Title: Growth Performances and Stability of Bacteriological Media Used for Clean-room Applications – a Comparative Study

Authors: Elke Lehmann, Andreas Dreusch*. MicroMol GmbH, Karlsruhe, Germany.

Keywords: ISO11133, European Pharmacopoeia, Bacteriology, TSA, clean-room, settlement plates

Abstract:

The scope of our study was the evaluation of nominally identical triple wrapped irradiated TSA agar-plates from four commercial manufacturers. The growth performances for the six test organisms devised by the European Pharmacopoeia was determined by comparison with a blood-containing medium at the date of delivery and at the date of expiry.
The recovery rates found with the four products differed vastly for some organisms and media. Some media showed only 16% of the recovery rate of the best performing ones. Especially the gram-negative bacteria reacted very fastidiously, showing also variation coefficients of up to 60%. At expiry date some organisms showed a better growth on the test media than on the media used for comparison. This effect could probably be due to the loss of water during storage or to pH variations.
The study reveals a mandatory necessity for a stringent validation even of pre-made media since a underscoring of the bacterial burden by the factor of ten is possible.

Zusammenfassung:

Ziel dieser Studie war die Evaluierung nominell identischer, dreifach verpackter und bestrahlter TSA-Agarplatten vier kommerzieller Hersteller. Die Wachstumseigenschaften der sechs von der Europäischen Pharmakopoë vorgeschlagenen Testorganismen wurden im Vergleich zu einem bluthaltigen Medium zum Zeitpunkt der Auslieferung und zum Verfallsdatum geprüft.
Die mit den vier Produkten ermittelten Wiederfindungsraten unterschieden sich erheblich in Abhängigkeit von Organismus und Medium. Mit einigen Medien wurden nur 16% der Keime wiedergefundenen, die auf dem besten Medium gewachsen waren. Insbesondere die Gram-negativen Bakterien reagierten sehr anspruchsvoll und zeigten Variationskoeffizienten von bis zu 60%. Zum Verfallsdatum zeigten wiederum einige Organismen ein besseres Wachstum als auf dem Vergleichmedium. Dieser Effekt könnte Folge des während der Lagerung stattfindenden Wasserverlusts oder von Veränderungen des pH-Werts sein.
Die Studie belegt die Unabdingbarkeit der Validierung selbst vorgefertigter Medien, da sonst die tatsächliche Keimbelastung bis um den Faktor 10 unterschätz werden kann.

* corresponding author

1. Introduction

Testing the bacterial burden of the air, the equipment, staff and media is especially for clean rooms a well-established control measure. In this environment the detection of the lowest amount of bacteria is necessary since the limits for grade A and B rooms are very low (<1 cfu/4 hours or 5 cfu / 4 hours on settlement plates in grade A rooms or grade B rooms respectively) [1]. Usually TSA-agar plates are used, which are conveniently supplied by several producers in clean room compatible packing. To meet GMP demands these products are triple wrapped and irradiated.
For these tests neither type of medium nor the performance criteria for it are proposed by the EC GMP-Guide, but use of TSA-agar and compliance with the criteria given by the European Pharmacopoeia are best practice [2].
Our interest was to validate such media from several manufacturers on a basis much more accurate than the one mentioned. Also we also wanted to see evolution in the performance of the media from delivery to expiry. This seemed to be important as the shelf lives of the products vary. For this purposes we used the quantitative testing scheme of ISO/TS 11133-2:2003 with some changes [3]. We compared microbial counts on a TSA-blood agar and a Sabouraud-Blood agar with the TSA agar-plates of four commercial manufacturers.
There are only a very few recent reports about such validations. The colony count method has been inspected thoroughly by Fisher et al. [4] and the statistical backgrounds have been elucidated by Hattori [5]. With our report we wanted to assist those involved in the daily business of detecting viable micro-organisms with data on the performance characteristics of media and test strains actually in use. Our findings reveal an unexpected large difference in recovery depending on the manufacturer, the organism and the age of the medium.

2. Materials and Methods

Triple-wrapped TSA agar-plates sterilized by irradiation were used. Test plates were ordered with the premise to have a shelf live as long as possible and tested them a first time shortly after arrival. The lot was stored at 4-8 °C and tested again at its expiry date. The media are listed in table 1.

