International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:13, No:7, 2019

Effect of Different Salts on *Pseudomonas taetrolens'* Ability to Lactobionic Acid Production

I. Sarenkova, I. Ciprovica, I. Cinkmanis

***Abstract*—**Lactobionic acid is a disaccharide formed from gluconic acid and galactose, and produced by oxidation of lactose. Productivity of lactobionic acid by microbial synthesis can be affected by various factors, and one of them is a presence of potassium, magnesium and manganese ions. In order to extend lactobionic acid production efficiency, it is necessary to increase the yield of lactobionic acid by optimising the fermentation conditions and available substrates for *Pseudomonas taetrolens* growth. The object of the research was to determinate the application of K_2HPO_4, $MnSO_4$, $MgSO_4 \cdot 7H_2O$ salts in different concentration for effective lactose oxidation to lactobionic acid by *Pseudomonas taetrolens*. *Pseudomonas taetrolens* NCIB 9396 (NCTC, England) and *Pseudomonas taetrolens* DSM 21104 (DSMZ, Germany) were used for the study. The acid whey was used as the study object. The content of lactose in whey samples was determined using MilcoScan^{TM} Mars (Foss, Denmark) and high performance liquid chromatography (Shimadzu LC 20 Prominence, Japan). The content of lactobionic acid in whey samples was determined using the high performance liquid chromatography. The impact of studied salts differs, Mn^{2+} and Mg^{2+} ions enhanced fermentation instead of K^+ ions. Results approved that Mn^{2+} and Mg^{2+} ions are necessary for *Pseudomonas taetrolens* growth. The study results will help to improve the effectiveness of lactobionic acid production with *Pseudomonas taetrolens* NCIB 9396 and DSM 21104.

***Keywords*—**lactobionic acid, lactose oxidation, *Pseudomonas taetrolens*, whey.

I. Introduction

WHEY is cheese and curd production by-product. The main whey component is lactose (Lac). Lactose (4-O-b-D-galactopyranosyl-D-glucose) is a unique disaccharide widespread in the mammalian milk [1], [2]. The European Union each year acquires around $40 \cdot 10^6$ tons of whey. The yearly residue of acquired whey is around $13 \cdot 10^6$ tons, which contains around 619,250 tons of milk sugar [3]. Approximately 9 L of whey is gained from 1 kg of produced cheese [4]. Whey has been used as carbon and nitrogen source in varied biotechnological operations, but it is mainly used as animal feed [1], [4]. Low solubility (solubility in water 195 g L^{-1}), low sweetness, only about 30% of sucrose sweetness, and the intolerance problems of some population segments limit the application of lactose in the food industry [5]. Lactose modification in value added compounds is advisable [2], [6], [7].

Inga Sarenkova is with Latvia University of Life Sciences and Technologies, Rigas iela 22, Jelgava, Latvia, (corresponding author, e-mail: inga.sarenkova@ inbox.lv).

Inga Ciprovica and Ingmars Cinkmanis are with Latvia University of Life Sciences and Technologies, Rigas iela 22, Jelgava, Latvia, (e-mail: inga.ciprovica@llu.lv, ingmarsc@llu.lv).

Lactobionic acid is one of high value added organic acids and is obtained via lactose oxidation. This organic acid has lately come up into the market with plenty of applications in fields like food and medicine, because of its excellent – nontoxicity, antioxidant, biocompatibility, biodegradability amphiphilic and chelating properties [8]. Lately, the food industry has a large interest in the use of lactobionic acid as a food additive, like calcium carrier, acidity regulator, antioxidant, etc. The dairy industry has been now implicated to the development and implementation of new biobased production methods including lactobionic acid as one of the significant products in dairy technologies [9]. The growing commercial significance has evoked the production via a biotechnological method of lactobionic acid as a long-lasting alternative to the chemical production which is very expensive and requires energy [10]. The possibility of renewable material or waste utilization for lactobionic acid production further increases the interest of the biotechnological route to cost-effective and environmentally friendly production [11].

