

# Antibacterial Activity of PLA Fibres Estimated by Quantitative Methods

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## Abstract

PLA fibres with the addition of the microbicidal agent Sanitized were examined by the two quantitative standards JIS L 1902:2002 and ASTM: E2149-01. It was found that the two different methods used to assess the antibacterial activity of one sample may produce results that contrast with each other. The impact of the testing conditions upon the results obtained was deliberated. Discussed are criteria to interpret the results obtained by the use of the two methods. The antibacterial activity of lactic acid liberated from PLA in an aqueous medium was confirmed. It was also examined how the laundering of fibers affect the antibacterial activity of the samples tested.

**Key words:** PLA, antibacterial property, quantitative methods, fibres

## Introduction

Examination *in vitro* is currently being applied to assess the activity of micro-biologically active samples in their full life cycle. Quantitative and qualitative methods are amongst the tests to which materials are put to assess their antibacterial character [1, 2]. Both methods are specified in a variety of standards including European, American and Japanese. A Polish standard also exists (PN-EN ISO 20743:2013) relating to a quantitative method of antibacterial activity estimation which is very similar to the Japanese standard JIS L 1902.

The qualitative methods are based on the diffusion of the active component from the sample to a nutrient medium, and assessment of microbial growth around the sample. In quantitative methods the number of microbes is counted before and after contact with the sample.

Results obtained by the two methods may vary widely. A sample, for instance, showing high activity according to the quantitative method may appear in-

active when measured by the qualitative method, which may result from the insufficient diffusion of the active component to the agar nutrient medium. The qualitative methods (e.g. PN-EN ISO 20645) are applicable in the testing of samples containing antibacterial and antifungal agents that readily diffuse to agar.

The quantitative methods are rather complex to carry out; the final measurement results depend upon several factors like different nutrient media, different incubation conditions, the contact time between the microbes and sample tested, the variety of concentration and kind of microbe strain, and how the activity is calculated. Moreover precision in carrying out the testing and strict adherence to the standard methodology are preconditions of reliable and comparable results obtained when the sample is tested with the same method in different laboratories. No doubt, the exactness and adherence may affect the final test result. The adoption of a quality management system of adherence to ISO standards and/or accreditation and participation in inter-laboratory comparative examinations are ways toward reliable outcomes.

Unfortunately even strict adherence to standard methodology may lead to contrasting results when a sample is tested with the use of two different quantitative methods. It is therefore important, when assessing test results, to take into account the standard method used, since the measurements made by two different methods may appear incomparable.

With this work we tried to interpret the difference in results obtained in the testing of the microbiological activity

of PLA fibres taken as an example. We harnessed two quantitative methods commonly used for many years in the textile business and routinely in our laboratory. We would like to share our experience and inferences related with the application of the methods.

Extensive investigations are devoted to PLA, a polymer with a wide spectrum of actual and potential uses. It is applied, among others, in packaging materials and textiles.

Medicine is one more sector where PLA finds application in medical devices like dressings and implants like stents, sutures as well as in tissue engineering for scaffolds in bone reconstruction [3]. Research works are on-going in the preparation of PLA dressings with the addition of bioactive agents for use in wound healing [4, 5]. Sought are possibilities of using PLA modified with the addition of bioactive substances in sectors other than medical [6 - 9].

## Materials and methods

### Materials

PLA fibres were used, prepared on an experimental scale at the Institute of Biopolymers and Chemical Fibres, Łódź, Poland. 65 dtex multifilament yarn was melt-spun by consecutive drawing. Fibre-grade granulated PLA marked 6201D from Nature Works Co, USA was applied in the spinning, characterised by a 1.4% content of D isomer and melt flow index of 15 - 30 g/10 min.

The antibacterial agent Sanitized BC A 21-41 from the Swiss company Sanitized AG was admixed into the poly-

mer to confer bioactive properties. The agent contains silver ions deposited upon a ceramic matrix; particle size:  $D_{98\%} < 5 \mu\text{m}$ ;  $D_{50\%} = 2 \mu\text{m}$ . It was in the amount of 0.2 - 1.0 wt%, powdered on the granulated polymer, and both components were mixed in a tumbling dryer. Estesol-PF-790 (poly-ethylene-glycolester) from Bozzetto Group, Italy, was applied on the fibre as a spinfinish in the form of a 13% aqueous solution.

