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Short communication

Thermal inactivation of *Pseudomonas aeruginosa* 1244 in salted *Sardinella fimbriata* meat homogenate

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Abstract

This study established the thermal inactivation parameters, D and z values of *Pseudomonas aeruginosa* 1244 in a laboratory-prepared salted herring meat homogenate suspending medium. The food matrix was determined to have a pH equal to 5.85 and a_w of 0.73. The moisture and NaCl contents were 38.25% and 13.81%, respectively. The test microorganism exhibited first-order inactivation behavior ($R^2 \geq 0.90$) at all heating temperatures. Thermal inactivation rates decreased with increasing temperatures. The D values established were 70.94 s (60°C), 60.46 s (75°C), 31.11 s (80°C) and 22.14 s (90°C).The thermal resistance parameter z value of 57.10°C was determined. The results provide information on the behavior of the test organism in heated salted herring meat, and could provide a basis for challenge studies involving other microbial species and strains in similar food matrices. The thermal inactivation kinetic parameters may be used in the establishment or validation or both of process schedules for salted herring meats, including mechanical dehydration and sun drying protocols.

Introduction

Dried salted herring is one of the more well-known traditional food products of the Philippines. Espejo-Hermes (2004) explained that this product is commonly consumed due to its affordability, nutritional value, and sensory attributes. Dried salted fish processing is a common backyard livelihood activity in nearshore communities and is considered a very easy and straight forward means of extending the shelf life of fish meat. This food processing technique renders a preservative effect by lowering the water activity (a_w) of the food matrix due to moisture loss from evaporation and moisture binding due to the addition of salt (Sikorski and Sun Pan, 1994).

However, assurance of the microbiological quality of finished products could pose challenges to food processors. The microbiology of the finished product is dependent on the inherent microbial flora present in the fish from the environment in which they live, as well as the handling practices of processors along the production line. Bacterial species that are able to survive in environments with very low a_w values previously isolated from dried salted fishes include those belonging to the genera *Staphylococcus*, *Vibrio*, and *Pseudomonas* (Hernandez-Herrero et al., 1999).

Pseudomonas aeruginosa is a particular food safety concern, as it is known to cause scombroid poisoning; Visciano et al. (2012) explained that the term 'scombroid' is from the family Scombridae, which includes fish species that were first implicated regarding toxicity such as tuna and mackerel. However, other non-scombroid fish species have been similarly implicated in poisoning, including *Corypahaena* spp. (mahi-mahi), *Sardinella* spp. (sardines), *Clupea* spp. (herring), and *Engraulis* spp. (anchovies). Hungerford (2010), Stratta and Badino (2012) and Tortorella et al. (2014) described scombroid poisoning as one of the most common causes of morbidity associated with fish intake, due to the ingestion of products with high levels of histamine due to improper processing and storage. Histamine is liberated in fish meats when histidine in the fish muscle undergoes decarboxylation, which is induced by enzymes in bacteria such as *Morganella morganii, Klebsiella pneumoniae, Hafnia alvei, Proteus vulgaris, Proteus mirabilis, Enterobacter aerogenes, Enterobacter cloacae, Serratia fonticola, Serratia liquefaciens, Raoultella* (formerly *Klebsiella*) *planticola, Raoultella ornithinolytica, Providencia stuartii,* and *Citrobacter freundii* (Kim et al. 2003). *Pseudomonas aeruginosa* was also shown to convert the amino acid tyrosine into tyramine, another compound of public health significance (Beutling, 1993).

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Cognizant of the challenges that *P. aeruginosa* poses to dried fish processors, there is a need to determine the inactivation behavior of this common foodborne organism in salted herring meat. Thus, this study was conducted to determine the thermal resistance of *Pseudomonas aeruginosa* 1244 in salted meat herring homogenate. The results obtained in this work may be used in the development and validation of processes with the recommended level of lethality against the target organism.