Pseudomonas spp. develop lactobionic acid through the lactose oxidation pathway [12]. *Pseudomonas spp.* dehydrogenase catalyzes lactose oxidize to a lactobiono-δ-lactone using flavin adenine dinucleotide (FAD) as a system of electron transfer. The lactobiono-δ-lactone is hydrolyzed by lactonase to lactobionic acid [5]. *Pseudomonas taetrolens* strains are suitable for producing products that can be further used in food production [13]. Whey is a cheap substance for bioproduction of lactobionic acid by *Pseudomonas taetrolens*. Undoubtedly use of this high-strength polluting by-product as a very cheap source is advantageous [14].

Mostly all accessible articles refer to the use of sweet whey as substrate [14], [15]. Acid whey (pH 4.0 – 5.0) usage as a medium for production of lactobionic acid has not been reported yet. Lactobionic acid production via biobased synthesis can be affected by many factors, and one of the factors is the salts content in the medium, because salts play an important role in processes that provides growth of microorganisms.

The aim of this study was to determinate the application of K_2HPO_4, $MnSO_4$, $MgSO_4 \cdot 7H_2O$ salts in different concentration for effective lactose oxidation to lactobionic acid by *Pseudomonas taetrolens*.

II. Materials and Methods

A. Microorganisms

Freeze-dried *Pseudomonas taetrolens* DSM 21104 pure culture from the German Collection of Microorganisms and

Cell Cultures (DSMZ, Germany) and *Pseudomonas taetrolens* NCIB 9396 from the England National Collection of Type Cultures (NCTC, England) pure culture were used for the study. *Pseudomonas taetrolens* strains were inoculated in nutrient broth agar (1 L containing of 2.8 g agar, 1 g meat extract, 5 g peptone, 2 g yeast extract and 5 g NaCl, respectively). The pure culture was incubated at 30 °C for 48 h and then used directly for preparation of inoculum.

B. Preparation of Inoculum

A loopful with 10 µL capacity was resorted to inoculate strains at 100 mL of nutrient broth liquid medium (1 L containing of 5 g NaCl, 5 g peptone, 1 g meat extract and 2 g yeast extract). These samples were stored at 30 °C for 16 h in an environmental shaker-incubator ES-20 with 230 rpm. Biomass of *Pseudomonas taetrolens* was separated at 6000 rpm 10 min using centrifugation (Hermle Labortechnik, Z206) and further used as a bulk starter.

C. Preparation of Acid Whey

Acid whey permeate (manufacturer SC "Tukuma piens") with following composition: lactose 4.51±0.03%; fat 0.01 ± 0.01%, protein 0.39 ± 0.01%, total solids 4.89±0.28% and pH 4.74±0.01 was used for the experiment. The acid whey permeate was treated at 95 °C temperature for 30 min and concentrated in the rotating vacuum evaporator "Heidolph Laborota 4000 efficient" till total solids of 10.02±0.81 %. The concentration process was carried out at 60 °C temperature, 45 to 80 mbar pressure and with 130 rpm of the flask. The solids concentration was fixed by refractometer (Kruss, Germany). The concentrated acid whey permeate with following average composition was used for investigation: lactose 8.49 ± 0.12%; fat 0.01 ± 0.01%, protein 0.88 ± 0.03%, total solids 10.09 ± 0.81% and pH 4.29±0.01.

D. The Study Design

The samples were marked with letters and numbers (see Table I).

Shake flask conversion experiments were carried out in Erlenmeyer 500 mL flasks containing 100 mL of acid whey permeate and 2% of *Pseudomonas taetrolens* NCIB 9396 or DSM 21104 bulk starter. This medium was supplemented with K_2HPO_4, $MnSO_4$ or $MgSO_4 \cdot 7H_2O$ at concentration 0.01%, 0.005%, or 0.001%.

Samples were cultivated an environmental shaker–-incubator ES–20 at 230 rpm for 48 h at 30 °C temperature. Samples were withdrawn during cultivation for monitoring of colony forming units (CFU) of *Pseudomonas taetrolens* NCIB9396 and DSM21104 and substrate pH. *Pseudomonas taetrolens* dry cell weight was determined only in control samples to prove that microorganisms were growing. The content of lactose was measured at the beginning and the end of the cultivation process, but the content of lactobionic acid was measured at the end of the cultivation process.

