Effect of cultivation conditions on spore production from 
*Bacillus amyloliquefaciens* B128 and its antagonism to 
*Botrytis elliptica*

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**Abstract**

**Aims:** To maximize spore production by *Bacillus amyloliquefaciens* B128, and its antagonism to the fungal pathogen *Botrytis elliptica* B061.

**Methods and Results:** In the 5-l stirred-tank bioreactor (STR), with the 0·5 vvm aeration rate, an agitation rate of 200 rev min⁻¹ significantly enhanced the spore yield compared to the same in 300 rev min⁻¹ cultivations. In a 20-l airlift bioreactor (ALR) the maximal spore production was further increased with a controlled aeration rate of 2·5 vvm operated in a 24-mesh net-draft tube mode, and no pH control cultivation. This spore yield in the 20-l ALR was five- and eightfold higher; in addition the cultivation period was 19 h shorter, compared to that obtained from shaker flask and in the 5-l STR cultivations respectively.

**Conclusions:** Although culture conditions are still to be optimized, by using an ALR with net-draft tube, a scaling up from shaker flasks and STR to ALR of spore production by the strain B128 is technically feasible.

**Significance and Impact of the Study:** The spore yields obtained using bioreactors were much higher than those previously reported. The freshly produced spore preparations from the B128 strain significantly antagonized the grey mould pathogen *B. elliptica*.

**Keywords**

airlift bioreactor, antagonism, *Bacillus amyloliquefaciens*, *Botrytis elliptica*, spore production.

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**Introduction**

Chemical insecticides and herbicides have been used extensively over the past 50 years because of their effectiveness and ease-of-use. Undoubtedly, the widespread use of chemical pesticides will continue although public concern about the safety of these chemicals has led to more rigorous registration requirements. Many of the most effective chemical pesticides have failed to pass re-registration (Brar et al. 2006). The gradual replacement of these chemical pesticides from the marketplace coupled with the emergence of herbicide- and insecticide-resistant has heightened interest in the development of biologically based pest control strategies (Yezza et al. 2006). Insect and plant pathologists have identified literally hundreds of microbial agents, including bacteria, fungi, protozoa and viruses that showed good potential producers of biological pesticides (Brar et al. 2006). *Bacillus amyloliquefaciens* is a Gram-positive, spore forming bacteria, and is closely related to *Bacillus subtilis*. Indeed, *B. amyloliquefaciens* has been studied extensively as a producer of industrial enzymes, such as α-amylase, subtilisin (a protease which is used as laundry detergents and contact lens cleansers), barnase (a ribonuclease), and antibacterial and antifungal peptide antibiotics (Yu et al. 2002). In addition, it has been reported that several strains have a potential for the biological control of several plant diseases (Mari et al. 1996; Chen and Wu 1999; Chiou and Wu 2001; Yoshida et al. 2001). Importantly, the strain B128 was reported as a biological control agent against *Botrytis elliptica* B061, a fungal pathogen of lily grey mould (Chiou and Wu 2001). This pathogen destroys all
lilies in field when environmental conditions are conducive to the disease development. Moreover, effective formulations having a suspension (1 × 10^6 CFU ml⁻¹) of B. amyloliquefaciens for the control of lily grey mould in the field has been established (Chiou and Wu 2001). All these attributes make the B128 strain an ideal candidate for large-scale production of spores for field applications.

Mass production is one of the important aspects of the commercial development of a biocontrol product. At present, submerged cultivations are often preferred in industry, as they tend to be more economic, shorter and easier to automate (Vidyarthi et al. 2002; Jagannath and Tsuchido 2003). The production of antibiotics, amino acids, ethanol and organic acids by submerged cultivations has provided an extensive knowledge base for optimizing the bioprocesses of biocontrol agents (Adams et al. 1999; Liu and Tzen 2000; Vidyarthi et al. 2001). In addition, the ability of many microorganisms to produce high concentrations of spores over short cultivation times has highlighted commercial interest in this method of bio-insecticide production (Brar et al. 2006). Thus, our objective was to determine if submerged cultivations could be used to produce high concentration of spores from the strain B128, which might find use as a potential biological control agent against the fungal pathogen B. elliptica B061. With this aim in mind, shaker flask cultivations were carried out to determine the effect of antifungo agents (AFAs) and starting pH on the strain B128 spore production. In addition, the effect of agitation speed in a 5-l stirred-tank bioreactor (STR), and pH and aeration rate in a 20-l airlift bioreactor (ALR) were examined for the rapid production of maximal spores of the same strain. Additional studies were also conducted to determine biocontrol efficacy.

