
**Separation Science and Technology**
Publication details, including instructions for authors and subscription information:
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Online publication date: 04 January 2003

**To cite this Article** Lin, Dong-Qiang , Yao, Shan-Jing , Mei, Le-He and Zhu, Zi-Qiang(2003) 'Collection and Purification of Parasporal Crystals from *Bacillus thuringiensis* by Aqueous Two-Phase Extraction', Separation Science and Technology, 38: 7, 1665 — 1680
**To link to this Article: DOI:** 10.1081/SS-120019263
**URL:** http://dx.doi.org/10.1081/SS-120019263

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SEPARATION SCIENCE AND TECHNOLOGY
Vol. 38, No. 7, pp. 1665–1680, 2003

# Collection and Purification of Parasporal Crystals from *Bacillus thuringiensis* by Aqueous Two-Phase Extraction

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## ABSTRACT

Aqueous two-phase extraction was used for efficient separation of biological particles with similar size, density, or other physical properties. The partitioning behavior of spores and parasporal crystals of *Bacillus thuringiensi* were studied in two types of aqueous two-phase systems: polymer/salt systems and polymer/polymer systems. The spores and crystals showed strong tendency towards the top polyethylene glycol (PEG)-rich phase and interface in PEG/salt systems, and were hardly influenced by several factors, such as concentration of PEG and salt, phase separation time, and phase ratio. Using the appropriate system (PEG 6000 2%/$(NH_4)_2SO_4$ 16%), collection of both spores and crystals directly from fermentation broth was carried out with a yield of 96%

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DOI: 10.1081/SS-120019263 0149-6395 (Print); 1520-5754 (Online)

crystals on lab-scale. Separation of crystals from spores could be achieved using the polymer/polymer systems. It was found that phase-forming polymers, pH, and phase separation time showed significant influence on the partition of spores, and the optimum conditions were chosen as PEG 6000 6%/DEX60 7.5% system, pH 10.0, and 1.5 ~ 2.0 hr for the separation of crystals from spores. The yield of crystals was 83%, and more than 90% of spores could be removed after four cycles of extractions of the bottom phase with fresh top PEG-rich phase. Finally, a process scheme was developed that included an initial extraction of both spores and crystals using PEG/salt system and integrated with subsequent removal of spores by extraction in PEG/dextran system. The feasibility of the integrated separation process was verified by lab-scale application. The total yield of crystals could reach 81.4%, and more than 90% of spores could be removed.

*Key Words:* Aqueous two-phase system; Parasporal crystal; Purification; *Bacillus thuringiensis*.



## INTRODUCTION

*Bacillus thuringiensis* (*Bt*) is a widely distributed, spore-forming, aerobic, gram-positive bacterium. It is unique because it produces one or more parasporal crystals during the sporulation cycle. These parasporal crystals, protoxins, consist of protein molecules called δ-endotoxins that are toxic to the larvae of various insects, including lepidoptera, coleoptera, and diptera.[1] The insect pathogen *Bt* has a highly selective mode of action. In most case, the activity of an isolate of *Bt* is limited to a single order of insects.[2] The safety of these biological insecticide formulations based on *Bt* to nontarget organism and man is responsible for the growing interest in their use on a variety of crops, including pastures and forests. Despite the relatively large number of insect pathogens that have been proposed as insecticides, those products formulated with *Bt* are the most extensively and widely used today.[1,3]

Usually, bioinsecticides derived from *Bt* contain inclusion bodies, spores, cell debris, and other residual solids, which are all recovered from the broth at the end of the fermentation and then formulated, packed, or dried. To produce spore-free formulation and protect improved proprietary strains, purification of parasporal crystals from spores and other residual solids is necessary. It will also be useful to have an easy and fast method of crystals purification that allows δ-endotoxin characterization or the utilization of pure crystals for other purposes, including studies on the mode of action. Several methods have been

described for separation of parasporal crystals from the spores, including froth flotation and density gradient centrifugation using either CsCl,[4] Renografin,[5,6] NaBr,[7] Ludox,[8] or sucrose.[9] These methods tend to be laborious and are often only partially successful. Froth flotation is relatively inefficient. Various methods based on density gradient centrifugation are time consuming and low-yield; moreover, expensive special regents and equipment are required. Therefore some new and efficient methods should be developed.