E. Analytical Methods

Colony forming units (CFU) was measured using nutrient broth agar at 30 °C for 48 h and counted with the Acolyte colony counter (N°: 7510/SYN). *Pseudomonas taetrolens* growth curves were depicted in the graphic as a function of time by plotting lg CFU mL^{-1}.

TABLE I
DECODE OF INVESTIGATED SAMPLES

Content of medium			Sample mark
P.taetrolens strain	Salt	Concentration of added salt, %	
NCIB 9396	$MgSO_4 \cdot 7H_2O$	0.01	NMg1
		0.005	NMg2
		0.001	NMg3
	$MnSO_4$	0.01	NMn1
		0.005	NMn2
		0.001	NMn3
	K_2HPO_4	0.01	NK1
		0.005	NK2
		0.001	NK3
	No salt	0	N-Control
DSM 21104	$MgSO_4 \cdot 7H_2O$	0.01	DMg1
		0.005	DMg2
		0.001	DMg3
	$MnSO_4$	0.01	DMn1
		0.005	DMn2
		0.001	DMn3
	K_2HPO_4	0.01	DK1
		0.005	DK2
		0.001	DK3
	No salt	0	D-Control

Medium pH was determined using an InLab® Expert Pro-ISM pH electrode (METTLER TOLEDO, Switzerland). pH changes in samples during cultivation process were displayed in the graphic as a function of time.

Pseudomonas taetrolens dry cell weight was detected using 1 mL of cultivated medium. Samples were centrifuged for 10 min at 13,000 rpm, right after that liquid was spilled out and precipitated cells were dehydrated by drying until constant weight. *Pseudomonas taetrolens* dry cell weight curves were displayed in the graphic as a function of time by plotting g L^{-1}.

The lactose amount was measured in the acid whey permeate and concentrated whey permeate samples before cultivation using MilcoScanTM Mars (Foss, Denmark). The lactobionic acid and residual lactose amount were determined via high–performance liquid chromatography (Prominance HPLC system, Shimadzu LC-20, Torrance, California, USA). All samples were filtered before the analysis through a 2 µm filter paper and right after that centrifuged to remove the cell debris, water–insoluble substances and other impurities. Samples were centrifuged for 5 min at 13,000 rpm. Detection of lactobionic acid was realized in a refractive index detector RID-10A; YMC–C18, 4.6 mm×250 mm, 5 µm column. Mobile phase isocratic elution on 2 L solution (1.15 mL H_3PO_4, 14.36 g KH_2PO_4, 20 mL acetonitrile and deionized water). The volume of injection sample was 10 µL, temperature 40 °C and flow rate 1 mL min^{-1}, detection of lactose was realized in a detector DAD SPD- M20 A; Alltech NH_2, 4.6 mm×250 mm, 5 µm column. Mobile phase is

isocratic elution (84% of acetonitrile, 16% of deionized water). The volume of the injected sample was 10 µL, temperature 35 °C and flow rate 1 mL min^{-1}). Samples were quantified according to HPLC-grade external analytical standards, lactose (Sigma Aldrich, Germany) and lactobionic acid (Acrōs Organics, India).

F. Data Analyses

Data analysis and acquisition were completed with Microsoft Excel 2010 programme. Statistical analyses were completed using Analysis of Variance (ANOVA) and t-test at significance level of $p<0.05$. All results are displayed as the average data from three independent tests.

III. Results and Discussion

A. pH Changes in the Substrate During the Cultivation Process in Shake Flask

Fig. 1 depicts pH changes by the time during the lactose oxidation process in acid whey permeate. At the beginning pH value was the same among all samples. Results showed that pH in some samples containing $MnSO_4$, $MgSO_4 \cdot 7H_2O$ slowly increased (NMg1, NMg2, NMg3, DMg1, DMg2, DMg3, NMn1, NMn2, NMn3, DMn1, DMn2, DMn3) but in some samples containing K_2HPO_4 and in control samples (NK1, NK2, NK3, N-Control, DK1, DK2, DK3, D-Control) slowly decreased during fermentation process.

