrifugal ultrafiltration against *Streptococcus mutans*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, and *Lactobacillus casei*. Bromelain enzyme showed effectivity of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for all cariogenic organisms. Ultrafiltrate Bromelain (UFB) enzyme had significant antibacterial activity against *S. mutans* with a MIC value of 250 µg/ml and complete susceptibility at an MBC value of 1000 µg/ml. Biofilm inhibition against *S. mutans* increased from 45.98% to 57.48% with ultrafiltration. A 3.25 purification fold increase was observed in the ultrafiltrate sample. In conclusion, the ultrafiltration technique is efficient in purifying Bromelain enzyme and effectively inhibits *S. mutans* biofilm formation and thus possesses a potential inhibitory effect on dental caries *in vitro*.

Keywords: Bromelain, Biofilm, Pineapple, Dental Caries, Enzyme

Introduction

Periodontal disease is a disease that affects the tissues surrounding the teeth. Common symptoms of periodontal diseases are gingivitis and periodontitis. Gingivitis refers to gum infections that manifest as bleeding and swollen gums from inflammation that can develop into periodontitis, characterized as gum detachment from the tooth and, finally, tooth loss. 50% of the global population suffers from periodontal disease, and the negative impact of periodontal disease on the quality of life is significant

(1). People suffering from periodontal disease suffer from physical pain, functional limitation, psychological discomfort, physical disability, and psychological disability (2). The main factor contributing to the high prevalence of periodontal disease is a bacterial plaque which progresses to dental caries if not treated.

Active dental caries is the formation of lesions resulting from pH imbalances from biofilm formation (3). Homeostatic changes of pathogenic oral biofilm due to sugar consumption will result in a cariogenic, acidogenic,

and aciduric bacterial population which induces caries lesions formation (4). The absence of proper treatment may lead to severe caries lesions, which require drastic measures such as tooth extraction. Cariogenic bacterial species, predominantly *Streptococcus mutans*, cause pH fluctuations by forming lactic acid resulting from its physiological metabolism. Evidence of other bacteria, such as *Lactobacillus spp.*, was also demonstrated, especially in deep lesions contributing to caries progression (5). This genus contributed to 0.1% of the total microbial inhabitant of the saliva (6). Regardless, controlling the growth of cariogenic biofilm would help reduce disease progression.

Countless measures have been hypothesized to deal with caries-associated dental biofilm. Current clinical strategies are prone to restoring pre-formed lesions rather than prevention (7). It is crucial to prevent dental caries from progressing to a much irreversible severe lesions that requires radical counter measure such as tooth removal. Common caries preventative measures include oral care products containing xylitol, chlorhexidine, and fluoride (8). Chlorhexidine is an effective antiplaque removal agent and has been the gold standard in plaque removal; however, chlorhexidine usage has been shown to lead to calculus formation and staining of the teeth. In addition, oral pathogens, especially *S. mutans*, have been reported to gain resistance to chlorhexidine (9).

Biofilm eradication via plant-derived enzymes offers an alternative green strategy to combat dental plaque. Plant-derived bioactive compounds were found to have fewer side effects and economic value than the chemically synthesized anti-cariogenic agents, with the added benefits of being low-cost and readily available (10). For instance, Papain from papaya fruit and Ficin from figs are enzymes studied for their anti-biofilm properties. Both enzymes degrade the biofilm matrix, thus successfully inhibiting biofilms; however, they do not exhibit antibacterial properties, proving that antibacterial does not interrelate (11, 12). Bromelain, an enzyme from pineapples, has been studied for its antibacterial activities due to its proteolytic, anti-oxidative, and anti-inflammatory properties (13, 14). The antibiofilm properties of Bromelain remain vague to this day. Bromelain exists in all parts of pineapple, including the wastes such as the peel, core, and leaves (15). Due to their high commercial value and medicinal properties, the pineapple industry in Malaysia is rapidly evolving, especially hybrid pineapples. The Malaysian Pineapple Industry Board (MPIB) stated that pineapple cultivars such as Smooth Cayenne, Spanish, Queen, and hybrids, including MD2 and Josephine, are primarily grown. Among the other cultivars, the Bromelain content in the cores in the MD2 variety is the highest (16).