Materials and Methods

Salted Sardinella meat homogenate preparation and decontamination

Freshly caught herrings were obtained from a public wet market in Quezon City, the Philippines, placed in an ice chest, and immediately transported to the laboratory. In the laboratory, the fish were thoroughly washed with running water for 2–3 min. Salting in ice was done following the generic process for dried salted herring production reported by Gabriel and Budiao (2015). Briefly, fish, ice and salt were mixed in a sanitized ice chest at a 3:1:0.9 ratio, respectively. The mixture was allowed to equilibrate for 24 hr. The meat homogenate was then prepared following steps described by the Association of Official Analytical Chemists (1990). The salted fish were descaled, filleted and deboned using a sterile scalpel, and the salted herring fillets were comminuted using a bench top blender set at minimum speed. The homogenates were separated into 150 g portions, placed in zipper bags and immediately stored at -18°C prior to background microflora decontamination. To eliminate the background microflora of the salted *Sardinella* meat homogenates, the samples were subjected to gamma ionizing radiation as similarly done by Sevilla and Gabriel (2010) using the Gammabeam 651 PT (MDS Nordion; Kanaka, ON, Canada) of the Philippine Nuclear Research Institute, University of the Philippines, Diliman, Quezon City, the Philippines. The samples were subjected to an irradiation dose of 25 kGy using ^{64}Co and the irradiated samples were immediately stored at -18°C until the inoculation and challenge studies. The sterility of the irradiated samples was also evaluated and compared to a non-treated control.

Physicochemical analysis

The salted *Sardinella* meat homogenate was subjected to pH, a_w, and moisture and NaCl content analyses. The pH measurements were conducted using pH meter (Cyberscan 500; Eutech Instruments; Singapore) with the electrode calibrated using pH 4.00 and 7.00 buffer solutions (Merck; Darmstadt, Germany). The Novasina ms1 set a_w (Novasina; Pfaffikon, Switzerland) was used to measure the a_w of the samples following the manufacturer-detailed procedures. The moisture content was determined using a Yeasten (Matsushita Electric Works Ltd.; Tokyo, Japan) Infrared Moisture Analyzer while the Mohr $AgNO_3$ titrimetric method described by Day and Underwood (1991) was used for the determination of the NaCl content.

Inoculum preparation and salted herring meat inoculation

Pseudomonas aeruginosa 1244 was obtained from the Culture Collection Division of the Natural Sciences Research Institute (NSRI), University of the Philippines, Diliman, Quezon City, the Philippines. The inoculum was prepared by first activating a loopful of cells obtained from the original culture slant in 10 mL sterile nutrient broth (NB; HiMedia; Mumbai, India), incubated at 37°C for 24 hr. A 1 mL aliquot of the activated culture was then enriched in 100 mL NB that was subsequently incubated at 37°C for 24 hr. Cells were then harvested from the enriched culture by spinning 50 mL aliquots at 2,419×*g* for 20 min. The supernatant liquids were decanted and the pelletized cells were resuspended in sterile phosphate buffered saline (PBS) to make a final volume of 250 mL. The PBS suspension was kept at ambient temperature (25°C) for not longer than 30 min, until inoculation into the test food matrix.

Prior to inoculation, frozen irradiated salted *Sardinella* meat homogenate samples were thawed at 10 °C for 8–12 hr and equilibrated to room temperature for 1–2 hr under a laminar flow hood. The irradiated meat homogenate was mixed with the PBS *P. aeruginosa* suspension at a 1:10 ratio, respectively, and the inoculated homogenate was thoroughly mixed using a metal spatula and manual pressing of the zipper bag. The inoculated sample was set aside for not longer than 30 min to allow the inoculated organisms to acclimatize to the food matrix. An initial population between 6 and 7 log colony forming units (CFU)/g was introduced to the salted *Sardinella* meat homogenate prior to inactivation studies.

Thermal inactivation studies

The study adapted the method reported by Sevilla and Gabriel (2010) for thermal inactivation studies in tilapia meat homogenate. Briefly, 5 g portions of the inoculated salted *Sardinella* meat homogenate were placed in sterile screw-capped tubes. A negative control tube containing meat homogenate inoculated with sterile PBS was also prepared and used for temperature monitoring during heat treatment. A probe thermometer was inserted into the geometric center or cold point of the homogenate. The cold point is a region within the heated food matrix that receives the desired temperature last (Heinz and Hautzinger, 2007). The tubes were immersed in a hot water bath (Büchi; Essen, Germany) to expose the inoculated matrix to 60, 75, 80, and 90°C challenge temperatures at predetermined time intervals between 0 and 2.0 min. In the heating studies, microbial counts reported at time zero (t_0) were those enumerated when the heated matrix reached the target temperature. Prior optimization works allowed the investigators to determine water bath temperature settings that minimized the lag or come up time (CUT). For example, for heating studies at 60°C, a water bath temperature setting of 63°C allowed for the minimization of CUT to less than 2 min. Bath temperatures of 77, 82 and 91°C also minimized the CUT at 75°C, 80°C and 90°C, respectively to less than 2 min. Upon exposure to the predetermined heating time, tubes were immediately removed from the hot water bath and immersed for not longer than 15 min in iced water.

