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Latvijas Lauksaimniecības universitāte
Pārtikas tehnoloģijas fakultāte

***Mg.sc.ing.* Kristīne Majore**

promocijas darbs

SŪKALU VALORIZĀCIJA LAKTOZES PĀRSTRĀDES PRODUKTU IEGUVEI
VALORISATION OF WHEY FOR LACTOSE RECYCLING PRODUCTS PRODUCTION

Zinātnes doktora (**PhD**) zinātniskā grāda iegūšanai
pārtikas un dzērienu tehnoloģijā

Promocijas darba vadītājs
Prof. Dr.sc.ing. Inga Ciproviča _____

Promocijas darba autore _____

Jelgava
2021

ANNOTATION

The doctoral thesis "Valorisation of whey for lactose recycling products production" was developed from 2016 to 2021. Experiments were carried out in the research laboratories of the Faculty of Food Technology, Latvia University of Life Sciences and Technologies; Dairy Innovation Institute, California Polytechnic State University (USA); Institute of Microbiology and Biotechnology, Latvia University; Faculty of Chemistry, Latvia University; Institute of Solid State Physics, Latvia University and J.S. Hamilton Baltic Ltd.

The **aim** of the doctoral thesis was to improve the lactose hydrolysis process for obtaining glucose-galactose and oligosaccharide syrups.

The **hypothesis** of the doctoral thesis – the two-stage fermentation increases the sweetness of glucose-galactose syrup.

The hypothesis of the doctoral thesis has been confirmed by the **defended thesis:**

1. The presence of cations affects the β-galactosidase activity in the sweet and acid whey permeate.
2. The chemical composition and quality of whey affect the physical properties of lactose.
3. Enzymatic reactions affect the functional and sensory properties of syrups.

The **research objects** – sweet and acid whey permeates, glucose isomerase, commercial β-galactosidases and glucose-galactose syrup.

The following **tasks** were set to achieve the aim of the doctoral thesis:

1. To evaluate the effect of cation concentration to ensure the β-galactosidase activity in substrate.
2. To investigate the physical properties of whey lactose in order to better understand its behaviour.
3. To study the changes of monosaccharide concentration in the lactose hydrolysis, varying with the solids concentration of the substrates and enzyme units.
4. To assess the possibilities of glucose isomerase to increase the sweetness of glucose-galactose syrup.
5. To evaluate the sensory properties of the developed syrups.

The **novelty** of the doctoral thesis:

1. The study of the relationship of lactose hydrolysis process in the formation of galacto-oligosaccharides and lactulose.
2. The combination of β-galactosidase and glucose isomerase increases the sweetness of glucose-galactose syrup.

The **economic significance** of the doctoral thesis:

1. The studies have shown the possibility to obtain syrup that can be used as sugar and sweeteners replacer in the food industry and to produce functional products.
2. A technological solution for hydrolysis of lactose is proposed, comprehensively evaluating the physical properties of lactose, fermentation parameters and whey composition. The doctoral thesis consists of three chapters:

Chapter 1 describes the composition of whey and the possibilities of using it. An overview of the chemical and physical properties of lactose, lactose hydrolysis methods, the application of β-galactosidases and the properties of glucose-galactose syrup are provided.

Chapter 2 summarises the materials and methods used in the thesis.

Chapter 3 provides a summary of the results obtained in the study, the properties of commercial enzymes in different cation concentrations, the stability of enzymes in the gastrointestinal tract model, methods for the determination of lactose, the properties of dehydrated permeates are evaluated. The influence of factors on the hydrolysis of permeates

and the profile of the obtained sugars was analysed. Possibilities for lactulose synthesis are considered. Sensory analysis of glucose-galactose syrups and syrups obtained in the two-stage fermentation are given.

During the PhD studies the author had an internship at the Dairy Innovation Institute California Polytechnic State University (USA), where the experimental work was done. Internship was provided by the Baltic – American Freedom Foundation (BAFF) and the Council on International Education Exchange (CIEE).

The study was partly financed by the LLU programme "Strengthening Research Capacity at the Latvia University of Agriculture" grant (Contract No. 3.2.-10/2017/LLU/27) "The optimization of bioprocesses for lactose recycling products".

The study was partly financed by the doctoral studies grant "Transition to the new doctoral funding model at the Latvia University of Life Sciences and Technologies" (Contract No. 3.2.-10/90).

The thesis is written in English, it consists of 111 pages, 32 tables, 41 figures, 3 appendixes, and 233 bibliographic sources.

ANOTĀCIJA

Promocijas darbs "Sūkalu valorizācija laktozes pārstrādes produktu ieguvei" izstrādāts laika posmā no 2016. līdz 2021. gadam. Eksperimenti veikti Latvijas Latvijas Lauksaimniecības universitātes Pārtikas tehnoloģijas fakultātes zinātniskajās laboratorijās: Kalifornijas Politehniskās Universitātes Piena inovācijas institūtā; Latvijas Universitātes Mikrobioloģijas un Biotehnoloģijas institūta un Ķīmijas fakultātes laboratorijās; Latvijas Universitātes Cietvielu fizikas institūtā; SIA Hamilton Baltic testēšanas laboratorijās.

Promocijas darba **mērķis**: pilnveidot laktozes hidrolīzes procesu glikozes-galaktozes un oligosaharīdu sīrupa iegūšanai.

Promocijas darba **hipotēze**: divpakāpju fermentācija palielina glikozes-galaktozes sīrupa salduma pakāpi.

Promocijas darba hipotēzi pierāda ar šādām **tēzēm:**

1. Katjonu klātbūtne ietekmē β-galaktozidāzes aktivitāti siera un biezpiena sūkalu ultrafiltrātā.
2. Sūkalu ķīmiskais sastāvs un kvalitātes rādītāji ietekmē laktozes fizikālās īpašības.
3. Enzimātiskās reakcijas būtiski ietekmē sīrupu funkcionālās un sensorās īpašības.

Pētījuma **objekti** – biezpiena un siera sūkalu ultrafiltrāts, glikozes izomerāze, komerciālās β-galaktozidāzes un glikozes-galaktozes sīrups.

Promocijas darba mērķa sasniegšanai izvirzīti šādi **uzdevumi**:

1. Novērtēt katjonu ietekmi uz β-galaktozidāzes aktivitāti fermentējamā substrātā.
2. Izpētīt laktozes fizikālās īpašības, tās sekmīgai hidrolīzei.
3. Analizēt monosaharīdu satura izmaiņas laktozes hidrolīzē, variējot ar substrātu sausnu un pievienotā enzīma aktivitātes vienībām.
4. Izvērtēt glikozes izomerāzes izmantošanas iespējas glikozes-galaktozes sīrupa salduma palielināšanai.
5. Novērtēt izstrādāto sīrupu sensorās īpašības.

Darba **novitāte**:

1. Laktozes hidrolīzes procesa likumsakarību izpēte galakto-oligosaharīdu, tostarp laktulozes veidošanā.
2. β-Galaktozidāzes un glikozes izomerāzes kombinācija glikozes-galaktozes sīrupa salduma palielināšanai.

Tautsaimnieciskā nozīme:

1. Īstenotie pētījumi ļauj iegūt sīrupu, kuru var izmantot saldvielu un saldinātāju aizstāšanai pārtikas rūpniecībā un funkcionālo produktu ieguvei.
2. Piedāvāts tehnoloģiskais risinājums laktozes hidrolīzei, vispusīgi izvērtējot laktozes fizikālās īpašības, fermentācijas parametrus un sūkalu sastāvu.

Promocijas darbs apkopots 3 nodaļās:

1. nodaļā aprakstīts sūkalu sastāvs un sūkalu izmantošanas iespējas. Sniegts pārskats par laktozes ķīmiskajām un fizikālajām īpašībām, hidrolīzes metodēm, β-galaktozidāzes enzīmu izmantošanu un glikozes - galaktozes sīrupa īpašībām.

2. nodaļā apkopots promocijas darbā izmantoto materiālu un metožu kopsavilkums.

3. nodaļā sniegts pētījumā iegūto rezultātu apkopojums, izvērtētas komerciālo enzīmu īpašības dažādās katjonu koncentrācijās, pētīta enzīmu stabilitāte kuņģa-zarnu trakta modeļvidē, vērtētas dažādas laktozes noteikšanas metodes, dehidrēto ultrafiltrātu īpašības. Analizēta dažādu faktoru ietekme ultrafiltrāta hidrolīzei un iegūto cukuru profilam. Izskatītas iespējas laktulozes sintēzei. Veikta sīrupu sensorā analīze, salīdzinot ar divpakāpju fermentācijā iegūtajiem.

Trešās nodaļas beigās formulēti secinājumi.

Studiju laikā doktorante praktizējusies un īstenojusi pētnieciskā darba izstrādi Kalifornijas Politehniskās universitātes Piena inovāciju institūtā (ASV) ar Baltijas - Amerikas Brīvības fonda (BAFF) un Starptautiskās izglītības apmaiņas padomes (CIEE) piešķirtās **stipendijas** atbalstu.

Promocijas darba izstrāde veikta ar daļēju "Zinātniskās kapacitātes stiprināšana Latvijas Lauksaimniecības universitātē" projekta Nr. 3.2.-10/2017 / LLU / 27 "Bioprocesu optimizācija laktozes pārstrādes produktu ieguvei" atbalstu.

Promocijas darba izstrāde veikta ar daļēju "LLU pāreja uz jauno doktorantūras finansēšanas modeli" projekta Nr. 3.2.-10/90 atbalstu.

Promocijas darbs uzrakstīts angļu valodā, apjoms 111 lpp, ieskaitot 32 tabulas, 41 attēlus, 233 bibliogrāfiskos nosaukumus un 3 pielikumus.

Approbation of the research work / *Zinātniskā darba aprobācija*

Research results are published in 6 research articles, which are indexed in international SCOPUS and Web of Science databases / *Pētījuma rezultāti publicēti 6 zinātniskajos izdevumos, kas indeksēti SCOPUS un Web of Science datu bāzēs.*

Publications indexed in the international SCOPUS and Web of Science databases / *Publikācijas, kas ir indeksētas SCOPUS un Web of Science datubāzē*:

1. Majore, K., Ciproviča, I. (2020) Optimisation of lactose hydrolysis by combining solids and β-galactosidase concentrations in whey permeates. *Proceedings of the Latvian Academy of Sciences. Section B Natural Exact and Applied Sciences*, 74, 4, p. 263-269. DOI: 10.2478/prolas-2020-0041
2. Žolnere, K., Ciproviča, I. (2019) The study of physical properties of spray dried whey and milk permeates lactose. *Agronomy Research*, 17, 2, p. 1501-1510. DOI: 10.15159/AR.19.054
3. Žolnere, K., Ciproviča, I. (2019) Lactose hydrolysis in different solids content whey and milk permeates. *13th Baltic Conference on Food Science and Technology, FOODBALT 2019 joined with 5th North and East European Congress on Food NEEFood 2019 "FOOD. NUTRITION. WELL-BEING"*, Conference proceeding, p. 35-39. DOI: 10.22616/FoodBalt.2019.011
4. Žolnere K., Ciproviča I., Ķirse A., Cinkmanis I. (2018) A study of commercial β-galactosidase stability under simulated in vitro gastric conditions. *Agronomy Research*, 16, 2, p. 1555-1562. DOI: 10.15159/AR.18.075
5. Žolnere K., Ciproviča I. (2017) The comparison of commercially available β-galactosidases for dairy industry: a review. *Research for Rural Development 2017, The Annual 23th International Scientific Conference Proceeding*, Volume: 1, Latvia University of Agriculture, Jelgava, Latvia, p. 215-222. DOI: 10.22616/rrd.23.2017.032
6. Žolnere K., Liepiņš J., Ciproviča I. (2017) The impact of calcium ions on commercially available β-galactosidase. *11th Baltic Conference on Food Science and Technology "Food science and technology in a changing world" FOODBALT-2017*, Conference proceedings, p. 27 – 30. DOI: 10.22616/foodbalt.2017.017

Results have been presented at **the international conferences and symposiums in the USA, Czech Republic, Estonia, Italy and Latvia.**

1. Žolnere, K., Ciproviča, I. (2019) The study of physical properties of spray dried whey and milk permeates lactose. The Biosystems Engineering conference, Tartu, Estonia, 2019, May 8 – 10 (Oral presentation / Mutiskā prezentācija).
2. Žolnere, K., Ciproviča, I. (2019) Lactose hydrolysis in different solids content whey and milk permeates". 13th Baltic conference on Food Science and Technology, FOODBALT 2019 joined with 5th North and East European Congress on Food NEEFood 2019 "FOOD. NUTRITION. WELL-BEING", Jelgava, Latvia, 2019, May 2 – 3 (Oral presentation / Mutiskā prezentācija).
3. Žolnere, K., Ciproviča, I., Ķirse, A., Cinkmanis, I. (2018) A study of commercial β-galactosidase stability under simulated in vitro gastric conditions. The Biosystems Engineering conference, Tartu, Estonia, 2018, May 9 (Poster presentation / Stenda referāts)
4. Žolnere, K., Ciproviča, I. (2018) Optimization of bioprocesses for lactose recycling products. CAFES Research Seminar Series, San Luis Obispo, California, USA, 2018, May 10 (Oral presentation / Mutiskā prezentācija).
5. Žolnere, K., Ciproviča, I. (2018) The effect of whey salts on enzymatic lactose hydrolysis. ICBFE 2018: 20th International Conference on Bioprocessing and Food

Engineering, Roma, Italy, 2018, March, 5 – 6 (Oral presentation / Mutiskā prezentācija).

6. Žolnere, K., Liepiņš, J., Ciproviča, I. (2017) Development of easy to use analytical method for determination of commercial β-galactosidase activity. 8th International Symposium on Recent Advances in Food Analysis, Praha, Czech Republic, 2017, November 7 – 8 (Poster presentation / Stenda referāts).
7. Žolnere, K., Ciproviča, I. (2017) The comparison of commercially available β-galactosidases for dairy industry: Review. 23rd Annual International Scientific Conference "Research for Rural Development 2017", Jelgava, Latvia, 2017. May, 17 - 19 (Oral presentation / Mutiskā prezentācija).
8. Žolnere, K., Liepiņš, J., Ciproviča, I. (2017) The impact of calcium ions on commercially available β-galactosidase. 11th Baltic Conference on Food Science and Technology “Food science and technology in a changing world” FOODBALT-2017. Jelgava, Latvia, 2017, April 27 – 28 (Oral presentation / Mutiskā prezentācija).

CONTENT / *SATURS*

List of included tables / *Darbā iekļauto tabulu saraksts*

List of included figures / *Darbā iekļauto attēlu saraksts*

List of designations, abbreviations and main terms included in the doctoral thesis /
Darbā izmantotie apzīmējumi, saīsinājumi un galvenie termini

ANOVA	Analysis of variance / *Dispersijas analīze*
AOAC	Association of Official Analytical Chemists / *Oficiālā analītiskās ķīmijas asociācija*
BLU	Bifido lactase unit / *Bifidolaktāzes vienība*
BOD / *BSP*	Biological oxygen demand / *Bioloģiskais skābekļa patēriņš*
Brix%	Soluble solids / *Šķīstošā sausna*
COD / *ĶSP*	Chemical oxygen demand / *Ķīmiskais skābekļa patēriņš*
EPS	Exopolysaccharides / *Eksopolisaharīdi*
EU / *ES*	European Union / Eiropas Savienība
GD	GODO-YNL2
GGS	Glucose – galactose syrup / *Glikozes – galaktozes sīrups*
GIT / *KZT*	Gastrointestinal tract / *Kuņģa – zarnu trakts*
GMA–MMA	Glycidyl methacrylate–methylmethacrylate / Glicidil metakrilāts–metilmetakrilāts
GOS	Galacto-oligosaccharides / *Galakto-oligosaharīdi*
GOS2	Galacto-oligosaccharide disaccharide / *Galakto-oligosaharīdu disaharīds*
GOS3	Galacto-oligosaccharide trisaccharide / *Galakto-oligosaharīdu trisaharīds*
GOS4	Galacto-oligosaccharide tetrasaccharide / *Galakto-oligosaharīdu tetrasaharīds*
HA	Ha-Lactase 5200
HPLC / *AEŠH*	High-performance liquid chromatography / *Augstas efektivitātes šķidruma hromatogrāfija*
ISO	International Standard Organisation / *Starptaustiskā Standartu Organizācija*
KMO	Kaiser-Meyer-Olkin test / *Kaisera-Meijera-Olkina tests*
NF	NOLA^{TM}Fit5500
NLU	Neutral lactase unit / *Neitrālā laktāzes vienība*
PHA-PHB	Polyhydroxyalkanoates – Polyhydroxybutyrate / *Polihidroksialkanoāti – Polihidroksibutirāts*
PCA	Principal component analysis / *Galveno komponentu analīze*
p	p-value / *p-vērtība*
pH	the negative logarithm of the hydrogen ion concentration / *ūdeņraža jonu koncentrācijas negatīvais logaritms*
rpm	Revolutions per minute / *Apgriezieni minūtē*
SEM	Scanning electron microscopy/ *Skenējošā elektronmikroskopija*
SGF	Simulated gastric fluid / *Imitēta kuņģa sula*
SIF	Simulated intestinal fluid / *Imitēts zarnu šķidrums*
TGA/DSC / *TGA/DSK*	Thermogravimetric Analyser / Differential Scanning Calorimeter / *Termogravimetriskais ananalizators / Diferenciālās skenēšanas kalorimetrs*

Dairy permeates / *Ultrafiltrāts*	milk by-products characterised by a high content of lactose and obtained by removing fats and protein from milk, whey, cream and/or sweet buttermilk, and/or from similar raw materials by ultrafiltration or other processing techniques / *piena pārstrādes produkts ar augstu laktozes saturu, iegūtu ar ultrafiltrācijas vai citādas apstrādes metodi, atdalot taukus un olbaltumvielas no piena, sūkalām, krējuma un/vai paniņām* (Regulation (EU) 2018/1602)
Whey / *Sūkalas*	by-product obtained during the manufacture of cheese or casein. In the liquid state, whey contains natural components (on average 4.8 % lactose, 0.8 % protein and 0.2 % fats) which remain when the casein and the majority of the fat have been removed from milk / *blakusprodukts, kas iegūts siera, biezpiena vai kazeīna ražošanā. Sūkalas satur piena sastāvdaļas (vidēji 4.8 % laktozi, 0.8 % olbaltumvielas un 0.2 % tauku), kuras pāriet tajās, atdalot no kazeīnu un taukus.* (Diretive 96/16/EC, 1996).