At the end of the process significant differences ($p<0.05$) were not established among samples with $MnSO_4$ and $MgSO_4 \cdot 7H_2O$ (NMg1, NMg2, NMg3, DMg1, DMg2, DMg3, NMn1, NMn2, NMn3, DMn1, DMn2, DMn3). It shows that both strains provide similar pH changes.

We noticed that pH was increased in control samples and in samples with added K_2PO_4 (NK1, NK2, NK3, N-Control, DK1, DK2, DK3, D-Control). In Fig. 1. (A), at the end of the process, significant differences ($p<0.05$) were not established among N-control and NK1, NK2, NK3 samples. In Fig. 1. (B), significant differences ($P<0.05$) were not established among D-control and DK1, DK2, DK3 samples. It seems that K_2HPO_4 presence does not affect *Pseudomonas teatrolens* behaviour while $MnSO_4$ and $MgSO_4 \cdot 7H_2O$ presence affected *Pseudomonas taetrolens* behaviour.

The pH changes during cultivation process with *Pseudomonas taetrolens* in sweet whey have been established by several authors [10], [12], [14], [16], [17]. During studies with sweet whey pH decreases very fast, because creation of lactobionic acid provides acid medium in substrate. The lowest pH 3.6 has been reached during lactose oxidation into lactobionic acid with sweet whey. During lactose oxidation process in sweet whey with *Pseudomonas taetrolens* pH value usually decreases, but authors have established that pH could increase a little bit.

The presence of nitrogen compounds in substrate promotes pH increasing. It could be explained with *Pseudomonas taetrolens* ability to produce proteins splitting enzymes [10].

B. Monitoring of Microbial Pathway of Pseudomonas taetrolens During Shake Flask Cultivation

Pseudomonas taetrolens DSM 21104 and NCIB 9396 multiplies in all investigated samples during the cultivation process at 30 °C for 48 h and reached around 10^{10} CFU mL^{-1} (Fig. 2). There were no significant differences ($p<0.05$) among all investigated samples at the end of the cultivation process.

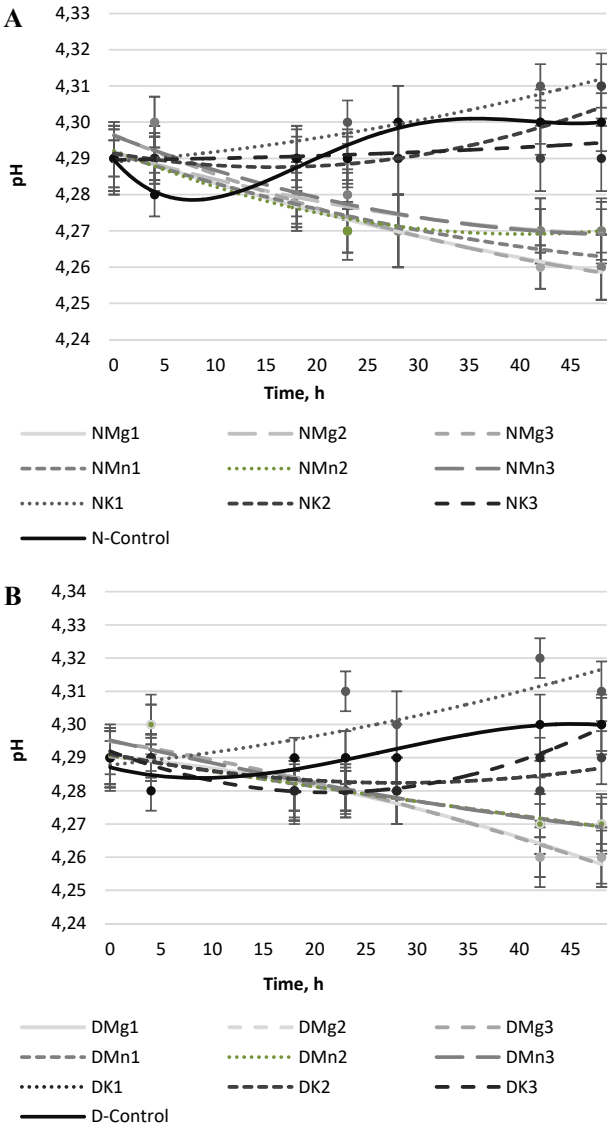

Fig. 1 Time-course graphic of pH determination during cultivation with different salts and their concentrations (A) *Pseudomonas taetrolens* NCIB 9396, (B) *Pseudomonas taetrolens* DSM 21104 strains in substrates