Currently, limited studies illustrate using Bromelain enzyme as a potential antibacterial and anti-biofilm agent towards cariogenic bacteria. Thus, this study is focused on elucidating the effect of Bromelain enzyme isolated from MD2 pineapple core against cariogenic bacterial strains such as *Streptococcus mutans*, *Staphylococcus aureus*,

Lactobacillus casei, and *Lactobacillus acidophilus*. The present study also includes the characterization of the Bromelain enzyme alongside its preliminary antibacterial and antibiofilm ability.

Materials and Methods

Plant material

Cores of the MD2 pineapples were obtained courtesy of our collaborative partner, SMART KJ Agro (Asia) PLT, Sungai Petani, Kedah, Malaysia. The cores were the result of industrial pineapple processing, as noted by the company.

Bacterial strains

Purchased organisms (Brand: American Type Culture Collection, ATCC), including *Streptococcus mutans* (ATCC 25175), *Staphylococcus aureus* (ATCC 25923), *Lactobacillus acidophilus* (ATCC 4356), and *Lactobacillus casei* (ATCC 393) were in ATCC specified media. All organism was incubated at optimal temperature and time, respectively.

Culture media

Brain Heart Infusion agar, Brain Heart Infusion broth, DE MAN, ROGOSA, SHARPE (MRS) broth, DE MAN, ROGOSA, SHARPE (MRS) agar was purchased and prepared according to OXOID. The biofilm growth media was made by dissolving sucrose in the Brain Heart Infusion broth making a 2% sucrose solution.

Pre-enzymatic treatment

Fifty ml of juice made by blending pineapple cores with distilled water in a 1:1 ratio. 0.01% of pectinase derived from *Aspergillus niger* (Brand Name: Nacalai Tesque) was mixed with the blended pineapple core juice before any extraction and purification steps were implemented. This was the crude enzyme which appears to be a slightly cloudy liquid with no sediments. The "crude" sample is Bromelain enzyme that has no treatment other than pectinase to digest pectin.

Ammonium sulphate precipitation and centrifugal ultrafiltration

This study used 50% (w/v) of ammonium sulphate for the precipitation step. Ammonium sulphate powder (Brand Name: Nacalai Tesque) was incorporated into 100 ml of pineapple core juice slowly pinch by pinch. The precipitation commenced for 12 hours at 4°C. The mixture was transferred into 50 ml falcon tubes and centrifuged at 13,000 rpm for 10 minutes. The obtained supernatant was removed, and the sediment was resuspended in 5% ethanol cooled at -20°C prior to the experiment. This sample was called the "fractionate" that did not undergo the centrifugal ultrafiltration step after the ammonium sulphate precipitation. The ultrafiltration of precipitated Bromelain enzyme was carried out using Millipore 10 kDa MW cut-off centrifugal units. The ultracentrifugation was

performed at 10,000 rpm for 20 minutes at 4°C. The final product obtained is Ultrafiltrated Bromelain (UFB).

Protein quantification

Total protein concentration was determined by colorimetric detection at 595 nm with Bovine Serum Albumin (BSA) as a protein standard. The concentration range was from 0 mg/ml to 1.2 mg/ml. Test samples (crude, fractionate and UFB) were mixed with Bradford's reagent and incubated at room temperature (~25°C) for 5 minutes. The color produced from the reaction results from Coomassie Brilliant Blue G-250 dye with the protein present in the sample under acidic pH.

Enzymatic activity

The method used was developed by Sigma Aldrich (17), which was targeted to evaluate protease activity in a sample. Casein was used as a substrate and any enzyme present in a test sample should hydrolyse the peptide bonds present in casein and produce tyrosine (amino acid). Briefly, 3 ml of 0.65% casein was added to 0.5 ml of test sample and incubated at 37°C for exactly 10 minutes. Then, 3.2 ml trichloroacetic acid (TCA) was added and incubated for 30 minutes to stop the reaction. Remaining unhydrolyzed casein residue then removed by filtration. Then, the filtrate was reacted with Folin's Ciocalteu's (Brand Name: Merck) reagent, a blue-coloured compound will be produced. 1ml of the reaction product was transferred to a plastic cuvette and the absorbance reading was recorded at 660 nm wavelength using a spectrophotometer. The enzymatic activity was calculated by comparison with the L-tyrosine graph and equation stated below:

$$\text{CDU/ml enzyme} = \frac{(\text{tyrosine equivalents released}) \times \text{dilution factor}}{\text{volume of enzyme used (ml)} \times \text{Cuvette Volume (ml)} \times \text{Assay time (min)}}$$

