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How to Recovery of Damaged Microorganisms by Supplying Several Sorts of Nutrients

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Abstract

Final sterilization was conducted to health care products immediately prior to shipping and sterility assurance OF 10-6 of SAL (sterility assurance level) must be confirmed from the result of Biological indicators (BIs). The survived microorganisms after sterilization may be mostly damaged and injured microorganisms. The auxotrophic characteristics of injured microorganisms are different from those of healthy microorganisms. In that sense, the cultivation conditions and culture medium constituent of injured microorganisms must be used for confirming reproducible sterilization validation study, otherwise it may result in false negative at shipping test. This is indispensable to attain safety assurance of health care products. The evaluation of several sorts of nutrients 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 lot to lot or batch to batch 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, gucose 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; Damaged microorganisms; Sterility assurance; Auxotrophic characteristics; Biological indicator

Introduction

Before shipping healthcare products must be exposed final sterilization, if applicable, to obtain Sterility Assurance Leve1 (SAL) of 10^{-6} for 7 days incubation. lf damaged and injured microorganisms after sterilization may survive, they may cause a false negative and grow after shipping, which cause contamination to healthcare products. 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 (mostly 10 days) incubation period at relatively 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 (enriched) 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 lot of SCDA, 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 heating, dry heating, ethylene oxide gas (EOG) exposure, gamma-ray irradiation and electron beam irradiation was 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

BIs for dry heating use (*Bacillus atrophaeus* ATCC 9372, 3×10^6 CFU, colony forming unit/carrier) were purchased from Raven Co. Ltd. They were exposed at 160°C for 3 and 6 min to attain injured and damaged spores. They apply to SCDΛ culture medium (M, D, B, ND, E, N companies), incubate for 7 days and count colony. M, D, BD, E, and N companies stand Merck, Dainihon, Becton Dickinson, Eiken and Nissui companies.

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 (ISO 14161). 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/nutrients are commercially available with pure grade.

Moist heat sterilization

BIs for moist heat sterilization use (*Geobacillus 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, BD, E and 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

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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 (ISO 14161).

Several sorts of recovery agents were added to original SCDA culture medium. 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 (*B. 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 (BD, N, M companies) incubate and count colony.

Colony count can be done by using 3 sheets 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-told diluted to become 30-100 CFU/plate (ISO 14161). 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 the original SCDA culture medium. They are calcium carbonate ($CaCO_3$), L-alanine, D-alanine, amino adds 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 *B. megaterium* spore at 1.4×10^6 CFU/carier were purchased from Raven Co. Ltd. *B. pumilus* and *B.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 the original SCDA culture medium. 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 (*B. pumilus* ATCC 9372, 2.0×10^6 CFU/carrier or *B. megaterium* spore at 1.4×10^6 CFU/carrier) were purchased from Raven Co. Ltd. *B. pumilus* and *B. 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 the original SCDA culture medium. They are calcium carbonate ($CaCO_3$), calcium lactate, D-alanine, L-alanine, amino adds 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 5 companies is presented. No significant difference of colony count can be observed when healthy spores were used. ISO 11138-1 approved –50 to +30% deviations from the labeled population.

Manufacturer of SCDA	Average of initial population (n=3), CFU/carrier
D	3.11×10^6
BD	3.52×10^6
M	3.43×10^6
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 and/or EOG sterilization use (*B. atrosphaeus* ATCC 9372).

Manufacturer of SCDA	Average population (n=3), CFU/carrier Exposure time (min)	
	3 min	6 min
D	1.21×10^5	1.56×10^3
BD	1.18×10^5	1.48×10^3
M	1.20×10^5	9.50×10^2
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.

Manufacturer of SCDA+0.5% glucose	Average population (n=3), CFU/carrier Exposure time (min)	
	3 min	6 min
D	1.47×10^5	1.75×10^3
BD	1.42×10^5	1.82×10^3
M	1.47×10^5	1.66×10^3
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 constituent plus 0.5% glucose.

Agents added	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 agents added into SCDA culture medium constituents from N company.

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 6 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, for example 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 2a), were used 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 compared with control. L-alanine presented different effect 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 add mixtures. Alanine rasemase produce both of L-alanine and D-alanine, so synergitic effect of both L and D-alanines will be much more favorable. Concerning Mg effect, our previous experiment indicated no effect to increase population contrary to calcium [1,4].

Moist heat sterilization

In (Table 4), the initial population from 5 companies is presented. No significant difference could be observed, which was identical to (Table l). 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, respectively, was observed. This variation is much over the approved range of ISO 11138-1 (-50-300%). 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 addition might be required to increase population and decrease the variation. These results indicated culture medium with rich agents for recovery of injured spores was necessary to attain reproducible sterilization validation.

Manufacturer of SCDA	Average population (n=3), CFU/carrier
D	1.67×10^6
BD	1.77×10^6
M	1.53×10^6
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).

