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# Fine bubble technology — Water treatment applications —

# Part 2:
# Test methods using *Escherichia coli* as a test micro-organism



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## Foreword

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~~The committee responsible for this~~This document ~~is~~was prepared by Technical Committee ISO/TC 281, *Fine bubble technology*.

A list of all parts in the ISO 20304 series can be found on the ISO website.

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## Introduction

In the last decade, fine bubble technology generated by hydrodynamic cavitation methods has been applied as part of water treatment facilities for water disinfection purposes. However, absence of ~~international standards~~International Standards in relation to the efficiency of water disinfectant properties on fine bubbles has been an obstacle to trading and technology transfer. This document describes a method for the determination of water disinfection efficiency of fine bubbles generated by a hydrodynamic cavitation.

Fine bubbles act as a disinfectant through chemical (i.e. generation of OH radicals) and physical mechanisms caused by presence of shock waves, pressure gradients and shear forces. The function as a disinfectant of fine bubbles was also found in that fine bubbles provide a more effective means for cleaning and disinfecting both, the bath and the reservoir, than traditional ultrasonic vibrator.~~[1]~~ [1]

Properties of fine bubbles and their disinfection mechanisms differ according to applied generation processes, bubble size distributions and bubble densities. However existing studies showed that fine bubbles generated have a significant water disinfection effect. Particularly, high deactivation efficiency of *Escherichia coli* (*E. coli*) has been achieved in water disinfection by microbubbles generated using hydrodynamic cavitation as well.~~[2,3 and 4]~~ [2]-[4]

Generation of highly reactive free radicals and turbulence associated with collapsing micro bubbles provides great potential for water disinfection. The effect of ozone microbubble on *E. coli* has often been found more effective and with faster disinfection kinetics of *E. coli*, when the ozone gas is activated as the micro or ultrafine bubble forms.~~[5 and 6]~~ [5],[6]

The test methods on the antibacterial activity of textile products have been standardized by ISO/TC 38, *textiles*. The present standard operation procedure (SOP) for the evaluation of disinfection efficiency is based on the quantitative measurement by ATP luminescence method for the evaluation of antibacterial activity.

Therefore, under the test conditions ~~which are configured by ISO/TC 281~~, establishment of ~~international standards~~International Standards to measure the water disinfection efficiency of fine bubbles is essential to promote the relevant trading technology transfer.

# Fine bubble technology — Water treatment applications — ~~Part 2:~~ ~~Test methods using *Escherichia coli* as a test micro-organism~~

# Part 2: Test methods using *Escherichia coli* as a test micro-organism

## 1 Scope

This document specifies a test method for assessing bactericidal viability of *Escherichia coli* as a test micro-organism, in dispersions of various fine bubbles generated by the hydrodynamic cavitation of water medium.

## 2 Normative references

~~The following document is referred to in the text in such a way that some or all of their content constitutes requirements of this document. For undated references, the latest edition of the referenced document (including any amendments) applies.~~

~~ISO/IEC Directives, Part 2.~~ www.iso.org/directives

There are no normative references in this document.



## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:



- ~~IEC Electropedia: available at https://www.electropedia.org/~~
- ~~ISO Online browsing platform: available at https://www.iso.org/obp~~https://www.iso.org/obp
- IEC Electropedia: available at https://www.electropedia.org/

**3.1**
**hydrodynamic cavitation**
fine bubble generation method used by physical hydrodynamic structures and pressures

**3.2**
**disinfection efficiency**
ratio of viable cell count to the initial number of the test bacteria

**3.3**
**control suspension**
suspension used to validate the growth condition of test bacteria and validate the test, i.e. bacterial test suspension produced without using fine bubble generation

**3.4**
**test solution**
solution samples obtained for the analysis of antibacterial activity after operating the test facility

**3.5**
**test gas**
gas mixed in the middle of operating the cavitation unit

**3.6**
**relative light unit**
**RLU**
unit used for adenosine triphosphate (ATP) luminescence measurement

## 4 Safety precautions

The test methods specified in this document ~~requires~~require the use of bacteria and conditions that promote bacterial growth. Since the bacteria can be pathogenic, the tests should be carried out by persons with training and experience in the use of microbiological techniques. The laboratory facilities should be designated as appropriate biosafety level~~[7].~~.[7]

Appropriate safety precautions should be observed with due consideration given to country-specific.

## 5 Requirements

This document is to provide the measurement method of disinfection efficiency for fine bubbles enhanced waters by test hydrodynamic cavitation units. The disinfection efficiency is measured by the test fine bubble solution generated by each test hydrodynamic cavitation unit.

