The adherence of *Pseudomonas fluorescens* to marble, granite, synthetic polymers, and stainless steel

Adesão de Pseudomonas fluorescens em mármore granito, polímeros sintéticos e aço inoxidável

Roberta Torres CARELI[1], Nelio José ANDRADE[1]*, Nilda Fatima SOARES[1],
José Ivo Ribeiro JÚNIOR[2], Marcilía Santos ROSADO[1], Patrícia Campos BERNARDES[1]

Abstract

The adherence of *Pseudomonas fluorescens* cells to nine food-processing contact surfaces was evaluated using the plate-count method. The surfaces include marble, granite, stainless steel, polyvinyl chloride, polyurethane, and silicone-coated cloth, which have been used only in a few studies concerning bacterial adherence. The number of cells adhered to the surfaces increased with contact time reaching 5.0-6.1 log CDM.cm^{-2} after 10 hours, which can be considered a well established adherence process. The number of adhered cells doubled in 29.5 minutes and 23.5 minutes on stainless steel and thin polyvinyl chloride-coated cloth, respectively. For the other surfaces, this value was 9.8 minutes on average. Marble, granite, thick polyvinyl-coated cloth, double-faced rugous polyurethane, and silicone-coated cloth were not different ($p \leq 0.05$) in their ability to adhere cells (CFU/cm^2) after 2 and 10 hours. The surfaces that had higher percentage of similarity in the adhesion level and higher log CFU/cm^2 of adhered cells were double-faced rugous polyurethane, silicone-coated cloth, and granite. The surfaces showed very different microtopography characteristics when viewed using scanning electron microscopy. This experiment showed the importance of using appropriate materials for food contact during processing, which will affect the cleaning and sanitation procedures.

Keywords: bacterial adherence; Pseudomonas fluorescens; marble; granite; synthetic polymers; stainless steel.

Resumo

A adesão de *Pseudomonas fluorescens* foi avaliada em nove tipos de superfícies de processamento de alimentos por contagem padrão em placas. Superfícies de mármore, granito, aço inoxidável, poli(cloreto de vinila), poliuretano e silicone revestidos com tecido foram avaliadas. O número de células aderidas aumentou com o tempo de contato, alcançando 5,0-6,1 log UFC.cm^{-2} após 10 horas, o qual pode ser considerado um processo de adesão bem estabelecido. O número de células aderidas duplicou em 29,5 minutos em aço inoxidável e 23,5 minutos em poli(cloreto de vinila) revestido com tecido. Nas outras superfícies, em média, esse valor foi 9,8 minutos p ≥ 0,05) no grau de adesão de células após 2 e 10 horas. As superfícies avaliadas com elevadas porcentagens de similaridade no nível de adesão e contagens de células aderidas foram poliuretano rugoso dupla face, silicone revestido com tecido e granito. As superfícies apresentaram características de microtopografias diferentes quando observadas por microscopia eletrônica de varredura. O experimento mostrou a importância da escolha adequada de materiais para contato com alimentos durante o processamento, os quais irão afetar os procedimentos de limpeza e sanitização.

Palavras-chave: adesão bacteriana; Pseudomonas fluorescens; granito; mármore; polímeros; aço inoxidável bacterial.

1 Introduction

There are a few studies on the adherence of pathogens to several surfaces that are extensively used for food processing. For example, marble and granite are commonly used in food services and domestic kitchens, places which have been recently considered responsible for foodborne diseases. There are also some types of polyvinyl chloride (PVC), polyurethane, and silicone, which are bottom face-coated with cloth and have been used as conveyors in several food industries such as fruits and vegetables processors, meat and poultry, bakery, confectionery, and pasta processing, which should be more carefully studied with regards to microbial adherence (LELIEVELD et al., 2005). Microbial adhesion has been evaluated in a large number of food processing contact surfaces including stainless steel, glass, rubber, formic, polypropylene, and polycarbonate (SILVA, et al., 2003; ZAQUEUS et al., 2000). Teflon and cast iron can support microbial growth if a thin layer of food residue is present conditioning the surface to allow bacterial growth (JULLIEN et al., 2002; WONG et al., 2002).

