

Growth and Recovery of Selected Gram-Negative Bacteria in Reconditioned Wastewater

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ABSTRACT

Previous reports indicate that *Escherichia coli* O157:H7, *Salmonella* spp., and *Vibrio cholerae* can grow in nutrient-limited, reconditioned wastewater over the temperature range of 4 to 46°C when the biological oxygen demand of this water is <2, while its coliform growth response (CGR) is >2. In the current study, we investigated the growth response of *Vibrio parahaemolyticus*, *Shigella* spp., *Vibrio vulnificus*, and *Pseudomonas aeruginosa* in water samples with a CGR of >2 over the temperature range of 4 to 50°C. Both the nonselective media, tryptic soy agar, and the selective media used to identify the pathogen were used for their recovery. The selective media were thiosulfate-citrate-bile-sucrose (TCBS), MacConkey agar (MAC), and *Pseudomonas* isolation agar (PIA) for the *Vibrio*, *Shigella*, and *Pseudomonas* spp., respectively. *V. parahaemolyticus* numbers declined rapidly after surviving for 6 days under the nutrient-limiting growth conditions. *Shigella* spp. did not grow but survived for >28 days at 4 to 25°C. *V. vulnificus* grew over the narrow temperature range of 12 to 21°C and survived for >21 days at the higher and lower temperature ranges. *P. aeruginosa* survived and grew during the 14-day test period at 13 to 35°C. Recovery on the nonselective agar gave statistically ($P > 0.05$) higher numbers than the respective selective media commonly used for these pathogens. These results indicate that caution should be used in attempting direct recoveries using selective media of the four gram-negative bacteria species used in this study from the nutrient-limited water environment.

Food and agricultural processors are reconditioning and reusing wastewater within various approved operations. Smith and Palumbo (40) reviewed water reuse in agriculture and industry and showed that this is a way to increase water supply and decrease water fees. Palumbo et al. (26) reviewed water reuse in the food industry, again showing decreased water cost without compromising safety. The usage of reconditioned wastewater in the food and agricultural operations is not standardized in the United States; therefore, approval for use of reconditioned water or wastewater in food plants is granted on a process-by-process basis by the U.S. government agency, such as the U.S. Department of Agriculture for use in meat-processing plants (6, 34). With increased approval for use of reconditioned wastewater, microbial safety is a concern.

The survival of pathogenic bacteria associated with a waterborne disease outbreak of hemorrhagic colitis in drinking water was reported by Rice et al. (32). Rice et al. (33) were able to predict coliform growth in drinking water using the bioassay procedures. *Escherichia coli*, *Campylobacter jejuni*, and *Yersinia enterocolitica* can survive in agricultural surface water, which might be a way of transmission of these bacteria (41). Rajkowski et al. (29–31) analyzed the reconditioned pork-processing wastewater by the bioassay procedure and demonstrated that *Salmonella* spp., *Vibrio cholerae* spp., and *E. coli* O157:H7 spp. grew when the bioassay coliform growth response (CGR) was >2, but

recovery of the microorganism was lower using the recommended selective agars. Palumbo et al. (24, 25) showed that *Aeromonas hydrophila* can grow in reconditioned wastewater with a CGR >2, whereas *Listeria monocytogenes* Scott A, *Staphylococcus aureus* (196E), and *Enterococcus faecium* (ATCC 19433) just survived.

Pseudomonas aeruginosa survives under carbon-starved conditions in soils (43) and in the water environment—tap, river, and technical (4, 7, 10, 14, 44, 45). *Pseudomonas* remained viable for long periods of time in the various water systems, and it was concluded that unlike other enteric bacteria, this species did not enter the viable nonculturable state (5, 24). Clegg et al. (5) and Gurijala and Alexander (11) suggested that the bacteria were injured due to the stress of the water environment (nutrient limiting) and that the injury could affect the recovery. Using selective media to recover *P. aeruginosa*, both groups (13, 24) indicated that lower recovery may result.

It is well documented that both *Vibrio parahaemolyticus* and *Vibrio vulnificus* can grow and survive in the water environments (15–17, 21, 22). Under starvation conditions, as in water, these *Vibrio* spp. change morphologically from the straight rod to a “V”-shaped cell, indicating stress or injury. Some researchers have shown that these pathogens under the starvation condition may become viable but nonculturable, and their recovery is lower using selective agars when under stress (23).