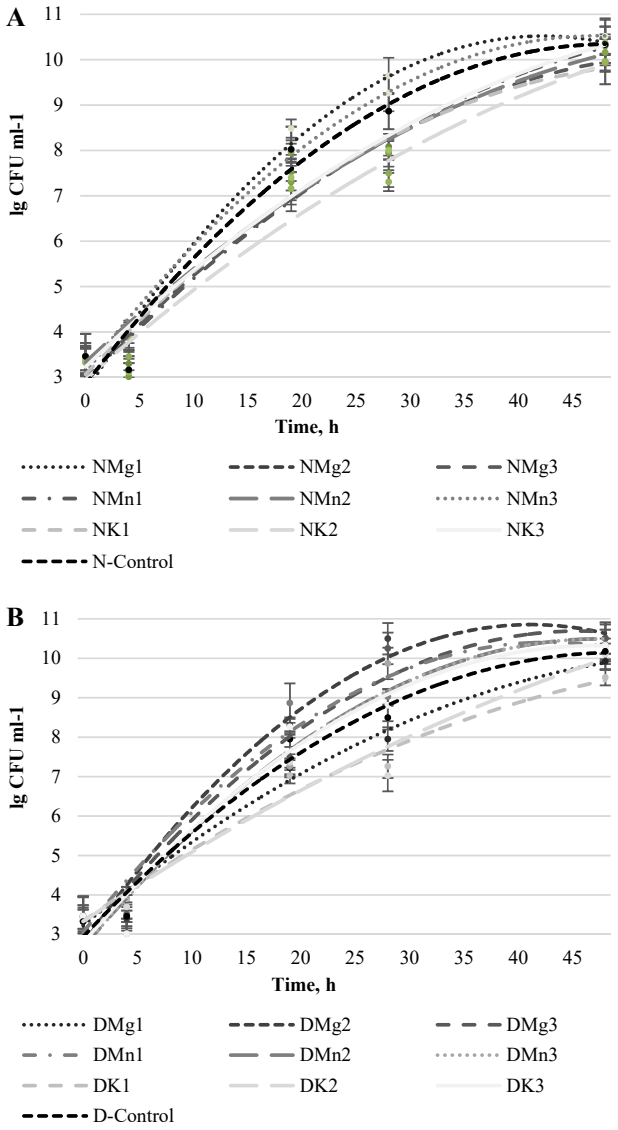

Fig. 2 Time-course graphic of total plate count determination during cultivation with different salts and their concentrations and (A) *Pseudomonas taetrolens* NCIB 9396, (B) *Pseudomonas taetrolens* DSM 21104 strains in substrates

Fig. 2 shows that microorganisms during the cultivation process had gone through the exponential phase. Both *Pseudomonas taetrolens* strains grow very similar in acid whey permeate with added salts.

The growth of *Pseudomonas taetrolens* in whey has been examined by some studies [14], [18]. It has been determined that in sweet whey *Pseudomonas taetrolens* mostly reach the highest amount 10^7 till 10^{10} CFU mL^{-1} and stays stable of this area for a while (stationary phase) until reaching the decline phase. In the studies, it has been mentioned that CFU of *Pseudomonas taetrolens* depends on inoculum amount, pH and cultivation time, as well as of solid content in the medium. It has been established that *Pseudomonas taetrolens* in sweet whey reach the stationary phase after 30 h of the cultivation. In this investigation after 30 h of cultivation, microorganisms were still in the exponential phase. It seems that in acid medium *Pseudomonats teatrolens* need more time to reach the stationary phase.