Survivor enumeration and heat resistance determinations

Surviving *P. aeruginosa* cells were enumerated from the heated salted *Sardinella* meat homogenate by subjecting the meat to 10-fold serial dilution with Proteose Peptone Saline (PPS). The PPS was compounded by preparing 0.1% peptone (HiMedia; Mumbai, India) solution from 0.85% NaCl solution. Appropriate dilution levels were surface plated on nutrient agar (NA, HiMedia; Mumbai, India) plates, which were subsequently incubated at 37°C for 24 hr. Emerging colonies were counted and survivor populations were expressed as log CFU per gram (log CFU/g).

The thermal resistance of *P. aeruginosa* in the salted *Sardinella* meat homogenate was determined by calculating the decimal reduction times (D values) per heating temperature, and the z value, which

quantified the temperature dependence of *P. aeruginosa* inactivation in the test food matrix. In a plot of heating time versus the surviving population, the D value is graphically equivalent to the negative inverse of the best-fitted line interpolated from the plot. Similarly, the z value was determined as the negative inverse of the slope of the best-fitted line in the log D value versus the heating temperature plot.

All heat inactivation challenges were done with at least two external replications. In each of the external replicates, a duplicate plating scheme was used to enumerate surviving *P. aeruginosa* populations. The D value per heating temperature was calculated from all heating time-survivor populations obtained from replicated runs. Hence at each heating temperature, only one D value was calculated.

Results and Discussion

The selected physicochemical properties of the salted *Sardinella* meat homogenate used in this study are presented in Table 1. The results showed that the suspending medium had a pH of 5.85., which was comparable to the pH of tilapia (*Oreochromis niloticus*) meat homogenate reported by Sevilla and Gabriel (2010) and typical of fish muscles in most species (Jay, 2000; Ross et al., 2000). Furthermore, the a_w, moisture content, and NaCl contents of the meat homogenate were 0.73, 38.25%, and 13.81%, respectively.

Table 1 Selected physicochemical properties of salted *Sardinella* meat homogenate

Physicochemical property	Mean ± SD (n = 3)
pH	5.85 ± 0.04
Water activity (a_w)	0.73 ± 0.05
Moisture content (%)	38.25 ± 3.20
NaCl (%)	13.81 ± 1.50

Increasing the heating temperature resulted in increasing population reduction within the 120 s exposure time. As shown in Fig. 1, an average population reduction of 1.88 log CFU/g was observed after 120 s of heating at 60°C. Population reductions of 2.11 log CFU/g, 4.04 log CFU/g and 5.31 log CFU/g were observed after 120 s exposure at 75, 80, and 90°C, respectively. The plots generated from the *P. aeruginosa* survivor populations and heating exposure times showed that the microorganism had a log-linear inactivation behavior in the food matrix with coefficient of determination (R^2) values of 0.90–0.99. A log-linear inactivation curve has also been described as an indicator of a first-order kinetics, where inoculated cells are assumed to have homogenous heat resistance or susceptibility (Moats, 1971).

At 60°C, the test organism had a D value of 70.94 s in salted *Sardinella* meat homogenate (Table 2). Increasing the heating temperature to 75°C and 80°C decreased the heat resistance of *P. aeruginosa*, as indicated by D values of 60.46 s and 31.11 s, respectively. The lowest heat resistance was observed at 90°C**,** where the test organism had a D value of 22.14 s. The substantial effect of exposure temperature on the lethality of heat treatments was similarly observed by Murphy et al. (2003a) and Murphy et al. (2003b) in their study with *Salmonella*, *Listeria innocua* and *Listeria monocytogenes* in fully cooked chicken breast meat.

The values and trend in the thermal resistance of *P. aeruginosa* 1244 in salted *Sardinella* meat homogenate established in the current study were comparable to those previously established by Sevilla and Gabriel (2010) for *E. coli* ATCC 25922 in tilapia meat homogenate. At 65°C, 70°C, and 75°C heating temperatures, *E. coli* had D values of 67.8 s, 53.4 s and 30 s, respectively. Furthermore, the D values of *Salmonella* Typhimurium E1366 in golden snail (*Pomacea conaliculata*) and coconut cream mixture at 60°C, 75°C and 90°C were reported by Gabriel and Ubana (2007) as 25.20 s, 23.40 s and 13.2 s, respectively. Moreover, the D values of *Pseudomonas paucimobilis* at 70°C in a number of seafood products reported by Mulak et al. (1995) varied from 69.6 s to 201.6 s.

