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Importance Considering Increased Recovery of Injured Microorganisms to Attain Reproducible Sterilization Validation

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Abstract

Final sterilization was conducted to health care products immediately prior to shipping and sterility assurance must be confirmed from the result of biological indicator (BI). The survived microorganisms after sterilization may be mostly injured microorganisms. The auxotrophic characteristics of injured microorganisms are different from those of healthy microorganisms. In that sense, the cultivation conditions of injured microorganisms must be used for confirming reproducible sterilization validation study, otherwise it may result in false negative at shipping test. This is important to attain correct and reproducible sterility assurance. This is indispensable to attain safety assurance of health care products. The evaluation of several sorts of agents must be studied to recover injured microorganisms and support growth reproducibly. It is also important to study for diminishing variation of culture medium performance among culture medium suppliers and that lotto lot variation to attain reproducible sterility assurance. These were studied by adding several sorts of agents to the culture medium inoculating injured microorganisms exposed by moist heating, dry heating, ethylene oxide gas exposure, gamma-ray and electron beam irradiation. Even though sterilizing method may differ, when amino acids mixture, glucose and calcium were added to the culture medium, cultivation performance of injured spores was significantly improved and reproducible sterilization validation can be attained in success.

Keywords: Sterilization validation; Injured microorganisms; Sterility assurance; Auxotrophic characteristics; Biological indicator

Introduction

Before shipping medical devices and medicines must be exposed final sterilization, if applicable, to attain sterility assurance level (SAL) of 10^{-6} for 7 days incubation. If damaged and injured microorganisms after sterilization may survive, they may cause false negative and grow after shipping, which cause contamination to medical devices and medicines. In appearance it seems to be successful for sterilization validation at that time of shipping. Damaged and injured microorganisms in the culture medium requires in general more than 7 days incubation at lower temperature, so validation study in several factors must be conducted. To avoid overlooking damaged and injured microorganisms and unsuccessful sterilization validation, the use of the appropriate constitute of culture medium is indispensable.

For sterilization validation, in general, soybean casein digest (SCD) agar (SCDA) is so often used. However, depending on supplier or difference of lot, cultivation performance significantly differed for injured spores. So, in order to attain reproducible cultivation performance and sterilization validation, we studied for what constitute of culture medium may cause differ to the injured spores in SCDA.

In this study, moist heat, dry heat, ethylene oxide gas (EOG), gamma-ray and electron beam irradiation were used to prepare damaged spores. In SCDA, several sorts of substances such as calcium, amino acids and glucose are required to add to attain reproducible SAL and successful sterility assurance [1-5].

Experimental

Dry heat sterilization

Biological indicators (BLs) for dry heat use (*Bacillus atrophaeus* ATCC 9372, 3×10^6 CFU/carrier) were purchased from Raven Co. Ltd. They were exposed at 160°C for 3 and 6 min to attain injured spores. They apply to SCDA culture medium (M, D, B, ND, E, N companies), incubate for 7 days and count colony.

Colony count can be done by using 3 sheet of BI and agitating with 30 ml of 0.1% Tween 80 under cooling. The solution recovered and the procedure repeated three times and combined the test solutions. The test solution was step-wise 10-fold diluted to become 30-100 CFU/plate. Colony count can be done by incubating with SCDA culture medium at 35 ± 2°C for 7 days.

Several sorts of agents were added to test for evaluating recovery of colony. They are calcium carbonate ($CaCO_3$), sodium pyruvate, D-alanine, L-alanine, vitamin mixture, amino acids mixture, L-serine, lysozyme, casamino acid, magnesium chloride ($MgCl_2$), ATP, and glucose. These agents are commercially available with pure grade.

Moist heat sterilization

BIs for moist heat sterilization use (Geo*bacillus stearothermophilus* ATCC 7953, 1×10^6 CFU/carrier) were purchased from Raven Co. Ltd. They were exposed at 121.1°C for 3 and 5 min to attain injured spores and apply to SCDA culture medium (M, D, B, ND, E, N companies), incubate and count colony at 55 ± 2°C for 7 days.

