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Growth requirements for production of stable cells of the bioherbicidal bacterium *Xanthomonas campestris*

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Xanthomonas campestris **MB245, a specific pathogen of the weedy grass** ***Poa annua*** **(annual bluegrass), is being developed as a bioherbicide to control this pest in turf. Nutritional and environmental factors were evaluated based on their ability to support rapid submerged culture growth and high cell yield. Temperature optima for the growth of** ***X. campestris*** **cells in submerged culture were between 27 and 30°C. At 30°C, optimal nutritional conditions for** ***X. campestris*** **growth supported generation times of 150–175 min and cell yields after 24 h growth of 1–2 $\times$ 10^{10} cells ml^{-1}. Media containing sucrose or glucose as the carbon source and various organic nitrogen sources supported optimal** ***X. campestris*** **growth and cell yield. The addition of vitamin mixtures to complex and defined media had no significant effect on growth or cell yield. The age of** ***X. campestris*** **cultures had a significant impact on cell survival after freeze drying. Following freeze drying, log phase cell survival (44%) was significantly lower than early and late stationary phase cell survival, 62% and 68%, respectively. Cells harvested in stationary phase, freeze dried and stored under vacuum at 4°C, showed no significant loss in viability after 6 months. Thus, high cell concentrations of the bioherbicide** ***X. campestris*** **can be rapidly produced in submerged culture and stabilized as freeze-dried preparations.**

Keywords: bacterial bioherbicide; freeze drying; growth phase; liquid culture production; shelf life; biological control

Introduction

Over the past 25 years, more than 100 microbial bioherbicides have been identified which are capable of controlling various weedy pests [3]. The overall lack of commercial success in using living microbial biocontrol agents stems from difficulties in producing and stabilizing these agents and the lack of consistent weed control in field situations [10,23]. Production methods for bioherbicidal agents must be low-cost and yield high concentrations of viable, highly effective propagules. These propagules must also be amenable to long-term storage as dry preparations. The development of liquid culture fermentation processes which overcome these problems is essential for the commercialization of microbial biocontrol agents.

Various *Xanthomonas* spp are specific pathogens of various grasses [6,17,18]. Selected strains of *Xanthomonas campestris* pv *poannua* are highly specific bacterial pathogens of *Poa annua* (annual bluegrass), a weedy pest of turf grasses [8,12,25]. In general, effective chemical herbicides against this weedy grass are unavailable [2]. Field trials in the US and Japan have shown that spray application of *X. campestris* on freshly mown grass infects and kills *P. annua* [9,19,24]. The high value of turf grasses, coupled with the ability to create an environment favorable to infection of *P. annua* by *X. campestris* through irrigating and mowing, makes this organism a promising biocontrol agent. As with many other Gram-negative bacterial biocontrol agents, methods are needed to maximize cell viability and stability after drying.

In this study, we measured the impact of various nutritional and environmental factors on *X. campestris* growth. Generation time during log phase growth and cell density after 24 h growth were the parameters used to assess medium efficacy for cell production. Freeze drying was employed to evaluate the desiccation tolerance and shelf-life of *X. campestris* cells harvested at different phases of growth. Growth and cell harvest conditions are presented which maximized *X. campestris* cell density and cell stability as a dried preparation.

Materials and methods

Culture maintenance

A stock culture of *X. campestris* pv *poannua* strain MB245 was obtained from Mycogen Corporation (San Diego, CA, USA). The stock culture was used to streak a nutrient agar (Accumedia, Baltimore, MD, USA) plate and a single colony from this plate was used as inoculum for 100 ml of nutrient broth in a 250-ml baffled Erlenmeyer flask. The liquid culture was grown overnight at 30°C and 300 rpm in a rotary shaker incubator. The whole culture was mixed 1:1 (v/v) with a sterile 20% glycerol solution and 2.0-ml aliquots were stored in cryovials at –80°C for use as stock cultures. Weekly, nutrient agar plates were streaked with *X. campestris* stock cultures to produce inocula for growth experiments.

