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ORIGINAL PAPER

Effects of physical culture parameters on bacteriocin production by Mexican strains of *Bacillus thuringiensis* after cellular induction

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Abstract We have shown previously that in the presence of inducer *Bacillus cereus* 183, significant increases in bacteriocin production and bactericidal activity of *B. thuringiensis* occur when the latter is cultivated at pH 7.2, 28°C, and 180 rpm. Here we show that this activity can be further improved when *B. thuringiensis* is induced with *B. cereus* 183 and then cultivated with modification of pH, temperature, and agitation. Five native strains of *B. thuringiensis*, LBIT 269, LBIT 287, LBIT 404, LBIT 420, and LBIT 524 which synthesize, respectively, morricin 269, kurstacin 287, kenyacin 404, entomocin 420, and tolworthcin 524, were cultivated in four different fermentation media. Of these, fermentation in tryptic soy broth (TSB) yielded the highest level of bacteriocin activity (~100–133 FU). Bacteria grown in TSB were induced with *B. cereus* 183 and cultivated at different pH (6.0, 7.2, 8.0), temperature (26, 28, 30°C), and agitation (150, 180, 210 rpm). Full factorial design was performed and results were analyzed with analysis of variance (ANOVA) and Tukey multiple comparison tests at significant level of $\alpha \leq 0.05$ to study the influence of the three variables on bacterial growth and bacteriocin production. Our data show that the highest bacteriocin activity was found with LBIT 269 and LBIT 404 with an increase of ~95–100% compared with induced *B. thuringiensis* strains cultivated under fixed conditions (pH 7.2, 28°C, 180 rpm), for which the data were set at 0%. The optimal conditions for morricin 269 and kenyacin 404 production were, respectively, pH 8, 30°C, 210 rpm and pH 7.2, 26°C, 210 rpm.

Keywords *Bacillus thuringiensis* · Bacteriocins · Physical factors · Induction

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Introduction

The ability of pathogenic microorganisms to adapt to sublethal doses of toxic compounds [20] necessitates the development of new and efficient strategies to control them. In particular, such strategies could incorporate bacteriocins, a class of antimicrobial peptides known to inhibit growth of diverse microorganisms, including clinically significant foodborne bacterial pathogens [1, 4]. Optimizing bacteriocin yield either through enhanced fermentation or genetic engineering techniques is required for large-scale production and applied use of these antimicrobial peptides, particularly those produced by *Lactobacillus* and *Bacillus* species [2, 13, 17]. Whereas several studies on the physical and biochemical parameters that affect bacteriocin production in *Lactobacillus* species have been reported [6, 18], similar studies are notably lacking for *Bacillus* species. In fact,

only one such report has been published on the physicochemical culture conditions (aeration, carbon source, C/N ratio) that affect bacteriocin production in *B. thuringiensis* [15].

Recently, the synthesis of five bacteriocins from Mexican strains of *B. thuringiensis* (morricin 269, kurstacin 287, kenyacin 404, entomocin 420, and tolworthcin 524) was reported [1, 11, 12]. Our studies showed a strong potential for use of these bacteriocins in industry as food preservatives because they exhibited a broad range of inhibitory activity against gram-positive and gram-negative pathogenic bacteria such as, respectively, *Staphylococcus aureus* and *Enterococcus faecium*, and *Klebsiella pneumoniae*, *Salmonella* spp., and *Enterobacter cloacae* [9, 11]. Furthermore, we demonstrated that bacteriocin production by the Mexican *B. thuringiensis* strains was enhanced by a proteinaceous component(s) secreted by or liberated from a susceptible bacterium (*B. cereus*) [11]. In the present study, we show that bacteriocin production by the Mexican strains of *B. thuringiensis* following induction by *B. cereus* could be significantly enhanced in culture medium by modification of the pH, temperature, and agitation.

Materials and methods

Bacterial strains

Bacillus thuringiensis strains were obtained from a native bacterial stock collection held in the laboratory of Dr. Jorge Ibarra (CINVESTAV-Irapuato, Guanajuato, Mexico). *B. thuringiensis* subsp. *morrisoni* (LBIT 269), *B. thuringiensis* subsp. *kurstaki* (LBIT 287), *B. thuringiensis* subsp *kenyae* (LBIT 404), *B. thuringiensis* subsp. *entomocidus* (LBIT 420), and *B. thuringiensis* subsp. *tolworthi* (LBIT 524) were employed for the production of the bacteriocins, respectively, morricin 269, kurstacin 287, kenyacin 404, entomocin 420, and tolworthcin 524 [1]. *B. cereus* 183 was obtained from a collection of *Bacillus* strains maintained in the International Entomopathogenic Bacillus Centre, Institut Pasteur, Paris, France. This strain was used as indicator bacterium for the determination of bacteriocin activity [1, 11].