INTRODUCTION / *IEVADS*

The new methods and technologies for the processing of food by-products into valuable and functional products are becoming increasingly important. There are mainly two types of whey - sweet and acid, which are constantly being studied by researchers to gain a new information and acquire new knowledge. One of the methods for processing a lactose in whey is biotechnology, in particularly β-galactosidase application, to hydrolyse lactose into monosaccharides. This is the first step in glucose-galactose syrup production. Today, some dairies in the world produce and offer glucose-galactose syrup with solids concentration 70% which is made from sweet whey.

The hydrolysis of lactose in glucose and galactose is considered to be an excellent alternative to sucrose, as the resulting sugars are sweeter and can be used as additives for food and animal products production. Over the last two decades, this process has motivated scientists and manufacturers to conduct research and analyse its benefits (Das et al., 2015).

There is a possibility to further increase the sweetness of the syrup by adding glucose isomerase, which converts glucose into fructose. It is important to emphasize that galacto-oligosaccharides are also formed during enzymatic reactions, which are especially valuable as prebiotics. In turn, there are methods to produce lactulose during the lactose hydrolysis adding a certain amount of fructose to the substrate. Lactulose adds value to the final product and has been shown to perform a number of biological activities in the human body.

Finally, the new syrup can be a great alternative with a high level of sweetness that could be used as a sweet substance for the manufacturing of food products, thus reducing the energetic value of the product.

The **aim** of the doctoral thesis was to improve the lactose hydrolysis process for obtaining glucose-galactose and oligosaccharide syrups.

The **hypothesis** of the doctoral thesis – the two-stage fermentation increases the sweetness of glucose-galactose syrup.

The hypothesis of the doctoral thesis has been confirmed by the **defended thesis:**

1. The presence of cations affects the β-galactosidase activity in the sweet and acid whey permeate.
2. The chemical composition and quality of whey affect the physical properties of lactose.
3. Enzymatic reactions affect the functional and sensory properties of syrups.

The **research objects** – sweet and acid whey permeates, glucose isomerase, commercial β-galactosidases and glucose-galactose syrup.

The following **tasks** were set to achieve the aim of the doctoral thesis:

1. To evaluate the effect of cation concentration to ensure the β-galactosidase activity in substrate.
2. To investigate the physical properties of whey lactose in order to better understand its behaviour.
3. To study the changes of monosaccharide concentration in the lactose hydrolysis, varying with the solids concentration of the substrates and enzyme units.
4. To assess the possibilities of glucose isomerase to increase the sweetness of glucose-galactose syrup.
5. To evaluate the sensory properties of the developed syrups.

The **novelty** of the doctoral thesis:

1. The study of the relationship of lactose hydrolysis process in the formation of galacto-oligosaccharides and lactulose.
2. The combination of β-galactosidase and glucose isomerase increases the sweetness of glucose-galactose syrup.

The **economic significance** of the doctoral thesis:

1. The studies have shown the possibility to obtain syrup that can be used as sugar and sweeteners replacer in the food industry and to produce functional products.
2. A technological solution for hydrolysis of lactose is proposed, comprehensively evaluating the physical properties of lactose, fermentation parameters and whey composition. The doctoral thesis consists of three chapters:

Chapter 1 describes the composition of whey and the possibilities of using it. An overview of the chemical and physical properties of lactose, lactose hydrolysis methods, the application of β-galactosidases and the properties of glucose-galactose syrup are provided.

Chapter 2 summarises the materials and methods used in the thesis.

Chapter 3 provides a summary of the results obtained in the study, the properties of commercial enzymes in different cation concentrations, the stability of enzymes in the gastrointestinal tract model, methods for the determination of lactose, the properties of dehydrated permeates are evaluated. The influence of factors on the hydrolysis of permeates and the profile of the obtained sugars was analysed. P ossibilities for lactulose synthesis are considered. Sensory analysis of glucose-galactose syrups and syrups obtained in the two-stage fermentation are given. Conclusions are presented at the end of Chapter 3.

During the PhD studies the author had an **internship** at the Dairy Innovation Institute at California Polytechnic State University (USA), where the experimental work was continued. Internship was provided by the Baltic – American Freedom Foundation (BAFF) and the Council in International Education Exchange (CIEE).

The study was partly financed by the grant "Strengthening Research Capacity at the Latvia University of Agriculture" (Contract No. 3.2.-10/2017/LLU/27 "The optimization of bioprocesses for lactose recycling products").

The study was partly financed by the doctoral studies grant "Transition to the new doctoral funding model at the Latvia University of Life Sciences and Technologies" (Contract No. 3.2.-10/90).

The thesis is written in English, it consists of 111 pages, 32 tables, 41 figures, 3 appendixes and 233 bibliographic sources.

1. PROBLEM STATEMENT / *PROBLEMĀTIKAS RAKSTUROJUMS*

1.1 An overview of whey application / *Pārskats par sūkalu izmantošanu*

Whey, which has been primarily considered as a waste product, has been valued as a raw material for a wide range of production, starting from fuel to functional food. Reuse of whey has attracted industrial interest not only because it has high nutritional value and is a cheap raw material for producing value-added product, but whey also has a high risk of environmental pollution (Pescuma *et al.*, 2015). Current whey processing vary depending on the amount of whey and the capabilities of the company. While large companies often divert it to secondary processing (concentrated protein, etc.) or animal feed (mainly pig farms), smaller plants mainly divert them to the domestic sewage system or biogas production. In total, about 50% of whey produced in the world (especially acid whey) is considered as waste. This causes serious environmental problems due to the high content of organic matter (especially lactose) and high levels of biological and chemical oxygen demand (BOD and COD). Whey contains valuable fertilisers, such as ammonia and phosphates, and their release into the environment can cause eutrophication of water bodies, thus disturbing the natural aquatic ecosystem (Bentahar *et al.*, 2019). These challenges open up the possibility of exploring the various properties of whey in different food systems to transform whey nutritional value into a novel and functional food product (Walzem *et al.*, 2002). Lactose, the largest component of whey, can act as a source of bacterial growth and be used for many biotechnological processes (Ryan & Walsh, 2016). In this way, with the significant advances in biotechnology, whey is becoming the substrate for various food and pharmaceutical sectors (Simović *et al.*, 2019).

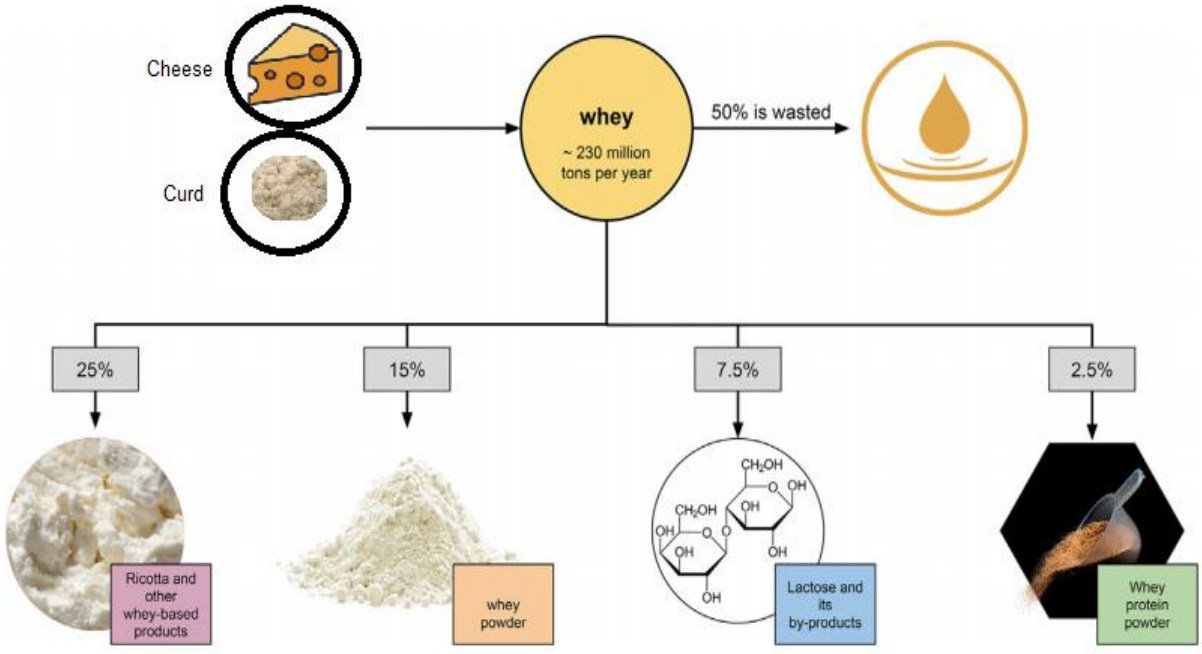


Fig. 1.1. Industrial application of whey / *1.1. att. Sūkalu pielietojums rūpniecībā* (Rama *et al.*, 2019)

Worldwide approximately 50% of whey has been reused and turned into different food and animal products. Half of these products are in liquid form and the other half - whey powder, lactose and its by-products, and residual protein concentrate (Spălățelu, 2012). The average whey amount increases equally with the amount of milk in the world >2% in a year (Smithers, 2008). Until 2019, it was expected that the amount of whey in the world will be around 230 million tons per year and in Europe 40 million tons per year (Mollea *et al.*, 2013). According

to the World Cheese Market 2000-2023, world production of whey is estimated at 230 million tonnes in 2023 (Rama *et al.*, 2019). It also increases the amount of acid whey by around of 4 million tonnes per year, nonetheless, acid whey recycle is limited. Dairy producers mostly drain acid whey into wastewater treatment plants or return it to farmers, who in turn cultivate their land or feed their livestock (Lindsay *et al.*, 2018). Based on the European statistics database Fig. 1.2 demonstrates the whey production data in the Baltic states.

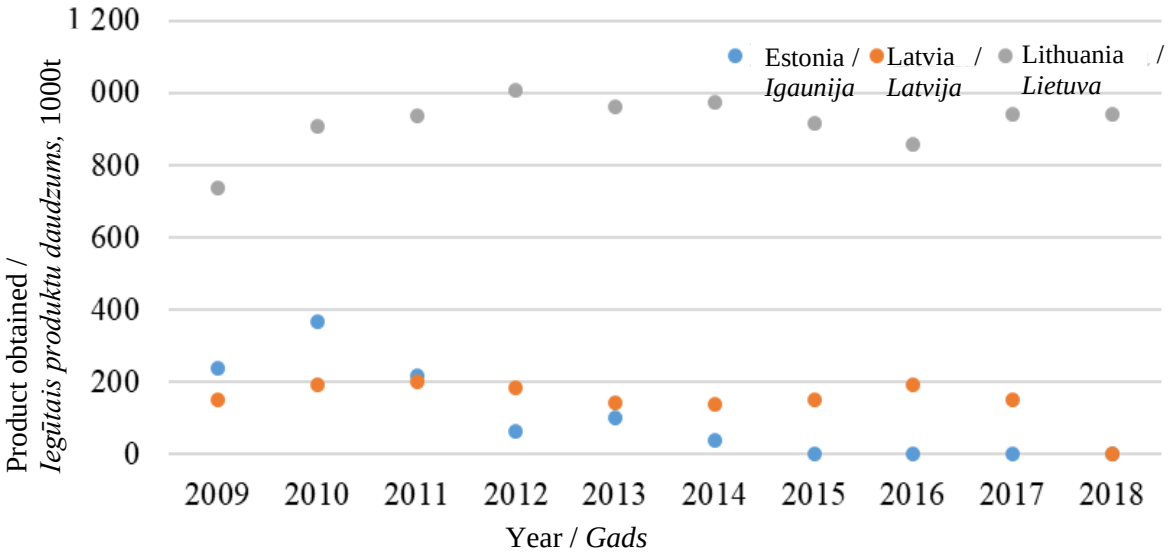


Fig. 1.2 The amount of whey produced in the Baltic States /
1.2. att. Iegūto sūkalu apjoms Baltijas valstīs
(Eurostat - Data Explorer, 2019)
(Value 0 means that certain data are confidential and not available)

The use and processing of whey in the Baltic States became popular only during the last decade, before that whey was mainly diverted to livestock feed. Countries are now looking for ideas for processing acid and sweet whey into a functional products. Mainly for the purpose of products development manufacturers cooperate with scientists, who during scientific research obtain all the necessary results for establishing a processing line. There are several Latvian factories such as SC "Smiltenes piens", Ltd "Baltic Dairy Board", SC "Tukuma piens" that have collaborated with scientists to promote whey processing and use in food.

Today, the dairy industry is still searching for alternative ways to use whey in production because it causes significant residues the elimination of which requires large capital investments (Lindsay *et al.*, 2018). Most dairy companies do not have a proper system for whey utilization, which does not promote whey as a potential source of new food and energy products. Whey is not just a cheap raw material (Królczyk *et al.*, 2016), but its degradation is unfavourable to the environment, affecting the physical and chemical properties of the soil, leading to the reduction of crop yields (Panesar *et al.*, 2007) and decomposing oxygen in water tanks, creating anaerobic conditions, promoting the extinction of the aquatic environment (Das *et al.*, 2015). This problem is more pronounced due to the fact that whey residues are often discharged into the sea, for example, in the regions of Northern Italy, where several dairies are located and approximately 1 million litres of whey was disposed daily (Koller *et al.*, 2012).

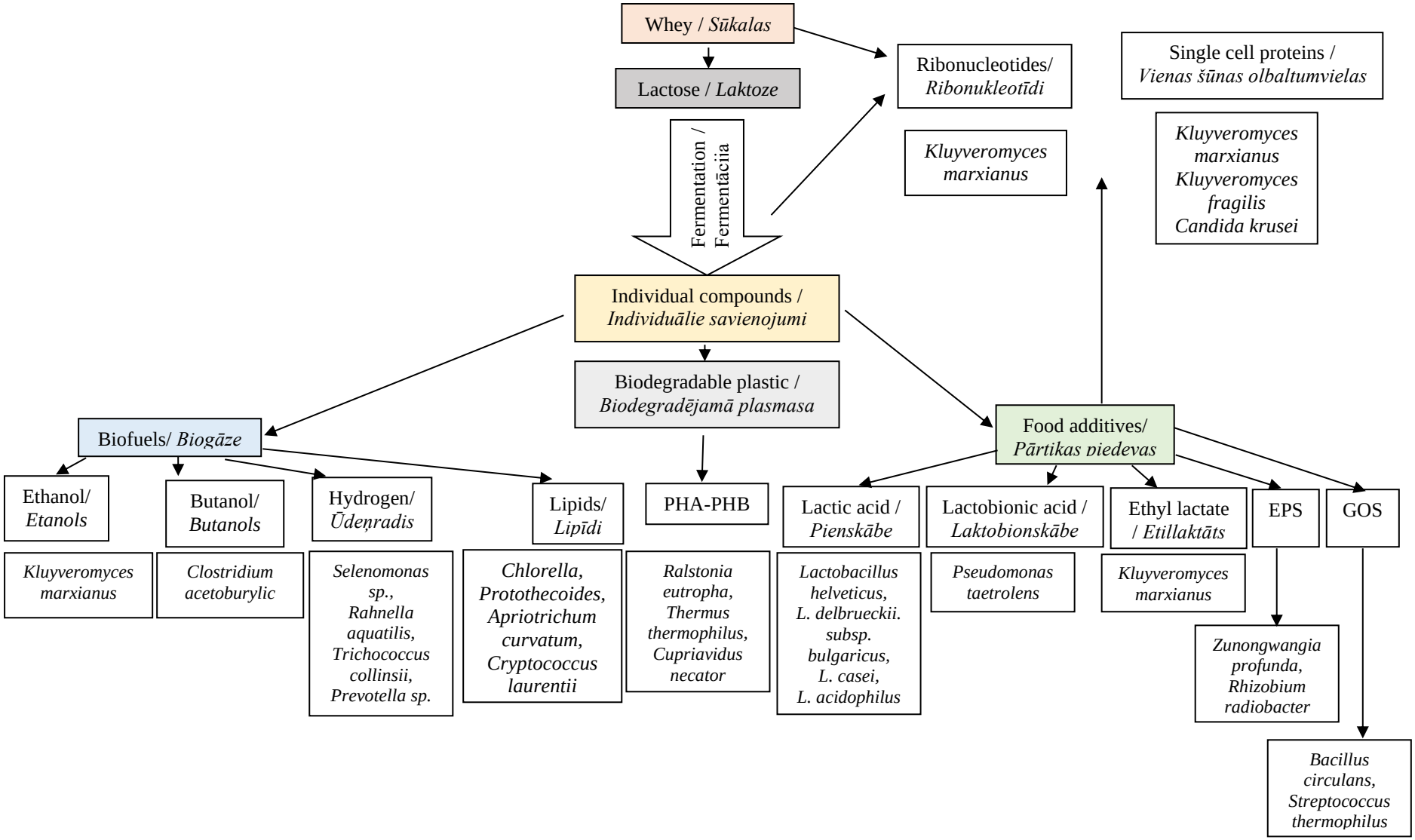


Fig. 1.3. An overview of the production of various compounds in whey fermentation /
1.3. att. Pārskats par dažādu savienojumu ieguvi sūkalu fermentācijā
(Pescuma *et al.*, 2015)

Finding the most appropriate technological solution should reduce the environmental pollution and increase the value of whey as a processing ingredient (Volpato *et al.*, 2016). The diversity of whey fermentation products is shown in Fig. 1.3. Fermentation is a cheap recycling process compared to chemical synthesis, moreover during fermentation none of toxic products (depending on the nature of the microorganisms) have been developed, which in turn is a frequent phenomenon in chemical reactions (Pescuma *et al.*, 2015).