Colony count can be done by using 3 sheet of BI and agitating with 30 ml of 0.1% Tween 80 under cooling. The solution recovered and the procedure repeated three times and combined the test solutions. The test solution was step-wise 10-fold diluted to become 30-100 CFU/plate.

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Several sorts of recovery agents were added to test. They are glucose, sodium pyruvate, calcium lactate, calcium carbonate, L-alanine, vitamin mixture, amino acids mixture, yeast extract, soluble starch, catalase, and milk. These agents are commercially available with pure grade.

EOG sterilization

BIs for EOG sterilization use (*Bacillus atrophaeus* ATCC 9372, 3.5×10^6 CFU/carrier) were purchased from Raven Co. Ltd. They were exposed at 600 ± 30 mg/L, 60 ± 10% RH, 54 ± 1°C for 3 min and 10 min, respectively, to attain injured spores and apply to SCDA culture medium (B, N, M companies), incubate and count colony.

Colony count can be done by using 3 sheet of BI and agitating with 30 ml of 0.1% Tween 80 under cooling. The solution recovered and the procedure repeated three times and combined the test solutions. The test solution was step-wise 10-fold diluted to become 30-100 CFU/plate. Colony count can be done by incubating with SCDA culture medium at 35 ± 2°C for 7 days.

Several sorts of recovery agents were added to test. They are calcium carbonate ($CaCO_3$), L-alanine, D-alanine, amino acids mixture, vitamin mixture, sodium pyruvate, and glucose. These agents are commercially available with pure grade.

Gamma-ray irradiation sterilization

BIs for gamma-ray irradiation sterilization use (*Bacillus pumilus* ATCC 9372, 2.0×10^6 CFU/carrier or *Bacillus megaterium* spore at 1.4×10^6 CFU/carrier) were purchased from Raven Co. Ltd. *Bacillus pumilus* and *Bacillus megaterium* were exposed at 2 kGy and 5 kGy, respectively. Injured spores were cultivated with SCDA from N company. Other procedures were identical to those of EOG.

Several sorts of recovery agents were added to test. They are calcium carbonate ($CaCO_3$), calcium lactate, D-alanine, L-alanine, amino acids mixture, sodium pyruvate, soluble starch, activated carbon, vitamin mixture, and glucose. These agents are commercially available with pure grade.

Electron beam irradiation sterilization

BIs for electron beam irradiation sterilization use (*Bacillus pumilus* ATCC 9372, 2.0×10^6 CFU/carrier or *Bacillus megaterium* spore at 1.4×10^6 CFU/carrier) were purchased from Raven Co. Ltd. *Bacillus pumilus* and *Bacillus megaterium* were exposed at 4.5 kGy. Injured spores were cultivated with SCDA from N company. Other procedures were identical to those of gamma-ray.

Several sorts of recovery agents were added to test. They are calcium carbonate ($CaCO_3$), calcium lactate, D-alanine, L-alanine, amino acids mixture, sodium pyruvate, vitamin mixture, and glucose. These agents are commercially available with pure grade.

Result and Discussion

Dry heat sterilization

In table 1, the initial population from 6 companies is presented. No significant difference of colony count can be observed when healthy spores were used. ISO 11138 approved –50 to +300% deviation from labeled population.

In table 2a, BI population exposed at 3 min and 6 min at 160°C, respectively, was presented. In table 2a 6 min exposure, the population differs significantly depending on culture medium supplier (i.e. between D and N, 380% difference). In table 2b, BI population after addition 0.5% glucose to SCDA presented.

By adding 0.5% glucose, population significantly increased (Table 2b, i.e., 205% recovery at 6 min in N company).

In table 3, BI population exposed at 160°C for 5 min to N company SCDA culture medium, which presented minimum population in table 2, were used for incubation and added several agents to evaluate what agents may present increasing population. By adding 0.1% $CaCO_3$, 0.2% calcium lactate, L-alanine, amino acid mixtures, and sodium pyruvate indicated increased population. L-alanine presented different effects to D-alanine and L-serine. L-alanine presented an identical effect to amino acids mixtures. L-alanine exists in nature, but D-alanine does not, which is favorable result [6,7]. L-alanine may be the major contributor for increasing colony count among amino acid mixtures.