The occurrence of foodborne disease outbreaks has been increasing considerably every year despite the continuous advances in food processing, hygiene, and cleaning and sanitizing procedures. Currently, food safety has been the greatest concern of consumers, food industry, and health public authorities. Food processing contact surfaces have been incriminated as a source of pathogens responsible for foodborne diseases. Under

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favorable conditions, microorganisms can attach and grow on surfaces and can contaminate the food being processed. Such microorganisms can start microbial adherence and biofilm formation when not removed from the surfaces by adequate cleaning and sanitizing procedures. These occurrences mean that complex microbial communities can contaminate and spoil the food being processed. Microorganisms grow and develop, and when they reach large numbers in the presence of extracellular polymeric substances, biofilm formation is established during food production. Even one single adhered cell can start these processes making it difficult to meet hygiene standards, as the adhered cells are more resistant to detergents and sanitizing actions (WIRTANEN; SALO, 2005).

It has long been recognized that equipment and utensil surfaces can help microorganisms adhere, grow, and become a source of spoilage and pathogenic microorganisms (BEECH, 2004; MIDELET; CARPENTIER, 2004; O'TOOLE et al.; 2000; STOODLEY et al., 2002). *Pseudomonas aeruginosa* (PARSEK; GREENBERG, 2000; HEYDOM et al., 2002; SAUER et al., 2002), *Pseudomonas fluorescens* (MASSON et al., 2002; ROSSONI; GAYLARDE, 2000; TYRER et al., 2004; VALCARCE et al., 2002), and *Shewanella putrefaciens* (HJELM et al., 2002) are members of the spoilage bacteria. *Yersinia enterocolitica*, *Salmonella thyphimurium*, (HOOD, 1996; LEJEUNE, 2003), *Listeria monocytogenes* (BERESFORD et al., 2001; LEJEUNE, 2003), *Escherichia coli*, *Staphylococcus aureus* (POMPERMAYER; GAYLARDE, 2000; LEREBOUR et al., 2004), *Vibrio parahaemolyticus* (WONG et al., 2002), and *Klebsiella pneumoniae* (DI MARTINO et al., 2003) are included among pathogenic bacteria. Spores of *Bacillus cereus* (FAILLE et al. 2001; JULLIEN et al., 2002), *Bacillus stearothermophilus* (FLINT et al., 2001), and *Bacillus sporothermodurans* (AKUTSU, 2001) are also involved in adherence processes and biofilm formation.

The aim of this research was to evaluate the adhesion of *Pseudomonas fluorescens* to nine food surfaces used in the food industry, domestic kitchens, and restaurants. In addition, the microtopography of the surfaces was analyzed by scanning electron microscopy.

2 Materials and methods

2.1 Surfaces

The samples (10 x 10 x 1 mm coupon-tests) of food processing contact surfaces evaluated were: marble, granite, polyurethane-coated cloth, double-faced smooth polyurethane, double-faced rugous polyurethane, silicone-coated cloth, thin polyvinyl chloride (PVC) coated cloth, thick PVC-coated cloth, and AISI 304 #4 stainless steel.

2.2 Bacterial suspensions and culture media

Suspensions of *Pseudomonas fluorescens* ATCC 13525 were used in the experiments. One milliliter of each of these cultures was kept at − 80 °C in nutrient broth (OXOID®) added to glycerol (80:20). A working culture was prepared by inoculating 100 µL of the frozen culture into 10 mL of nutrient broth and incubating at 28 °C for 24 hours. The culture was sub-cultured three times before use.

2.3 Attachment of cells

The coupon tests for *P. fluorescens* attachment were first cleaned by washing with liquid detergent and water, rinsing with distilled water, and immersing them in 70% ethyl alcohol for 1 hour to remove fat. After that, they were rinsed with distilled water, air-dried, and sanitized by UV exposure of $100 \, \mu W.cm^{-2}$ for 1 hour at 254 nm (PARIZZI et al., 2004).

