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Effect of physical factors on the production of bacteriocin from *Pediococcus acidilactici* ITV 26

M Calderón-Santoyo, PG Mendoza-García, MA García-Alvarado and BI Escudero-Abarca

Departamento de Ingeniería Química–Bioquímica, Instituto Tecnológico de Veracruz, Veracruz, Ver. A.P. 1420 C.P. 91860, México

The effect of pH, temperature and agitation on growth and bacteriocin production by *Pediococcus acidilactici* ITV 126 was investigated. Experiments were made in flasks containing MRS medium at 30 to 40°C, pH 5 to 7 and agitation 0 to 200 rpm. Factor levels were arranged in a 2^3 factorial design with central and axial points. Anova and Tukey paired comparison tests showed that a temperature of 35°C favored bacteriocin production, whereas 40°C was best for cell growth. A statistical interaction of temperature and agitation was observed affecting microbial growth. pH 5 favored both cell growth and bacteriocin production. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 191–195.

Keywords: Bacteriocin; *Pedioccoccus*; physical factors

Introduction

Bacteriocins, peptides produced by lactic acid bacteria are effective against some foodborne pathogens and offer a good alternative to chemicals used as food preservatives. Nisin is the only bacteriocin that has been approved by the U.S. Food and Drug Administration to be used in food preservation, although its use is limited to acidic foods [7]. In recent years the bacteriocins produced by *Pediococcus*, named pediocins, have received much attention as potential food biopreservatives [1,10,11,13]. The inhibitory spectrum of pediocins has been reported and shows that they are effective against most lactic acid bacteria and various Gram-positive pathogens [16,18], the latter including *Listeria monocytogenes* [6,13,15], *Clostridium perfringens* and *Staphyloccus aureus* [2]. Thus, pediocins may be useful in control of food pathogens. Bacteriocin production is affected by pH, temperature and agitation [3,19]. However, little is known about the interactions between them. The aim of this work was to study the influence of pH, temperature agitation and their interactions on pediocin production by *Pediococcus acidilactici* ITV 26.

Materials and methods

Microorganisms

The bacteriocin producer *P. acidilactici* ITV 26 was isolated from fermented sausages and vegetables; 5 g of sample was homogenized in 10 ml of 0.1 M phosphate buffer, pH 7.0. A 1-ml aliquot was transferred to 10 ml of MRS broth (pH 6.0) and Elliker broth (pH 6.5), and incubated under anaerobic conditions at 37°C for 24 h. Tubes that showed growth were used to plate on MRS agar (37°C, 24 h). A total of 150 strains was screened by inhibitory activity using the agar spot test and *Enterococcus faecalis* NRRL-B537 as indicator strain. *P. acidilactici* ITV 26, producing bacteriocin with the highest potency and stability on MRS medium, was selected and identified as a Gram-positive, nonspore-forming and catalase negative. It was identified using the API 50 CHL gallery and Bergey's Manual of Determinative Bacteriology [5]. The strain was deposited at the collection of the Technological Institute of Veracruz. Stock cultures were maintained on refrigerated MRS agar slants or frozen in 10% glycerol at −18°C. *E. faecalis* NRRL-B537 was kindly supplied by the ARS Culture Collection, U.S. Department of Agriculture, Peoria, IL, USA and was maintained under the same conditions as *Pediococcus* in nutrient broth. Cultures were transferred to new agar slants every 6 months.

Culture conditions

P. acidilactici ITV 26 was grown in 10×120 mm culture tubes containing 10 ml of MRS broth at 37°C for 24 h. A 10-μl aliquot (O.D. 0.26 at 660 nm) was inoculated in 250-ml Erlenmeyer flasks

