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Effect of thermal treatment of whey contaminated with antibiotics on the growth of *Kluyveromyces marxianus*

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Research Article

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Abstract

The objective of the studies reported in this research communication was to investigate the use of whey contaminated with antibiotics such as cephalosporins, quinolones and tetracyclines as a nutrient medium for the growth of *Kluyveromyces marxianus* with particular attention to the effect of thermal treatment used to overcome the inhibitory effects of antibiotic concentrations close to the Maximum Residue Limits. The heat treatments at 120 °C for 40 min, 120 °C for 83 min, and 120 °C for 91 min caused total inactivation of cephalosporins, tetracyclines and quinolone residues in whey respectively.

Antibiotics are used for the treatment of numerous diseases of dairy cows, such as mastitis, metritis, pneumonia, and enteritis. However, some antibiotic molecules are not fully metabolized and can thus be found as antibiotic residues in milk (Siljanoski et al., 2018). The presence of antibiotic residues in milk and whey is a problem for the dairy industry and the environment. Whey can be utilized for the production of yeast biomass by whey fermentation. Among the various yeasts that can be used, *Kluyveromyces marxianus* is important because of its good ability to assimilate lactose. However, Althaus et al. (2014) emphasize that the fermentative capacity of *K. marxianus* decreases when residues of cephalosporins, quinolones and tetracyclines are present at levels close to the Maximum Residue Limits (MRLs) (European Community, 2009) established in milk. The information available on the inactivation of antibiotics in whey is limited, and for this reason it is necessary to better understand thermal degradation of antibiotics in whey to enable its use as a growth matrix. Therefore, the objective of this study was to evaluate the effect of the thermal treatment time at 120 °C of whey fortified with cephalosporins, quinolones and tetracyclines, through the growth of *Kluyveromyces marxianus* and its lactose consumption.

Materials and methods

Antibiotics

The antibiotics (Sigma-Aldrich, St. Louis, USA) used were three cephalosporins (cephalexin, cefoperazone and ceftiofur), three quinolones (ciprofloxacin, enrofloxacin and marbofloxacin), and three tetracyclines (chlortetracycline, oxytetracycline and tetracycline). In all cases, a concentration of antibiotics equal to the MRLs established by the European Community (2009) and Codex Alimentarius (2010) was used, with a limit of 100 µg/l for the antibiotics tested, except for cefoperazone and marbofloxacin for which the limit was 75 µg/l and 50 µg/l, respectively.

Yeast

Kluyveromyces marxianus (ATCC 8554) obtained from the collection of strains of the Department of Microbiology of the Facultad de Ingeniería Química, Universidad Nacional del Litoral (Santa Fe, Argentina) was used.

Culture medium

Antibiotic-free whey was deproteinized by heat treatment at 120 °C for 20 min followed by filtration of precipitated proteins, and then fortified with 5 g/l yeast extract (Merck Millipore, USA) and 25 g/l peptone casein (Biokar Diagnostics, Allonne, France). Then, the pH was initially adjusted to 7·5 (with 1 M NaOH) for the fermentation to develop for a time of 12 h (final pH 5·5).

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Experimental design

Five aliquots of the above-described medium were used for each treatment, which were: [Control], without antibiotic or heat treatment; [ATB], with antibiotic and without heat treatment; [TT_{20}], [TT_{40}], [TT_{60}], with antibiotic and heat-treated at 120 °C for 20, 40 or 60 min, respectively. In total, 45 fermenters (five aliquots for nine antibiotics) were used in borosilicate glass flasks. Each fermenter was inoculated with 20% of a *K. marxianus* suspension, to obtain uniformity in the initial optical density ($OD_0 = 0{\cdot}210 \pm 0{\cdot}015$). Subsequently, each fermenter was stirred at 42 °C for 12 h. Triplicates of samples from each fermenter were taken every 2 h (21 samples per treatment) to determine yeast biomass and residual lactose concentrations.

Biomass

Cell growth was determined by OD readings at 620 nm by using a Boeco Model S-20 Vis & S-22 UV/Vis spectrophotometer (Hamburg, Germany). With the biomass data obtained for each sample, the relative growth (RG) was calculated.