The concentration of parasporal crystals in fermentation broth is usually about 1%, thus a large amount of waste water has to be removed. Meanwhile, the size, density, and some physical properties of parasporal crystals are very similar to that of spores, which enhances the difficulty of separating crystals free from spores. Liquid–liquid extraction in aqueous two-phase systems (ATPS) has been demonstrated to be a powerful tool for recovery of intracellular proteins from disrupted cells[10,11] and fractionation of inclusion bodies from cell homogenate.[12] The applications of ATPS for spore recovery and crystals purification have also been reported in a few literatures. Sacks and Aderton[13] achieved spore isolation from vegetative cells of *Bacillus subtilis* species using polyethylene glycol (PEG)/potassium phosphate ATPS. Goodman et al.[14] and Pearson and Ward[15] reported insecticidal crystal purification from spores by PEG/sodium dextran sulphate ATPS. To obtain pure crystals, 3 ~ 4 consecutive extractions of the bottom phase with fresh top PEG-rich phase had to be carried out. A total of 90% of the spores could be removed, but the total recovery of crystals was very low. Gucreca et al.[16] purified crystals from spores using PEG/potassium phosphate ATPS with a yield of 42%. However, their method based on the simultaneous control of centrifugation time and centrifugation rate was difficult in practical process.

In the present work, two types of ATPS—polymer/polymer systems (PEG/dextran and PEG/hydroxypropyl starch) and polymer/salt systems (PEG/$(NH_4)_2SO_4$ and PEG/potassium phosphate)—were explored for effectively collection and purification crystals from spores.

## MATERIALS AND METHODS

### Bacterial Isolate and Culture Methods

*Bt* strain (Bt 8010) was provided by Biotechnology Center, Fujian Agricultural University (Peoples Republic of China). One cell could produce only one parasporal crystal during its sporulation cycle. The microorganism was cultivated in a liquid medium containing 10 g/L polypepton, 5 g/L nutrient broth, and 10 g/L NaCl (pH 7.4 ~ 7.5) at 30°C in a gyrator shaker at 200 rpm.

Incubation was performed 36 hr for cell growth, and continued for 36 hr to complete sporulation and autolysis. The final concentration of spore (or parasporal crystal) in the broth was $3 \sim 5 \times 10^9\,mL^{-1}$. The amount of parasporal crystals was about $12 \sim 17\%$ of wet biomass. The broth was used directly for crystal recovery without any pretreatment.

## Materials

Polyethylene glycol with average molecular weights of 2000 (1900–2200) and 6000 (5500–6500) and dextran, DEX40 ($M_w$ =40,000) and DEX60 ($M_w$ =60,000), were purchased from Shanghai Chemical Reagent Factory (Peoples Republic of China). Hydroxypropyl starch (PES), Reppal PES100 ($M_w$ = 100,000) and Reppal PES 200 ($M_w$ = 200,000), was a kind gift from Carbamyl AB (Sweden). All other chemicals were of analytical grade.



## Spores and Crystals Assay

Spores and crystals were monitored by optical microscopic observation of crystal violet stained smears. The numbers of spores and crystals were counted in a Neubauer chamber (0.1 mm depth, $0.0025\,mm^2$) by Olympus BH-2 optical microscope. The samples were measured triplicate with the error less than $\pm 5\%$, and the average values were obtained.

## Aqueous Two-Phase Partitioning

The aqueous two-phase systems were prepared from stock solutions: 30% PES, 30% dextran, 30 ~ 50% PEG, and 40% $(NH_4)_2SO_4$. All concentrations are given in weight per weight percentage (w/w%). Detailed description of the preparation of aqueous two-phase systems has been given elsewhere.[10,11] In the present work, the system was 10 g for normal partition experiments, and 40 ~ 500 g for lab-scale partitioning. The systems were well mixed by a vortex mixer and left in a water bath for partitioning. After appropriate separation time, the samples was carefully withdrawn from the top phase and bottom phase, and diluted to appropriate concentration for spores and crystals counting. During all partition experiments the temperature was 25°C.