The eastern Mediterranean area is regarded as an endemic area for shigellosis where there are limited guidelines for reconditioned wastewater use (2). Shigellosis is a wa-

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TABLE 1. CGR and assimilable organic carbon analysis of unchlorinated reconditioned pork-processing wastewater

Sample	AOC (μg of carbon equivalents/liter)			CGR ($\log(N_t/N_0)$)	BOD ^c
	P-17 ^a	NOX ^b	Total		
1	68	600	668	2.96	<2
2	76	355	431	3.41	<2
3	54	620	674	2.79	<2
4	61	717	778	2.93	<2
5	56	510	566	2.86	<2
6	61	528	589	1.90	<2

^a *P. fluorescens* P-17.

^b *Spirillum* NOX.

^c BOD determined by the company's processing plant.

terborne illness in both developed and underdeveloped areas when water supplies become contaminated, usually with sewage (8, 36). A 1987 review indicated that in the United States, *Shigella* was primarily a foodborne illness (38), but shigellosis is now waterborne. In the United States, four waterborne outbreaks of shigellosis occurred between 1986 and 1988 (18), and *Shigella sonnei* was associated with two waterborne outbreaks during 1995–1996 (19). These outbreaks were traced to sewage contamination of the drinking water supply. Since *Shigella* can survive in the nutrient-limited water environment, starvation injury can occur. When recovering injured cells from the water environment, ingredients in the selective agars or media (particularly bile salts) may be too harsh for *Shigella* recovery (12, 39, 43), whereas the healthy cells grow on MAC (37). *Shigella dysenteriae* type 1 was capable of surviving for more than 6 months in a water environment as viable nonculturable still remaining potentially virulent, which poses a public health problem (27).

The purpose of this study was to determine the survival and growth potential over the temperature range of 4 to 50 \pm 1°C for *V. parahaemolyticus* and *V. vulnificus*, *Shigella* spp., and *P. aeruginosa* in water with limited nutrients having a CGR >2.0, comparing the recovery on a nonselective and selective agar used for each species.

MATERIALS AND METHODS

Microorganisms. A cocktail containing selected strains of either *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, or *Shigella* spp. was used for the studies. *V. parahaemolyticus* strains 1318 Kanagawa phenomenon negative and ATCC 17802 Kanagawa phenomenon positive were obtained from the Food and Drug Administration (FDA), Washington, D.C., and strain E-10 was obtained from the FDA, Dauphin Island, Ala. The stock cultures of each strain were maintained in brain heart infusion broth (Difco Laboratories, Inc., Detroit, Mich.) and stored at room temperature. *V. vulnificus* strains A-9 and J-7 were obtained from the FDA, Dauphin Island, Ala., and strain LAM-624 was obtained from the FDA, Washington, D.C. The stock cultures of each strain were maintained in brain heart infusion broth and stored at room temperature. *P. aeruginosa* strains ATCC 7700, DAR41352, J799, and H788 were obtained from the Microbial Food Safety Research Unit's culture collection. *Shigella flexneri*, *S. sonnei*, *S. dysenteriae*, and *Shigella boydii* were obtained from the FDA, Dauphin Island, Ala. The stock cultures of each strain of *Shigella* and *Pseu-*

domonas were maintained in brain heart infusion broth and stored at 4°C.

A cocktail of *P. aeruginosa*, *V. parahaemolyticus*, *V. vulnificus*, or *Shigella* spp. was used for the growth study. The individual strains were cultured overnight by transferring 0.1 ml of each strain to 50 ml of brain heart infusion broth in a 250-ml Erlenmeyer flask and were placed on a rotary shaker set at 150 rpm (Model 3520, Lab-Line Instrumentation, Inc., Melrose Park, Ill.) at 37°C. The cells were concentrated by centrifugation at 3,300 \times g for 15 min, and the supernatant fluid was discarded. The cells were washed once to remove any nutrients in sterile deionized water (SDW), then resuspended in sterile deionized water and combined to make up the inoculating cocktail. The cocktail was diluted in sterile deionized water then added to the test water to achieve a final concentration of 10³ to 10⁴ CFU/ml, which was confirmed by plate count on tryptic soy agar (TSA; Difco) and incubated at 37°C for 24 h.