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Broth microdilution was used to determine the minimum inhibitory concentration (MIC) with minor modifications (18). Bromelain samples were serially diluted (2000 µg/ml-1.9531 µg/ml) and tested. Standardized organism suspension (0.5 McFarland) was added to all wells except the negative control. Sterile water was dispensed into one of the tubes, termed the negative control. 96 wells of sterile microplate were incubated in an incubator with optimal temperature and time. The turbidity of the broth indicates the growth of organisms. The lowest concentration of Bromelain with no turbidity in the test wells were determined as the MIC. MBC was performed using the "streak plate" method. Wells that were visibly clear were inoculated. After incubation, the presence of any bacterial colonies was observed. The lowest concentration of the Bromelain enzyme sample that revealed no bacterial growth was taken as the MBC value.

Biofilm inhibition activity

A method was followed, and the partially purified fractionate and UFB Bromelain enzyme was subjected to biofilm analysis against *S. mutans* and *S. aureus* (19). Bromelain extract with concentrations between 1000 µg/ml to 62.5 µg/ml were tested by mixing the extract with sterile biofilm media (BHI with 2% sucrose) with a final volume of 4.9 ml. Control tubes do not consist of any Bromelain. 100 µl of overnight bacterial culture (18-24 hours) were added into the culture. The tubes were incubated overnight in a 37°C incubator. The formed biofilm was stained by adding 0.1% Crystal Violet (CV) to the tube and incubated for 20 minutes at room temperature. The CV was removed and rinsed three times with sterile distilled water gently. The stained biofilm was then dissolved with 100% ethanol and 1ml of the solution was transferred into a cuvette. Quantitative determination of biofilm mass was done by absorbance reading at 570 nm. Then, the percentage (%) of biofilm inhibition was calculated using the formula below:

$$\text{Percentage Biofilm Inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of Sample}}{\text{Absorbance of negative control}} \times 100\%$$

Statistical analysis

All experiments were performed in triplicates. The statistical significance was determined by Student's t-test with $p < 0.05$ taken as significant. Further, all results will be reported as mean \pm SD. A two-way ANOVA was used to compare each treatment group for the biofilm inhibition test. Bonferroni's multiple comparison tests were employed to find out the significance. The measure of significance was set at $p < 0.05$.

Results

Ultrafiltrated Bromelain using membrane-based filtration

To conclude that the alternative ultrafiltration method is compatible and efficient in extracting Bromelain enzyme, a set of parameters was investigated from two primary biochemical assays. Considering the classification of Bromelain as a protease enzyme, a protease assay was performed utilizing casein as a substrate. Further, Bradford's assay was used to quantify the protein concentration. From these assays, the enzymatic activity, protein concentration, specific activity, purification fold, and yield of Bromelain were investigated. As a result, a significant increase in these parameters after the ultrafiltration step was employed, as shown in Table 1. This result was concluded after statistically comparing each parameter ($p < 0.001$). Regarding a purification step applied, the highest yield of Bromelain was observed in UFB (222.50%) compared to fractionate (64.54%) sample. Fractionate sample is

the unpurified form of Bromelain obtained after the precipitation process by ammonium sulphate. Further,

a higher yield of Bromelain was also obtained after the ultrafiltration step compared to the fractionate.

Table 1: Parameters of Bromelain enzyme extraction (protein concentration, enzyme activity, specific activity, purification fold, and yield)

Sample	Final Volume (ml)	Protein Concentration (mg/ml)	Enzyme Activity (CDU/ml)	Specific Activity (U/mg)	Purification Fold	Yield (%)	P value
Crude	35	1.40±0.007	0.79±0.001	0.56±0.002	1.00	100	< 0.001
Fractionate	27	1.663±0.001	0.67±0.000	0.402±0.004	0.22	64.54	
Ultrafiltrate	15	2.41±0.069	4.41±0.004	1.83±0.052	3.25	222.50	