Phase ratio was defined as the ratio of top phase volume to bottom phase volume.

$$\text{Phase ratio} = \frac{\text{top phase volume}}{\text{bottom phase volume}} \quad (1)$$

The yield of top phase, $Y_{top}$, was defined as the ratio of the number of biological particles (spores or parasporal crystals) in the top phase to that added to the system.

$$Y_{top} = \frac{\text{particle number in top phase}}{\text{particle number added to the system}} \times 100\% \quad (2)$$

The yield of top phase and interface, $Y_{top+interface}$, was the ratio of the number of bioparticles in the top phase and interface to that added to the system. Because it was difficult to directly determine the particle number in the interface, the number in the bottom phase was used for the definitions of $Y_{top+interface}$ as the following:

$$Y_{top+interface} = \left(1 - \frac{\text{particle number in bottom phase}}{\text{particle number added to the system}}\right) \times 100\% \quad (3)$$



## RESULTS AND DISCUSSION

### Partitioning Behavior in Polymer/Salt Systems—Collection of Both Spores and Crystals from Broth

#### Partitioning Behavior in PEG/Salt Systems

In PEG 2000/$(NH_4)_2SO_4$ and PEG 6000/$(NH_4)_2SO_4$ aqueous two-phase systems, most vegetative cells, spores, and crystals showed strong attraction to the top PEG-rich phase and interface after static phase separation of 30 min; meanwhile, most of the debris and nucleic acid was left in the bottom salt-rich phase. The pH of systems was adjusted to 7.2. The effects of concentration of PEG and salt, molecular weight of PEG, and separation time were investigated. The results indicated that the partition tendency of spores and crystals toward the top phase was too strong to be influenced by factors listed previously (data not shown). $Y_{top+interface}$s of spores and crystals were usually more than 95%. The partitioning behavior in PEG/potassium phosphate systems (pH 7.0) was similar to that in PEG/$(NH_4)_2SO_4$ systems. These results

may be due to the significant differences of density and hydrophobicity between the two phases of PEG/salt systems.

## Effect of Phase Ratio

To investigate the capacity of top phase and interface for biological particles, the effect of phase ratio was studied. The data in Table 1 showed that spores and crystals still tended to concentrate in the top PEG-rich phase and interface when the phase ratio decreased to 0.1, which indicated that the operating volume could be reduced significantly after aqueous two-phase partitioning. The appropriate compositions of the separation system were chosen as PEG6000 2.0% and $(NH_4)_2SO_4$ 16.0%, so that the phase ratio was about 0.12. Using this optimum ATPS, spores and crystals could be directly collected from broth, and the volume for next-step separation process could be effectively reduced because of the excellent partition behavior and low phase ratio.



## Collection of Spores and Crystals from Broth in Lab-Scale

Based on these results, collection of spores and crystals from fermentation broth was carried out on lab-scale. The broth was obtained directly from shake flask culture without any pretreatment, and the amount of the broth adding in the extraction systems was as high as possible. On the other hand, to reduce the total volume of phase system, solid powder of $(NH_4)_2SO_4$ was used. The details are listed in Table 2. For 400 g broth, the yield of crystals could

***Table 1.*** Effect of phase ratio on partition of spores and crystals in PEG 6000/$(NH_4)_2SO_4$ aqueous two-phase system at 25°C (pH7.2).

| | System composition (w/w%) | | | | $Y_{top+interface}$ (%) | |
|---|---|---|---|---|---|---|
| No. | PEG6000 | $(NH_4)_2SO_4$ | Broth | Phase ratio | Spore | Crystal |
| 1 | 1.0 | 16.0 | 50 | 0.10 | 96.8 | 98.3 |
| 2 | 1.5 | 16.0 | 50 | 0.11 | 98.6 | 99.2 |
| 3 | 2.0 | 16.0 | 50 | 0.12 | 98.8 | 99.2 |
| 4 | 3.0 | 16.0 | 50 | 0.18 | 98.9 | 99.4 |
| 5 | 4.0 | 16.0 | 50 | 0.21 | 99.0 | 99.6 |
| 6 | 5.0 | 16.0 | 50 | 0.28 | 99.0 | 99.7 |

***Table 2.*** Collection of spores and parasporal crystals from fermentation broth on lab-scale (25°C, pH7.2).

| | Materials (g) | | | | $Y_{top+interface}$ (%) | |
|---|---|---|---|---|---|---|
| No. | Broth | PEG6000 40% | $(NH_4)_2SO_4$ | Phase ratio | Spore | Crystal |
| 1 | 40 | 2.5 | 8 | 0.12 | 86.6 | 97.0 |
| 2 | 400 | 25 | 80 | 0.11 | 82.2 | 96.0 |

reach 96%; meanwhile, the volume of solution was reduced to about one-eighth of the initial broth, which was suitable for the purification step.