Lactose consumption

The lactose concentration (L) was determined using a colorimetric enzymatic technique based on the hydrolysis of lactose into galactose and glucose in the presence of β-galactosidase according to the technique proposed by Aktaş et al. (2006). The lactose relative consumption (LRC) was determined in relative terms to the initial concentration of lactose.

Statistical analysis

The results were analysed using the stepwise option of the General Linear Regression Model procedure included in the statistical package StatGraphics Centurion XVI (StatGraphics®, 2008). Eq. (1) describes the effects of time, antibiotic and heat treatment on the fermentation of *K. marxianus*:

$$Y_{ijkl} = (\beta_1 - \beta_2 \times \mathrm{ATB}_j + \beta_3 \times \mathrm{ATB}_j \times \mathrm{TT}_k) \times t_i + \varepsilon_{ijkl} \quad (1)$$

where: Y_{ijkl} = variable response (RG_{ijkl}: relative growth, LRC_{ijkl}: lactose relative consumption); β_1, β_2, β_3 coefficients estimated by the model; t_i: effect of fermentation time ($i = 7$: 0, 2, 4, 6, 8, 10 and 12 h); ATB_j: effect of the antibiotic in terms of dummy variable ($j = 2$, ATB = 0: without antibiotic, ATB = 1: with antibiotic); TT_k: effect of the heat treatment time at 120 °C ($k = 4$, TT = 0, 20, 40 and 60 min) and ε_{ijkl}: residual error of the model.

Subsequently, the percentage of relative degradation (% RD) for each antibiotic (ATB = 1) and thermal treatment ($TT_k = 20$, 40 and 60 min) was calculated using Eq. 1 for the relative growth (RG). The percentage of relative degradation due to the heat treatment was calculated according to the following equation:

$$\%\mathrm{RD} = \frac{(\mathrm{RG}_{(ATB=1,TT=k)} - \mathrm{RG}_{(ATB=1,TT=0)})}{(\mathrm{RG}_{(ATB=0,TT=0)} - \mathrm{RG}_{(ATB=1,TT=0)})} \times 100 \quad (2)$$

Replacing the values of RG for their predictor variables (Eq. 1) yields the following equation:

$$\%\mathrm{RD} = \frac{(\beta_1 - \beta_2 \times \mathrm{ATB}_1 + \beta_3 \times \mathrm{ATB}_1 \times \mathrm{TT}_k) \times t_i - (\beta_1 - \beta_2 \times \mathrm{ATB}_1 + \beta_3 \times \mathrm{ATB}_1 \times \mathrm{TT}_0) \times t_i}{(\beta_1 - \beta_2 \times \mathrm{ATB}_0 + \beta_3 \times \mathrm{ATB}_0 \times \mathrm{TT}_0) \times t_i - (\beta_1 - \beta_2 \times \mathrm{ATB}_1 + \beta_3 \times \mathrm{ATB}_1 \times \mathrm{TT}_0) \times t_i} \times 100 \quad (3)$$

After replacing the dummy variables by their respective values ($ATB_0 = 0$, $TT_0 = 0$ and $ATB_1 = 1$) and simplifying 't_i' (note that the degradation percentage is independent of the fermentation time), the following equation is obtained:

$$\%\mathrm{RD} = \frac{(\beta_1 - \beta_2 \times 1 + \beta_3 \times 1 \times \mathrm{TT}_k) - (\beta_1 - \beta_2 \times 1 + \beta_3 \times 1 \times 0)}{(\beta_1 - \beta_2 \times 0 + \beta_3 \times 0 \times 0) - (\beta_1 - \beta_2 \times 1 + \beta_3 \times 1 \times 0)} \times 100 \quad (4)$$

Simplifying terms allows reaching the reduced expression for the calculation of degradation (Eq. 5), which in turn allows estimating the treatment times to achieve the total inactivation of each antibiotic:

$$\%\mathrm{RD} = \frac{\beta_3 \times \mathrm{TT}_k}{\beta_2} \times 100 \quad (5)$$