Log phase *X. campestris* cells were used to inoculate flasks for growth studies. Log phase cells were obtained by inoculating complex or basal media with *X. campestris* cells obtained from streaked nutrient agar plates and growing

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these cultures for 12–16 h in a rotary shaker incubator at 30°C and 300 rpm. Prior to use, the generation time for the *X. campestris* cultures was determined by measuring optical density at 1-h intervals. Only cells in log phase (generation times 150–200 min) were used as inocula. Cell inocula were prepared by centrifuging whole cultures at $8000 \times g$ for 10 min at 23°C, decanting the supernatant, and resuspending the cell pellet in 0.004% sterile phosphate buffer. Cell suspensions with an optical density of 1.5 at 620 nm were used as a 10% inoculum in growth studies, providing an initial cell concentration of 1.6×10^8 cells ml^{-1} in the growth medium.

Culture conditions and media composition

Growth studies were conducted using various complex and defined media. Medium fitness was evaluated by measuring culture generation times during log phase growth and by determining cell density after 24 h growth for *X. campestris* cultures. The standard complex (SC) medium was composed of nutrient broth, 8 g L^{-1}; yeast extract (Difco Laboratories, Detroit, MI, USA), 10 g L^{-1}; sucrose, 10 g L^{-1}; and a vitamin mix composed of *m*-inositol, 62.5 mg L^{-1}; pyroxidine, 3.1 mg L^{-1}; Ca-d-pantothenate, 3.1 mg L^{-1}; thiamine-HCl, 27.5 mg L^{-1}; nicotinic acid, 2.5 mg L^{-1}; and biotin (Sigma Chemical, St Louis, MO, USA), 625 μg L^{-1}. Nutrient broth is composed of 37.5% beef extract and 62.5% pancreatic digest of gelatin. This SC medium was used to produce cells for freeze-drying studies, temperature and aeration studies, carbon concentration studies, and as a standard for nitrogen source studies.

The defined medium used for nitrogen source studies contained sucrose, 10 g L^{-1}, and minerals, trace metals, and vitamins as previously described [11]. The nitrogen sources tested included nutrient broth, yeast extract, Casamino acids (Difco), l-glutamate (free acid) (Sigma), urea (EM Science, Gibbstown, NJ, USA), ammonium sulfate, ammonium nitrate and a synthetic amino acid mixture. To approximate the nitrogen content of the SC medium, each nitrogen source was added to the defined medium in an amount that provided the medium with 1.4 g nitrogen L^{-1}. The amino acid mixture mimicked the typical amino acid composition of soy protein and was added to the defined medium (g L^{-1}): lysine 0.71; histidine 0.27; arginine 0.81; aspartate 1.40; threonine 0.45; serine 0.60; glutamate 2.30; proline 0.56; glycine 0.49; alanine 0.48; cystine 0.13; valine 0.55; methionine 0.11; isoleucine 0.49; leucine 0.83; tyrosine 0.34; phenylalanine 0.49; and tryptophan 0.08 (Sigma).

Nutritional studies were conducted to evaluate the importance of various components of the SC medium relative to generation time and 24-h cell density for *X. campestris* cultures. In nutrient omission studies, the requirement for vitamins, nutrient broth, and yeast extract was tested. In media where the nitrogen source, yeast extract or nutrient broth was omitted from the medium, the remaining nitrogen source was augmented to compensate for the reduction in nitrogen. Experiments were also conducted to determine the impact of sucrose concentration (10–80 g L^{-1}) or yeast extract concentration (18–32 g L^{-1}) on culture growth. In addition, the replacement of sucrose with glucose in the SC medium was examined.

Growth measurements

Growth parameters used in this study were generation time (GT) during balanced growth and cell density after 24 h. Growth was monitored by optical density (A) measurements at 620 nm and by dilution plate counts on nutrient agar. 1 A unit = 1.1×10^9 *X. campestris* cells ml^{-1}. Dilution plate counts on nutrient agar were used to measure final viable cell densities after 24 h growth and to measure *X. campestris* survival during storage as freeze-dried preparations. Dilution plates were incubated for 2–3 days at 28°C. The growth phase of cells harvested for freeze-drying studies was determined by taking hourly optical density readings of the individual cultures and determining the generation time. In this study, *X. campestris* cultures with generation times lower than 200 min were considered to be in log phase; 200–400 min, early stationary phase; and greater than 400 min, late stationary phase.

