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Heat Resistance of *Acanthamoeba sp.* Cysts in Green Mussel Broth and Phosphate-Buffered Saline

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Cysts propagated by the agar block method with heat-killed *Escherichia coli* as nutrient overlay were inoculated into *Perna viridis* broth (PVB) and phosphate-buffered saline (PBS) prior to exposure to 60, 75 and 100°C for 0, 3, 5, and 10 min. The heat resistance of *Acanthamoeba sp.* cysts expressed in of D- and Z-values were found to be greater in the complex organic PVB than in the aqueous PBS. The established D-values in the PVB were 81.20, 44.59, and 8.83 min at 60, 75, and 100°C, respectively. The calculated Z-value of *Acanthamoeba sp.* cysts in PVB was 40.28°C.

Keywords: D-value, Z-value, protozoon cysts, shellfish

Inroduction

Application of heat treatment is one of the more traditional modes for microbial decontamination of food products (Murphy *et al.*, 2002). The efficiency of heat treatment against microorganisms is expressed commonly in terms of the heat resistance parameters, D- and Z-values (Stumbo 1973, Jay 1992). Although numerous studies have already been done about the heat resistance of bacterial contaminants of food, there is still paucity of studies on protozoon heat resistance in food products.

Protozoa have been proven to be positive transvectors of human diseases (Martinez 1985). Jackson (1990) included protozoa to the list of foodborne parasites of public health significance. The number of deaths per annum resulting from food- and waterborne-amoebae infections gives adequate justification for studies of protozoon in food systems (Campbell and Chadee 1997, Petri *et al.*, 2000).

Acanthamoeba sp. is among the free-living amoebae found to have the ability to become opportunistic parasite and cause diseases to humans (Griffin 1972, Martinez 1985). This amoeba species has been found to cause an array of diseases, especially to immunocompromised individuals (Griffin 1972). Fatal granulomatous amoebic meningoencephalitis, skin lesions, transient diarrhea, and keratitis are some of the many diseases that *Acanthamoeba sp.* cause in humans. Unfortunately the route of human infection of *Acanthamoebae* has not been fully established. Volk and Wheeler (1988) included free-living amoebae to the list of parasites infecting humans via the digestive tract. Some authors also suggested a route implicating the blood stream flowing through a primary infection site (Martinez 1985, Todd 2001).

Molluscan bivalves, like the green mussels (*Perna viridis*), filter feed on particles suspended in their water environment including several pathogenic bacteria and protozoon cysts (Hawkins and Bayne 1992). A number amoebic species were found to inhabit the mantel cavities of mussels (Bower 1992). Cheng (1970) reported that contaminated bivalve mollusks could become vehicles of transmission of soil amoebae to humans.

The survival of amoebae in environments with harsh conditions could be attributed to the ability of the cells to form resistant cysts (Griffin 1972). Moreover, the survival of microbial contaminants in edible animal tissues could possibly be enhanced by the proteinaceous and fatty components of the food that could offer protection to microorganisms. Jay (1992) explained that both the protein and lipid components of a food system could reduce heat transfer within the system and thus delimit its thermal cidal activity against microorganisms. The objective of this study was to establish the thermal resistance characteristics, expressed in D- and Z-values, of *Acanthamoeba sp.* cysts in PVB and PBS. The results of this study may be considered in thermal processing or even in home-scale preparation of green mussels that maybe contaminated with *Acanthamoeba sp.* cysts.

Methodology

Microbial culture Two-month old monoxenic cyst culture of *Acanthamoeba sp.* (IMA strain) on biphasic bacteriological agar and heat-killed *E. coli* nutrient overlay in Petri plate was procured from the Institute of Biology, University of the Philippines, Diliman (IB-UPD), Philippines. The *Acanthamoeba sp.* cysts was originally isolated from the soil of Mount Arayat in Central Luzon, Philippines and maintained as a part of the culture collection of IB-UPD. *Escherichia coli* (ATCC 25922) agar slant was obtained from the Culture Collection of the Natural

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E. coli culture propagation The *E. coli* culture was maintained in nutrient agar (NA) slants (BBL, USA) at 35°C. Subcultures were done every 7 d to propagate *E. coli* cells needed as nutrient source for *Acanthamoeba* cyst culture. The *E. coli* overlay of the biphasic *Acanthamoeba sp.* propagating medium was prepared by suspending a loopful of *E. coli* culture in 7 ml *Escherichia coli* broth (ECB) (BBL, USA) and incubating at 35°C for 24 h. The *E. coli* cultures in ECB were deactivated by dipping the tubes in a water bath (Buchi, Germany) to attain tube temperature of 100°C for 10 min. The heat-killed cells were then prepared to be used as nutrient overlay for *Acanthamoeba sp.* cyst propagation.

