# Changes in physiological properties as a criterium for detection of plasmid loss in *Bacillus thuringiensis*

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***Abstract***
Recent technological improvements have extended the application range of dielectric permittivity biomass measurements to on-line monitoring of physiological changes during bacterial fermentations. In an industrial fermentation of *Bacillus thuringiensis,* it is important to verify the intactness of bacterial plasmid content in all steps of culture. Changes in certain plasmid resident properties of this bacterium affect the permittivity measurements and could represent the potential signs of change in the plasmid content. In order to study this, the permittivity measurements of cultures of two plasmid-containing ($Cry^+$) and plasmid-free ($Cry^-$) phenotype strains of *B. thuringiensis* H14 were followed and compared throughout the fermentation. They showed different profiles of permittivity during vegetative growth and sporulation phases which related to the inability of the $Cry^-$ phenotype strain to form aggregates and proteinaceous crystals, respectively. $Cry^-$ strain grew faster than $Cry^+$ strain, but both of them were able to sporulate. The respiratory quotients of strains were similar during the growth phase but during sporulation phase the $Cry^-$ strain had a lower respiratory quotient than the $Cry^+$ strain.
***Keywords:*** Aggregation behaviours; *Bacillus thuringiensis* H14; Bioinsecticide production; Fermentation; Permittivity; Plasmid

## INTRODUCTION

During the last two decades, extensive efforts have been made to control insect vectors by *Bacillus thuringiensis* as the most important microbial control agent. *Bacillus thuringiensis* is a gram-positive and spore-forming soil bacterium distinguishable from the closely related species *Bacillus cereus* by its ability to form large proteinaceous crystalline inclusions that appear during sporulation (Buchanan and Gibbons 1974). These parasporal inclusions, which are composed of insecticidal crystal proteins (also called δ-endotoxins), are the basis for the commercial use of *B. thuringiensis* as a control agent of various insect pests (Feitelson *et al.*, 1992). A large number of studies have revealed correlations between the production of δ-endotoxins and the presence of plasmids (Bulla *et al*., 1980; Carlton and Gonzalez, 1985). Often plasmids confer an obvious advantage to the host, or encode traits that favour their own maintenance and survival. Stahly *et al.* (1978) were the first to attempt to correlate the presence of plasmids with crystal production in *B. thuringiensis*. They made the important observation that $Spo^+Cry^-$ (spore containing, crystal free) mutants, induced by heat shock, have lost the six plasmids detected in the parental $Cry^+$ strains of subsp. *kurstaki* HD-1, which implicated one or more of the plasmids in crystal production. Later investigations (Gonzalez and Carlton, 1980; Gonzalez *et al*., 1981) of $Spo^+Cry^-$ mutants indicated the involvement of specific large plasmids in the production of crystals by several strains. These investigations also demonstrated that successive cultures of some strains can result in a sequential loss of plasmids and/or in plasmid rearrangement. *B. thuringiensis* can lose and exchange plasmids during culturing (Gonzalez *et al*., 1982). Therefore, manufacturers try to limit the number of passes of the bacterium from the initial flask culture to the fermentation tank (Couch, 2000). However plasmid loss is a real risk in the industrial fermentation of

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this bacterium that can waste money and time by producing a non-active product. *B. thuringiensis* subsp. *israelensis* (serotype H14), which is the most produced bioinsecticide in large-scale fermentation, is the bioinsecticide of choice in worldwide programs to control mosquitoes and blackfly vectors (Margalith and Ben-Dov, 2000). The conjugative plasmid pXO16 (250 kb) and the toxin-encoding plasmid pBtoxis (137 kb) are two known large plasmids in this mosquitocidal bacterium. The genetic basis of the aggregation-mediated conjugation system is located on the first plasmid (Andrup *et al*., 1993; Faust *et al*., 1983; Jensen *et al*., 1995) whereas the insect pathogenicity of this bacterium depends on the presence of the second plasmid (Berry *et al*., 2002; Faust *et al*., 1983).

