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Full Length Research Paper

Growth of *Enterococcus durans* E204 producing bacteriocin-like substance in MRS Broth: Description of the growth and quantification of the bacteriocin-like substance

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Bacteriocin-like substance E204 is an antimicrobial compound produced by *Enterococcus durans* E204 isolated from camel milk of Morocco that shows a broad spectrum of inhibitory activity against taxonomically related microorganisms. It is sensitive to digestive proteases. In the first study, de Man, Regosa and Sharpe (MRS) broth was inoculated by E204 strain, incubated for 30°C at 200 rpm and monitored by checking dry cell weight, nutrients consumption, lactic acid and bacteriocin-like productions. The maximum biomass (2.3 g/l) and antimicrobial activity (32 AUml^{-1}) were obtained at 12 h of incubation. No increase in the production of bacteriocin-like was recorded after the exponential phase. In the other hand, the quantification of the antimicrobial activity was carried out by a photometric assay on culture tubes based on the determination of the ID50 dose causing 50% growth inhibition of Enterococcus faecium 410 CECT in 6 h of incubation. The highest bacteriocin-like titre (279.71 BUml^{-1}) was obtained at acidic pH (3.5) and at 70°C after 10 min of incubation.

Key words: *Enterococcus durans*, bacteriocin-like, MRS broth, nutrients consumption, quantification.

INTRODUCTION

Enterococci are Gram-positive cocci among lactic acid bacteria (LAB). LAB produces diverse antimicrobial substances, including organic acid, hydrogen peroxide and bacteriocins, which are of importance in food preservation. Bacteriocins are antimicrobial proteinaceous substances secreted by some bacteria against microorganisms that are usually closely related to the producer organism (Klaenhammer, 1988). They are used as natural food additives for the elimination of spoilage and pathogeinic microflora. This study was undertaken firstly to determine the profile of the principal compounds (sugar, protein, nitrogen and phosphorus) remaining in the MRS broth (de Man Regosa and Sharpe, 1960) during the growth of *Enterococcus durans* E204 isolated from the camel milk of Morocco (Khay et al., 2011). In addition, the biomass and the lactic acid were also determined. Secondly, bacteriocin-like produced by *Enterococcus durans* E204, was quantified by a turbidimetry assay in order to evaluate the growth inhibition of *Enterococcus faecium* 410 CECT. The turbidimetry assay has considerable advantages compared to common methods used to estimate bacteriocin in culture supernatant such as the agar diffusion tests (Tramer and Fowler, 1964; Richardson et al., 1968; Wolf and Gibbons, 1996) and the microtitration (Daba et al., 1994) that are time-consuming and with limited specificity and accuracy. In this study, the procedure used request only 6 h for quantification of

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bacteriocin-like. The dose-response model, proposed by Cabo et al. (1999), was used to calculate the 50% inhibittory dose (ID_{50}) by interpolation to provide a continuous scale for bacteriocin-like activity and a complete inhibition curve was generated by a mathematical model. *Enterococcus durans* is found in various habitats. However, only a few papers have been published on bacteriocins produced by *Enterococcus durans*, such as strain L28-1 (Yanagida et al., 2005) and strain QU49 (Hu et al., 2008). Little is know about the growth conditions required for optimal growth and bacteriocin production by the corresponding bacteria.

A number of studies have been conducted on the influence of parameters on the production and the stability of bacteriocins. Nisin and pediocin productions were lower on whey media than on MRS broth and both bacteriocins were heat-stable particularly at low pH (Guerra et al., 2001). The pediocin titres on whey were 9.7 times lower than those obtained on MRS broth (Guerra and Pastrana, 2002). This suggests that the whey media lacks some essential nutrients for growth and bacteriocins production.

In this paper, we report on bacteriocin-like produced by *Enterococcus durans* E204 camel milk isolate on MRS broth. The effects of nutrients consumption and lactic acid production on the production of bacteriocin-like are discussed.