All growth experiments were performed using triplicate flasks for each treatment and all experiments were repeated at least twice. For statistical analysis, mean values for cell yield and generation time were subjected to analysis of variance (ANOVA) using Fisher's protected least significant difference at $P < 0.05$ (Statistix Version 4.0, Analytical Software, St Paul, MN, USA). For data not suitable for ANOVA, standard error values are provided as a measure of variance.

Preparation of cytoplasmic pool extracts for ^{13}C NMR spectroscopy

Cells (500 μl) were harvested by centrifugation and the cell pellet was extracted twice with 5 ml of cold 7% (v/v) perchloric acid by resuspension of the pellet and centrifugation [20]. The combined extracts were neutralized with 4 M KOH, and the salt was removed by centrifugation. The supernatant was lyophilized, dissolved in D_2O and 100 μl of standard phosphate buffer, pH 7.0. The mixture was filtered through a 0.22-μm syringe Millipore filter into the sample tube. Methanol (10 μl) was added as the internal standard and ^{13}C NMR spectra were recorded at 25°C using a Varian Gemini 300FT NMR spectrometer.

Drying experiments

For freeze-drying experiments, suspensions of *X. campestris* cells produced in the SC medium were mixed (1:1) with a solution containing 20% lactose and 2% bovine serum albumin (BSA) to produce a cell suspension in 10% lactose, 1% BSA. Freeze drying was performed in a tray dryer (Durastop-MP, FTS Systems, Stone Ridge, NY, USA) using an automatic-eutectic drying program. This program determined the eutectic point of the sample and set drying conditions based on this information, monitored the primary and secondary drying process, and determined when the drying process was completed. Ten-milliliter vials containing 2-ml cell suspensions were used in all studies. At the end of the freeze-drying cycle, vials were sealed under vacuum and stored at 4°C.

Results and discussion

Since the commercial production of *X. campestris* for use as a bioherbicide requires short fermentation times and high

Table 1 Growth of *X. campestris* in the SC basal liquid medium with sucrose (10 g L^{-1}) and various nitrogen sources at concentrations which provide 1.4 g nitrogen L^{-1}

Nitrogen source	Generation time*,† (min)	Yield‡ (CFU ml^{-1})
Nutrient broth: yeast extract (SC medium)	154[a]	1.2×10^{10}
Yeast extract	153[a]	1.4×10^{10}
Nutrient broth	175[a]	9.5×10^{9}
Synthetic amino acid mixture	160[a]	1.1×10^{10}
Casamino acids	222[b]	4.2×10^{9}
Urea	No growth	
$(NH_4)_2SO_4$	No growth	
NH_4NO_3	No growth	

* Mean values in columns followed by different letters are significantly different using Fisher's protected LSD, $P \leq 0.05$.
† Generation times are measured during log phase growth.
‡ Cell yields were determined after 24 h growth.

cell densities, optimization of nutritional and environmental conditions for the production of this bacterium was based on minimizing generation time during log phase growth and cell density after 24 h growth. Our nutritional studies demonstrated that various complex media supported the rapid growth of *X. campestris*. The standard complex (SC) medium used in these studies contained nutrient broth, yeast extract, sucrose and a vitamin mixture and produced generation times of 150–175 min and 24-h cell yields of approximately 1–2 $\times 10^{10}$ cells ml^{-1} (Table 1). Omission of the vitamin mixture in the SC medium or substitution of sucrose with glucose did not significantly alter cell densities or generation time (data not shown). Increasing the concentration of sucrose in the medium decreased cell density and increased generation times (Table 2). In previous studies with *X. campestris* strains utilized in the production of xanthan gum, increasing the concentration of sucrose or glucose decreased generation times or had no effect on *X. campestris* growth while increasing xanthan gum production [16,21]. Under the conditions of our study, increased glucose concentrations did not noticeably increase the viscosity (ie, xanthan gum concentration) of the *X. campestris* cultures.

Table 2 *Xanthomonas campestris* growth in SC medium with various amounts of sucrose

Sucrose (g L^{-1})	Generation time* (min)	Yield† ($\times 10^9$ CFU ml^{-1})
10	152[a]	12.0[a]
20	156[a,b]	11.0[b]
30	160[a,b]	10.5[b]
40	166[a,b,c]	9.5[c]
50	172[b,c]	8.8[c,d]
60	182[c]	8.0[d,e]
70	182[c]	6.4[f]
80	208[d]	6.4[f]

* Mean values followed by different letters are significantly different using Fisher's protected least significant difference ($P = 0.05$).
† Cell density after 24 h incubation at 28°C and 300 rpm in a rotary shaker incubator.