Thus, the lactose separation from whey would not only reduce the amount of organic matter in it, but also promote and supplement the application of lactose in the food and pharmaceutical industries due to its functional properties (Mukhopadhyay *et al.*, 2003).

Three main alternatives are distinguished in the use of whey, taking into account the complexity and cost of the processes: direct application, whey concentration and drying, and fractionation and/or fractionation of whey ingredients (Henriques *et al.*, 2013). The demand for whey products in the food sector has been increasing for years. Combining biochemistry, microbiology, and sophisticated technology would make it possible to produce high quality and safe foods. In recent years, information on the composition, production, and use of whey and whey products has increased dramatically. The research on whey has been conducted in various fields, such as nutrition, pharmaceuticals, medicine, process technology, etc., and the articles pertaining to this subject have been published in various food journals (Wit, 2001).

1.2 Whey characteristics / *Sūkalu raksturojums*

Whey is greenish yellow in colour and in some cases, it can be even blue, mainly depending on the quality and origin of milk. Whey is obtained only from milk processing. The most popular in western countries is cow's milk, while in other parts of the world it is goat's, sheep's, and even camel's milk (Smithers, 2008).

Whey also contains salts and water-soluble vitamins (Reddy *et al.*, 2016). The chemical composition of whey depends on the chemical composition of the milk, which varies within the lactation period, feeding, breeding, and due to individual differences of the animals, and climate change (Polat, 2009).

There are usually two main sources of obtaining whey: cheese making – sweet whey and casein production – acid whey. Acid whey is obtained after acidification of milk using lactic acid bacteria, organic or mineral acids. Sweet whey forms after adding proteolytic enzymes, such as rennet, to milk and treatment of coagulum by different techniques (Carvalho *et al.*, 2013; McSweeney & Fox, 2009). Whey yield is 75 – 85% of the total milk volume and contains approximately 55% of milk nutrients - lactose, proteins, vitamins, salts and fats (Shankar *et al.*, 2015). This indicates that whey can be considered a valuable by-product with multiple uses in the food and pharmaceutical industries (Mollea *et al.*, 2013). Although whey contains an impressive number of organic substances (Królczyk *et al.*, 2016), it has high biochemical oxygen demand (BOD, 40000 - 60000 ppm) and chemical oxygen demand (COD, 50000 - 80000 ppm), making its disposal relatively expensive (Koller *et al.*, 2012). According to European standards, it can involve a very high level of pollution that can be associated with a city of 50,000 residents (Ostojić *et al.*, 2005). Lactose, which constitutes 70-72% of the total dry matter in whey, is the main component responsible for the amount of BOD and COD (Ryan & Walsh, 2016).

The origin and composition of whey mainly depend on the processing method used for casein precipitation. Cheese production involves the use of proteolytic enzyme complexes. The coagulation process occurs at pH 6.5 resulting in the production of sweet whey (Shankar *et al.*, 2015). Coagulation of casein results in the formation of acid whey, which has pH 5.1 and contains lactic acid bacteria, mineral salts, and organic acids (Oliveira *et. al*, 2011). Some whey proteins become insoluble in acid whey and therefore it is difficult to perform membrane filtration (Anand *et al.*, 2013). In general, this indicates that the difference between whey types is due to acidity, salts and protein content (Shankar *et al.*, 2015).

Table 1.1. /*1.1. tabula*

Characteristics of acid, casein and sweet whey /
Biezpiena, kazeīna un siera sūkalu raksturojums (Бугаева, 2014)

Type of whey and permeate / *Sūkalu un ultrafiltrāta veids*	**Content / *Sastāvdaļas*, %**					**Acidity / *Skābums*, °T**	**Density / *Blīvums*, kg m^{-3}**
	Solids / *Sausna*	**Lactose / *Laktoze***	**Protein / *Olbaltumvielas***	**Fat / *Tauki***	**Salts / *Sāļi***		
Sweet / *Siera*	4.5 – 7.3	3.9 – 5.2	0.4 – 1.1	0.04 – 0.6	0.3 – 0.8	15 - 25	1018 - 1027
Acid / *Biezpiena*	4.2 – 7.4	3.2 – 5.1	0.5 – 1.0	0.05 – 0.4	0.5 – 0.8	50 - 85	1019 - 1026
Casein / *Kazeīna*	4.5 – 7.5	3.5 – 5.2	0.5 – 1.0	0.02 – 0.3	0.3 – 0.9	50 - 120	1020 - 1025
Sweet whey permeate / *Siera sūklau ultrafiltrāts*	5.1 – 5.4	4.2 – 4.8	0.2 – 0.24	-	0.5 – 0.75	8 - 18	1012 – 1018
Acid whey permeate / *Biezpiena sūkalu ultrafiltrāts*	5.2 – 5.6	4.2 – 4.8	0.2 – 0.24	-	0.6 – 0.9	80 - 100	1016 - 1018

Low lactose content in acid whey is achieved by the lactic acid fermentation in milk, with a significant increase of lactic acid concentration and decrease in pH. Higher content of salts in acid whey is due to the elimination of calcium phosphate bonds in casein micelles (Wit, 2001) or salt addition to milk during cheese production. It shows that acid whey has higher content of salts, lower protein content and higher acidity than sweet whey, making its usage in foods more limited (Oliveira *et al.*, 2015).

- Proteins

Whey proteins are one of the two main components of bovine milk proteins, the second is casein. The major components of whey proteins are β-lactoglobulins (50%), α-lactalbumins (20%), bovine serum albumin (10%) and immunoglobulins (10%). Whey also contains other important proteins such as lactoferrin, protease peptone fraction, and osteopontin (Bansal & Bhandari, 2016). The important properties of whey proteins are the ability to form gels, act as emulsifiers and provide texture properties, etc. (Argenta & Scheer, 2019). Therefore, whey proteins are included in processed meat, dairy, and bakery products. Another feature is its foaming property, which is mostly dependent on the degree of protein denaturation. This ability is also affected by the protein concentration in the whey protein concentrate. For example, whey protein concentrate containing 34-35% protein (WPC35) has good emulsifying properties, is highly soluble, and has a mild milky taste (Bacenetti *et al.*, 2018).

- Fats

The concentration of fat in whey is low. Nevertheless, the major lipid class is triacylglycerol and the amount of fat in whey mainly depends on the fractionation technique (Walzem *et al.*, 2002).

- Salts

The relatively high content of salts in whey solids can also be considered as a problem for whey recycling since these salts create mainly bitter taste. The acid whey with high content of lactic acid and salts increases its solubility (Macwan *et al.*, 2016).

Table 1.2. / *1.2. tabula*

The amount (g L^{-1}) of salts and phosphate ions in sweet and acid whey / *Minerālvielu un fosfāta jonu saturs (g L^{-1}) siera un biezpiena sūkalās*

Ions / *Joni*	**Sweet whey / *Siera sūkalas***	**Acid whey / *Biezpiena sūkalas***	**Reference / *Atsauce***
Na^+	0.4-0.5	0.4-0.5	(Gésan–Guiziou, 2013) (Waldron, 2009) (Koller *et al.*, 2012) (Theoleyre, Gula, 2004) (Cataldi *et al.*, 2003) (Gernigon, *et al.*, 2010)
K^+	1.4-1.6	1.4-1.5	
Ca^{2+}	0.4-0.6	1.1-1.6	
Mg^{2+}	0.1-0.2	0.1-0.2	
Cl^-	1.0-1.2	0.9-4.2	
PO_4^{3-}	0.5-1.3	0.6-2.4	

The data where the amount of salts varies depending on the type of whey summarises in Table 1.2. Acidic medium provides higher separation of minerals (mainly calcium phosphate) due to their mineralisation, and calcium salts at a low pH are more soluble.

The differences in whey content give options of where and for what it can be used for extraction.

1.3 Description of lactose / *Laktozes raksturojums*

Lactose (4-O-β-D-galactopyranosyl-D-glycopyranose) with its empirical formula $C_{12}H_{22}O_{11}$ is a disaccharide, one of the best known and important sugars in milk (Illanes, 2016). The hydrolysis of lactose in the intestinal tract occurs slowly (Parker & Watson, 2017), it is a substrate for lactic acid or other acids, and it also increases calcium bioavailability (Pérez *et al.*, 2008). Lactose also stimulates the growth and development of lactic acid in the intestines (Anand *et al.*, 2013).

Lactose is a reducing sugar and reacts with protein amino groups. Lactose can participate in the Maillard's reaction, it causes significant loss of nutrients by creating by-products which are capable of changing the product colour to a darker one as well as changing the taste (Anand *et al.*, 2013). It is important to point out that raw milk also contains mono- and oligo-saccharides, but only in small quantities (Huppertz & Gazi, 2016).

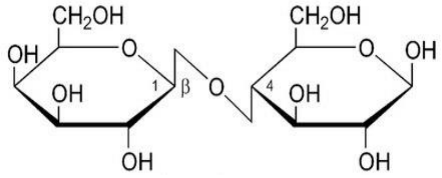


Fig. 1.4. α- and β- Lactose / *1.4. att. α- un β- laktozes*
(Carpin *et al.*, 2016)

The presence of chiral carbon in the lactose anomer indicates that it may exist in two forms of α- and β-anomers (Wong & Hartel, 2014), because the placement of -OH and -H groups at the glucose first carbon atom may be different (Illanes, 2016). Optical rotation of both anomers α- and β- are $[\alpha]^{20}_{D}$ +89.4° and +35° and solubility 70 and 500 g L^{-1} (20 °C) respectively (Wong & Hartel, 2014; Shendurse & Khedkar, 2016).

In aqueous solutions, the rate of transformation and its balance between lactose anomers (Carpin *et al.*, 2016) is hard to control and determinate, and it depends on the temperature, concentration, pH and the presence of other substances (minerals, riboflavin, traces of fat and protein) (Wong & Hartel, 2014). For example, when lactose dissolves in an aqueous solution at 20° C, 62.7% β-lactose and 37.3% α-lactose is formed (Kuusisto *et al.*, 2007). In the crystallisation process, α-lactose converts into monohydrate, but β-lactose in an anhydrite form (Illanes, 2016). Lactose anomeric forms may characterise the complexity of lactose functional and physical properties, such as melting point, solubility, etc. (Gambelli, 2017; Chandrapala *et al.*, 2016).

The introduction of membrane technology in whey processing improves commercial lactose production technology. Several crystalline lactose forms are also available on the market with different physical properties. The dominant commercial form is non-hygroscopic α-lactose monohydrate (Anand *et al.*, 2013). Lactose in milk may exist in various crystalline and non-crystalline forms. This indicates that the quality and stability of certain foods can be significantly affected by the physical properties of lactose (Chandrapala *et al.*, 2016). Lactose is hygroscopic sugar and has strong abilities to absorb taste and aroma, as well as it can cause sandy texture and sediments in food products (Panesar *et al.*, 2010a). The presence of lactose in milk, milk products, and whey limits the consumption of milk products for people with

lactose intolerance. Lactose intolerance may be caused by the absence of the enzyme β-galactosidase in the intestinal wall of small intestines or by a reduced enzyme activity (Dutra Rosolen, *et al.*, 2015). Figure 1.8 illustrates the possible lactose forms.

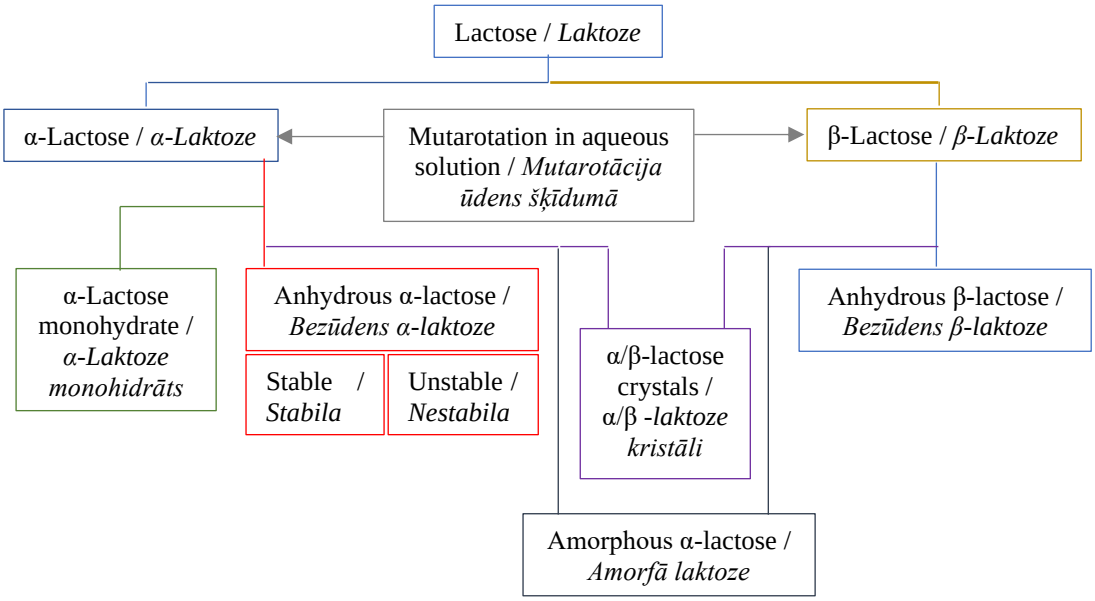


Fig. 1.5. An overview of lactose form diversity / *1.5. att. Pārskats par laktozes formu daudzveidību* (MacFhionnghaile *et al.*, 2017)

- **α-Lactose**

α-Lactose is gained from α-lactose monohydrate, where water is released from the crystal during crystallisation. α-Lactose may exist in a stable and unstable form. When the water is released from the crystals and it does not change its shape, a porous and unstable α-lactose structure is formed. Unstable α-lactose is highly hygroscopic and rapidly converts into α-lactose monohydrate, whereas stable α-lactose has a different crystalline form that does not change at room temperature and relative humidity below 50%. Both forms can be obtained from α-lactose monohydrate at 100 ° C to 190 ° C temperature under vacuum conditions. Stable α-lactose can also be obtained using organic solvents that do not need to be mixed with water (Carpin *et al.*, 2016).

- **β-Lactose**

β-Lactose is more stable at a temperature of 25° C and relative humidity below 95% than α-lactose (Salameh *et al.*, 2006). The form of lactose crystals depends on the environment from which it is crystallised. Lactose crystallisation in water makes the crystal forms into an irregular diamond shape, but when crystallising from the alcoholic substrates (methanol, ethanol etc.), the crystal forms a convex needle, similar to a prism. There are different ways of producing β-lactose from saturated lactose solution or α-lactose monohydrate. Most of these methods include heating the saturated solution at high temperature as lactose crystallises in β form at a temperature higher than 93.5° C. Regardless of the method used, it is particularly difficult to obtain a single anomeric powder that is completely free from other isomeric lactose form. Commercial anhydrous lactose contains up to 80% of β-lactose (Carpin *et al.*, 2016).

- **α- Lactose monohydrate ($C_{12}H_{22}O_{11}\cdot H_2O$)**

Heating up the lactose monohydrate solution below 93.5° C, any form of lactose crystallises into α-monohydrate. In turn, at 93.5° C or lower lactose forms α-monohydrate containing 5% water. Lactose can be transformed into a number of crystalline forms that depend on the crystallisation conditions. Crystals with a tongue shape and less than 10 μm in size are invisible in food products, about 16 μm crystals give a grainy and sandy texture, while 30 μm crystals are very grainy. The term sandy is used to describe the texture of evaporated milk, ice

cream, and other dairy products due to various factors that cause the formation of large lactose crystals (Shendurse & Khedkar, 2016).

- **Amorphous lactose**

Amorphous lactose can exist in the glassy state in concentrated syrups. The form of this lactose is a mixture of α- and β-lactose molecules, forming an irregular grid. Amorphous lactose has a higher energy value than crystalline lactose. It is thermodynamically unstable and very hygroscopic (Carpin *et al.*, 2016).

These properties indicate that lactose has a number of application possibilities in various food sectors, such as an agglomeration agent to accentuate and / or enhance the taste of various foods, to improve functionality, also as a substrate for enzymes (Shendurse & Khedkar, 2016).

1.4 Lactose application in the food industry / *Laktoze pārtikas rūpniecībā*

Food and pharmaceutical industries have found multiple options for lactose usage, mainly in crystallised form. In most cases, lactose has been added to bakery and confectionary products (Carpin *et al.*, 2016). In bakery, lactose is engaged in the Maillard reaction to form a caramel taste (Parker & Watson, 2017; Shendurse & Khedkar, 2016).