*Values are expressed as Mean ± SD of triplicates measurements

*P-value of less than 0.05 is considered statistically significant

Antibacterial activity of Bromelain against dental caries pathogens

A standard set of antimicrobial tests was used in this study to find out its antibacterial potential against dental caries pathogens, including minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Both MIC and MBC are the minimum concentration of a test sample to suppress growth (MIC) or directly eradicate (MBC) bacterial cells. In this study, Bromelain was used as the test compound (fractionate & UFB) in order to assess its antibacterial activity against selected organism that is associated with dental caries. Overall, among the cariogenic strains tested, decreased concentration of

Bromelain was needed to inhibit bacterial growth in the UFB sample compared to the fractionate, as shown in Table 2. Though, *S. mutans* showed the most sensitivity with the lowest MIC value of 125 µg/ml. The *Lactobacillus spp.* used in this study showed moderate antibacterial activity compared to *S. aureus*, which was resistant to all concentrations of Bromelain. All organisms used in this study are gram-positive. A difference could be observed in the antibacterial activity of Bromelain if gram-negative strains are used. This is due to the difference of their cell wall structure which directly influences the ability of any compound to penetrate it. It is shown that the Bromelain enzyme is most effective towards *S. mutans*.

Table 2: Tabulation of MIC and MBC results

Organism	Bromelain concentration (µg/ml)			
	Minimum Inhibitory Concentration (MIC)		Minimum Bactericidal Concentration (MBC)	
	Fractionate	Ultrafiltrate	Fractionate	Ultrafiltrate
<i>S. mutans</i>	250	125	1000	500
<i>S. aureus</i>	1000	1000	Resistant	Resistant
<i>L.casei</i>	1000	500	Resistant	1000
<i>L.acidophilus</i>	500	250	1000	500

*All tests were performed in triplicates

Inhibition of static biofilm formation by Bromelain enzyme

Biofilm is one of the crucial factors of dental caries. As stated, biofilm is a complex framework of bacterial colonies that sits on top of the tooth surface. It will become pathogenic when there is a shift in population in which the number of pathogenic strains is much more than the normal flora. However, not all strains can form biofilm. In this study, *S. mutans* and *S. aureus* are chosen based on their ability to

form a biofilm to assess the ability of Bromelain to inhibit its formation. Bromelain was introduced into the static culture of the test organism, and the inhibition was observed after 18-24 hours of incubation. The inhibition of biofilm is based on the reduced biofilm mass quantified compared to the control in which no Bromelain was added to the culture. The value is calculated using a formula stated in equation 2.4. A significant difference was observed between the biofilm inhibition by fractionate and UFB samples for both

S. mutans and *S. aureus* ($p < 0.05$), in which UFB gave higher inhibition compared to just the fractionate. It is also observed that the inhibition activity of the Bromelain enzyme increased in a concentration-dependent manner

shown in Figure 1. The UFB with the highest concentration of Bromelain enzyme (2000 $\mu\text{g/ml}$) yielded the highest biofilm activity towards *S. mutans* (57.48%) and *S. aureus* (83.42%), respectively.