Bioassay determination of water sample. The bioassay for CGR utilizes *Enterobacter cloacae*, *Spirillum* NOX and *Pseudomonas fluorescens* P-17 are used for the assimilable organic carbon bioassay. The inoculum and test samples were prepared according to the method described by Rice et al. (33).

Gradient growth temperature study. Good-quality reconditioned wastewater was obtained from a local meat-processing plant with an in-house water treatment plant. The water was collected just before chlorinating. After arrival in the laboratory, it was filter sterilized using a 0.2- μm -pore Nalgene filter (Nalgene, Rochester, N.Y.) and kept refrigerated until used in the growth studies. A separate water sample, taken at the same time, was sent under ice by overnight express to the U.S. Environmental Protection Agency laboratory in Cincinnati, Ohio, for the CGR and assimilable organic carbon bioassays. Biological oxygen demand (BOD) results were obtained according to the prescribed method (1) and provided by the company.

One liter of the filtered unchlorinated reconditioned wastewater (FUR) was mixed with the inoculating cocktail to achieve a starting level of 10⁴ to 10³ CFU/ml. After mixing, 12 ml of the inoculated FUR was distributed into a duplicate set of sterile "L"-shaped test tubes placed in a temperature-gradient incubator (Model TN-3F, Advantec, Toyo Roshi Inter., Co., Dublin, Calif.). The temperature gradient was set between 3.5 and 55.4°C. Growth was monitored by spiral plating the samples on TSA and selective agar used to enumerate each bacteria group. TCBS (Difco), PIA (Difco), and MAC (Difco) were used for the *Vibrio*, *Pseudomonas*, and *Shigella* spp., respectively. The plates were incubated at 37°C for 18 to 24 h before counting. The actual gradient temper-

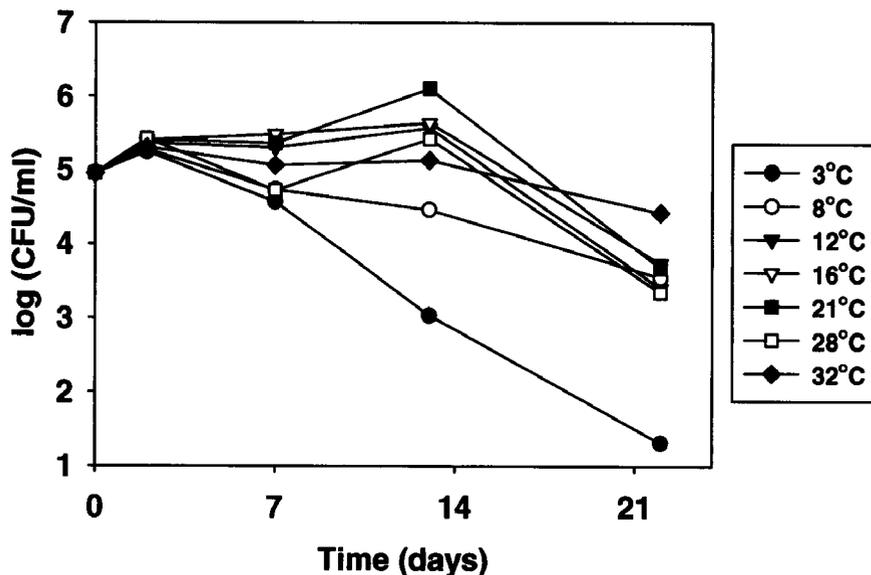


FIGURE 1. Growth/survival profiles of *V. vulnificus* cocktail in FUR after growth at 3, 8, 12, 16, 21, 28, and 32°C as recovered on TSA.

ature range was verified at the end of each study using the thermocouple sensor fitted in the gradient incubator. The growth study for each microbe was repeated.

At the termination of each growth study, the viability of the cultures was determined using the LIVE/DEAD BacLight Viability Kit (Molecular Probes, Inc., Eugene, Oreg.) as described earlier (32, 33).