Lactose also is used as a supplement for infant formula (Illanes, 2011). The price of lactose on the international market in 2014 reached US$1826 per ton. That has been emphasized as the highest value ever reached in the Global Dairy Trade auctions, but then it declined to an average value of about US$800 per ton. In September 2018, the price was US$917/t from the Global Dairy Trade, 2018, driven by the high demand for powder from Asia, but this demand slowed down with the financial crisis of the Chinese market (Costa *et al.*, 2019).

At this particular time in the European Union the labelling and composition rules for the absence or reduced amount of lactose in food are not coordinated, there are no strict rules of the lactose content in food. However, this information is essential for those who are lactose intolerant. Regulation (EU) No 1169/2011 lays down rules on the information that needs to be provided for substances which have scientifically proven allergenic or intolerant effects, so that consumers can make a safe choice. The provisions on the use of terms relating to the absence or reduction of lactose in foods should be governed by Regulation (EU) No 1095/2010 regarding the scientific opinion of 10 September 2010 on lactose limit values for lactose intolerance and galactosemia (Schulz & Creighton, 2013). Pulinas *et al.*, (2017) reported that Italian Ministry of Health allows in Italy labelling food as a lactose-free if lactose is less than 0.1 per 100 g or mL, moreover the label “low in lactose” is for fluid and fermented milk when the lactose amount is less than 0.5 per 100 g or mL. The same amount of lactose refers to products that do not naturally contain or are low in lactose in dairy products where lactose is hydrolysed during production (Pulinas *et al.*, 2017).

Numerous products contain lactose, starting with small concentration (caseinates, whey protein and milk protein powders) till high concentration (permeate, whey powder). Thereby, product physical properties (solubility, crystallisation, sensory), production process and storage time might be affected by lactose if it is more than 50% of product solids (Huppertz & Gazi, 2016).

Lactose usage as a food ingredient is limited, it is less soluble and has low sweetness (Batista *et al.*, 2017). Lactose can easily be crystallised which emphasizes the fact that the usage is limited for the production of other kinds of dairy products (Panesar *et al.*, 2010a).

In addition, lactose can be separated and used in a wide range of food as well as non-food products as a raw material for several derivatives (Huppertz & Gazi, 2016). Therefore, it is necessary to look for new processing technologies and methods which help to transform lactose into other products.

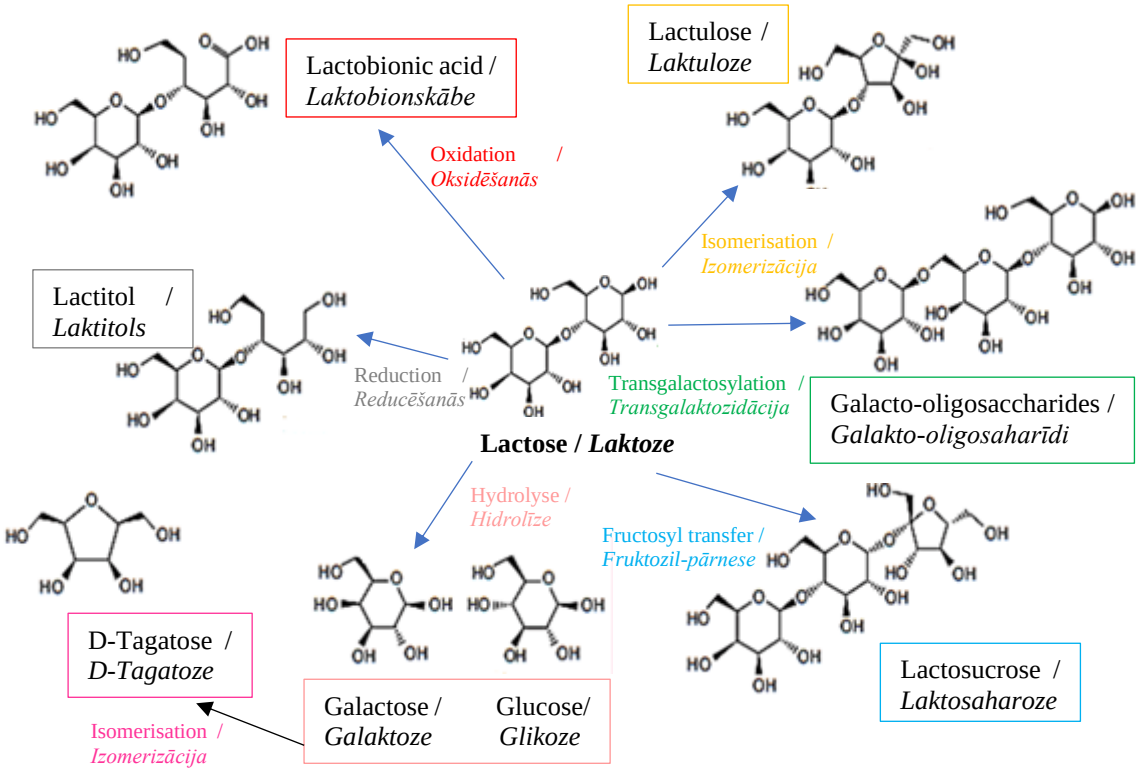


Fig. 1.6. Forms of lactose derivatives / *1.6. att. Laktozes atvasinājumu formas* (Paterson & Kellam, 2009)

Fig. 1.6 shows lactose derivatives which may be formed by appropriate treatment and most of them have become commercially successful. The formation of present products and by-products mainly depends on the temperature, pH, catalyst, agitation, and pressure (Cheng *et al.*, 2019). Lactose oxidation promotes the formation of **lactobionic acid** which is quite a new derivative that can be used in the food, pharmaceutical and chemical industries because of its chelating, moisturizing and emulsifying properties (Gutiérrez *et al.*, 2012). Glucose rearrangement in the fructose molecules causes lactose isomerisation to **lactulose**. Numerous complex reagents such as alkalis or enzyme can be used as catalyst for lactulose production (Panesar & Kumari, 2011). **Lactitol** is a sugar alcohol also known as a polyol obtained by lactose hydrogenation. Generally, lactitol is used as a low calorie sweetener, it is a multifunctional compound with various food, dairy, and pharmaceutical applications (Martínez & Monteagudo *et al.*, 2019).

The food industry is trying to develop products with a lower content of lactose or lactose free products. Therefore, lactose enzymatic hydrolysis using β-galactosidase is the major biotechnological process which is used in the dairy industry (Dutra Rosolen *et al.*, 2015). This technology will not only change the physical and chemical properties of the product but it will also help to make it more digestible (Saqib *et al.*, 2017). The most appropriate method to reduce the amount of lactose in milk and milk products is fermentation using β-galactosidase. The potential of fermented products on the market will increase due to avoided crystallisation and the increase in sweetness of the dairy products. Whey hydrolysis and appropriate process treatment transforms lactose into a valuable product such as sweet syrup which could be used in milk, bakery, cooked, and non-alcoholic product production. Therefore, hydrolysis of lactose would complement the range of products and solve the environmental problem (Panesar *et al.*, 2010b). In addition to lactose, it is also a valuable source of enzymatic or chemical treatment. For some of those transformations purified lactose can be used, but for some other whey or whey permeate might be used as a lactose source. The type of lactose processing depends mainly on the desired product, as well as economic considerations, which differ depending on the situation (Illanes, 2011).

Significant efforts are being made worldwide to improve the bioconversion of whey into high-nutrition products (Koller *et al.*, 2012).

1.5 Prospects of whey lactose hydrolysis / *Sūkalu laktozes hidrolīzes perspektīvas*

Food biotechnology, which began with the research into the effects of microorganisms in food fermentation (Muñiz-Márquez *et al.*, 2015) now can be described as a modern biotechnological technique to manufacture and process food. Biotechnology influences the production and preservation of raw materials, as well as changes in their nutritional and functional properties (Lee *et al.*, 2015). One such interest is the production of sugar which can be gained by hydrolysis of lactose (Das *et al.*, 2011). There are certain methods in which lactose can be used and which are currently in focus: one method is to use enzymes to convert lactose into various compounds, and the second is to use the hydrolysis of lactose to monosaccharides (glucose and galactose). Acid and sweet whey permeates can be used as a raw material to hydrolyse lactose into monosaccharides and after an appropriate treatment glucose-galactose syrup can be obtained (Shen *et al.*, 2019). In terms of sweetness, the relative sweetness level of lactose compared with sucrose (100%) is 16%, galactose 60% and glucose 74% (Joesten *et al.*, 2007). Glucose - galactose syrup has approximately 70% of sweetness compared with sucrose and it is 4-4.5 times sweeter than lactose (Whintaker & Wong, 2003). Glucose - galactose syrup is a thick sugar solution consisting of about 30% water, 68% glucose and galactose, 11% lactose and 1% salts (Lindsay *et al.*, 2018). The syrup can be used in ice cream, milk desserts, sauces, as caramel ingredient and a sweet substance in cereal bars (Roy *et al.*, 2015). There are multiple methods of stopping lactose hydrolysis reaction and enzyme activity. However, the information about β-galactosidase behaviour under *in vitro* digestion conditions is not sufficient.

The enzymatic method for the production of glucose-galactose syrup is highly selective for monosaccharides, and it is limited by excessively high enzyme costs (Lindsay *et al.*, 2018). It has been known for a long time that lactose can be hydrolysed into glucose and galactose using a strong mineral acid, followed by pure acid and ion exchange resin (Whintaker & Wong, 2003). Hydrolysis with acid is characterised by rapid catalytic action (80% of hydrolyzed lactose in 3 minutes) and lower catalyst costs. However, unwanted thermal and acid-catalysed degradation reactions limit production (Lindsay *et al.*, 2018). Hydrolysis with acid is more suitable for purifying the lactose solution. There are a number of effective methods available for obtaining glucose-galactose syrup and other lactose hydrolyses products, but research is still ongoing and will undoubtedly lead to improved methods and cost changes (Whintaker & Wong, 2003). Each commercial β-galactosidase is individual and acts differently during the hydrolysis of lactose. It is important to use an appropriate amount of enzyme, lactose concentration, and reaction time to obtain the highest hydrolysation rate in percentage. In addition, these factors also influence the content of final products and catalyse the hydrolysis of lactose and the synthesis of GOS during the reaction (Suárez *et al.*, 2018).

β-Galactosidase is designated for a number of processing operations in the dairy industry. Decreasing lactose concentration in milk and whey using β-galactosidase results in several products such as low-lactose milk, sweetened condensed milk, ice cream (Lima *et al.*, 2021), dulce de leche (Francisquini *et al.*, 2019), sugars syrups from whey permeate (Cervantes *et al.*, 2020), fermentation to ethanol (Whintaker & Wong, 2003).

To increase the sweetness of the resulting solution, glucose isomerisation was used, resulting in a solution of about 50% galactose, 29% glucose and 21% fructose (Belitz *et al.*, 2009). Another option is to consider the hydrolysis of lactose not only to form monosaccharides but also lactulose, giving the syrup higher nutritional value (McSweeney & Fox, 2009).

1.6 Characteristics of lactose hydrolysis methods / *Laktozes hidrolīzes metožu raksturojums*

The hydrolysis of lactose in whey is evaluated as a great alternative because the hydrolysed products are sweeter and can be used as additives or/and products for human and animal consumption. In the last two decades, this subject has motivated scientists and producers to do research and to analyse the benefits of the final product (Das *et al.*, 2015). Lactose hydrolysis promotes the solubility of milk and other products derived from milk, limiting the use of dairy products as ingredients in some recipes. (Budriene *et al.*, 2005). Moreover, as the sweetness level increases, it is necessary to add less sweeteners or sugars, thus reducing the amount of calories in the final product (Saqib *et al.*, 2017). Hydrolysis of 70% of lactose in milk and whey increases sweetness by an amount corresponding to an addition of about 2% sucrose (Harju *et al.*, 2012).

The benefits and advantages of lactose hydrolysis for industrial application are as follows:

- expanding the range of products with reduced lactose content minimizing the problem of lactose intolerance;
- during lactose hydrolysis, a galacto-oligosaccharide formation improves human microbiota and its maintenance;
- food processing technology and sensory characteristics improvement;
- providing better whey utilisation (Mlichová & Rosenberg, 2006).

Hydrolysis of lactose in milk and dairy products can be achieved with two methods (Şener *et al.*, 2006), as shown in Figure 1.7.

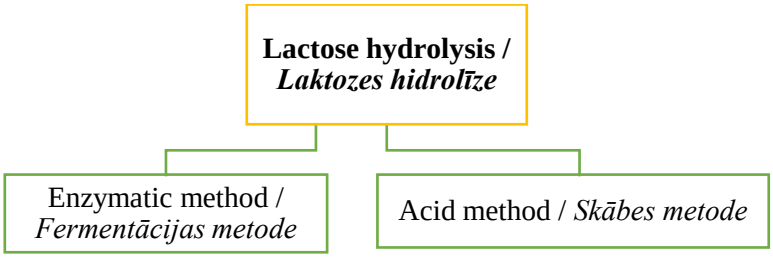


Fig. 1.7. Lactose hydrolysis methods / *1.7. att. Laktozes hidrolīzes metodes*

- **Acid method**

Lactose is resistant to acidic conditions, compared with other disaccharides, such as sucrose. Using organic acids such as citric acid, it is possible to hydrolyse sucrose but it does not work with lactose. The speed of lactose hydrolysis depends on reaction time, temperature and acid concentration (Holsinger, 1988; Üstok, 2007). The reaction conditions at which the highest lactose hydrolysis level can be reached showed in Table 1.3.

Table 1.3. / *1.3. tabula*

Lactose hydrolysis with hydrochloric acid / *Laktozes hidrolīze ar sālskābi* (Holsinger, 1988)

Lactose / *Laktoze*, %	**0.001 M HCl, mL**	**Reaction / *Reakcija***		**pH of medium after reaction / *Vides pH pēc reakcijas***	**Hydrolysis / *Hidrolīze*, %**	**Time for hydrolysis of 99.5% lactose - min / *Laiks, lai hidrolizētu 99.5% laktozes*, min**
		Temperature / *Temperatūra*, °C	**Time / *Laiks*, min**			
36.6	0.034	130	36	1.23	82.0	111.3
29.0	0.023	130	59	1.46	79.7	195.4
28.4	0.023	140	30	1.47	84.5	85.3
23.2	0.019	165	8	1.60	79.0	27.8

Coughlin and Nickerson (1975), Vujicic *et al.* (1977) and Lin and Nickerson (1977) conducted research where lactose hydrolysis was used with lactose solution at a concentration range from 5 to 40% as well as hydrochloric acid and sulfuric acid used at a concentration range from 1 to 3 M. The results showed that lactose can be hydrolysed till 90% at a temperature of 60 °C during 36 hours. The authors do not recommend using hydrolysis of lactose at some conditions in whey because acids cause by-products and dark colour (Holsinger, 1988).

Sulfonic acid exchange raisins which are reusable can also be used for lactose hydrolysis. Reaction time for hydrolysis is short and mineral acids do not impact the outcome. Boer and Robbertsen (1981) used whey permeate with solids concentration 10% and strong acid exchange raisins to reduce the pH to 1.2, the reaction was for 3 minutes at a temperature of 150 °C. The results showed 80% of lactose hydrolysis (Zadow, 1992).

The presence of salts in whey can neutralise acid and the obtained product needs to be demineralised (Üstok, 2007). Other disadvantages of final product are colour and aroma. The colour of hydrolysed lactose solution can be improved by demineralisation and decolourisation using ion exchange and filtration with active carbon. Unfortunately, this will rise the expenses for this kind of product production (Fox, 1997). Summarising all disadvantages of lactose hydrolysis by acid method shows that hydrolysis with β-galactosidase is a better choice (Üstok, 2007).

➢ **Enzymatic method**

It has been more than ten years that enzymatic hydrolysis is arousing attention (Volpato *et al.*, 2016). Enzymes have quite a few advantages, the first and the main one is that they can be used as an alternative instead of traditional chemical technology, replacing chemicals. Moreover, enzymes in their reactions are more specific than chemicals, less side reactions occur during enzymatic catalysis, and less by-products develop. As a result, a product with high quality and with minimal chemical contamination is obtained (Oort, 2010). β-Galactosidase is used for milk and whey lactose hydrolysis and this enzyme catalyses galacto-oligosaccharides formation, which are prebiotics (Grosová *et al.* 2008).

