

PRODUCTION OF A BIOCONTROL AGENT FOR CRUCIFERS BLACK ROT DISEASE

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Abstract - Of nine epiphytic isolates of the genus *Bacillus*, only *B. subtilis* R14, *B. pumilus* C116, *B. megaterium* pv. *cerealis* RAB7, and *B. cereus* C210 showed antibiotic activity when tested *in vitro* against the bacterium *Xanthomonas campestris* pv. *campestris* LFR-3. Aiming product a biocontrol agent for black rot in crucifers, the production of *B. subtilis* R14 was evaluated in a batch bioreactor. Rapid growth was observed in a medium containing molasses and yeast extract as C-source and N-source, respectively. During the exponential growth phase, the specific growth rate was 1.2 h⁻¹. A quick sporulation was also observed in a C/N well-balanced medium. After the sporulation phase, maximum viable spore concentrations around 10⁹ CFU/mL were obtained. Preliminary sedimentation tests at different pH values showed better biomass separation efficiencies at low pH values.

Keywords: biocontrol agent, antibiotic activity, epiphytic, genus *Bacillus*, bacterium *Xanthomonas*.

INTRODUCTION

Increasing the productivity of plants by improving their phytosanitary state with sustainability has been a great challenge for mankind in modern society. This objective has been achieved in many cases through the use of resistant varieties and chemical control. However, for some diseases, there is no source of resistance or new strains of pathogens, capable of infecting resistant varieties, may arise. Moreover, the indiscriminate use of agrochemicals leads to degradation of the ecosystem, may induce pathogen resistance to the pesticide, and may cause human and animal health problems (Huang, 1997).

Alternative strategies for disease management are necessary and include the use of bacteria that show benefic effects on plants, known as plant growth-promoting rhizobacteria (PGPR). The positive

effects of PGPR are normally divided into two categories: growth promotion and biological control (Kloepper, 1997). Of the PGPR, species of the genus *Bacillus* have shown promising results for the growth promotion of several cultures and the biological control of various plant pathogens (Mariano et al., 1997). This occurs specially due to the capacity of *Bacillus* spp. to form spores, which survive and remain metabolically active under harsh environmental conditions (Rodgers, 1989), making them appropriate for the formulation of stable and viable products (Kloepper, 1997).

One of the most efficient biocontrol agents is *B. subtilis*, which shows biological activity against several phytopatogenic bacteria and fungi. This antagonism has been attributed to the production of peptide antibiotics (McKeen et al., 1986; Loeffler et al., 1986) and to the capacity for colonizing the plant. Recently, the use of *Bacillus* spp. has been

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studied for control of the most important bacterial disease in the crucifers (e.g., cabbage, kale, and radish), known as black rot, which is caused by the bacterium *Xanthomonas campestris* pv. *campestris*. In greenhouse experiments, Assis et al. (1996) observed that *B. subtilis* R14, isolated from the surface of kale leaves, completely controlled the disease on cabbage when tested against three strains of *X. campestris* pv. *campestris* in all application periods tested, suggesting its utilization as a preventive treatment for the disease. Besides, a 73% reduction in the disease in field tests corroborated the efficiency of this same isolate in controlling cabbage black rot (Assis et al., 1997).

The genus *Bacillus* includes a variety of important species used in the fermentation industry. *Bacillus* spp. are nonpathogenic, good secretors of proteins and metabolites, and easy to cultivate. Products currently available commercially include enzymes, antibiotics and insecticides. The topics addressed by this work were *in vitro* evaluation of the antimicrobial activity of nine epiphytic *Bacillus* isolates against *X. campestris* pv. *campestris*, a study of the production of *B. subtilis* R14 in a batch bioreactor using a molasses medium, and a preliminary study of separation of biomass from the fermentation broth by sedimentation.

MATERIAL AND METHODS

Microorganisms

The *Bacillus* spp. used were *B. subtilis* R14, *B. megaterium* pv. *cerealis* RAB7, *B. megaterium* pv. *cerealis* C211, *B. pumilus* C116, *Bacillus* sp RAB9, *B. cereus* C240, *Bacillus* sp C11, *B. cereus* C210, and *Bacillus* sp C21. These organisms were isolated from crucifers. *Xanthomonas campestris* pv. *campestris* LFR-3 was used as the test microorganism for determination of *in vitro* antibiotic activity. All microorganisms belong to the Laboratory of Phytobacteriology of the Department of Agronomy at the Federal Rural University of Pernambuco, Brazil.