In a defined basal medium, various nitrogen sources were evaluated. The use of yeast extract or a synthetic amino acid mixture as the sole nitrogen source provided 24-h cell densities and generation times comparable to those obtained with the SC medium (Table 1). Among the nitrogen sources tested, yeast extract and a synthetic amino acid mixture were comparable to SC medium for optimal cell yield and generation time. In studies where various concentrations (18–32 g L^{-1}) of yeast extract were used as the sole nitrogen source, no differences in generation times or cell density were noted (data not shown). These results corroborate previous studies with xanthan gum-producing strains of *X. campestris* that showed that lower nitrogen concentrations optimized growth [5,7]. Urea and the inorganic nitrogen sources tested did not support growth by *X. campestris* (Table 1). These results suggest that the use of various organic nitrogen sources support rapid growth and high cell yields of *X. campestris* cells.

Using the SC medium, we examined the growth of *X. campestris* cultures at temperatures between 24 and 36°C. Temperature optima for the growth of *X. campestris* were 27–30°C. Shake flask aeration studies were performed by growing *X. campestris* at agitation rates of 100, 200, and 300 rpm. During the early stages of growth (0–6 h), all rates of agitation gave similar growth rates. The higher agitation rates of 200 or 300 rpm maximized growth in the later stages of growth.

The ability to produce desiccation-tolerant bacterial biocontrol agents is an important commercial consideration. Gram-negative bacteria like *X. campestris* are generally less tolerant to drying compared to Gram-positive organisms [22]. In our study, the desiccation tolerance of *X. campestris* MB 245 cells harvested during different growth phases was evaluated by measuring survival after freeze drying. Cells harvested during log phase showed significantly less survival following freeze drying compared to cells harvested during the early or late stationary phases (Table 3). In contradiction to this study, log phase cells of the Gram-negative bacterium *Rhizobium meliloti* survived freeze drying better than stationary phase cells [1]. Nuclear magnetic resonance analysis of solutes extracted from *X. campestris* cells demonstrated that early and late stationary phase cells contained trehalose (Figure 1b) as evidenced by signals (δ 93.27, 72.57, 72.19, 71.07, 69.74 and 60.60 ppm) characteristic of trehalose ([14], Figure 1c). Log phase cells lacked this compound or other dominant carbon compounds (Figure 1a). Trehalose enhances the survival of bacteria

Table 3 The influence of harvesting *Xanthomonas campestris* cells at different growth phases on cell survival after freeze drying. Cells were grown in the SC medium and suspended in a 10% lactose/1% bovine serum albumin solution during freeze drying

Generation time (min)	% Survival after drying*	No. of samples
<200 (Log phase)	44.1[a]	16
200–400 (Early stationary)	62.2[b]	24
>400 (Late stationary)	69.7[b]	14

* Values followed by different letters are significantly different using Fisher's protected LSD, $P \leq 0.05$.

