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Selection of batch process conditions for microbiological production of lactic acid using waste whey

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The research was focused on the selection of the best conditions for the lactic acid production. As the organic source diluted waste whey was used. Two facultative anaerobic bacteria strains were examined: *Lactobacillus rhamnosus* and *Lactococcus lactis*. The neeed of anaerobic conditions as well as mineral supplementation of cultivation were investigated. It turned out that the oxidation was not the key parameter, but cultivation medium needed a supplementation for higher process efficiency. Finally, *Lactobacillus rhamnosus* strain was selected, for which LA production was app. 45% higher than for *Lc. lactis*. On the other hand, *Lactobacillus rhamnosus* was active at higher lactose concentration, thus waste whey needed to be less diluted. Additionally, high values of product/substrate yield coefficient make the process very efficient.

Keywords: lactic acid bacteria, whey, MRS, Monod equation, biomass

1. INTRODUCTION

Lactic acid (LA), one of the organic acids, is widely applied in pharmaceutical industry, mainly to obtain water soluble lactates or to produce biodegradable capsules. It has moisturized properties and thanks to its disinfectant and keratolytic properties at higher concentration it is used in peeling production (Smith, 1996). Only its L–form is biologically active (Wang et al., 2015).

LA fermentation is carried out with lactic acid bacteria – LAB (Rattanachaikunsopon and Phumkhachorn, 2010), which convert simple carbohydrates such as glucose, galactose and even lactose to LA. These bacteria strains grow at anaerobic conditions and they can be divided into three groups (Ganzle, 2015):

- homofermentatives – ferment sugar to LA as the only product;
- heterofermentatives – ferment sugar to LA but produce also some by-products such as acetic acid or ethanol;
- facultative heterofermentatives-depending on conditions produce either LA or LA together with by-products.

The aim of this work was to select the most appropriate conditions of LA production using waste whey. At the beginning more efficient strain was selected. In the presented work two of LAB strains: *Lactobacillus rhamnous* and *Lactococcus lactis* were tested. These are facultative heterofermentative strains (Claesson et al., 2008; Drider et al., 2004) that produce LA only in L-form (Kwon et al., 2001; Narayanan et al., 2004). The bacteria were tested in respect to the efficiency of LA production in a quantitative and qualitative

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approach. Whey, a medium with high concentration of lactose was used as an organic compound source. In effect, this work joins two important aspects – microbial production of valuable acid (LA) and utilization of waste (whey). A concentration of lactose (LC) in whey, the need for whey mineral supplementation and application of strict anaerobic conditions were also tested.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Microorganisms

Lactococcus lactis (PCM 476, Institute of Immunology and Experimental Therapy, Polish Academy of Science, Poland) and *Lactobacillus rhamnnosus* isolated from pharmaceutical preparation "Gynomed" (Probionov, France) were used.

2.1.2. Medium

In order to verify whether or not supplementation was necessary, a post-production whey diluted to lactose content varying from 0.8 to 9.5 g·dm^{-3} was enriched with the mineral solution. In respect to inorganic compounds the medium responded to the MRS broth: (in g·dm^{-3}) CH_3COONa (5), Tween (1), K_2HPO_4 (2), Triamonium citrate (2), $MgSO_4 \cdot 7H_2O$ (0.2), $MnSO_4 \cdot 4H_2O$ (0.05) (De Man et al., 1960). As a control, whey with water diluted was used for cultivation (without supplementation).

2.2. Methods

During the process the concentrations of biomass, lactose and LA were evaluated spectrophotometrically (Hitachi, USA). The content of biomass was measured at λ = 550 nm using the standard curves based on the dry mass method: $X_{Lc.\ lactis}$ [g·dm^{-3}] = 1.01·A_{550} and $X_{Lb.\ lactis}$ [g·dm^{-3}] = 0.86·A_{550}. The concentration of LA was determined by Lawrence colorimetric method (Lawrence, 1970) with the standard curve C_{LA} [g·dm^{-3}] = 0.22·A_{570}. LC concentration was measured by DNS method (Miller, 1959) with the standard curve. C_{LC} [g·dm^{-3}] = 2.36·A_{550}.