## Partitioning Behavior in Polymer/Polymer Systems—Purification of Crystals from Spores

In PEG/salt systems, spores and crystals concentrated together in the top PEG-rich phase and interface thus could not be isolated from each other. Therefore, the polymer/polymer ATPSs were introduced for separating crystals from spores.

### Effect of Phase-Forming Polymer

Several kinds of polymer/polymer ATPSs, PEG/PES, and PEG/dextran with different molecular weights of polymers were investigated systematically. As the samples shown in Figs. 1 and 2, for PEG/PES systems $Y_{top}$ of spores was a little lower than that of crystals, whereas $Y_{top}$ of spores was a little higher than that of crystals for PEG/DEX40 systems. The similar partitioning behavior between spores and crystals indicated that the crystals could not be isolated effectively from spores in the systems. It was interesting to find that the partition in PEG6000/DEX60 systems was of some differences (as shown in Fig. 3). With the decreasing of concentration of PEG 6000, the $Y_{top}$ of crystals maintained less than 5%, whereas the $Y_{top}$ of spores increased significantly from 9% to 34%. The results indicated that crystals might be purified from spores using appropriate PEG6000/DEX60 systems.

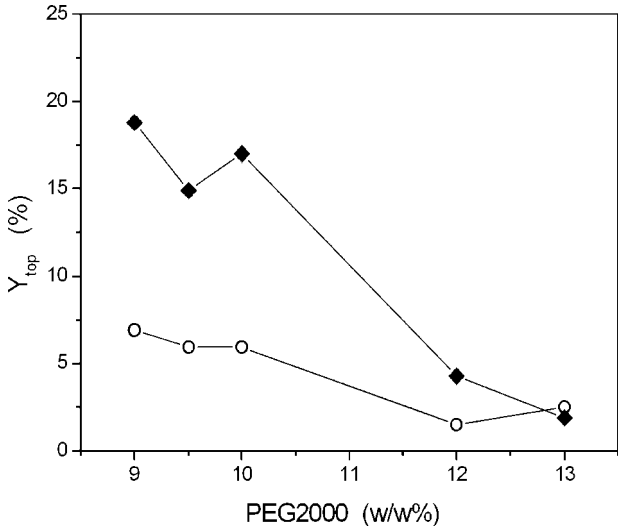


***Figure 1.*** Partition of spores (○) and crystals (◆) in PEG2000/PES100 systems at 25°C (pH 7.2). The concentration of PES100 was 15%. The separation time was 2 hr.

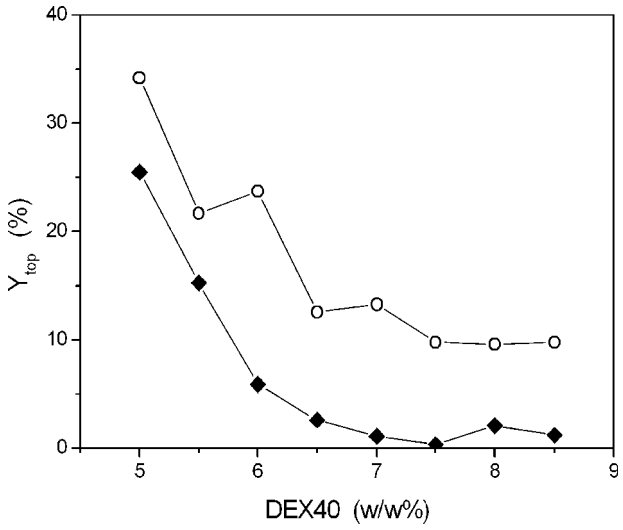


***Figure 2.*** Partition of spores (○) and crystals (◆) in PEG6000/DEX40 systems at 25°C (pH 7.2). The concentration of PEG6000 was 7.0%. The separation time was 2 hr.