Materials and methods

Culture maintenance, inoculum development and medium preparation

The strain B. amyloliquefaciens B128 used in this study was isolated from an infected lily plants provided by one of the authors, Prof. Wen-Shi Wu. Stock cultures of the strain B128 were maintained on NB (0.8% nutrient broth and 0.3% yeast extract) agar at 4°C and ~70°C for short-term and long-term storage, respectively, at the Institute of Biotechnology, Chaoyang University of Technology, Wufeng, Taiwan, ROC.

The micro-organisms were cultivated in a 500-ml Erlenmeyer flask containing 200 ml of complex medium (3 g l⁻¹ yeast extract, 8 g l⁻¹ nutrient broth) on a rotary shaker at 200 rev min⁻¹ and 30°C for 12 h. The standard medium used for all shaker flask and bioreactor production studies consisted of (g l⁻¹): 12.7 of lactose, 16.7 of tapioca, 1.8 of ammonium sulfate and 8.0 of peptone (Difco, Becton Dickinson, Sparks, MD, USA), as optimized by response surface methodology (Rao et al. 2007). In addition, minerals (g l⁻¹): KH₂PO₄ 2; K₂HPO₄ 1; MgSO₄·7H₂O 2; CaCl₂·2H₂O 0·01; FeSO₄·7H₂O 0·045; ZnSO₄·7H₂O 0·001; MnSO₄·4H₂O 0·001 (Sigma, St Louis, MO, USA) were also added to the cultivation medium. The cultivation media were autoclaved at 121°C for 15 min before use. Cell and spore concentrations were determined microscopically with a haemocytometer.

Shaker flask cultivations

A 2% (v/v) solution of inoculums was used for shaker flask cultivations to evaluate the effects of AFAs and starting pH on the production of spores from B. amyloliquefaciens B128. Cultures were grown at 30°C and 200 rev min⁻¹ in a rotary shaker incubator (Yih-Der, LM-530R, Taipei, Taiwan), in 500-ml baffled Erlenmeyer flasks with 200 ml of standard production medium. A few seconds before the start of an experiment, and again after 24-h cultivation, a 0.4% (v/v) AFA was added, and no pH control was applied for all cultivations. The AFAs used in this study include RC-232 and RC-234 (Rich Chemical Co., Taipei, Taiwan), KM-72 (Shin-Etsu Chemical Co., Ken Nakamura, Japan), SD-22 (Ywan-Shan Chemical Co., Taipei, Taiwan), F and F-9 (Saint Chemical Co., Taichung, Taiwan). For pH studies, the starting pH was adjusted to 9.0, 8.0, 7.0, 6.0 or 5.0 by the addition of 2 N HCl or 2 N NaOH, and 0.4% (v/v) of KM-72 was used as AFA. The final pH of the cultures was measured 48 h postinoculation.

Bioreactor cultivations

Cultivations were carried out at 30°C in a 5-l STR, and in 20-l ALR equipped with accessories and automatic control systems for dissolved oxygen (DO), pH, antifungo, impeller speed, aeration rate and temperature. Unless otherwise stated, the cultivation medium used the same as in shaker flask cultivations. The 5-l STR (BTF-600T; Bio-Top Inc., Taichung, Taiwan) cultivations were conducted with 5% inoculum in 3 l of the desired medium; the aeration rate was controlled at 0.5 vvm, and varied the agitation rate 200 and 300 rev min⁻¹.