2.2.1. Clarification of whey

In order to preserve a natural turbidity of whey (including residue of casein clot and fat), the whey was centrifuged at 9000 rpm and 4 °C for 20 min. Then, $CaCl_2$ was added to supernatant at 2–5 °C and next 6M NaOH was used to increase pH value to 7.3. At the end, the solution was heated to 55 °C and maintained in this temperature for 8 min. Upon cooling, the suspension was centrifuged at 9000 rpm for 20 min (Rinn et al., 1990).

2.2.2. Cultivation in flask

The *Lc. lactic* and *Lb. rhamnosus* strains were cultivated in a rotary shaker (Elpin plus, Poland) at 37 °C (as a mesophilic strain (Ahmed et al, 2006; Chen et al., 2013) and 40 °C (as a thermophilic strain (Kok et al., 2012; Valik et al., 2013)), respectively. The temperatures were selected based on literature, because

cultivation conditions of these bacteria have been intensively investigated (Akerberg et al., 1998; Chaisu et al., 2014; Hofvendahl and Hahn-Hägerdal, 2000). A medium based on natural whey and additional minerals was heated to the appropriate temperature and after that 1 cm^3 of inoculum (prepared according to the same procedure as standard culture) was transferred to the medium. During cultivation, at regular intervals samples were collected and the content of microorganisms was directly measured, while before Lawrence and DNS analysis samples was centrifuged at 6000 rpm for 5 min (Hettich Zentrifugen Eba 20, Germany). Analysis was performed in duplicate. The influence of lactose concentration, type of strain application and mineral supplementation were tested.

2.2.3. Cultivation in batch stirred tank reactor

The final part of research was performed in batch stirred tank bioreactors (New Brunswick, USA) working at 70 rpm and at 37 °C and 40 °C, respectively for *Lc. lactic* and *Lb. rhamnosus*. 0.5 dm^3 of medium consisting of whey and minerals, like in flask procedure, was heated and inoculated with 5 cm^3 of inoculum. Purity of culture was monitored by daily inoculation on agar plate (N9405, Fluka Analitycal, USA).

In bioreactors the need for nitrogen application was tested. To verify the necessity of inert gas dosing (obligatory anaerobic condition applying), compressed nitrogen at the stream of 1.5 $dm^3 \cdot min^{-1}$ was pumped into the bioreactors.

3. RESULTS AND DISCUSSION

3.1. Mineral supplementation

Crude whey is an aqueous solution rich in lactose (38–46 $g \cdot dm^3$), proteins (immunoglobulins, albumins, lactoferrin, α-lactoalbumin, β-lactoglobulin) and peptides, lipids, minerals, vitamins (Gonzalez-Chavez, 2009). For batch process the load of organic matter is too high and crude whey has to be diluted before fermentation (Lech and Trusek-Holownia, 2015).

The results of measurements for cultures supplemented with MRS medium were compared to these without mineral salts as presented in Fig. 1a.

As shown in Fig. 1b, the decrease of pH value during cultivation was observed. The most intensive acidification of culture was noticed for two first days but it did not slow down the growth of strains and LC conversion rate.

3.2. Anaerobic conditions

Both tested bacterial strains prefer anaerobic conditions but the presence of oxygen should not inhibit their growth (Edwards-Jones V., 2016). The processes proceeding under anaerobic conditions are much more expensive and complicated, due to the sophisticated equipment, difficulties in sampling, etc. Therefore, the influence of inert gas pumping to the bioreactor was tested. The rate of nitrogen dosing was 1.5 $dm^3 min^{-1}$ and that should be the value ensuring anaerobic conditions.

It was noticed (Fig. 2) that the difference between cultures growing under aerobic and anaerobic conditions differed in the range of analytical error.