Culture Media

During the experiments, *Bacillus* spp. were preserved by subculturing on a NA (nutrient agar) medium: meat extract, 3 g/L; meat peptone, 10 g/L; agar, 20 g/L. For *X. campestris* pv. *campestris* LFR-3, the medium used was YMA (yeast-malt agar): glucose, 10 g/L; peptone, 5 g/L; yeast extract, 3 g/L;

malt extract, 3 g/L. Fermentations of *B. subtilis* R14 were carried out in molasses media, which are described in Table 1.

Experimental Procedures

For preliminary fermentation tests, the inoculum was grown in a test tube containing nutrient broth (NA without agar) at 30°C for 24 hours. This culture (2.5 mL) was transferred to a 250mL shaking flask, containing 25 mL of medium M1 or M2, which was incubated for 12 hours at 30°C and 200 rpm. Media with components sterilized together and separately were used.

Fermentations for spore production were carried out in a 5L bioreactor (BIOFLO III, New Brunswick Scientific) at 37°C and 700 rpm. Pre-inoculum was grown in a 250mL shaking flask, containing 25 mL of medium M3 or M4, for 12 hours at 30°C and 200 rpm. The culture was transferred to a Fernbach flask, containing 300 mL of medium M3 or M4, and after 24 hours at 30°C and 200 rpm, was inoculated in the bioreactor.

To study the influence of pH on biomass sedimentation, the pH values of broth fermentation samples were adjusted to 3.0, 5.0, and 7.0 with addition of 15 M HCl. Sedimentation tests were performed in a 100 mL graduated cylinder, containing 75 mL of the fermentation broth. Sedimentation efficiency was determined according to the following equation:

$$E(\%) = \frac{X_T - X_S}{X_T} \times 100$$

where X_T and X_S are the dry weight concentrations of the suspension (total biomass) and of the supernatant, respectively. To evaluate biomass concentration in the supernatant, samples were harvested when sediment reached 20% of the total volume (15 mL).

Analytical Methods

The agar diffusion method was used to measure the antibiotic activity of *Bacillus* spp. Blocks of NA containing *Bacillus* spp., grown at 30°C or 37°C for 48 hours, were placed over Petri dishes containing YMA that had been previously inoculated with *X. campestris* pv. *campestris*. The dishes were incubated at 28°C and after 24 hours the inhibition halos were measured.

Viable cell and spore concentrations were

determined as CFU (colony-forming units) on the NA medium before and after thermal shock at 80°C for 12 minutes, respectively. Biomass concentration was determined as dry weight after filtering samples through millipore membranes and drying at 80°C for 24 hours. Reducing sugars in the filtrate were assayed using the DNSA (dinitrosalicylic acid) method (Miller, 1959). The broth filtrate activity was measured by the agar diffusion method, using a 6mm paper disc containing 20 µL of sample, under the same conditions as those described above for *Bacillus* spp. antibiotic activity tests.

RESULTS AND DISCUSSION

Antimicrobial Activity of *Bacillus* spp.

The results of antibiotic activity tests, which are the averages of four repetitions, are presented in Figure 1. From the nine *Bacillus* tested, only *B. pumilus* C116, *B. megaterium* pv. *cerealis* RAB7, *B. subtilis* R14, and *B. cereus* C210 showed antibiotic

activity.

While testing 32 epiphytic *Bacillus* against *X. campestris* pv. *campestris* on kale, Assis et al. (1996) showed that strains R14, C210, and C240 were able to achieve 100% control of the disease in two greenhouse experiments. In a third experiment, *B. subtilis* R14 also showed 100% control in three periods of application: three days before, simultaneously, and three days after inoculation with *X. campestris* pv. *campestris*. Besides, in field tests R14 as well as C210, C116, and C240 were also able to control this same bacterium on kale, in different periods of application and different kale cultivars (Assis et al., 1997). Correlating the results obtained *in vitro* in this work with those *in vivo* results reported in the literature, it can be found that at least isolates R14 and C210, which consistently control crucifers black rot disease in field tests, could have antibiosis as one mechanism of action. However, other mechanisms may also be involved (Kloepper, 1997)

Due to its performance *in vivo* and *in vitro*, *B. subtilis* R14 was selected for studies on spore production and separation.

