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High Pressure Processing of Honey: Preliminary Study of Total Microorganism Inactivation and Identification of Bacteria

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Abstract

Due to the demand for better quality and safety food as it provides health benefits, the study of innovative high pressure processing (HPP) is currently one of the most interesting researches in food processing and preservation. As an alternative to classical thermal processing, HPP has potential to produce high quality foods that are microbiologically safe with 'fresh-like' characteristics and improved functionalities. In present work, the use of HPP will be investigated for its ability to inactivate bacteria spores, the heat resistance microbes. Preliminary results show that it is possible to use HPP to inactivate microorganisms present in high sugar content foods, particularly in Manuka honey. Further investigation will be carried out to find an optimal combination of treatment pressure, temperature and time. This project will generate a new approach in honey processing which can guarantee the safety of honey without a compromise on its quality and natural freshness.

Keywords: honey; high pressure processing; microorganism inactivation; bacteria

1. INTRODUCTION

Honey is the world's primary sweetener and nature's original sweetener prepared by honey bees. Honey has been used as a food for at least 6000 years. The popularity of honey is largely due to it being a natural sweetener evolved from the nectar mainly consisting of glucose and fructose. The honey in the nutshell is flower nectar which has been collected, regurgitated and dehydrated by honey bees to enhance its nutritional properties and ready to be consumed by humans. The consumption of honey is on the rise due to the unique combination of components in honey which makes it a prized addition to the diet. According to Food and Agriculture Organization of the United Nations (FAO), total honey production in 1961 was 0.7 million tonnes and it was steadily increased to about 1.5 million tonnesin 2009.The demand for high quality honey is attracting a lot of attention as it provides health benefits and has been shown to possess antimicrobial, antiviral, antiparasitory, anti-inflammatory, antioxidant, antimutagenic and antitumor effects [1]. Diseases prevention through consumption of honey is probably due to the presence of more than 181 substances with a wide range of health promoting phytochemicals, some with antioxidant properties. Honey antioxidant compounds include phenolic acids, flavonoids [2] and amino acids. The importance of these honey bioactive compounds and antioxidants on human health therefore generates a great interest in honey processing research.

Although consumption of honey has remarkably increased in the last years all over the world, the safety of this product is not regularly assessed. One common problem faced by honey producers is its deterioration in quality with storage due to fermentation. Yeasts are spoilage microorganisms of concern in honey and have been reported to grow to very high numbers [3]. *Saccharomyces cerevisiae*was found as the dominant yeast fermenting honey, even at the limited level of water available. It is also known as osmophilic or sugar tolerant yeast because it can withstand the low water content (around 16 - 21 %) and high sugar concentration in honey. Food grade, honey with a very high yeast count (more than 100 000 CFU/g) is not likely to be marketable [3]. With respect to medical health applications, yeast count must be less than 500 CFU/g.

Thus, commercial thermal processing of honey is an important operation which potentially eliminates spoilage microorganisms and decreases the moisture content to a level that retards the microbial growth. Additionally, the use of heat is found to be essential at different stages of honey processing for fast handling, to dissolve large sugar granules and to sustain quality. Conventionally, there are two stages of heating applied in the honey industry which are liquefaction and pasteurization. Liquefaction is operated at approximately 55°C and has a purpose to ensure the honey can stay in liquid form for as long as possible for filtering. Pasteurization is done at 60°C and above to inactivate yeast and other spoilage microorganisms. However, heat treatment above 75°C is not suitable because it

causes degradation of bioactive compounds which could affect the quality of the honey. Phenolic compounds were reported as the main honey bioactive compounds which would be negatively affected by the thermal treatment.

High pressure processing (HPP) is a non-thermal treatment which have an optimal effect on product quality and capable to inactivate microorganisms in various food matrices [4]. Although past research has proven that HPP can inactivate most microorganisms such as *Escherichia coli*, *Salmonella sp.*, *Listeria* and *Vibrio* pathogens without additional heat processing in various food matrices [3], it is not known yet if HPP can inactivate bacterial spores and yeasts especially in high sugar content foods. Hence the effect of HPP on inactivation kinetics of bacterial spores should be carried out. There has been no research on the potential application of HPP to obtain higher quality of honey with 'fresh-like' characteristics and improved functionalities.

Besides, a combination of HPP and heat has been used to investigate the efficiency of bacterial spore inactivation [5]. Although HPP is termed a nonthermal process, there is a relationship between pressure and temperature in physical compression due to the adiabatic heating caused by fluid compression at high pressure can cause significant temperature distribution throughout the treated food.