Table 1. Media manufacturers and lots of TSA 90 mm agar-plates, triple wrapped and irradiated

Product; Manufacturer	Ref. No.	Lot	Shelf-life*
A; BD Diagnostic Systems	254955	3338410	4 months
B; BioMerieux	43131	782707301	3 months
C; BioRad	46025	44175-1	6 months
D; Biotest Heipha Dr. Müller	3073e	60439	4 months

*maximum shelf life according to the manufacturer

The test plates were compared with reference plates, which comprised Sabouraud dextrose, agar-plates for *A. niger* or TSA plates with 5% sheep blood (ref. no. 43555 respectively ref. no. 43001, both BioMerieux) for the other organisms. A fresh lot of these plates was used for each test.

All organisms used were prepared from lyophilized discs (Microtrol, BD Diagnostic Systems), which had a nominal content of 10^5 to 10^6 cfu/disc, except for *A. niger*, which had only 500 spores/disc (table 2).

Table 2. Test organisms and strains used for this work. The incubation conditions listed are taken from the European Pharmacopoeia

Organism	Strain	Incubation conditions	Reference plate
Aspergillus niger	ATCC 16404	72h at 22 °C ± 3 °C	Sabouraud dextrose agar
Candida albicans	ATCC 10231	48h at 22 °C ± 3 °C	TSA with 5% sheep blood
Bacillus subtilis	ATCC 6633	24h at 30 °C ± 1 °C	TSA with 5% sheep blood
Pseudomonas aeruginosa	ATCC 9027	24h at 30 °C ± 1 °C	TSA with 5% sheep blood
Staphylococcus aureus	ATCC 6538	24h at 30 °C ± 1 °C	TSA with 5% sheep blood
Escherichia coli	ATCC 8739	24h at 30 °C ± 1 °C	TSA with 5% sheep blood

The discs were dissolved in 10 ml of buffered peptone-water (two discs of the bacteria and 10 discs of the mould, respectively) for 1 hour at room temperature and diluted to a germ concentration of nominally 100 to 1000 cfu/ml, which was held on ice. From these suspension 100 µl were plated on the 12 test plates for each manufacturer and organism and on 12 reference plates for each organism. The conditions for incubation are indicated in table 1. The plates with the bacteria were counted after 24 h and 48 h except for *B. subtilis*, which was recounted after 72 h. The plates with *C. albicans* were counted after two and four days, the ones with *A. niger* after three, four and five days.
From the raw data the arithmetical mean was calculated.
The fertility coefficient (according to ISO/TS11133-2 called productivity) is the division of the mean of the tested plate type by the mean of the reference plate,

$$F = \frac{\overline{X}_{test}}{\overline{X}_{reference}}$$

Where $\bar{x}$ is the arithmetical mean. The standard deviation was calculated by the formula:

$$s = s = \sqrt{\frac{n\sum x^2 - \left(\sum x\right)^2}{n^2}}$$

where n is the number of plates and x the individual counts.
$Conf_{95}$ assigns the confidence interval at a probability of 95%, which means that 5% of all results lay outside of the mean ± $Conf_{95}$. It was calculated with the formula

$$Conf_{95} = 1.95996\left(\frac{s}{\sqrt{n}}\right)$$

3. Results and Discussion

The incubation times according to the pharmacopoeia revealed to be not optimal for the test purposes. Whereas the counts for *E. coli*, *S. aureus*, and *P. aeruginosa* after 48 h derived from the counts after 24 h by roughly 10%. As expected, the *B. subtilis* colonies developed delayed. After 48 h 30% to 50% more colonies were counted then after 24 h. For *Candida* and *Aspergillus* the incubation temperatures are suboptimal which resulted in a retarded growth (data not shown). To obtain stable results we used the counts from the longest incubation time indicated under Materials and Methods.
The results for *B. subtilis* showed virtually no difference between the reference and the test plates. Overall a somewhat higher standard deviation was observed. The fertility coefficients for all plates was <1 at the first experiment, but >1 at the expiry date.