In a previous investigation it was shown that changes in these resident plasmid properties could be considered as the simple potential signs of changes in the plasmid content (Sarrafzadeh *et al*., 2007). However the need for a faster technique still remains and thus has stimulated this work. Dielectric permittivity measurement is one of the most recent techniques used for, on-line biomass quantification and qualification (Yardley *et al*., 2000). The basic principles and the theory underlying the measurements were published 50 years ago (Schwan, 1957): When exposed to an electric field, living cells behave as tiny capacitors, accumulating charges at the cell surface. The permittivity of living cell suspensions is dependent on the frequency, and falls in a series of steps, called dispersions, as the frequency increases. The β-dispersion, between 0.1 and 100 MHz, results from the build-up of charges at cell membranes. The difference between permittivity measurements made at two frequencies, on either side of the β-dispersion range, is proportional to the viable biomass concentration. However the variation of the dielectric permittivity of cell suspensions is dependent not only on the cell concentration but also on medium and cell cytoplasm conductivity, cell size and membrane capacitance. The latter parameters are dependent on the physiological state of the cells and make this technique a highly promising tool for on-line monitoring of physiological changes in cell state. Use of the permittivity signals as an indicator of the sporulation in combination with optical density measurements has already been reported (Sarrafzadeh, 2005a). On the other hand, it has been shown that significant changes in insecticidal activity occur as a result of sporulation and synthesis dynamics of the toxin (Manonmani and Balaraman, 1987). Study of certain changes in the physiological properties of *B. thuringiensis* especially the kinetic behaviors of growth and sporulation could reveal some differences between plasmid-containing and plasmid-free strains of this bacterium. The present investigation describes the fermentations of two plasmid-containing and plasmid-free strains of *B. thuringiensis* H14 and explores the results of on-line permittivity measurements of these fermentations in order to evaluate its pertinence as an on-line method for detection of loss of plasmid during fermentation.

## MATERIALS AND METHODS

### Microorganisms

Two plasmid-containing ($Cry^+$) and plasmid-free ($Cry^-$) phenotype strains of *B. thuringiensis* serotype H14 (Ecautec S.A., Tahiti, French polynesia) were used as described previously (Sarrafzadeh *et al.*, 2007). The lyophilized form of $Cry^+$ strain was considered as the mother strain.

### Fermentation procedure

The strains were cultivated in a 20 l fermentor (Biolafitte) in which 9 l of a semi-synthetic culture medium containing essentially glucose, hydrolyzed casein, yeast extract, ammonium sulphate and other salts as presented previously (Sarrafzadeh *et al*., 2005b), were sterilized *in situ.* Fermentations were initiated under identical conditions in batch mode at 30ºC. Fed-batch cultures were started from the 4$^{th}$ h to the 24$^{th}$ h of fermentation at a constant feed rate of 150 ml/h. Dissolved oxygen (DO) was monitored continuously by an Ingold electrode. The pH was maintained at 6.8 during the growth phase by 3 M NaOH. The fermentor was equipped with an on-line BT65 optical sensor (Wedgewood, USA). The response of this sensor is not linear, and had to be corrected using the empirical equation described previously (Sarrafzadeh *et al.,* 2005a). The $CO_2$ concentration at the off-gas was measured by an infrared gas analyzer (Abiss, France).

### Dielectric permittivity

Absolute permittivity increment and conductivity, respectively expressed in picoFarads/cm (pF/cm) and milliSiemens/cm (mS/cm), were measured using a Biomass System (FOGALE Nanotech, France), fitted with a standard 25 mm diameter probe. The Biomass System is a multi-frequency capacitance device operating in the range of 0.3-10 MHz. It computes the

medium permittivity from the difference between capacitance measurements made at two frequencies. The higher frequency (10 MHz) is used as a reference, while the middle frequency is chosen as the characteristic frequency ($f_c$), in the middle range of the β-dispersion to minimize the influence of cell size variations. The third lowest frequency is used to compensate the electrode polarization in conductive media. The $f_c$ frequency (2 MHz) was calculated according to the β-dispersion theory, assuming an average bacterial cell size of 5 micron. Data were recorded on a personal computer, using the X-system program provided by the manufacturer.

