Food Research 6 (Suppl. 2) : 49 - 57 (2022)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

High pressure inactivation of *Escherichia coli* and *Staphylococcus aureus* in pineapple puree (variety MD2) and preliminary quality evaluation during chilled storage

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Article history:
Received: 16 September 2021
Received in revised form: 19 October 2021
Accepted: 26 November 2022
Available Online: 8 December 2022

Keywords:
High-pressure processing, Inactivation, *Escherichia coli, Staphylococcus aureus,* Pineapple puree, Safety

DOI:
https://doi.org/10.26656/fr.2017.6(S2).021

Abstract

The increased demand in the global pineapple market from Malaysia makes the focus on downstream processing even more important. However, contamination of fruit purees with foodborne pathogens could take place during processing and post-processing handling. In this study, the efficiency of high-pressure processing (HPP) was investigated against aseptically inoculated *Escherichia coli* ATCC® 25922™ and *Staphylococcus aureus* ATCC® 25923™ in pineapple puree. Pineapple (*Ananas comosus*) variety MD2 was chosen for HPP treatment due to Malaysia's competitive advantage in the global pineapple market. An 8-log reduction of *E. coli* and *S. aureus* ($p<0.05$) in pineapple puree during HPP (400 MPa for 5 mins at 30°C processing temperature) in this research making HPP an effective technology. In this study, *S. aureus* showed a higher inactivation effect than *E. coli* in HPP-treated pineapple puree. Untreated pineapple puree samples with inoculated pathogens were kept as control samples. Knowledge of food safety is necessary for a reliable HPP process; hence a preliminary storage study was added to compare the chemical and microbiological quality between HPP-treated pineapple puree and heat-treated pineapple puree. Interestingly, HPP-treated samples effectively retained vitamins A, B1, B2 and C close to fresh samples (control) during 0 months, and vitamin A was retained after 6 months of storage. The microbiological results also showed that HPP-treated samples were microbiologically safe during chilled storage for a minimum of 6 months based on international microbiological standards. In summary, *E. coli* and *S. aureus* inactivation can be accelerated with pressure higher than 400 MPa at 30°C processing temperature within 5 mins and HPP successfully maintained the safety of pineapple puree for a minimum of 6 months.

1. Introduction

Thermal processing is recognized as a significant food preservation method in the food industry. However, it can also cause a loss of food flavours, vitamins, fatty acids, proteins and other essential nutrients (Patras *et al*., 2009). To overcome or minimise these disadvantages, non-thermal processing such as high-pressure processing (HPP) can be a promising alternative method (Farkas and Hoover, 2000; Cullen *et al*., 2012). In the fruit juices and beverages processing, HPP technology with pressures between 400 and 600 MPa at ambient or chilled temperatures, and processing times under 10 mins, has been used commercially as described by Cheftel (1995). Globally, food processing is rapidly adapting to the consumer's awareness towards safe, nutritious and high-quality food products.

The potential growth of microbial pathogens in pineapple puree could affect its quality and safety. A microbial challenge test can be performed to determine the growth of specific microorganisms in food products in case of contamination. Joyce *et al*. (2018) reported that puree products could be contaminated in real scenarios during processing at a small-scale level such as *E. coli* through the use of non-potable water and *S. aureus* if good hygiene practices are not followed. HPP treatment can be used as an emerging effective non-thermal technology to inactivate these microorganisms and increase shelf life (Tan *et al*., 2019). With regards to food safety, precise information about HPP efficiency to

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

kill pathogens with the correct pressure-time-temperature combinations is vital to be affirmed (Cheftel, 1992; Smelt, 1998). Studies also revealed that the pressure resistance margin of microorganisms could also be subjected to strain diversity (Isaacs and Chilton, 1995; Benito *et al*., 1999).

Pineapple (*Ananas comosus*) was chosen as the product to be subjected to HPP treatment in this study, due to its high nutritional properties, and Malaysia's competitive advantage in the global pineapple market (Chubashini *et al*., 2011). The MD2 variety was preferred due to its availability and sweet taste making it the main variety for export (Chubashini *et al*., 2017). However, puree products are also subject to changes in quality attributes during storage (Snehasis *et al*., 2016) and limited information on the survival potential of *E. coli* and *S. aureus* in HPP-treated pineapple puree making this study important to technical knowledge in food safety. In addition, the chosen HPP treatment parameter used to inactivate *E. coli* and *S. aureus* was hypothesized would be enough to inhibit other, low-resistant microorganisms due to their relatively higher resistance to pressure based on Alpas *et al*. (1999) and Bayindirh *et al*. (2006).