Table 3. Results for *B. subtilis*. In the upper part of the table the results for the initial testing are shown, beneath the values for the tests at each expiry date. Mean, arithmetical mean (cfu/plate); SD, standard deviation (cfu/plate); F, fertility coefficient; Min, minimum result as percentage of the mean; $Conf_{95}$, see *Materials and Methods*; Max, maximum result as percentage of the mean. All percentages are rounded.

Medium	Mean	SD	F	Min	95% conf.	Max
Start						
Reference	31.8	21.1	1	50%	38%	264%
Product A	26.9	12.2	0.846	33%	26%	227%
Product B	31.3	11.1	0.982	48%	20%	166%
Product C	30.3	6.8	0.950	60%	13%	136%
Product D	30.9	11.2	0.971	58%	21%	188%
Expiry						
Reference	107.7	17.2	1	87%	9%	150%
Product A	114.6	17.4	1.064	79%	9%	130%
Reference	93.8	24.3	1	63%	15%	157%
Product B	94.3	20.8	1.006	74%	12%	155%
Reference	72.8	9.8	1	84%	8%	126%
Product C	73.0	9.2	1.003	81%	7%	125%
Reference	115.4	26.1	1	79%	13%	158%
Product D	102.8	15.6	0.890	79%	9%	126%

The fertility of all plates for *P. aeruginosa* was acceptable at the first experiment, but decreased dramatically for the plates of product C and D at expiry date. At this time point for the best performing medium F was 0.938 ± 0.062 (deviation at 95% confidence level, calculated from F * $Conf_{95}$ with not rounded values), for the worst performing one 0.122 ± 0.016. With an identical dose of bacteria (e.g. 50 cfu) this could result in a count of 50 in the best or 5 in the worst case.

Table 4. Results for *P. aeruginosa*, for explanations see table 3

Medium	Mean	SD	F	Min	95% conf.	Max
Start						
Reference	13.0	4.1	1	54%	18%	162%
Product A	9.8	1.9	0.756	71%	11%	132%
Product B	12.7	2.6	0.974	71%	11%	134%
Product C	9.3	2.3	0.712	65%	14%	151%
Product D	9.0	2.4	0.692	56%	15%	144%
Expiry						
Reference	95.1	8.9	1	83%	5%	119%
Product A	11.6	2.8	0.122	60%	13%	138%
Reference	42.8	4.7	1	87%	6%	122%
Product B	40.1	4.8	0.938	80%	7%	120%
Reference	131.0	8.2	1	89%	4%	108%
Product C	100.3	13.5	0.765	74%	8%	119%
Reference	24.8	7.9	1	48%	18%	153%
Product D	9.6	4.9	0.386	31%	29%	177%

With *E. coli* a marked discrepancy of recovery was seen at both tests (table 5). At the first test point product C detected of the inoculate about one fifth, as did the product D at expiry date. Unexpectedly the recovery increased for the product C at expiry, which was true also for the product A.

Table 5. Results for *E. coli*, for explanations see table 3

Medium	Mean	SD	F	Min	95% conf.	Max
Start						
Reference	75.7	7.6	1	83%	6%	123%
Product A	36.1	19.7	0.477	39%	31%	233%
Product B	71.5	12.9	0.945	77%	10%	131%
Product C	16.4	6.9	0.217	30%	24%	207%
Product D	11.8	7.7	0.156	17%	37%	211%
Expiry						
Reference	114.6	10.2	1	87%	4%	120%
Product A	61.4	37.4	0.812	31%	34%	186%
Reference	78.2	7.2	1	84%	5%	114%
Product B	77.5	9.7	0.991	71%	7%	120%
Reference	210.0	10.0	1	93%	3%	110%
Product C	137.8	34.8	0.656	70%	14%	139%
Reference	86.1	21.3	1	69%	14%	153%
Product D	17.9	3.6	0.208	56%	11%	134%

As a quite stress tolerant organism the recovery of *S. aureus* was good. F-values between roughly F=0.95 and 0.85 were found at both test points. The deviation from the mean was also quite low.