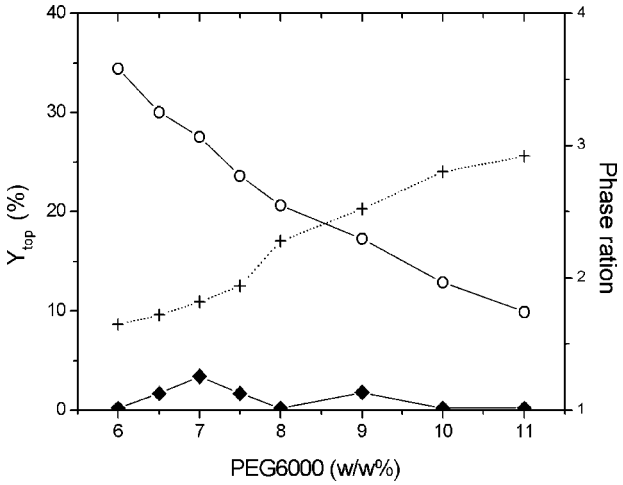


***Figure 3.*** Partition of spores (○) and crystals (◆) in PEG6000/DEX60 systems at 25°C (pH 7.2). The concentration of DEX60 was 7.5%. The phase ratio for different systems is also indicated as symbol (+). The separation time was 2 hr.



## Effect of Separation Time

During the experiments, it was found that the phase separation time influenced the partition of spores, thus the effect of the time of static phase separation was studied carefully. The results are showed in Fig. 4. At the range from 0.5 to 2.5 hr, the partitioning behavior of spores was relatively stable. When time was longer than 2.5 hr, the $Y_{top}$ of spores decreased significantly, and spores concentrated mostly to interface after 4.5 hr. Therefore, the appropriate time of static phase separation was chosen as $1.5 \sim 2.0$ hr. One explanation of these facts is that the possibility of aggregation between spores after a period of flotation that may result in higher sedimentation rate to the interface.

## Effect of pH and Addition of Salts and Surfactants

For biological particles, the changes of surface characters, especially for surface charges, always influences the partitioning behavior significantly. Therefore, some factors (pH, addition of salts and surfactants) were investigated to enhance the efficiency of separation. Figure 5 showed

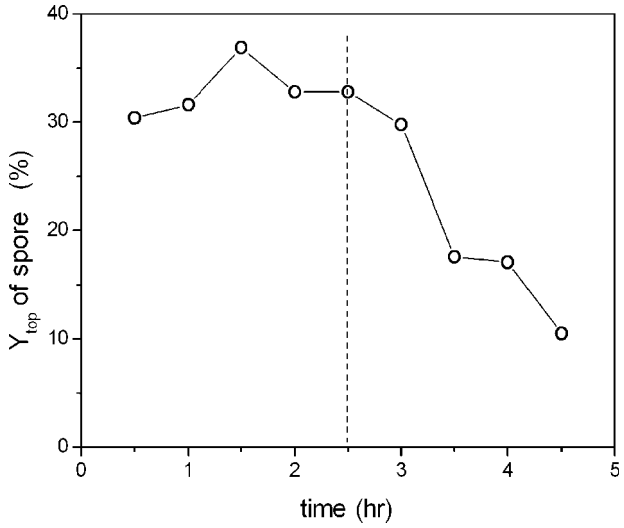


***Figure 4.*** Effect of separation time on partition of spores in PEG6000/DEX60 systems at 25°C. System: PEG6000 6.0%, DEX60 7.5%, pH 7.2.

