Rapid quantification of viable spore by a simple spectrophotometric method

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ABSTRACT

Bacteria used in self-healing of concrete crack need to be embedded into the concrete in the form of dormant spores. However, quantification of viable spores is still a challengeable task due to the lack of rapid, efficient and accurate methods. Considering that viable spores have a higher refractive index than vegetative cells and the refractivity issubject to decrease during the germination process, we attempted to develop a simple spectrophotometric method for the detection of viable spores. By comparison of the results obtained by both spectrophotometric method and colony counting method, a good linear correlation (R^2=0.99) was achieved between viable spore concentration and OD loss under appropriate conditions. The calibration equation developed could be used to predict the viable spore yield produced in a series of fermentation experiments. Our experiment proved that this novel spectrophotometric method was sensitive, rapid, and easy to perform compared to conventional colony counting method.

1. INTRODUCTION

As one of the most resistant life forms on the Earth exhibiting remarkable longevity , bacterial spore (endospore) is a dormant cellular structure during the stationary phase in the life cycle of spore-forming bacteria such as *Bacillus* sp.[1]. Spores of *Bacillus* species are used commercially as probiotics in humans, animals, and agriculture[2]. For the commercial production of spore, the viability of endospores is a key factor, as not all dormant spores can germinate and grow[3]. Therefore, an effective method to evaluate the viability of dormant spores in spore production is important.

Hitherto, the standard method for enumerating viable endospores is based on colony forming unit (CFU) quantification technique[4]. This conventional method requires serial dilutions, plating, incubation and colony counting, apparently being time-consuming and labor-intensive[5].

Spores have a higher refractive index than vegetative cells. As long as the environment becomes appropriate, germination of viable spores can be initiated on a timescale of minutes. During the germination process, refractivity of the viable spores is gradually diminished due to both the release of dipicolinic acid (DPA) and water uptake. Ungerminable spores cannot activate such process, thus not leading to any change of refractivity. The decline of refractivity of the germinating spores can be detected by monitoring the optical density of spore suspension and expressed as OD loss[6]. Therefore, if the refractivity decline of the spore suspension during

germination can be correlated to the quantity of viable (germinable) spores, then the spore viability can be simply indicated by OD loss.

In this study, we attempt to establish a rapid and simple spectrophotometric method for the first time to detect the viability of the spore samples. Firstly, we demonstrate that OD loss of spore germination process can be reliably converted to the concentration of viable spores; secondly, we investigate the influence of vegetative cells and ungerminable spores on the new method. Finally, the validation of the spectrophotometric method will be further evaluated by comparing the results of spore production detected by this new method and traditional plate counting method under different spore production conditions. This novel spectrophotometric method can hopefully be a good substitute for traditional plate counting method.

2. MATERIALS AND METHODS

Strains. An alkaliphilic spore-forming strain *Bacillus sp*. H4 CGMCC 9629 was isolated from the sediment samples. The spore of *Bacillus sp*. H4 can germinate at pH 10.5 and was used to establish and validate the novel assay of viable spores. A neutrophilic strain *Bacillus subtilus* ATCC6051 was used to provide “dead” or ungerminable spores in the experiment because its spores cannot germinate as pH value of the culture is more than 10.0.

Endospore preparation. A modified sporulating medium (MSP medium) based on that in Jonkers’ study[7] was used for spore production of strain H4, which contained (1L): NH_4NO_3 0.3 g, KH_2PO_4 0.02 g, $CaCl_2{\cdot}2H_2O$ 0.225 g, KCl 0.476 g, $MgCl_2{\cdot}6H_2O$ 0.2 g, $MnSO_4{\cdot}2H_2O$ 0.01 g, yeast extract 3 g, soluble starch 1 g, $NaHCO_3$ 4.2 g and Na_2CO_3 5.3 g. The pH of the medium was about 9.7. Strain H4 was grown overnight at 30 °C in alkaline LB broth containing $NaHCO_3$ (4.2 g l^{-1}) and Na_2CO_3 (5.3 g l^{-1}), and 8 ml grown culture was inoculated into 100 ml fresh MSP medium. The cultivation was carried out in a series of 500 ml Erlenmeyer flasks with 100 ml working volume in a rotary incubator shaker at 30 °C, 150 rpm. *Bacillus subtilus* ATCC6051 was point inoculated on YA agar (yeast extract 15 g l^{-1}, NaCl 5 g l^{-1}) to yield single colony. After 7 days, the spores were harvested from the incubation liquid or the plate surface. The spore stock suspensions of strain H4 and strain ATCC6051 were both prepared to contain about 2.0×10^9 spores ml^{-1}, as determined by direct spore counting with a Helber bacterial counting chamber (Hawksley, Lansing, UK). The stock solution could be diluted to different concentrations according to experimental requirements.

Preparation of the calibration curve. H4 spore stock suspension was diluted with ATCC6051 spore stock suspension to prepare working standard suspensions of H4 spore at concentrations ranging from 1×10^8 to 2×10^9 spores ml^{-1}.In all above spore suspensions, the total spore concentrations kept the same, while the concentrations of H4 spores exhibited gradient arrays. The CFU per milliliter of spore suspensions was determined using plate counting method.

For assay of spore germination, the standard spore suspensions were heat activated in water at 60 °C for 30 min. Six aliquots (25 µl) of various standard spore suspensions were transferred into individual well of 96-well microplate. 175µl of alkaline germination solution (AGS, 100 mmol l^{-1} 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 200 mmol l^{-1} NaCl, 10 mmol l^{-1} L-inosine, pH 10.5) was added to each well and then the plate was incubated at 30 °C for 2 hours. Germination was monitored by the change of optical density (OD) of samples at 490 nm in a microplate reader (Molecular device MX190 plus 384, US). The average values of OD loss obtained were plotted against viable spore concentrations obtained by plate counting method to get a linear calibration equation.