Therefore, the main objective of this study was to investigate the effect of HPP against aseptically inoculated *Escherichia coli* (ATCC® 25922™) and *Staphylococcus aureus* (ATCC® 25923™) in pineapple puree (variety MD2). Following that, a preliminary storage study was done between uninoculated, HPP-treated and heat-treated (canning) pineapple puree to determine their chemical and microbiological quality. Apparently, there is relatively little published literature on the efficiency of HPP for the inactivation of *E. coli* and *S. aureus* in pineapple puree in Malaysia and its quality impact during chilled storage, making these findings valuable for potential technology transfer in local food industry.

2. Materials and methods

2.1 Materials

Pineapple (variety MD2) was obtained from the local market, washed, peeled, cut and blend. High-acid foods require less heat treatment for their stability and therefore 20% citric acid (food grade) was added during processing to maintain their pH at 3.85. The puree was filled into 6.5 cm (width) and 17 cm (length) polyethylene/ nylon/ aluminium/ cast polypropylene laminate packaging (20 g per pouch). Samples were then held in ice until further treatment. For bacterial strains, *Escherichia coli* ATCC® 25922™ and *Staphylococcus aureus* ATCC® 25923™ were used in this study. *Escherichia coli* and *S. aureus* were streaked on Eosin Methylene Blue Agar (CM0069, OXOID) and Baird-Parker Agar Base (CM0275, OXOID), respectively and incubated at 37°C for 24±2 hrs. Then, each bacterium was cultivated in Tryptone Soy Broth (CM0129, OXOID) supplemented with 0.6% Yeast Extract (212750, BD) at 37°C for 16 - 18±2 hrs and transferred to fresh broth every 48±2 hrs.

2.2 Preparation of inoculated pineapple puree, pressurization study and microbiology analysis

The pineapple puree samples were autoclaved at 121°C for 5 mins to ensure its sterility. Then, the samples were inoculated with *E. coli* and *S. aureus*, respectively from Tryptic Soy Broth that contained approximately 10^8 CFU/mL colonies (1 mL of bacteria into 20 g of pineapple puree). The pineapple puree was then sealed aseptically and held in ice before HPP treatment. The aluminium-laminated pouches were then placed into a secondary pack of nylon plastic and sealed to avoid cross -contamination between the pressure chamber and laminate. In this research, HPP treatments were performed in a Stansted Mini Foodlab FPG5620 (Standsted Fluid Power Ltd, UK) (capacity: 30 cm^3, maximum P: 900MPa). The time to maximum pressure is less than 1 min with an enhanced drive pump for the system. A mix of propylene glycol (Meilun Food Chemical, Thailand) and distilled water (3:7) was used as a pressure transmitting medium. The high pressure was generated by a cylindrical pressure chamber, a pressure pump and a hydraulic unit inside the HPP equipment system.

In the pressurization study, the pouches composed of inoculated pineapple puree samples were exposed to 100 MPa, 200 MPa, 300 MPa, 400 MPa, 500 MPa, 600 MPa pressure for 5, 10, 15 and 20 mins, respectively maintained at 30°C processing temperature as a non-thermal temperature set with a water bath. Before pressurization, the chamber was closed for 1 min to achieve temperature equilibration with the pouches being positioned inside. Controls were represented by unpressurized samples, maintained at 30°C processing temperature with all the samples duplicated in the experiment. After pressurization, the samples were immediately removed from the HPP vessel and cooled in ice before microbial evaluation. A microbiological test was carried out 1 hr after HPP treatment. Ten grams of treated samples were added with 90 mL 0.1% peptone solution (CM0009, OXOID) and homogenized using a stomacher for 60 s. Next, serial dilution was carried out using peptone solution prior to plating. Following the standard spread plate method, 0.1 mL from each serial dilution was plated in duplicate on pre-poured Tryptone Soy Agar (CM0131, OXOID) plates. In this work, 0.6%

https://doi.org/10.26656/fr.2017.6(S2).021

Yeast Extract (212750, BD) was supplemented into Tryptone Soy Agar, appropriately. The plates were then incubated at 37°C for 48±2 hrs, and plates with 25 to 250 CFU/g were used for enumeration.