MATERIALS AND METHODS

Bacterial cultures and medium

Enterococcus durans E204, isolated from camel milk of Morocco, was used as bacteriocin-like-producing strain. *Enterococcus faecium* CECT 410 (Spanish Type Culture Collection) was used as target organism. The two bacteria were grown in MRS broth (de Man, Regosa and Sharpe, Panreac, Barcelona, Spain) and maintained as frozen stock held at -40°C in MRS broth plus 35% (v/v) glycerol. The cultures were maintained as slants on MRS agar at 4°C, and propagated twice in liquid culture at 30°C before use.

Analytical methods

Enterococcus durans E204 was grown in a 1 L Erlenmeyer flask containing 200 ml of sterile MRS broth at 30°C with agitation at 200 rpm orbital shaking. Each 2 h, cells of culture samples were harvested by centrifugation at 15000 *g* for 15 min at 4°C, washed twice and re-suspended in saline solution (0.8% NaCl) to measure the absorbance at 700 nm. The dry cell weight was estimated from a previous calibration curve. The corresponding supernatants were collected and pH was measured. Thereafter, these supernatants were used to determine total sugars by phenol–sulphuric acid method (Dubois et al., 1956) according to Strickland and Parsons (1968) with glucose (Panreac, Barcelona, Spain) as standard, phosphorous by molybdate reaction (Murphy and Riley, 1962) according to Strickland and Parsons (1968) with potassium dihydrogen phosphate (Panreac, Barcelona, Spain) as standard, total nitrogen by micro-kjeldahl substituting distillation for the spectrophotometric method of Havilah et al. (1977) with ammonium sulphate (Panreac, Barcelona, Spain) as standard and proteins by the method of Lowry et al. (1951) with bovine serum albumin (Sigma, St. Louis, MO, USA) as standard. Lactic acid was quantified in culture supernatants by high-performance liquid chromatography (HPLC) using an ION-300 organic acid column (length 300 mm, internal diameter 7.8 mm) with a pre-column IONGUARD^{TM} (polymeric guard column), both obtained from Teknokroma S. Coop. Ltda, Barcelona, Spain. The mobile phase consisted of 6 mM sulphuric acid at a flow rate of 0.4 ml min^{-1} at 60 to 65°C and the refractive index of the peaks was measured (Guerra and Pastrana, 2003). Alls assay were carried out in triplicates.

The antimicrobial activities of the corresponding supernatants were determined using the well diffusion method (Tagg and McGiven, 1971). The supernatants adjusted to pH 6 (to prevent the inhibitory effect of lactic acid) were filtrated with 0.22 µm pore-size syringe and serial two-fold dilutions were prepared. Aliquot of each dilution was placed in wells (6mm) cut out in cooled MRS agar plate overlaid with soft agar (7.5 g/l) seeded with an overnight culture of indicator strain. After incubation at 30°C for 24 h, the arbitrary units of activity (AU/ml) of the bacteriocin-like were determined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain (Barefoot and klaenhammer, 1983).

Effects of temperature and pH on the activity of bacteriocin-like E204

Extraction of antibacterial compound

Enterococcus durans E204 culture from 18 h old was adjusted to pH 3.5 with 5 N HCl to avoid the adsorption of molecules of bacteriocin onto the producer cell surfaces (Yang et al., 1992). Subsequently, it was heated for 3 min to kill the cells and centrifuged at 15000 *g* for 15 min at 4°C. The supernatant (containing bacteriocin-like extract) was adjusted to pH 6, divided into three aliquots and frozen until further use.

Bacteriocin-like activity assay

Aliquots of bacteriocin-like extracts were adjusted at different pH values with 5 N HCl or NaOH, and incubated for 5, 10 and 20 min at the temperatures defined in the experimental matrix (Table 1). After incubation, the samples were adjusted to pH 6.0 and monitored for the remaining activity. Controls consisted of samples of untreated supernatant.