Acanthamoeba sp. cyst propagation The agar-block technique described by Matias (1993) was followed in the propagation and subsequent maintenance of *Acanthamoeba* cyst cultures. For the initial propagation of the cysts, plates of biphasic medium consisting of a solidified 1.5% bacteriological agar (BA) (Pronadisa, Spain) as base overlaid with 1 ml aliquots of heat killed *E. coli* cultures in ECB were prepared for cyst propagation and maintenance. Approximately 1 cm^3 blocks of the *Acanthamoeba* cyst culture procured from the IB-UPD were excised and placed inverted with the *Acanthamoeba sp.* cyst-inoculated surface touching the freshly prepared biphasic plates. The cysts were lawned by gently pushing the agar blocks on the surface of the biphasic medium with a sterile glass hockey stick. The resulting inoculated plates were incubated at 25°C and used as the stock culture for the thermal resistance studies of *Acanthamoeba* cysts. Two-week old cyst cultures were prepared as needed for the thermal inactivation studies.

Suspending media for thermal resistance study *Perna viridis* broth was prepared based on the procedure by APHA (1966). Chopped fresh green mussel meat were suspended in PBS at 4.5:10 wt/vol ratio and cooked by steaming for 1 h. The broth was then filtered through filter paper (Whatmann No. 113 Qualitative, Wet-Strengthen) and the pH of the filtrate was adjusted to 6.3 using 0.1N HCl. The filtrate was then sterilized following the protocol cited by Rowbotham (1980) for sterilizing *Acanthamoeba sp.* propagating medium. Briefly the sterilization of PVB involved exposure of the medium to ultraviolet light with a generated radiation wavelength of 254 nm. A lamp-to-medium distance of 15 cm was maintained with exposure time of 3 h. Phosphate-buffered saline was prepared by dissolving NaCl, KCl, KH_2PO_4, and $NaHPO_4$ with 8.0:0.2:0.2:1.2 wt ratios in distilled water to make 1000 ml solution. The pH of the buffered saline was then adjusted to 6.3 with 1 N HCl. The PBS was sterilized at 121.5°C at 15 psi for 15 min.

Heat treatment and cyst viability determination Two-week old cyst cultures incubated at 25°C were harvested for thermal resistance studies by flooding the inoculated biphasic plates with 2.5 ml sterile PBS or PVB and gently scraping the flooded surface with a sterile glass hockey stick. The cyst suspensions were then aseptically aspirated and collected in a 50 ml sterile beaker. Two ml portions of PVB or PBS were aseptically aspirated into 5 ml sterile screw-capped tubes.

Triplicate tubes containing 2 ml aliquots of 10^6 *Acanthamoeba sp.* cysts/ml suspension in PVB or PBS were exposed to heat treatments for 0, 3, 5 and 10 min at 60, 75 and 100°C test temperatures using a water bath (Buchi, Germany). The heat-treated tubes were immediately immersed into ice bath after exposure to the desired time and temperature protocols. Viable heat-treated cysts were distinguished from the non-viable ones by direct microscopic examination coupled with nigrosine dye exclusion procedure (Capati 2001 and McAteer and Davis 1994). Direct microscopic counts of cysts were conducted using a haemacytometer (New Improved Neubower Counting Chamber, Germany) and a compound light microscope (Meiji, Japan) under 100 × magnification. Viable cysts were observed to be colorless against a dark background while non-viable cysts were darkly colored. Percentages viability of cysts were calculated against the total number of the cysts per suspending medium.

D- and Z-value determination The decimal reduction times, D-values, per exposure temperature per suspending medium were calculated from the survivor curves. The $\log_{10}$ of the percentages viable cysts against time of exposures were plotted and the equation of the best-fitted line was obtained through linear regression. The D-values were determined as the number of unit time required for the survivor curve to traverse 1 $\log_{10}$ cycle (Mossel *et al.*, 1985, Jay 1992) and graphically equal to the negative inverse of the slope of the regressed straight-line equation. The Z-values, the temperature increase required to increase the death rate 10-fold or reduce the D-value 10-fold, were calculated by plotting the $\log_{10}$ of the D-values against exposure temperatures. The calculated Z-values were graphically equal to the negative inverse of the slope of the regressed straight-line equation from the $\log_{10}$ of D-values against temperature plots.