The cleaned and sanitized coupons were placed into 250 mL flasks containing 100 mL of nutrient broth previously inoculated with suspensions of *P. fluorescens*. The initial number of cells ranged from 10^3 to 10^4 $CFU.mL^{-1}$, and the flasks were incubated statically in a water bath at 28 °C. The number of adhered cells on the different surfaces was evaluated by the plate count method after 2, 4, 6, 8, or 10 hours of contact time. The results were expressed in CFU/cm^2.

2.4 Counting adhered cells using the plate count method (PCM)

The coupons with adhered cells were immersed in 10 mL of 0.1% peptone water to remove planktonic cells. Then, the coupons were immersed in separated tubes containing 5 mL of the same solution and swirled in a vortex mixer for 1 minute to release the sessile cells. (PARIZZI et al., 2004). Appropriate dilutions were prepared and transferred to Petri dishes containing Plate Count Agar (OXOID®), which were incubated at 28 °C for 48 hours, and colonies were counted using the method of Swanson et al. (2001). Each colony counted in the plate was equivalent to 2.5×100 $UFC.cm^{-2}$, as determined by the diluent volume used for rinse (5 mL), aliquot plated (0.1 mL), average of colony counting after incubation time (M), decimal dilution, and area of the coupon-test (2 cm^2). For this purpose, the following equation was applied (Equation 1):

$$UFC.cm^{-2} = \frac{(5/1) \times M \times D}{2} = 2{,}5 \times M \times D \qquad (1)$$

2.5 Adherence ratio

The time in minutes to duplicate the number of adhered cells to surfaces (t_a) was calculated using the following Equation 2:

$$Y = kx + b \qquad (2)$$

where Y = number of adhered cells on surfaces, x = contact time, k = velocity of adherence in minutes, and b = constant (Equation 3):

$$at = \frac{\log 2}{k} \qquad (3)$$

2.6 Photomicrography

The photomicrography of the microorganisms adhered to different surfaces was obtained by using an epifluorescence

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microscope coupled to a computerized image analysis system (PARIZZI et al., 2004).

2.7 Microtopography surfaces

To analyze differences in surface microtopographies, photomicrographs of coupons that were not submitted to bacterial adhesion were obtained using Scanning Electron Microscopy (SEM) with a LEO 1430 VP microscope. Double-faced adhesive tape was attached to a stub and a thin layer of colloidal silver was applied to improve electrical conductivity. Then, the coupons were attached to a stub, subjected to a vacuum for metallization (Balzers FDU 010) with a thin gold layer of 20 nm (to improve electrical conductivity as well), and observed by SEM.

2.8 Statistical analyses

Data were analyzed as a split plot design consisting of two treatments – surfaces or contact time. The CFU/cm^2 means of the surfaces were compared by the Scott-Knott test. The results were analyzed by analysis of variance using the Statistical Analysis System - SAEG version 9.0 (Ribeiro Júnior, 2001). The coefficients of linear regressions for contact time were analyzed by the T test at 5% probability for each surface. A dendrogram based on log CFU/cm^2 means after 2, 4, 6, 8, and 10 hours was obtained to verify the similarity of adherence in the different surfaces using the SAEG.

3 Results and discussion

3.1 Adherence of P. fluorescens

P. fluorescens was able to adhere to all surfaces evaluated. Similar to our results, the ability of a number of microorganisms to adhere to different surfaces has been investigated in several studies (AKUTSU, 2001; BERESFORD et al., 2001; DI MARTINO et al., 2003; FLINT et al., 2001; HJELM et al., 2001; LEREBOUR et al., 2004; MASSON et al., 2002; MOREIRA, 2002; PARIZZI et al., 2003; POMPERMAYER; GAYLARDE, 2000; SILVA et al., 2003; TYRER et al., 2004; WONG et al., 2002; ZAQUES et al., 2000).