Statistical analysis. Analysis of variance was performed separately for each time period to determine the effect of temperature zone on the percentage difference in bacteria growth (based on log CFU/ml) from inoculation. The calculations were performed using the General Linear Model procedure of the SAS/STAT software system (37).

RESULTS AND DISCUSSION

Good-quality unchlorinated reconditioned wastewater with a BOD <2 was tested using the more sensitive bioassays, and the results are presented in Table 1. All water samples with a CGR >2 supported the growth/survival of the gram-negative pathogens studied. Water sample 6 had a CGR <2 and did not support the growth/survival

of the gram-negative pathogens used in this study. Rice et al. (32) reported that potable water supported the growth of coliforms when the CGR was ≥ 1 . In previous studies, water samples with a CGR >2 supported the growth/survival of *Salmonella* spp., *V. cholera*, and *E. coli* O157:H7 (29, 30).

V. vulnificus grew over the temperature range of 12 to $21 \pm 1^\circ\text{C}$ and survived at the other temperature ranges, as shown in Figure 1. Growth was determined to be an increase of 1 full log unit in counts on TSA from the initial counts. The difference in recovery after 13 days of growth over the temperature range of 3.1 to 46.6°C is represented in Figure 2, with any point above the solid line indicating growth. Recovery using TCBS for *V. vulnificus* was statistically ($P > 0.05$) lower in counts than when using TSA, which is consistent with previously reported data for *V. cholerae* (29). This difference in recovery is an indicator of starvation stress. The growth/survival of *V. vulnificus* at lower temperature is also consistent with data obtained by others for *V. vulnificus* (3, 21). Growth at the lower tem-

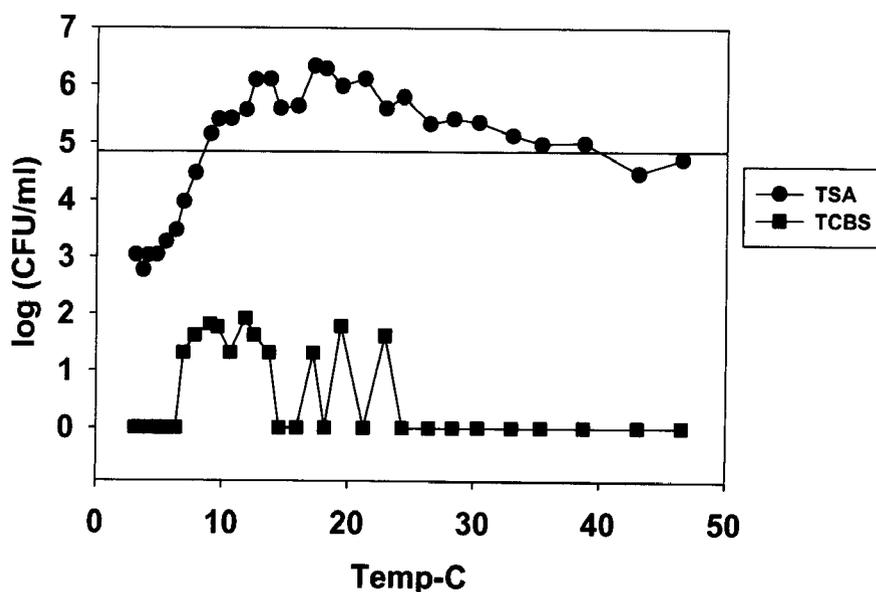


FIGURE 2. Growth/survival of *V. vulnificus* cocktail in FUR after 13 days of growth over the temperature range of 3.1 to 46.5°C as recovered on TSA and TCBS.

TABLE 2. Survival of *V. parahaemolyticus* spp. in the reconditioned pork-processing wastewater recovered on TSA

Temp °C	0 time	6 days	13 days
16.3	4.96 ^a	2.94	— ^b
17.7		3.36	—
19.1		3.24	1.32
20.6		4.09	—
22.0		4.73	1.32
23.2		4.37	—
24.7		4.90	—
26.4		4.52	—
28.3		4.67	1.32
29.8		4.70	—
30.9		4.79	2.80
32.0		4.44	2.32
33.7		3.10	2.27
36.5		3.98	2.47
37.7		4.64	—
38.8		4.39	—
40.7		4.74	—
42.2		3.66	—
44.2		4.15	—

^a Reported as log CFU/ml.