The antibacterial (bactericidal) activity shall be evaluated using the following test organism: *E. coli* (see ~~Table A.1).~~Table A.1). The test tank and test facility should be cleaned by appropriate methods like triple-wash with sterilized water after chlorination before the test.



## 6 Test methods

### 6.1 Principle

The test tank is filled with a test suspension of bacteria and the test hydrodynamic cavitation (HC) unit is operated under the required initial test condition (~~15~~approximately 15 °C to 24 °C ± 1 °C). The fine bubbles-enriched water will be contacted to the test suspension of bacteria in the test tank (see ~~Figure 1).~~Figure 1).



At a specific contact time, an aliquot is taken; the bactericidal action in this portion is immediately measured using the luminescence photometer (see ~~6.4.8). Firstly~~6.4.8). First, the relationship between the measurement unit of the luminescence photometer, ~~RLU (~~relative light unit~~;~~ (RLU) (see ~~Annex C).~~Annex C) and counted ~~CFU (~~colonies forming unit (CFU) of the initial density of the test microorganism is measured to check the initial cell density of the bacterial test suspension.

The test is performed using *E. coli* (see ~~Table 3).~~Table A.1).

### 6.2 Test facility

Fine bubbles are generated by circulating the liquid in a fine bubble generating system with the following characteristics: a 125~~l~~-litre tank, a 0,4- kW centrifugal multistage pump made of stainless steel, PVC pipes and a cavitation unit with the test fine bubble generating unit (see ~~Figure 1).~~Figure 1). Test gas can be chosen for the appropriate test purposes.

### 6.3 Culture media and reagents

#### 6.3.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes.

~~NOTE~~ To improve reproducibility, dehydrated material should be used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

### 6.3.2 Water

Water used in tests shall be analytical-grade water for microbiological media preparation, which is freshly distilled and/or ion-exchanged and/or ultra-filtered and/or filtered with ~~RO (~~reverse osmosis (RO). It shall be free from substances that are toxic or inhibiting to the bacteria.

### 6.3.3 Tryptone ~~Soya Agar~~soya agar (TSA)

TSA used in the test is for maintenance of bacterial strains and performance of viable counts.

| | |
|---|---|
| Tryptone, pancreatic digest of casein | 15,0 g |
| Soya peptone, papaic digest of soybean meal | 5,0 g |
| NaCl | 5,0 g |
| Agar | 5,0 g |
| Water (see ~~6.3.2)~~6.3.2) | to 1,0 l |

Sterilize in the autoclave ~~(see 6.4.1).~~(6.4.1). After sterilization, the pH of the medium shall be 7,2 ~~±~~ ± 0,2 when measured at 20 °C.

### 6.3.4 Tryptone soya broth (TSB)

| | |
|---|---|
| Tryptone, pancreatic digest of casein | 17 g |
| Soya peptone, papaic digest of soya | 3 g |
| Sodium chloride (NaCl) | 5 g |
| Glucose | 2,5 g |
| Dipotassium hydrogen phosphate | 2,5 g |
| Water | 1 000 ml |

Mix well and adjust pH, 7,2 ~~±~~ ± 0,2, then sterilize by autoclave ~~(6.4.1).~~(6.4.1).



### 6.3.5 Lysogeny broth (LB)

| | |
|---|---|
| Tryptone, pancreatic digest of casein | 10 g |
| ~~Yest~~Yeast extract | 5 g |
| NaCl | 10 g |
| Water | 1 000 ml |

Mix well and adjust pH, 6,9 ~~±~~ ± 0,2, then sterilize by autoclave ~~(6.4.1).~~(6.4.1).

### 6.3.6 Cryoprotective solution for bacterial species

For freezing, a cryoprotective solution containing 150 g/l of glycerol or 100 g/l of dimethylsulfoxide shall be used and prepared as follows:

| | |
|---|---|
| TSB (see ~~6.3.4)~~6.3.4) or LB (see ~~6.3.5);~~6.3.5): | 1 000 ml |
| Add Glycerol: 150 g or dimethylsulfoxide: | 100 g |

Mix well and sterilize by autoclave ~~(6.4.1).~~(6.4.1).

For solutions containing glycerol, sterilize the mixed solution by autoclave ~~(6.4.1).~~(6.4.1). For solutions containing dimethylsulfoxide, sterilize the mixed solution by using a 0,22 µm membrane filter.

NOTE Any commercially available product can be used as long as it is a cryoprotective solution or preserving system that contains glycerol or dimethylsulfoxide.

#### 6.3.7 Physiological saline

| | |
|---|---|
| Sodium chloride (NaCl) | 8,5 g |
| Water | 1 000 ml |

Mix well, then sterilize by autoclave ~~(6.4.1).~~(6.4.1).