C. Pseudomonas taetrolens Biomass Changes During the Cultivation Process in Shake Flask

Bacteria dry cell weight was measured (Fig. 3) to evaluate the effect of the *Pseudomonas teatrolens* bacteria strains NCIB 9396 and DSM 21104 growth in the acid whey permeate.

In the acid whey permeate samples cells reached highest amounts of dry cell weight around of 23 h of incubation. In the sample with *Pseudomonas taetrolens* NCIB 9396, higher cell density was gained (around 0.545 g L^{-1} in 23 h of incubation), but in sample with *Pseudomonas taetrolens* DSM 21104 (around 0.507 g L^{-1} in 23 h of incubation) between N-Control and D-Control data was set the significant differences ($p>0.05$). There were no significant differences ($p<0.05$) between both samples during incubation after 23 h. *Pseudomonas taetrolens* DSM 21104 strain needs more time to grow in acid whey permeate. *Pseudomonas taetrolens* NCIB 9396 strain faster adopts acid whey permeate as substrate.

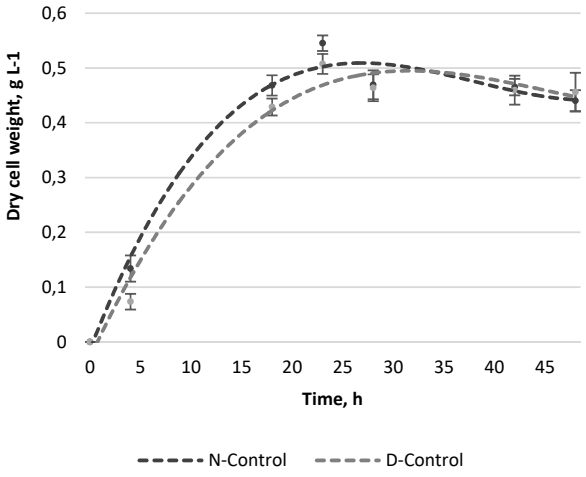

Fig. 3 Time-course graphic of *Pseudomonas taetrolens* dry cell weight during cultivation process in control samples

The growth of *Pseudomonas taetrolens* in whey has been examined by several authors [10], [16], [17]. They reported that using sweet whey (pH 6.5) as substrate and adding 10% or more of inoculation amount of bacteria can reach around 1.8 g L^{-1} of *Pseudomonas taetrolens* dry cell weight approximately after 20 h of incubation.

It has been reported that the maximum of gained *Pseudomonas taetrolens* dry cell weight is 0.55 g L^{-1} in sweet whey if all incubation time the medium is adjusted around pH 4.5 [17]. These results are similar to this investigation results.

To obtain more dry cell weight of *Pseudomonas taetrolens*, it would be necessary to add more bacteria and adjust pH to 6.5.

D. Effect of the Salts on Lactobionic Acid Production in Acid Whey Permeate

Fig. 4 represents results of lactobionic acid and lactose quantity in samples after 48 h of cultivation at 30 °C temperature. The lactobionic acid conversion yield was reached from 13.02 g L^{-1} to 21.65 g L^{-1}, lactose was converted from 15.46% till 25.71 %.

The significant differences were not established ($p<0.05$) among N-Control and NMg3, NMn2, NMn3, NK1, NK2, NK3 sample (Fig. 4 (A)). But among N-Control and NMg1, NMg2, NMn1 samples are significant differences ($p>0.05$).