**Verification of presence of plasmid**

For DNA preparation, both strains were inoculated in to 25 ml of Luria-Bertani broth, and grown overnight (14 to 17 h) at 28ºC to a final optical density (540 nm) of 7 to 10. Three ml of the above cell cultures were pelleted by centrifugation and plasmid DNA extracted using the QIAGEN plasmid mini kit, as described previously (Sarrafzadeh *et al.,* 2007). The presence of plasmid was also verified by amplifying a *cry* gene in a PCR analysis.

**Microscopic and other off-line methods**

Sporulation and formation of inclusion bodies in the $Cry^+$ and $Cry^-$ phenotypes of strains were studied during fermentation by using a phase contrast microscope (Olympus BX60) and a Thoma's chamber as previously described (Sarrafzadeh *et al*., 2005b). The vegetative and sporulated cells were distinguishable due to the refractile nature of spores. Optical density of the medium was measured at 540 nm ($OD_{540}$) after dilution with distilled water to remain in the 0-0.3 linear range. Dry biomass weight was determined by drying the cells obtained from 25 ml cultures (centrifuged at 5000 ×*g*, 15 min), overnight at 100ºC.

## RESULTS

**Preliminary evaluation**

The first step was to evaluate the sensitivity of the Biomass System, as a capacitive detection instrument, by establishing the correlation between permittivity and biomass concentration in different physiological cell states. The performance of the permittivity measurements was compared against two off-line biomass measurements: optical density at 540 nm ($OD_{540}$) and total cell count ($C_T$). As seen in Figure 1, three different relations can be established between permittivity and these measurements with respect to three distinct physiological phases detected previously as $\Phi_1$: rapid growth, $\Phi_2$: transition phase and $\Phi_3$: sporulation phase (Sarrafzadeh *et al.*, 2005a). The most straightforward relation can be observed during $\Phi_1$ when only vegetative cells are present. In this phase the following linear correlations between permittivity (ε) and other measurements were obtained: $\varepsilon = 0.28\ OD_{540}$; $\varepsilon = 3.5 \times 10^{-9}\ C_T$. Whereas in the two other phases, the presence of cells in diverse physiological states and different physical perceptions of them by these biomass quantification techniques, don't let such simple linear correlations to be obtained.

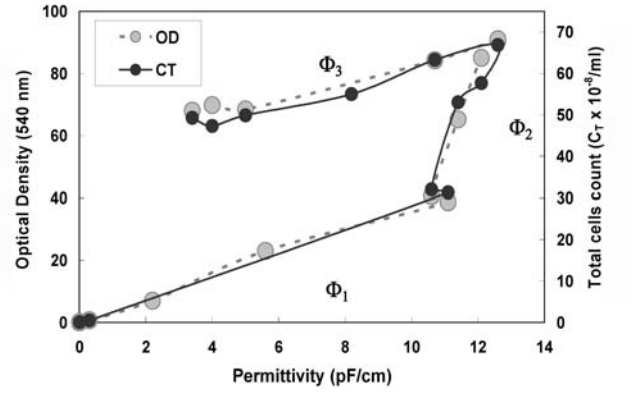


**Figure 1.** Time-independent plot of total cell counts ($C_T$) and optical density at 540 nm as a function of permittivity during cultures of the plasmid containing strain ($Cry^+$) of *Bacillus thuringiensis* H14. The three culture phases ($\Phi_{1\text{-}3}$) have been specified on the figure.

**Growth, sporulation and inclusion bodies**

Comparisons between permittivity and conductivity profiles during the fed-batch cultures of two strains of *B. thuringiensis* in the fermentor have been presented in Figures 2 and 3. The growth of the $Cry^-$ strain with respected to permittivity measurements started with a longer lag phase than its parental $Cry^+$ strain due to its more sporulated preculture (Fig. 2). However its specific growth rate during $\Phi_1$ was higher than $Cry^+$ strain ($0.71\ h^{-1}$ against $0.51\ h^{-1}$). A similar faster growth has been observed during the cultvation of two strains in flasks containing Luria-Bertani (LB) media, in which the OD 540 nm reached 7 for $Cry^-$ strain and 4.5 for $Cry^+$ strain, after a 12 h cultivation period. In agreement with this faster growth, the concentration of DO became limiting more quickly in the culture of the $Cry^-$ strain and the $CO_2$ concentration in the off-gas line of the fermentor also attained a value of 6% during the