Table 1: Molasses media compositions.

Medium	Composition (g/L)
M1	molasses, 20.0; yeast extract, 6.1; KH ₂ PO ₄ , 1.0
M2	molasses, 20.0; urea, 1.3; KH ₂ PO ₄ , 1.0
M3	molasses, 20.0; yeast extract, 9.2; KH ₂ PO ₄ , 1.0
M4	molasses, 12.0; yeast extract, 12.0; KH ₂ PO ₄ , 1.0

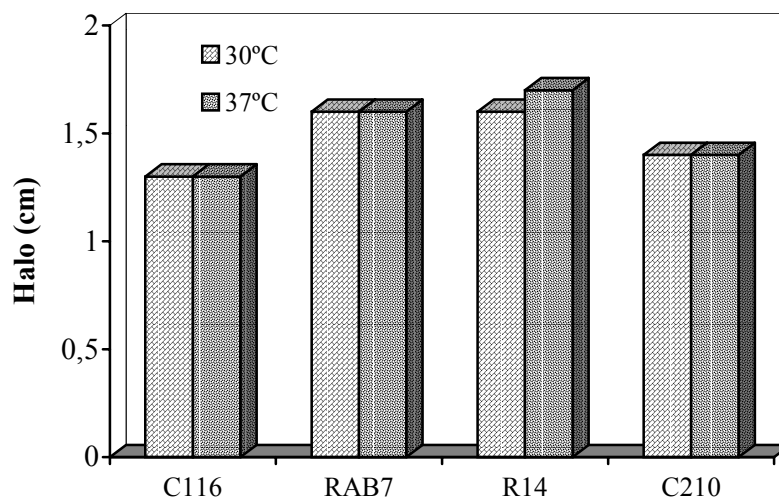


Figure 1: Inhibition halos for *Bacillus pumilus* C116, *B. megaterium* pv. *cerealis* RAB7, *B. subtilis* R14, and *B. cereus* C210 against *Xanthomonas campestris* pv. *campestris*. *Bacillus* spp. were tested after growth at 30°C or 37°C.

Preliminary Fermentation Experiments

The aim of these fermentation experiments in shaking flasks was a preliminary study of the growth of *B. subtilis* R14 on a molasses-based medium. Media M1 and M2 - having, respectively, yeast extract and urea as nitrogen sources - were used. Whose experiments were carried out using media components had been sterilized together or separately to check for the occurrence of undesirable reactions when all components were autoclaved together (Cote and Gherna, 1994).

The results for these fermentations, which are averages of duplicates, are presented in Table 2. The data show that the different autoclaving conditions had no apparent negative effect on biomass accumulation. Table 2 also shows the suspended solid contents of the media, which are relatively low. This characteristic is important when one considers the biomass separation process and product formulation.

The formulation of media M1 and M2 was such that the amounts of molasses, nitrogen and phosphorous in their compositions were enough to produce a final biomass concentration around 5 g/L. The biomass obtained in 12 hours using yeast extract was more than three times that obtained using urea as the nitrogen source, indicating a much faster growth rate. This is not surprising since yeast extract is a rich source of amino acids, vitamins, and other growth factors.

Production of *B. subtilis* R14

Media with yeast extract as the nitrogen source were used for biomass production in the bioreactor. Figures 2 to 4 show the results for production of *B. subtilis* R14 in medium M3. In Figure 2, a rapid growth rate can be observed during the exponential phase, which ended after four hours of fermentation, when reducing sugars were still present in the bioreactor. A decrease in biomass after 10 hours can

be associated with cell lyses and sporulation, when a residual amount of reducing compounds can still be observed, but which were not fermentable by the microorganism and remained in the medium throughout fermentation. After 24 hours of fermentation, a maximum amount of spores, around 10^9 CFU, had already been attained, when fermentable reducing sugars had already been absent from the medium for about 14 hours (Figure 3). Figure 4 shows the variations in pH and dissolved oxygen (%DO) during fermentation. The rapid decrease in pH and %DO before 3 hours is associated with the exponential growth phase of the microorganism. Deceleration of growth is followed by increases in both pH and %DO. A second phase of decrease occurs while fermentable sugars are still being assimilated. A later rise in pH may be associated with sporulation.