Selection of culture medium for bacteriocin production

Bacterial strains were cultured overnight at 28°C with 180 rpm, and aliquots of 5 ml ($\sim 1 \times 10^9$ cells/ml) were mixed with 45 ml of tryptic soy broth (TSB) (BDBioxon) [(1.7% (w/v) casein peptone, 0.3% (w/v) soy peptone, 0.5% (w/v) NaCl, 0.25% (w/v) K_2HPO_4, 0.25% (w/v) dextrose], Luria–Bertani (LB) (Difco) [(1% (w/v) Bacto tryptone, yeast extract 0.5% (w/v), 0.5% (w/v) NaCl], NB plus salts (NBS) [0.8% (w/v) nutrient broth Bioxon (beef extract, gelatin peptone), 0.15% (w/v) extract yeast, 0.005% (w/v) $MnCl_2$, 50 mM NaH_2PO_4, 1.22 mM $MgSO_4$, 0.68 mM $CaCl_2$], and NBS plus 0.5% (w/v) glucose (NBSG) [1, 8]. Duplicate aliquots of 1.0 ml were taken at 4-h intervals over a 24-h period. One sample was used for turbidity measurement at 600 nm and the other for bacteriocin activity using the fluorogenic method previously described by de la Fuente-Salcido et al. [12].

Table 1 Values of experimental variables for the application of the full factorial design

Variables	Level			
	Units	Low (−1)	Medium (0)	High (+1)
pH		6.0	7.2	8.0
Agitation	rpm	150.0	180.0	210.0
Temperature	°C	26.0	28.0	30.0

Bacteriocin production following modification of physical conditions

In order to ensure that *B. thuringiensis* strains used for the induction assays were growing in the exponential phase of the growth curve, bacterial strains were cultured ~8 h during the night in TSB at 28°C with orbital shaking at 180 rpm [1]. From these cultures, an aliquot of 5 ml of each strain with cell numbers of $\sim 1 \times 10^9$ cells/ml [1] was mixed with 45 ml of fresh TSB and 1% (v/v) of *B. cereus* 183 culture containing $\sim 1 \times 10^7$ cells/ml [11]. Then, the pH of the medium was adjusted, and the culture was incubated under different temperature and agitation conditions (Table 1) for 24 h. Duplicate aliquots of 1.0 ml were taken after 24 h, and one sample was used for turbidity measurement at 600 nm (SmartSpec 3000, Bio-Rad, Hercules CA), and the other was centrifuged, filtered through a 0.22-µm filter, and used for bacteriocin detection using the fluorogenic method previously described [12].

Evaluation of bacteriocin activity

Bacteriocin activity was evaluated by the fluorogenic method [12]. *B. cereus* 183 was grown in TSB overnight at 28°C, then 1 volume of this culture was added to 4 volumes of fresh TSB and incubated at 28°C at 200 rpm for 2 h. Cells were centrifuged at 10,000 × *g* for 15 min and the pellet was resuspended in 50 mM phosphate buffer with 5% (v/v) glycerol (PBG) to adjust the bacterial concentration to $\sim 4 \times 10^8$ cells/ml. Then 20 µl of this sample was mixed with 0–50 µl of bacteriocin preparation and volumes were adjusted to 100 µl with 50 mM PBG. Reactions were incubated for 5 min at room temperature. Subsequently, 6 µl of