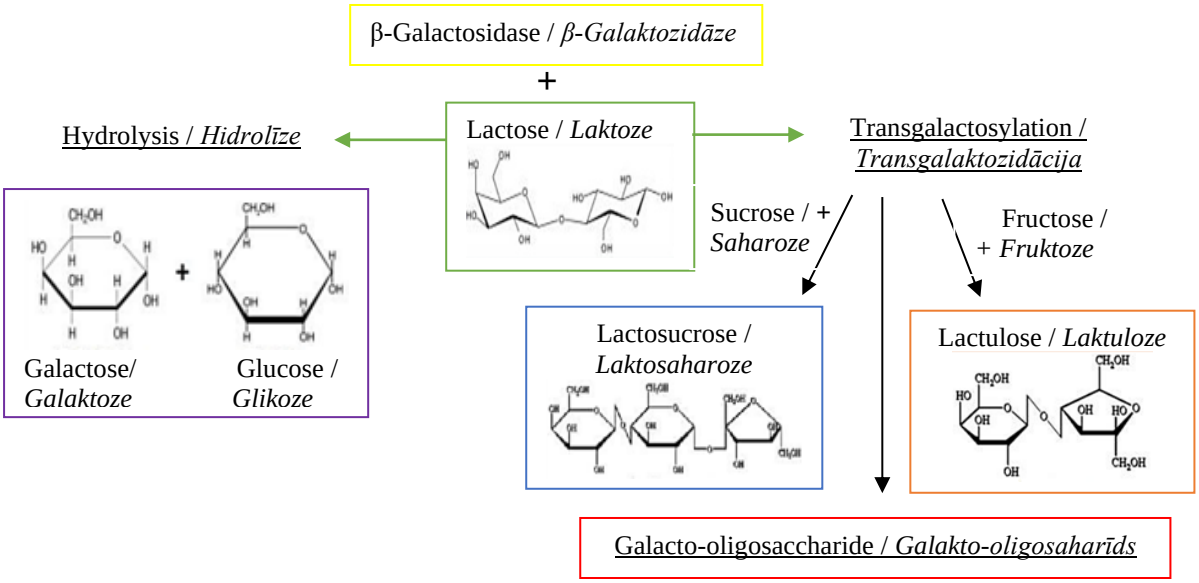


Fig. 1.8. Products of lactose hydrolysis / *1.8. att. Laktozes hidrolīzes produkti* (Reddy *et al.*, 2016)

β-Galactosidase is one of the enzymes which is used in free or immobilized form for recycling a large quantity of food (Grosová *et al.*, 2008). Milk and whey are the most common substrate for enzymatic lactose hydrolysis (Rastall, 2007). Ultrafiltration yields both whey protein and permeate, where permeate contains almost all of the initial amount of lactose from milk. Permeate can be considered as an excellent fermentation (with β-galactosidase) substrate

for obtaining glucose-galactose syrup (Illanes *et al.,* 2016). This technological process is used by Snampogetti in Italy, where lactose is reduced in milk, Corning Glass Companys in the UK, France and the USA, and Valio in Finland use whey for lactose hydrolysis (Rastall, 2007). There is a greater demand for the products with a low lactose content and they are more appreciated by consumers. Hydrolysed dairy products are valuable not only as products for a certain group of people, but also, they are viewed as an option to avoid the crystallisation of lactose in concentrated and frozen milk products (Illanes *et al.,* 2016).

The hydrolysis of lactose can be considered as a simple reaction process and there is no need for a special equipment. Therefore, if a single enzyme is used for the hydrolysis of lactose, several factors must be taken into account, such as substrate composition, operating pH, temperature and treatment time, enzyme activity and total costs. In order to reduce costs, lactose hydrolysis can occur for a short period of time at 35-45 °C, whereas with milk it usually causes a widespread microbial growth, so it is optimal to keep it overnight at 4 °C. Lactose hydrolysis will increase the sweetness of the product, which in many cases can reduce the amount of added sugar (Harju *et al.*, 2012).

Despite enzyme specification and high catalyse, its direct use in food production is limited because of the high costs. Other factors which impact enzyme usage are its solubility and low stability during production (Volpato *et al.,* 2016).

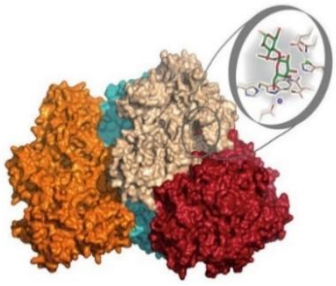


Fig. 1.9. *Kluyveromyces lactis* β-galactosidase in 3D /
1.9. att. Kluyveromyces lactis β-galaktozidāzes 3D attēls
(Plou *et al.*, 2016)

The zoomed image (see Fig. 1.9.) illustrates the mechanism of complexes showing how such substrate as lactose in green colour interacts with the enzyme in beige colour. In this substrate binding, sodium in purple colour and magnesium in green colour are also involved (Plou *et al.,* 2016). Galactose and lactose bind to the enzyme at its active site, which explains the reason of the reaction changes. In addition, glucose is less competitive because it binds to an enzyme site away from the active site and has less effect on the reaction when lactose is involved (Seok *et al.,* 2013).

Nowadays numerous manufacturers are still using free enzymes but recently they have started focusing more on β-galactosidase immobilization for enzyme recycling. Providing the necessary technique, immobilisation can improve such properties of β-galactosidase as stability of the enzyme at a low or high pH and temperature which depend on each enzyme's characteristics. Enzyme immobilisation technology prevents the possibility of low enzyme activity, thus ensuring higher lactose hydrolysis rate (Vasileva *et al.*, 2016).

β-galactosidase has been immobilized using various carriers – enzyme immobilization by glycidyl methacrylate–methylmethacrylate on magnetic poly (GMA–MMA) beads, by bioaffinity on concanavalin A-cellulose, by polyethylenimine on cotton cloth, by glutaraldehyde on chitosan particles, etc. (Bayramoglu *et al.* 2007; Klein *et al.*, 2013). In recent years, membranes such as nylon, polyvinylidene fluoride, cellulose acetate membranes, and polyether sulfone have been used to immobilise β-galactosidase. This method showed that the most suitable membranes for β-galactosidase immobilization are those which provide low protein absorption, less pollution, and concentration of polarisation (Vasileva *et al.,* 2016).

Hydrolysis reaction with a free and immobilised enzyme can be carried out in different kind of reactor configurations such as filled bottom reactor, relative reactor and membrane

reactor. The most popular reactors which are used for enzymatic hydrolysis are filled bottom and membrane reactors (Vasileva *et al.*, 2016). The ingredients of milk as lactose and soluble salts in membrane reactor easily get to the section where the enzyme is located and starts the hydrolyse reaction. Lactose has been hydrolysed without changing other milk ingredients. The technological methods of fructose production using lactose hydrolysis and whey permeate hydrolysis with immobilized β-galactosidase and glucose oxidise have been developed to obtain a product with similar sweetness as sucrose (Illanes, 2011).

1.7 β-Galactosidase in food industry / *β-Galaktozidāze pārtikas rūpniecībā*

β-Galactosidase (EK 3.2.1.23) is an enzyme catalysing glycosidic bond for lactose hydrolysis (Adalberto *et al.*, 2010). β-Galactosidase consists of four identical polypeptide chains, each chain consisting of 1023 amino acids and combining structural domains (Saqib *et al.*, 2017).

In the manufacturing process of dairy products, the enzyme has been used to obtain a product which can be consumed by people with lactose intolerance, to control product quality, to prevent lactose crystallisation storing at low temperatures, transporting the product, as well as to improve the sweetness of the product (Adalberto *et al.*, 2010).

β-Galactosidase can be obtained from plants, animals and microorganisms. There are several conditions for the enzyme that control the process and its recovery (Adalberto *et al.*, 2010). Plant and animal origin enzymes have small commercial value, while β-galactosidase from various microorganisms has great technological significance. Microorganisms offer various advantages over other available sources, such as easy handling, higher propagation speed, and high yield (Panesar *et al.*, 2010b).

It is important to note that in many cases the use of β-galactosidase is limited because the enzyme may have low activity, thermostability, and high inhibition under the required conditions in the presence of reaction by-products (Benavente *et al.*, 2015). Therefore, it is important to know as much information as possible about the β-galactosidase for industrial use. Currently, enzymes derived from *Aspergillus oryzae*, *Aspergillus niger*, *Kluyveromyces lactis* un *Kluyveromyces fragilis* are used in industry and GRAS status is guaranteed (Garcia *et al.*, 2011).

Depending on the extraction source (plant, animal, or micro-organisms), β-galactosidase may have different properties with different possible technological applications (Bosso *et al.*, 2016). β-Galactosidases from mesophilic microorganisms, predominantly from *Aspergillus* and *Kluyveromyces* strains, are most commonly used for lactose hydrolysis. Moreover, β-galactosidases from psychophilic and thermophilic microorganisms are also studied and tested because compared to enzymes from mesophilic microorganisms they are resistant to aseptic conditions. Salts are also used during hydrolysis to make the reaction more productive and beneficial. Several immobilised enzymes and reactor designs are made directly for lactose hydrolysis in milk and whey. The progress of the process and optimisation are described in several articles by Illanes *et al.*, 2000; Szczodrak, 2000; Fontes *et al.*, 2001; Hatzinikolaou *et al.*, 2005; Şener *et al.*, 2006; Mariotti *et al.*, 2008; Demirhan *et al.*, 2010; Olafadehan *et al.*, 2009, where scientists give a broader insight into the question of where more attention should be focused on and how to achieve better results (Illanes, 2016).

Besides, β-galactosidase plays an important role in human lifestyle and diet. The low level of this enzyme in the human intestinal wall of the small intestines causes lactose intolerance. Therefore, supplemental β-galactosidase preparation is available on the market. The enzyme is coated to prevent inactivation in gastric juice (pH 1.0 to 3.0) but in the intestine the enzyme release starts, as well as activation. The activity of β–galactosidase can be influenced by several factors, such as temperature, pH, gastric enzymes, and bile acids. Moreover, the strain from which β-galactosidase has been extracted may affect enzyme properties in different applications

(Žolnere *et al.,* 2018). Further in the work the characteristics of factors influencing enzyme activity will be described.

➢ **Bacteria source**

The enzyme can be obtained from numerous bacteria - for instance, *Bifidobacterium infantis* CCRC14633, *Bifidobacterium longum* CCRC15708, and *Bifidobacterium longum* CCRC15708 strains - showing high enzyme activity but *Lactobacillus* and *Bifidobacterium* species are effective probiotics and they are widely used as potential β-galactosidase source (Saqib *et al.,* 2017). It should be taken into consideration that β-galactosidases which are obtained from *Escherichia coli* serve only for experiments and research, they cannot be included in food production because there is a high possibility that the enzyme might cause toxicity problems (Panesar *et al.,* 2010b).

➢ **Yeast source**

β-Galactosidase, which is obtained from yeasts, is an intracellular enzyme (Khare & Prakash, 2017). Looking from industrial point of view yeasts are considered an important source of β-galactosidase production. The enzyme with the highest activity can be reached at a neutral pH that leads to consider that it is suitable for lactose hydrolysis in milk and has been widely recognised as safe for food production (Panesar *et al.,* 2010). In the process of hydrolysis of milk, dairy products or sweet whey lactose mostly yeasts (*Kluyveromyces lactis* and *Kluyveromyces fragilis*) β-galactosidases are used where the optimal pH medium is within the interval of 6.0 to 7.0. *Kluyveromyces lactis* is considered one of the most commercially significant sources of β-galactosidase, which is the most used in food industry (Dutra Rosolen *et al.,* 2015; Saqib *et al.,* 2017).

➢ **Fungal source**

Fungal β-galactosidase is mostly thermostable, although it is sensitive to reaction products, mainly galactose (Husain, 2010). *Aspergillus oryzae* and *Aspergillus niger* β-galactosidase has the optimal pH interval from 2.5 to 5.4, which shows that this enzyme is more suitable for lactose hydrolysis in an acidic condition for example acid whey (Dutra Rosolen *et al.,* 2015). The optimal temperature for hydrolysis reaction is 50 – 60 °C which is higher than it is for yeasts enzyme (Panesar *et al.,* 2010b).

A summary of the optimal environmental factors for β-galactosidase from different sources of origin is shown in Table 1.4.

Table 1.4. / *1.4. tabula*

Characteristics of β-galactosidase / *β-Galaktozidāzes raksturojums*

(Zolnere & Ciprovica, 2017)

Source / *Avots*	**Microorganisms / *Mikroorganismi***	**Optimal pH of the medium/ *Optimālais vides pH***	**Optimal temperature / *Optimālā temperatūra*, ◦C**
Fungi / *Pelējumi*	*Aspergillus niger*	4.0 – 4.5	55 – 60
	Aspergillus oryzae	5.0	50 – 55
	Penicillium simplicissimum	4.0 – 4.6	55 – 60
Bacteria / *Baktērijas*	*Bacillus subtilis*	8.0 – 8.5	35
	Bacillus licheniformis	6.5	50
	Bacillus circulans	6.0	60
	Bacillus stearothermophilus	7.0	70
	Escherichia coli	7.0	55
Yeasts / *Raugi*	*Kluyveromyces lactis*	7.0	40
	Kluyveromyces fragilis	6.6	37
	Kluyveromyces marxianus	7.5	40

The various microorganisms are used for enzyme production, the optimal temperature of enzymatic hydrolysis is in the range of 10 to 60 °C and pH from 4.0 to 8.5 showed in Table 1.4.

Therefore, it is essential to know what conditions in the manufacturing process must be ensured that the reaction is carried out with the greatest efficiency of hydrolysis.

- **Physical and chemical characteristics**

Enzyme activity and thermostability depend on such factors as temperature, pH, pressure, concentration of chemicals, and the presence of metal ions. All these factors can seriously impact the enzyme three-dimensional structure. The combination of factors can cause enzyme deactivation or opposite enzymes become active and provide a successful enzymatic reaction. Depending on the source of extraction (plant, animal, microorganisms) β-galactosidase has multiple properties in different technological processes. According to the research findings of Şener *et al.*, 2006, enzymes which were obtained from certain fungi have higher activity at low pH, but enzymes from yeasts are active at the neutral level of pH and medium high temperature (Bosso *et al.*, 2016; Jurado *et al.*, 2004).

- **Temperature**

Thermostable β-galactosidase is a remarkable enzyme which is capable of lactose hydrolysis at high temperatures where other microorganisms slowly develop or do not exist (Whintaker & Wong, 2003). Enzymes derived from *Thermus sp.*, *Aspergillus niger*, *Bacillus stearothermophilus* are relatively stable at 35 – 80 °C temperature. The β-galactosidase for hydrolysis can be obtained from psychrophilic microorganisms such as *Arthrobacter psychrolactophilus, Pseudoalteromonas haloplanktis* which are relatively stable within the temperature range of 0 - 25 °C (Sheik Asraf *et al.*, 2010).

- **pH**

The profile of the pH of the medium depends on several factors, such as the concentration of organic acids and mineral salts. As the pH of the medium changes, the ionization groups of the enzyme and the substrate swich places, thus affecting the rate at which the substrate attaches to the active site of the enzyme (Robinson, 2015). Depending on the source, β-galactosidase has a definite pH range in which it has the highest activity, see Table 1.4. According to this, enzymes can be divided into two groups, β-galactosidase derived from fungi, the highest activity is in acidic conditions, but neutral pH is suitable for yeasts and bacteria (Üstok, 2007).

- **Enzyme concentration**

β-Galactosidase catalyzes hydrolysis and transglycolysis reactions by changing the equilibrium of the reaction, depending on the type and concentration of the enzyme, substrate concentration, and reaction conditions (Tokošová *et al.*, 2015). The concentration of β-galactosidase significantly affects the amount of glucose and galactose, as well as the intensity of the GOS production and the amount of lactulose with fructose in the substrate (Carrazco-Escalante *et al.*, 2019; Van De Voorde *et al.*, 2014).

- **Presence of metal ions**

Regulation of enzyme activity with metal ion complexes (Ca^{2+}, Mg^{2+}, Na^{+}, NH_4^{+} and K^{+}) is one of the key factors of many catalysis reactions. The molecular mechanism of metal ions and their influence is an important aspect of the characterisation of biological macromolecules (Page & Di Cera, 2006). The effect of metal ions on enzyme activity depends mainly on the source of β-galactosidase. The Ca^{2+} ion inhibit β-galactosidase activity, but Mg^{2+} ion increase enzyme activity. It should be noted that the effect of monovalent ions NH_4^{+}, K^{+} and Na^{+} on enzymatic activity and stability may differ. The effect of these ions appears to be related to the radius of monovalent ions, so smaller ions such as Na^{+}, can enter the protein structure, causing significant changes (thermal and pH of the medium resistance, strength) in the enzyme structure that can deactivate the enzyme (Jurado *et al.*, 2004).

Table 1.5. /*1.5. tabula*

Summary of cationic effects on β-galactosidase activity /
Kopsavilkums par katjonu ietekmi uz β-galaktozidāzes aktivitāti
(Zolnere *et al.*, 2017)

β-Galactosidase source / ***β-Galaktozidāzes avots***	**Activator /** ***Aktivators***	**Inhibitor /** ***Inhibitors***
Kluyveromyces lactis	K^+; Mg^{2+}	Ca^{2+}; Na^+
Lactobacillus reuteri	K^+; Na^+; Mn^{2+}	Fe^{2+}; Ca^{2+}; Cu^{2+}
Bacillus licheniformis	Ca^{2+}; Mn^{2+}; Mg^{2+}	Cu^{2+};Zn^{2+}; Fe^{2+}
Kluyveromyces fragilis	Mn^{2+}, Mg^{2+}, K^+	Ca^{2+}
Amygdalus communis	Ca^{2+}; Mn^{2+}	K^+; Na^+

It was determined that β-galactosidase, derived from the *Kluyveromyces lactis* and *Kluyveromyces fragilis*, was activated by the presence of manganese (Mn^{2+}), potassium (K^+) and magnesium (Mg^{2+}) ions, while calcium (Ca^{2+}) ion is an inhibitor of the enzyme (Saqib *et al.*, 2017).

It has been a task for dairy facilities to try to reduce the amount of sugar in products avoiding flavour loss (McCain *et al.*, 2018). The hydrolysing milk, milk products, and whey, it is important to predict the final products and conditions to ensure that the lactose hydrolysis is completed.

➢ **β-Galactosidase on commercial scale**

In order to increase the industrial demand for β-galactosidase, cost-effective production methods are needed. The total costs of enzyme production are a major obstacle to the efficient use of any technology in the enzyme production. Fermentation conditions, in particularly the optimisation of physical and chemical parameters, are important processes which affect the economy and practicality of the process (Gouripur & Kaliwal, 2013).

Over the last decade, a considerable number of research papers have been published to analyse the activity and potential of the enzyme obtained from various sources for the hydrolysis of lactose (Erich *et al.*, 2015). Today, commercial β-galactosidase is predominantly derived from *Kluyveromyces lactis*, as yeast can produce relatively high levels of enzyme and is suitable for the milk environment (You *et al.*, 2017). It is still important to find a solution to the dairy industry, where products such as D-galactose, which is one of the substances, deactivate the enzyme during the lactose hydrolysis reaction. It is also important from an economic point of view to introduce improvements and innovations in the use of the enzyme for the hydrolysis of lactose (Erich *et al.*, 2015).