We found differences in the adherence of *P. fluorescens* depending on the surface and contact time (p < 0.05). Linear equations showing the adherence of *P. fluorescens* as a function of contact time for each surface are shown in Table 1. Our findings for the attachment of *Listeria innocua* to polypropylene, stainless steel, and polycarbonate are close to those observed by Parizzi and co-workers (2004) for the attachment of *Listeria innocua* to polypropylene, stainless steel, and polycarbonate, and close to those from Silva and co-workers (2003) for the attachment of *Escherichia coli* and *Staphylococcus aureus* to low density polyethylene.

As shown in Table 2, the number of adhered cells increased as a function of time as expected ranging between 1.6 log CFU/cm^2 on stainless steel surfaces after 2 hours and 6.1 log CFU/cm^2 on marble and granite surfaces after 10 hours.

After 4 and 6 hours of contact time, silicone-coated cloth, double-faced rugous polyurethane, granite, and polyurethane-coated cloth surfaces showed higher adherence. After 6 hours, the number of adhered cells reached 4.8 log CFU/cm^2 on granite

and polyurethane-coated cloth, and after 8 hours this value was 5.5 log CFU/cm^2 on granite (Table 2).

Marble, granite, thick polyvinyl chloride-coated cloth, double-faced rugous polyurethane, and silicone-coated cloth did not show differences (p > 0.05) in log CFU/cm^2 after 2 and 10 hours (Table 2).

After 10 hours, stainless steel (5.0 log CFU/cm^2), double-faced smooth polyurethane (5.0 log CFU/cm^2), and thin PVC chloride-coated cloth (5.1 log CFU/cm^2) surfaces showed the lowest number of adhered cells, which were different (p > 0.05) from the other surfaces (Table 2).

3.2 Ratio of adherence

There were significant differences (p < 0.05) in the ratio of adherence which means difference in the time (minutes) for

Table 1. Adherence of *Pseudomonas fluorescens,* growing in nutrient broth at 28 °C, to different surfaces evaluated by the plate count method.

Surfaces	Equations	R^2 (%)
SS	$\hat{Y} = 0.874 + 0.402 * TC$	99.31
PU$_{DFS}$	$\hat{Y} = 1.371 + 0,402 * TC$	96.45
PUcc	$\hat{Y} = 2.132 + 0,365 * TC$	94.95
PU$_{DFR}$	$\hat{Y} = 2.387 + 0.361 * TC$	99.80
PVC$_{TCC}$	$\hat{Y} = 0.773 + 0.455 * TC$	95.59
PVC$_{TCT}$	$\hat{Y} = 1.710 + 0.414 * TC$	99.18
SIL$_{cc}$	$\hat{Y} = 2.276 + 0.374 * TC$	98.10
GRA	$\hat{Y} = 2.052 + 0.422 * TC$	99.04
MAR	$\hat{Y} = 1.585 + 0.451 * TC$	99.24

*Significant at 5% of probability level by F test (p < 0.05); $\hat{Y}$ = log CFU/cm^2; CT = contact time (hours); SS = stainless steel; PU$_{DFS}$ = double-faced smooth polyurethane; PUcc = polyurethane coated with cloth; PU$_{DFR}$ = double-faced rugous polyurethane; PVC$_{TCC}$ = polyvinyl chloride thin coated with cloth PVC$_{CTC}$ = polyvinyl chloride coated with thick cloth; SIL$_{cc}$ = silicon coated with cloth; GRA = granite; and MAR = marble.

Table 2. Log CFU/cm^2 of *Pseudomonas fluorescens* ATCC 13525 adhered to nine food contact processing surfaces after growing in nutrient broth at 28 °C and incubated for different Contact Time (CT), as evaluated by the plate count method.