vegetative growth phase against only 4% for the Cry$^+$ culture. The permittivity profiles of the two cultures differed deeply during the next phases of fermentation. The observation of the first refractile cells indicated that sporulated cells appeared after 10 h of fermentation in the culture of the Cry strain whereas for Cry$^+$ strain this time was about 14 h means that the Cry- culture entered earlier in the transition phase 2. Although sporulation started earlier during the culture of the Cry strain, its development appeared to be slower and for this reason its permittivity decline during the sporulation phase was less important. While in the Cry$^+$ strain sporulation reached approximately 85% at the end of fermentation, the final sporulation level of the Cry$^-$ strain was approximately 75%. The conductivity profiles of the two cultures, in general had the same trends, however their increment during the sporulation phase were higher for the Cry$^+$ culture than that of Cry$^-$ strain due to the higher levels of sporangial lysis (Fig. 3).

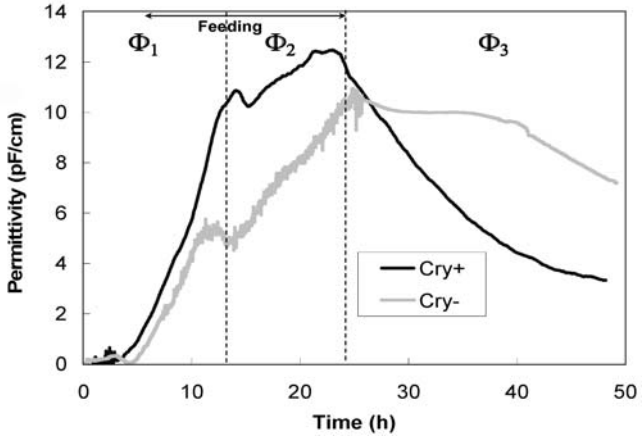


**Figure 2.** Growth profiles during fed-batch cultivation of the Cry$^+$ and Cry$^-$ strains of *B. thuringiensis* H14 with respect to on-line measurements of dielectric permittivity. The three culture phases ($\Phi_{1\text{-}3}$) have approximately been specified on the figure for both cultures.

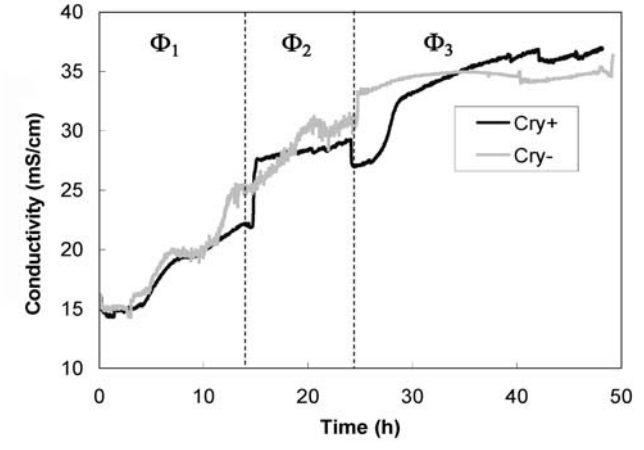


**Figure 3.** Variation of conductivity during fed-batch cultivation of two Cry$^+$ and Cry$^-$ strains of *B. thuringiensis* H14. The three culture phases ($\Phi_{1\text{-}3}$) have approximately been specified on the figure for both cultures.

