Curr Microbiol (2013) 66:279–285
DOI 10.1007/s00284-012-0269-2

Bacillus subtilis Endospores at High Purity and Recovery Yields: Optimization of Growth Conditions and Purification Method

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Received: 4 June 2012 / Accepted: 9 November 2012 / Published online: 27 November 2012

Abstract *Bacillus subtilis* endospores have applications in different fields including their use as probiotics and antigen delivery vectors. Such specialized applications frequently require highly purified spore preparations. Nonetheless, quantitative data regarding both yields and purity of *B. subtilis* endospores after application of different growth conditions and purification methods are scarce or poorly reported. In the present study, we conducted several quantitative and qualitative analyses of growth conditions and purification procedures aiming generation of purified *B. subtilis* spores. Based on two growth media and different incubations conditions, sporulation frequencies up to 74.2 % and spore concentrations up to 7×10^9 spores/ml were achieved. Application of a simplified spore isolation method, in which samples were incubated with lysozyme and a detergent, resulted in preparations with highly purified spores at the highest yields. The present study represents, therefore, an important contribution for those working with *B. subtilis* endospores for different biotechnological purposes.

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Introduction

Bacillus endospores represent the most resilient life form on our planet and reflect the outcome of precisely regulated genetic networks leading to unique cellular structures [11]. In addition, *Bacillus* spores, particularly those produced by *Bacillus subtilis*, have received growing interest as cellular tools with different biotechnological applications. For instance, *B. subtilis* spores have been successfully used as probiotics for humans and animals, as bioindicators for the validation of sterilization processes and as heat-stable live vaccine vectors, in which target antigens are genetically fused to spore coat proteins or expressed after spore germination [1, 3, 7, 9, 13]. Nonetheless, contamination of spore preparations with viable or inactivated vegetative cells, or even cell debris, may cover the immune responses induced by the spore-associated antigens. Therefore, production of highly purified spores at high yields represents an essential process in the development of spore-based vaccine vectors.

Production and purification of *B. subtilis* spores, as well as other *Bacillus* species, involve two major steps. The first one is the generation of a good spore crop that can be achieved only at specific growth conditions, and the second step is the spore purification procedure itself in which spores must be separated from non-sporulating cells and cellular debris. Several spore purification protocols have been reported using different *Bacillus* species but quantitative data of the recovery yields and purity levels, regarding contamination with vegetative cells and cellular debris, are usually poorly reported [2, 5, 10]. In fact, the *Bacillus* spore purification methods, current described in the literature, make use of laborious and rather expensive technologies [6, 8].

In an attempt to improve the reproducibility of the method and obtain samples at high purity and reasonable recovery yields, we performed a systematic quantitative

analysis of sporulation conditions and spore purification methods using the most widely used *B. subtilis* laboratory strain cultivated under ordinary growth conditions. Based on the results generated during this study, we established a methodology which allowed high sporulation frequencies and defined the most important steps required for the purification of spores.

Materials and Methods

Bacterial Culture and Sporulation

The *B. subtilis* 1012 strain (*leuA8 metB5 trpC2 hsdRM1 amyE::neo)* was kindly provided by Dr. Wolfgang Schumann from University of Bayreuth, Germany. This strain is registered in *Bacillus* Genetic Stock Center (code BGSC 1A982) and has been maintained as frozen stock in our bacterial strain collection during the last 10 years. The *B. subtilis* 1012 strain was initially cultivated in Luria–Bertani (LB) liquid medium with neomycin sulfate (25 μg/ml) for 16 h at 37 °C in an orbital incubator shaker. Sporulation was performed in two growth media, without the addition of antibiotics: Difco sporulation media (DSM), described by Nicholson and Setlow [12], and the F medium, modified from Foerster and Foster [4] composed of 1 % glucose, 0.1 % L-glutamate, 0.05 % yeast extract, 0.5 % KH_2PO_4, 0.1 % $(NH_4)_3PO_4$, 0.02 % $MgSO_4$, 0.01 % NaCl, 0.005 % $CaCl_2$, 0.0007 % $MnSO_4$, 0.001 % $ZnSO_4$, and 0.001 % $FeSO_4$. Sporulation in liquid media was performed in Erlenmeyer flasks containing 20 ml of medium inoculated with 0.2 ml of an overnight grown culture. Flasks were kept at 37 °C in an orbital shaker at 200 rpm for 24 h or 72 h. Sporulation on plates was performed using 0.2 ml culture samples spread on agar plates (1.5 % agar in 20 ml of medium) in a regular Petri dish (8 cm in diameter) and incubated at 37 °C for 24 or 72 h. After incubation time, spores in the plates were resuspended in 5 ml of phosphate-buffered saline (PBS) by pipetting, transferred to a 50 ml polypropylene tube, and the volume was adjusted to 20 ml.