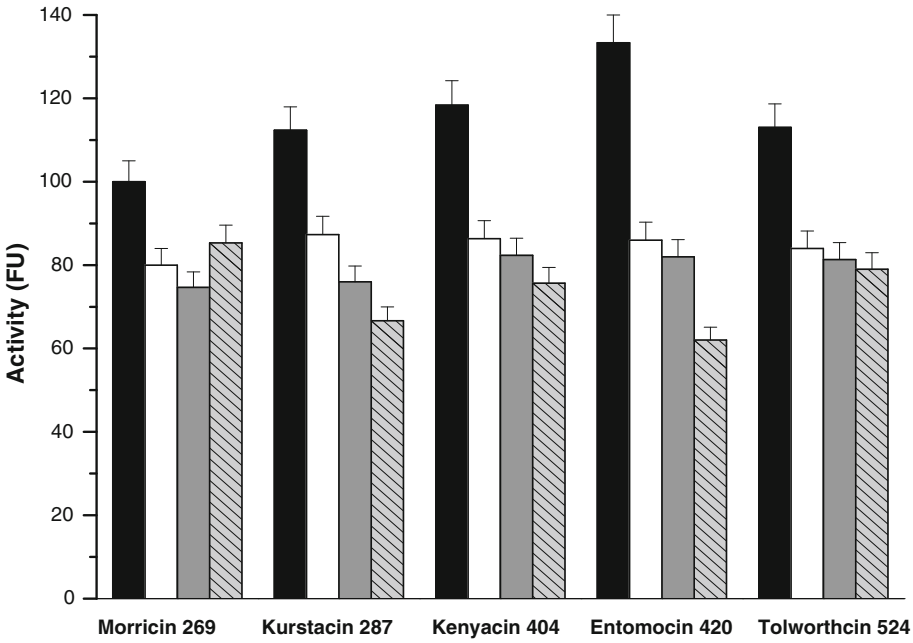

Fig. 1 Antibacterial activity of *B. thuringiensis* strains cultivated in four different media. *B. thuringiensis* subsp. *morrisoni* (LBIT 269), *B. thuringiensis* subsp. *kurstaki* (LBIT 287), *B. thuringiensis* subsp. *kenyae* (LBIT 404), *B. thuringiensis* subsp. *entomocidus* (LBIT 420), and *B. thuringiensis* subsp. *tolworthi* (LBIT 524) synthesize bacteriocins morricin 269, kurstacin 287, kenyacin 404, entomocin 420, and tolworthcin 524, respectively. Bacteria were grown in TSB (*black bars*), LB (*white bars*), NBS (*gray bars*), and NBSG (*hatched bars*) media. Standard errors were calculated based on three replicates. Bacteriocin activity in supernatants was determined with a fluorogenic method

4 mM berberine sulfate (Sigma) dissolved in ethanol was added and adjusted to 1,000 µl with 50 mM PBG, for a final berberine concentration of 24 µM. The mixtures were incubated for 1 min at room temperature and fluorescence (FU, fluorescence units) was determined in a Turner fluorometer (model 450; 340-nm interference filter and 415-nm cut filter). With this protocol membrane damage induced by bacteriocins causes the entrance of berberine into the cells. Berberine fluoresces immediately when it binds to different biomolecules (e.g., DNA and glycosaminoglycans); and bacteriocin activity is measured as relative fluorescence [12].

Statistical analyses

Full factorial analysis was developed to study the influence of three variables on bacterial growth and bacteriocin production [7, 24]. In this study the variables were pH (X), temperature (X), and agitation (X) and the responses were the bacterial growth (optical density (OD), Y1) and antibacterial activity (FU, Y2). Each variable was studied at three coded levels (1, 0, −1) (Table 1). A series of 27 experiments (3^3) were carried out in triplicate. The effect of each variable and their interactions on cell growth and antibacterial activity were studied through statistical analysis using the analysis of variance (ANOVA) and Tukey multiple comparison tests performed with STATGRAPHICS Plus version 5 (Statistical Graphic Corp., Warrenton VA, USA). Results were considered significant at a level of $\alpha \leq 0.05$.

Results

Selection of media for bacteriocin production

The effect of culture media on bacteriocin production by Mexican strains of *B. thuringiensis* was evaluated using TSB, LB, NBS, and NBSG; the last two media are routinely used in the laboratory for high-level production of the insecticidal crystal (Cry) proteins by *B. thuringiensis* [8, 23]. All cultures were incubated under the same physical conditions. In general, the highest level of bacteriocin activity by the five strains of *B. thuringiensis* was obtained when bacteria were grown for 24 h in TSB, for which the values obtained were ∼100–133 FU, followed by LB, NBS, and NBSG with activities of ∼80–87, ∼74–82, and ∼62–85 FU, respectively (Fig. 1).