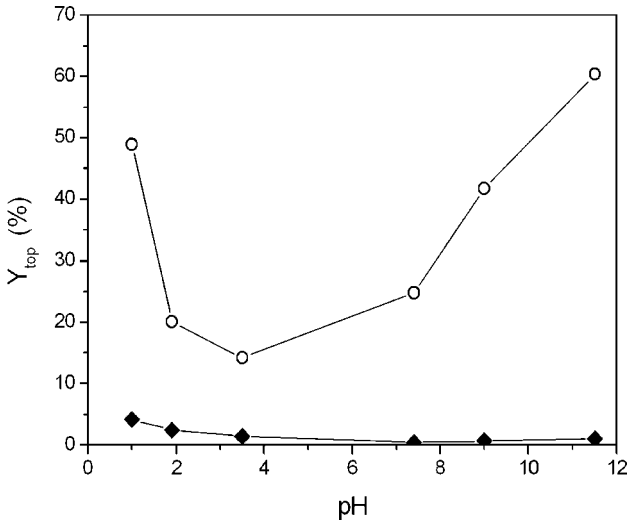


***Figure 5.*** Effect of pH on partition of spores (○) and crystals (◆) in PEG6000/DEX60 system at 25°C. System: PEG6000 6.0%, DEXX60 7.5%. The separation time was 2 hr.

the effect of pH on partition of spores and crystals. At the range of pH from 1.0 to 12.0, the effect of pH on the partition of crystals could be ignored, but partition of spores was influenced significantly. Increasing pH value from 1.0 to 12.0, the $Y_{top}$ of spores showed a concave-up curve, and the minimum value reached at pH 3.8. Therefore, it might be at an advantage in acid (pH $<$ 2.0) or alkaline (pH $>$ 8.0) solution for separation crystals from spores.

Two kinds of salts, KCl and $Na_2SO_4$, showed different effects on the partitioning behavior of spores (Fig. 6). However, the results indicated the addition of salts could not enhance the partition of spores toward the top phase. The addition of surfactants caused the $Y_{top}$ of spores to decrease with the increase of surfactant concentration (as shown in Fig. 7). Two kinds of surfactants, sodium dodecyl sulphate and hexadecyl trimethyl ammonium bromide, were chosen as the typical representation of anionic and cationic surfactants, respectively.



## Purification of Crystals from Spores

Using the appropriate ATPS system (PEG6000 6.0%, DEX60 7.5%), the top phase would contain large quantities of spores whereas the bottom phase

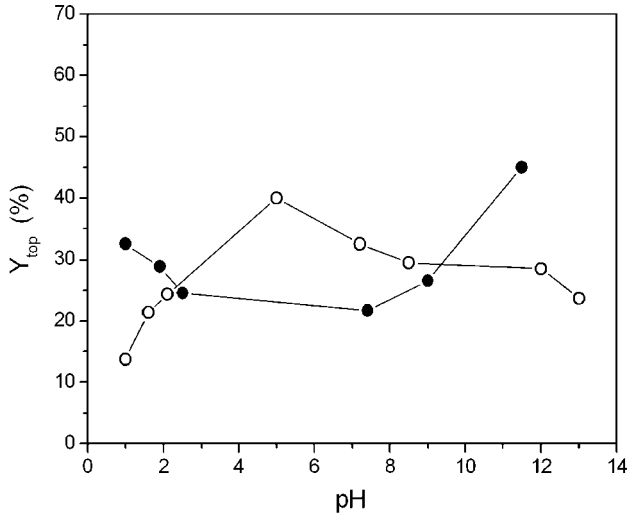


***Figure 6.*** Effect of addition of salts, 50 mM $Na_2SO_4$ (○) and 100 mM KCl (●), on partition of spore in PEG6000/DEX60 system at 25°C. System: PEG6000 6.0%, DEX60 7.5%. The separation time was 2 hr.