Determination of Sporulation Frequencies

Spore-containing *B. subtilis* cultures were serially diluted and plated on LB plates with neomycin before and after applying a heat treatment that eliminates the vegetative cells but not the spores (65 °C for 1 h). Plates were incubated for 16 h at 37 °C, and the visible colonies were counted. Sporulation frequencies were calculated as the titers of colony forming units (CFU/ml) before and after the heat treatment. Values are expressed as: (Spores titer after the heat treatment/Spores titer before the heat treatment) × 100. The experiments were independently performed at least three times, and each experiment was performed in duplicate.

B. subtilis Spore Purification Methods

We compared two spore purification procedures: (1) Method 1, described by Nicholson and Setlow [12], which consists in centrifugation of cells for 10 min at 10,000×*g* at 4 °C, and washing the cells with 1 M KCl and 0.5 M NaCl. The cells were suspended in 50 mM Tris HCl (pH 7.2), and lysozyme was added to a final concentration of 50 μg/ml followed by incubation at 37 °C for 1 h. After incubation, the cells and spores were pelleted and washed once with distilled water and once with 0.05 % sodium dodecyl sulfate (SDS) solution. After suspension by vortexing, spores were pelleted and homogenized with TEP buffer (50 mM Tris–HCl (pH 7.2) and 10 mM EDTA), centrifuged for 10 min at 10,000×*g* at room temperature and washed three times (10 min, 10,000×*g*) with distilled water and suspended in 1 volume of water. Method 2, consists in centrifugation of the cells for 10 min at 10,000×*g* at 4 °C and suspending the pellets with 50 mM Tris–HCl (pH 7.2) containing 50 μg/ml of lysozyme. Samples were incubated for 1 h at 37 °C and washed once with 1 volume of distilled water. Pellets were centrifuged, and suspended with 0.05 % SDS solution by vortexing. Samples are washed three times with distilled water and suspended in 1 volume of distilled water.

Determination of Spore Purity and Recovery Yields

Purity of the spore preparations was evaluated after serial dilutions of the spore suspensions and determination of the Spores titers before and after the heat treatment (65 °C, 1 h) that inactivates only vegetative cells. The purity values were expressed as: (Spores titer after the heat treatment/Spores titer before the heat treatment) × 100. Spore recovery yields were determined as the percentages of spores recovered after application of the purification method with regard to the initial number of spores in the culture. The values were expressed as: (Spores titer at the end of the purification procedure/Spores titer before application of the spore purification procedure) × 100. The determinations were independently repeated at least three times, and each experiment was carried out in quadruplicates. Overall efficacy values of each spore purification method were calculated as the arithmetic means of spore purity and the recovery yield of each tested procedure.

Image Analysis

Samples (50 μl) of each preparation or culture were smeared onto glass slides, air-dried, and analyzed using a

phase-contrast microscope (EVOS fl, AMG, Bothell, USA). At least ten images of each preparation were acquired, and one representative image of each preparation was presented.

Statistical Analyses

The results were analyzed with GRAPHPAD PRISM 5 software and were expressed as the means ± SD.