Based on the results obtained above, the fermentation medium was reformulated. The ratio between molasses and yeast extract was reduced in medium M4, since sporulation is intensified after exhaustion of fermentable sugars. Figure 5 shows the results obtained for biomass accumulation and sugar consumption in medium M4. The results are similar during the exponential phase of growth as expected, and in this case the amount of samples taken allowed determination of the maximum specific growth rate, μ_{max} . In Figure 6, the plot of biomass concentration, X, versus time is shown for the exponential phase and the μ_{max} value presented. Sporulation increased after 6 hours when fermentable sugars were completely depleted (Figure 7). After 10 hours of fermentation, a spore concentration around 10^8 had already been attained. As predicted, production time could be considerably reduced in this case.

No antibiotic activity against *X. campestris* pv. *campestris* was detected in the fermentation broth sample filtrates during the fermentations in media M3 and M4. This could be expected since conditions favorable to growth are normally unfavorable to secondary metabolite production.

Table 2: Biomass concentration after growth of *Bacillus subtilis* R14 in media M1 and M2 for 12 hours.

Medium	Autoclaving	Suspended Solids (g/L)	Biomass after 12 hours (g/L)
M1	together	0.49	4.05
	separately	0.77	3.97
M2	together	0.71	1.27
	separately	0.56	1.32

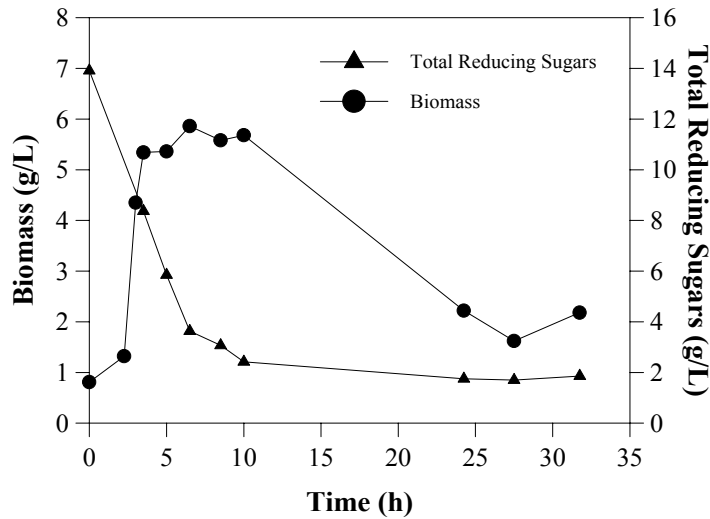


Figure 2: Time course of biomass accumulation and sugar consumption during growth of *Bacillus subtilis* R14 in medium M3.

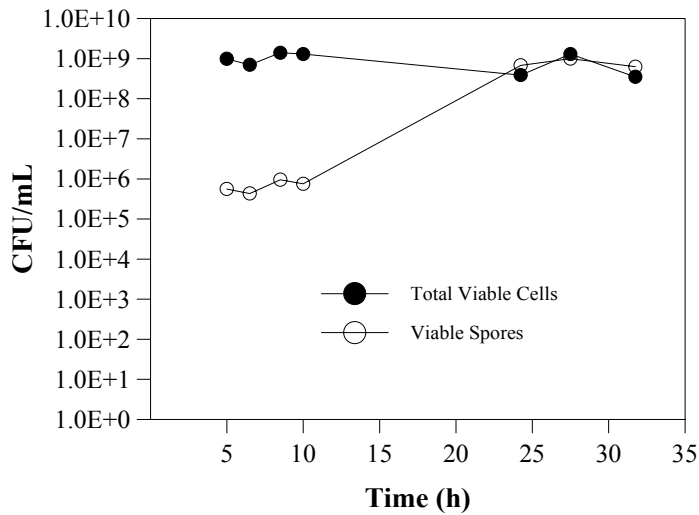


Figure 3: Time course of viable spore accumulation during growth of *Bacillus subtilis* R14 in medium M3.