Quantitative activity of bacteriocin-like from culture of E204 strain was estimated by using a photometric assay on culture tubes (Cabo et al., 1999). *Enterococcus faecium* 410 CECT was used as target organism. The method consists the determination of growth inhibition at 700 nm of the target organism caused by serial dilutions of supernatants.

The supernatants were diluted as needed in distilled sterile water. 1 ml of the diluted bacteriocin-like extracts was added in sterile culture tubes. Each tube was inoculated with 1 ml of a culture of *Enterococcus faecium* 410 CECT diluted to an absorbance of 0.2 at 700 nm with sterile buffered MRS broth at pH 6. Controls consisted of three culture tubes in which the diluted bacteriocin-like extract was substituted by distilled sterile water. The tubes were incubated for 6 h at 30°C. Growth inhibition was measured spectrophotometrically at 700 nm. Dose–response curves were obtained from these data. Bacteriocin-like activity was calculated as Bacteriocin Units ($BUml^{-1}$); $1BUml^{-1}$= amount of bacteriocin needed to obtain 50% growth inhibition of indicator strain (lethal dose 50: LD_{50} obtained from duplicate samples) compared to the control tubes.

Table 1. Experimental domain and codification of the variables.

Coded values	Natural values	
	Temperature (°C)	pH
- 1.414	59.4	2.8
- 1	70	3.5
0	95.9	5,3
1	121.0	7
1.414	131.6	7.7
Increments		
1	25.5	1.8

Statistical method

A multifactorial composite rotatable design (Akhnazarova and Kafarov, 1982), based on five levels and two variables, was used to study the combined effect of pH and temperature on the stability of bacteriocin-like E204. The design consisted of 16 experiments with four (2^2) factorial points, four axial points to form a central composite design with α=1,414 and five centre points for replication. Response surfaces were depicted from empirical equations derived from the design. The range and codification of both variables are shown in Table 1.

RESULTS AND DISCUSSION

Biomass, lactic acid and antimicrobial activity productions and nutrients consumption

The experimental results are shown in Figure 1. The concentrations of nutrients (total sugars, protein, nitrogen and phosphorous) decreased with an increase in biomass production in MRS broth.

In addition, the profiles described by the production of lactic acid and antibacterial activity appeared in parallel to the biomass synthesis. *Enterococcus durans* E204 consumed practically the entirety of the glucose on commercial MRS medium. The maximum production of lactic acid was observed at 12 h. The corresponding value was 6.58 g/l and the glucose consumption was about 60%. Thus, the lactic acid production and the glucose consumption were affected by the use of MRS medium whose glucose was converted to lactic acid (only lactic acid produced). The biomass curve revealed an exponential phase and a stationary phase that began at 12 h of incubation at the time the biomass reached 2,3 g/l. These values are similar than reported for normal fermentations in MRS broth for several strains growth (Vázquez and Murado, 2008; Vázquez et al., 2004, 2008). During 20 h of growth, the maximal production of bacteriocin-like (32 $AUml^{-1}$) was recorded after 10 h. The pH of culture decreased from 6.63 to 4.2.

The E204 strain utilized the glucose and the proteins contained in the medium, as carbon and nitrogen sources. Carbon source was used to form cellular material (cellular growth as biomass formation), metabolic products (in this study lactic acid) and to maintain the remaining cellular functions and nitrogen source for antimicrobial activity production. As well, the bacteria need a source of nitrogen to grow, for this reason the total nitrogen was proportioned. With regards to nutrient consumption, *Enterococcus durans* E204 consumed important amounts of total sugars, protein, nitrogen and phosphorous indicating that MRS medium had a good affinity for cell growth. Also, it was observed that the metabolic activity was very important in the first 12 h of incubation when the pH of the culture was between 6.5 and 4.5.