One of the three methods mentioned below is used for enzymatic hydrolysis of lactose:

1. "Single use" or "Throw away" batch system;
2. Recovery system (re-use of β-galactosidase);
3. Immobilized enzymes that are systems in which the enzyme is chemically linked to an inert matrix (Üstok, 2007).

A summary of the commercial preparations used for various studies of lactose hydrolysis is given in Table 1.6.

Table 1.6. / *1.6. tabula*

Commercial preparations of β-galactosidase /
β-Galaktozidāzes komerciālie preparāti
(Zolnere & Ciprovica, 2017)

Source / *Avots*	**Name of the preparation / *Preparāta nosaukums***	**Activity / *Aktivitāte***	**Yield / *Hidrolīzes pakāpe*, %**	**Manufacturer / *Ražotājs***
Yeast / Raugs				
Kluyveromyces sp.	Enzeco Lactase NL	NM*	95	EDC, New York, US
Kluyveromyces lactis	GODO-YNL2	5000 NLU g^{-1}	99	Danisco A/S, Denmark
	Maxilact® LX5000	5000 NLU g^{-1}	100	Sedim Cedex, France
	Maxilact-L/2000	2000 NLU g^{-1}	90	Gist-Brocades
	Lactozym 2600L	2600 LAU g^{-1}	NM*	Novozymes
	Lactomax Pure	NM*	75	Prozyn, Brazil
	Lactozym pure 6500 L	1320 U mL^{-1}	95	Novozymes
	Ha-Lactase 5200	8040 LAU g^{-1}	90	Chr. Hansen, Denmark
Kluyveromyces fragilis	Lactozym 3000L HPG	3000 LAU mL^{-1}	72	Novo Nordisk
Fungal / *Pelējumi*				
Aspergillus oryzae	Lactomax F30	NM*	50	Prozyn, Brazil
	Bio-Cat	5000 NLU g^{-1}	41	INC/USA
Bacteria / *Baktērijas*				
Bacillus circulans	Biolactase NTL	553 U mL^{-1}	50	Biocon, Spain

*NM – not mentioned / *nav minēts*

The advantage of the immobilized enzyme is easier recovery of the enzyme, its reuse, and its continuous function (Panesar *et al.*, 2010a). The enzyme batch system method is easier to use for a new product development, but the second method is better suited for large-scale production. The choice of the method mainly depends on several factors - product pH, max temperature and reaction time, enzyme activity, substrate and cost (Zadow, 1992).

There are also commercially available β-galactosidase enzymes that contribute to lactose digestion eaten with food. Many people may consume yogurt even though they are lactose intolerant because the yogurt contains an "active culture" that contains live lactic acid bacteria that produce β-galactosidase (Khare & Prakash, 2017).

Despite the fact that commercial β-galactosidase preparations are grouped according to the source of origin, there is another aspect that is no less important - the degree of purity. This feature is more relevant to the hydrolysis process when milk is used as a substrate. Pure enzyme preparation should be used for milk processing, since there is a risk that the preparation may contain proteases and milk coagulation may occur during the reaction (Ranken & Kill, 1993). The market for immobilized enzymes in 2014 amounted to a total of about $ 3.2 billion. Market leaders were Novozymes (Denmark), with a share of 48%, followed by Danisco (Denmark) (21%), DSM (The Netherlands) (6%), AB Enzymes (Finland) (5%) and BASF (Germany) (4%) (Spohner *et al.*, 2016).

Many research efforts focus on a method of reducing or completely eliminating lactose in dairy products. That is why more research into the development of lactose hydrolysed products is required because it is important to know how the physical and chemical properties of the product change (Üstok, 2007).

Summary / *Kopsavilkums*

The hydrolysis of lactose in whey is evaluated as a great perspective because hydrolysed products are sweeter and can be used as additives for human and animal consumption. In the last two decades, this subject has motivated scientists and manufacturers to do research and analyse the benefits of the final product. Lactose hydrolysis gives an opportunity to improve the solubility of milk and its products, as well as their other properties, such as texture and flavour.

One of the ways to use acid and sweet whey is to produce glucose-galactose syrup using an enzymatic method for lactose hydrolysis. Glucose - galactose syrup is a viscousus sugar solution consisting of about 30% water, 34% glucose and 34% galactose, 11% lactose and 1% salts.

The enzymatic method for the production of glucose-galactose syrup is highly selective, however, it is limited by excessively high enzyme costs. It has been known for a long time that lactose can be hydrolysed into glucose and galactose using a strong mineral acid, followed by pure acid and ion exchange resin. There are a number of effective methods available for glucose-galactose syrup and other lactose hydrolysed products production, but research is still ongoing and will undoubtedly lead to improved and more cost-effective methods. The aim of the research was to improve the lactose hydrolysis process for obtaining glucose-galactose and oligosaccharide syrups.

Laktozes hidrolīze monosaharīdos ir lieliska alternatīva cukuram, jo tās hidrolīzes produkti ir saldāki, tos var izmantot uzturā un dzīvnieku barībā. Pēdējās desmitgadēs šī tēma ir motivējusi zinātniekus un ražotājus veikt pētījumus un vispusīgi analizēt šī produkta priekšrocības. Laktozes hidrolīze rada iespēju uzlabot arī citas produktu īpašības, tostarp šķīdību.

Viens no veidiem, kā izmantot biezpiena un siera sūkalas, ir glikozes-galaktozes sīrupa ražošana, izmantojot fermentatīvo metodi. Glikozes - galaktozes sīrups ir biezs šķīdums, kas sastāv no 30% ūdens, 34% glikozes un 34% galaktozes, 11% laktozes un 1% minerālvielām.

Glikozes-galaktozes sīrupa ieguve ar fermentatīvo metodi ir selektīva, to ierobežo pārāk augstās enzīmu izmaksas. Jau ilgu laiku ir zināms, ka laktozi var hidrolizēt glikozē un galaktozē, izmantojot minerālskābes. Ir pieejamas vairākas metodes glikozes-galaktozes sīrupa un citu hidrolizētu laktozes produktu iegūšanai, taču eksperimenti joprojām turpinās ar mērķi pilnveidot ieguves metodes un samazināt izmaksas. Pētījuma mērķis ir pilnveidot laktozes hidrolīzes procesu glikozes-galaktozes un oligosaharīdu sīrupa ieguvei.

2. MATERIALS AND METHODS/ *MATERIĀLI UN METODES*

2.1 Time and location of the research / *Pētījuma laiks un vieta*

Experimental work was conducted during the time period from 2016 to 2021. Analyses and research were performed at the following institutions:

- Faculty of Food Technology, Latvia University of Life Sciences and Technologies;
- Institute of Microbiology and Biotechnology, Faculty of Chemistry, Institute of Solid State Physics at the University of Latvia;
- Dairy Innovation Institute, California Polytechnic State University;
- J.S. Hamilton Baltic Ltd. Laboratory.

2.2 Description of materials/ *Materiālu raksturojums*

Sweet and acid whey permeates which were used for the experiments were kindly donated by local dairy manufacturers SC "Smiltenes piens" and SC "Tukuma piens". Table 2.1 represents the composition and pH of the permeates.

Table 2.1. / *2.1. tabula*

Composition of sweet and acid whey permeates and pH /
Biezpiena un siera sūkalu ultrafiltrāta sastāvs un pH

Permeate / *Ultrafiltrāts*	Fat, % / *Tauki*, %	Proteins, % *Olbaltumvielas*, %	Lactose, % / *Laktoze*, %	Total solids, % / *Sausna*, %	pH
Sweet whey / *Siera sūkalu*	0	0.2±0.1	3.8±0.1	4,6±0.1	6.1±0.1
Acid whey / *Biezpiena sūkalu*	0	0.5±0.1	4.2±0.2	5.2±0.2	4.6±0.1

At the beginning of the experiments, the composition of permeates was analysed by MilkoScan^{TM} Mars (Foss Analytical, Denmark) and pH by pH-meter (ISO 5546:2010) inoLab pH 7110 (WTW, Germany).

Commercial β-galactosidases in Table 2.2 were used for lactose hydrolysis.

Table 2.2. / *2.2. tabula*

Commercial β-galactosidase enzymes used in the study /
Pētījumā lietotie komerciālie β-galaktozidāzes enzīmi

Enzyme / *Enzīms*	NOLA^{TM}Fit5500	Ha-Lactase 5200	GODO-YNL2
Activity / *Aktivitāte*	5500 BLU g^{-1}	5200 NLU g^{-1}	5000 NLU g^{-1}
Source / *Avots*	*Bacillus licheniformis*	*Kluyveromyces lactis*	*Kluyveromyces lactis*
Supplier / *Piegādātājs*	Chr.HANSEN Denmark / *Dānija*	Chr.HANSEN Denmark / *Dānija*	Danisco Denmark / *Dānija*
Optimal pH / *Optimālais pH*	5.4 – 7.0	6.5 – 8.0	7.5 – 8.0
Optimal temperature / *Optimālā temperatūra*, °C	35 – 50	35 – 45	40 – 45
Reference / *Literatūras avots*	NOLA™Fit5500 Product Information	Ha-Lactase 5200 Product Information	GODO-YNL2 Product Information

Immobilized glucose isomerase was isolated from *Streptomyces murinus* and purchased from Sigma–Aldrich (Germany). The enzyme has a cylindrical shape, with an approximate 0.6–0.8 mm diameter and 1.4–1.8 mm length. According to the manufacturer data, the specific dry

activity of glucose isomerase was more than 350 U g^{-1}, optimal media pH 7.5 and temperature 70 °C (Gaily *et al.*, 2010; Glucose Isomerase Product Information).

Fructose from Sigma-Aldrich (Germany).

Glucose – galactose syrup SC "Smiltenes piens".

During the research, the enzymes and products were stored at a temperature of + 4 °C to keep the high activity and quality.

2.3 The structure of the research / *Pētījuma struktūra*

The research work is structured in five stages, illustrated in Table 2.3, where each stage contributes to increasing the sweetness of syrup. All stages are further divided into numerous steps that are discussed in a more detailed way.

Table 2.3. / *2.3. tabula*

The stages of the research / *Pētījuma posmi*

Posms/Stage	Description / *Raksturojums*
Stage I / *I posms*	Research on the stability of commercial β-galactosidases / *Komerciālo β-galaktozidāžu stabilitātes izpēte*
Stage II / *II posms*	The study of physical properties of lactose in whey permeates / *Laktozes fizikālo īpašību izpēte ultrafiltrātos*
Stage III / *III posms*	Dynamics of the hydrolysis of lactose in the various permeates solid concentrations / *Laktozes hidrolīzes dinamika dažādā ultrafiltrāta sausnas saturā*
Stage IV / *IV posms*	The study of glucose-galactose syrup sweetness in two-stage fermentation / *Glikozes-galaktozes sīrupa salduma paaugstināšanas izpēte divpakāpju fermentācijā*
Stage V / *V posms*	The sensory evaluation of syrups / *Sīrupu sensorā novērtēšana*

2.4 Stage I of research / *Pirmais pētījuma posms*

Physical and chemical properties of commercial β-galactosidases were analysed in a more detailed way:

- the effect of metal ions (calcium, sodium, potassium and magnesium) on enzyme activity;
- study of lactose hydrolysis in gastrointestinal model environment;
- evaluation of a rapid method for the determination of enzyme activity and lactose hydrolysis level.

There are listed experimental steps of the particular stage for a comprehensive analysis of the commercial β-galactosidases. The pros and cons of the selected enzymes were studied.

2.4.1 Materials / *Materiāli*

The materials used in Stage I are listed in Table 2.4. and the equipment in Table 2.5.

Table 2.4. / *2.4. tabula*

List of materials and chemicals used in the study/
Pētījumā izmantoto materiālu un ķīmisko vielu saraksts

Material or chemical / *Materiāls vai ķimikālija*	**Brand / *Ražotājs***	**Country / *Valsts***
$MgCl_2$ (Magnesium chloride / *Magnija hlorīds*)	Sigma	Germany / *Vācija*
$CaCl_2$ (Calcium chloride / *Kalcija hlorīds*)		
KCl (Potassium chloride / *Kālija hlorīds*)		
NaCl (Sodium chloride / *Nātrija hlorīds*)		
KH_2PO_4 (Monopotassium phosphate / *Monokālija fosfāts*)		
$NaHCO_3$ (Sodium bicarbonate / *Nātrija hidrogēnkarbonāts*)		
$MgCl_2(H_2O)_6$ (Magnesium chloride hexahydrate / *Magnija hlorīda heksahidrāts*)		
$(NH_4)_2CO_3$ (Ammonium carbonate / *Amonija karbonāts*)		
NaOH (Sodium hydroxide / *Nātrija hidroksīds*)		
HCl (Hydrochloric acid / *Sālsskābe*)		
Bile salt / *Žults sāļi*		
Porcine pepsin / *Cūkas pepsīns* (EC 3.4.23.1)		
Porcine trypsin / *Cūkas tripsīns* (EC 3.4.21.4)		
Bovine chymotrypsin/ *Liellopu himotripsīns* (EC 3.4.21.1)		
Porcine pancreatic α-amylase / *Cūku aizkuņģa dziedzera α-amilāze* (EC 3.2.1.1)		
Porcine pancreatic lipase / *Cūku aizkuņģa dziedzera lipāze* (EC 3.1.1.3)		
α-Lactose monohydrate / *α-laktozes monohidrāts*		
D-Glucose/*D-Glikoze* (≥98%)		
D-Galactose / *D-Galaktoze* (≥98%)		
o-Nitrophenol / *o-Nitrofenols* (o-NP)		
2-Nitrophenyl-galactoside / *2-Nitrofenil-galaktozīds* (o-NPG)		
Tris-HCl (Tris hydrochloride / *Tris hidrohlorīds*)		
96-well plate / *96-lauciņu mikroplate*		
Column / *Kolonna* SUPELCOSILTM LC-NH2, (250 mm × 4.6 mm × 5 µm)		
Acetonitrile / *Acetonitrils* (≥99.93%)		
1.5 mL eppendorf tubes/ *1.5 ml Ependorfa mēģene*		
1.5 mL vial / *1.5 mL pudelīte*		
8-channel pipette / *8 kanālu pipete*		
Test strip / *Testa plāksnītes*	GLUCOSENSE	Poland / *Polija*

Table 2.5. / *2.5. tabula*

List of equipment used in the study / *Pētījumā izmantoto iekārtu saraksts*

Equipment / *Iekārta*	**Brand / *Ražotājs***	**Country / *Valsts***
Multimode plate reader/ *Multifunkcionāls plašu lasītājs*	Infinite 200 M Pro	Switzerland / *Šveice*
pH meter / *pH metrs*	720 pH meter	The Netherlands / *Nīderlande*
HPLC /*AEŠH*	Shimadzu LC-20	USA /*ASV*
Glucometer / *Glikometrs*	GLUCOSENSE pro	Poland / *Polija*
GIT system / *GIT sistēma*	Labfors 5	Switzerland / *Šveice*
Cryoscope / *Krioskops*	CryoStar 1	Germany / *Vācija*
Digital Refractomer / *Digitālais refraktometrs*	KR ÜSS GmbH	Germany / *Vācija*

2.4.2 Assessment of β-galactosidase kinetics / *β-Galaktozidāzes kinētikas izvērtēšana*

Kinetics measurements of commercial enzymes were necessary in order to evaluate at which dilution each enzyme will be the most suitable to use.

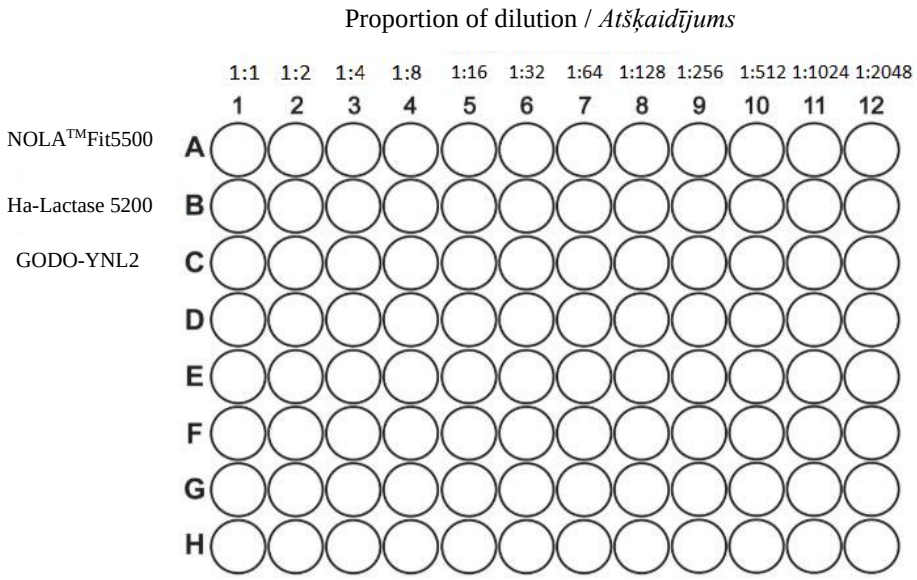


Fig. 2.1. Enzyme preparation for activity measurement in 96-well plate /
2.1. att. Enzīmu sagatavošana aktivitātes noteikšanai 96 lauciņu mikroplatē

Enzymatic reactions were measured using a 96-well plate in a multimode plate reader, and the total reaction volume was 200 µL per well. In the beginning, enzyme samples with different dilution factor in several 1.5 mL eppendorf tubes were prepared. Dilution proportions can be seen in Figure 2.1., the enzymes were diluted using 0.1 M Tris-HCl buffer, pH 6.6. After 180 µL of enzyme samples were injected in wells, the plate was transferred into the reader. Automatically 20 µL of 20 mM 2-nitrophenyl-galactoside (o-NPG) was added before taking the measurement. The data were acquired every 3 seconds for 20 minutes in the spectrophotometer at 410 nm (Xu *et al.*, 2020).