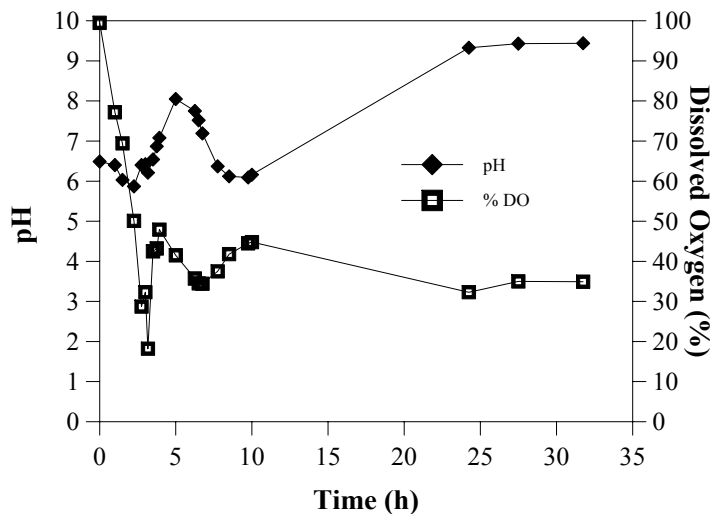


Figure 4: Time course of dissolved oxygen and pH variations during growth of *Bacillus subtilis* R14 in medium M3.

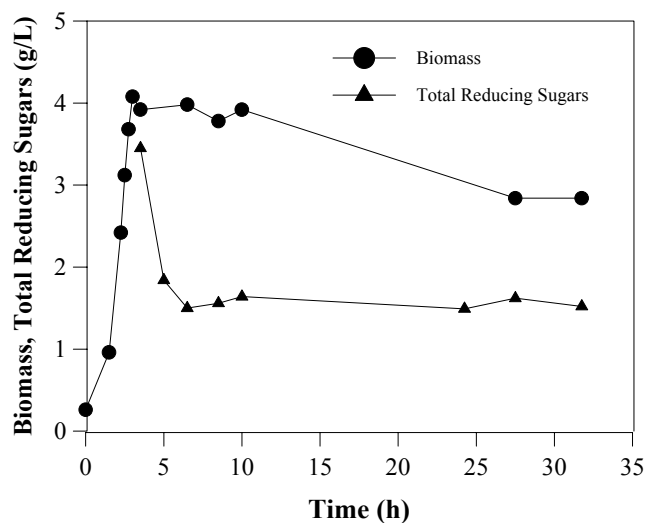


Figure 5: Time course of biomass accumulation and sugar consumption during growth of *Bacillus subtilis* R14 in medium M4.

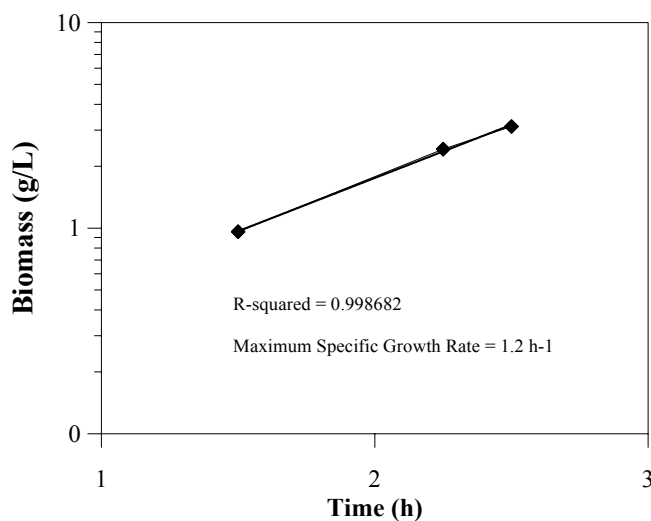


Figure 6: Time course of biomass accumulation during the exponential growth of *Bacillus subtilis* R14 in medium M4.