Therefore, it is of paramount importance to establish processing conditions which not only can inactivate the spore forming bacteria and yeasts but also be able to preserve the nutritional quality and bioactive compounds present in honey. This research aims at developing the optimum condition of pressure, temperature and processing time for the inactivation of spore forming bacteria and yeasts to ensure the best honey quality and extended shelf life.

This paper reports pre experimental results of HPP inactivation of total microorganism in Manuka honey at different pressure levels and at different treatment time. The identification of bacterial spore which dominantly found in Manuka honey was also investigated.

2. MATERIALS AND METHODS

2.1 Honey Sample and Packaging

Unprocessed Manuka honey used in this study (with pH of 4.3, 79° Brix and water content of 19 %) was kindly donated by honey producer in New Zealand. Manuka Honey is sourced from New Zealand's remote and pollution free forests. The bees feed of the Manuka plant (*Leptospermum scoparium)* which has delicate pink or white flowers and is native to New Zealand.

Honey of 2 g, in duplicate, were packed in 5 cm x 5 cm transparent plastic film pouches (Cas-pak plastic vacuum pouch, New Zealand) and thermosealed under vacuum. The plastic film is made of cast polypropylene for excellent transparency and heat sealing qualities which can withstand temperatures up to 125 °C.

2.2 High Pressure Processing Equipment

The HPP unit used in this research was 2L- 700 Laboratory Food Processing System (Avure Technologies, Columbus, Ohio, USA). The equipment consists of a 2-L cylindrical shape pressure treatment chamber, water circulation, a pumping system and the control system operated through a computer with software supplied by the manufacturer. Distilled water was used as the medium in the chamber where the honey samples were placed. The equipment can operate at maximum pressure and temperature of 600 MPa and 90 °C, respectively.

2.3 Thermal Treatment

The water bath used for thermal treatment in this research was Water Bath W28 (Grant Instruments (Cambridge, Ltd, England). The capacity of tank is 28 litres and made from stainless steel with an easy to clean, solvent resistant plastics outer case. The control units, which are mounted on a bridge plate, offer digital temperature setting and display. This provides powerful stirring to ensure good temperature uniformity throughout the bath. Each bath can be fitted with a pump allowing temperature controlled liquid to be circulated through external apparatus. The consistent temperatures control over the range 0 to 99.9 °C. For the treatment, the vacuum-sealed samples were placed into the tank fully submerged in the distilled water. In this experiment, the temperature of 60 °C, 70 °C and 80°C were chosen considering the standard temperatures to which honey is exposed in industrial liquefaction and pasteurization processes which is 45 °C and 80°C, respectively [6].

2.4 Preparation of Agar Plates

A 39 g Difco^{TM} Potato Dextrose Agar (PDA) powder form (Fort Richard, Auckland, New Zealand) was weight using analytical balance (Sartorius Germany CP225 D, Germany) and dissolved in 1L of distilled water and autoclaved at 121 °C for 15 minutes (Tomy Autoclave SS-325, Germany). Thereafter, the hot solution was held at 45 °C in water bath. Under controlled environment, laminar flow hood (Airstream ESCO Class II, Biological Safety Cabinet, ACZ 4E1, Germany), the hot solution was then poured into series of petri dish. Once the agar solidified, the cover was inverted to prevent plate contamination due to condensation build during cooling the plates. The plates were then stored under sterile condition at 4 °C for later use.

2.5 Microbial Enumeration

The microbial enumeration in honey samples was determined as for enumeration of yeasts and moulds. Two grams of each sample was mixed and homogenized with 18 mL of saline water (8.5 g/L) to prepare the initial dilution which is used as the mother dilution. A serial dilution 1/100 to 1/100000 of sample was done. Then, 1 mL of each dilution was inoculated onto PDA agar plates were incubated by spread-platting technique. PDA was used for total microbial counts. The platting was done in duplicate for each series of dilution. The inoculated plates were compute using colony counter (Suntex Colony Counter 570, Taiwan). Only plates presenting 30 – 300 colonies were used for analysis. The colonies were counted as CFU/g.