Results and discussion

To visualize the significant effects of the antibiotics and times of the thermal treatment on the growth of *K. marxianus* and the relative lactose consumption, please refer to Fig. 1 (cephalosporins), Fig. 2 (quinolones) and Fig. 3 (tetracyclines). In each Figure the logistic equation obtained by applying the stepwise option of Eq. 1 is shown. The coefficients of treatment time (β_1) indicate an increase in cell growth and lactose consumption as the fermentation time increased. The inhibitory action of the antibiotics on *K. marxianus* was manifested by the negative coefficients of parameter 'β_2', indicating a decrease in biomass production and lactose consumption as the fermentation time increased. In addition, the thermolability of antimicrobials is manifested by the positive terms of 'β_3', indicating an increase in biomass and lactose consumption as the heat treatment time increased. The figures show that the relative cell growths increased as the fermentation time increased. In addition, the presence of antibiotics at levels equivalent to their respective MRLs caused a significant decrease ($P < 0{\cdot}05$) in cell growth, whereas the heat treatments inactivated the antibiotics, thus improving the cell growth of these samples.

Regarding lactose consumption by *K. marxianus*, it decreased ($P < 0{\cdot}05$) due to the presence of the antibiotics studied, whereas samples heated at 120 °C for 60 min showed an increase in lactose consumption ($P < 0{\cdot}05$) (Figs 1–3). However, shorter heating times (20 and 40 min) did not significantly ($P > 0{\cdot}05$) improve lactose consumption. This indicates that the analysis of lactose consumption is a less sensitive parameter than cell growth.

Cephalosporins

These antibiotics showed a great thermolability, because the heat treatment at 120 °C for 20 min was statistically different from the

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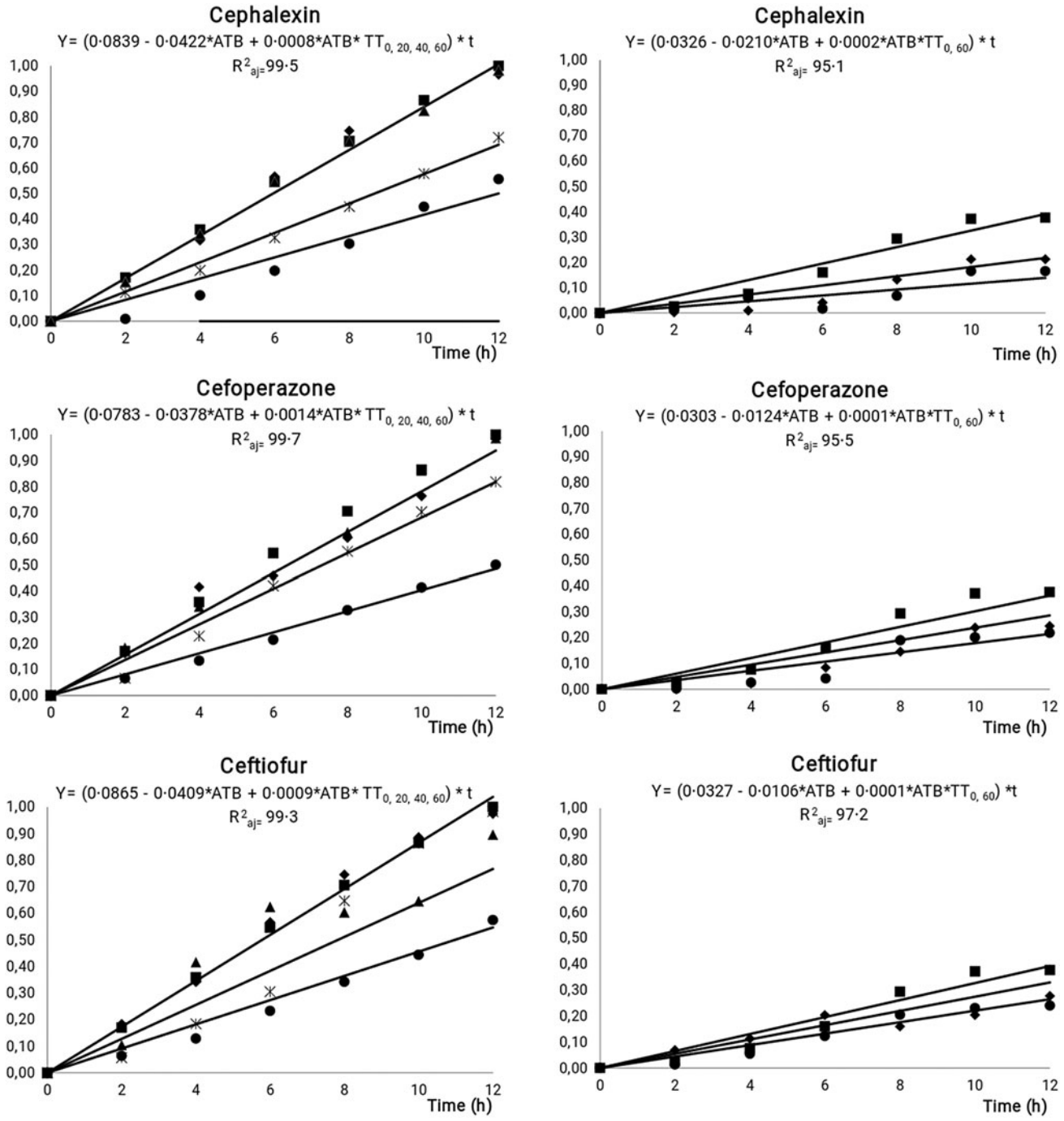