2.3 Colour evaluation, statistical analysis and storage study

Colour measurement of pineapple puree samples was assessed visually after the HPP treatment and recorded in L* a* b* values using a chromameter (Minolta, Japan). The effects of HPP on the inactivation of *E. coli* and *S. aureus* in pineapple puree were statistically analysed by using two-way Analysis of Variance (ANOVA) (SAS version 9.3) and Duncan Multiple Range Test (DMRT) at a significance level of $p<0.05$ (SAS Institute, 1985). The estimated safety margin was derived from the estimated marginal margin that indicated the level for the inhibition of both *E. coli* and *S. aureus*. In addition, an estimated marginal margin was obtained from ANOVA output (IBM SPSS version 25) that specified interaction within factors such as pressure and time. For the storage study, a new batch of HPP-treated samples was produced based on the recommended combination parameters achieved during the pathogen inactivation study. The same packaging was used during HPP but without inoculation of bacteria. Production of control samples was also made namely, heat-treated pineapple puree (canning at 110°C for 10 mins) and fresh pineapple puree. All samples were stored at a chilled temperature (4±2°C) for 6 months for comparison purposes. During storage study, samples were sent through a cooler box periodically (0 months and 6 months for chemical analysis whilst 0 months, 3 months and 6 months for microbiology analysis, respectively) and analysed at Food and Agriculture Analysis Laboratory (MARDILab), Technology Commercialization and Business Centre, MARDI Serdang. Chemical analysis was inclusive of proximate elements and vitamin tests, whilst microbiology analysis was inclusive of Total Plate Counts, Yeast and Mould Counts, Psychotropic Counts, Coliform Counts, *Escherichia coli* and *Staphylococcus aureus* according to MARDILab MS ISO/IEC 17025 In-house Methods, based on AOAC Official Method of Analysis (2000) and American Public Health Association (2001), respectively.

3. Results and discussion

3.1 Pathogen inactivation by high-pressure processing in pineapple puree

The efficiency of HPP pressure-time-temperature combinations on *E. coli* and *S. aureus* in pineapple puree are demonstrated in Tables 1 and 2, respectively. Even though most of the heat-resistant bacterial spores did not germinate and grow in the acidic environment ($pH<4.6$) of the fruit juices (Silva and Gibbs, 2004), several researchers have reported that some strains of *E. coli*, *Shigella* and *Salmonella* can grow several days or even weeks in acidic foods, thus making its important to study the effect of these foodborne pathogens (Leyer *et al*., 1995). Based on our findings, the HPP treatment has shown more than 8-log cycles viability loss for both microorganisms studied. A significant log reduction ($p<0.05$) was obtained on *E. coli* between 300 MPa (4.107) and 100 MPa (8.208) for 5 mins as shown in Table 1, whilst significant reduction ($p<0.05$) on *S. aureus* from HPP treatment at 300 MPa, 15 mins (2.501) to 100 MPa, 5 mins (7.953) in Table 2, at the processing temperature of 30°C, respectively in pineapple puree samples. This was in agreement with (Wilson *et al*., 2008; Evelyn and Silva, 2015; Sarker *et al*., 2015) to support that HPP is an effective tool to inhibit microorganisms in food products.

Due to the factor difference between pressure resistance bacteria and food matrices, it is become more realistic to validate pressurization and processing parameters in foods, rather than in laboratory media and buffers (Bayindirh *et al*., 2006). Therefore, this work has shown that *E. coli* was more pressure-sensitive than *S.*

Table 1. Effect of HPP treatment at selected pressure and time at 30°C processing temperature on survival of *Escherichia coli* ATCC® 25922™ in pineapple puree (variety MD2)

Pineapple puree	Log_{10} CFU/g			
	Escherichia coli Control = 8.257 (Control sample – Unpressurized)			
Time (min)	5	10	15	20
Pressure (MPa)				
600	ND	ND	ND	ND
500	ND	ND	ND	ND
400	ND	ND	ND	ND
300	4.107^{dD}	ND	ND	ND
200	7.238^{eD}	6.374^{eE}	6.370^{eE}	5.192^{eD}
100	8.208^{fD}	8.076^{fE}	8.042^{fE}	7.857^{fD}

Values are presented as mean log_{10} CFU/g (n = 8). Values with different lowercase superscript with within the same column are significantly different ($p>0.05$) while values with different uppercase superscript within the same row are significantly different ($p>0.05$). ND, not detected.