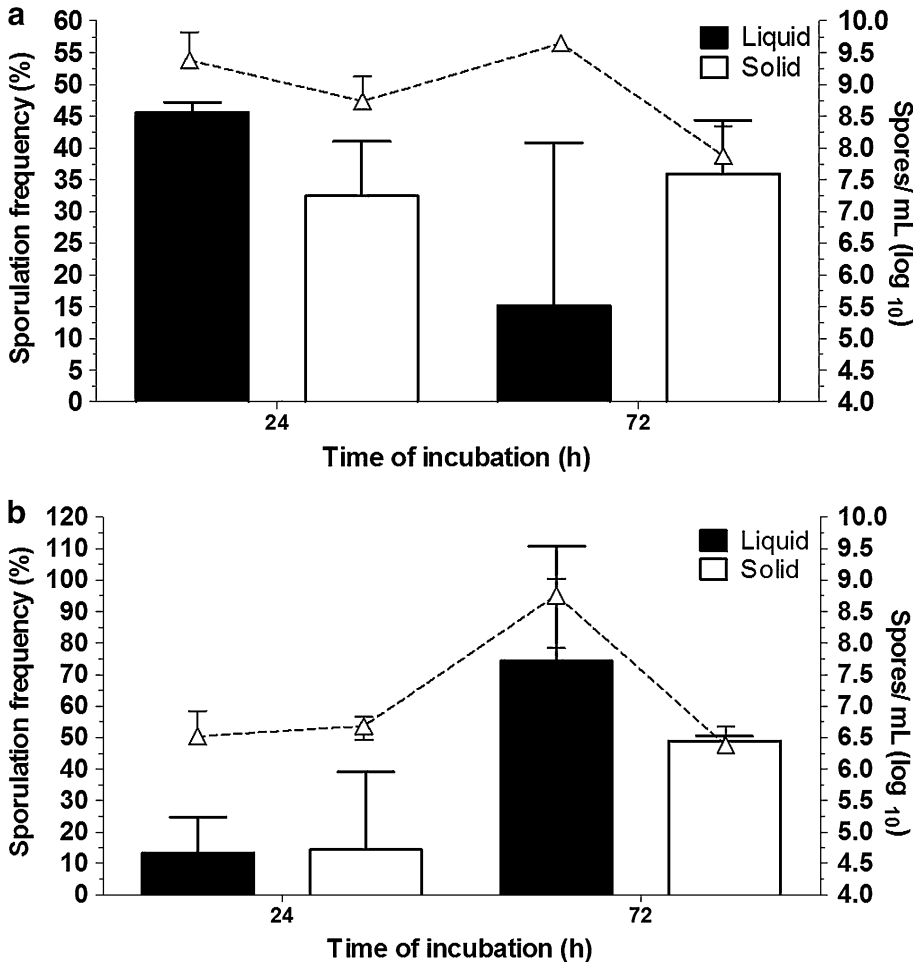

Fig. 1 Effects of different growth conditions on the sporulation frequency of the *B. subtilis* 1012 strain. **a** Cultivation in DSM medium. **b** Cultivation in F medium. *Bars values* represent percentage of sporulation frequency. Total number of viable spores are indicated by *symbols* (Δ) connected by a *dotted line*. Values represent the averages of three independent experiments

Table 1 Comparison of spore purification methods regarding purity and recovery yields using the *B. subtilis* 1012 strain

Medium (broth)	Incubation (h)	Sporulation frequency (%)[a]	Purity (%)		Yield (%)		Efficacy (%)	
			Method[b]		Method[b]		Method[b]	
			1	2	1	2	1	2
DSM	24	45.5	89	93.5	32	100	60.5	96.7
	72	15.1	87.9	97.7	3.6	4.7	45.7	51.2
F	24	13.2	47.1	95.6	0.14	6.1	23.6	50.9
	72	74.2	82.4	99.1	0.7	100	41.5	99.6

[a] Sporulation frequencies represent the number of vegetative cells giving rise to a viable spore. The values were expressed as (spores titer/culture titer) × 100 before application of the tested spore purification methods. Culture titers (vegetative cells and spores) and spores titers were determined before and after application of the heat treatment at 65 °C for 1 h, respectively

[b] Spore purification procedures: method 1 as previously described by Nicholson and Setlow [12] and method 2, the lysozyme/SDS treatment as described in the "Materials and methods" section

Statistically significant differences ($^{**}P < 0.01$) were determined with two-way ANOVA and Student′s *t* test.

Results and Discussion

Effect of Growth Conditions on *B. subtilis* Sporulation Frequencies

Initially, we determined the sporulation frequencies of the tested *B. subtilis* strain submitted to different growth conditions. We tested two growth media (DSM and F), two incubation times (24 and 72 h), and cultures prepared in broth or plates. Under the testing conditions, the *B. subtilis* 1012 strain reached sporulation frequencies of 45.5 and 15.1 % (with spore concentrations of 4×10^8 and 6.7×10^8 spores/ml) in DSM broth after incubation for 24 or 72 h, respectively (Fig. 1a; Table 1). On the other hand, samples cultivated in plates reached a maximum sporulation frequency of 35.9 % after incubation for 72 h and concentrations up to 10^8 spores/ml after incubation for 24 h (Fig. 1a).