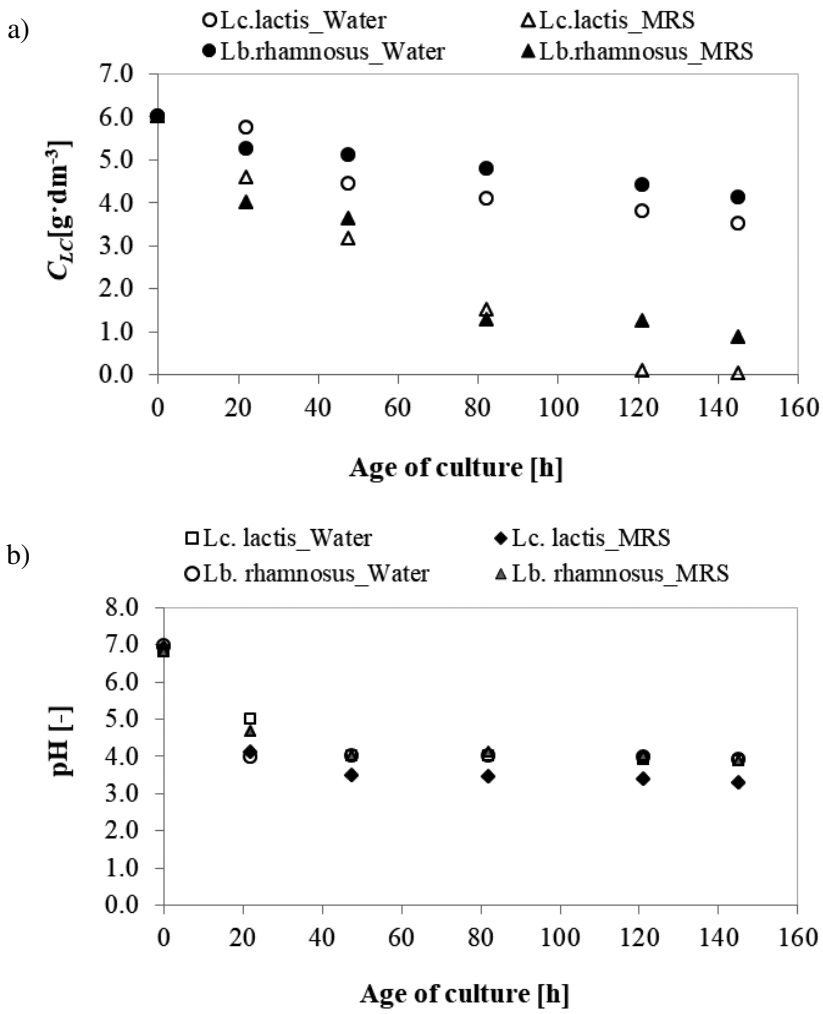

Fig. 1. Lactose (LC) concentration a) and pH value changing b) during *Lc. lactis* (37 °C) and *Lb. rhamnosus* (40 °C) cultivation – the case of whey dilution with water and MRS ($C_{LC}(t = 0) = 6.0$ g·dm^3)

Based on the data obtained during 2 days of cultivation (after this time lactose concentration was very low and the process was not effective), the rate of LC consumption and LA production were calculated as follows:

$$r = \pm\frac{\Delta C}{\Delta t} \tag{1}$$

where: ΔC – change of substrate/product concentration (g·dm^{-3}), Δt – time of substrate/product concentration changes [h].

Calculated values are presented in Table 1.

Table 1. The rate of LC consumption and LA production under aerobic and anaerobic conditions (the values calculated for the most intensive growth corresponded to the culture age 48 h); C_{LC} $(t = 0) = 2.6$ g·dm^{-3}, pH = 7.0

$\frac{\Delta c}{\Delta t}\left[\frac{\text{g}}{\text{dm}^3\cdot\text{h}}\right]$	Lactose		Lactic acid	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Lc. lactis (37 °C)	6.15×10^{-2}	5.21×10^{-2}	4.94×10^{-2}	4.92×10^{-2}
Lb. rhamnosus (40 °C)	4.53×10^{-2}	3.61×10^{-2}	2.75×10^{-2}	2.37×10^{-2}