Effects of pH, temperature, and agitation on bacteriocin production

After the culture medium (TSB) for bacteriocin production was selected, we determined if it was possible to obtain higher incremental increases of both bacterial growth and bacteriocin production (measured as an increment of the antibacterial activity) by modifying the pH, temperature, and agitation of cultures after inducing the five *B. thuringiensis* strains with *B. cereus* 183 [11]. Various combinations of these physical factors were used to culture the bacteria, and these experiments were performed in triplicate for statistical analysis. We obtained different values of cellular growth and antibacterial activity depending on the strain

Table 2 Selection of the physical parameters that allow the optimization of bacterial growth and bacteriocin production by Mexican strains of *B. thuringiensis*

Parameters	Bacterial growth (OD)					Antibacterial activity (UF)				
	Bacterial strains (LBIT)					Bacteriocins				
	269	287	404	420	524	Morricin 269	Kurstacin 287	Kenyacin 404	Entomocin 420[a]	Tolworthcin 524
pH	8.0	7.2	6.0	7.2	6.0	8.0	8.0	7.2	6.0	7.2
Agitation (rpm)	210.0	180.0	180.0	210.0	210.0	210.0	180.0	210.0	150.0 (210)	150.0
Temperature (°C)	28.0	26.0	26.0	30.0	30.0	30.0	28.0	26.0	26.0 (28)	28.0

[a] LBIT 420 grown at pH 6, 150 rpm and 26°C has the same entomocin 420 activity than bacterium cultivated at pH 6, 210 rpm and 28°C

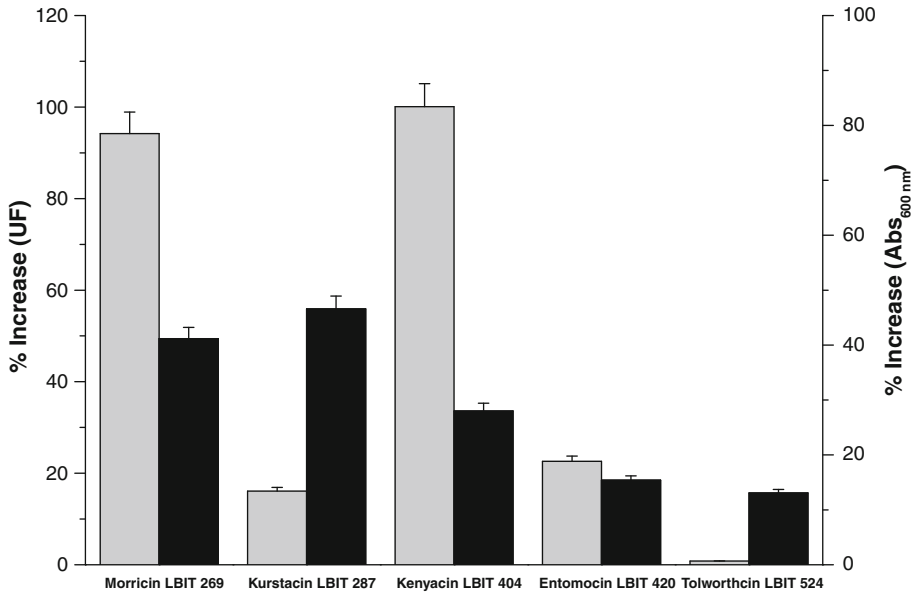

Fig. 2 Increment of the antibacterial activity of bacteriocins synthesized by native strains of *B. thuringiensis*. Antibacterial activity (*gray*) and bacterial growth (*black*) were measured after inducing *B. thuringiensis* strains with *B. cereus* 183 and cultivating under different conditions that afforded the highest activities (Table 2). *B. thuringiensis* strains induced with *B. cereus* 183 and cultivated at pH 7.2, 28°C, and 180 rpm were used as baseline controls and are represented at 0% of increase. Standard errors were calculated based on three replicates. Bacteriocin activity (Y axis, *left*) in supernatants was determined with a fluorogenic method whereas the bacterial growth was determined using optical density (Y axis, *right*) measured at 600 nm

and conditions used in the assays; in some samples those results were lower (data not shown) or higher than the controls. We selected the conditions (Table 2) under which all strains showed the highest increment in cellular growth and antibacterial activity compared with induced *B. thuringiensis* strains cultivated under a fixed condition (pH 7.2, 180 rpm, 28°C) [11], for which the data were set at 0% (Fig. 2). The highest increment in bacterial growth was obtained with LBIT 287 and the lowest with LBIT 524 with values of ~56 and 16%, respectively (Fig. 2); whereas the highest yield in the bacteriocin activity was found with LBIT 269 and LBIT 404 with an increment increase of ~95–100% in the antibacterial activity of morricin 269 and kenyacin 404, respectively (Fig. 2). Interestingly, although LBIT 287 had an increment in the bacterial growth of ~56% it had only an increase of 16% in antibacterial activity (kurstacin 287). Otherwise, LBIT 404 had an increase of 33% in the optical density but an increment in the bacteriocin activity (kenyacin 404) of 100%. These results indicated that for each strain the condition for optimal growth and bacteriocin production were different. Likewise, when data from all strains were analyzed, bacteria that showed the highest increase in bacteriocin production (i.e., LBIT 404) did not show the highest increment in bacterial growth or vice versa.