https://doi.org/10.26656/fr.2017.6(S2).021

Table 2. Effect of HPP treatment at selected pressure and time at 30°C processing temperature on survival of *Staphylococcus aureus* ATCC® 25923™ in pineapple puree (MD2)

Pineapple puree	Log_{10} CFU/g			
	Staphylococcus aureus Control = 8.310 (Control sample – Unpressurized)			
Time (min)	5	10	15	20
Pressure (MPa)				
600	ND	ND	ND	ND
500	ND	ND	ND	ND
400	ND	ND	ND	ND
300	5.692^{dE}	5.637^{dE}	2.501^{dD}	ND
200	7.909^{eE}	7.860^{dE}	7.886^{eD}	7.720^{eF}
100	7.953^{eE}	7.804^{eE}	7.901^{eD}	8.000^{eF}

Values are presented as mean log_{10} CFU/g (n = 8). Values with different lowercase superscript with within the same column are significantly different ($p>0.05$) while values with different uppercase superscript within the same row are significantly different ($p>0.05$). ND, not detected.

aureus bacteria in the HPP combination studied. From Table 1, the significant changes in *E. coli* at each level of pressure were observed from 100 to 300 MPa for 5 mins with a log reduction of 8.208 and 4.107, respectively. This was in contrast with *S. aureus* which only showed significant changes at pressure from 200 MPa, 5 mins (7.909) to 300 MPa, 5 mins (5.692), but not from 100 MPa, 5 mins (7.953) at 30°C processing temperature, respectively. Likewise, this was proven when ND (not determined) level, was first detected for *E. coli* at 300 MPa, 10 mins. However, ND level was first detected for *S. aureus* at a much higher level at 300 MPa, 20 mins. It has been described that Gram-negative bacteria are more pressure-sensitive than Gram-positive bacteria, probably due to their thinner cell wall (Hauben *et al*., 1996; Kalchayanand *et al.,* 1998). In theory, peptidoglycan polymer layers in Gram-positive gave more structural protection, in contrast to the cell walls of Gram-negative (Bruslind, 2019). Researchers who have studied HPP in different juices, mostly have used bacteria such as *E. coli* O157: H7 and *Salmonella* (Linton *et al*., 1999; Teo *et al*., 2001).

Based on Duncan Multiple Range Test (DMRT) statistics (Figure 1) that indicates an estimated marginal mean value line from 0 – 8, which means 0 for inactive microbial and 8 for active microbial. HPP treatments (300 MPa, 20 mins; 400 MPa, 20 mins; 400 MPa, 15 mins; 400 MPa, 10 mins; 400 MPa, 5 mins), respectively at 30°C processing temperature have resulted in the inactivation of *E. coli* and *S. aureus*, significantly ($p<0.05$) with more than 8-log units. These five sets of combined treatments, respectively gave the adequate margin of safety for the inhibition of both pathogens, effectively in the pineapple puree studied. However, apart from microbial safety aspects, the economic feasibility aspects of HPP processing also need to be commercially concerned. Optimization of the lowest pressure and shortest time is needed to provide the safest HPP formula, with the best sensory properties of the treated food. However, in our findings, we only concluded based on preliminary chromameter results. The colour tends to be bleached [decrease in b* (yellowness), increase in a* (less green), increase in L* (lightness)] when samples were subjected to HPP at a longer time and higher pressure. Ideally, time should be no greater than 10 mins and pressure should be no greater than 400 MPa in this study to achieve the best colour quality at the same time reduce capital equipment costs, in agreement with Cheftel (1995).

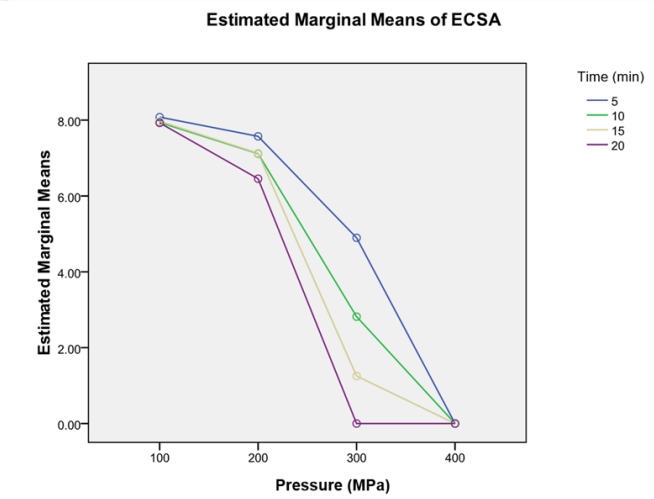

Figure 1. Estimated safety margin on the effects of HPP treatment on *Escherichia coli* ATCC® 25922™ and *Staphylococcus aureus* ATCC® 25923™ survival in pineapple puree (variety MD2)