Manufacturer of SCDA	Average population (n=3), CFU/carrier Exposure time (min)	
	3 min	5 min
D	1.89×10^4	2.37×10^3
BD	1.66×10^4	1.75×10^3
M	5.40×10^3	3.92×10^2
E	2.36×10^4	2.84×10^3
N	2.99×10^4	5.13×10^2

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.

Manufacturer of SCDA + 0.5% glucose	Average population (n=3), CFU/carrier Exposure time (min)	
	3 min	5 min
D	1.96×10^4	2.95×10^3
BD	1.91×10^4	2.23×10^3
M	1.19×10^4	1.25×10^3
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 constituent plus 0.5% glucose.

Agents added	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% Panvutan 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% skin 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 agents added into SCDA culture medium constituents from M company.

Manufacturer of SCDA	Average population (n=3), CFU/carrier Exposure time (min)	
	3 min	10 min
BD	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 qith EOG gas exposure for 3 min or 10 min, respectively.

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 the 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), indicating that cultivation at lower temperature may be more favorable than the temperature applied for healthy spores. Therefore, for damaged spores, low temperature and longtime incubation may attain more colony count result [8,9].

EOG sterilization

BI as EOG sterilization, *B. atrophaeus* ATCC 9372, was exposed at 600 ± 90 mg/L, RH 60 ± 10% and temperature at 54 ± 1°C for 3min and 10 min, respectively, and the population is presented in (Table 7). From (Table 7), 3 min is one log reduction, indicating D vale is 3 min and 10 min exposure indicated ca 3 log reduction, indicating that the survivor curve is straight line, not curved or tailing phenomenon (Figure 1). D (decimal reduction) value indicates the time or does to decrease 1 log reduction of colony count.

In (Table 8), BI population exposed at 3 mill and 10 min,

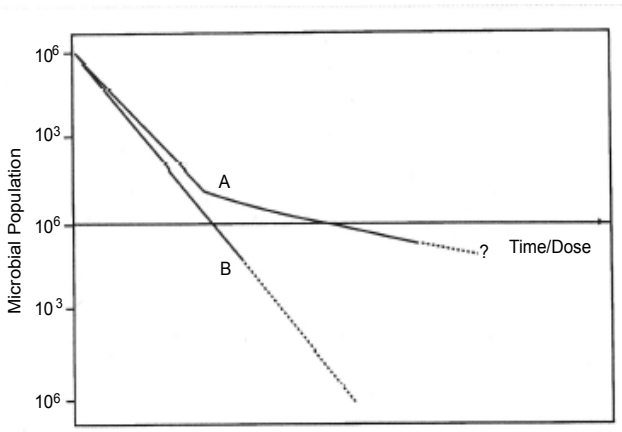

Figure 1: A: Survivor curve tailing phenomenon, **B:** straight line up to SLR of 10^{-6} SLR stands spore log reduction.

Added agents	Average population (n=3), CFU/carrier Exposure time (min)	
	3 min	10 min
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% Amono 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 agents added into SCDA culture medium constituents from N company.

Sots of microorganisms	Average population (n=3), CFU/carrier Exposure condition (kGy)	
	2 kGy	5 kGy
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.

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 may be used in place of sodium pyruvate, the result may favorably differ. The result of D-alanine was identical to (Table 3).

Gamma-ray irradiation sterilization

In (Table 9), the population exposed at 2 and 5 kGy for *B. megaterium* and *B. pumilus* was 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 1og reduction can be observed, indicating curved survival curve can be observed. On the contrary, D value of *B. pumilus* was around l kGy, therefore 5 kGy presented around 5 log reduction and therefore straight line of survivor curve can be confirmed (Figure 1A).

Added chemicals	Average population (n=3), CFU/carrier Exposure condition (kGy)	
	2 kGy	5 kGy
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 onto SCDA constituents from N company using *B. megaterium*.

Added chemicals	Average population (n=3), CFU/carrier Exposure condition (kGy)
	5 kGy
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	6.92×10^2
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 added to SCDA culture medium constituent from N company by using *B. pumilus* spore as BI.

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 play present increased population. By adding all agents except vitamin mixture (panvitan) and sodium pyruvate, population was increased. In place of sodium pyruvate, use of calcium pyruvate may cause more 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* was presented by adding into N company culture medium and added several agents. It was conducted 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 add mixture were found to be favorable recovery factors. Calcium was also major candidate, but magnesium was not. Glucose will be necessary for EMP (Embden-Meyerhof-Parnas) pathway and TCA (tri carboxylic acid) cycle to attain ATP energy and several intermediate components. Amino adds and calcium cannot successfully explain. Other agents

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
0.52% 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* and that of *B. pumilus* was 1.4×10^6 CFU/carrier and 2.0×10^6 CFU/carrier, respectively.

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.

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 of 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 recovery of injured spores.

5. More than 0.5% glucose would be more favorable.

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