Results and Discussion

Thermal resistance of Acanthamoeba sp. cysts in PVB and PBS The established D-values of *Acanthamoeba* cysts in PBS were always lower than in the PVB for all test temperatures utilized in the study (Table 1). The D-value of any biological entity in a food system is the number unit time required to reduce the initial population by 1 $\log_{10}$ unit (Mossel *et al.*, 1985, Jay 1992). The physiological characteristics and morphological structures of the *Acanthamoeba* cysts can help explain their natural thermal resistance. The cysts were previously reported to retain viability in culture incubation temperatures as high as 56°C (AWWA 1997). The morphology of *Acanthamoeba* cysts indicating double wall protection prevents cellular damages even in extreme environmental conditions like severe desiccation and low atmospheric redox potential (Martinez 1985, AWWA 1997). The single-walled *Entamoeba histolytica* cysts was found to have lower thermal resistance than *Acanthamoeba* cysts (Jones and Newton 1950) with thermal death times at 65 and 100°C of 5 min and 10 sec in water, respectively. In

Table 1. Thermal parameters* of *Acanthamoeba sp.* cysts in *Perna viridis* broth and PBS, pH 6.36 and A_w 0.98.

Temperature (°C)	Time (min)	PBS		*PvB*	
		% Survival	D** (min)	% Survival	D (min)
60	0	100.00 ± 0.00	**72.73 ± 2.06**	100.00 ± 0.00	
	3	94.54 ± 1.06		99.45 ± 0.36	**81.20 ± 2.80**
	5	86.93 ± 0.47		88.45 ± 0.59	
	10	75.52 ± 0.32		81.95 ± 0.49	
75	0	100.00 ± 0.00	**13.65 ± 0.33**	100.00 ± 0.00	**44.59 ± 2.83**
	3	66.99 ± 1.36		72.99 ± 1.00	
	5	46.76 ± 0.74		63.63 ± 0.58	
	10	19.87 ± 0.96		50.54 ± 0.89	
100	0	100.00 ± 0.00	**6.88 ± 0.30**	100.00 ± 0.00	**8.83 ± 0.32**
	3	14.51 ± 0.25		18.99 ± 1.03	
	5	3.55 ± 0.21		9.85 ± 0.70	
	10	1.76 ± 0.09		3.19 ± 0.12	
Z* (°C)**			**35.61 ± 0.79**		**40.28 ± 0.47**

*values given are means of three trials ± standard deviation
** Decimal reduction time is the number of minutes that will effect a single $\log_{10}$ cycle change in the original amoeba cyst population
*** Z value is temperature increase required to increase the death rate 10-fold or reduce the D value 10-fold.

this study, the established D-values of *Acanthamoeba* cysts in the aqueous PBS were 72.73, 13.65, and 6.88 min at 60, 75 and 100°C, respectively. The D-values were higher in PVB with values of 81.20, 44.59, and 8.83 min at 60, 75, and 100°C, respectively. Normal green mussel cooking protocol which exposes green mussel meats to temperatures only as high as 100°C for 10 min (Arroyo-Staub, 1989) can only effect approximately 1 $\log_{10}$ cycle change in the concentration of *Acanthamoeba sp.* cysts.

Phosphate-buffered saline may have imparted lesser protective effect to the cysts in suspension than in PVB since the buffer is composed mainly of water. The PVB, on the other hand, is a complex food-based medium composed mainly of protein, fat and carbohydrates. The Philippine Food and Nutrition Research Institute (FNRI 1997) cited that raw Philippine green mussels contain 13.6%, 7.5% and 11.0% of protein, fat and carbohydrates, respectively. Mulak *et al.*, (1994) similarly showed that the D-values for several bacteria inoculated in homogenized fish terrine were higher than those in phosphate buffer. It has been reported that the proteinaceous, fatty and several ionic components of food systems have buffering or protective effects to thermal action (Stumbo 1973, Jay 1992). Furthermore, physical properties of the food system like viscosity tend to slow down thermal transfer. The denaturation of green mussel proteins in PVB during heating may have also caused increase in viscosity of the broth, slowed down heat transfer rates and thus possibly enhanced cyst survival.

The calculated Z-values of *Acanthamoeba sp.* cysts in PBS and PVB were 35.61 and 40.28°C, respectively. The Z-value is numerically equivalent to the temperature increase needed to effect a 10-fold change in lethality of a process against a target agent. The calculated Z-values established for the *Acanthamoeba sp.* cysts in both test media may have significant impact to processing schedules that maybe applied to green mussels if higher orders of destruction would be required to ensure safety of the processed product. The heat sensitivity of green mussels would affect the overall acceptability of the thermally processed products if longer heating time would be required as dictated by the Z-value factors.