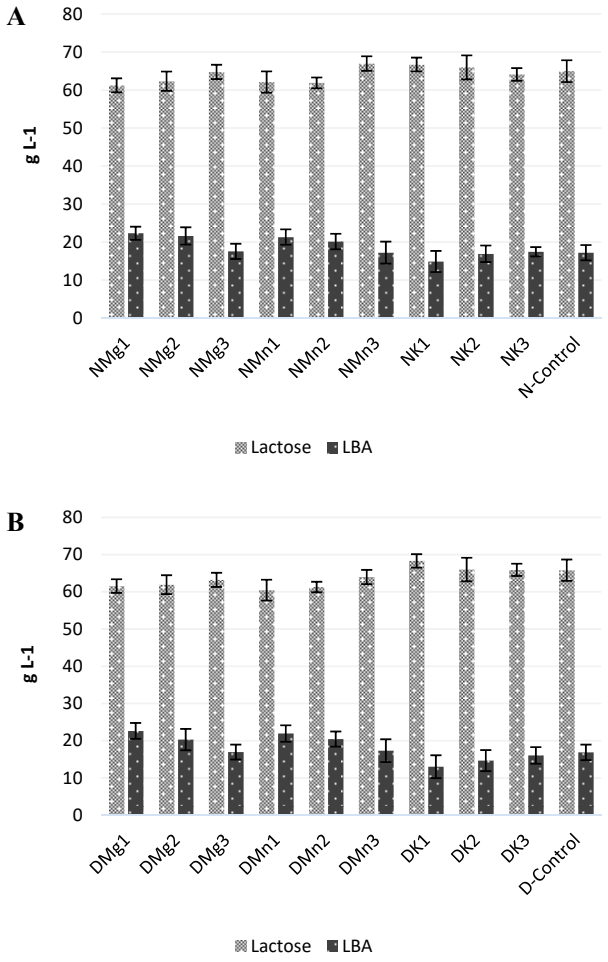

Fig. 4 Yield of Lactobionic acid and Lactose after 48 h of cultivation process with (A) *Pseudomonas taetrolens* NCIB 9396, (B) *Pseudomonas taetrolens* DSM 21104

The significant differences were not established ($p<0.05$) among D-Control and DMg2, DMg3, DMn2, DMn3, DK1, DK2, DK3 sample (Fig. 4. (B)). But among D-Control and DMg1, DMn1 sample are significant differences ($p>0.05$), the presence of Mg^{2+} and Mn^{2+} ions positively affected *Pseudomonas taetrolens* activity to convert lactose and to get more lactobionic acid. Presence of K^{+} did not give any changes in lactobionic acid production process.

The effect of salts on *Pseudomonas taetrolens* activity to produce lactobionic acid has been not reported yet. The lack of Mg^{2+} in the substrate can cause the emergence of fibrous bacteria, delaying cell division and growth. The growth of bacteria in the presence of Mg^{2+} is explained by the fact that Mg^{2+} acts as an activator of various metabolic responses for cell division. Mg^{2+} also stabilizes nucleic acids (DNA and RNA) and participates in peptide hydrolysis. Mn^{2+} is needed in many enzymatic functions of microorganisms. Mn^{2+} is involved in RNA polymerase, initiates lactic acid enzyme, xylose isomerase, manganese catalase, manganese superoxide dismutase and NADH oxidase activity. Manganese ions can increase the pH range of enzyme activity [19]. Mg^{2+} and Mn^{2+} ions are necessary for *Pseudomonas taetrolens* grown.

IV. Conclusion

The most suitable salts are $MnSO_4$ and $MgSO_4 \cdot 7H_2O$ at concentration 0.01% for lactose oxidation to lactobionic acid with *Pseudomonas taetrolens* NCIB 9396 and DSM 21104. Mn^{2+} and Mg^{2+} ions are necessary for *Pseudomonas taetrolens* growth. The study suggests that adjusting of the acid whey pH prior to lactose oxidation increases *Pseudomonas taetrolens* activity and gains higher lactobionic acid yield.

Acknowledgment

We acknowledge Dr.sc.ing. Ruta Galoburda, Dr.sc.ing Asnate Kirse-Ozolina and Dr.sc.ing. Liga Skudra from Latvia University of Life Sciences and Technologies for valuable consultation during investigation.

Research was funded by the grant „Strengthening Research Capacity in the Latvia University of Life Sciences and Technologies" project Nº Z23.

References

[1] A. R. Prazeres, F. Carvalho, J. Rivas, "Cheese whey management: a review," *J. Environ. Manag.* Vol 110, pp. 48–68. 2012.

[2] N. Seki, H. Saito, "Lactose as a source for lactulose and other functional lactose derivatives," *International Dairy Journal,* vol. 22, pp. 110–115. 2012.

