

Effect of Microwave Heating in Media Containing Preservatives on Viability and Caseinase Production of *Pseudomonas aeruginosa*

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Pseudomonas spp. (including *P. aeruginosa*) can cause spoilage of many types of food. This study examined the effect of the combination of microwave heating and addition of low doses of preservatives on viability and caseinase production of this microorganism. Microwave exposure at high power for 20 sec in nutrient broth (pH 7.2 or 5.9) alone, or in nutrient broth containing potassium sorbate (0.05%, 0.09%) significantly reduced ($p < 0.05$) number of viable cells (reduction of *P. aeruginosa* cells ranged between 3.84 and 4.88 log CFU/ ml). Presence of NaCl (3%) in nutrient broth (pH 7.2) increased the sensitivity of *P. aeruginosa* to microwave action, no viable cells were detected after 20 sec exposure. Microwave exposure for 30 sec of *P. aeruginosa* in NB (pH 7.2 or 5.9), NB (pH 5.9) + 0.05% potassium sorbate, or NB (pH 5.9) + 0.09% potassium sorbate killed all cells in the media. Presence of *T. vulgaris* extract (0.3%) alone in nutrient broth (pH 7.2) slightly decreased the sensitivity of *P. aeruginosa* to microwave action, exposure for 20 or 30 sec reduced number of viable cells by 3.81 and 5.85 logs, respectively. Caseinase production by *P. aeruginosa* in all media tested was abolished after 20 sec exposure although viable cells could still be detected. Microwave heating for 20 sec in chicken homogenate or chicken homogenate + 3% NaCl reduced number of viable cells by ~ 3.2 logs in both media, and significantly reduced ($p < 0.05$) caseinase titer. Exposure for 30 sec reduced number of viable cells by ~ 5.33 logs in both media and inhibited production of caseinase.

Key words: Microwave, *P. aeruginosa*, caseinase, chemicals.

Pseudomonas aeruginosa is Gram-negative non-spore forming bacterium that is able to grow effectively in simple and complex media, water and many types of food. For example, in USA, Kim and Wei (2007)¹ reported that *P. aeruginosa* was isolated from ground beef, ground turkey, and ground pork in retail stores. Also, Venieri *et al.* (2006)² reported that in Greece the prevalence of *P. aeruginosa* in bottled mineral water samples analyzed during the period 1995-2003 was 0-17%.

Pseudomonas spp. (including *P. aeruginosa*) are the most frequently reported cause of spoilage of meat, poultry, fish, and milk stored at low temperature. Sundheim *et al.* (1998)³ reported that the percentage of Pseudomonads increased from initial 2-15% of the microbial flora (immediately after slaughtering) to 63-97% after storage of chicken carcasses at 4-5°C for 3 days. Arnaut-Rollier *et al.* (1999)⁴ reported that the predominant bacteria on chicken carcasses after slaughtering and wrapping were Pseudomonads and 2.3% of these were *P. aeruginosa*. Eribo and Jay (1985)⁵ reported that *Pseudomonas* spp. and *Acinetobacter-Moraxella* spp. were the primary spoilage organisms of ground beef and hamburger meat. Also, *P. aeruginosa* has been reported to grow and multiply in bottled natural mineral water.

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For example, Legnani *et al.* (1999)⁶ observed that counts of *P. aeruginosa* were increased by 3 log units after 4- 5 days of inoculation into samples of mineral water, and this level was maintained until 70- 100 days after inoculation. Also, Leclerc and Moreau (2002)⁷ reported that although number of bacteria recovered at the source of mineral water is generally very low, viable counts increase after 1- 3 weeks of storage.