Table 1 2^3 Factorial design with central and axial points

Run	pH	T (°C)	Rpm	Codified variables (Equations 2 to 4)		
				X_1	X_2	X_3
1	5	30	0	−1	−1	−1
2	7	30	0	+1	−1	−1
3	5	40	0	−1	+1	−1
4	7	40	0	+1	+1	−1
5	5	30	200	−1	−1	+1
6	7	30	200	+1	−1	+1
7	5	40	200	−1	+1	+1
8	7	40	200	+1	+1	+1
9	6	35	100	0	0	0
10	7	35	100	+1	0	0
11	5	35	100	−1	0	0
12	6	40	100	0	+1	0
13	6	30	100	0	−1	0
14	6	35	200	0	0	+1
15	6	35	0	0	0	−1

Correspondence: BI Escudero-Abarca, Departamento de Ingeniería Química–Bioquímica, Instituto Tecnológico de Veracruz, Veracruz, Ver. A.P. 1420 C.P. 91860, México
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containing 30 ml of MRS broth and incubated for 12 h at different temperatures (30, 35 and 40°C), agitation (0, 100 and 200 rpm) and pH (5, 6 and 7). pH was controlled by 0.1 M phosphate buffer and adjusted with NH_4OH. The final pH found in all experiments was 4.6–4.9.

Bacteriocin assay

Culture samples were centrifuged at 5000×*g* for 15 min to obtain cell-free supernatants that were neutralized with 0.1 N NaOH to pH 6.8, and microfiltered using 0.45-μm pore size sterile membranes (MFS). Pediocin activity was determined using serial dilutions (1:100 to 1:5000) of the cell-free supernatant in 5 ml of MRS broth and 50 μl of an overnight culture of the sensitive strain *E. faecalis* NRRL-B537 adjusted to an O.D. of 0.2 at 660 nm (approximately 10^5 cfu/ml). Inoculated tubes were incubated 12 h at 37°C undisturbed, and O.D. 660 nm was recorded. One activity unit (AU) was expressed as the inverse of the dilution that produced a 50% growth inhibition of the sensitive strain.