^b —, no growth.

perature range is similar to data obtained for *V. cholerae* (31) and is consistent with those previous reports for *E. coli* 0157:H7, *Salmonella* spp., and *A. hydrophila* (25, 30) using the FUR samples with a CGR level >2.0 as noted in Table 1.

Using the FUR sample, both *V. cholerae* (31) and *V. vulnificus* grew/survived depending on the incubation temperature. However, *V. parahaemolyticus* survived for only 6 days in the FUR, then the number of cells declined rapidly as recovered on TSA (Table 2). There were no recoverable cells using TCBS at 6 days, indicating stress.

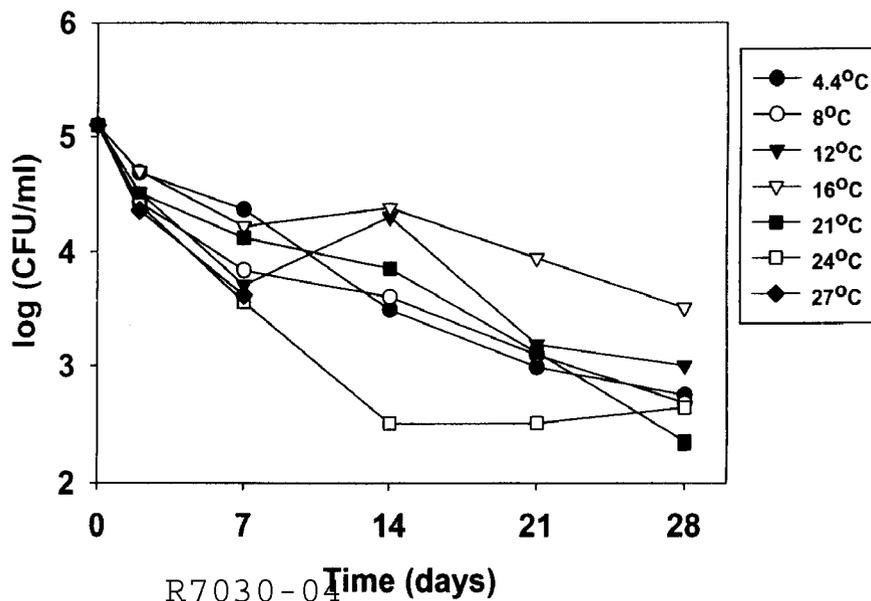
Shigella did not grow in the FUR over the temperature range studied (4.4 to 45.8°C), but the organisms did survive at the temperature range <27°C for the 28 days of this

study. The survival profiles over the temperature range of 4.4 to 27°C as recovered using TSA are presented in Figure 3. By day 14, no survival of *Shigella* was observed in samples incubated at temperatures >27°C. The difference in recovery between TSA and MAC for the *Shigella* spp. after 14 days of growth over the temperature range of 4.4 to 45.8°C is presented in Figure 4, which is statistically lower ($P < 0.05$) using the MAC.

The viable noncultured state was reported for *Shigella* (28) and *Vibrio* spp. (15, 25, 46). When no recoverable cells were obtained on TSA for either the *Shigella* or the *Vibrio* spp., the cultures were tested to determine if the viable noncultured state occurred. Using the BacLight LIVE/DEAD test, we observed only dead cells for the *Shigella* spp., *V. vulnificus*, and *V. parahaemolyticus*.