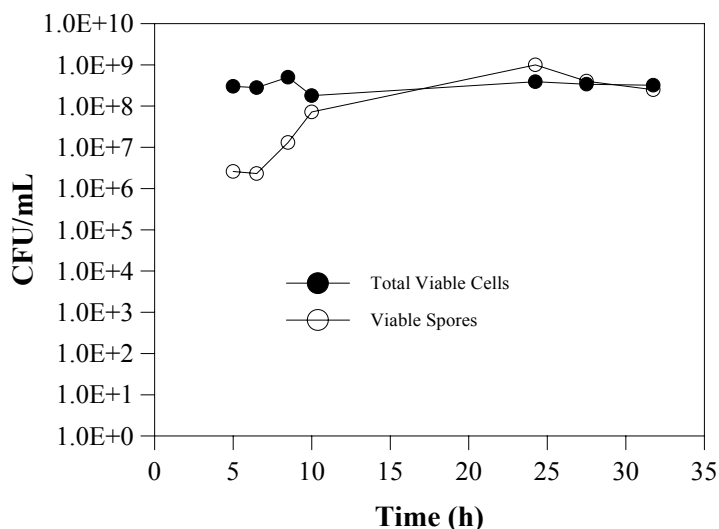


Figure 7: Time course of viable spore accumulation during growth of *Bacillus subtilis* R14 in medium M4.

Biomass Separation by Sedimentation

The curves in Figure 8 show the influence of fermentation broth pH on biomass sedimentation.

The highest value obtained for sedimentation efficiency was 92% at pH 3.0, decreasing to 45%

and 18% at pH 5.0 and pH 7.0, respectively (Table 3). When studying the sedimentation of *B. sphaericus* 2362, Luna (1999) also found the best results at a pH value of 3.0. Further studies are necessary to verify the mechanism involved in this separation process.

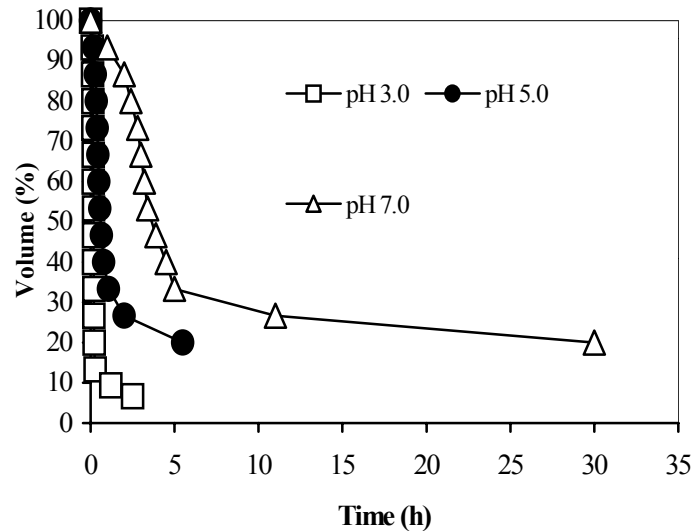


Figure 8: Sedimentation curves of *Bacillus subtilis* R14 at different pH values.

Table 3: Sedimentation efficiency for *Bacillus subtilis* R14 in fermentation broth at different pH values.

pH	Sedimentation Efficiency (%)
3.0	92
5.0	45
7.0	18

CONCLUSIONS

B. subtilis R14, *B. megaterium* pv. *cerealis* RAB7, *B. pumilus* C116, and *B. cereus* C210 show antibiotic activity *in vitro* against the bacterium *X. campestris* pv. *campestris*, the causative agent of black rot in crucifers. Spores of *B. subtilis* R14, which consistently control black rot disease in field tests, may be produced in a batch bioreactor using a molasses-based medium for the purpose of biocontrol. In a C/N well-balanced medium, production around 10^8 CFU/mL can be achieved in 10 hours of fermentation. Biomass separation from fermentation broth by sedimentation is enhanced when the pH value is decreased from neutral to acid.

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