2.6 Microbial Inactivation in Manuka Honey by HPP and Thermal Treatment

After vacuum-packing the samples, the pouches were subjected to HPP (except for the control samples) within pressure range of 250 MPa to 450 MPa, temperature between 20° C to 60 °C and processing time between 3 min to 40 min. These ranges were chosen based on the previous works carried out with honey as well as other high sugar food such as fruit jam. Two replicates of honey samples were carried out for the pressure, temperature and time as designed by Design Expert 8.0.1. The treatment time only includes the holding time and does not include the come up time and the decompression time. Pressure come up times were approximately 1.5 min to reach the desired pressure and the decompression time was < 20 s. The adiabatic heating was observed during pressurization phase. A slight decrease in the chamber temperature was observed in particular at higher pressure. The temperature of the pressure medium (distilled water) during pressure treatmentwas measured using a thermocouple. After treatment, the samples were immediately in cooled ice water before the microbial enumeration. For the thermal treatment, the vacuum-sealed samples submerged in the hot distilled water. All the honey samples were taken from the same honey batch.

2.7 Identification of Bacteria in Manuka Honey

The identification for the type of bacteria present in Manuka honey was conducted at School of Biological Sciences (SBS), Thomas Building, and The University of Auckland. The bacteria colonies on the agar plate were viewed under light microscope to get an overview of the shape and size. Then, the samples were identified using PCR technique and DNA sequencing. The sequenced DNA from the colonies tested was run through a program called BLAST found on NCBI. The genus and species of the bacteria was then determined. This experiment was successfully done with Prof Richard Gardner and Whitney from SBS.

3. RESULTS AND DISCUSSION

3.1 Microbial Inactivation in Manuka Honey by HPP and Thermal Treatment

Twenty different experimental conditions (pressure, temperature and time) were tested according to Response Surface Methodology design. The results were analysed using Design Expert 8.0.1 software. Five from 20 runs were found to have incomplete inactivation of microorganism in the samples tested.

Treating samples at 300 MPa showed microbial survivors particularly at lower temperature 30 °C, regardless of treatment time, as tabulated in Table 1. However, the microorganisms were totally inactivated when the temperature increased up to 50 °C. The same pattern was found for treatment at 350 MPa. As comparison with Run 1 (350 MPa, 25 °C, 20 min), increasing temperature up to 40 °C with the same pressure and time (as represented in Run 9 – Run 14) shows total inactivation of the microorganisms present in honey samples. Treatment at 270 MPa and 40 °C for 20 min (Run 6) gave the lowest inactivation of microorganism as compared to other treatments. The result also shows that the pressure of more than 350 MPa and temperature above 40 °C is needed to inactive microorganism present in Manuka honey.

Table 1: Log of microorganism (CFU/g honey) in unprocessed Manuka honey after HPP treatments at different pressure, temperature and time conditions.

Sample Run	Pressure (MPa)	Temp (°C)	Time (min)	log N± SD (N in CFU/g)
Un processed	Atmospheric pressure (0.101)			6.1 ± 0.1
6	270	40	20	4.0 ± 0.1
2	300	30	10	3.1 ± 0.1
3		30	30	3.1 ± 0.1
16		50	10	not detected
17		50	30	not detected
1	350	25	20	3.6 ± 0.1
7		40	3	3.0 ± 0.1
9		40	20	not detected
10		40	20	not detected
11		40	20	not detected
12		40	20	not detected
13		40	20	not detected
14		40	20	not detected
8		40	37	not detected
20		60	20	not detected
4	400	30	10	not detected
5		30	30	not detected
18		50	10	not detected
19		50	30	not detected
15	450	40	20	not detected

Based on the ANOVA analysis of experimental designed, the value of "Prob > F" is 0.008 (less than 0.05), indicates that the pressure and temperature are significant. However, time was found to be insignificant. The 3D model graph in

Figure 1 showed the relationship between pressure and temperature. Increasing the treatment temperature as well as the treatment pressure will reduces the total microorganisms present in the samples.

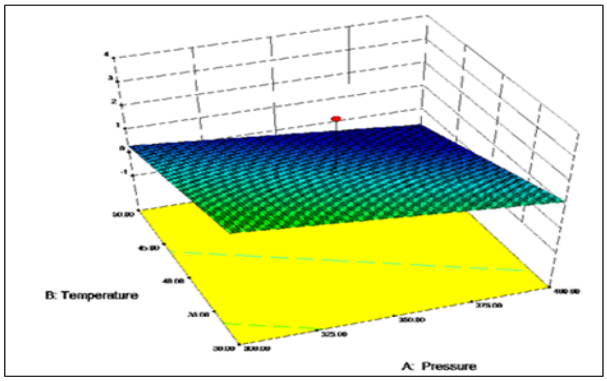

Figure 1: 3-D graph of interaction between treatment temperature and pressure using HPP