Representative growth/survival profiles as recovered on TSA for *Pseudomonas* are represented in Figure 5. At the lower temperature range (10 to 22 ± 1°C), the *Pseudomonas* cell count declined but survived (lag) before increasing in number (regrowth) as measured by a full log increase in number from the lowest count. This was the only gram-negative organism studied that exhibited this regrowth (Figure 5, at 12°C). *Pseudomonas* spp. grew, as measured as a full log cycle increase from the initial inoculum count over the narrow temperature range of 23 to 30 ± 1°C, but survived for over 21 days at the other temperature ranges 4.1 to 9 and 30 to 45 ± 1°C. This growth in the lower temperature range under nutrient-limiting conditions was reported for other gram-negative organisms under the same growth conditions (30, 31). The differences in recovery between the selective and nonselective agars are illustrated in Figure 6 after 14 days of growth over the temperature range of 4.1 to 45°C. The results above the solid line represent growth of the *Pseudomonas* in the FUR. The recovery of *Pseudomonas* using the selective agar (PIA) was statistically ($P > 0.05$) lower than the nonselective agar (TSA) and is consistent with reported data on the recovery of gram-negative pathogens after growth in FUR (29, 30). This lower recovery was

FIGURE 3. Survival profiles of *Shigella* spp. cocktail in FUR after growth at 4.4, 8, 12, 16, 21, 24, and 27°C as recovered on TSA.



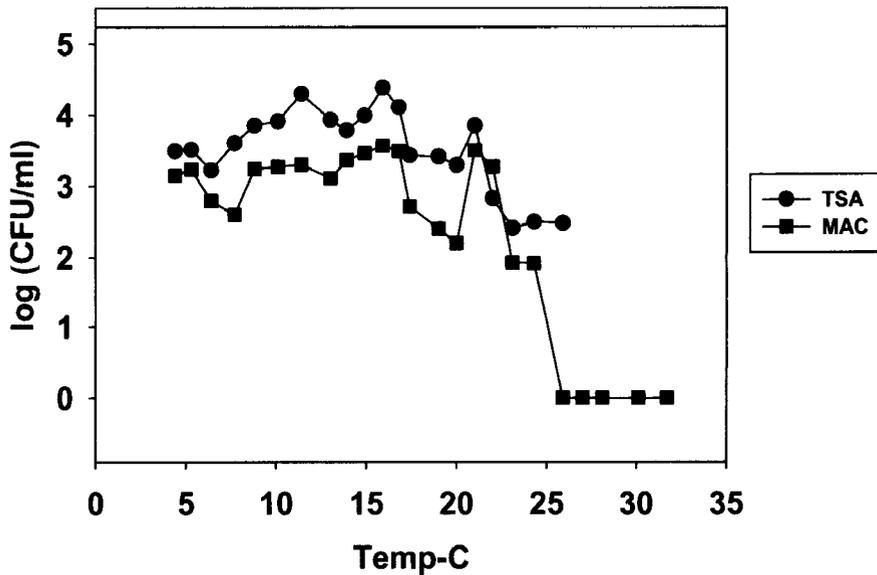


FIGURE 4. Survival of *Shigella* spp. cocktail in FUR after 14 days of growth over the temperature range of 4.4 to 45.8°C as recovered on TSA and MAC.

probably due to nutrient-deprived stress injury, as indicated by other researchers (5, 11, 13). When no counts were recovered using TSA, the cells were tested for viability using the BacLight LIVE/DEAD test, and only dead cells were observed.

For all four classes of bacteria studied, the selective agar, which is routinely used in recovery of these pathogens from samples with mixed microflora, gave statistically lower counts. In this study, attempts to recover the bacteria by enrichment procedures before using the selective agar were not made. Previous results indicate that enrichment recovery will not increase the numbers when the bacteria are under starvation stress (30) from the water environment. With the increase of infectious disease now occurring from contaminated drinking water, Ford (9) reviewed the microbiological safety of drinking water globally and in the United States. He stated two concerns: biofilm formation by *Pseudomonas* within water lines and chlorine resistance waterborne pathogens. This study showed that *Pseudomonas* survived and even increased in numbers in the water environment. Another concern is that there is need for fur-

ther understanding of starvation stress injury and the mechanisms by the pathogens for survival under this stress condition (35, 40, 42).