The 20-l ALR cultivations were conducted in a net draft-tube modified bioreactor (ALF-20; Bio-Top Inc.). Preculture was cultivated as stated above, then 1.5 l of the vegetative seed culture was aseptically transferred to the bioreactor previously filled with 13.5 l of sterile medium. For effect of pH studies aeration was controlled at
1.5 vvm, whereas for influence of aeration rate studies, pH left free to change and varied the aeration rate from 1.5 to 3.0 vvm. The biogas (CO₂) production was monitored by a gas flow meter (Guardian 3000; Avensys Inc., Montreal, QC, Canada). All cultivations were performed in three replicate experiments, and the analyses were carried out at least in duplicate. The values reported here are mean values with standard deviations being <5% in all cases.

Antifungal activity

The strain B128 cultivation broth was spun down and filtered through a 0.2-μm sterile membrane. The supernatant was serially diluted with sterile distilled water (10²–10⁸ spores per millilitre) and filtered through a sterile millipore membrane filter and stored at 4°C. Botrytis elliptica B061 was cultivated on the PDA plate for 5 days (7.5 × 10⁵ spores per millilitre). A cork borer (6 mm diameter) was used to make a disk, from margin of the fungal colony every disk was placed in the centre of another PDA plate. Approximately 2 cm away, a filter paper soaked with c. 100 μl of B. amyloliquefaciens B128 culture broth or supernatant without cells or spores was laid down, then incubated at 24°C for 5–7 days (Chiou and Wu 2001). The growth of B. elliptica B061 on plates with and without the B. amyloliquefaciens B128 crude extract was compared. A concentration–response curve was obtained in which the percentage of inhibition was plotted against the spore yield.

Electron microscopy

A scanning electron micrograph of the B. elliptica was recorded by electron microscopy (EM; Zeiss DSM 950; Carl Zeiss, Jena, Germany) as we reported previously (Rao et al. 2007). After 5 days of B. amyloliquefaciens B128 spores treatment to B. elliptica B061, the fungal pathogen cell growth morphological changes were studied by transmission EM (TEM). Spores from the B128 strain were fixed at 4°C in 0.1 mol l⁻¹ sodium cacodylate buffer (pH 7.0) with 6% (v/v) glutaraldehyde, and then with 1% (w/v) osmium tetroxide (OsO₄) for 3 h, and overnight respectively. The samples were then dehydrated in a graded series of ethanol concentrations and embedded in Epon–Araldite resin. Sections were cut with an LKB Ultratome III ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a Philip CM 100 transmission electron microscope at an accelerating voltage of 100 kV. The TEM of B. elliptica B061 sections without the treatment of B. amyloliquefaciens B128 spores were cut with a Reichert-Jung ultramicrotome, and observed with JEOL JEM-1010 electron microscope.

Statistical analyses

Statistical analysis was performed using one-way ANOVA followed by Dinettes post hoc test, and the significant difference was set at P < 0.05.

Results

Shaker flasks cultivations

The effect of various AFAs on the production of spores from the strain B128 was presented in Fig. 1. The presence of AFA had a positive effect on spore production compared to the controlled cultivations (i.e without AFA in the cultivation medium). It is evident from Fig. 1 that the effectiveness in increasing spore production by the antifoaming agents follows the order KM-72 > RC-232 > SD-22 > F > F-9 > RC-234. Therefore, AFA KM-72 was chosen for further cultivations.

Next, the effect of starting pH on the maximal spore yield was investigated in shaker flask cultivations. Typical data extracted from the multiple experiments are presented in Table 1, where significant differences (P < 0.05) were observed in spore yield after 48 h of cultivation with the starting pH ranging from 9.0 to 5.0. The initial alkaline pH value of 8.0 was impressive where the concentration of spore production increased moderately from 7.96 × 10⁸ to 10.30 × 10⁸ (spores per millilitre) compared to control cultivations (no pH control). In contrast to this, the acidic pH (6.0 and 5.0) values significantly (P < 0.05) suppressed maximal spore production. Furthermore, with a starting pH of 9.0 and 8.0, cultivation medium pH ended at 7.34 and 7.08, respectively, while
no significant reduction \((P < 0.05)\) was noted in the starting pH of 7.0 or lower cultivations. On the basis of these results, an alkaline pH value was chosen as the starting point for further cultivations.