Conclusion

This study established the D-values of *Acanthamoeba sp.* cysts in PVB to be 81.20, 44.59 and 8.83 min at 60, 75 and 100°C, respectively. The calculated Z-value of the cysts suspended in the green mussel broth medium was 40.28 min. The calculated heat resistance parameters of *Acanthamoeba sp.* cysts maybe considered in the establishment of thermal process schedules for contaminated green mussel meat. Further investigations should be undertaken to establish the heat resistance of protozoon cysts in bivalve meat from other strains of *Acanthamoeba sp.* to validate the results obtained in the study.

References

American Public Health Association. (1966). Recommended methods for the microbiological examination of foods. Ed. J.M. Sharf. American Public Health Association, Inc., New York.

American Water Works Association. (1997). Drinking water inspectorate fact sheet – Acanthamoeba. (1) March 1997.

Arroyo-Staub, Patricia and Staub, H.E. (1989). Cooking Filipino food in Germany. Karlsruhe, Germany.

Bower, S.M. (1992). Diseases and parasites of mussels. In "The mussel *Mytilus*: ecology, physiology, genetics and culture." ed. by E. Gosling. Elsevier Science Publishers, Netherlands. pp. 543–559.

Campbell, D. and Chadee, K. (1997). Survival strategies of *Entamoeba histolytica:* modulation of cell-mediated immune response. *Parasitology Today* **13**, 184–189.

Capati, C. (2001). *In vitro* response of *Perna viridis* haemocytes to *Pyrodinium bahamense var. compressum.* B. Sc. Thesis. University of the Philippines, Diliman, QC.

Cheng, T. (1970). *Hartmanella tahitinensis* sp. n., an amoeba associated with mass mortalities of oyster, *Crassostrea commercialis*, in Tahiti, French Polynesia. *Journal of Invertebrate Pathology.* **15**, 405–419.

Griffin, J.L. (1978). Pathogenic free-living amoebae. In "Parasitic protozoa", ed. by J.P. Krier. Academic Press, New York. pp. 455–497.

Philippine food composition tables. (1997). Food and Nutrition Research Institute, Department of Science and Technology. Bicutan, taguig, Metro Manila.

Hawkins, A.J. and Bayne, B.L. (1992). Physiological interrelations and the regulation of production. In "The mussel *Mytilus*: ecology, physiology, genetics and culture", ed. by E. Gosling. Elsevier Science Publishers, Netherlands. pp. 171–212.

Jackson, T. (1990). Parasitic protozoa and worms relevant to the U.S. Food Technology **44**, 106–112.

Jay, J. (1992). Modern food microbiology. Chapman and Hall. New York.

Jones, M. and Newton, W. (1950). Survival of cysts of *E. histolytica* in water at temperatures between 45 and 55°C. *Am. J. Tropical Med.*, **30**, 53–58.

Martinez, J. (1985). Free-living amoebas. CRC Press, USA.

Matias, R. (1993). Biology of *Acanthamoeba sp.* III. Restriction fragment polymorphism of DNA from seven *Acanthamoeba spp.* isolates. Natural Science Research Institute technical report. University odf the Philippines, Diliman, Quezon City.

McAteer, J. and Davis, J. (1994). Basic cell culture technique and maintenance of cell lines. In "Basic cell culture: a practical approach." ed. by J.M. Davis. Oxford University Press. New York.

Mossel, D.A.A., Corry, J.E., Struijk, C.B. and Baird, R.M. (1995). Essentials of microbiology of foods: a textbook for advanced studies. John Wiley and Sons, New York.

Mulak, V., Tailliez, R., Eb, P. and Becel, P. (1994). Heat resistance of bacteria isolated from preparations based on seafood products. *Journal of Food Protection* **58**(1), 49–53.

Murphy, R.Y., Duncan, L.K., Johnson, E.R., Davis, M.D., and Smith, J.N. (2002). Thermal inactivation D- and Z values of *Salmonella* serotypes and *Listeria innocua* in chicken patties, chicken tenders, franks, beef patties, and blended beef and turkey patties. *Journal of Food Protection* **65**, 1, 53–60.

Petri, W.A. Jr., Haque, R., Lyerly, D., and Vines, R.R. (2000). Estimating the impact of amoebiasis on health. *Parasitology Today* **16**, 320–321.

Rowbotham, T.J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoeba. *J. Clin. Pathol.* **33**, 1179–1183.

Stumbo, C. (1973). Thermobacteriology in food processing 2nd ed. Academic Press. New York.

Todd, Melissa. (2001). Amoeba: the trojan horse of the water treatment process? Water Quality News. Cooperative Research Center for Water Quality and Treatment, Australian Water Quality Center – University of South Australia. **13**, 3–5.

Volk, W.A. and Wheeler, M.F. (1988). Basic Microbiology 6th ed. Harper and Row Publishers. New York.