#### 6.3.8 Tryptone ~~Sodium Chloride Solution~~sodium chloride solution (TSCS)

| | |
|---|---|
| Tryptone, pancreatic digest of casein | 1 g |
| NaCl | 8,5 g |
| Water | 1 000 ml |

### 6.4 Laboratory apparatus and glassware

The usual laboratory apparatus and, in particular, the following shall be used.

**6.4.1 Autoclave,** capable of sterilizing at 121 °C ~~±~~ ± 2 °C and 103 kPa ~~±~~ ± 5 kPa.

**6.4.2 Incubator,** capable of being controlled at either 36 °C ~~±~~ ± 1 °C or 37 °C ~~±~~ ± 1 °C. An incubator at 37 °C ± 1 °C may be used if an incubator at 36 °C ~~±~~ ± 1 °C is not available.

**6.4.3 pH meter**, having an accuracy ±0,1 in calibration on pH units at 25 °C.

**6.4.4 Stopwatch**.

**6.4.5 Container**, test tubes or flasks of suitable capacity.

**6.4.6 Pipettes**, having the most suitable volume for each use, with a tip made of glass or plastic, and with a tolerance of 0,5 % or less.

**6.4.7 Petri dishes**, that have been sterilized, made of glass or plastic, in diameter sizes of 90 mm to 100 mm.

**6.4.8 Luminescence photometer,** with ATP kit for water test**,** capable of measuring ATP of $10^{-12}$ mol/l to $10^{-7}$ mol/l at 300 nm to 650 nm with a luminescence-measuring reagent.

**6.4.9 Vials, 250 ml plastic bottles,** with screw openings, polytetrafluoroethylene or silicone packing and caps made of polypropylene, polycarbonate or another suitable material.

**6.4.10 Glass beads,** with a diameter of 3 mm to 4 mm.

**6.4.11 Freezers,** one adjustable to a temperature below −70 °C and another to a temperature below −20 °C.

**6.4.12 Homogenizer**, capable of speeds of ~~6~~six blows per second to ~~8~~eight blows per second, with the corresponding disposable containers.

**6.4.13 Disposable plastic bags,** sterile bags suitable for containing food products, to be used for one of the shaking methods of the specimens.

**6.4.14 Sterilized stirring rod,** with a 6 mm (±2 mm) diameter and a 300 mm (±50 mm) length.

## 6.66.5 6.5 Preparation of bacterial test suspension

### 6.6.36.5.1 Storage of strains

#### 6.6.3.16.5.1.1 General

The strains shall be stored in accordance with the supplier's recommendations. The experiment instruments should be sterilized by the autoclave (6.4.1)(6.4.1) for a minimum of 15- min.

#### 6.6.3.26.5.1.2 Preparation method

Obtain a sample of the freeze-dried bacterial strain in accordance with Annex A,Annex A. following the recommendations supplied with the culture and resuspend it in 5- ml of TSB (see 6.3.4).6.3.4). Obtain a sample of the suspension and isolate it in a plastic bottle (6.4.10)(6.4.10) containing LB (see 6.3.5).6.3.5). Incubate the cultures for 18- h to 24- h at 37 °C ± 2 °C.

After incubation, use the culture isolated in the Petri dish to verify the purity of the strain.

After verification, prepare the stock cultures to check the density of *E. coli* of the bacterial test suspension.

Sample 0,7- ml of the broth culture and spread it over the surface of the Petri dish containing the TSA. Incubate the culture on plates for 18- h to 24- h at 37- °C ± ± 2- °C.

Add 10– ml of cryoprotective solution (see 6.3.6)6.3.6) to the surface of the TSA plate culture and resuspend the cells in the solution using a sterile glass spreader. Sample the suspended cells from the surface of the agar, dilute them in 100 ml of cryoprotective solution and incubate for 30- min at 20 °C.

Using a pipette (6.4.6),(6.4.6), sample 1– ml of the suspension and transfer it to a cryogenic vial (6.4.9)(6.4.9) containing the beads (6.4.10).(6.4.10). Shake the vial in order to spread the suspended cells around the beads.

— Where a cryoprotective solution containing dimethylsulfoxide is used, do not let it stand longer than 1 min at ambient temperature.

— Where a cryoprotective solution containing glycerol is used, let it stand for 30- min at 20 °C.

— Withdraw the excess cryoprotective solution with a sterile pipette. Place the cryogenic vials in a freezer (6.4.11)(6.4.11) set at −70 °C or lower.