Fig. 1. Effect of thermal treatment and cephalosporins on the growth of *K. marxianus* and consumption of lactose (*t*, test time; ATB , antibiotic; TT, thermal treatment. ▬: control; ◆: TT 60 min; ▲: TT 40 min; ✳: TT 20 min; ●: sin TT).

control group ($P < 0{\cdot}05$), while longer heating times (TT = 40 min) allowed total degradation of these antibiotics (% RD = 100). Therefore, in Fig. 1, the relative growth lines for the treatments for 40 and 60 min are superimposed with those of the control group ($P > 0{\cdot}05$), whereas heating at 120 °C for 20 min, inactivated 69% of cephalexin, 82% of cefoperazone and 77% of ceftiofur. Zorraquino et al. (2008*b*) reported values of thermal inactivation of cephalosporins slightly higher (>92% for cefoperazone and >90% for cephalexin) than those obtained in this work when sterilizing milk samples at 120 °C for 20 min. Similarly, Roca et al. (2011) achieved high thermal degradations (99% for cephalexin and 100% for cefoperazone) with a sterilization process of 120 °C for 20 min and a quantification of these molecules by HPLC.

Quinolones

The high effect of quinolones on cell growth due to the high values of coefficient 'β_2' ($\beta_{2,\text{ciprofloxacin}} = 0{\cdot}0689$, $\beta_{2,\text{enrofloxacin}} = 0{\cdot}0682$ and $\beta_{2,\text{marbofloxacin}} = 0{\cdot}0686$) completely prevented the growth of *K. marxianus*. This inhibitory action was also manifested in a decrease in lactose consumption ($\beta_{2,\text{ciprofloxacin}} = 0{\cdot}0052$, $\beta_{2,\text{enrofloxacin}} = 0{\cdot}0028$, and $\beta_{2,\text{marbofloxacin}} = 0{\cdot}0094$).An increase in the heat