Although the successful commercial application has used higher pressure, at 500 MPa and 550 MPa, respectively (Linton *et al*., 1999; Jordan *et al*., 2001) than our study, we have chosen 400 MPa, 5 mins at 30°C processing temperature as our recommended combination parameter and for our further testing during storage study. This was due to the safe parameter for both *E. coli* and *S. aureus* studied in the microbial challenge test, and preliminary colour results for pineapple puree. The interpretation of the decreased quality of colour was enzyme activation and acid

https://doi.org/10.26656/fr.2017.6(S2).021

sensitization effects. This might be due to the proportion of cells that survived pressurization being injured, therefore reducing their tolerance to the unfavourable organic acids and pH (Garcia-Graells *et al*., 1998). In light of related HPP studies (Landl *et al*., 2010; Tan *et al*., 2019), the sensorial evaluation shall be performed to further validate this finding in terms of the best combination parameter. The storage at room temperature could make this research more impactful but it was also interesting to note that Snehasis *et al.* (2016) reported the overall sensory acceptability was higher at 5°C than 15°C and 25°C during storage of high-pressure pineapple puree. Also noteworthy, the possibility of surviving dead bacteria cells could additionally study with several methods such as ethidium monoazide DNA-based quantification to confirm ND (not determined) values. *E. coli* and *S. aureus* pathogens (Wang *et al*., 2009).

3.2 Chemical and microbiological results for pineapple puree during storage study

All uninoculated samples of pineapple puree (HPP-treated, heat-treated and control) generally demonstrated a similar amount of value for proximate results namely total dietary fibre, energy, fat, moisture, ash, carbohydrate and crude protein (g/100 g) at 0 months of storage study as shown in Table 3. A similar trend was also demonstrated for vitamin results in this study whereby HPP at 400 MPa, 5 mins at 30°C processing temperature managed to preserve vitamin A (Beta carotene), vitamin B1 (Thiamine), vitamin B2 (Riboflavin) and vitamin C (Ascorbic acid) in HPP treated samples (222.6 ug/100 g; 1.0 mg/100 g, 1.0 mg/100 g, 22.4 mg/100 g), almost similar as control (fresh) samples (246.5 ug/100 g; 1.0 mg/100 g; 1.0 mg/100 g; 22.4 mg/100 g) in contract to heat-treated (canning) samples during 0 months. Interestingly, vitamin A was also retained (218.5 ug/100 g) in HPP-

Table 3. Chemical analysis results for chilled pineapple pure

Sample	Chilled pineapple puree (0 month)		
Code	AH	AC	AF
Proximate			
Total dietary fibre	0.4 g/100 g	0.2 g/100 g	0.1 g/100 g
Energy	52 kcal/100 g	52 kcal/100 g	52 kcal/100 g
Fat	0.0 g/100 g	0.0 g/100 g	0.0 g/100 g
Moisture	86.7 g/100 g	86.7 g/100 g	86.6 g/100 g
Ash	0.4 g/100 g	0.3 g/100 g	0.4 g/100 g
Carbohydrate	12.0 g/100 g	12.2 g/100g	12.2 g/100 g
Crude protein	0.9 g/100 g	0.8 g/100g	0.8 g/100 g
Vitamin			
Vitamin A (Beta carotene)	222.6 ug/100 g	0.0 ug/100 g	246.5 ug/100 g
Vitamin B1 (Thiamine)	1.0 mg/100 g	0.0 mg/100 g	1.0 mg/100 g
Vitamin B2 (Riboflavin)	1.0 mg/100 g	0.0 mg/100 g	1.0 mg/100 g
Vitamin C (Ascorbic acid)	22.4 mg/100 g	0.0 mg/100 g	22.4 mg/100 g
Sample	Chilled pineapple puree (6 months)		
Code	AH	AC	AF
Vitamin A (Beta carotene)	218.5 ug/100 g	0.0 ug/100 g	-
Vitamin B1 (Thiamine)	0.0 mg/100 g	0.0 mg/100 g	-
Vitamin B2 (Riboflavin)	0.0 mg/100 g	0.0 mg/100 g	-
Vitamin C (Ascorbic acid)	0.0 mg/100 g	0.0 mg/100 g	-