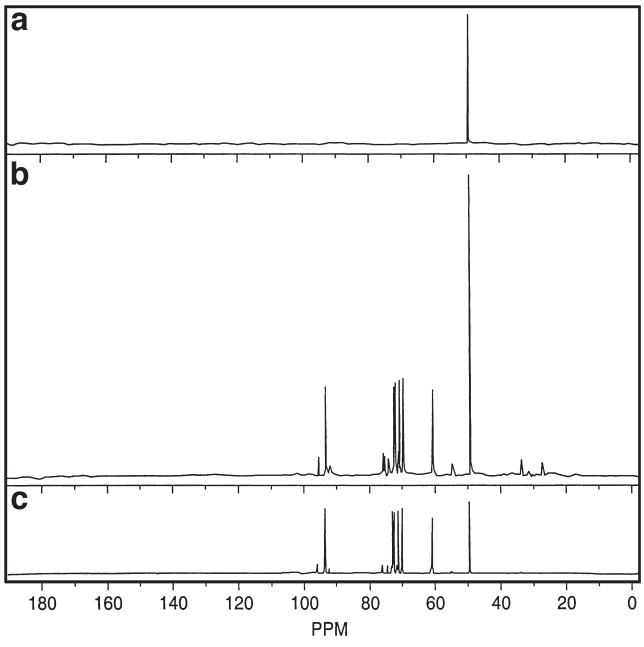

Figure 1 Measurement of trehalose in extracts from (a) log phase cells and (b) stationary phase cells of *Xanthomonas campestris* grown in a defined medium using ^{13}C nuclear magnetic resonance (NMR). The NMR spectra (δ 93.27, 72.57, 72.19, 71.07, 69.74 and 60.60 ppm) of authentic trehalose standard (c) corresponds to the spectra seen in extracts from stationary phase cells of *X. campestris*. Ten microliters of methanol (δ 49.10) were added to each sample as an internal standard.

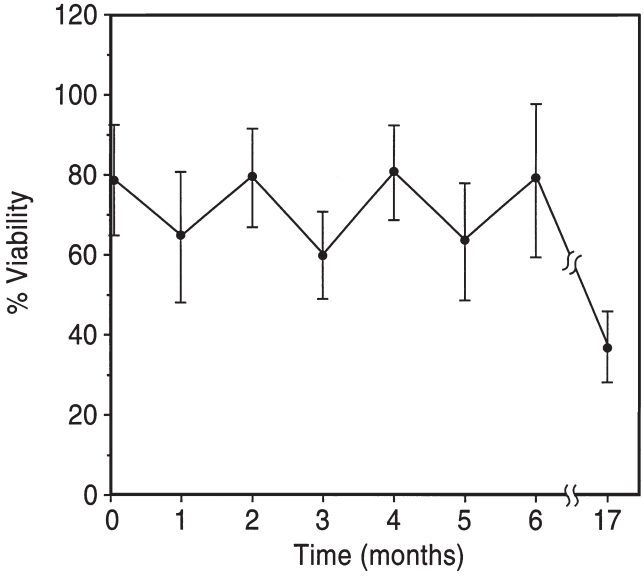

Figure 2 Survival of freeze-dried *Xanthomonas campestris* cells stored under vacuum at 4°C. *X. campestris* cells were grown in the standard complex (SC) medium and harvested in stationary phase. Cell viability was measured as colony forming units (CFU) on nutrient agar after 3 days incubation at 28°C. Percent viability for freeze-dried cells was calculated as the CFU of freeze-dried cells at various stages of storage per CFU of the cells' suspension prior to freeze drying × 100.

after freeze drying, presumably due to stabilization of the cell membrane during the freezing and drying processes and by stabilizing proteins in the anhydrous state [15]. The hydroxy groups of trehalose are believed to participate in extensive hydrogen bonding to phospholipids and proteins during freezing and drying according to the water replacement hypothesis [4]. This association of trehalose content and relatively high cell survival after drying (~65%) suggests that, with this strain of *X. campestris*, trehalose may play a role in enhancing desiccation tolerance. Also, stationary phase bacterial cells are morphologically and biochemically significantly different from exponential phase cells, and typically are more resistant to a variety of chemical and physical stresses [13].

An additional requirement for commercial biocontrol agents is product shelf life. In our study, the stability of the freeze-dried cell preparations was evaluated by storing stationary phase *X. campestris* cells at 4°C under vacuum. No significant loss in viability in the freeze-dried cell preparations occurred after 6 months storage (Figure 2). After 17 months storage, we observed a 50% loss in cell viability. These results are encouraging and suggest that the cells which survive the drying process are very stable under the storage conditions tested.

These studies have demonstrated that *X. campestris* cells can be produced rapidly in submerged culture and can be stabilized as a dry preparation. High cell concentrations are produced in simple media using various organic nitrogen sources and inexpensive carbohydrates like glucose or sucrose. Although *X. campestris* has a relatively long generation time of 150–175 min, a 24-h growth period theoretically allows *X. campestris* time for a 500-fold increase in cell concentration. In our study using a relatively high inoculum of 1.6×10^8 cells ml^{-1}, a 100-fold increase in cell density is obtained after 24 h growth and the cells are in the stationary phase of growth. Our data suggest that harvesting stationary phase cells is desirable to enhance cell survival following drying.

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