### Effect of agitation in 5-l STR

Further studies on the maximal spore production by \textit{B. amyloliquefaciens} B128 were investigated in a 5-l STR. The cultivations were carried out at a constant temperature of 30°C and agitation rate of 0.5 vvm but with different agitation speed of 200, and 300 rev min\(^{-1}\). On the basis of the shaker flasks result, the foaminess could be controlled by appropriate feeding of AFA KM-72. The effect of agitation on spore production from the strain B128 is shown in Fig. 2. The spore production was started after 30 h and reached its maximal value of \(4.65 \times 10^8\) (spores per millilitre) in 48 h of inoculation with a relatively mild agitation rate of 200 rev min\(^{-1}\). From this time onwards there was no substantial increase in spore production (Fig. 2). In contrast to this, in an agitation speed of 300 rev min\(^{-1}\), the spore production was started after 50 h and reached \(3.56 \times 10^8\) (spores per millilitre) in 100 h after inoculation. The residual sugar concentration profiles were different for the two levels of agitation tested, where the assimilation of sugars decreased with agitation speeds shifting from 200 to 300 rev min\(^{-1}\) (Fig. 2).

### Cultivations in 20-l ALR

Further studies on the production of spores from the strain B128 were investigated in a 20-l net-draft tube modified ALR. In this study, the effect of pH was investigated for spore production process at two different controlled-pH operations (pH 7.0 and 8.0), and at one uncontrolled-pH operation, at 1.5 vvm aeration rate. As shown in Fig. 3a, among the three pH values studied, no significant difference \((P < 0.05)\) was found in spore yield at the end of cultivations. However, in uncontrolled pH experiments, at the end of the run, the vegetative cells concentration was significantly \((P < 0.05)\) increased to \(8.6 \times 10^9\) cells per millilitre, which corresponds to a sixfold increase when compared to the batch performed with pH controlled cultivations. Furthermore, in uncontrolled pH experiments, the DO concentration decreased and reached to a minimum around 10% while increasing the CO\(_2\) concentration. This is in contrast in the pH controlled experiments. From all these results, we have chosen the uncontrolled pH cultivations for further studies on \textit{B. amyloliquefaciens} B128 spore production in 20-l ALR.

To optimize aeration rate in 20-l ALR, cultivations were carried out in a range of aeration rate from 1.5 up to 3.0 vvm (Fig. 3b). Among the aeration rates studied, maximal concentration of \(3.82 \times 10^9\) (spores per millilitre) spores are favoured by 2.5 vvm after 29 h of inoculation, which is fivefold higher than that aeration rate of 1.5-vvm cultivation (Fig. 3b). Furthermore, through investigating the effect of net-draft tube on the spore production, we performed cultivations for four runs under an aeration rate of 2.5 vvm, a liquid volume of 15 l in the ALR. The highest average spores were only \(5.63 \times 10^8\) (spores per millilitre) after 29 h of cultivation. Thus, the net-draft tube has significant effect on \textit{B. amyloliquefaciens} B128 spore production. During these cultivations, the DO levels at all aeration rates were reduced from 100% saturation from the beginning of culture, to a minimum of around 10–20%, then profiles were gradually increased as the spore production growth shifted to the stationary

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**Table 1** Production of \textit{Bacillus amyloliquefaciens} B128 cells and spores in shaker flask cultures at various pH levels*

<table>
<thead>
<tr>
<th>Starting pH</th>
<th>Cells (no. per millilitre)</th>
<th>Spores (no. per millilitre)</th>
<th>pH after 48 h cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>(5.25 \times 10^8)</td>
<td>(7.00 \times 10^6)</td>
<td>7.34</td>
</tr>
<tr>
<td>8.0</td>
<td>(9.13 \times 10^6)</td>
<td>(10.30 \times 10^6)</td>
<td>7.08</td>
</tr>
<tr>
<td>7.0</td>
<td>(6.88 \times 10^6)</td>
<td>(8.88 \times 10^6)</td>
<td>6.85</td>
</tr>
<tr>
<td>6.0</td>
<td>(4.75 \times 10^6)</td>
<td>(4.00 \times 10^6)</td>
<td>6.04</td>
</tr>
<tr>
<td>5.0</td>
<td>(2.00 \times 10^7)</td>
<td>(2.50 \times 10^6)</td>
<td>4.94</td>
</tr>
<tr>
<td>No control</td>
<td>(6.42 \times 10^6)</td>
<td>(7.96 \times 10^6)</td>
<td>6.95</td>
</tr>
</tbody>
</table>

*For experimental details, see the Materials and methods section.