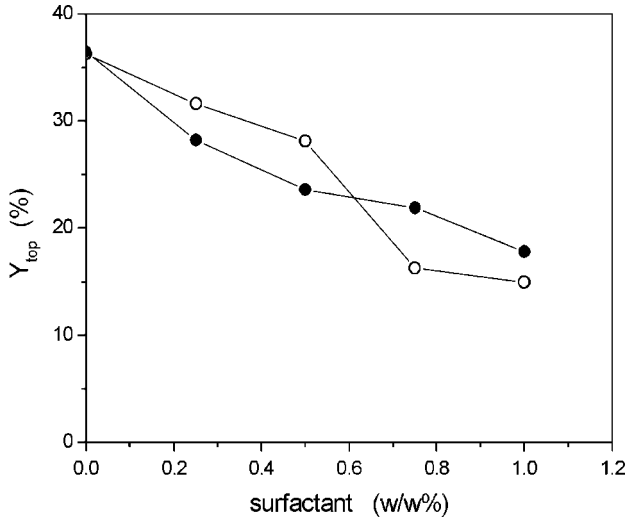


***Figure 7.*** Effect of addition of surfactants, sodium dodecyl sulphate (○), and hexadecyl trimethyl ammonium bromide (●) on partition of spore in PEG6000/DEX60 system at 25°C. System: PEG6000 6.0%, DEX60 7.5%, pH 7.2. The separation time was 2 hr.



and interface still contained crystals and some residual spores. To obtain highly purified preparations of crystals from spores, the multistep aqueous two-phase technique was used. After first partition in PEG 6000/DEX60 system, the top PEG-rich phase was carefully aspirated off without disturbing the interface, then the fresh top phase of PEG 6000 6.0%/DEX60 7.5% system was added and the process ("pass") repeated. The number of passes required depended on the quantity of starting material and the degree of purity desired in the final crystal preparation; usually four passes were required. The results of lab-scale separation (40 g) at different pH values are presented in Fig. 8. The data indicated that the separation results were comparable to that of tube-scale experiments (10 g), and the best preparations were obtained at pH 1.8 and pH 10.0. It was found that large number of flocculates increased in the top phases at pH 1.8, which multiplied the difficulties of separation operation, so the separation conditions based on pH 10.0 were more suitable for practical case. About 93.5% of spores could be removed, and the yield of crystals reached more than 83%. Crystals isolated and purified in this manner have been demonstrated to retain their toxicity.

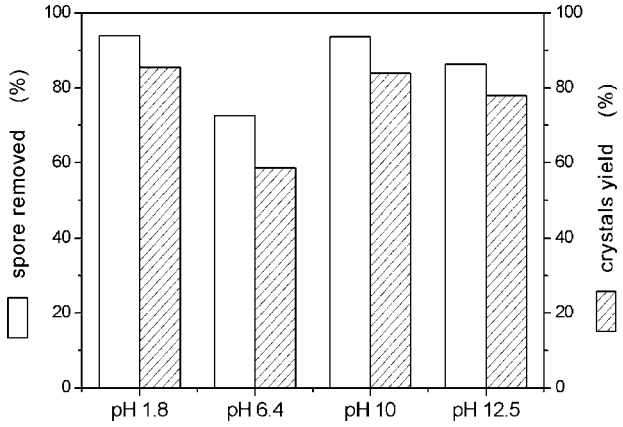


***Figure 8.*** Effect of pH on the purification of crystals free from spores on lab-scale. The experiments were performed in four-step extraction with successive top phase of a single bottom phase. The total weight of system was 40 g.



## Process Integration of Collection and Purification

Based on the previous results, two separation passes, collection of spores and crystals from broth and purification of crystals from spores, were integrated into one separation process, as shown in Fig. 9. First, using PEG6000/$(NH_4)_2SO_4$ system (PEG6000 2.0%, $(NH_4)_2SO_4$ 16.0%), spores and crystals concentrate mostly in the top PEG-rich phase and interface and can be collected directly from the fermentation broth, meanwhile the volume for next-step pass is reduced effectively. Second, the top-rich phase containing spores and crystals can be carried out in two ways: (1) after

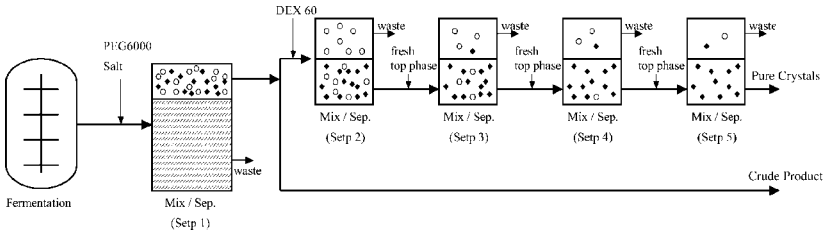


***Figure 9.*** Scheme of integration of collection and purification of parasporal crystal from *Bt* fermentation broth. Mix = mixing; Sep = separation.