Table 6. Results for *S. aureus*, for explanations see table 3

Medium	Mean	SD	F	Min	95% conf.	Max
Start						
Reference	80.9	5.4	1	89%	4%	115%
Product A	77.7	5.1	0.960	86%	4%	109%
Product B	71.3	9.8	0.881	81%	8%	124%
Product C	74.4	11.8	0.920	69%	9%	117%
Product D	73.3	11.2	0.905	74%	9%	119%
Expiry						
Reference	36.7	7.2	1	76%	11%	145%
Product A	30.6	7.4	0.834	65%	14%	144%
Reference	32.8	4.4	1	79%	8%	125%
Product B	31.8	7.2	0.969	54%	13%	142%
Reference	121.1	13.3	1	83%	6%	122%
Product C	112.8	12.8	0.932	87%	6%	123%
Reference	44.1	8.5	1	68%	11%	132%
Product D	37.2	10.2	0.843	54%	15%	151%

With C. albicans the results were nearly the same as for S. aureus, with F-values near 1, low standard deviation and only slight variations between the first and the second test point (table 7).

Table 7. Results for *C. albicans*, for explanations see table 3

Medium	Mean	SD	F	Min	95% conf.	Max
Start						
Reference	50.2	8.3	1	74%	9%	130%
Product A	54.7	7.9	1.090	82%	8%	123%
Product B	60.8	8.9	1.213	60%	8%	127%
Product C	40.3	7.6	0.802	73%	11%	129%
Product D	53.4	8.3	1.065	70%	9%	118%
Expiry						
Reference	174.0	9.8	1	93%	3%	107%
Product A	162.8	11.3	0.935	85%	4%	110%
Reference	105.9	11.8	1	84%	6%	121%
Product B	116.8	7.8	1.103	87%	4%	110%
Reference	28.9	3.9	1	73%	8%	118%
Product C	26.8	4.7	0.928	78%	10%	134%
Reference	56.8	4.8	1	86%	5%	118%
Product D	58.9	7.8	1.037	64%	7%	121%

TSA is not a medium recommended for the detection of filamentous fungi. This was confirmed by the comparison with the Sabouraud dextrose agar reference. Showing only about half of the recovery, growth was on TSA also retarded. A remarkably

improvement of recovery rates at the second test point was observed for nearly all but the plates of manufacturer B (table 8).

Table 8. Results for *A. niger*, for explanations see table 3

Medium	Mean	SD	F	Min	95% conf.	Max
Start						
Reference	38.5	7.9	1	73%	12%	143%
Product A	12.3	2.6	0.318	73%	12%	155%
Product B	15.6	1.9	0.405	77%	7%	122%
Product C	14.0	3.1	0.364	64%	13%	136%
Product D	12.7	2.1	0.329	79%	10%	134%
Expiry						
Reference	38.5	4.0	1	83%	6%	119%
Product A	19.9	3.3	0.517	75%	9%	126%
Reference	35.6	4.1	1	84%	7%	118%
Product B	16.8	2.7	0.471	60%	9%	119%
Reference	60.3	5.8	1	90%	5%	126%
Product C	38.4	3.9	0.638	81%	6%	115%
Reference	32.8	3.3	1	82%	6%	119%
Product D	26.2	2.5	0.799	84%	5%	115%

The results can be summarized by two points of view. The first one is the relative behaviour of the organisms irrespective of the origin and age of the medium. *B. subtilis, S. aureus* and *C. albicans* showed the best recovery rates and most stable results with F-values of 0.96 ± 0.06, 0.91 ± 0.05, and 1.02 ± 0.12 respectively (mean of all F-values ± 95% confidence interval of the standard deviations).

The mould and the both gram negative species showed a bad recovery and unreliable results with F-values of 0.48 ± 0.15, 0.67 ± 0.26 and 0.56 ± 0.31 for *A. niger*, *E. coli* and *P. aeruginosa* respectively.
Especially for *E. coli*, the molecular biologists' guinea pig, the results are sobering since the recovery of a colony count for *E. coli* can vary between 0.87 and 0.25. Adding the mean of the confidence intervals of all experiments (21% for *E. coli*) a specific inoculate can be recovered from a 100% to a 19% level, depending on the medium used.