The z value of *P. aeruginosa* in the salted *Sardinella* meat homogenate was 57.10°C (Table 2). The z value is also equivalent to the increase in heating temperature that will reduce the D value of *P. aeruginosa* by 10-fold (Forsythe, 2000). This value was almost 2-fold larger than that reported by Sevilla and Gabriel (2010) for *E. coli* ATCC 25922 in tilapia meat homogenate at 28.5°C. Moreover, the z values of *Pseudomonas paucimobilis* in different seafood products were reported to range from 5.8°C to 9.1°C (Mulak et al., 1995).

The thermal inactivation kinetic parameters (D and z values) obtained in the current study differed from those reported in the literature because microbial resistance or susceptibility toward heat is dependent on intrinsic food properties, extrinsic thermal process variables and implicit microbial characteristics (Gabriel, 2012). Jay (2000) reported that microbial thermal resistance is relatively higher in suspended food media with high pH, low a_w, and low moisture levels. Essential cellular proteins are more effectively denatured by heat under acidic conditions; hence the basis for the severity of thermal process lethality (Alabastro, 1987). Furthermore, moisture in the food system is an effective heat transfer agent, so that microorganisms are more easily inactivated in high moisture and high a_w food products (Jay, 2000).

Furthermore, differences in the food composition, including fat, protein and salt contents significantly caused variation in the heat transfer within the suspending medium, and eventually affected microbicidal activity (Murphy et al., 2003a; Murphy et al., 2003b).The presence of non-moisture food components protects microorganisms from thermal inactivation through several hypothesized mechanisms including changes in viscosity, the heat transfer rate and cellular dehydration (Jay 2000). Previous studies have also demonstrated that other factors such as suspending medium species, muscle type, challenge organism culture age, culture growth temperature, and initial inoculum population significantly influenced microbial heat resistance (Mulak et al., 1995; Juneja et al., 2001; Smith et al., 2001; Abe et al., 2004).

Furthermore, the effect of cellular physiology on microbial resistance has been similarly demonstrated in previous studies. For example, previous work reported by Gabriel and Nakano (2011), Gabriel (2012), Gabriel (2013), and Gabriel and Arellano (2014) involving different test microorganisms and suspended food matrices showed that previous exposure of microbial cells to physicochemical stresses significantly affected the thermal inactivation rates. For example, sublethal exposures to acidity were previously explained to result in an adaptive mechanism to heat through complex molecular machineries. Thus, this multifactorial dependence of thermal resistance or susceptibility makes the application of generic process schedules for unique food products and specific reference microorganisms dangerous. Underestimation of inactivation rates could result in underprocessing that compromises food safety, while overestimation of microbial inactivation rates could result in overprocessing and eventual quality deterioration.