Sporulations performed in F broth were more dependent of the incubation time (Fig. 1b; Table 1). A sporulation frequency of 13.2 % was achieved following incubation in broth for 24 h (with a spore concentration of 6.5×10^6 spores/ml) while higher sporulation frequencies (74.2 %) and spore concentrations (1.2×10^9 spores/ml) were observed after incubation in broth for 72 h. Cultures prepared on plates incubated, either for 24 h or 72 h, showed sporulation frequencies that ranged from 14.4 to 48.8 %, respectively, and spore concentrations up to 9.5×10^6 spores/ml after incubation for 24 h (Fig. 1b). Collectively, these results indicate that for the widely used *B. subtilis* 1012 laboratory strain higher sporulation frequencies and spore concentrations are obtained in cultures prepared in F broth incubated for 72 h at 37 °C.

Purity of Spores Prepared with Optimized Purification Method

In order to improve the spore purity and simplify the overall spore purification procedure, we compared a

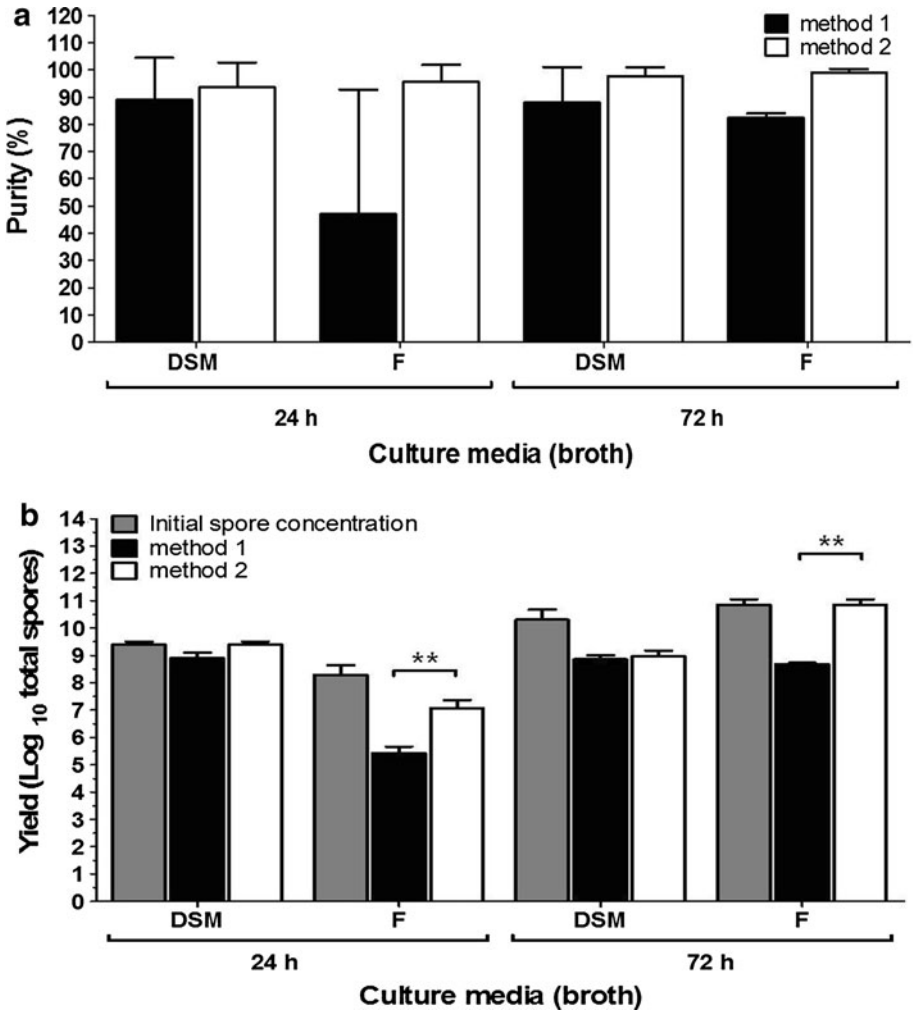

Fig 2 Evaluation of the spore preparations after application of the purification methods 1 (*black bars*) or 2 (*open bars*). **a** Purity of the spore preparations after purification. Bacterial cultures were incubated for 24 or 72 h in DSM or F medium broth. **b** Spore recovery yields of purified samples. The values were expressed as total of viable spore in 20 ml cultures. The results represent the average of three independent experiments carried out in duplicate. Statistically significant difference ($^{**}P < 0.01$)