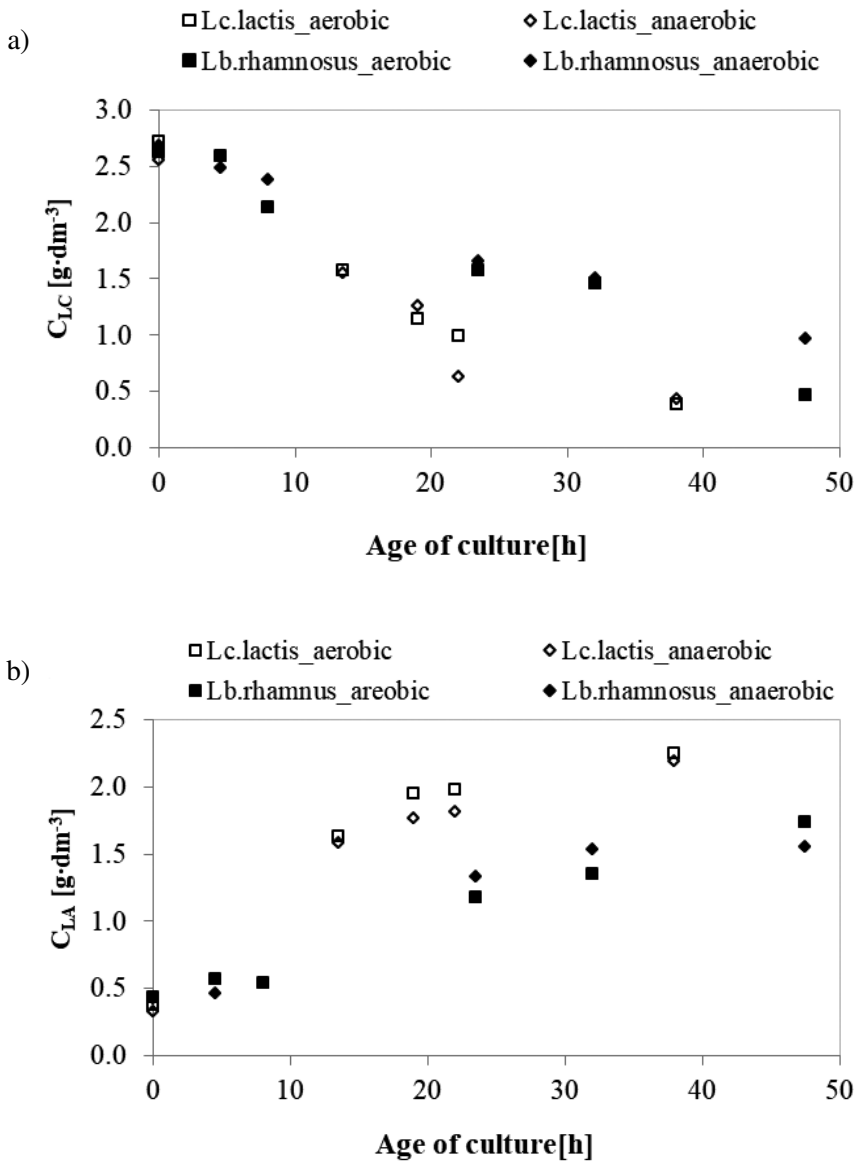

Fig. 2. *Lc. lactis* (37 °C) and *Lb. rhamnosus* (40 °C) cultivation in aerobic and anaerobic conditions: a) changes in LC concentration; b) changes in LA concentration ($C_{LC}(t = 0) = 2.6$ g· dm^{-3})

LC utilization was higher under aerobic conditions but the rate of LA synthesis was similar under aerobic and anaerobic conditions for each culture and slightly higher for *Lc. lactis* than for *Lb. rhamnosus*. Thus, further research was carried out without nitrogen that reduced the process costs.

3.3. Growth conditions

Whey was diluted with the mineral solution to obtain LC concentrations ranging from 0.8 to 9.5 g·dm^{-3}. Then, the concentrations of LC, LA and biomass were monitored during the cultivation process. Fig. 3 presents selected data.

For both tested microorganisms it was observed that the bacterial strains very quickly entered the logarithmic growth phase; almost after inoculation, the linear decrease of LC content accompanied by the increase of biomass concentration was observed. It concerns the processes carried out at the LC initial concentration below 4.3 g·dm^{-3}. At higher concentrations the processes run at a strong substrate inhibition