However, when the pH value decreased below 4.5, the metabolic activity was very low. When the cells reached the stationary phase, growth and antimicrobial activity ceased. It has been reported that the activity of bacteriocin in broth cultures was stable after the exponential phase (Floriano et al., 1998). Growth of lactic acid bacteria is inhibited by the production of lactic acid and by the limited concentrations of nutrients present in the medium (Biswas et al., 1991; Leroy and De Vuyst, 2001).

In the other hand, transport nutrient is a function of pH levels. The limitation of the bacteriocin-like production is a result of this growth inhibition due to the growth-associated character of bacteriocin-like production. The activity of bacteriocin-like E204 did not decrease during the 3 days of incubation at 30°C (data not shown), suggesting that the E204 strain did not produce peptidases active against the bacteriocine-like which could be a primary metabolite of *Enterococcus durans* E204 strain. Similar results were reported for bacteriocins ST461BZ and ST462BZ produced by *Lactobacillus rhamnosus* (Todorov and Dicks, 2005) and bacteriocin produced by *Lactobacillus rhamnosus* GP1 (Sakira et al., 2010). In Figure 1, it can be noted that at 12 h of incubation, the concentration of nitrogen increased in the medium due certainly to the relative amounts of essential amino acids generated from proteins degradation observed after 12 h of incubation by proteases produced in the medium.

Effects of temperature and pH on the activity of bacteriocin-like E204

After analysis of the main nutrients and metabolites that influence the production of bacteriocin-like and important titre in the experience of production on MRS medium at 30°C and without controlled pH are obtained, it seemed reasonable to study the behaviour of this antimicrobial compound in the different conditions of pH and temperature, as well as on the time treatment at levels commonly used in food treatments on the same medium.