P. aeruginosa has been implicated in food- borne and water- borne diseases^{8, 9}. It is resistant to many preservatives and disinfectants¹⁰, and also produces toxins and hydrolytic enzymes (such as caseinase) which cause spoilage of food. Microwave is used at home and in industry for many purposes such as cooking, reheating, decontamination or sterilization of water, food and food products. Because the combination of microwave heating and addition of chemical preservatives at low doses to food is nowadays frequently used to prevent or reduce growth of food spoilage and food poisoning microorganisms, and there are no published data on the effect of presence of preservatives on behavior of *P. aeruginosa* upon microwave heating, this study was undertaken to examine the effects of some preservatives (e g sodium chloride, potassium sorbate, *Thymus vulgaris* extract) on growth and caseinase production by *P. aeruginosa* subjected to microwave heating.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas aeruginosa strains were isolated from water. The strains were maintained on nutrient agar slants at 4° C. To prepare the inoculum, nutrient broth (20ml) was inoculated with bacterial culture and incubated static at 31°C for 20h.

Media and chemicals

Nutrient broth was obtained from Scharlau Chemie (Barcelona, Spain). Crude extract of *Thymus vulgaris* (extracted by hydrodistillation) was obtained from Systema Co. Ltd. Potassium sorbate and sodium chloride were obtained from Gainland Chemical Co. (Hampshire, UK).

Microwave exposure

Exposure in nutrient broth

A 0.5 ml of the overnight grown bacterial

culture was transferred to sterile flasks containing 20 ml of nutrient broth (pH7.2 or 5.9), NB (pH 7.2) plus 3% NaCl, NB (pH 7.2) plus 0.3% *Thymus vulgaris* extract, NB (pH 5.9) plus 0.05% potassium sorbate, NB (pH 5.9) plus 0.09% potassium sorbate, NB (pH 5.9) plus 0.05% potassium sorbate + 3% NaCl, NB (pH 5.9) plus 0.05% potassium sorbate + 0.3% *Thymus vulgaris* extract. The flasks were exposed to microwave (Sharp) at high power (1000 W). At time zero and after 10, 20 and 30 sec, samples were withdrawn, and diluted in 9-ml aliquots of 0.85% NaCl. Appropriate dilutions were plated in duplicate onto nutrient agar to enumerate bacterial cells. Plate counts were recorded after a 24h incubation at 31°C. The lower limit of detection for this method was 10 CFU/ml.

Exposure in chicken or fish homogenate

Skin and fat (50 g) were obtained aseptically from fresh chicken, and chicken homogenates were prepared by blending with 400 ml distilled water in a sterile blender. The homogenate was sterilized by heating at 70°C for 10 min, then cooled. A 20 ml portion of the homogenate was dispensed into flasks, and sodium chloride was added aseptically. A 0.5 ml of the overnight grown bacterial culture was added to flasks, and the flasks were exposed to microwave at high power (1000 W). At 0, 10, 20 and 30 sec, samples were withdrawn and treated as previously described to enumerate bacterial cells. For growth in fish homogenates, skin and fat (50 g) were obtained aseptically from fresh fish, and fish homogenates were prepared and treated as mentioned above.

Caseinase assay

Caseinase activity was semiquantified by the method described by Paniagua *et al.* (1990)¹¹. In brief, doubling dilutions of the cell- free culture filtrates (0.1 ml) in 0.1 M Tris buffer (pH 7.2) were placed in 8-mm wells made on a petri dish filled with 30 ml of 2% skim milk and 2% agar. Plates were maintained at 4°C to permit diffusion of the enzyme, then incubated at 32°C for 48h. Caseinase activity was recorded as the highest dilution showing a halo of hydrolysis.

Statistical analysis

All experiments were done four times with consistent results. Statistically significant differences between means of log number of viable cells of bacteria in different experimental

conditions were tested by two- way analysis of variance (ANOVA). Student's t-test was used to determine the significant differences ($p < 0.05$) in caseinase production by bacteria.