Thermal treatment with higher temperature (above 50 °C) was performed. The temperature of 60 °C, 70 °C and 80°C were chosen considering the standard temperatures to which honey is exposed to in industrial liquefaction and pasteurization processes which is 45 °C and 80 °C, respectively [6]. The result obtained shows that as temperature increases, the microorganism levels decrease, even though only a slight decrease in the microorganism numbers was observed particularly at lower temperature, 60 °C. As expected, a higher temperature leads to more microbial inactivation. The log of microorganism survival and their reduction in Manuka honey after being treated at three different temperatures for 30 min were tabulated in Table 2. As shown in the table, the reduction of microorganism counts after treatment at 60 °C, 70 °C and 80 °C were not significant. In addition, the effect of increasing temperatures on the reduction of total microorganism count was also not apparent.

Table 2: Log of microorganism (CFU/g) in Manuka honey after heat-treated with different temperatures for 30 minutes

	log N± SD (N in CFU/g)	**Log reduction N± SD (N in CFU/g)**
Unprocessed	4.0 ± 0.01	
60 °C	3.8 ± 0.01	0.2± 0.03
70 °C	3.6 ± 0.02	0.4 ± 0.02
80 °C	3.5 ± 0.05	0.5 ± 0.01

From both HPP and thermal treatment preliminary test, it clearly shows that thermal treatment gave a minor effect on the microbial inactivation as compared with HPP treatment. This has been proven when 60 °C was applied in thermal treatment, the applied heat was not sufficient to inactivate all microorganism (with 3.8 ± 0.01 log CFU/g of microorganism survival), but when 350 MPa was applied in combination with 60 °C, the microorganism in the samples were not detected.This preliminary result suggests, by using mild heat in combination with high pressure, a higher quality safe and durable honey can be produced. Based on the amount of microorganism surviving after thermal treatment at 80 °C (with 3.5 ± 0.01 log CFU/g), it is necessary to find out what is the species of microorganism present in the samples. The next section was then discuss on the identification of bacterial species.

3.2 Identification of Bacterial Spore in Manuka Honey

The ability of microorganism to survive after heat treatment, even at higher temperature (80 °C for 30 min), leads to the hypothesis that the microorganisms present in Manuka honey were spore forming bacteria. The hypothesis made was based on the characteristics of the bacterial spores which are resistant to high temperature as noted by previous works [3,8]. The bacterial spores are highly resistant to heat compared to vegetative cells due the endospore content.

The identification of microorganism present in Manuka honey started with cultivation of microorganism on Potato Dextrose Agar (PDA) after heat treatment of samples. The plates were then incubated at 25 °C for 5 days. After 5 days incubation, the white and creamy with round shape colonies were observed on the plate agar as shown in Figure 2.

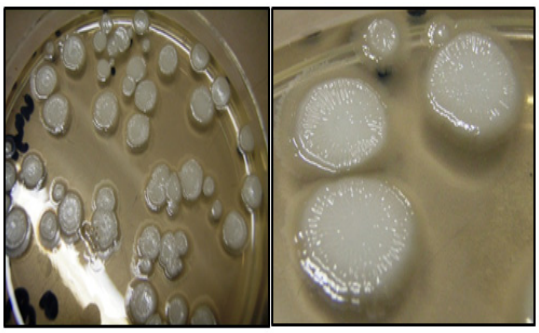

Figure 2: (a) Microorganism colonies found in Manuka honey samples after treated at 80 °C for 30 min and (b) the colonies were well grown on the Potato Dextrose Agar (PDA) after 5 days incubation at 25° C.

The investigation proceeded with a general observation on the shape and size of the bacteria. The colonies were viewed under light microscope by putting drops of distilled water on a glass with needle tip of colony. The glass was then covered on top and under microscope with a drop of emission oil to get a clearer image. The shape and size of colony viewed under light microscope were shown in Figure 3. It was found that the microorganism were in rod-shape, elongated chain with the size of more than 4 µm. [7] revealed the bacteria size was in range from 0.2-2 µm in width or diameter, and up to 1-10 µm in length for the nonspherical species. Thus, this observation gives a clear assumption that the microorganisms were from bacteria due to the size. Regarding to the shape, the observation of rod-shaped with 4- 6 µm long in size, make a closer to the prediction that the bacteria found was in genus *Bacillus*. [7,9] reported the word *bacillus* (plural *bacilli*) is used to describe any rod-shapedbacterium and the size of *bacillus* of about average size 2.0 to 6.0 m long.