The fibre was laundered in laboratory conditions in an ultrasonic washer at 30 °C with Persil powder, and 5-times rinsed in the same washer with distilled water. The action was repeated three times. One full cycle of laundering and rinsing lasted 15 minutes.

Compositions of the fibres examined are compiled in **Table 1**.

A cotton fabric was used as a reference sample in the JIS L 1902: 2002 standard [10]. It is typically applied in laboratories as standard material devoid of microbicidal properties; 0.26 mm thick and of 97.7 g/m<sup>2</sup> surface density. The absence of fabric activity is testament to the correctness of the test.

**Table 1.** Composition of PLA fibres put to microbiological examination.

Fibre marking	Composition of fibre
B	PLA 6201D / Estesol PF 790
B/P	PLA 6201D / Estesol PF 790/washed
B/S0.2	PLA 6201D / Estesol PF 790/0.2% Sanitized BCA 21-41
B/S0.2/P	PLA 6201D / Estesol PF 790/washed/0.2% Sanitized BCA 21-41
B/S0.6	PLA 6201D / Estesol PF 790/0.6% Sanitized BCA 21-41
B/S0.6/P	PLA 6201D / Estesol PF 790/washed/0.6% Sanitized BCA 21-41
B/S1.0	PLA 6201D / Estesol PF 790/1.0% Sanitized BCA 21-41
B/S1.0/P	PLA 6201D / Estesol PF 790/washed/1.0% Sanitized BCA 21-41

**Test strains**

Bacteria strains derived from the American Type Culture Collection (ATCC) were used in the examination. Selected were microbes most popular in testing: Gram (-) *Escherichia coli* ATCC 11229 and Gram (+) *Staphylococcus aureus* ATCC 6538. The strains were deep-frozen stored in CrioBanks; their biochemical features were regularly controlled, and before the test they were multiplied on nutritive agar.

**Test methods**

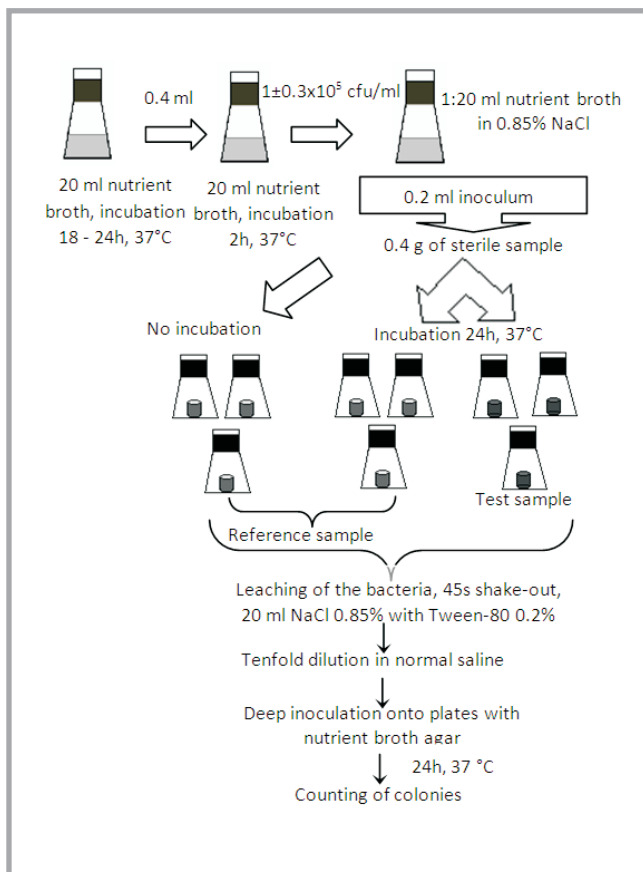
Two methods were adopted for comparison of the methods to examine PLA microbiological activity:

- one from the Japanese standard JIS L 1902: 2002 and

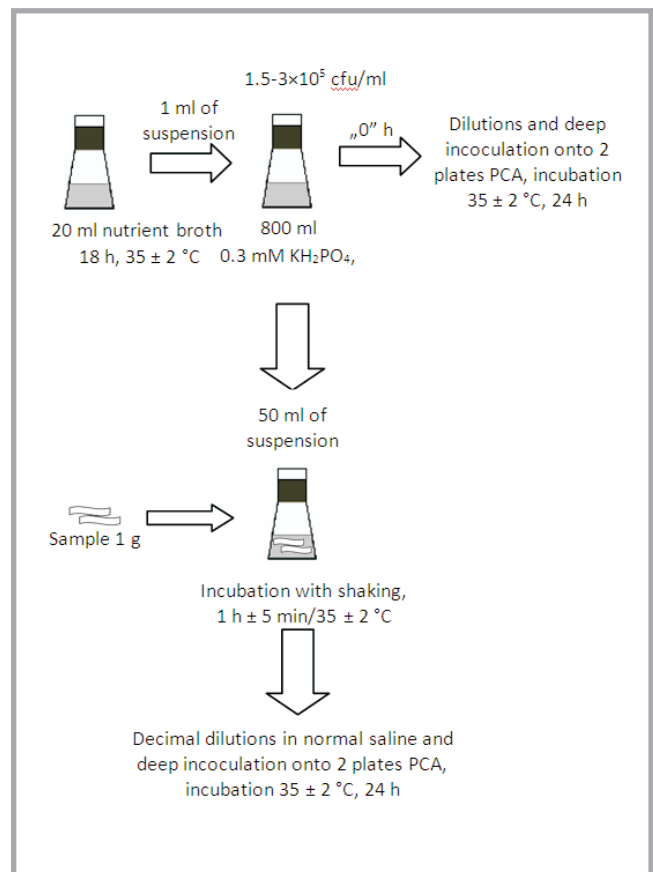
- the other from the American standard ASTM: E2149-01 [11].

**JIS L 1902: 2002 Testing for antibacterial activity and efficacy on textile products**

Two samples of PLA, 0.5 g each, with and without the biocidal agent and a cotton reference fabric were used in the examination. All three materials were inoculated in three repetitions with a suspension of bacteria at an adequate density at a volume of 0.2 ml. The microbes were leached from the materials by means of standard saline with the addition of a surface-active substance and then planted on agar plates straight after inoculation and after 24 hours of incubation at 37 °C. The method is illustrated in **Figure 1**.



**Figure 1.** Scheme of method JIS L 1902: 2002.



**Figure 2.** Scheme of the method as per ASTM: E2149 – 01.

**Table 2.** Antibacterial activity of unwashed PLA fibres with z Sanitized estimated by standard JIS L 1902: 2002 against *S. aureus*.

Symbol of sample	Time, h	Number of bacteria, cfu/sample	Bacteriostatic activity	Bactericidal activity	Growth value
reference	0	$2.7 \times 10^4$	-	-	-
reference	24	$8.5 \times 10^6$			2.5
B	24	< 20	5.6	3.1	-
B/S0.2	24				
B/S0.6	24				
B/S1.0	24				

**Table 3.** Antibacterial activity of unwashed PLA fibres with z Sanitized estimated by standard JIS L 1902: 2002 against *E. coli*.

Symbol of sample	Time, h	Number of bacteria, cfu/sample	Bacteriostatic activity	Bactericidal activity	Growth value
reference	0	$5.3 \times 10^4$	-	-	-
reference	24	$1.5 \times 10^8$			3.5
B	24	< 20	6.9	3.4	-
B/S0.2	24				
B/S0.6	24				
B/S1.0	24				

Knowing the number of bacteria on the reference sample before incubation and that of bacteria that survived on the reference and test samples after the incubation with microbes, the bacteriostatic and bactericidal activity is calculated from the formulae:

$$S = M_b - M_c$$

$$L = M_a - M_c$$

where: S – value of bacteriostatic activity, L – value of bactericidal activity,  $M_a$  – common logarithm of the bacteria number on the sample without the active agent straight after inoculation,  $M_b$  – common logarithm of the bacteria number on the sample without the active agent after 24-hours of incubation,  $M_c$  – common logarithm of the bacteria number on the sample with the active agent after 24-hours of incubation