In general, this study was directed to determine

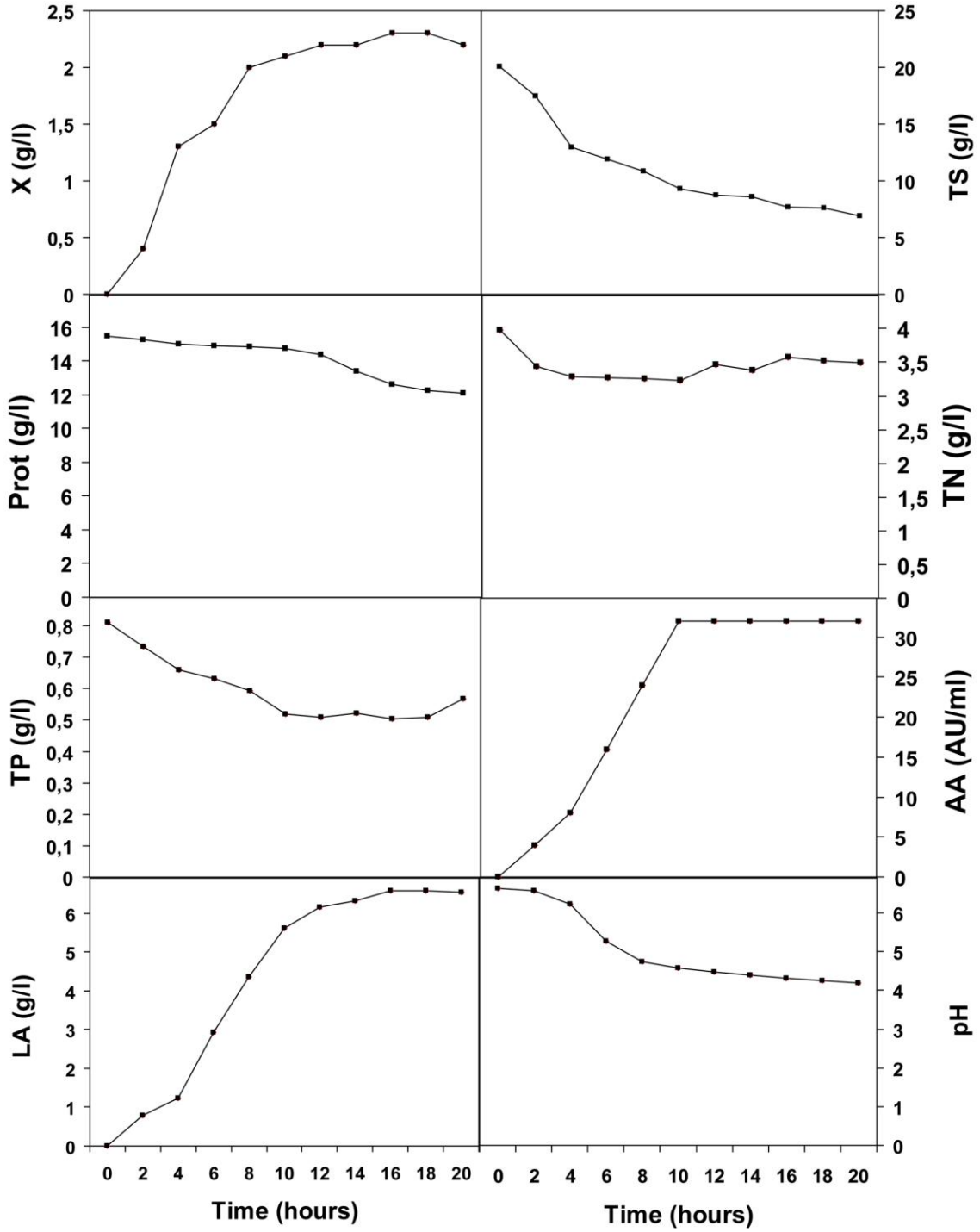

Figure 1: Time course of *Enterococcus durans* E204 on MRS medium. X: biomass, TS: total sugars, LA: lactic acid, TP: total phosphorous, AA: antimicrobial activity, Prot: proteins, TN: total nitrogen. Data are from one experiment with three replicates.

thermostability to a certain value of pH or to determine the stability against pH, maintaining the samples to a constant temperature, as it happened to the nisin and the pediocin (Guerra et al., 2001).

Table 2 shows the experimental matrix, as well as the corresponding results for antimicrobial activity of bacteriocin-like E204 remained in the samples. The empirical equations, obtained for antimicrobial activity ($BUml^{-1}$) for bacteriocin-like E204 samples after temperature (T) and pH treatment for 5, 10 and 20 min,

Table 2. Experimental matrix and bacteriocin-like titres obtained after pH and temperature treatment for 5, 10 and 20 min of bacteriocin-like E204 samples.

Natural value		Coded value		Bacteriocin-like titre ($BUml^{-1}$)		
T(°C)	pH	T(°C)	pH	5 min	10 min	20 min
121.0	7.0	1	1	212.86	153.55	118.08
121.0	3.5	1	-1	176.47	271.71	174.20
70.0	7.0	-1	1	241.05	179.22	179.23
70.0	3.5	-1	-1	230.45	279.71*	180.98*
131.6	5.3	1.41	0	182.45	230.34	124.93
59.4	5.3	-1.41	0	249.19*	224.05	119.74
95.5	7.7	0	1.41	172.12	188.16	83.46
95.5	2.8	0	-1.41	191.88	240.34	127.61
95.5	5.3	0	0	243.67	250.34	169.89
95.5	5.3	0	0	240.54	249.67	170.34
95.5	5.3	0	0	242.67	258.92	172.32
95.5	5.3	0	0	243.87	254.32	168.34
95.5	5.3	0	0	243.95	252.12	173.34
95.5	5.3	0	0	245.87	253.23	174.23
95.5	5.3	0	0	244.65	255.45	172.23
95.5	5.3	0	0	243.98	253.76	170.78

*Maximal bacteriocin-like titres.

were as follows:

$BT\ (BUml^{-1})_{5min} = 243.65 – 22.07T + 2.38pH + 6.45TpH – 9.84T^2 – 26.75pH^2$

$BT\ (BUml^{-1})_{10min} = 253.48 – 3.1T – 53.56pH – 4.42TpH + 13.06\ T^2 – 19.53pH^2$

$BT\ (BUml^{-1})_{20min} = 171.43 – 7.57T – 15.04pH + 13.59TpH + 12.25T^2 – 20.65pH^2$

The coefficients of model were found to be significant according to the Fisher F test ($\alpha < 0.05$) and Student's test ($\alpha < 0.05$). The two independent variables had negative coefficients for bacteriocine-like stability except for pH (5 min). On the contrary, the coefficients of the interactions between variables had positive effects on response except for 10 min. The response surfaces corresponding to the remaining activity of bacteriocin-like E204 samples after pH and temperature treatment for 5, 10 and 20 min are shown in Figure 2. It can be noted that the temperature has a high negative effect on the bacteriocin-like stability, however the pH had a slight positive effect (after 5 min of incubation). The positive interaction between temperature and pH showed that increased bacteriocin-like E204 stability was obtained with neutral pH at low temperature. In fact, the increase in temperature inhibited the antimicrobial activity of bacteriocin-like. In parallel, the bacteriocin-like showed high activity at neutral pH. However, after 10 min of incubation, the two variables possessed negative effects. The negative interaction between the two variables was explained by the fact that the increase in pH levels displayed a dramatic restriction of bacteriocin-like activity. After 20 min of incubation, it was observed that both variables temperature and pH produced negative effects on the bacteriocin-like activity and forming positive interaction.

Interactions between the two variables were discussed for bacteriocin-like extracts after 5, 10 and 20 min of incubation. At the three incubation times, the remaining activity of bacteriocin-like extracts seemed to be similar at different combinations of the two variables (Table 2). These findings indicate that the bacteriocin-like E204 are heat-stable over the wide range of pH values and could be applied in fermented food products usually processed at the levels of pH and temperature chosen in this study. This character is common for all enterocins (Giraffa et al., 1994). The maximal bacteriocin-like titre (279.71 $BUml^{-1}$) was recorded at 70°C and at pH (3.5) after 10 min of incubation. The heat-stability of bacteriocin-like studied depended on the pH. At acidic pH values, it was more heat-stable than at neutral or alkaline pH. This can be attributed to bacteriocin-like solubility that increases with decreasing pH while, bacteriocin-like E204 was most sensitive to temperature and pH after 20 min of incubation, respectively. Lower bacteriocin-like activities recorded after 20 min of incubation can be attributed to the time of incubation. In effect, the highest bacteriocin-like titre (180.89 $BUml^{-1}$) was obtained at acidic pH (3.5) and at 70°C. The results obtained show similar behaviour than enterocin A reported by Rehaiem et al. (2011).

Conclusion

In this study, it has been shown that the bacteriocin-like producing milk isolate *Enterococcus durans E204 was*