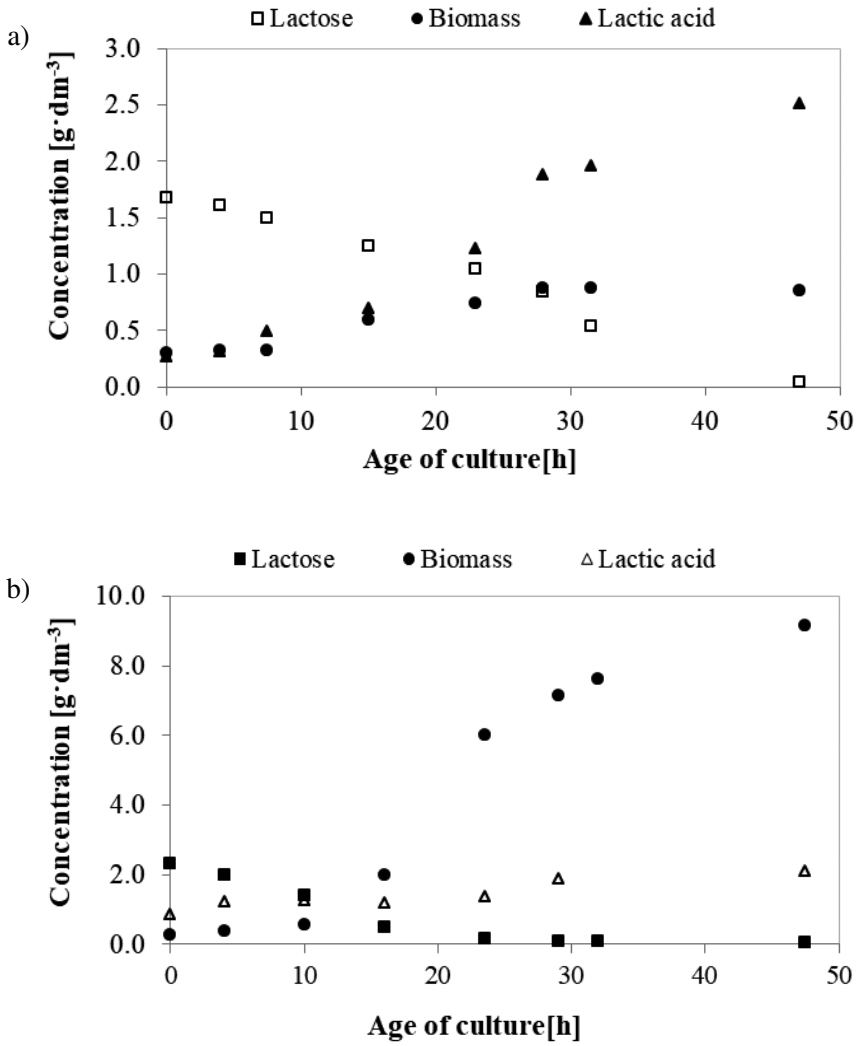

Fig. 3. LC, LA and biomass concentration changes: a) *Lb. rhamnosus* (40 °C, $C_{LC}(t = 0) = 1.7$ g·dm^{-3}), b) *Lc. lactis* (37 °C, $C_{LC}(t = 0) = 2.2$ g·dm^{-3})

but having in mind LA production in continuous mode, the range of substrate inhibition can be omitted (Trusek-Holownia and Noworyta, 2015). According to the steady-state theory, a state corresponding to lower substrate concentration is preferred for a given residence time (Zhang et al., 1999). Up to the LC concentration of 4.3 g·dm^{-3}, the kinetics could be described by Monod's equation (Monod, 1949) – Fig. 4:

$$\mu = \frac{\mu_{\max} \cdot C_S}{K_M + C_S} \tag{2}$$

The values of constants in the above equation are presented in Tab. 2.

Table 2. The values of constants of Monod's equation (valid till the $C_{LC} = 4.3$ g·dm^{-3})

	$\mu_{\max}$ [h^{-1}]	K_M [g·dm^{-3}]	R^2 [–]	S_x [·10^{-3}] [h^{-1}]	$RMSE$ [·10^{-3}] [h^{-1}]	SEE [·10^{-3}] [h^{-2}]
Lc. lactis (37°C)	0.136	0.330	0.896	4.27	5.51	0.18
Lb. rhamnosus (40°C)	0.200	4.822	0.899	9.33	6.63	2.64