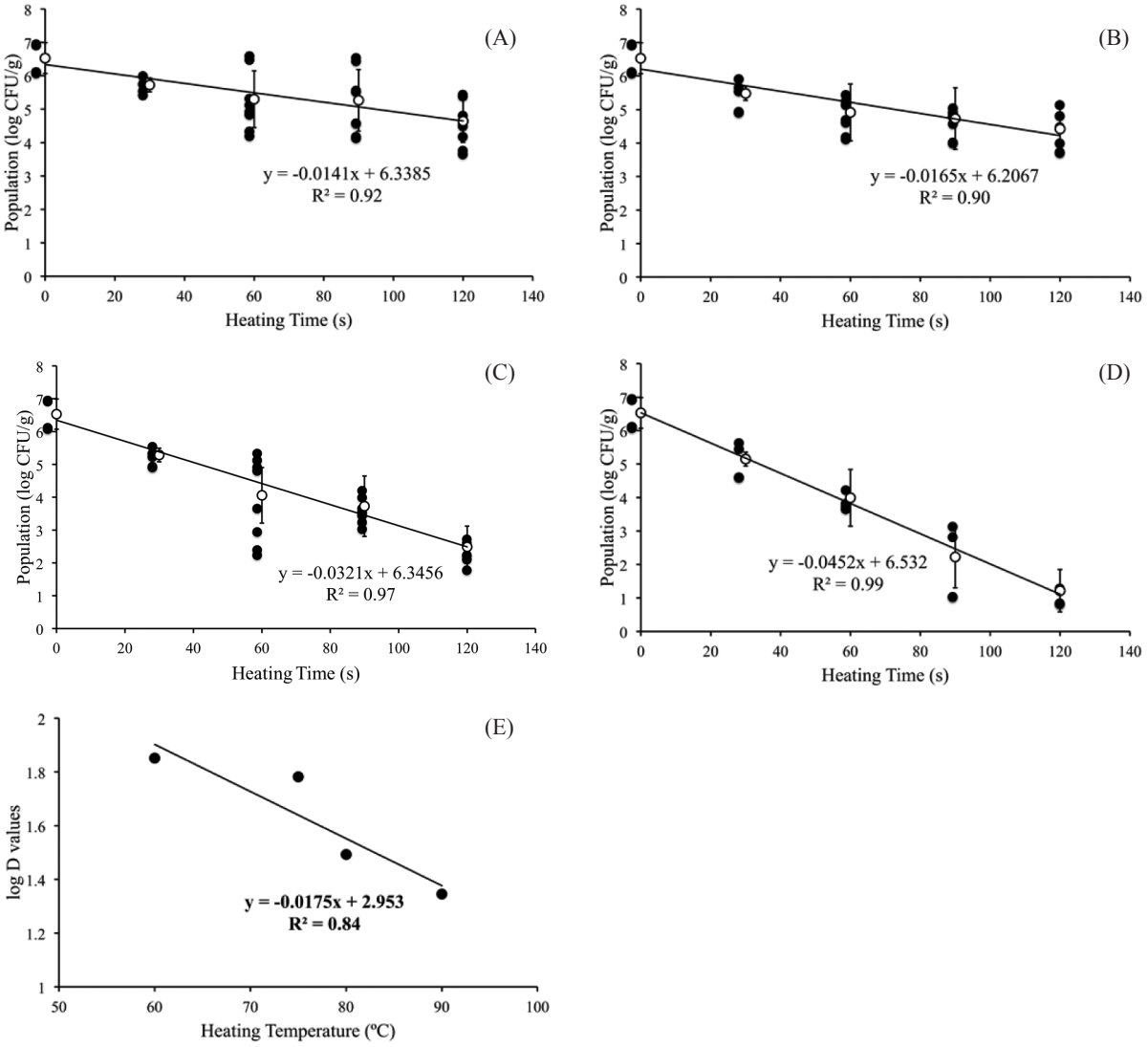

Fig. 1 Thermal inactivation parameters of *Pseudomonas aeruginosa* in salted *Sardinella* meat homogenate. Decimal reduction times (D values) at (A) 60°C, (B) 75°C, (C) 80°C and (D) 90°C were calculated from population versus time plots (CFU = colony forming units, R^2 = coefficient of determination. Solid circles represent raw data points while unfilled circles represent average values from which the D values were calculated, error bars represent standard deviations

Table 2 Thermal inactivation parameters (D- and Z-values) of *Pseudomonas aeruginosa* in salted *Sardinella* meat homogenate

Heating temperature (°C)	Inactivation rate (log CFU/s)	D value (s)	R^2
60	0.014	70.94	0.92
75	0.016	60.46	0.90
80	0.032	31.11	0.97
90	0.045	22.14	0.99
	0.017 log D/°C[1]	Z = 57.10 °C[1]	0.84[2]

CFU = colony forming units; D value = graphical equivalent of the negative inverse of the best-fitted line interpolated from the plot of heating time versus the surviving population; R^2 = coefficient of determination; Z value = thermal resistance parameter calculated from the log D value versus a heating temperature plot ([1]Determined from the plot of log D versus heating temperature; [2]R^2 for Z-value fitting)

The current study established thermal inactivation parameters (D and z values) of *Pseudomonas aeruginosa* in salted *Sardinella* meat homogenate. These inactivation parameters provide information on how the test microorganism behaves in heated test food matrix. These results also suggested that the test organism may be used in the establishment and validation of appropriate thermal process schedules, including fish dehydration processes that can be done mechanically by dehydrators or by sun drying. The thermal resistance parameters established in this study may be compared with those that may be determined through inactivation studies that utilize other forms of heating such as dry-air heating. Further studies should also be made to evaluate process schedules that could be established using the thermal resistance parameters determined in this study. Process schedule evaluation should include the effects of calculated thermal processes on the safety and quality attributes of finished products.

Conflict of Interest

The authors declare there are no conflicts of interest.

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