***Table 3.*** The integrated process for collection and purification of parasporal crystals from fermentation broth on lab-scale at room temperature.

| Step[a] | System | Total weight[b] (g) | pH | Phase ratio | Spores removed (%) | Crystal recovery (%) |
|---|---|---|---|---|---|---|
| 1 | PEG6000/$(NH_4)_2SO_4$ | 505 | 7.2 | 0.11 | 18 | 96 |
| 2 | PEG6000/DEX60 | 275 | 10.0 | 1.6 | 28 | 92 |
| 3 | PEG6000/DEX60 | 275 | 10.0 | 1.7 | 22 | 95 |
| 4 | PEG6000/DEX60 | 275 | 10.0 | 1.7 | 15 | 98 |
| 5 | PEG6000/DEX60 | 275 | 10.0 | 1.7 | 8 | 99 |
| Total | | | | | 91 | 81.4 |

[a]The number of step is corresponding to extraction step in Fig. 9.
[b]The total weight of the system.



centrifuged or filtrated to remove PEG then yields the crude product; and (2) directly combines with appropriate amount of DEX60 solution (30%) yielding PEG6000/ DEX60 system (PEG6000 6.0%, DEX60 7.5%, pH10.0), then after washing with fresh top PEG-rich phase for three times, most of spores are removed and the pure crystals are obtained. The integrated process has been carried out in lab-scale. The initial amount of broth was 400 g. In step 1, 25 g of PEG6000 40% solution and 80 g of $(NH_4)_2SO_4$ solid powder were added to form the system of PEG6000 2.0%/$(NH_4)_2SO_4$ 16.0%. After partitioning in PEG/salt system, 55 g solution combining top phase and interface was obtain. Then 70 g of DEX60 30% solution and 150 g distilled water was added to form the system of PEG6000 6.0%/DEX60 7.5%. The pH was adjusted to 10.0. After partitioning and three times washing by the fresh top phase, the total yield of crystals could reach 81.4%, and more than 90% of spores could be removed. The results are list in Table 3. It demonstrated that the integrated separation process is feasible and suitable for large-scale purification of parasporal crystals free from spores.

## CONCLUSION

Effective solid-liquid separation (i.e., cell mass), cell debris, and insoluble, is usually troublesome to achieve in downstream processing of biological products. The isolation and purification of parasporal crystals of *Bt* directly from fermentation broth is a good typical case. Using appropriate

aqueous two-phase extraction, the parasporal crystals could be collected directly from broth and be purified effectively from spores. In PEG/$(NH_4)_2$ $SO_4$ system, spores and crystals concentrated mostly in the top PEG-rich phase and interface. The results of lab-scale extraction with PEG6000 2.0%/$(NH_4)_2SO_4$ 16.0% indicated that the collection of spores and crystals directly from broth was successful. The yield of crystals could reach 96%, and the volume for next-step separation was reduced significantly. Furthermore, crystals could be purified from spores using optimum PEG6000/DEX60 system. The appropriate system was PEG6000 6.0%/DEX60 7.5%. The pH and separation time were found as two main factors to influence the partitioning of spores, and were chosen as 10.0 and 1.5 ~ 2.0 hr, respectively. Three consecutive extractions of the bottom phase with fresh top PEG-rich phase had to be carried out to ensure 93.5% removing of spores with a yield of 83% of crystals. Finally, two separation passes, collection in polymer/salt system and purification in polymer/polymer system, were integrated into one separation process. The integrated process was implemented in lab-scale with a total yield of 81% of crystals and more than 90% removing of spores. It demonstrates that separation techniques based on aqueous two-phase systems could offer a rapid and selective extraction of biological particles with similar physical sizes. The integrated separation process will have a better application for isolation and purification of some recombinant exogenous proteins, such as inclusion bodies, from cell mass, and cell debris. Work is in progress in this direction.



## ACKNOWLEDGMENTS

The authors are grateful to Professor Xiong Guan and Mr. Zhi-Peng Huang for providing the *Bt* strain and many helpful discussions. The authors also thank the National Natural Science Foundation of China for the financial support and Carbamyl AB for the kind gift of Reppal PES samples.

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Received April 2002
Revised September 2002