Concerning Mg effect, our experiment indicated no effect to increase population contrary to calcium [1,4].

Moist heat sterilization

In table 4, the initial population from 6 companies is presented. No significant difference could be observed, which was identical to table 1.

In table 5a, BI population exposed at 3 min and 5 min at 121.1°C, respectively, presented. In table 5a, 3 min and 5 min exposure, population between M and N indicated 554% and 1309% difference,

Manufacture of SCDA	Average population (n=3), CFU/carrier
D	3.11×10^6
B	3.52×10^6
M	3.43×10^6
ND	3.23×10^6
E	3.61×10^6
N	3.14×10^6

Labeled population was 3×10^6 CFU/carrier

Table 1: Initial population of BI for dry heat sterilization use (*B. atrophaeus* ATCC 9372).

Manufacture of SCDA	Average population (n=3), CFU/carrier	
	Exposure time (min)	
	3	6
D	1.21×10^5	1.56×10^3
B	1.18×10^5	1.48×10^3
M	1.20×10^5	9.50×10^2
ND	1.08×10^5	7.70×10^2
E	1.02×10^5	1.14×10^3
N	8.42×10^4	4.10×10^2

Table 2a: Injured population of BI of *B. atrophaeus* ATCC 9372 exposed with dry heating at 160°C for 3 min or 6 min, respectively.

Manufacture of SCDA	Average population (n=3), CFU/carrier	
+0.5% glucose	Exposure time (min)	
	3	6
D	1.47×10^5	1.75×10^3
B	1.42×10^5	1.82×10^3
M	1.47×10^5	1.66×10^3
ND	1.23×10^5	1.37×10^3
E	1.29×10^5	1.45×10^3
N	1.20×10^5	8.40×10^2

Table 2b: Injured population of BI of *B. atrophaeus* ATCC 9372 exposed with dry heating at 160°C for 3 min or 6 min, respectively and cultured in SCDA+0.5% glucose.

Chemicals	Population (n=3) CFU/carrier
Not added (Control)	2.80×10^3
0.1% $CaCO_3$	4.61×10^3
0.2% Calcium lactate	4.33×10^3
0.1% Panvitan powder	2.76×10^3
L-alanine at 100 µg/ml	4.44×10^3
D-alanine at 100 µg/ml	1.95×10^3
L-serine at 100 µg/ml	2.50×10^3
0.2% $MgCl_2$	2.86×10^3
0.1% Amino acid mixture	4.17×10^3
0.5% Sodium pyruvate	4.73×10^3
Lysozyme from egg white at 100 µg/ml	2.14×10^3
ATP at 100 µg/ml	2.66×10^3

BI of *B. atrophaeus* ATCC 9372 was treated with dry heating at 160°C for 5 min

Table 3: Recovery effect of several sorts of chemicals on SCDA culture medium from N company.

Manufacture of SCDA	Average population (n=3), CFU/carrier
D	1.67×10^6
B	1.77×10^6
M	1.53×10^6
ND	1.56×10^6
E	1.79×10^6
N	1.61×10^6

Labeled population was 1×10^6 CFU/carrier

Table 4: Initial population of BI used for moist heating (*G. stearothermophilus* ATCC 7953).

respectively, was observed. This variation is much over the approved range of ISO 11138 (-50 to +300%).

In table 5b, BI population after addition 0.5% glucose to SCDA presented. By adding 0.5% glucose, population significantly increased. Especially at 3 min exposure at M company and 5 min at M company, colony count increased 220% and 319%. However, the difference of the performance of the company was observed 290% between M and N companies at 3 min and 459% between M and N companies at 5 min of table 5b. This is not negligible. 0.5% glucose addition may not be enough to recover and more than 0.5% glucose may be required to decrease the variation. These results indicated culture medium with rich agents for recovery of injured spores was necessary to attain reproducible sterilization validation.