According to standard JIS L 1902:2002, a value of bactericidal activity (L) higher than zero is an indication of bactericidal activity, while bacteriostatic properties begin with (S) values exceeding 2,

**ASTM: E2149 – 01 "Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions"- Shaking Flask Method**

A sample of 1 g of fibres was put into a 250 ml conical flask and flooded with buffer 0.3 mM  $KH_2PO_4$  with bacteria at a density of  $3.0 \times 10^5$  cfu/ml. Samples in two repetitions and a reference without a sample were incubated for 1 hour

at 37 °C. 1 ml of the suspension was collected from each of the flasks, and the number of bacteria was estimated by the method of decimal dilution on nutrient agar. Incubation was made from either dilution onto two parallel plates.

The result of the analysis is presented as a reduction in the number of bacteria calculated from the formula:

$$\text{Reduction, \% CFU/ml} = \frac{B - A}{B} \times 100$$

or

$$\text{Reduction, log} = (\log B - \log A)$$

where:

A = cfu of 1 ml in the tested sample after 1 hour, B = cfu of 1 ml estimated in the starting suspension before the addition of the sample (time 0).

Standard ASTM: E2149 – 01 does not quote any criteria to define the level of antibacterial properties in the textiles tested. A scheme of the method is presented in **Figure 2** (see page 127).

**Results of investigation**

**Tables 2 & 3** present results of the examination of unwashed PLA fibres tested according to JIS L 1902: 2002 against *E. coli* and *S. aureus*.

The PLA fibres tested revealed high antibacterial activity. With three concentrations of the bactericidal agent: 0.2, 0.6, 1.0% as well as with the PLA sample without Sanitized, maximal bactericidal and bacteriostatic activity was achieved,

which means that the level of bacteria was undetectable after incubation. The activity against *S. aureus* in the fibre tested amounted to 3.1 and 5.6, respectively, and 3.4 & 6.9 against *E. coli*. The results obtained are proof that pure PLA exerts biocidal properties. In continuation of the analysis, the fibres were laundered three times. Results are presented in **Tables 4 & 5**.

Bactericidal and bacteriostatic activity of PLA fibres with and without the Sanitized additive was high or very high against both strains. It was only PLA holding 0.2% of the additive where activity against *E. coli* was down after laundering.

The activity of the same fibres against the same strains was tested by another quantitative shaking flask method according to Standard ASTM: E2149 – 01. Presented are the numbers of bacteria for both repetitions and the arithmetic mean of logarithmic and percentage reduction. The results are compiled in **Tables 6 & 7**.

Unwashed PLA fibres containing the bactericidal agent showed antibacterial activity, which was highest for a 1% and lowest for a 0.2% content of Sanitized. Pure PLA used as reference did not reveal activity when tested by the Shaking Flask Method. The average number of *E. coli* cells after 1 hour of incubation was close to that in the buffer without the sample *i.e.* in the inoculum.

Fibres with the bactericidal agent washed three times were much less antibacterial than those unwashed.

A higher content of Sanitized produced higher antibacterial activity.

The highest reduction in activity was after laundering with fibres containing 0.2% of the bactericidal agent. Activity against *S. aureus* dropped from 98.94% to 8.49% reduction, and against *E. coli* from 99.51% to 2.64%. The order of magnitude of the number of units making the colony is thus decreased by two orders. The decrease was lower in the sample with 0.6% content of Sanitized in the case of *S. aureus*. The sample with 1% Sanitized attained, in spite of laundering, a high percentage of reduction

## Discussion

Differing results were attained in the examination by different methods for the same PLA samples with a low content of the antibacterial additive Sanitized and without it. Only fibres with the highest content of Sanitized showed in both tests the same high antibacterial activity.