commonly used spore purification method (method 1) with a simplified procedure that included the lysozyme/SDS treatment (method 2). The purity of spores obtained with method 1 ranged from 47.1 % (cultures prepared in F medium for 24 h) to approximately 90 % (cultures prepared in DSM medium both after 24 or 72 h) (Fig. 2a). The results showed that procedures based on successive washing steps carried out only with water, detergent or lysozyme resulted in negligible to very low spore purity values (data not shown).

In contrast, treatment with method 2 resulted in highly purified spore samples (above 90 % purity values in all tested conditions and cultivation times) with values ranging from 93.5 % (in DSM broth after cultivation for 24 h) to 99.1 % (in F broth after cultivation for 72 h) (Fig. 2a; Table 1), which were statistically superior to the results obtained with method 1 using the same samples. Noticeably, cultures prepared in plates showed drastically different results with lower spore yields and heavier contamination with vegetative cells that tend to forms clumps hard to solubilize during the spore purification process. These results indicated that the purification of *B. subtilis* spores can be significantly improved after application of a simplified variation of the original spore isolation technique and appropriate growth conditions leading to spore preparations with negligible contamination with vegetative cells (Fig. 2a; Table 1).

Spore Recovery Yields

The two spore purification methods were compared regarding the final spore recovery yields after cultivation in DSM or F broth. The spore yields ranged from 8.9×10^3 to 2.7×10^7 to spores/ml with method 1, which corresponded to 0.1 to 32 % of the total number of spores initially present at the cultures. On the other hand, application of method 2 resulted in 100 % spore recovery in two cultivation conditions (cultivation in DSM broth for 24 h and F broth for 72 h) with final spore yields ranging reaching values above 10^9 spores/ml (maximum value of 2×10^9