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**Figure 2** Effect of agitation rate on the \textit{Bacillus amyloliquefaciens} B128 spore production in a 5-l stirred-tank bioreactor cultivations: cells in 200 rev min\(^{-1}\) (—■—) and in 300 rev min\(^{-1}\) (—□—); spores in 200 rev min\(^{-1}\) (—●—) and in 300 rev min\(^{-1}\) (—○—); and residual sugars in 200 rev min\(^{-1}\) (—▼—) and in 300 rev min\(^{-1}\) (—▽—). The cultivation temperature was set at 30°C with fixed aeration rate of 0.5 vvm. Results presented are the means of three different determinations from two replicated experimental runs.
phase. On the other hand, the variations in residual sugar concentration, at an initial concentration of 30 g l\(^{-1}\), decreased until the 48 h of the cultivation. In an aeration rate of 2–5 vvm cultivation, the spore formation rate, and consequently the oxygen and glucose consumption rates were high.

Antifungal activity

The antagonistic effect of \textit{B. amyloliquefaciens} B128 spores against \textit{B. elliptica} B061 was demonstrated by dual culture assay. Scanning electron microscopic observation of the growing cultures that \textit{B. elliptica} B061 grew well in the medium after 5 days of incubation as shown in Fig. 4a. However, the dual culture of spores from the strain B128 and \textit{B. elliptica} B061 showed that the bacterial spores inhibited the growth of fungal pathogen, as indicated by the formation of an inhibition zone. The results showed that the higher the concentrations of the antagonist, the lower the fungal viability. At the concentrations of \textit{B. amyloliquefaciens} B128 at 1 \(\times\) 10\(^8\), 1 \(\times\) 10\(^7\) and 1 \(\times\) 10\(^6\) spores per millilitre, the \textit{B. elliptica} B061 (spore suspension of 7.5 \(\times\) 10\(^5\) spores per millilitre) was inhibited by 85\%, 50\% and 25\%, respectively, after a 5-day incubation at 24\(^\circ\)C. This was further supported by TEM analyses. TEM sections of \textit{B. elliptica} B061 hyphae (H) interacting with \textit{B. amyloliquefaciens} B128 spores (1 \(\times\) 10\(^8\) spores per millilitre) are shown in Figs 4d,e. After 5 days of interaction of the hyphae of B061 with B128 revealed some invaded host cells was occluded with a heterogeneous material (HM) made of vesicles and was presumed to be degenerated vesicles, and organelles and electron-dense (ED) inclusions. In addition, changes in mitochondrial shape (MS), and necrosis in the hyphae (NH) growing towards the bacterial colony were also observed. Furthermore, nonmembranous electron-transparent inclusion bodies (empty cells, ES) were often irregularly distributed in the affected hyphae.

Discussion

Foam formation is an undesirable feature in many submerged cultivation processes. It results in an increase in the working volume, oxygen transfer rate during \textit{Bacillus} spp cultivation and limit the production of spores or biopesticidal activity (Vidyarthi \textit{et al.} 2001). These problems could be tackled to a certain extent by the addition of AFAs which could help to reduce the surface tension and to enhance oxygen transfer (Vidyarthi \textit{et al.} 2001; Ishikawa \textit{et al.} 2002). The shaker flask cultivation results indicate that AFAs can affect the maximal spore yield of the strain B128. Depending on which AFAs are used, however, the effects are markedly different. The use of KM-72 acted as an activator of bacterial spore germination, which would account for the increased spore production.