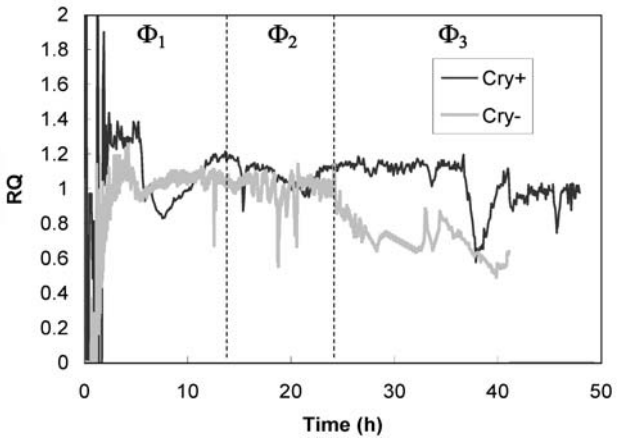


**Figure 4.** variation of respiratory quotient (RQ) during cultivation of the two Cry$^+$ and Cry$^-$ strains of *B. thuringiensis* H14. The three culture phases ($\Phi_{1\text{-}3}$) have approximately been specified on the figure for both cultures.

The spores of the Cry$^-$ strain were slightly bigger than the spores of the Cry$^+$ one. Inclusion bodies were observed in both cases by phase contrast microscopy, with a slightly smaller size in the Cry$^-$ strain. However, a further analysis by transmission electron microscopy of inclusion bodies in the Cry$^-$ strain showed that they are not proteinaceous inclusions.

The respiratory quotients (RQ) of both cultures have also been compared. During the growth phases both of them had an RQ of approximately 1 or higher, but during the sporulation phase the Cry$^-$ showed a more significant decline of RQ to approximately 0.8 (Fig. 4).

**Aggregation properties**

It has been shown previously that the Cry$^+$ strain can form cell aggregates (Agr$^+$) but the Cry$^-$ phenotype fails to do this due to loss of the 250-kb plasmid pXO16 (Sarrafzadeh *et al.*, 2007). On the other hand, cell aggregation can affect the permittivity measurements (Mas *et al.*, 2001). Therefore, the cultures of the two strains were inspected for their ability to form large aggregates throughout the fermentation. The inspections were made during the different phases of fermentation: in the first hours of cultures, in the middle of rapid growth phase, at the beginning and end of the transition phase and during the sporulation phase.

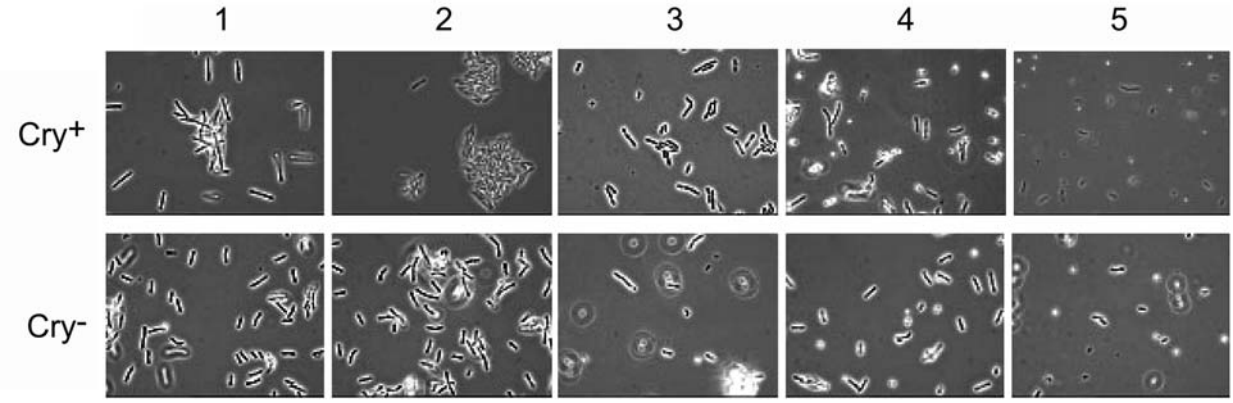


**Figure 5.** Comparison between the aggregation properties of the two strains. The $Cry^+$ strain was able to form aggregates during vegetative growth but the aggregation behavior was not observed for the $Cry^-$ strain. The numbers at the top of the figure refers to the different times of fermentation as follows: (1) Vegetative cells at the beginning of the fermentation. (2) in the middle of rapid growth phase. (3, 4) at the beginning and end of the transition phase and (5) during the sporulation phase.