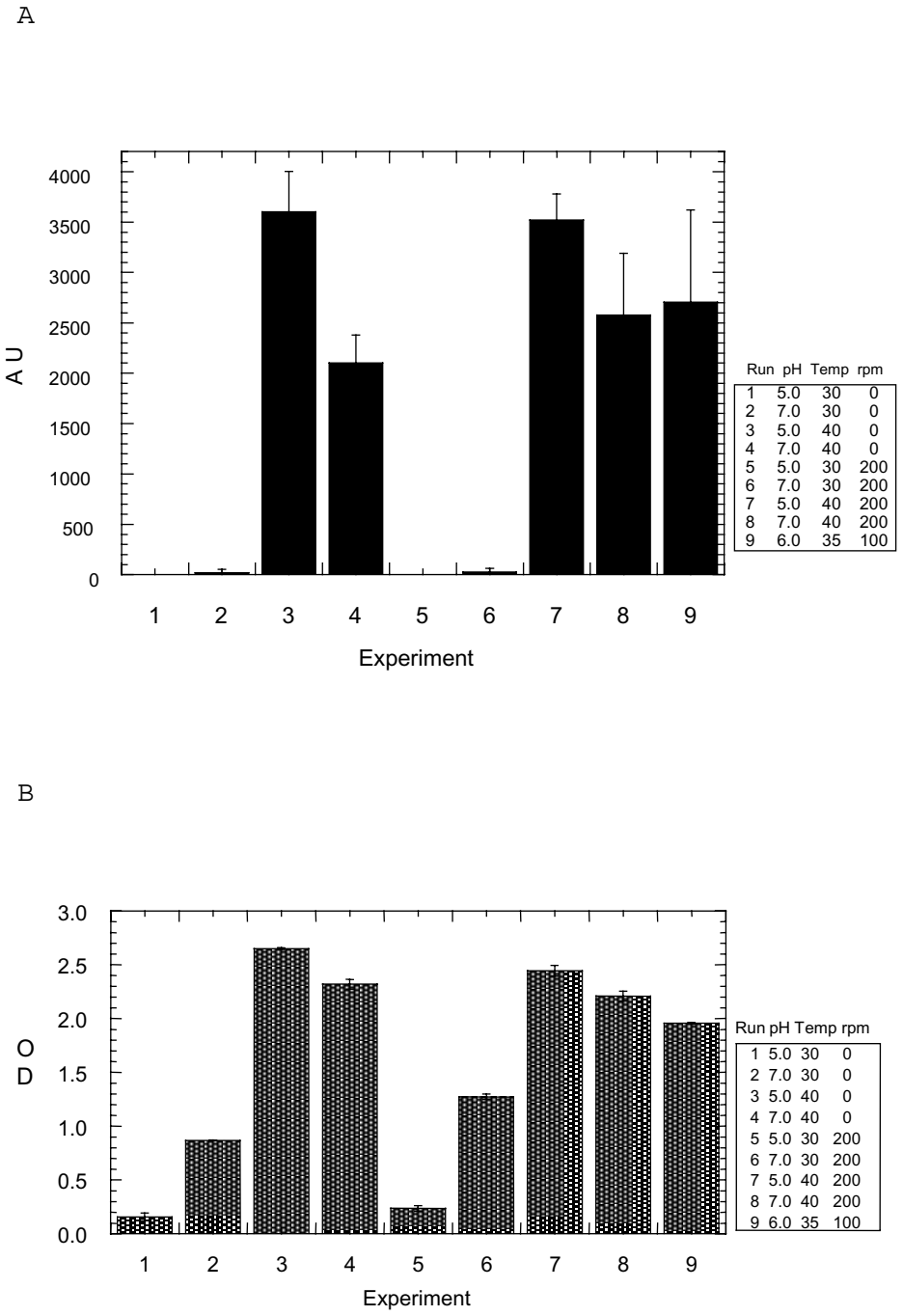

Figure 1 (A) Bacteriocin activity (AU) and cell growth of *P. acidilactici* ITV 26 monitored as optical density (B) at different conditions of pH, temperature and agitation, according to the statistical design 2^3 (described in Table 1). The fermentation medium was MRS and the incubation time for all cases was 12 h. Bars represent standard deviations.

Modeling

A surface response model was used to evaluate numerically the effect of pH, temperature and agitation on bacteriocin activity and cell growth yield. This type of model was suggested by Box *et al* [4] to model phenomena that do not have any theoretically obtained relationship. This statistical method has been applied in the biotechnological production of glucosyltransferase by *Aspergillus niger* and synthesis of trehalose and sucrose, for example [12,17]. In this work, bacteriocin activity and cell growth yield (Y) are represented as a second-order polynomial of the codified variables for pH, temperature and agitation, named X_1, X_2 and X_3, respectively. The polynomial model is represented as

$$X = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Experimental design

The orthogonal numerical evaluation of the polynomial model parameter estimators requires a 2^3 experimental design with six axial points (Box *et al* [4]). The experimental design structure for the codified and noncodified variables are presented in Table 1. The relation between these variables are expressed in the following equations,

$$X_1 = (\mathrm{pH} - 6)/1.0$$

$$X_2 = (T - 35)/5$$

$$X_3 = (\mathrm{rpm} - 100)/100$$

These last equations are stated in a manner that the limits for codified variables (X_1, X_2, X_3) are between -1 to $+1$.

Results and discussion

In preliminary studies, it was shown that *P. acidilactici* ITV 26 grows optimally at pH 5–7, producing bacteriocin in variable amounts (data not shown). However, Biswas *et al* [3] reported that pediocin AcH was detected at pH 4 although the highest production was observed when the initial pH was 6.4. The difference could be due to the different strain used and medium composition (MRS for *P. acidilactici* ITV 26 and TGE for *P. acidilactici* H). In this study we selected MRS medium because a greater bacteriocin production was obtained.