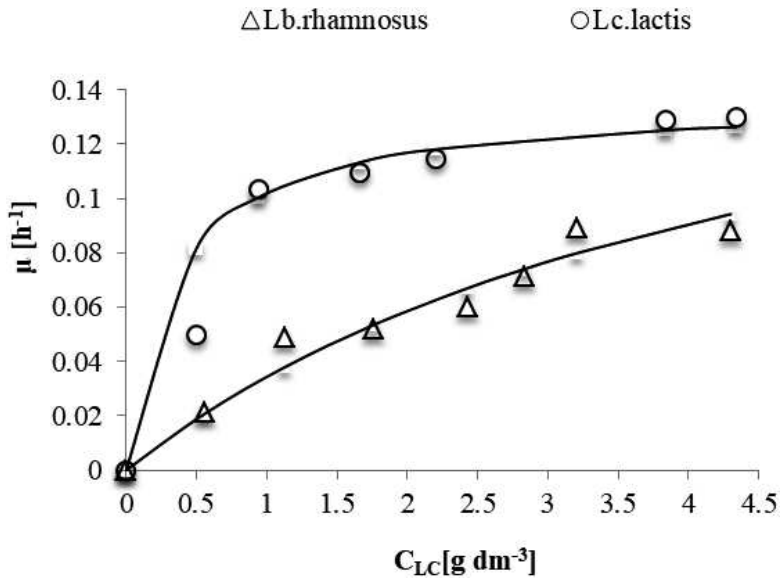

Fig. 4. Kinetics of *Lc. lactis* (37 °C) and *Lb. rhamnosus* (40 °C) growth based on the LC concentration (the continuous lines correspond to Monod's equation with the estimated values of constants)

The dependence of LC conversion rate on its concentration (in the range up to 3.0 g·dm^{-3}) could be described by a first-order kinetics (Fig. 5) with the constant value estimated for k_{LC} = 0.057 ± 0.05 h^{-1} for *Lc. lactis* and 0.038 ± 0.04 h^{-1} for *Lb. rhamnosus*, respectively.

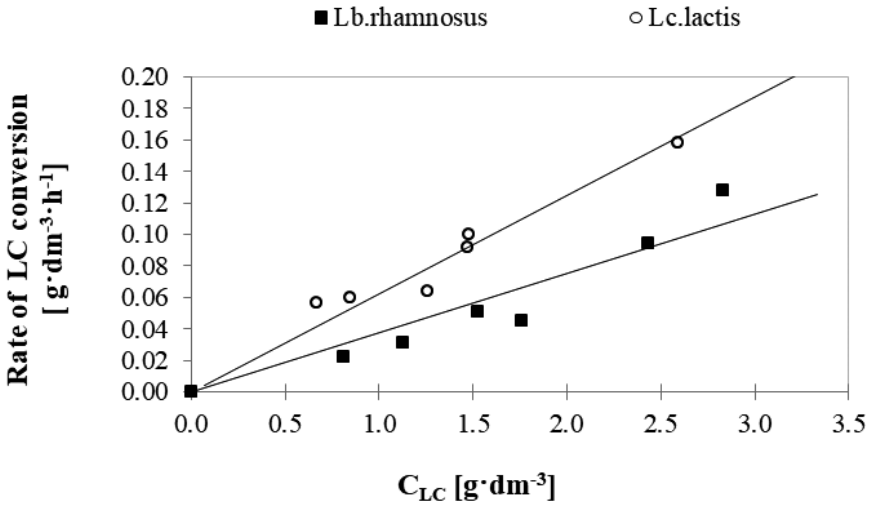

Fig. 5. The rate of LC conversion as a function of its concentration at 37 °C for *Lc. lactis* and 40 °C *Lb. rhamnosus*

3.4. Lactic acid production

The dependence of LA production rate on LC concentration is presented in Fig. 6. The high LC concentration had negative influence on the rate of LA production. This negative effect was particularly observed for *Lc. lactis*, for which above the concentration of around 2.0 g·dm^{-3}) the rate of LA production decreased. For *Lb. rhamnosus* the rate of LA production was the highest (around 0.070–0.075 g·dm^{-3}·h^{-1}) at the concentration close to 4.0 g·dm^{-3}).

For both tested microorganism, the greatest amount of LA was formed when the initial LC concentration was 4.6 g·dm^{-3}) (Fig. 7).