As seen in Figure 5 the vegetative cells of the $Cry^+$ strain were able to form cell aggregates especially during rapid growth phase in which aggregation was very significant. But no evident aggregation was observed with the $Cry^-$ phenotype all throughout the fermentation.

## DISCUSSION

Two plasmid-containing and plasmid-free strains of *B. thuringiensis* H14 were compared in order to elucidate how the loss of plasmid can affect the many properties of this bacterium. The plasmid-free strain was in many physiological points different from the plasmid-containing strain. Therefore, by studying some physiological properties and the changes in the behavior of a *B. thuringiensis* strain during fermentation, certain points about the alteration in the plasmid content of this bacterium could be raised. In fact by having a simple and fast method to verify regularly the presence of the δ-endotoxin encoding plasmid is indispensable during the fermentation of *B. thuringiensis*. It becomes more important when on the basis of the obtained results we now know that with competition with the Cry+ strain the $Cry^-$ strain can grow faster and can become the predominant population during an industrial fermentation. Many methods could be applied for this purpose. One may simply follow the presence of inclusion bodies during culture or fermentation process by microscopic observation. But it has been shown that the observation of inclusion bodies could not always guarantee their proteinaceous nature. The granules that can easily be confused which crystalline inclusion bodies have frequently been reported in many prokaryotes (Shively 1974). In addition the crystalline inclusion bodies normally appear during sporulation whereas we need to be able to detect the loss of insecticidal activity hours before and avoid the production of huge amounts of inactive biomass. Capacitance on-line sensors have recently demonstrated a high ability for detection of changes in physiological properties by measuring dielectric permittivity (Neves *et al.*, 2000; Sarra *et al.*, 1996). Here, in the special case of *B. thuringiensis* H14, this technique has been applied to show its usefulness for prediction of plasmid loss. The aggregated phenotype ($Agr^+$) of *B. thuringiensis* H14 is correlated with a conjugation-like plasmid transfer and characterized by the formation of aggregates when the bacteria are socialized during exponential growth (Jensen *et al.,* 1995). These authors have shown that $Agr^+$ phenotype is associated with the presence of the large (250 kb) self-transmissible plasmid pXO16. In our experiments, we have found that the $Cry^+$ and $Cry^-$ strains correspond to the $Agr^+$ and $Agr^-$ phenotypes respectively, suggesting that the loss of the pBtoxis plasmid is normally accompanied by the loss of the pXO16 plasmid. We could not find the $Cry^+Agr^-$ or $Cry^-Agr^+$ phenotypes during the course of fermentation, and thus were unable to confirm if the insecticidal activity and the aggregation property are always closely correlated or not. But it can be concluded clearly that the absence of large aggregates in the fermentation culture of this bacterium, at least, could proclaim the risk of the loss of certain plasmids and among them pBtoxis. Since cell aggregation affects permittivity measurements, a different profile for $Cry^-$ strain will be expected. In addi-