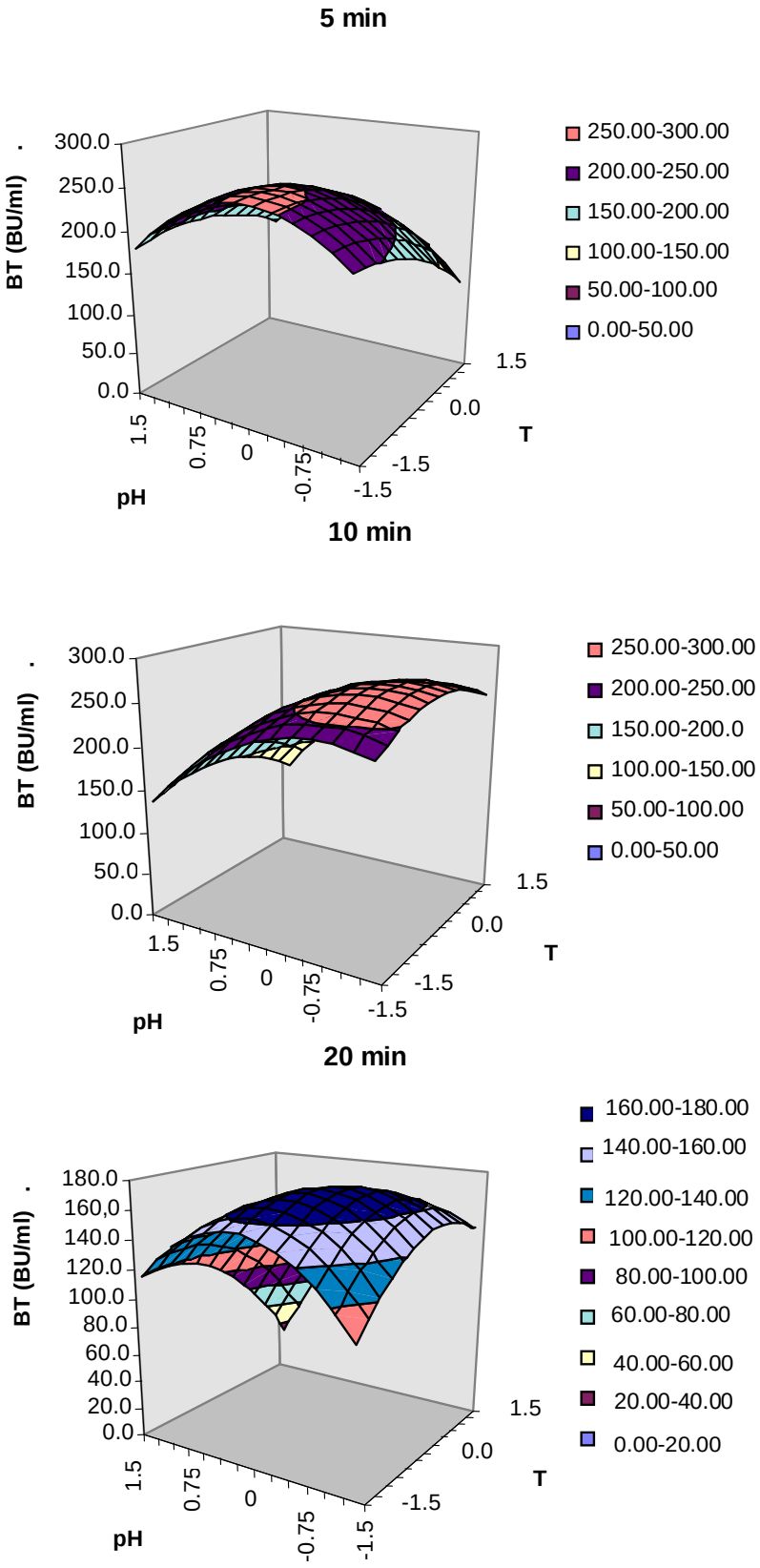

Figure 2. Response surfaces corresponding to antimicrobial activity of bacteriocin-like E204 samples after pH and temperature treatment for 5, 10 and 20 min according to the experimental plan defined in Table 1.

limited in its growth in MRS medium due to the nutrients depletion. Thus, this strain will be adapted to other environment contained components stimulating biomass production and resulting in higher bacteriocin-like titres at low production cost. MRS is a complex growth medium used for the normal growth of lactic acid bacteria and the production of bacteriocins, but it is not economical. Adoption of raw materials like some wastes from the food industry as a culture media for a large scale production is recommended. To find out a suitable culture medium for cell growth and antimicrobial extracellular products (AECP) synthesis in *Enterococcus durans* E204, cultures would be grown in others media adjusted to an initial pH of 7.0.

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