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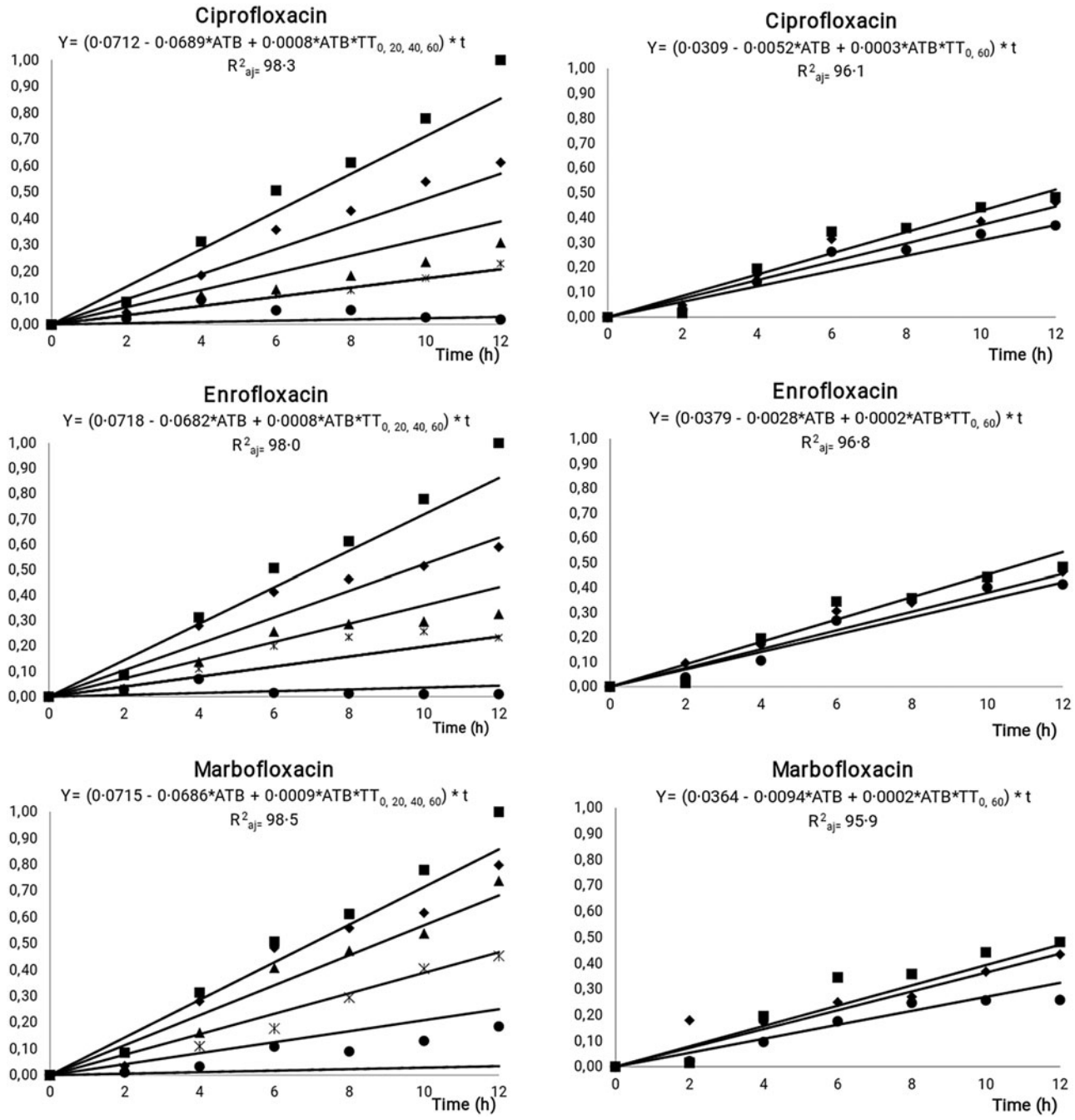

Fig. 2. Effect of thermal treatment and quinolones on the growth of *K. marxianus* and consumption of lactose (*t*, test time; ATB, antibiotic. TT, thermal treatment. ▬: control; ♦: TT 60 min; ▲: TT 40 min; ✳: TT 20 min; ●: sin TT).