In table 6, BI population exposed at 121.1°C for 4 min to M company SCDA culture medium, which presented minimum population in table 5a, was used and added several agents to evaluate what agents may present increased population. By adding all agents except only yeast extract, population is increased. Behavior of panvitan (vitamin mixture) differs between tables 3 and 6. The reason is not sure. One speculation is the difference of sterilization mechanism. Dry heat is oxidation of air and moist heat is denaturation of protein. As calcium is effective, so calcium salt is more effective than sodium salt.

Amino acid mixture is effective to increase population and among amino acid, L-alanine may be most effectively contributed for increase of population.

For culture temperature, 47°C culture was 4 times greater population than 55°C cultivation (data not shown).

For damaged spores, low temperature and long time incubation may attain more colony count result [8,9].

EOG sterilization

BI as EOG sterilization, *B. atrophaeus* ATCC 7953, was exposed at 600 ± 90 mg/L, RH 60 ± 10% and temperature at 54 ± 1°C for 3 min and 10 min, respectively, and the population is presented in table 7. From table 7, 3 min is 1 log reduction, indicating D vale is 3 min and 10 min exposure indicated 3 log reduction, indicating that the survivor curve is straight line, not curved or tailing phenomenon (Figure 1). D value indicates the time or does to decrease 1 log reduction of colony count.

In table 8, BI population exposed at 3 min and 10 min, respectively, were presented. BI population of N company SCDA culture medium, which presented minimum population in table 7, were used and added several agents to evaluate what agents may present increasing population. By adding all agents except sodium pyruvate and D-alanine, population was increased. If calcium pyruvate is used in place of sodium salt, the result may favorably differ. The result of D-alanine was identical to table 3.

Manufacture of SCDA	Average population (n=3), CFU/carrier	
	Exposure time (min)	
	3	5
D	1.89×10^4	2.37×10^3
B	1.66×10^4	1.75×10^3
M	5.4×10^3	3.92×10^2
ND	1.43×10^4	1.76×10^3
E	2.36×10^4	2.84×10^3
N	2.99×10^4	5.13×10^3

Table 5a: Injured population of BI of *G. stearothermophilus* ATCC 7953 exposed with moist heating at 121.1°C for 3 min or 5 min, respectively.

Manufacture of SCDA	Average population (n=3), CFU/carrier	
+0.5% glucose	Exposure time (min)	
	3	5
D	1.96×10^4	2.95×10^3
B	1.91×10^4	2.23×10^3
M	1.19×10^4	1.25×10^3
ND	1.72×10^4	2.54×10^3
E	2.60×10^4	3.72×10^3
N	3.45×10^4	5.74×10^3

Table 5b: Injured population of BI of *G. stearothermophilus* ATCC 7953 exposed with moist heating at 121.1°C for 3 min or 5 min, respectively and cultured in SCDA+0.5% glucose.

Chemicals	Population (n=3) CFU/carrier
Not added (Control)	1.02×10^3
0.1% $CaCO_3$	2.56×10^3
0.2% Calcium lactate	1.75×10^3
0.1% Panvitan powder	1.64×10^3
0.1% Amino acid mixture	1.72×10^3
ATP at 100 µg/ml	1.15×10^3
0.5% Yeast extract	9.73×10^2
0.5% Soluble starch	1.34×10^3
Catalase at 150 µg/ml	1.15×10^3
L-alanine at 100 µg/ml	1.92×10^3
0.5% sodium pyruvate	1.84×10^3
1% fresh milk	1.16×10^3
1% skim milk	1.55×10^3

BI of *G. stearothermophilus* ATCC 7953 was treated with moist heating at 121.1°C for 4 min

Table 6: Recovery effect of several sorts of chemicals on SCDA culture medium from M company.