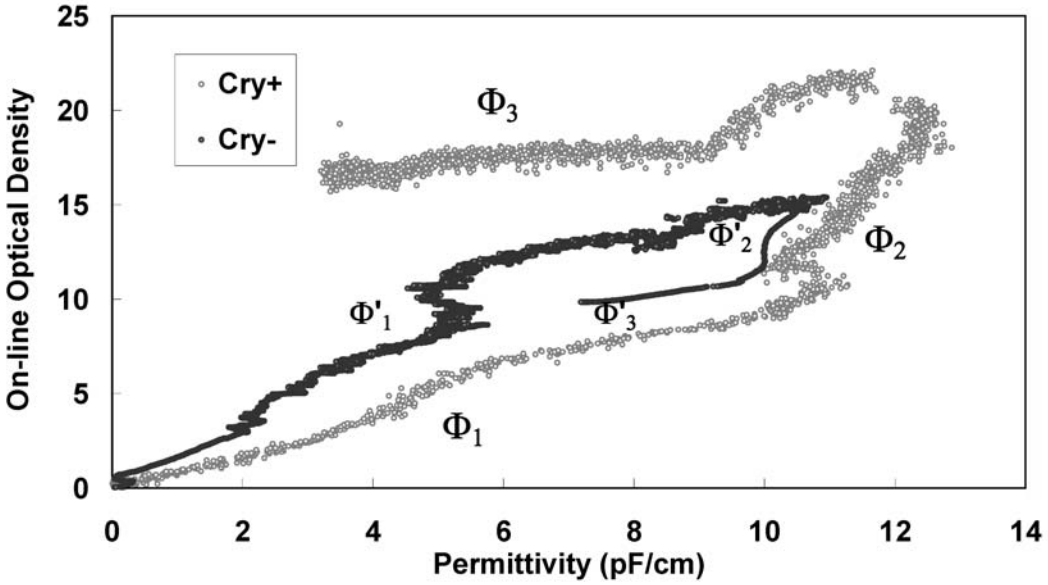


**Figure 6.** Comparison of profiles representing on-line measurements of OD versus permittivity of $Cry^+$ and $Cry^-$ strains of *B. thuringiensis* throughout the fermentation proces. The three distinct culture phases for $Cry^+$ have been specified by ($\Phi_{1\text{-}3}$) and for $Cry^-$ by ($\Phi_{1\text{-}3}$) on the figure.

tion, an obvious characteristic of a *Bacillus* culture is the presence of three distinct cell types: vegetative cells, sporangia, and spores (Fordyce *et al*., 1996). When the nutrients are abundant, the vegetative cell can grow quickly by division. When the nutrients are depleted, some of the cells will die, while other cells will survive by differentiating to form sporangia. Capacitance measurement could only sense vegetative cells and sporangia (because these two populations had functional, insulating cytoplasmic membranes). However the signal amplitude responses of the two populations are different, because the structure of these cells differ deeply (Sarrafzadeh *et al*., 2005b). In the *B. thuringiensis* case, due to the formation of some proteinaceous crystals concomitantly with prespore maturation within the sporangia, permittivity will be affected more strongly. The optical density perception of these phenomena is completely different and therefore its relation with permittivity during vegetative growth where the cells form aggregates and during sporulation where the cells form crystal proteins will be different. In Figure 6 the changes of OD versus permittivity have been illustrated throughout the fermentation of both strains. They showed different profiles in all growth phases. During the vegetative growth phase ($\Phi_1$) the differences could be related to aggregation properties. In this phase until about the $12^{th}$ h of fermentation the following relationships were found between on-line optical density (OD) and ($\varepsilon$): OD= $\varepsilon$ for $Cry^+$ and OD= 1.7$\varepsilon$ for $Cry^-$. During the sporulation phase the OD/$\varepsilon$ for $Cry^+$ tended to increas while the opposite behavior was observed for $Cry^-$. However for the main objective of on-line and early detection of plasmid-related changes, the observed differences in the vegetative growth phase are more useful. Therefore by analyzing the effect of cell aggregation on permittivity measurements a highly promising technique could be established for on-line detection of plasmid loss. It should be noted that aggregation is not a simple phenomenon with a regular effect on permittivity. Because the type, size and numbers of aggregates could affect permittivity measurements and thus result in diverse signal amplitude. In addition, since the permittivity increment depends on the electrical field frequency, the selection of the most sensible frequency to cell aggregation is critical. Instead of using a dual frequency measurement, performing a scan over the whole frequency range of the $\beta$-dispersion, could be more informative, thus providing additional information, such as an indirect estimation of the aggregates size which could be the purpose of future research.

In conclusion, the lack of certain resident plasmid properties in *B. thuringiensis* can be used to conduct a rapid prediction of the loss of many plasmids in this bacterium. The aggregation property could be a useful sign for revealing the changes in the plasmid content of *B. thuringiensis* and could prevent the production of a non-active culture during the industrial fermentation of this bacterium. This work is also another demon-

stration of the usefulness of on-line dielectric permittivity measurements in fermentation. Not only can the biomass concentration be readily monitored, but physiological changes can also be detected. The benefits of the combination of on-line optical density and permittivity measurements should not be limited only to the monitoring and control of *B. thuringiensis* H14 culture but should be of more general applicability.

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