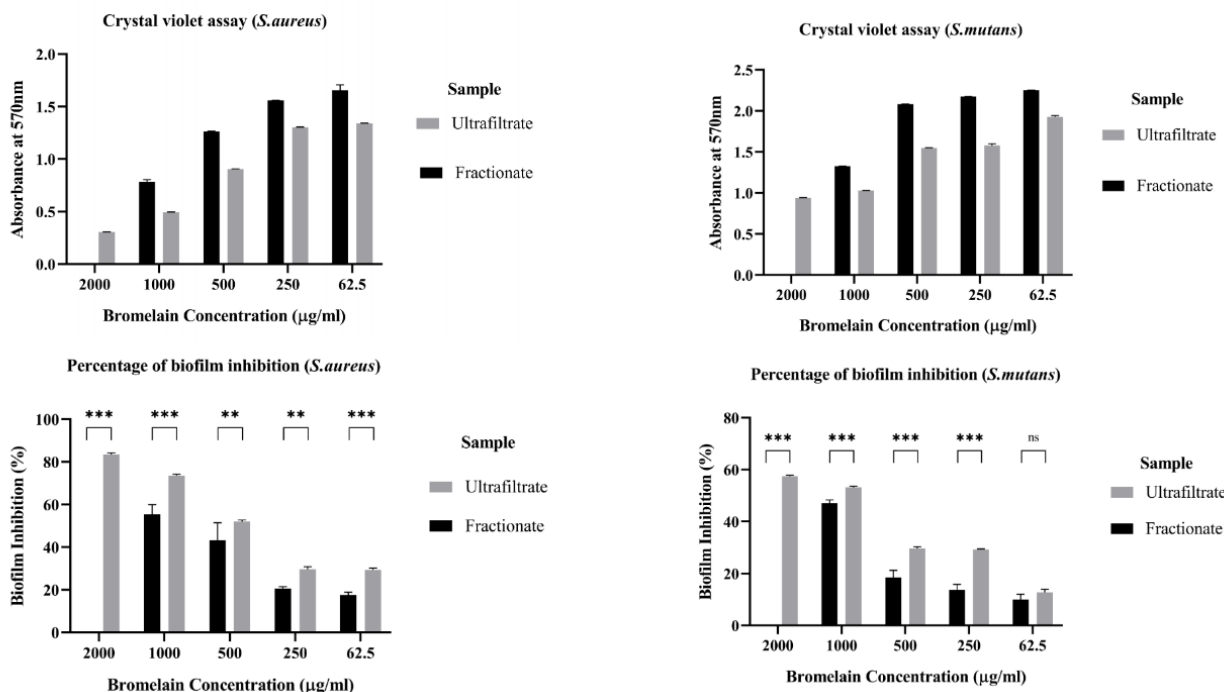


Figure 1: Crystal violet assay and biofilm inhibition of Bromelain enzyme against a) *S. aureus* and b) *S. mutans* (*) indicates significant mean differences ($p < 0.05$) when compared to the control (0%)

Discussion

The present study demonstrated the potential of Bromelain isolated from MD2 pineapple core towards its antibacterial and anti-biofilm activities. Roughly, pineapple cores represent about 10% w/w of the pineapple itself (20). Bromelain’s specific enzyme activity was used as the main parameter to evaluate the purification step’s efficacy. Recovery of enzymatic activity indicates a high extraction yield. The Bromelain sample that underwent ultrafiltration yielded the highest enzymatic activity of 1.83 ± 0.052 (U/mg) compared to the crude and fractionated sample. This result aligns with a recent study by Gul et al. (21) that used *Phu Lae* pineapple core and centrifugal ultrafiltration. However, differences in pineapple cultivars and extraction buffer used will affect the extraction of Bromelain, and the parameters concluded in between studies (22). Further, the Bromelain enzyme purification fold of the present study lies close to Sari et al. (23), which achieved a 1.7-2.32-fold. The difference was that their study used the traditional salting out method by dialysis. Generally, ultrafiltration membranes are more selective and feasible than dialysis with molecular weight cut-off between 3-100kDa (24). It is also a straightforward process after the precipitation step, which is quicker and more feasible than conventional methods.

To date, there is limited study presenting the potential of Bromelain as an antibacterial and antibiofilm agent specifically for dental caries pathogens. Most studies conclude that the antibacterial activity was attributed to its enzymatic properties. Dental caries is an oral disease that has been neglected due to its slow progression and diverse etiology. Though, it is inevitable that the pathogenic bacterial population consisting of largely *S. mutans* fluctuates oral pH by producing lactic acid (25). Subsequently, changes in oral pH cause the demineralization of tooth layers leading to caries lesions. Conventional formulation of oral care products with chlorohexidine is effective in fighting caries. Though, localized side effects correlated to its long-term usage have been reported (26). The present study proposed the antibacterial ability of Bromelain enzyme from MD2 pineapple core against cariogenic bacterial strains. MIC and MBC tests showed lower concentrations of Bromelain enzyme were needed to inhibit bacterial growth for UFB compared to the fractionate, regarding its higher enzymatic activity (Table 2). For instance, approximately 125 $\mu\text{g/ml}$ was needed to inhibit the growth of *S. mutans* by the ultra-purified Bromelain enzyme compared to 250 $\mu\text{g/ml}$ of the fractionate sample that did not go through the purification step by ultrafiltration after precipitation by ammonium sulphate. A similar understanding was also observed in

a previous study that mentioned the ability of Bromelain to hydrolyze bacterial cell walls, which eventually swell, leak, and open, which result in death (27). Previous studies reported the antibacterial activity of Bromelain in which a higher concentration of Bromelain enzyme extract (2 mg/ml) was needed to inhibit *S. mutans* (28). In addition, Goudarzi et al. (29) concluded a MIC value of 25 mg/ml and MBC of 1000 mg/ml of pineapple extract against *S. mutans*. Growth conditions and parts of the pineapple used do interfere with the properties of the Bromelain enzyme isolated (30). In turn, this brings disparities in the antibacterial activity produced.