Surface	CT$_2$	CT$_4$	CT$_6$	CT$_8$	CT$_{10}$
SS	1.6^b	2.6^c	3.2^c	4.0^c	5.0^b
PU$_{DFS}$	1.9^b	3.1^b	3.9^b	4.8^b	5.0^b
PUcc	2.7^a	3.5^a	4.8^a	5.1^b	5.6^b
PU$_{DFR}$	3.1^a	3.9^a	4.6^a	5.3^a	5.9^a
PVC$_{TCC}$	1.8^b	2.6^c	3.2^c	4.9^b	5.1^b
PVC$_{CTC}$	2.7^a	3.2^b	4.2^b	5.0^b	5.9^a
SIL$_{CC}$	2.8^a	3.9^a	4.7^a	5.3^a	5.9^a
GRA	2.8^a	3.8^a	4.8^a	5.5^a	6.1^a
MAR	2.6^a	3.2^b	4.2^b	5.3^a	6.1^a

Averages followed by the same lower case letter in the column did not differ according to the Scot-Knott test (p > 0.05). SS = stainless steel; PU$_{DFS}$ = double-faced smooth polyurethane; PUcc = polyurethane coated with cloth; PU$_{DFR}$ = double-faced rugous polyurethane; PVC$_{TCC}$ = polyvinyl chloride thin coated with cloth PVC$_{CTC}$ = polyvinyl chloride coated with thick cloth; SIL$_{cc}$ = silicon coated with cloth; GRA = granite; and MAR = marble.

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doubling the number of cells adhered to surfaces. A higher ratio of adherence was observed for stainless steel and thin polyvinyl chloride-coated cloth (Table 3) reaching 29.5 minutes and 23.5 minutes, respectively. In addition, these surfaces showed the lowest number of adhered cells after the evaluated contact time, except after 8 hours (Table 2). There were no differences ($p \geq 0.05$) in the ratio of adherence for the other surfaces. In those surfaces, the number of adhered cells doubled, on average, after 9.8 minutes.

The dendrogram (Figure 1) shows the similarity between the surfaces used for evaluating the adhesion of *P. fluorescens* and the similarity of surface groups concerning the number of adhered cells.

Table 3. Ratio of adherence* (minutes) of *Pseudomonas fluorescens* to different surfaces.

Surfaces	Ratio of adherence (minutes)	
SS	29.50	a
PU$_{DFS}$	13.38	b
PUcc	8.58	b
PU$_{DFR}$	7.45	b
PVC$_{TCC}$	23.50	a
PVC$_{CTC}$	10.56	b
SIL$_{CC}$	8.10	b
GRA	9.01	b
MAR	11.41	b

*Time for doubling the bacterial population.

1 - Stainless steel
2 - Double-faced smooth polyurethane
3 - Polyurethane coated with cloth
4 - Double-faced rugous polyurethane
5 - Polyvinyl chloride thin coated with cloth
6 - Polyvinyl chloride coated with thick cloth
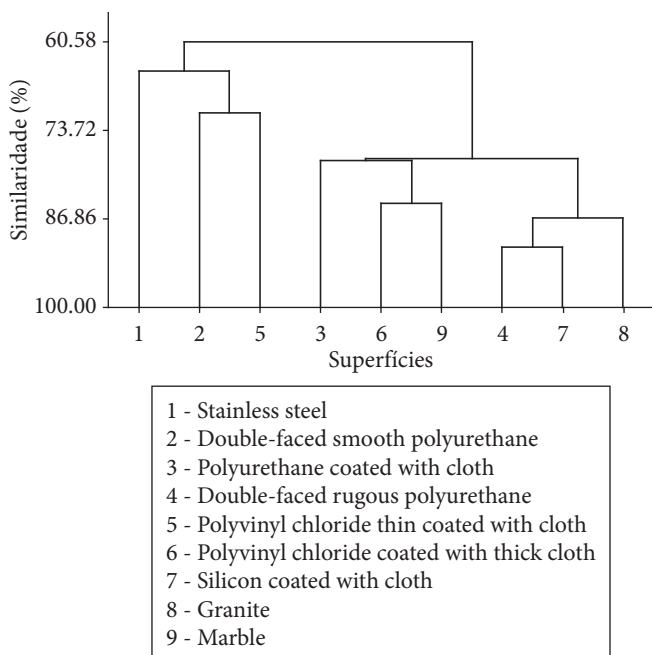7 - Silicon coated with cloth
8 - Granite
9 - Marble

Figure 1. Dendrogram of *Pseudomonas fluorescens* ATCC 13525 adherence to nine food processing contact surfaces by the plate count method (expressed in log CFU/cm²).