RESULTS

Four different strains were examined in this study and similar results were obtained, the results of a representative strain are shown

Survival of *P. aeruginosa* in nutrient broth during microwave heating

The effect of microwave heating on *P. aeruginosa* in nutrient broth (pH 7.2) and nutrient broth (pH 5.9) is presented in Tables 1 and 2. The rate of decrease in number of viable cells was

exponential during the first 10 sec of microwave exposure, D values for *P. aeruginosa* in nutrient broth (pH 7.2) or nutrient broth (pH 5.9) were 3 and 4.25 sec, respectively. After microwave exposure for 20 or 30 sec, a slower death rate was observed. This may be because the viable cells used compounds released from dead cells (such as amino acids and nucleotides) as nutrients, thus causing a decrease in their death rate. Or there may be a small number of cells that are more resistant (e g because of their stage in the cell cycle). Exposure of *P. aeruginosa* in nutrient broth (pH 7.2) or nutrient broth (pH 5.9) to microwave heating for 20sec reduced number of cells by 4.87 or 3.84 log CFU/ml, respectively. Heating in the microwave for 30 sec killed all bacterial cells in

Table 1. Effect of microwave heating on growth and caseinase production of *P. aeruginosa* in nutrient broth (pH 7.2) containing chemicals

Exposure time (sec)	NB (pH 7.2)		NB plus 3% NaCl		NB plus 0.3% thyme extract	
	Log CFU/ml	Caseinase titer	Log CFU/ml	Caseinase titer	Log CFU/ml	Caseinase titer
0	6.95	640	6.95	640	6.95	640
10	3.43	4	2.82	4	5.95	400
20	2.08	0	0	-	3.14	0
30	0	-	-	-	1.1	0

nutrient broth (pH 7.2) and nutrient broth (pH 5.9) (Table 1& 2A). The temperature of nutrient broth after 10, 20 or 30 sec of microwave exposure was 59.5 °C, 84 °C or 94 °C, respectively.

Effect of sodium chloride and *T. vulgaris* extract on inactivation of *P. aeruginosa* by microwave

Addition of 3% NaCl to the heating medium greatly reduced ($p < 0.05$) number of bacteria tested. Microwave heating for 10 or 20 sec reduced number of viable bacteria by 4.13 logs and killed all cells, respectively (Table 1). Exposure of *P. aeruginosa* in nutrient broth (pH 7.2) + 0.3% thyme extract to microwave heating for 10, 20, or 30 sec reduced ($p < 0.05$) number of viable bacteria by 1, 3.81 or 5.85 logs, respectively (Table 1).

Effect of potassium sorbate on inactivation of *P. aeruginosa* by microwave

Exposure of *P. aeruginosa* in nutrient broth (pH 5.9) + 0.05% potassium sorbate to microwave heating reduced ($p < 0.05$) number of

cells, number of viable cells was reduced by 2.34 and 4.5 logs after heating for 10 or 20 sec, respectively. Heating for 30 sec killed all cells in the medium (Table 2A). Presence of 0.05% potassium sorbate + 3% NaCl or 0.05% potassium sorbate + 0.3% thyme extract in the heating medium did not further decrease number of viable cells of *P. aeruginosa* (Table 2B). Heating of *P. aeruginosa* in NB (pH 5.9) containing 0.05% potassium sorbate + 3% NaCl for 10, 20, or 30 sec decreased number of cells by 1.95, 4.52, or 5.32 logs, respectively. Microwave exposure of the tested organism in NB (pH 5.9) containing 0.05% potassium sorbate + 0.3% thyme extract for 10, 20, or 30 sec decreased number of cells by 1.8, 4.53, or 5.95 logs, respectively (Table 2B).

Exposure of *P. aeruginosa* in nutrient broth (pH 5.9) + 0.09% potassium sorbate greatly increased the death rate ($p < 0.05$) of the tested organism. Number of viable cells was reduced by

Table 2(a) : Effect of microwave heating on growth and caseinase production of *P. aeruginosa* in a) nutrient broth (pH 5.9) containing potassium sorbate and b) nutrient broth (pH 5.9) containing potassium sorbate plus other chemicals

Exposure time (sec)	NB (pH 5.9)		NB plus 0.05% potassium sorbate		NB plus 0.09% potassium sorbate	
	Log CFU/ml	Caseinase titer	Log CFU/ml	Caseinase titer	Log CFU/ml	Caseinase titer
0	6.95	640	6.95	640	6.95	640
10	4.45	20	4.61	20	3.32	0
20	3.11	0	2.45	0	2.07	0
30	0	0	0	-	0	-

Table 2(b).