When the effect of varying physical factors on bacteriocins production for the two strains showing the highest yield (LBIT 269 and LBIT 404) was analyzed, we observed that in order to optimize bacterial growth of *B. thuringiensis* subsp. *morrisoni* LBIT 269, it was necessary to cultivate

Table 3 Statistical analysis of the full factorial design for bacterial cell growth of *Bacillus thuringiensis* subsp. *morrisoni* and antibacterial activity of morricin 269

Sources	Bacterial growth				Antibacterial activity			
	SS	df	*F* ratio[a]	Probability >*F*	SS	df	*F* ratio[a]	Probability >*F*
pH	60.44	2	22.20	0.0005*	1,274.0	2	60.44	0.0000*
Agitation rpm	3.78	2	13.92	0.0025*	1,331.55	2	3.78	0.0698
Temperature	1.79	2	2.52	0.1417	631.227	2	1.79	0.2263
pH–rpm	7.30	4	11.54	0.0021*	5,141.36	4	7.30	0.0088*
pH–temperature	0.26	4	2.22	0.1567	184.751	4	0.26	0.8940
rpm–temperature	2.02	4	0.37	0.8223	1,418.92	4	2.02	0.1851
Residual	3,955.33	8	494.16		1,407.88	8		

SS sum of squares, *df* degree of freedom

* Significant at $\alpha \leq 0.05$

[a] *F* ratios were based on the residual mean square error

Table 4 Statistical analysis (ANOVA) of the full factorial design for bacterial cell growth of *Bacillus thuringiensis* subsp. *kenyae* and antibacterial activity of kenyacin 404

Sources	Bacterial growth				Antibacterial activity			
	SS	df	*F* ratio[a]	Probability >*F*	SS	df	*F* ratio[a]	Probability >*F*
pH	33,007.6	2	84.32	0.0000*	8,882.48	2	14.53	0.0022*
Agitation rpm	698.66	2	1.78	0.2286	322.276	2	0.53	0.6095
Temperature	595.887	2	1.52	0.2753	2,168.5	2	3.55	0.0789
pH–rpm	5,363.44	4	6.85	0.0107*	993.188	4	0.81	0.5514
pH–temperature	678.681	4	0.87	0.5234	8,133.0	4	6.65	0.0116*
rpm-temperature	759.68	4	0.97	0.4741	538.844	4	0.44	0.7766
Residual	1,565.9	8			2,445.63	8		

SS sum of squares, *df* degree of freedom

* Significant at $\alpha \leq 0.05$

[a] *F* ratios were based on the residual mean square error

the bacterium at pH 8.0, 210 rpm, and 28°C; whereas for a higher increment in the antibacterial activity of morricin 269, it was necessary to grow the bacterium at the same pH (8.0) and agitation (210 rpm), but at a higher temperature of 30°C (Table 2; Fig. 2). It was evident that the range of temperature (28–30°C) had an effect on both bacterial growth and bacteriocin production. For example, by modification of the temperature at 28 or 30°C under fixed pH and agitation (8, 210 rpm) an increment in the bacterial growth of, respectively, 49 and 40% was obtained. However, when the interaction of temperature with the other two parameters was analyzed, only pH, agitation, and the interaction of pH–agitation had an effect on both bacterial growth and antibacterial activity of morricin 269 (Table 3). Alternatively, when *B. thuringiensis* subsp. *kenyae* (LBIT 404) was cultivated in TSB, a lower temperature of 26°C was necessary to optimize its growth and antibacterial activity. Additionally, agitation at 180 and 210 rpm was required for, respectively, higher values cellular growth and bacteriocin activity. Values of pH of 6.0 and 7.2 were required for optimal cell growth and kenyacin 404 activity. Furthermore, it was demonstrated that varying pH and pH–agitation influenced bacterial growth, whereas kenyacin 404 activity was affected by varying pH and pH–temperature (Table 4). This indicated that the main physical factor that affects bacterial growth and antibacterial activity in LBIT 404 is the pH, but the modification of this parameter simultaneously with agitation or temperature could affect cellular division and production of the antimicrobial peptide, respectively.