Treatments that produced the highest cell growth also exhibited the highest production of bacteriocin. The two experiments that showed the highest production of bacteriocin and cell growth were, respectively, treatments 3 (pH 5, 40°C, no agitation) and 7 (pH 5, 40°C, 200 rpm) (Figure 1A and B); thus it was deduced that the best conditions for *P. acidilactici* to grow and produce bacteriocin are pH 5 and 40°C, and that agitation has no effect. Under treatments 1, 2, 5 and 6, *P. acidilactici* ITV 26 was unable to grow and to produce bacteriocin. In all four cases the temperature was 30°C. These results do not agree with those found for *P. acidilactici* H [3], which showed a maximum cell mass and pediocin production at 30 and 37°C, respectively, and a reduction of both at 40°C.

We also found that cell growth was affected by the interaction between agitation and temperature (Table 2). Because agitation alone did not have any effect on growth and production, we suggest that the effect found is due to an increase in temperature caused by agitation. However, fermentor level studies are needed to support this.

As statistical significance in the central point was found (treatment 9, Figure 1A), we made measurements of bacteriocin activity for the execution of axial points to find out a curvature in the response caused by temperature (Figure 2).

Although bacteria grew well in treatment 13 (pH 6, 30°C, 100 rpm) they failed to produce bacteriocin (Figure 2A and B), confirming that an incubation temperature of 30°C is unfavorable for bacteriocin production. The level of bacteriocin in treatment 11 (pH 5.0, 35°C, 100 rpm) was similar to that in treatments 3 and 7, which were made at 40°C. This suggests that curvature effect exists associated with temperature, since $\alpha = 4.7 \times 10^{-4}$ for ${X_2}^2$ (Table 2). The polynomial model fitted for AU is a linear regression model ($r^2 = 0.9666$) and is,

$$\mathrm{AU} = 2323.9 - 481.44X_1 + 1419.3X_2 + 19.63X_3 - 311.55X_1X_2 + 69.76X_1X_3 + 48.61X_2X_3 + 101.39X_1^2 - 1009X_2^2 + 41.09X_3^2.$$

The results of model parameters (Table 2) provides additional evidence that agitation had no effect on the production of bacteriocin. Similar findings are reported in the literature for other kinds of bacteriocins [8,14]. pH presented a significant negative effect on bacteriocin production, and a negative interaction with temperature. In contrast, temperature alone had a positive effect on bacteriocin production. It is interesting that at pH 5, the range of temperature that affected bacteriocin production was 30–35°C whereas at pH 7, the range was wider (30–40°C). This is probably related to posttranslational processing, as has been described for bacteriocin AcH [9,19]. Consequently, we suggest that activation of pediocin occurs efficiently at pHs close to 5 and that activation is affected by small changes in temperature. Temperature showed a significant negative curvature effect, which means that optimum temperature may be located within the range we examined.

In conclusion, physical conditions play an important role on cell growth and production of pediocin of *P. acidilactici* ITV 26. The experimental design used in this study was suitable for analyzing the effect of physical factors and their interactions on the production

Table 2 Values obtained and statistical significance (α) for model parameters of for bacteriocin production (AU) and for cell growth (OD) Equation 1

	AU		OD	
	Value	α	Value	α
β_0	+2323.90	5.00E−7	2.1013	1.35E−8
β_1	−481.44	7.38E−4	0.0912	3.14E−2
β_2	+1419.30	4.50E−7	0.8273	4.48E−8
β_3	+19.63	9.64E−1	0.0206	6.16E−1
β_{12}	−311.55	2.40E−2	−0.2890	2.77E−5
β_{13}	+69.76	5.99E−1	0.0524	2.52E−1
β_{23}	+48.61	7.22E−1	−0.0999	3.46E−2
β_{11}	+101.39	6.66E−1	−0.0317	6.99E−1
β_{22}	−1009.00	4.70E−4	−0.2931	1.69E−3
β_{33}	+41.06	9.54E−1	−0.2472	8.33E−3