Method validation. The validity of this spectrophotometric method was confirmed by accuracy of the estimation of viable spore production under different medium and fermentation conditions. In the experiment, some components of MSP medium including soluble starch (0, 2.5, 5,10 and 20 g l^{-1}), yeast extract (0, 0.5, 1, 2, 3, 4, 6, 8, 10 g l^{-1}) and sodium chloride (0, 1, 2, 4, 8, 16, 32, 64, 128 g l^{-1}) , and cultivation conditions such as initial pH (11.0, 10.5, 10.0, 9.5, 9.0) and working volumes (25, 50, 75, 100, 125, 150 ml in 250 ml Erlenmeyer flasks) were separately adjusted to various levels.Strain H4 was cultivated under different conditions at 30°C for 7 days, and the spores were centrifuged (6000 ×g, 10 min) and resuspended in equal volumes of deionized water. 1.5 ml of such spore suspension was transferred into a 5 mm light-path cuvette and the OD_{490} value was recorded with a spectrophotometer (Molecular device MX190 plus 384, US). Added adequate aliquots of ATCC6051 spore suspension (containing about 4.0×10^{10} spores ml^{-1}in water) into the cuvette, mixed the whole suspension thoroughly by pipetting back and forth several times and then read the OD_{490} value, and repeated the operation until the OD_{490} value reached approximately 1.7 (total spore concentration was about 2×10^{9} spores ml^{-1}). Six aliquots (25 µl) of the spore mixture were transferred into individual well of 96-well microplate. 175 µl of AGS was added to each well and the plate was incubated at 30 °C for 2 hours. The OD_{490} reduction value was detected by the spectrophotometric method and converted to the number of cfu ml^{-1} by means of the calibration equation. The achieved value of cfu ml^{-1}, representing viable spore concentration of the suspension, was further compared with the result obtained by the plate counting method.

3. RESULTS AND DISCUSSION

A linear correlation (R^2=0.9989) of OD loss to CFU-forming spore concentration was determined in the range of viable spore concentrations from 0.5×10^{8} to 9×10^{8} cfu ml^{-1}(Fig. 1a). The calibration curve in Fig.2 can be expressed by a linear equation:

$OD_{loss} = 0.0246 \times C_{vs} + 0.001$

Where C_{vs} is viable spore concentration of the sample ($\times10^{8}$ cfu ml^{-1}), and OD_{loss} is the OD loss of spore suspension in AGS after 2 h.

To validate this spectrophotometric method, we performed parallel germination experiments with spores obtained under different cultural conditions. The spore yield was quite different with the change in the concentration of medium components such as yeast extract, soluble starch and sodium chloride, and in cultivation conditions including pH and working volume. For all the samples, the plate count values and the detected viable spore yields by spectrophotometric measurements displayed a good linear correlation (y=0.9679x+ 0.3363, r^2=0.9981, n=34; Fig. 1b).The slope for the correlation was 0.9697. For all the samples, the viable spore concentrations measured with the spectrophotometric method were consistent with that obtained by plate counting method Compared to the plate counting method, the spectrophotometric assay doesn't require time-consuming and labor-intensive procedures such as serial dilution and colony formation, and accordingly is sensitive, rapid and easy to perform. Consequently, this rapid method can show the potential in biotechnological application, for example, facilitating the optimization of operational conditions in spore production.

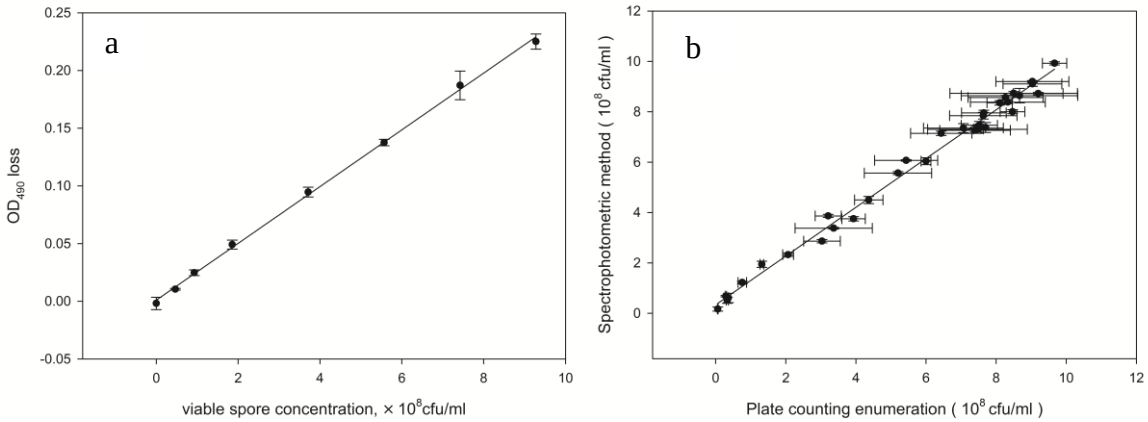

Figure 1. Calibration curve of OD loss to viable spore concentration in sample(a) and correlation between plate counting enumeration and the spectrophotometric method for quantification of viable spore concentration (b).

4. CONCLUSION

Based on endospore's property of losing refractivity during germination process, a novel spectrophotometric method was developed for rapid detection of viable spores. Our experimental results showed that under suitable conditions, the viable spore concentrations measured by the spectrophotometric method were in good agreement with that obtained by plate counting method. Furthermore, without serial dilution and colony formation process, this novel method is sensitive, rapid and easy to perform. Therefore, this spectrophotometric method is an effective alternative to conventional colony counting method in viable spore determination, especially for large-scale detection of spore samples.

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