A final concern is the analytical method used to determine water quality and safety. The water used in this study, although reconditioned, had a BOD <2, which indicates good quality. Rice et al. (33) and Miettinen et al. (20) showed that the bioassay procedures for CGR and assimilable organic carbon were better indicators of water quality. In this study, we found that the bioassay procedure (CGR) predicted survival/growth for the pathogenic gram-negative bacteria, which can survive in drinking and environmental water. Using a water sample with a CGR >2, our data showed that this type of water supported the growth of *V. vulnificus* and *Pseudomonas* and the survival of *Shigella* and *V. parahaemolyticus* under the nutrient-limiting condition. Research should continue to understand the growth response under starvation conditions of bacteria capable of surviving in any water environment. To ensure public safety, the residual chlorine level of the water must be maintained, and it should be noted that stress of the water en-

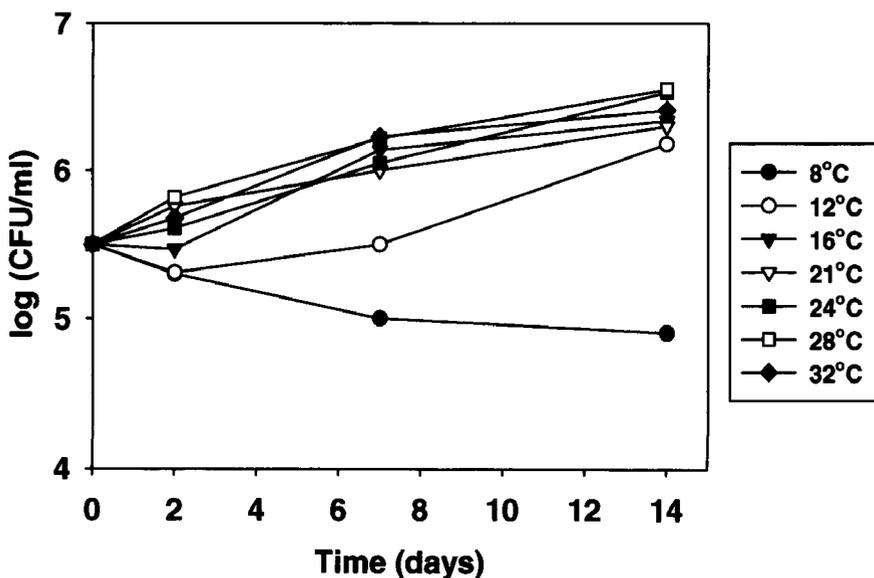
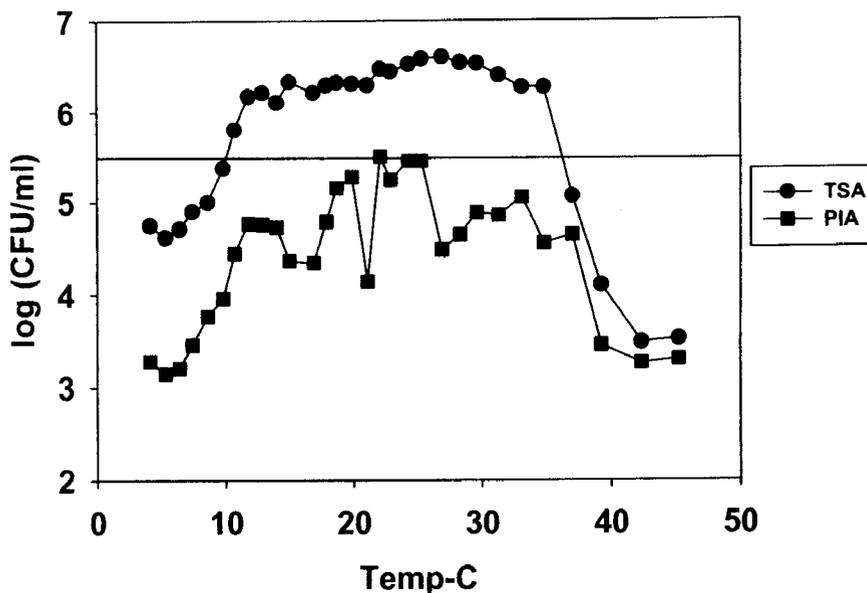


FIGURE 5. Growth/survival profiles of *P. aeruginosa* cocktail in FUR after growth at 8, 12, 16, 21, 24, and 28°C as recovered on TSA.

FIGURE 6. Growth/survival of *P. aeruginosa* cocktail in *FUR* after 14 days of growth over the temperature range of 4.1 to 45°C as recovered on TSA and PIA.



environment could affect the recoverable number of bacteria when using any selective agar.

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