3.3 Similarity in adherence ability

The nine surfaces were clustered in three groups according to similarities in adherence ability (Figure 1 and Table 3). The first group was composed of stainless steel, double-faced smooth polyurethane, and thin polyvinyl chloride-coated cloth, with 65% similarity and a mean of 3.5 log UFC.cm^{-2}. The second group included surfaces of polyurethane with cloth, thick polyvinyl-coated cloth, and marble, with 78% similarity and a mean of 4.27 log CFU/cm². The third group was composed of double-faced rugous polyurethane, silicone-coated cloth, and granite, with 87% similarity and mean of 4.55 log CFU/cm². According to the PCM, double-faced rugous polyurethane, silicone-coated cloth, and granite showed a higher percentage of similarity and higher means of adhered cells.

The Scott-Knott test and the similarity test did not show good agreement. Only the surfaces included in group III (Table 3), according to the similarity test, were not different according to the Scott-Knott test ($p > 0.05$).

A few studies in the literature focus on the adherence process of marble and granite. Several microbial species were isolated from these surfaces in healthcare services including *Bacillus* spp., *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemoliticus*, *Proteus vulgaris* and *Shigella sonne*. *Pseudomonas aeruginosa* (15%) and *Staphylococcus haemoliticus* (15%) were found to be the main microorganisms adhered to surfaces (MOREIRA, 2002). The other microorganisms were responsible for 70% of the adhesion observed.

3.4 Surface microtopography

Figure 2 illustrates the microtopography and bacterial cells adhered to marble and granite surfaces. Some surface imperfections, as shown by scanning electron microscopy, were large enough to hold and protect *P. fluorescens* bacterial cells which are straight and rod-shaped with dimensions of 0.5 – 1.0 μm x 1.5 – 5.0 μm (HOLT et al. 1994). Before this experiment, commercial sample surfaces were not submitted to chemical agents such as detergents and sanitizers commonly applied in hygiene procedures. It is known that chemicals such as acids, alkalis, or oxidants cause abrasion and increase the microscopic porosity of rock surfaces such as granite and marble. These materials are considerably damaged by use over time. Figure 2 shows the surfaces of granite and marble as observed by SEM. These surfaces are extremely rough showing the presence of cracks and crevices, undulations and depressions (26.46 μm, Figure 2a) in diameter, Figure 2a), and pores (6.118 and 13.44 μm in length, Figure 2c). Figures 2b and 2d show the adherence of *Pseudomonas fluorescens* to granite and marble as seen under epifluorescence microscopy.