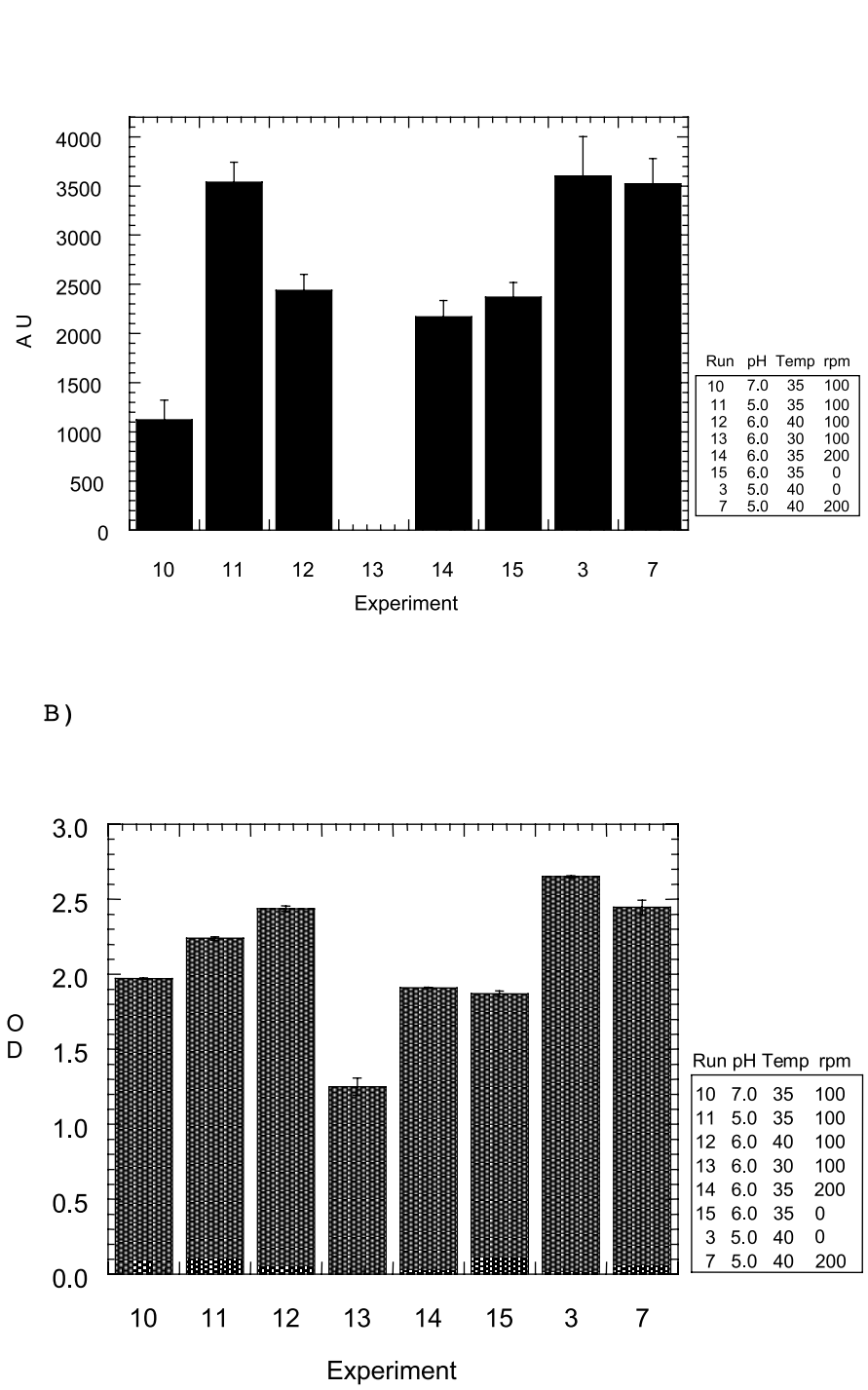

Figure 2 (A) Bacteriocin activity (AU) of *P. acidilactici* ITV 26 corresponding to axial points (see Table 1) and (B) bacterial growth (optical density). Growth was in MRS broth for 12 h. Bars represent standard deviations.

of bacteriocin. The best conditions for the production of bacteriocin were pH 5 and 35°C and for growth were pH 5.0 and 40°C, and without agitation for both cases.

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