The divergence ensues from the different methods in both standards, which provide for dissimilar contact of bacteria with the sample. As per ASTM: E2149 – 01, the samples tested are immersed for 1 hour in a voluminous buffer with a suspension of microbes, while according to Standard JIS L 1902:2002, the textiles are incubated for 24 hours with a small amount of bacteria suspension.

Longer incubation time and a higher amount of the active substance in proportion to the number of cells and volume of the bacteria suspension are the reasons for a stronger impact of the active substance upon the microorganisms. The activity of pure PLA against bacteria, revealed particularly in the test according to JIS L 1902:2002, probably results from the migration of lactide from the fibre to the medium. It is known that lactide exerts a strong antibacterial and antifungal activity [3, 12]

All PLA samples were also tested after laundering, which was to remove the lactide and examine the durability of the Sanitized application.

In method JIS L 1902:2002, a decrease in activity in the washed samples was observed only in the case of *E. coli* for samples without Sanitized or with its lowest content of 0.2%, while in method ASTM: E2149 a substantial fall of activity in the same samples occurred both with *E. coli* and *S. aureus*. According to both methods, the washing did not affect the activity of the samples with 1% of Sanitized; it was not leached to the extent which would imply a decrease in antibacterial activity.

The drop in activity in samples without Sanitized probably results from the removal of lactide in the course of washing. The decrease in activity in washed samples with 0.2% Sanitized could have been caused by the removal of both lactide and the active agent.

**Table 4.** Antibacterial activity of washed PLA fibres with Sanitized estimated by standard JIS L 1902: 2002 against *S. aureus*.

Symbol of sample	Time, h	Number of bacteria, cfu/sample	Bacteriostatic activity	Bactericidal activity	Growth value
reference	0	$4.60 \times 10^4$	-	-	-
reference		$4.30 \times 10^6$			1.9
B/P	24	< 20	5.3	3.4	-
B/S0.2/P					
B/S0.6/P					
B/S1.0/P					

**Table 5.** Antibacterial activity of washed PLA fibres with Sanitized estimated by standard JIS L 1902: 2002 against *E. coli*.

Symbol of sample	Time, h	Number of bacteria, cfu/sample	Bacteriostatic activity	Bactericidal activity	Growth value
reference	0	$1.0 \times 10^5$	-	-	-
reference		$1.1 \times 10^8$			3.0
B/P	24	$2.9 \times 10^3$	4.5	1.5	-
B/S0.2/P		$1.1 \times 10^3$	5.0	2.0	
B/S0.6/P		< 20	6.7	3.7	
B/S1.0/P					

**Table 6.** Bactericidal activity against *S. aureus* of unwashed and washed PLA fibres containing Sanitized estimated according to Standard ASTM: E2149 – 01.

Symbol of sample	Unwashed fibre			Washed fibre		
	cfu/ml	Average reduction, %	Average reduction, log	cfu/ml	Average reduction, %	Average reduction, log
Inoculum	$1.78 \times 10^5$	-	-	$1.09 \times 10^5$	-	-
B	$6.87 \times 10^4$	61.40	0.52	$1.39 \times 10^5$	-27.52	-0.11
B/S0.2	$1.83 \times 10^3$	98.94	2.00	$9.98 \times 10^4$	8.49	0.04
B/S0.6	$5.25 \times 10^0$	100.00	4.53	$2.75 \times 10^2$	99.75	2.60
B/S1.0	< 1	100.00	5.25	$9.25 \times 10^0$	99.99	4.07

**Table 7.** Bactericidal activity against *E. coli* of unwashed and washed PLA fibres containing Sanitized estimated according to Standard ASTM: E2149 – 01.