Exposure time (sec)	NB plus 0.05% potassium sorbate + 3% NaCl		NB plus 0.05% potassium sorbate + 0.3% thyme extract	
	Log CFU/ml	Caseinase titer	Log CFU/ml	Caseinase titer
0	6.95	640	6.95	640
10	5	20	5.15	400
20	2.43	0	2.42	0
30	1.63	0	1	0

Table 3. Effect of microwave heating on growth and caseinase production of *P. aeruginosa* in chicken homogenate

Exposure time (sec)	Chicken homogenate		Chicken homogenate plus 3% NaCl	
	Log CFU/ml	Caseinase titer	Log CFU/ml	Caseinase titer
0	7.28	640	7.28	640
10	5.92	128	5.96	128
20	4.08	2	3.86	2
30	1.95	0	1.48	0

3.63 or 4.88 logs after heating for 10 or 20 sec, respectively; and heating for 30 sec killed all microorganisms (Table 2A).

Statistical analysis and interaction between type of chemical and microwave

Numbers of viable cells in different experimental conditions were analyzed by ANOVA. According to time (i.e. heating for 10, 20 or 30 sec) there were statistically significant differences ($p < 0.05$) in number of viable cells in all experiments. Also, to determine the interaction between the type of chemical added (i.e. NaCl, thyme extract or potassium sorbate) and *P. aeruginosa* inactivation by microwave, numbers of viable cells in different experimental conditions were analyzed by ANOVA.

In most experiments, there were statistically significant differences ($p < 0.05$) according to the interaction between the added chemical and microwave inactivation of *P. aeruginosa*. For example, after heating for 10 sec, high difference was between log number of viable cells in NB (pH 7.2) + 3% NaCl and log number of viable cells in NB (pH 7.2) + 0.3% thyme extract. Also, high difference was between log number of viable cells in NB (pH 5.9) + 0.05% potassium sorbate and log number of viable cells in NB (pH 5.9) + 0.09% potassium sorbate; and between log number of viable cells in NB (pH 7.2) + 3% NaCl and log number of viable cells in NB (pH 5.9) + 0.05% potassium sorbate.

Survival of *P. aeruginosa* during microwave heating in chicken and fish homogenates

The effect of microwave heating on *P. aeruginosa* in chicken homogenate is presented in Table 3. Heating of *P. aeruginosa* in chicken homogenate for 10, 20, or 30 sec reduced ($p < 0.05$) number of cells by 1.36, 3.2, or 5.33 logs, respectively. Addition of 3% NaCl to chicken homogenate did not further decrease number of viable cells ($p > 0.05$). Number of viable cells after 10, 20, or 30 sec of microwave heating in chicken homogenate + 3% NaCl was nearly similar to number of viable cells after 10, 20, or 30 sec of microwave heating in chicken homogenate alone (Table 3). Similar results were obtained for heating in fish homogenates (data not shown).

Effect of microwave heating on caseinase production by *P. aeruginosa*

Microwave heating of the tested organism in nutrient broth (pH 7.2), NB (pH 7.2) + 3% NaCl, or NB (pH 7.2) + 0.3% thyme extract markedly decreased ($p < 0.05$) production of caseinase by cells. Caseinase titer of the heated cells for 10 sec in NB, NB + 3% NaCl, or NB + 0.3% thyme extract was reduced by 160, 160, or 1.6 – folds, respectively (Table 1). Microwave heating for 20 sec completely inhibited caseinase production by bacterial cells even though viable cells were detected after heating (Table 1).