AH: HPP-treated, AC: Heat-treated (canning), AF: Control (fresh)

Table 4. Microbiology analysis results for chilled pineapple puree

Sample	Chilled pineapple puree					
Test/Time	0 month		3rd months		6th months	
Code	BH	BC	BH	BC	BH	BC
Total Plate Counts (CFU/g)	<1×10	<1×10	<1×10	<1×10	<25×10 est. (1.0×10^2)	<1×10
Yeast and Mould Counts (CFU/g)	$<1\times10^2$	$<1\times10^2$	-	-	$<1\times10^2$	$<1\times10^2$
Psychotropic Counts (CFU/g)	$<1\times10^2$	$<1\times10^2$	-	-	$<1\times10^2$	$<1\times10^2$
Coliforms (3-MPN) (MPN/g)	<3	<3	<3	<3	<3	<3
E. coli (3-MPN) (MPN/g)	<3	<3	-	-	<3	<3
S. aureus (CFU/g)	$<1\times10^2$	$<1\times10^2$	-	-	$<1\times10^2$	$<1\times10^2$

BH: HPP-treated, BC: Heat-treated (canning)

https://doi.org/10.26656/fr.2017.6(S2).021

Table 5. Microbiological limit based on international standards

Food Category	Chilled pineapple puree						
Main reference/method	Microbiological Guidelines for Food (Centre for Food Safety Hong Kong)			Compendium of Microbiological Criteria for Food (Food Standards Australia New Zealand)			
Microbiological limit	Colony forming unit (CFU/g)			Colony forming unit (CFU/g)			
	Satisfactory	Borderline	Unsatisfactory	Satisfactory	Marginal	Unsatisfactory	Potentially hazardous
Total Plate Counts	$<10^4$	10^4 -$<10^7$	$^3 10^7$	$<10^4$	10^4 -$<10^6$	$^3 10^6$	-
Coliform Counts	$<10^2$	10^2 - $\leq 10^4$	$>10^4$	$<10^2$	10^2 - 10^4	$>10^4$	-
Escherichia coli	<20	20 - $\leq 10^2$	$>10^2$	<3	3 -$<10^2$	$>10^2$	-
Staphylococcus aureus	<20	20 - $\leq 10^4$	$>10^4$	$<10^2$	10^2 -$<10^3$	10^3 - $\leq 10^4$	$>10^4$

https://doi.org/10.26656/fr.2017.6(S2).021

treated pineapple puree after 6 months of storage. This could be related to Evrendilek (2018) and Abera (2019) that cited HPP ability to give minimal loss of nutritional properties, to provide general positive effects on bioaccessibility without compromising food safety. Furthermore, microbiological results (Table 4) showed HPP-treated pineapple puree gave satisfactory a microbiology level as effective as heat-treated (canning) puree from 0 months until 6 months of storage study. Almost zero counts were recorded for all bacteria tested especially for *E. coli* and *S. aureus* in both samples, which compliant with international standards. Centre for Food Safety, Food and Environmental Hygiene Department, Hong Kong (2014) and Food Standards Australia New Zealand (2018) (Table 5) were used as our microbiological satisfactory limit reference because there was no reference yet in Malaysian Food Act 1983 for this category.

4. Conclusion

In summary, the high-pressure inactivation of *E. coli* and *S. aureus* in pineapple puree (variety MD2) was determined. Both bacteria were significantly reduced after HPP treatment in pineapple puree. Based on the current data, the HPP parameter of 400 MPa in 5 mins at 30°C processing temperature was chosen as the recommended pressure-time-temperature combination for pineapple puree in this study. During chilled conditions, vitamin A was retained in HPP-treated pineapple puree after 6 months of storage and HPP-treated samples showed safe microbiological results for a minimum of 6 months. Hence, future research can be explored to other food matrices to further contribute to a better understanding of HPP mechanisms for food safety.

Conflicts of interest

The authors declare no conflicts of interest or any personal or financial relationships that could have apeared to influence the work reported in this paper.

Acknowledgement

This work was supported with a grant from MARDI, under the Centre of Excellence for Aquaculture and Fruit Products Processing (The Potential of High-Pressure Processing for Fruit Puree Preservation: Project No. P21003001590001-C). The support from laboratory staff from Food Science and Technology Research Centre, MARDI was much appreciated.

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