An increment in the bacteriocin activity after induction with *B. cereus* 183 was observed with LBIT 420 and LBIT 287 (Fig. 2), with values of 22 and 16%, respectively. In contrast, only a minor increase (1%) in bacteriocin production of LBIT 524 was detected. With these strains, bacterial growth and antibacterial activity were affected by pH. Variations in pH and agitation affected cellular growth and antibacterial activity of LBIT 287, whereas in LBIT 420 and

LBIT 524 these were affected by varying pH and temperature (data not shown).

Discussion

Overproduction of proteins with applied value is of crucial concern to industry, because higher yields per unit of culture lower the production costs [14]. In this regard, different strategies have been developed to improve the production of proteins with biotechnological potential, such as insecticidal proteins, antimicrobial peptides, and chitinases [1, 23, 24]. Because we are interested in enhancing bacteriocin yield for both basic studies and for potential applied use as preservatives in food and medicine [1, 19], our purpose was to increase the synthesis of bacteriocins produced by five Mexican strains of *B. thuringiensis* [1, 11, 12]. Recently, we found that these strains do not require the presence of a susceptible bacterium to induce synthesis of bacteriocins, although significant increases in yield occurred in the presence of *B. cereus* 183 [11]. To our knowledge, only one report on the improvement of the bacteriocin activity by *B. thuringiensis* has been published. However, in that study only aeration as a physical parameter was modified to study the effect on antimicrobial peptide production [15]. Here, we studied the effect of fermentation media, pH, temperature, and agitation on five Mexican strains of *B. thuringiensis*, each producing a different bacteriocin in the presence of an enhancer strain *B. cereus* 183.

Firstly, we selected a single culture medium for our studies after testing four media (TSB, LB, NBS, NBSG), because our purpose was to study the effect of media composition on bacteriocin production without testing another factor (i.e., induction), and subsequently used the selected medium in induction assays. We expected to obtain higher bacteriocin production in NBS or NBSG because of their composition. These media contain a rich source of organic nitrogen and organic carbon, and also salts such as Mn^{2+} and Mg^{2+}, two important ingredients known to enhance bacteriocin and insecticidal crystal (Cry) protein yields [22, 23]. We show that for the strains under study, the highest bacteriocin yields were obtained in TSB medium, a medium formulated with digest of casein and soybean meal and other carbon–nitrogen sources.

We previously demonstrated that the addition of *B. cereus* 183 cells to cultures of *B. thuringiensis* grown at pH 7.2, 180 rpm, and 28°C (baseline condition) significantly enhanced LBIT 269, LBIT 287, LBIT 404, LBIT 420, and LBIT 524 yield by, respectively, 42, 65, 68, 90, and 79% [11]. The results in the present study clearly demonstrated that if Mexican strains of *B. thuringiensis* strains are induced with *B. cereus* and then cultivated under conditions using different physical parameters (pH, temperature, and agitation), an increment or decrement (data not shown) in the bacteriocin activity is observed compared with bacteria cultured under baseline conditions. We detected that the physical parameters that produced the highest cell growth do not necessarily correspond to the highest yield in bactericidal activity (Table 2; Fig. 2). Similar results have been observed elsewhere [5, 24].

When we analyzed the physical conditions (Table 2) that optimized the growth and antibacterial activity of the five strains of *B. thuringiensis*, we found that LBTI 269 and LBIT 404 had the highest increment in antibacterial activity (100%) when compared with induced *B. thuringiensis* strains grown under baseline conditions, even though these did not yield the highest bacterial growth (Fig. 2). Although LBIT 287 showed the highest growth (Fig. 2), this strain had a lower increment in the antibacterial activity compared with LBIT 268 and LBIT 420, which suggests that the increment in the bacteriocin production depends on the physiology of the particular strain [3, 5].

Finally, statistical analysis indicates that pH was the most important physical factor that affected not only growth of the five *B. thuringiensis* strains, but also bacteriocin production. The effect of pH on the production of other bacteriocins has been demonstrated experimentally and by predictive models [10, 16, 21]. In conclusion, our studies show that incremental production of bacteriocin synthesized by *B. thuringiensis* can be significantly enhanced by using two strategies simultaneously: induction with a susceptible bacterium and modification of the physical culture conditions.

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