Scale-up is an important step in process development. Expanding a cultivation process from a laboratory-scale unit to a commercial one is a challenge because of the difficulty in assessing the factors affecting the scale-up process during the cultivation. As a result, many large-scale cultivation processes give a lower yield than was expected from the laboratory. Hence, much work has been carried out on studying the transient behaviour of these bioprocesses (Tzeng and Young 1996; Rao \textit{et al.} 2006; Yezza \textit{et al.} 2006). In the present study, the
cultivation of the strain B128 for the spore production with pH, agitation and aeration as the environmental state variable were demonstrated.

Agitation could be beneficial to the growth and performance of micro-organisms by improving mass transfer characteristics with respect to substrates, product/byproduct and oxygen (Riscaldati et al. 2000; Liu et al. 2003). The maximal production of spores was facilitated at certain agitation rates. The present study suggested that the agitation speed of 200 rev min$^{-1}$ was best for \textit{B. amyloliquefaciens} B128 maximal spore production in 5-l STR. Such trends have been reported earlier for antibiotic production by \textit{Xenorhabdus} sp. (Wang and Zhang 2007). Moreover, the STR adapted with a baffle was relatively unfavourable for the high yield of spore production (data not shown). However, the reality of the effect of agitation rate needs to be further elucidated. As expected, the concentration of residual sugars decreased as the culture progressed, with corresponding increases in spore production. In general, significant biomass accumulation is necessary for optimal spore yield as spore production is usually dependent on the endogenous nutrients accumulated by the bacterial strain during vegetative growth (Monteiro et al. 2005). Nutritional factors such as carbon sources, nitrogen sources, trace metals, vitamins, carbon loading and carbon-to-nitrogen ratio can all have an influence on growth, propagule formation and biocontrol efficacy (Brar et al. 2006). Once a defined medium has
been developed which supports adequate growth, nutrients are varied in a directed way and their impact on spore yield and spore fitness can be assessed. During this study with a defined medium (Rao et al. 2007), the minimal residual sugars and maximal spore yield were observed at agitation speeds of 200 rev min⁻¹, whereas maximal residual sugars and minimal spores were obtained at a high agitation speed of 300 rev min⁻¹ in 48-h cultivation (Fig. 2). This could be due to increases in agitation rate producing higher shear stress in the STR, which may cause a decrease in the growth of shear-sensitive micro-organisms, and affect the spore yield under aerobic cultivations (Calik et al. 2000).

As ALR does not require mechanical agitation, the shear force is actually less than that in STR which makes them more advantageous for various cultivation applications (Fu et al. 2003; Selbmann et al. 2004). In this study, maximal yield of spore obtained in uncontrolled pH cultivations. This is in contrast to previous reports, where the pH was maintained at a constant value during the whole experiment in a 2-L bioreactor, a significant increase of the vegetative cells concentration and sporulation efficiency was achieved by B. subtilis (Jagannath et al. 2003; Monteiro et al. 2005). This is because of the difference in sizes of vessel and working volumes of bioreactor with different ranges of aeration rate. Oxygen transfer into microbial cells in aerobic cultivation processes strongly affects product formation, by influencing metabolic pathways and changing metabolic fluxes (Calik et al. 2000). Furthermore, the level of aeration rate in the cultivation medium plays a key role in the cell metabolism involved in spore production as was previously speculated (Foda et al. 1985). The influence of aeration rate was studied in the range between 1.5 and 3.0 vvm, and the best condition for the highest spore production found at 20-L ALR was 2.5 vvm. This agrees with the fact that sporulation of several species of the genus *Bacillus* has been shown to be related to oxygen supply in the cultivation medium (Dingman and Stauly 1983). From this study, in addition to the effects of pH, agitation speed and aeration rate, there are also many factors affecting spore production from the strain B128 which needs further study.

The ability of the *B. elliptica* B061 pathogen to rapidly develop resistance to a wide variety of fungicides has highlighted the increasing need for alternative forms of control including biological control. TEM analyses revealed that the spores from the strain B128 could affect the morphology and membrane system of *B. elliptica* cells.

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**References**