Symbol of sample	Unwashed fibre			Washed fibre		
	cfu/ml	Average reduction %	Average reduction log	cfu/ml	Average reduction %	Average reduction log
Inoculum	$2.01 \times 10^5$	-	-	$2.27 \times 10^5$	-	-
B	$1.77 \times 10^5$	12.19	0.06	$2.21 \times 10^5$	2.86	0.01
B/S0.2	$9.93 \times 10^2$	99.51	2.31	$2.21 \times 10^5$	2.64	0.01
B/S0.6	$1.20 \times 10^2$	99.94	3.22	$4.26 \times 10^2$	99.81	2.73
B/S1.0	< 1	100.00	5.30	< 2	100.00	5.05

As can be seen, the antibacterial activity of the samples tested may stem from the presence of substances that were not added as antibacterial agents. The presence of such substances may be typical for the sample material or they might have been generated by the process of fibre spinning. PLA 6201D, according to the manufacturer [13], contains up to 0.5% of unreacted lactide, which may go up in the course of melt spinning as a result of thermal degradation of the polymer. In an aqueous medium, lactide yields lactic acid, which confers antibacterial properties upon the fibre. It is

particularly manifested with the Japanese method, which is more sensitive due to the longer incubation time of the bacteria on the sample and the small volume of the suspension in proportion to the sample mass. Such conditions are the reason why PLA samples without the active additive or with its low content revealed strong antibacterial activity.

For the interpretation of test results according to Standard JIS L 1902:2002, criteria are available that indicate the sample is active if the value of bacte-

riostatic activity  $S > 2$  and that of bactericidal activity  $L > 0$ .

Standard **ASTM: E2149** does not contain criteria for the evaluation of results *i.e.* at which a reduction in the cell number of the sample can be recognized as active. The lack of criteria may lead toward false inferences. To give an example: evaluating the reduction in *S. aureus* cells at a level of 61.40%, one may deduce that the PLA sample without the Sanitized additive revealed strong antibacterial activity. It must, however, be noted that the decrease in the cell number is rather modest, from  $1.78 \times 10^5$  to  $6.87 \times 10^4$  cfu/ml on average, responding to the reduction at a level of 0.52 log. Such a value can be recognized as meaningless, since a high concentration of the bacteria cell was preserved in the medium. In the standard ASTM: E2149, high activity is observed when the bacteria number drops from the  $10^5$  level to  $10^1$ , responding to a decrease of 4 in the logarithm scale and reduction of around 99.9%.

The lack of criteria in the standard induces the adoption of own assumptions for the interpretation of results. Pinto *et al.* [14] consider a drop in the cell number by one order of magnitude in proportion to the initial value as testament of sample activity. Recognition of the samples tested as active according to such criteria may not necessarily mean sufficient efficiency in the intended use.

It is worth remembering that an infection may be initiated even when an insignificant cell number survives despite contact with the sample, for instance bioactive dressing. One infectious inoculum *i.e.* a minimal number of bacteria to start an infection cannot be established for all microorganisms. According to Schmidt-Hempel *et alii* [15], various pathogens are characterised by different minimal infectious doses of cells depending on the type and place where the infection develops. Some pathogens may begin infection from a small number of cells in the start phase. Only 10 cells constitute an infectious inoculum in the case of the enterhemorrhagic strain *Escherichia coli*, while others like *Vibrio cholerae* need a lot of cells (from  $10^3$  to  $10^8$ ) to effectively infect the host organism.

In investigations concerned with antibacterial properties, criteria are needed to evaluate the results. Such criteria unified

for a given method ought to constitute a part of all standards.

## Conclusions

1. Results of the testing of microbiological activity are comparable only if they were derived by the use of the same examination method.
2. Due to the multitude of available methods, information about the method applied must always be given whenever test results are announced concerning biological activity.
3. When the results of antibacterial activity examination are construed, except the activity factor indicated in the standard, the initial and final level of bacteria (cfu) on the sample tested must be taken into account. It is particularly important if no criteria exist to assess the results
4. Selection of the method for a given material is crucial in the assessment of antibacterial activity. As was shown with the instance of PLA fibres, testing according to Standard JIS L 1902:2002 allows the detection of activity resulting from the presence of lactide/lactide acid, in contrast to the ASTM method, which did not produce such results.
5. In the examination of PLA fibres, the Shake Flask Method (ASTM: E2149 – 01:2010) appeared adequate because it allowed to reveal the impact of Sanitized concentration upon the fibre activity.
6. Laundering caused a partial removal of lactide from the fibre, which was demonstrated by the decrease in activity of the PLA fibres in the ASTM method. In the Japanese method, the residual lactide preserved the activity of the washed fibres.

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