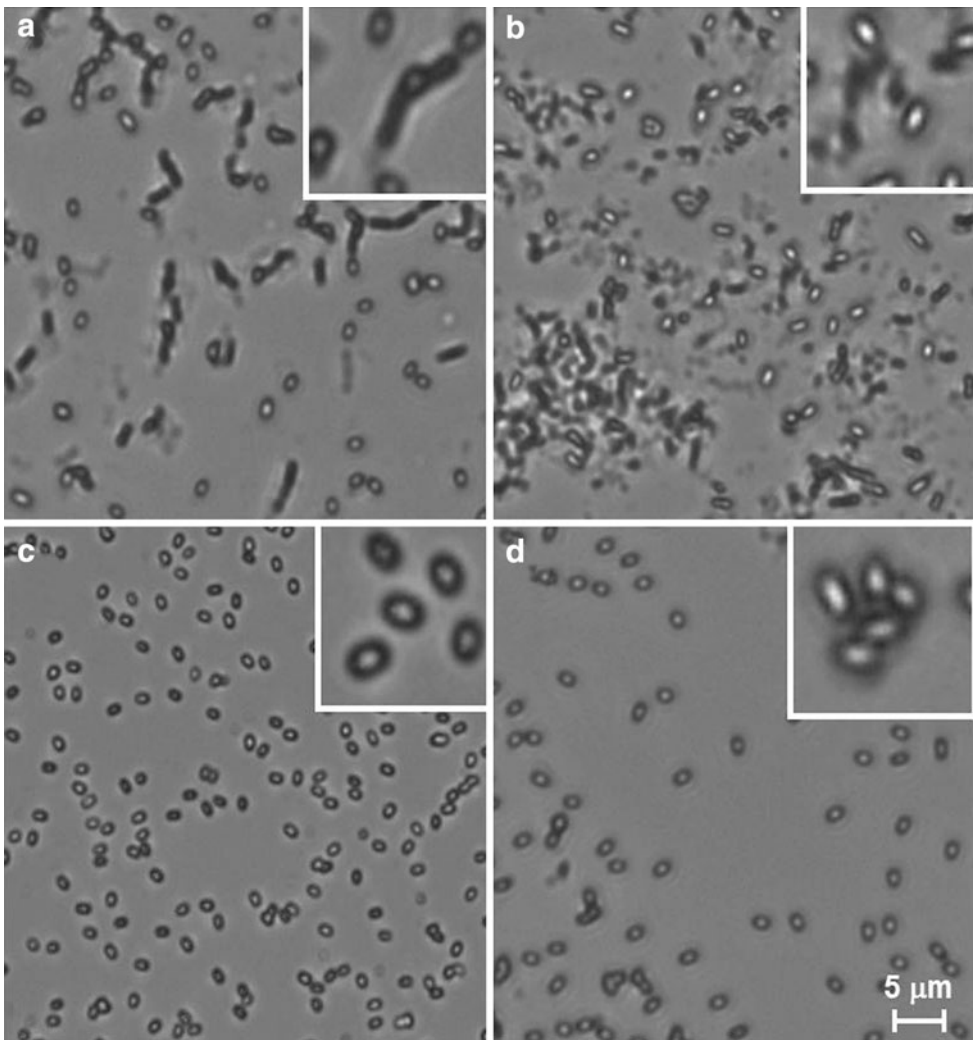

Fig. 3 Microscopic examination of the spore preparations after application of the different spore purification methods. All samples were cultivated in F broth for 72 h at 37 °C. Samples **a** initial bacterial culture before application of the spore purification methods, **b** spores prepared with method 1, **c** spores prepared with method 2, **d** *B. coagulans* spore preparation after purification using method 2. Magnitude: ×600. *Upper right side* digital zoom (additional amplification of ×5)

spores/ml in cultures cultivated in F broth for 72 h) (Fig. 2b; Table 1).

The purified spore preparations from cultures prepared in F broth for 72 h were also monitored for the presence of dead cells or cellular debris using phase-contrast microscopy. As shown in Fig. 3, before application of the spore purification procedures, cultures exhibited a large number of vegetative cells as well as free spores (panel A). Samples that were submitted to method 1 or 2 did not show residual vegetative cells. However, cellular debris was observed in spore samples prepared with method 1 (panel B) but not in samples prepared with method 2 (panel C). These results showed that method 2 could efficiently eliminate vegetative cells and cell debris with minimal losses of the spores with a concomitant reduction of workload and time required to complete the procedure.

Interestingly, in some samples, the final spore concentration of the purified preparations was superior to those determined in the initial cultures before application of the spore purification procedure. This fact was mainly attributed to the fact that cell filaments containing several spores were counted as a single colony during determination of the culture titer based on colony forming units (unpublished data).

Based on the purity and recovery yields of the different tested conditions procedures, we determined the overall efficacies of the two purification methods using the *B. subtilis* 1012 strain cultures cultivated in broth. As shown in Table 1, the overall efficacy of method 1 ranged from 23.6 to 60.5 % while the efficacy of method 2 ranged from 50.9 to 99.6 %. Altogether, these findings demonstrated that the spore purification method 2 gave better results than method 1, particularly for samples cultivated in F and DSM broth for 72 and 24 h, respectively.

We also evaluated the growth condition based on F medium for 72 h and spore purification method 2 with wild type *B. subtilis* strains and, spore purity values above 70 % were routinely achieved (data not shown). In conclusion, we observed that high sporulation values, and consequently high spore recovery yields, require definition of proper strain-specific growth conditions (unpublished observations). We also tested the reported conditions using a different *Bacillus* specie, the *B. coagulans* strain GanedenBC^{30} [1]. Based on the optimum growth conditions (cultivation in F broth for 72 h) and the purification method 2 spore samples with purity values of 72 %, concentrations above 10^9 spores/ml were achieved and no cellular debris was observed by microscopic analysis (data not shown and Fig. 3d).

In conclusion, this study showed that purification of *B. subtilis* spores can be significantly improved, regarding both purity and recovery yields by means of simple adjustments of growth conditions and purification method. Based on the proposed conditions, spores of the *B. subtilis* 1012 strain could be routinely obtained at concentrations above 10^9 spores/ml with cultures prepared in under ordinary laboratory conditions without the need of biofermentors or density-gradient centrifugation. Altogether, these results represent a relevant contribution for those aiming to work with *B. subtilis* spores at high purity including the use of spores as live vaccine vectors or as probiotic.

Acknowledgments The present study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grants. We gratefully acknowledge Dr. A. L. Sonenshein, Tufts University, for the critical reading the manuscript and helpful suggestions. We also acknowledge the skillful technical assistance of L.C. Silva.

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