The initial velocity (V_0) of reaction was calculated by equation:

$$V_0 = \frac{A_1 - A_0}{t_1 - t_0}, \text{ where:} \quad (1)$$

A_1 – the absorbance of sample or standard during reaction, at 410 nm;

A_0 – the initial absorbance of sample or standard before reaction, at 410 nm;

t_1 – the time of sample or standard when absorbance was measured, min;

t_0 – 0 min (Harris & Keshwani, 2009).

The enzymatic activity of β-galactosidase is measured by the production of o-nitrophenol (o-NP). The amount of o-NP in all samples was calculated using a calibration plot. Calibration curve was set up at 410 nm by using pure o-NP solution in the range of 0 to 3 mM.

Lineweaver-Burk plot was constructed to determine kinetic parameters (Lineweaver & Burk, 1934). Three independent experiments were averaged, each experiment included three replicates.

2.4.3 The impact of salts on β-galactosidase activity / *Sāļu ietekmes izpēte uz β-galaktozidāzes aktivitāti*

Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$) were used for the study.

For the preparation of salt stock solutions eppendorf tubes were used where each solution was prepared in a molar concentration of 1 M and for the determination of enzyme activity stock solutions were used in concentration range of 0 – 50 mM. As a substrate 20 mM o-NPG was prepared and used at the interval of 0 – 3 mM.

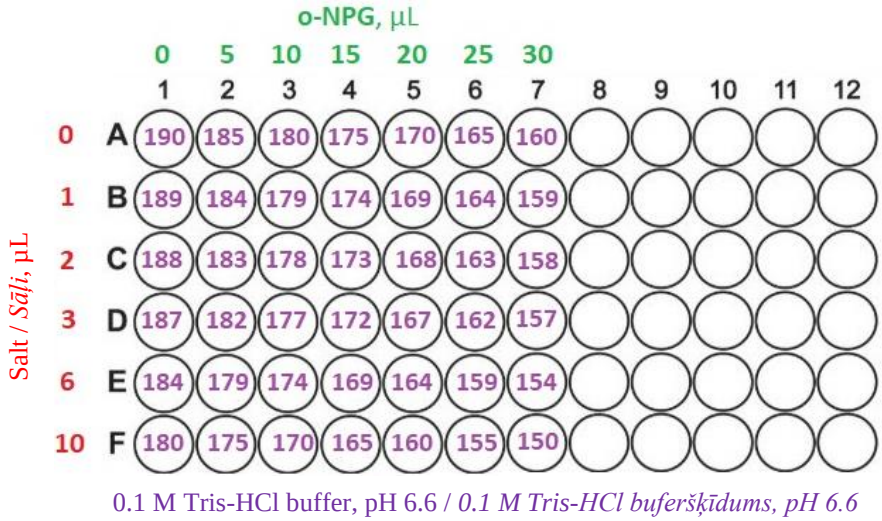


Fig. 2.2. Scheme of salt effects / *2.2. att. Vispārīga sāļu ietekmes shēma*

Fig 2.2. illustrates the volumes of several chemical solutions which are marked differently for easier understanding. The o-NPG volume in microlitres which is located above well column numbers are marked in green colour and points out how much of the substrate needs to be injected into particular column wells. In the same way the information about salts ($MgCl_2$, $CaCl_2$, KCl, NaCl, KH_2PO_4) is shown in red colour, regarding the volume to be injected in the wells of each row letter (A, B, C etc.). In purple colour the volume of Tris-HCl buffer is marked in each well. Following the described instruction, the total volume of one well is 190 µL. When all wells are filled up, the plate is placed into the reader. Absorption was measured at 410 nm and data were acquired every 3 seconds for 20 minutes. Before starting the measurement, 10 µL of enzyme was injected manually into all wells using an 8-channel pipette (Hamed *et al.*, 2020).

The concentration (C) of produced o-NP was calculated based on the Beer-Lambert Law:

$$A = C \cdot \varepsilon \cdot l, \text{ where:} \quad (2)$$

A – Absorbance;

ε – molar extinction coefficient (1.36 mM^{-1} cm^{-1});

l – pathlength of the well (0.5 cm).

Five independent experiments were averaged, each experiment included three replicates (Baltierra-Trejo *et al.*, 2015).

2.4.4 Determination of macroelements and phosphate / *Makroelementu un fosfātu noteikšana*

Determination of macroelements was done in laboratory of Hamilton Baltic Ltd. by atomic absorption spectrophotometry and phosphate by spectrophotometry.

2.4.5 Determination of lactose, glucose and galactose / *Laktozes, glikozes un galaktozes noteikšana*

The sample of study was transferred into 1.5 mL eppendorf tubes and centrifuged for 5 min at 10 000 rpm. Each sample was placed into the sampler vial and sealed for HPLC analysis. HPLC was used to determine lactose hydrolysis to glucose and galactose at the time range of 0 to 15 minutes. Detector: refractive index RID-10A; Column: Alltech NH2, 4.6 mm x 250 mm, 5µm; temperature: 30 °C; mobile phase: A – acetonitrile 85%, B – deionized water 15%; injection volume of the sample: 10 µL; isocratic elution regime; total time of the analysis: 15 minutes; rate of flow: 1.0 mL min^{-1} (Žolnere *et al.*, 2018). The calibration for HPLC was done using α-lactose monohydrate, D-glucose (>98%) and D-galactose (>98%) of concentration range 0.5 to 100 g/L. The equation was used to calculate the mass concentration of lactose ρ_{lac} (g L^{-1}) in the sample:

$$\rho_{lac} = \frac{M_{lac} \times \rho_{lac\,m}}{M_{lac\,m}}, \text{ where:} \quad (3)$$

M_{lac} – molecular weight of lactose (342.3 g mol^{-1});

$M_{lac\,m}$ – molecular weight of α-lactose monohydrate (360.3 g mol^{-1});

$\rho_{lac\ m}$ – mass concentration of α-lactose monohydrate (g L^{-1}) from HPLC analyse (Srivastava *et al.*, 2014).

Analyses were carried out in triplicate.

2.4.6 Costs of one assay for determination of β-galactosidase activity / *Vienas analīzes izmaksas β-galaktozidāzes aktivitātes noteikšanai*

The costs calculation was made based on the chemicals and consumables purchased for the research from local distributors. The list of materials is provided in Appendix 1, where the initial price of the material is shown in column A and the price of one test in column B.

2.4.7 Glucose strip test / *Glikozes noteikšana ar glikozimetru*

Lactose solution was prepared by dissolving lactose monohydrate in 0.1 M Tris-HCl buffer, pH 6.6 to the final concentration 5% (50 mg mL^{-1}). From lactose solution 180 µL were taken out and filled in separate eppendorf tubes, then 20 µL GODO-YNL2 diluted at ratio 1:8, and NOLA™Fit5500 diluted at ratio 1:16 were added. Those particular enzyme ratios were chosen based on the results obtained in Section 2.4.2., where enzymes showed the highest activity. Eppendorf tubes were vortexed for 5 sec and the measurement was taken with glucose meter after 0, 1, 2, 4 and 8 minutes (Pandalaneni & Amamcharla, 2018).

Glucose fermentation test system was used and for test strip 1.2 µL volume of sample for injection was used. The glucose concentration was confirmed using the HPLC method. Each analyse was carried out in triplicate.

2.4.8 Analytical methods for the determination of β-galactosidase activity / *Analītiskās metodes β-galaktozidāzes aktivitātes noteikšanai*

The activity of β-galactosidase was assayed with several methods:

- **Glucose strip test** (see 2.4.7),

The β-galactosidase activity (E) U g^{-1} was calculated by equation:

$$E = \frac{\rho_{sug} \times 1000}{t \times V \times M_{sug}}, \text{ where:} \quad (4)$$

ρ_{sug} – mass concentration of α-lactose monohydrate (g L^{-1}) from glucometer test;

t – time, min;

V – enzyme volume, mL;

M_{sug} – molecular weight of sugar, g mol^{-1} (Abd-Elhalem *et al.*, 2015).

- **HPLC method** (see 2.4.5), enzyme activity U g^{-1} calculation was done based on equation (4);
- **Spectrophotometric method** (see 2.4.2)

The β-galactosidase activity (E) U g^{-1} was calculated by equation:

$$E = \frac{((A_1 - A_0) \times V \times f)/(a \times i \times W)}{1.30}, \text{ where:} \quad (5)$$

A_1 – the absorbance of sample or standard during reaction, at 410 nm;

A_0 – the initial absorbance of sample or standard before reaction, at 410 nm;

V – total volume of sample, mL;

f – total dilution factor of test solution;

a – absorptivity;

i – incubation time, min;

W – weight of test portion, g (AOAC 998.04, 2000).

Each analyse was carried out in triplicate.

2.4.9 Cryoscopy method / *Krioskopijas metode*

The physical and chemical properties of permeate were tested with the cryoscopy method. The permeate sample was filled till the mark of the test tube, which is approximately 2 ml, and placed in a cryoscope reader (ISO 5764:2009).

First experiment: Sweet whey permeate was firstly concentrated till 24% and immediately the sample solids was measured by refractometer, then 2 mL of permeate was taken to detect the freezing point and 1 mL to HPLC for lactose determination (lactose concentration was calculated by equation (3)). The concentration of permeate was then gradually reduced five times with distilled water, and after each change the properties of the sample were determined. Each analyse was carried out in triplicate.

Second experiment: 500 mL of sweet whey permeate was concentrated till 20% and prepared for hydrolysis with Ha-Lactase 5200, hydrolyse procedure is described in Section 2.6.3. Before the hydrolysis, 2 ml of the permeate sample was taken for the initial measurement of the freezing point and the freezing temperature was measured every 30 minutes of the experiment. Each analyse was carried out in duplicate.

2.4.10 The study of of the comercial β- galactosidase stability in gastrointestinal model *in vitro* / *Komerciālās β-galaktozidāzes stabilitātes izpēte kuņģa-zarnu trakta modeļvide in vitro*

The modified method of Minekus *et al.* (2014) was used in order to determine enzyme activity. Concentration of simulated gastric (SGF) and intestinal (SIF) fluid, as well as gastrointestinal enzyme activity (U mL^{-1} digesta) were calculated according to Minekus *et al.* (2014).

In all experiments, the enzyme concentrations were 1 and 5 mL L^{-1}, calculated as 5,000 and 25,000 NLU mL^{-1} for GY, 5,200 and 26,000 NLU mL^{-1} for HA and 5,500 and 27,500 BLU mL^{-1} for NF enzyme.

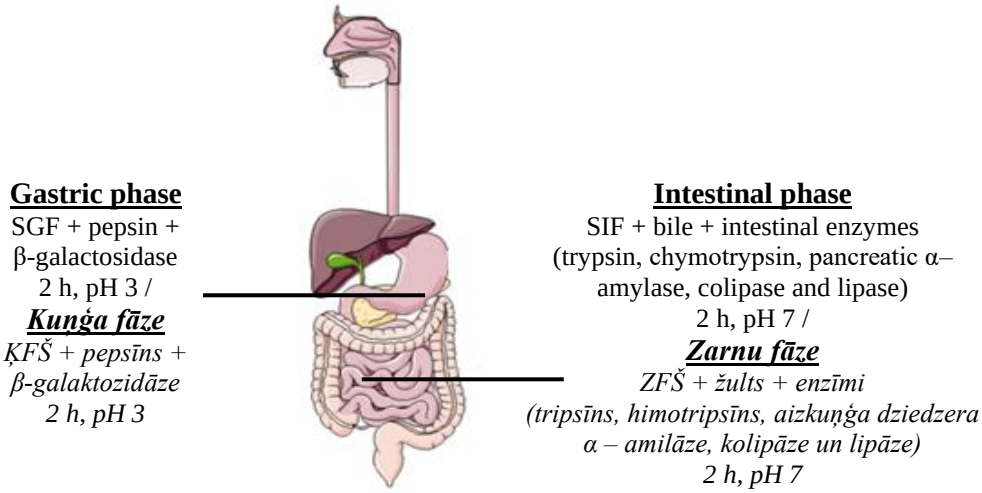


Fig. 2.3. Scheme of a simulated *in vitro* digestion /
2.3. att. In vitro fermentēšanas shēma
(Minekus *et al.*, 2014; Vertzoni *et al.*, 2019)

Gastric phase

β-Galactosidase samples were mixed with 120 mL of SGF, which consists of 120 mL water, pepsin 0.96 g, and 1 M HCl which was added to reduce the pH to 3.0. Solution was incubated at 37°C for 2 hours.

Intestinal phase

After gastric digestion, 160 mL of SIF, intestinal enzymes and bile salt were added. 1 M NaOH was used to increase the pH to 7.0 and the solution was incubated at 37°C for 2 hours.

Digested galactosidase activity measurement

The 1 ml of digested samples was taken after the intestinal phase and added to the 9 ml of 5% (50 mg mL^{-1}) α-lactose monohydrate solution to measure the yield of hydrolysis (Zárate *et al.*, 2000).

The degree of lactose hydrolysis H (%) was calculated by the following equation:

$$H(\%) = \frac{L_0 - L_1}{L_0} * 100, \text{ where:} \quad (6)$$

L_0 – initial lactose amount, g L^{-1};

L_1 – amount of lactose after hydrolysis, g L^{-1} (Vasileva *et al.*, 2016).

After hydrolysis samples were heated till 90 – 95 °C to inactivate the enzymes, and then cooled to 4 – 6 °C to analyse the amount of sugars by HPLC. Lactose amount was calculated based on equation (3). The experiments were performed with three replicates for each test.

2.5 Stage II of research / *Otrais pētījuma posms*

Researchers (Luzzi *et al.*, 2020; Pandalaneni & Amamcharla, 2018; Rico-Rodríguez *et al.*, 2020) had been used permeate and/or pure lactose powder to hydrolyse lactose. Thus, it is meaningful to study the physical state of lactose crystals, their stability and behaviour in spray – dried powders, comparing with pure α-lactose monohydrate as a control.

Main tasks for the second stage:

- crystallographic properties analyse;
- thermal properties analyse;
- lactose morphology determination.

Particular methods have been selected from scientific papers to be sure that the chosen methods are appropriate for the investigation of the effect of permeate composition on the physical state of lactose crystals, their glass transition properties and behaviour.

2.5.1 Materials / *Materiāli*

The materials used are listed in Table 2.6. and the equipment in Table 2.7.

Table 2.6. / *2.6. tabula*

List of materials and chemicals used in the study/
Pētījumā izmantoto materiālu un ķīmisko vielu saraksts

Material or chemical / ***Materiāls vai ķimikālija***	**Brand /** ***Ražotājs***	**Country /** ***Valsts***
Polymer membrane filter / *Polimēra membrānfiltrs, 92 mm*	Sterlitech	USA / *ASV*
α-Lactose monohydrate / *α-Laktozes monohidrāts*	Sigma	Germany / *Vācija*
Aluminium pan / *Alumīnija trauks*	MettlerToledo	Switzerland / *Šveice*
50 mL test tube / *50 mL mēģene*	Sarstedt	Germany / *Vācija*

Table 2.7. / *2.7. tabula*

List of equipment used in the study / ***Pētījumā izmantoto iekārtu saraksts***

Equipment / ***Iekārta***	**Brand /** ***Ražotājs***	**Country /** ***Valsts***
Cross–flow membrane / *Caurplūdes membrānas*	Armfield FT17	United Kingdom / *Apvienotā Karaliste*
Spiral membrane / *Spirālveida membrāna*	GEA	Germany / *Vācija*
MilkoScan™ Mars analyser / *MilkoScanTM Mars analizators*	Foss Analytical	Denmark / *Dānija*

Continuation of Table 2.7. / *2.7. tabulas turpinājums*

Equipment / *Iekārta*	**Brand / *Ražotājs***	**Country / *Valsts***
BÜCHI mini B–290 spray–drier / *BÜCHI mini B–290 izsmidzināšanas kalte*	Labortechnik AG	Switzerland / *Šveice*
Polarimeter / *Polarimetrs*	Polax–2L	USA / *ASV*
Diffractometer / *Difraktometrs*	D8 Advance	Germany / *Vācija*
Thermogravimetric Analyser / Differential Scanning Calorimeter (TGA/DSC)/ *Termogravimetriskais analizators / Diferenciālais skenējošais kalorimetrs (TGA/DSK)*	Mettler Toledo	Switzerland / *Šveice*
Scanning electron microscope / *Skenējošais elektronmikroskops*	Tescan Lyra	Czech Republic / *Čehija*
Coater / *Pārklājējs*	Quorum Q150R	United Kingdom / *Apvienotā Karaliste*
Diffractometer / *Difraktometrs*	Bruker AXS D8 Advance	Germany / *Vācija*

2.5.2 Preparation of permeate / *Ultrafiltrāta iegūšana*

Sweet whey was treated using cross–flow membrane filtration and polymer membrane (92 mm) filter GKSP with molecular weight cut-off of 3 kDa, the process was operated at a temperature of 4±2°C and pressure 2.8±0.2 MPa. Acid whey was ultrafiltrated using ultrafiltration equipment with spiral membranes (molecular size cut-off 10^{-1} to 10^{-2} µm) and the process was operated at a temperature of 4±2°C and pressure 1±0.2 MPa. Permeates were stored in the fridge at 4±2°C not longer than 24 h.