treatment time caused partial inactivation of the quinolones, which increased as the heating time increased (Fig. 2). Heating for 20 min caused mild inactivations, resulting in low % RD increases (% $RD_{ciprofloxacin} = 21$, % $RD_{enrofloxacin} = 24$, and % $RD_{marbofloxacin} = 25$). Similarly, Zorraquino et al. (2008*a*) reported low percentages of inactivation (18% for enrofloxacin and 34% for marbofloxacin) when sterilizing milk samples at 120 °C for 20 min, using *Escherichia coli* as a bacterium test. Also, Roca et al. (2010) reported low thermal inactivations (120 °C-20 min) for ciprofloxacin (13%) and enrofloxacin (5%) when using HPLC with UV detection. Our results showed that the treatment times used in this study were not enough to completely inactivate the quinolones. However, the use of Eq. 5 allowed us to estimate the time needed to reach the total degradation of ciprofloxacin (91 min), enrofloxacin (84 min) and marbofloxacin (76 min).

Tetracyclines

Figure 3 shows that heating at 120 °C for 60 min causes total inactivation of oxytetracycline and tetracycline, but is insufficient to inactivate chlortetracycline (72%). For this molecule, the total degradation time (83 min) can be estimated by Eq. 5. The thermal inactivations of the tetracyclines obtained in this study were similar to those reported by Zorraquino et al. (2010) in milk samples

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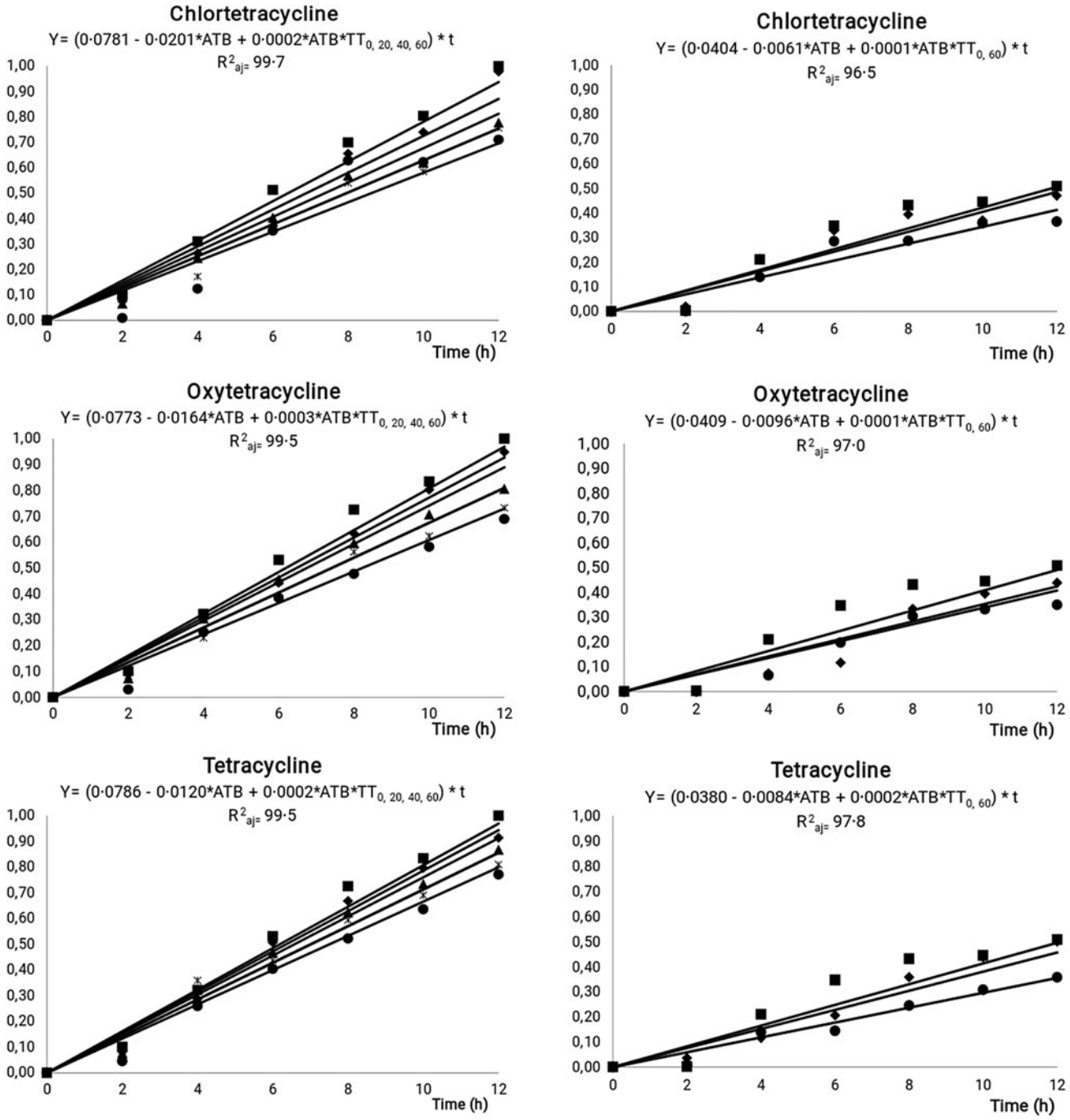