Destroying planktonic bacteria versus biofilm is a challenge to any active compounds, and this statement has been clearly described by various literature (31). About 750 species of bacteria exist in one milligram (mg) of biofilm. It represents approximately 10^6 bacterial colonies where in cases of severe dental caries, about 3 to 5 mg of biofilm would be produced (32). The bacterial biofilm is made up of proteins, nucleic acids, and exopolysaccharides (EPS) that exist in an enmeshed three-dimensional (3D) extracellular structure (33). Biofilm serves as a protection layer in which all bacteria continue to thrive. Destroying biofilm layers would decrease the risk of dental caries. In this study, a static biofilm was grown *in vitro*, and the efficacy of Bromelain in inhibiting its growth was assessed by quantifying its mass and comparing it with the control. The goal was achieved using a crystal violet stain that stains the bacterial biofilm. This study showed that the highest biofilm inhibition percentage was achieved at Bromelain enzyme concentration of 2000 $\mu\text{g/ml}$, at which *S. aureus* (83.42%) showed higher inhibition than *S. mutans* (57.48%).

Interestingly, *S. aureus* showed resistance in the susceptibility studies (Table 2). These differences may be attributed to the ability of each strain to produce biofilm. Pereira et al. (34) stated extracellular matrix produced by *S. mutans* was higher compared to *S. aureus*. The lower percentage of inhibition by Bromelain in *S. mutans* is hypothesized due to its virulence factors and ability to produce resilient biofilm. This may be attributed to glucosyltransferases (GTF) gene expression (35–37). On the other hand, *S. aureus* possesses intracellular adhesions (*ica*) genes that contribute to its biofilm formation ability (38). The differences in these genes controlling the formation of Biofilm might illustrate its susceptibility towards synthetic or natural intervention.

We observed a dose-dependent effect between Bromelain in ameliorating planktonic and biofilm bacterial culture. However, a difference in its activity was observed between culture techniques. A planktonic culture with a less favorable environment for biofilm growth showed moderate antibacterial activity. However, it can be seen in a sucrose-induced biofilm culture that pathogenic *S. mutans* responded less to Bromelain. This phenomenon has been agreed upon in various pieces of literature. Bacteria in a biofilm are harder to kill compared to planktonic cultures.

This study proposed that the antibacterial and antibiofilm of Bromelain is due to its ability to digest cell walls taking into its enzymatic nature, which eventually prevents the colonization of bacterial cells. Though, the molecular mechanism is yet to be discovered. A possible molecular mechanism is the quorum sensing (QS) pathway. QS is a cell density-dependent cellular communication pathway that triggers the virulence factor genes. QS promotes biofilm formation; thus, the inhibition of biofilm via quenching of the QS pathway by Bromelain is a possible inhibitory mechanism. In the future, the Bromelain enzyme would be an alternative and exciting strategy to promote overall oral health, especially in fighting dental caries. Naturally derived bioactive compounds are generally favorable due to biocompatibility and feasibility. Lastly, it could also boost the pineapple sector in venturing into dentistry and oral health.

Conclusion

Summarising, the data presented in this study emphasized the feasible approach to extracting Bromelain enzyme from MD2 pineapple core. Ammonium sulphate precipitation paired with membrane-based centrifugal ultrafiltration was proven to purify a high yield of the Bromelain enzyme compared to the crude, and just the fractionate. Furthermore, this study successfully attempted to demonstrate the antibacterial and antibiofilm of the bioactive Bromelain enzyme against the cariogenic organism *S. mutans*. Despite the promising preliminary data presented in this study, future research should include further fundamental analysis of the molecular mechanism to verify its full potential as an anti-biofilm agent.

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Competing interests

The authors declare that they have no competing interests.

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