A closer look on the recovery rates of the different media reveals an average total recovery of 73% for product A, 91% for product B, 74% for product C and 69% for product D. These values may be taken as a measure for the overall performance of the media for the recovery of a mixed microbial population. Nevertheless, as mentioned above, there are big differences of the recovery rates depending on the test organisms. This is very obvious for the gram-negative bacteria and the product A, C and D. The product B showed the most reliable results in overall and in detailed view.

The better recovery of *A. niger* and, in a more moderate manner, for *B. subtilis* with plates at expiry date may be due to a loss of water or a change of pH during the storage. Some products (at expiry) could not be dried before plating in order to avoid cracks (which some of them already had) Though not quantified it was observed, that these plate showed the higher recovery for *A. niger* and *B. subtilis*.
To illustrate the differences in performance, we related the mean of the fertility coefficients either for one organism and all media or one medium and all organisms to the mean of their $Conf_{95}$ (fig. 1A and 1B).

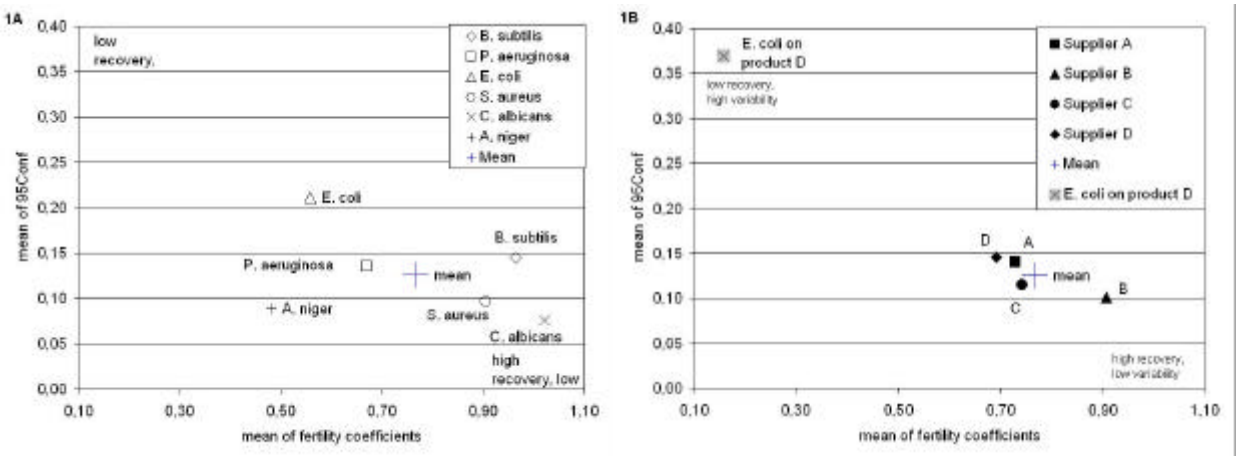

Figure 1A and 1B. Performance and stability of results with reference to either the test organism or the medium. The arithmetical mean of the F-values shown in tables 3 to 8 was calculated either for an organism (fig. 1A) or a manufacturer's product (fig. 1B) and correlated to the mean of the corresponding confidence intervals. The absolute mean of all F- and $Conf_{95}$-values is shown by the hair-cross. The result for one test (*E. coli* on product D at the first time point) is given as an example for strong deviation from the mean.

The graphical presentation clarifies the plain numbers. Every value to left of the hair-cross represents a bad recovery; every value above it stands for scattered results. Taken together, the values obtained in the left upper quadrant are somewhat unreliable, as exemplified by the single result of *E. coli* on the product D. Those in the right, lower quadrant are strongly reliable.

Depending on the medium and its age, for a specific organism there can be differences of recovery by a factor of roughly 4. If a medium newly introduced into the routine control is not validated, the results may be very distressing. In the case of organisms for which the medium notoriously gives strongly scattering results an out of specifications status may be systematically underscored. The studies underlines the mandatory necessity for evaluation of every medium, even pre-made plates, like it is required by ISO 17025 according to the amending paper of EA [6].

4. Literature

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Address for correspondence:
Dr. Andreas Dreusch, MicroMol GmbH, Seboldstrasse 3, 76227 Karlsruhe, Germany, e-mail: ahj.dreusch@micromol.com