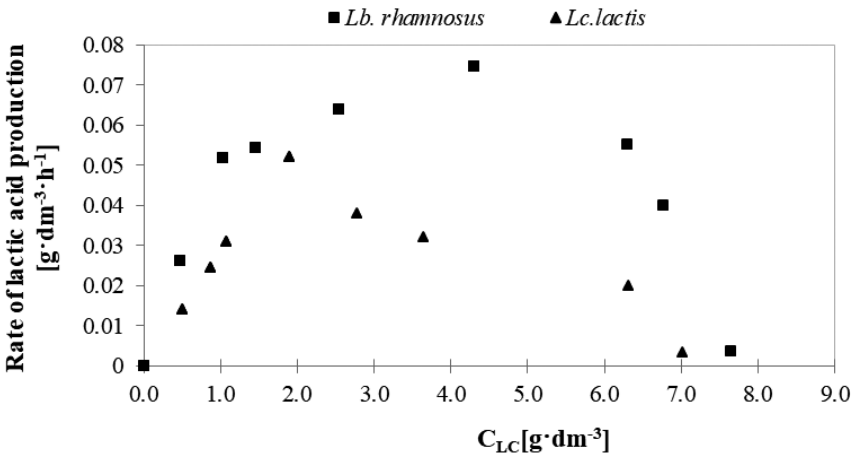

Fig. 6. Rate of LA production for *Lb. rhamnosus* (40 °C) and *Lc. lactis* (37 °C) as a function of LC concentration

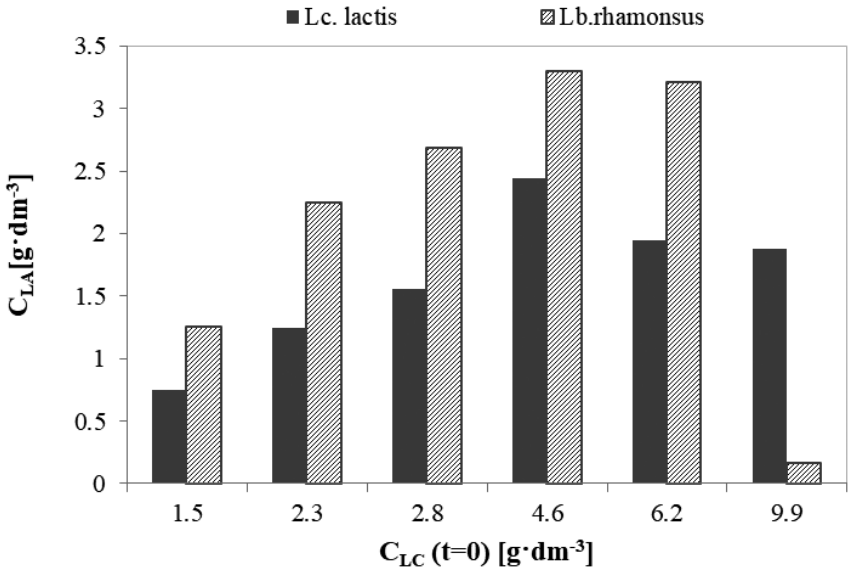

Fig. 7. The final LA concentration for *Lb. rhamnosus* (40 °C) and *Lc. lactis* (37 °C) depending on initial LC concentration

4. CONCLUSION

The aim of this research was to select the best conditions of batch process for LA production from waste whey using two bacterial strains: *Lc. lactis* and *Lb. rhamnosus*. *Lc. lactis* grows under mesothermal conditions, so it is associated with lower energy expenditure that generates lower cultivation costs compared to thermophilic *Lb. rhamnosus* strain. However, process temperature, based on literature data, was not so much different.

The growth rate was higher for *Lc. Lactis*, but LA production was much more efficient when *Lb. rhamnosus* was applied. This strain was more resistant on the higher substrate concentration (up to 4.3 g·dm^{-3}). Using the starting LC concentration close to this value, the highest final LA concentration was obtained as well. It means that post-produced whey should be diluted app. 10 times. The dilution should be made with a solution of mineral components. The valuable conclusion is that anaerobic conditions are not needed. This reduces costs and complexity of the equipment.

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SYMBOLS

C	concentration, g·dm^{-3}
K_M	Monod's constant, g·dm^{-3}
k	constant of conversion rate, h^{-1}
R^2	squared coefficient of determination, [–]
r	rate, g·dm^{-3}h^{-1}
t	time
X	concentration of biomass, g·dm^{-3}
S_x	standard deviation, [–]
RMSE	root mean square error, [–]
SSE	sum of squared errors of prediction, [–]

Greek symbols

μ	specific growth rate, h^{-1}
$\mu_{\max}$	logarithmic growth rate, h^{-1}

Subscripts

LA	lactic acid
LC	lactose

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