Prepare $10^{-6}$ and $10^{-7}$ dilutions of the suspension using the serial dilution method. Take a 1,0- ml sample of each dilution and transfer it to separate Petri dishes. Add 12- ml to 15- ml of nutritive solution, cooled down to 45 °C ± ± 1 °C. Incubate for 18- h to 24- h under the conditions specified for the strain. Enumerate the plate cultures and confirm that the cell density of the suspension is $1 \times 10^5$ CFU/ml to $5 \times 10^8$ CFU/ml using TSCS (see 6.3.8).6.3.8).

The other preparation details of initial bacteria stock culture are described in EN 1040[9]. [8]

### 6.6.46.5.2 Working culture of test bacteria

In order to prepare the working culture of strains, subculture from the stock culture by inoculation into LB broth and incubates. After 18- h to 24- h, prepare a second subculture from the first subculture in the same way and incubate for 18– h to 24– h. From this second subculture, a third subculture may be produced in the same way.

NOTE The second and/or third subculture are the working culture(s).

If it is not possible to prepare the second subculture on the same day, a 48 h subculture may be used for subsequent sub-culturing, provided that the subculture has been kept in the incubator during a 48 h period. In these circumstances, prepare a further 24 h subculture after proceeding. Do not take a fourth subculture.

### ~~6.6.5~~6.5.3 Bacterial test suspension

Take the working cultures and transfer into the test tank and adjust the final volume of the bacterial test suspension to 50 l, after it has been diluted with water (see ~~6.3.2)~~6.3.2) at room temperature and stir with a stirring rod for 30 s.

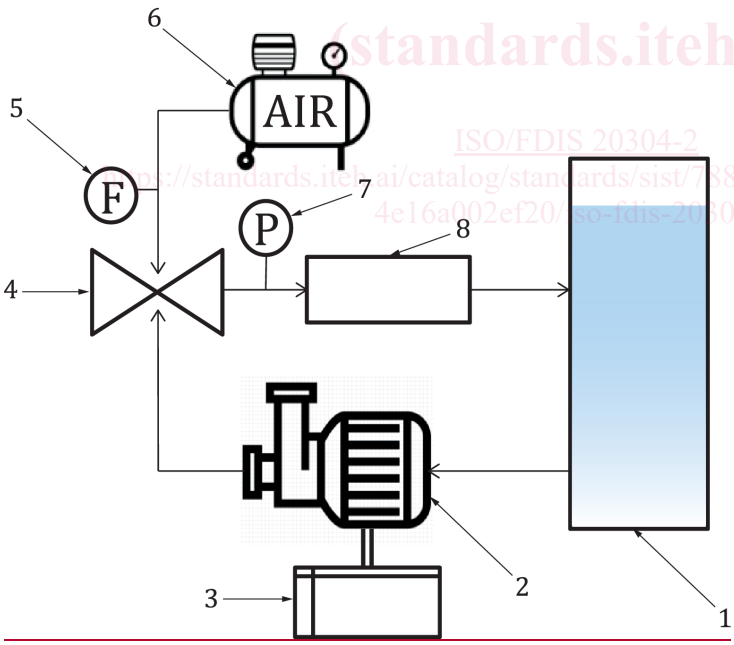
Prepare $10^{-1}$ and $10^{-2}$ dilutions of the suspension using the serial dilution method. Take a 1,0 ml sample of each dilution and transfer it to separate Petri dishes. Add 12 ml to 15 ml of nutritive solution, cooled down to 45 °C ± 1 °C. Incubate for 18 h to 24 h under the conditions specified for the strain. Enumerate the plate cultures and confirm that the suspension contains more than $1 \times 10^5$ CFU/ml. Measure RLU ~~(~~of the sample using luminescence photometer ~~(6.4.8).~~(6.4.8). The principle of RLU ~~is~~shall be as defined in ~~Annex C.~~Annex C.

Adjust the final concentration of *E. coli*, which is more than $1 \times 10^5$ CFU/ml using luminescence photometer ~~(6.4.8)~~(6.4.8) and working cultured suspension.

### ~~6.8~~6.6 Procedure

The capacity of the pump can be appropriately fixed to circulate the bacterial test suspension within the test facility (see ~~Figure 1).~~Figure 1). Test fine bubble generating unit shall be operated to produce fine bubbles (see ~~Figure 1).~~Figure 1). Bacterial test suspension shall be moved to the test tank within 1 h in accordance with ~~Annex B, B.3.~~Annex B, B.3. The produced fine bubble-enhanced water shall be contacted the bacterial test suspension during the operation of the test facility.






~~Key~~

1 test tank
2 pump
3 control panel
4 gas injector

Inserted Cells