Manufacture of SCDA	Average population (n=3), CFU/carrier	
	Exposure time (min)	
	3	10
B	1.33×10^5	3.13×10^3
N	1.03×10^5	1.95×10^3
M	1.13×10^5	2.18×10^3

Initial population was 3.5×10^6 CFU/carrier

Table 7: Injured population of BI of *B. atrophaeus* ATCC 9372 exposed with EOG gas for 3 min or 10 min, respectively.

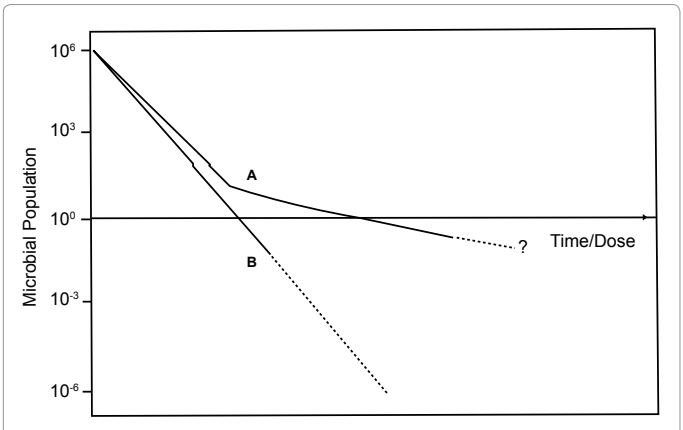

Figure 1: Survivor curve. A: tailing line, B: straight line.

Added chemicals	Average population (n=3), CFU/carrier	
	Exposure time (min)	
	3	10
Not added (Control)	1.10×10^5	1.67×10^3
$CaCO_3$	1.28×10^5	2.08×10^3
0.2% Calcium lactate	1.37×10^5	2.95×10^3
0.5% Glucose	1.26×10^5	1.98×10^3
0.5% Sodium pyruvate	1.07×10^5	1.41×10^3
L-alanine at 100 µg/ml	1.27×10^5	1.97×10^3
D-alanine at 100 µg/ml	1.07×10^5	1.28×10^3
0.1% Panvitan powder	1.22×10^5	1.68×10^3
0.1% Amino acids mixture	1.25×10^5	1.90×10^3

Initial population was 3.5×10^6 CFU/carrier

Table 8: Recovery effect of several sorts of chemicals on SCDA culture medium from N company.

Gamma-ray irradiation sterilization

In table 9, the population exposed at 2 and 5 kGy for *B. megaterium* and *B. pumilus* were presented. SCDA culture medium from N company, the least population in table 7, was used.

In *B. megaterium*, 2 kGy can be D value, however at 5 kGy, more than 2.5 log reduction can be observed, indicating tailing phenomenon was observed (Figure 1). On the contrary, D value of *B. pumilus* was around 1 kGy, therefore 5 kGy presented around 5 log reduction and straight line of survivor curve can be confirmed (Figure 1).

In table 10, BI population exposed at 2 and 5 kGy to *B. megaterium* in N company medium and added several sorts of agents to evaluate what agents may present increased population. By adding all agents except vitamin mixture (panvitan) and sodium pyruvate, population was increased. In place of sodium pyruvate, calcium pyruvate may cause favorable result.

In table 11, BI population exposed at 5 kGy to *B. pumilus* and added several agents were conducted to evaluate what agents may present increased population. By adding all agents except activated carbon, population was increased.

Electron beam irradiation sterilization

In table 12, BI population exposed at 4.5 kGy to *B. megaterium* and *B. pumilus*, in N company culture medium and added several agents was conducted to evaluate what agents may present increased population. By adding all agents except $CaCO_3$, population was increased.

All sterilization procedures

In all sterilization procedures, glucose and amino acid mixture were found to be favorable recovery factors. Calcium was also major candidate, but magnesium was not. Glucose will be necessary for EMP

Sorts of microorganisms	Average population (n=3), CFU/carrier	
	Exposure condition (kGy)	
	2	5
B. megaterium	1.93×10^5	4.13×10^2
B. pumilus	2.41×10^4	4.22×10^2

Initial population was 1.4×10^6 CFU/carrier

Table 9: Injured population of *B. megaterium or B. pumilus* exposed with gamma-ray at 2 or 5 kGy, respectively, cultivated in SCDA from N company.