Fig. 3. Effect of thermal treatment and tetracyclines on the growth of *K. marxianus* and consumption of lactose (*t*, test time; ATB, antibiotic. TT, thermal treatment. ▬: control; ◆: TT 60 min; ▲: TT 40 min; ✳: TT 20 min; ●: sin TT).

(90% for chlortetracycline, 89% for oxytetracycline and 91% for tetracycline).

In conclusion, thermal treatments at 120 °C for 40, 83 and 91 min are necessary for the total inactivation of cephalosporins, tetracyclines and quinolone residues in whey respectively. Therefore, we conclude that these thermal treatments to whey contaminated with these antibiotics could be applied for cell biomass production with *K. marxianus*.

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References

Aktaş N, Boyacı İH, Mutlu M and Tanyolaç A (2006) Optimization of lactose utilization in deproteinated whey by *Kluyveromyces marxianus* using response surface methodology (RSM). *Bioresource Technology* **97**, 2252–2259.

Althaus R, Nagel O and Molina P (2014) Efecto de antibióticos en suero de leche sobre la fermentación de *K. marxianus* [Effect of antibiotics in whey on the fermentation of *K. marxianus*]. Argentina. Concordia, Argentina.

https://doi.org/10.1017/S0022029919000098 Published online by Cambridge University Press

Libro. Resumen. Congreso. *Food Innova – International Conference in Food Innovation* –978-950698340-6.

European Community (2009) Council regulation No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union* **15**, 1–72.

Roca M, Castillo M, Martí P, Althaus RL and Molina MP (2010) Effect of heating on the stability of quinolones in milk. *Journal of Agricultural Food Chemistry* **58**, 5427–5431.

Roca M, Villegas L, Kortabitarte ML, Althaus RL and Molina MP (2011) Effect of heat treatments on stability of β-lactams in milk. *Journal of Dairy Science* **94**, 1155–1164.

Siljanoski A, Ciglarič R, Pezdir T, Lainšček PR, Dolenc J, Starič J and Šinigoj-Gačnik K (2018) Detection of tetracycline and other antimicrobial residues in milk from cows with clinical mastitis treated by combination therapy. *Journal of Dairy Research* **85**, 321–326.

Zorraquino MA, Roca M, Castillo M, Althaus RL and Molina MP (2008*a*) Effect of thermal treatments on the activity of quinolones in milk. *Milchwissenschaft* **63**, 192–195.

Zorraquino MA, Roca M, Fernandez N, Molina MP and Althaus RL (2008*b*) Heat inactivation of beta-lactam antibiotics in milk. *Journal Food Protein* **71**, 1193–1198.

Zorraquino MA, Althaus RL, Nagel OG, Roca M and Molina P (2010) Efecto de los Tratamientos Térmicos sobre la Actividad Antimicrobiana de Tetraciclinas en Leche [Effect of thermal treatments on the antimicrobial activity of tetracyclines in milk]. *Revista de la Facultad de Ciencias Veterinarias de la Universidad Nacional del Litoral* **9**, 47–56.

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