These results confirm the importance that must be given to the choice of material for equipment, utensils, and tables used in food processing. The choice of surfaces with the most appropriate characteristics is crucial for controlling the adherence process and biofilm formation. Effective control of cleaning and sanitizing procedures can possibly avoid or minimize food spoilage and outbreaks of foodborne diseases.
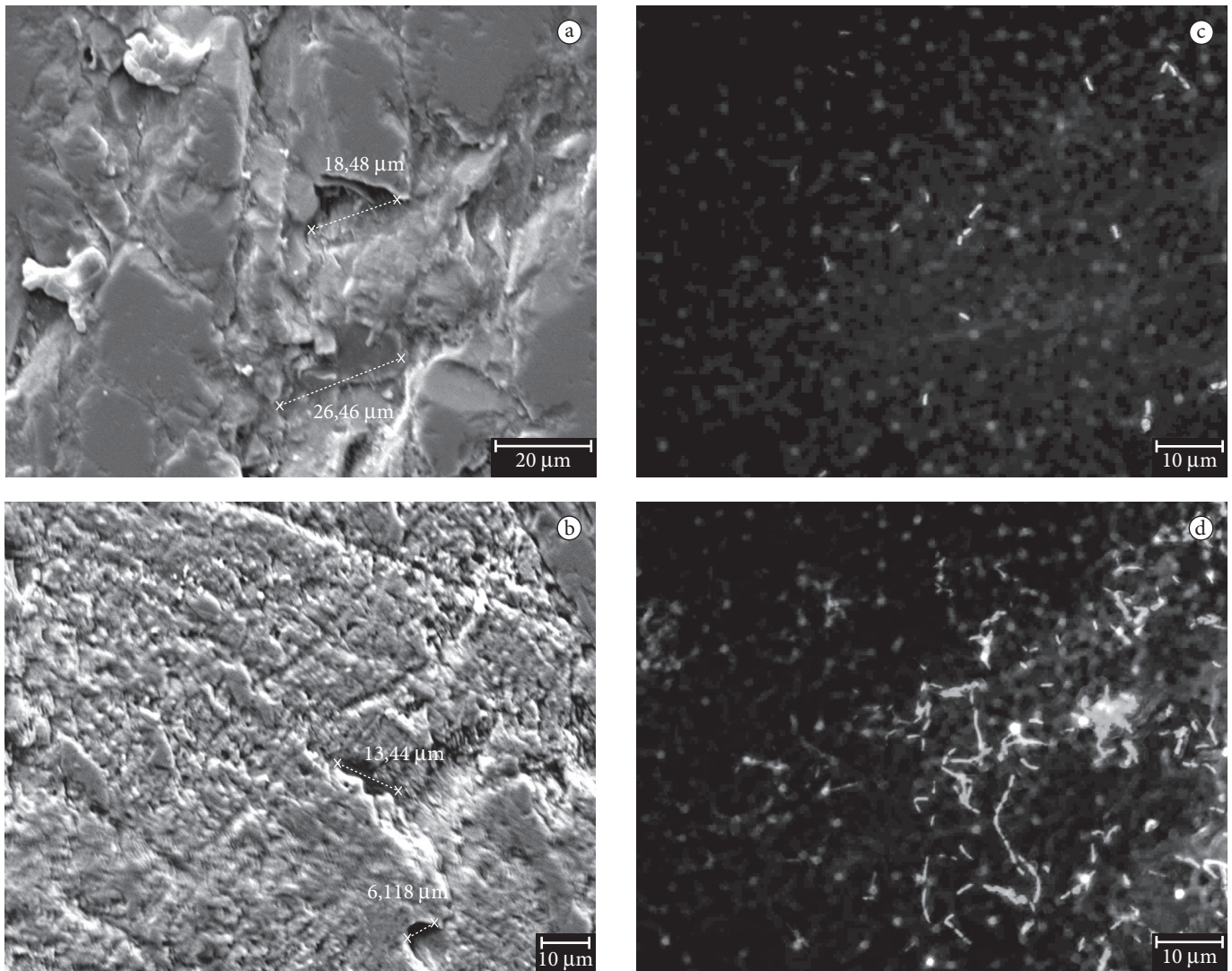
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Figure 2. Photomicrography of *Pseudomonas fluorescens* ATCC 13525 adhered to granite and marble after growing for 2 and 8 hours respectively, in nutrient broth at 28 °C. a) Microtopography of granite surface; b) adhered cells after 2 hours; c) microtopography of marble surface; and d) dhered cells after 8 hours.

4 Conclusions

All surfaces showed adherence of *Pseudomonas fluorescens,* reaching a maximum of 6.1 log CFU/cm². Marble and granite surfaces showed high porosity, cracks, crevices, undulations, and depressions large enough to hold and anchor high numbers of adhered cells, as observed under epifluorescence microscopy and scanning electron microscopy.

A higher adherence ratio was observed for stainless steel and thin polyvinyl chloride-coated cloth reaching 29.5 and 23.5 minutes, respectively. For the other surfaces, the adherence ratio mean was 9.8 minutes.

Surfaces coated with cloth showed occurrences of protuberances, elevations, microholes, cloth fraying, non-smooth surfaces, undulations and depressions with different diameters, and the occurrence of air bubbles due to protuberance defects that supported microbial growth. This resulted in a high number of adhered cells on all surfaces, which can be considered an adherence process.

Acknowledgements

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