[3] E. S. El-Tanboly, M. El-Hofi & Khorshid, "Recovery of Cheese Whey, a by-Product from the Dairy Industry for use as an Animal Feed," *J. Nutr. Health. Food. Eng,* vol 6(5), pp. 1–7. 2017.

[4] C. I. Onwulata & P. J. Huth, *Whey processing, functionality and health benefits.* USA: Wiley Blackwell, IFT Press. 2018.

[5] L. F. Gutiérrez, S. Hamoudi, K. Belkacemi, "Selective production of lactobionic acid by aerobic oxidation of lactose over gold crystallites supported on mesoporous silica," *Applied Catalysis A: General,* vol. 402, pp. 94–103. 2011.

[6] A. N. Silva, R. Perez, V. P. R. Minim, D. D. S. Martins, L. A. Minim, "Integrated production of whey protein concentrate and lactose derivatives: What is the best combination?" *Food Research International,* vol. 73, pp. 62–74. 2015.

[7] Y. S. Song, H. U. Lee, C. Park., S. W. Kim, "Optimization of lactulose synthesis from whey lactose by immobilized β-galactosidase and glucose isomerase," *Carbohydrate Research*, vol. 369, pp. 1–5. 2013.

[8] S. Alonso, M. Rendueles, M. Diaz, "Bio-production of lactobionic acid: Current status, applications and future prospects," *Biotechnology Advances,* vol. 31(8), pp. 1275–1291. 2013.

[9] R. K. Merrill, & M. Singh, U.S. Patent No. US8021704. United States Patent. Leprino Foods Co, 2011.

[10] S. Alonso, M. Rendueles, M. Díaz, "Efficient lactobionic acid production from whey by Pseudomonas taetrolens under pH-shift

conditions," *Bioresource Technology*, vol. 102, pp. 9730–9736.2011.
[11] D. Pleissener, D. Dietz, J. B. J. H. Duuren, C. Wittmann, X. Yang, C. L. S. Lin, J. Venus, "Biotechnological production of organic acids from renewable resources," *Advances in Biochemical Engineering / Biotechnology*, vol 166. pp. 373–410. 2017.
[12] S. Alonso, M. Rendueles, M. Díaz, "Role of dissolved oxygen availability on lactobionic acid production from whey by *Pseudomonas taetrolens*," *Bioresource Technology*, vol. 109, pp. 140–147. 2012.
[13] T.P. West, "Regulation of pyrimidine nucleotide formation in Pseudomonas taetrolens ATCC 4683," *Microbiol. Res,* vol. 159, pp. 29–33. 2004.
[14] S. D. Giorgi, N. Raddadi, A. Fabbri, T. G. Toschi, F. Fava, "Potential use of ricotta cheese whey for the production of lactobionic acid by *Pseudomonas taetrolens* strains," *New Biotechnology Journal,* vol. 42, pp. 71–76. 2018.
[15] S. Alonso, M. Rendueles & M. Díaz, "Tunable decoupled overproduction of lactobionic acid in *Pseudomonas taetrolens* through temperature-control strategies," *Process Biochemistry*, vol. 58, pp. 9–16. 2017.
[16] S. Alonso, M. Rendueles & M. Díaz, "Feeding strategies for enhanced lactobionic acid production from whey by Pseudomonas taetrolens," *Bioresource Technology,* vol. 134, pp. 134–142. 2013.
[17] S. Alonso, M. Rendueles & M. Díaz, "Selection method of pH conditions to establish Pseudomonas taetrolens physiological states and lactobionic acid production", *Appl Microbiol Biotehnol*, vol. 97(9), pp. 3843-54. 2013.
[18] K. Goderska, A. Szwengiel, Z. Czarnecki, "The utilization of *Pseudomonas taetrolens* to produce lactobionic acid," *Biotechnology and Applied Biochemistry,* vol. 173, pp. 2189–2197. 2014.
[19] I. C. Gunsalus, R. Y. Stanier, *The Bacteria.* A treatise on structure and functions. Volume 4: The physiology of growth. Academic press, New York. Elsevier. – 474 p. 2013.