Added chemicals	Average population (n=3), CFU/carrier	
	Exposure condition (kGy)	
	2	5
Not added (Control)	1.54×10^5	2.41×10^2
$CaCO_3$	1.45×10^5	2.53×10^2
0.2% Calcium lactate	1.72×10^5	3.09×10^2
0.5% Soluble starch	1.46×10^5	2.51×10^2
0.15% Activated charcoal	1.42×10^5	2.63×10^2
L-alanine at 100 µg/ml	1.78×10^5	3.12×10^2
D-alanine at 100 µg/ml	1.80×10^5	2.93×10^2
0.1% Panvitan powder	1.52×10^5	2.33×10^2
0.1% Amino acids mixture	1.80×10^5	2.82×10^2
0.5% Sodium pyruvate	1.48×10^5	2.34×10^2
0.5% Glucose	1.72×10^5	2.98×10^2

Initial population was 1.4×10^6 CFU/carrier

Table 10: Recovery effect of several sorts of chemicals on SCDA culture medium from N company by using *B. megaterium* spore.

Added chemicals	Average population (n=3), CFU/carrier
	Exposure condition (kGy)
	5
Not added (Control)	4.84×10^2
$CaCO_3$	6.12×10^2
0.2% Calcium lactate	6.33×10^2
0.5% Soluble starch	4.90×10^2
0.15% Activated charcoal	4.80×10^2
L-alanine at 100 µg/ml	6.41×10^2
D-alanine at 100 µg/ml	6.93×10^2
0.1% Panvitan powder	7.15×10^2
0.1% Amino acids mixture	7.22×10^2
0.5% Sodium pyruvate	6.34×10^2
0.5% Glucose	5.50×10^2

Initial population was 2×10^6 CFU/carrier

Table 11: Recovery effect of several sorts of chemicals on SCDA culture medium from N company by using *B. pumilus* spore.

Added chemicals	Average population (n=3), CFU/carrier	
	4.5 kGy exposure	
	B. megaterium	*B. pumilus*
Not added (Control)	1.78×10^3	2.51×10^3
0.1% $CaCO_3$	1.41×10^3	2.32×10^3
0.2% Calcium lactate	2.13×10^3	2.52×10^3
0.5% Glucose	2.17×10^3	2.70×10^3
0.5% Sodium pyruvate	1.88×10^3	2.68×10^3
L-alanine	1.86×10^3	2.61×10^3
D-alanine	2.62×10^3	2.56×10^3
0.1% Panvitan powder	1.93×10^3	3.06×10^3
0.1% Amino acids mixture	1.95×10^3	2.95×10^3

Initial population of *B. megaterium* was 1.4×10^6 CFU/carrier and that of *B. pumilus* was 2.0×10^6 CFU/carrier

Table 12: Injured population of *B. megaterium or B. pumilus* exposed with electron beam at 4.5 kGy, which cultivated in SCDA from N company.

cycle and TCA cycle to attain ATP energy and several intermediate components. Amino acids and calcium cannot successfully explain. Other agents indicated favorable result, but not always to other sterilization procedure. This may be the difference of the sterilization mechanism.

Conclusion

1. When used healthy spores, no significant difference of population can be observed among medium suppliers.
2. In order to recover population and reproducible validation study, calcium, glucose and amino acid mixture addition will be favorable.
3. For the incubation of injured spores, low temperature and long period incubation was more favorable.
4. The difference of population depending on sterilization procedures will be the difference of sterilization mechanism. Dry heat is oxidation of air, moist heat is denaturation of protein, EOG is alkylating agent, gamma-ray and electron beam are OH radicals, so each sterilization procedure has different mechanism for sterilization. The difference of sterilization mechanism might cause the difference of performance of agents for the recovery of injured spores.
5. More than 0.5% glucose would be required.

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