Microwave exposure of *P. aeruginosa* in NB (pH 5.9), NB (pH 5.9) + 0.05% potassium sorbate, NB (pH 5.9) containing 0.05% potassium sorbate + 3% NaCl, or NB (pH 5.9) containing 0.05% potassium sorbate + 0.3% thyme extract significantly decreased ($p < 0.05$) caseinase production by bacterial cells. Caseinase titer of heated cells (for 10 sec) in the above mentioned media was reduced by 32, 32, 32, or 1.6 – folds, respectively (Table 2). However, when the tested organism was exposed to microwave heating for 20 sec in the above mentioned media no detectable caseinase was observed (Table 2). Also, exposure for 10 sec of *P. aeruginosa* in NB (pH 5.9) + 0.09% potassium sorbate completely inhibited caseinase production (Table 2).

Microwave heating of *P. aeruginosa* in chicken homogenate or chicken homogenate + 3% NaCl significantly reduced ($p < 0.05$) caseinase production. Caseinase titer of cells heated for 10 sec in either media was reduced by 5- folds (Table

3). Heating for 20 sec in either media further decreased caseinase production, caseinase titer was reduced by 320- folds; and heating for 30 sec in either media completely inhibited caseinase production by cells (Table 3). Also, similar results were obtained for heating in fish homogenates (data not shown).

DISCUSSION

P. aeruginosa has been implicated in food spoilage and food poisoning outbreaks. The combination of addition of low doses of chemicals and microwave heating of food is nowadays frequently used to extend shelf life of food products. Therefore, the determination of behavior of *P. aeruginosa* upon microwave heating is important. In this study, microwave was effective in elimination of *P. aeruginosa* in nutrient broth; and in chicken homogenate *P. aeruginosa* was slightly more resistant to microwave inactivation. Similar results were obtained by other investigators. Apostolou *et al.* (2005)¹² reported that after 30 sec of microwave heating, 83 CFU/g *E. coli* O157: H7 cells were recovered from chicken breast portions (20 g) and elimination of the cells occurred only after 35 sec of microwave exposure at 73.7 °C. Jamshidi *et al.* (2010)¹³ reported that microwave radiation that leads to surface temperature more than 70 °C could eliminate the superficial contamination of beef slices with *E. coli* O157: H7. Shen *et al.* (2009)¹⁴ observed that microwave heating of frankfurters for 30 sec reduced *Listeria monocytogenes* numbers by 0.8-1.3 log CFU/cm². Also, Lu *et al.* (2011)¹⁵ reported that microwave at medium power reduced *Salmonella enterica* on grape tomatoes by ~ 1.45 and 1.7 logs after 40 and 50 sec heating, respectively; and at high power, 40 and 50 sec heating achieved ~ 1.6 and 2 log reductions of *S. enterica*, respectively.

In this study, presence of NaCl (3%) or potassium sorbate (0.09%) in the heating medium increased the sensitivity of *P. aeruginosa* to microwave action. These compounds may weaken bacterial cells so that they become more vulnerable to microwave inactivation. Presence of thyme extract (0.3%) in the heating medium increased the resistance of *P. aeruginosa* to microwave action. Fats may protect bacterial cells by contributing to

the non- uniform heating of the medium that occurs during microwave exposure. However, longer exposure time may kill the organism. Quesada *et al.* (2003)¹⁶ reported that for complete elimination of *E. coli* O157: H7 in minced meat, a prolonged exposure (e g 120 sec) was necessary even though it caused undesirable organoleptic characteristics in the food.

CONCLUSION

This study showed that presence of 3% NaCl or 0.09% potassium sorbate in nutrient broth increased the sensitivity of *P. aeruginosa* to microwave action. Presence of 0.3% thyme extract decreased the sensitivity of the tested organism to microwave action, and heating for longer time is needed to eliminate this microorganism. Microwave exposure for 30 sec abolished caseinase production by *P. aeruginosa* in all media tested.

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