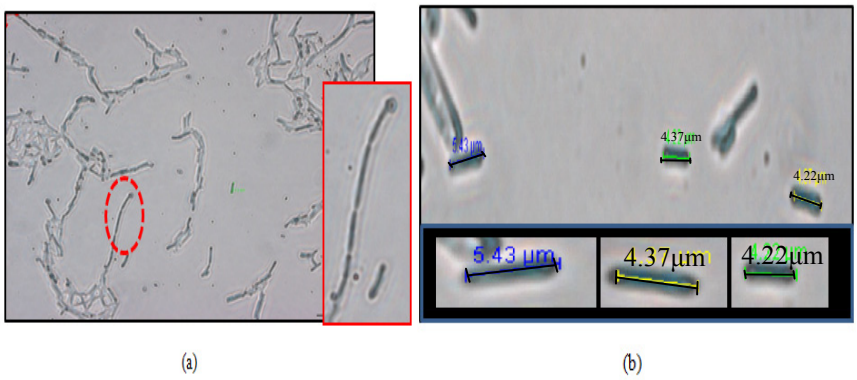

Figure 3: The structure of colony viewed under light microscope (a) shape of the microorganism from the colonies cultivated on the PDA. Small picture shows the clear image of the rod-shaped with elongated chain (b) size of the colonies found were in the range of 4- 6 µm long.

Polymerase Chain Reaction (PCR) technique was then performed together with DNA sequencing in order to get the DNA sequence of targeted bacteria and compare to other known sequences, using BLAST program. This will result in the identification of bacteria genus and species. A digital of gel electrophoresis for different restrictions digest run on a 1 % w/v agarose agar gel, 3 volt/cm, stained with ethidium bromide was shown in Figure 4. The DNA size marker is a commercial 1 kbp ladder. The position of the wells and direction of DNA migration is noted. In this experiment, yeasts and bacteria markers were used.

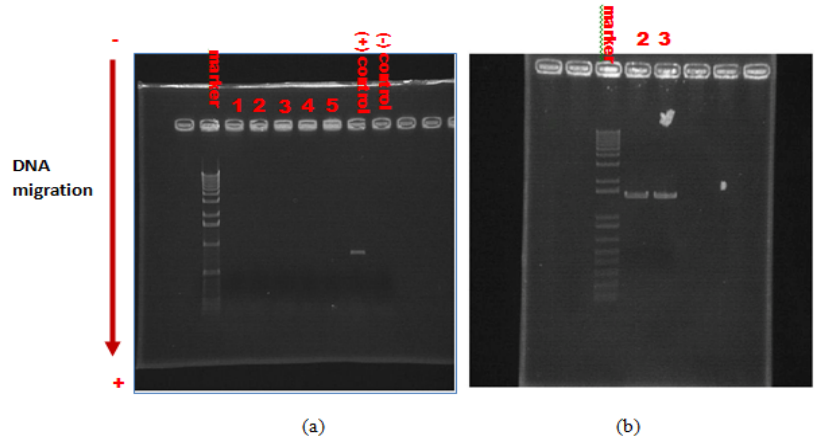

Figure 4: (a) Commercial DNA Yeasts Markers, (b)commercial DNA Bacteria Markers

Figure 4 (a) shows a Commercial DNA Yeasts Markers (1kbplus) in Lane 1, meanwhile Lane 2, 3, 4 and 5 were empty with no bands. Meanwhile, Figure 4 (b) shows another gel electrophoresis using commercial DNA Bacteria Markers (1kbplus) with no positive control this time as it is known they were most probably bacteria. Lane 2 and 3 shows a band showing that the target DNA sequences were compatible with bacteria DNA.From the PCR using 16S primers which amplify the ribosomal DNA from the colonies found on the plates, the sequences have been aligned and were run through a program called BLAST found on NCBI. This gave the result of the colony being a *Bacillus*, most likely the species *cereus*. The same finding was reported in Moroccon honey [10] and Ibadan honey [11]. They found that most of the samples tested were found from genus *Bacillus*. In addition, most of the 94 % spores found in commercial honey samples were from genus *Bacillus* and predominant species was *B. cereus* [12].

4. CONCLUSIONS

It is possible to use HPP to inactivate microorganisms present in high sugar content foods, particularly in Manuka honey. Pressure and temperature are found to be significant parameters that effect the inactivation of microorganism present in honey. The unknown microorganism which was resistant to high temperature (80 °C) in Manuka honey was identified as *Bacillus cereus* using PCR technique. Further works are necessary to find an optimal combination of treatment pressure, temperature and time to ensure the safety of honey without compromising its quality and natural freshness.

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