Sweet and acid whey permeates were analysed by MilkoScan™ Mars for lactose, proteins, fats, and total solids determination and also the pH by pH-meter (Pulinas *et al.*, 2017). Analyse was carried out in triplicate.

2.5.3 Production of dehydrated lactose and permeates / *Dehidratētās laktozes un ultrafiltrātu ieguve*

Spray–drier was used to transform sweet and acid whey permeate as well as 5% (50 g L^{-1}) aqueous α-lactose monohydrate solutions into powder form for research. To objectively analyse the impact of permeate origin on lactose physical and chemical parameters under the same conditions, dehydrated 5% aqueous α-lactose monohydrate solution was compared with spray–dried permeate powders.

The spray–dryer was used with the following conditions: aspirator rate 100%, the flow rate of the feed solution 40–50 mL min^{-1}, an inlet air temperature 170°C and an outlet temperature 115–120°C. The collected powders were immediately placed in 50 mL tubes, cooled down and stored in a desiccator at 0–3% relative humidity at room temperature till further analysis (Chandrapala & Vasiljevic, 2017; Islam & Langrish, 2010).

2.5.4 Optical rotation measurement / *Optiskās rotācijas noteikšana*

Polarimeter was used for measurement of lactose optical rotation. Each sample was dissolved in deionized water at a concentration of 1% and placed into 100 mm cuvettes. To calculate specific optical rotation [α]D, the following equation was used:

$$[\alpha]D = \frac{\alpha}{I \cdot C}, \text{ where:} \quad (7)$$

I – length of cuvette, dm;

α – measured optical rotation;

C – the sample concentration in g 100 mL^{-1} (Chandrapala & Vasiljevic, 2017). Measurement was carried out in triplicate.

2.5.5 X-ray diffraction analysis / *Rentgenstarojuma difrakcijas analīze*

Structural characterisation of samples was carried out using diffractometer with CuKα1 radiation at λ = 1.5418 Å and a position sensitive detector (PSD). The tube was operated at 40 kV voltage and current 40 mA, the scan range of 5°–60° with a rate of 5° per min was used according to Wu *et al.* (2014) method. The database ICDD PDF2 was used for pattern analysis.

2.5.6 Differential scanning calorimetry and thermogravimetric analyses / *Diferenciālās skenēšanas kalorimetrijas un termogravimetriskās metodes*

The thermogravimetric analyses (TGA) were performed with the STARe System Software. The instrument analyses the thermal transition and thermogravimetry of the sample. Sample (5–10 mg) was weighed into an aluminium pan and heated at the temperature range 30 to 300°C within a heating rate of 10°C min^{-1}. An empty aluminium pan was used as a reference in every test (Badal Tejedor *et al.*, 2018; Veldre *et al.*, 2011).

2.5.7 Scanning Electron Microscopy / *Skenējošā elektronmikroskopija*

The morphology of the lactose crystal samples was examined using scanning electron microscopy SEM-FIB with an accelerating voltage of 12 kV. Each sample was placed onto a carbon tape on an aluminium sample disc and a compressed gas was used to remove unfixed powder particles. Lactose samples were coated with a 27 nm gold layer using coater at 25 mA for 45 sec (Kougoulos *et al.*, 2010).

2.6 Stage III of research / *Trešais pētījuma posms*

The first and second stages were designed to evaluate and analyse research object, these results contain significant information used in stage III activities.

In this stage, each hydrolyses parameter such as, medium pH, solids, enzyme units was carefully analysed and evaluated to modulate the most effective factors for glucose-galactose syrup production with the highest sweetness (see Figure 2.4.).

Permeates were hydrolysed with a method which is practised on a commercial scale for glucose – galactose syrup production.

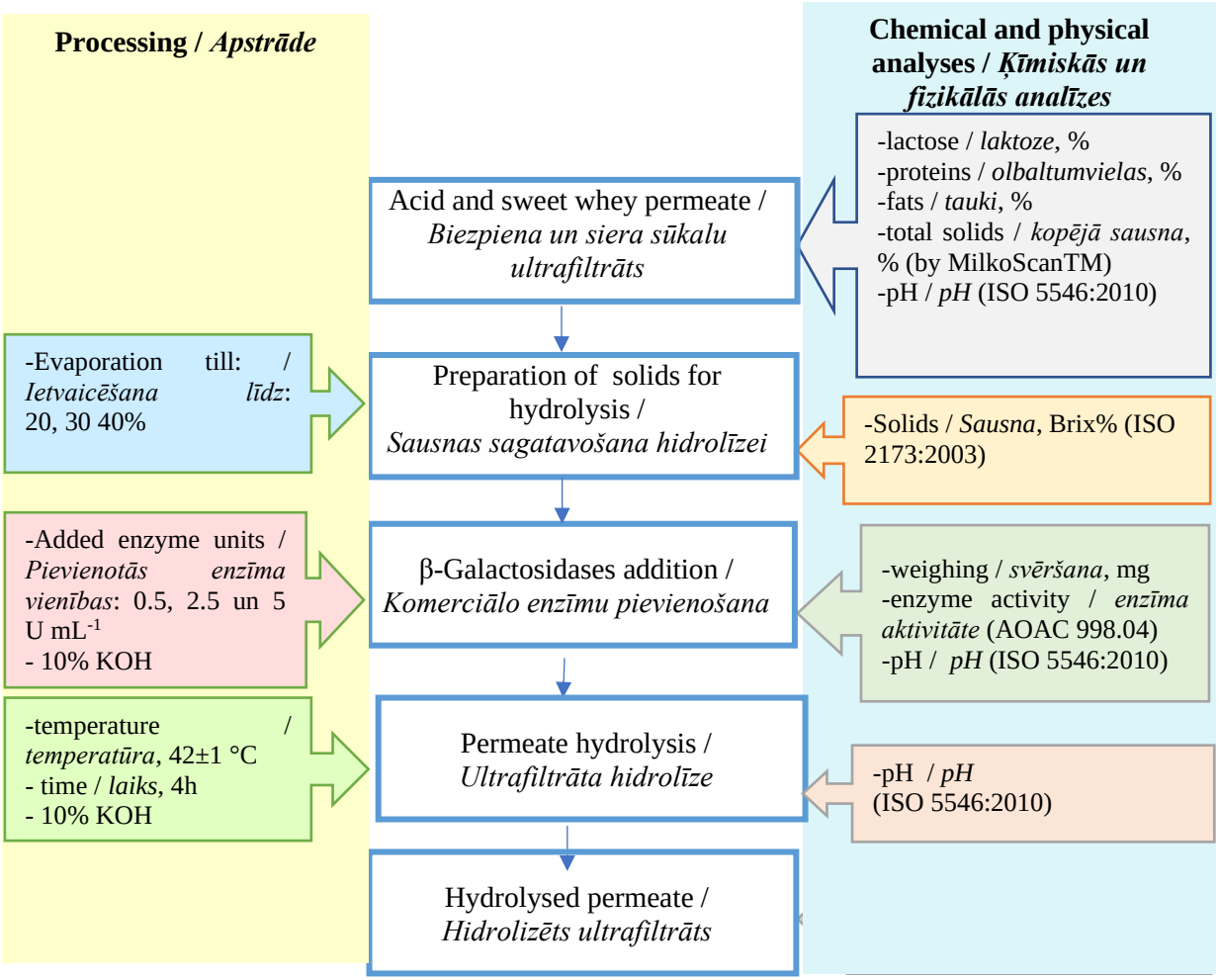


Fig. 2.4. Schema of acid and sweet whey permeate hydrolysis /
2.4. att. Biezpiena un siera sūkalu ultrafiltrāta hidrolīzes shēma

Several substrate solid concentrations 20, 30 and 40%, as well as commercial enzyme unit amounts 0.5, 2.5 and 5 U mL^{-1} were chosen for the experiment. Variations of the concentration and amount of enzymes are necessary for the evaluation of the yields of final sugars, the economic perspectives and to gain a better knowledge of commercial enzyme properties, as well as results will show which combination suits best for the highest lactose hydrolysis rate. Moreover, this research provides valuable data of the profile of monosaccharides giving information about the nutritional value of the glucose-galactose syrup.

2.6.1 Materials / *Materiāli*

The materials used in Stage III are listed in Table 2.8 and the equipment used in Table 2.9. Additionally, Table 2.10. includes the enzyme concentrations which were used for lactose hydrolysis.

Table 2.8. / *2.8. tabula*

List of materials and chemicals used in the study/
Pētījumā izmantoto materiālu un ķīmisko vielu saraksts

Material or chemical / *Materiāls vai ķimikālija*	**Brand / *Ražotājs***	**Country / *Valsts***
KOH (≥85%, pellets) / *KOH (≥85%, granulas)*	Sigma	Germany / *Vācija*
α-Lactose monohydrate / *α-laktozes monohidrāts*		

Continuation of Table 2.8. / *2.8. tabulas turpinājums*

Material or chemical / *Materiāls vai ķīmikālija*	**Brand / *Ražotājs***	**Country / *Valsts***
D-Glucose / *D-Glikoze* (≥98%)	Sigma	Germany / *Vācija*
D-Galactose / *D-Galaktoze* (≥98%)		
Acetonitrile / *Acetonitrils* (≥99.93%)		
1.5 mL eppendorf tubes/ *1,5 ml Epedorfa mēģenes*		
1.5 mL vial / *1.5 mL pudelīte*		
Column / *Kolonna* SUPELCOSILTM LC-NH2, (250 mm × 4.6 mm × 5 µm)		
50 mL test tube / *50 mL mēģene*	Sarstedt	Germany / *Vācija*
15 mL test tube / *15 mL mēģene*		

Table 2.9. / *2.9. tabula*

List of equipment used in the study/ *Pētījumā izmantoto iekārtu saraksts*

Equipment / Iekārta	**Brand / *Ražotājs***	**Country / *Valsts***
Evaporator / *Ietvaicētājs*	Armfield FT22	United Kingdom / Apvienotā Karaliste
Rotary vacuum evaporator / *Rotācijas vakuuma ietvaicētājs*	Laborota 4000 efficient	Germany / *Vācija*
Refractometer / *Refraktometrs*	30GS Mettler Toledo	Switzerland / *Šveice*
HPLC / *AEŠH*	Shimadzu LC-20	Japan / *Japāna*
Refractive index detector / *Refrakcijas indeksa detektors*	RID-10A	Japan / *Japāna*
Thermostat / *Termostats*	Memmert IN55	Germany / *Vācija*

Table 2.10. / *2.10. tabula*

Description of enzymes / *Lietoto enzīmu raksturojums*

Enzymes / *Enzīmi*	**Activity / *Aktivitāte***	**Weight / *Svars*, mg***	**Units / *Vienības****
Ha-Lactase 5200	5200 NLU g^{-1}	11±1	57±5 NLU
		53±2	290±12 NLU
		106±2	590±12 NLU
NOLA^{TM}Fit5500	5500 BLU g^{-1}	11±1	60±2 BLU
		51±2	280±11 BLU
		104±2	540±11 BLU
GODO-YNL2	5000 NLU g^{-1}	11±1	57±5 NLU
		51±2	280±10 NLU
		107±2	540±12 NLU

*- mean values ± SD (n = 3) / *vidējais aritmētiskais ± standartnovirze (n=3)*

2.6.2 Permeate solids concentration / *Ultrafiltrāta sausnas koncentrēšana*

Permeate solids concentration was performed by Palai *at al.* 2012 method with a slight modification. At first, approximately 20% of permeate solids was achieved using evaporator under vacuum conditions with the following parameters: flow rate 8 L h^{-1}, warming steam pressure 1 bar, permeate temperature 78±1 °C, cooling water rate 5 L h^{-1}, and vacuum 56 kPa. Then, to reach the permeate solids concentration of 30% and 40%, the rotary vacuum evaporator Laborota 4000 was used. Permeate solids was periodically measured with a refractometer.

2.6.3 Hydrolysis of permeates / *Ultrafiltrātu hidrolīze*

The hydrolysis of lactose was carried out by adding several amounts of β-galactosidases (see Table 2.10) to 100 mL of permeate. Permeates were placed into a thermostat at a temperature of 42.5 °C for 4 or 24 h. Before and during hydrolysis 10% (100 g L^{-1}) KOH solution was used for pH adjustment to provide pH corresponding to β-galactosidase activity - Ha-Lactase 5200 enzyme 6.5–6.6, for NOLA^{TM}Fit5500 enzyme 5.4–5.6 and for GODO-YNL2 enzyme 7.5–7.6. To inactivate β-galactosidase, permeates were placed in water bath at 90 °C

for 5 min. Then the permeates were transferred into 50 mL test tubes and afterwards put into a freezer at -18 °C for further analysis.

The degree of lactose hydrolysis was calculated by the following equation:

$$H(\%) = \frac{L_0 - L_1}{L_0} * 100, \text{ where:} \quad (8)$$

L_0 – initial lactose amount, g L^{-1};

L_1 – concentration of lactose after hydrolysis, g L^{-1} (Vasileva *et al.*, 2016).

2.6.4 Determination of lactose, glucose, and galactose by HPLC-RID / *Laktozes, glikozes un galaktozes noteikšana, izmantojot AEŠH-RI*

See 2.4.5.

2.7 Stage IV of research / *Ceturtais pētījuma posms*

The effect of added fructose amount for lactulose synthesis was investigated. Moreover, this research provides valuable data on sugar profile which definitely gives significant information about the sweetness of the final product.

In this study was investigated:

- the lactulose synthesis during lactose hydrolysis by adding fructose from 1 to 20 g to 100 mL permeate sample (see Figure 2.5.);
- two-stage fermentation, adding 1 g glucose isomerase to 100 mL permeate sample (see Figure 2.6.).

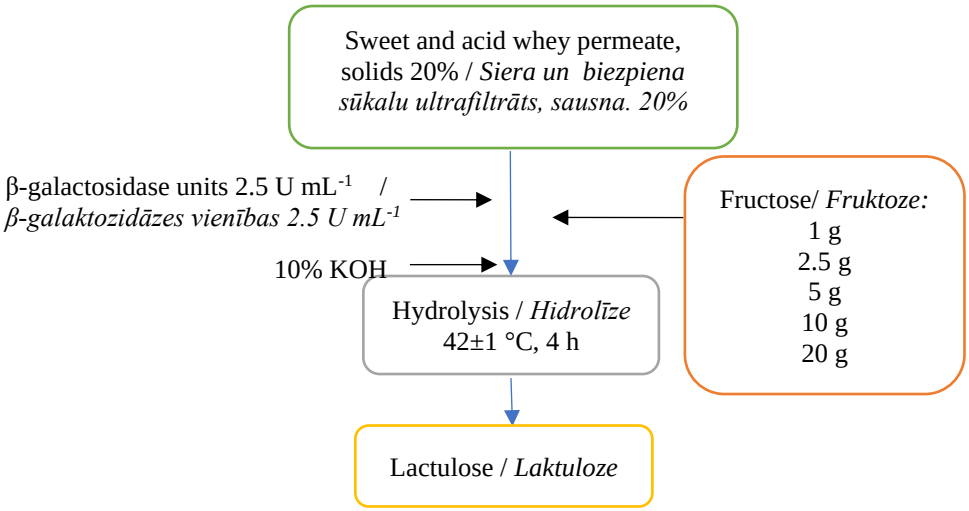


Fig. 2.5. Lactulose synthesis by adding fructose /
2.5. att. Laktulozes sintēze, pievienojot fruktozi

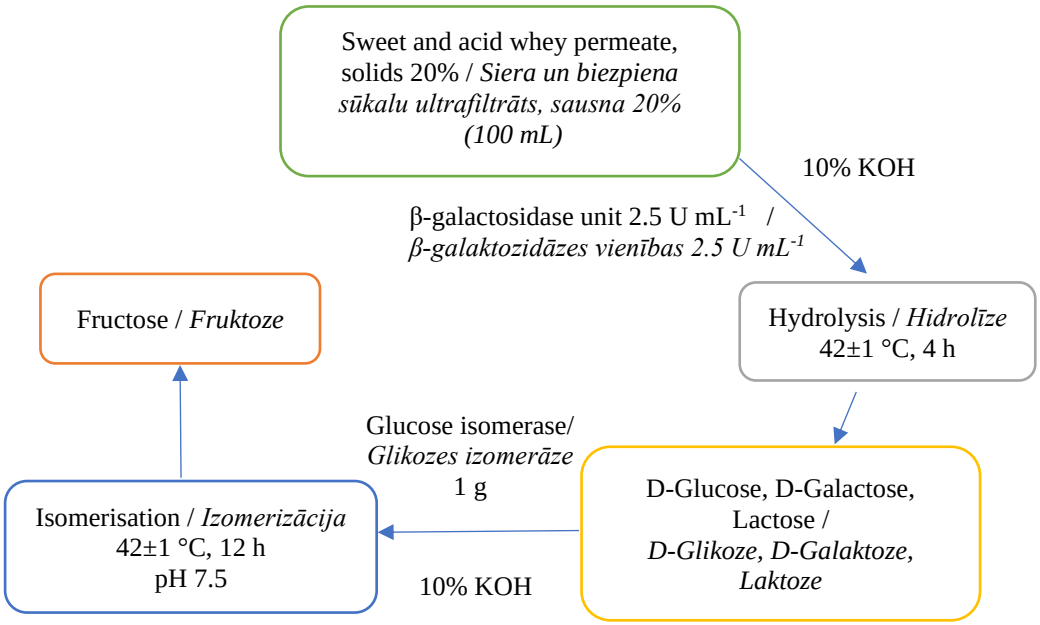


Fig. 2.6. Two-stage enzymatic hydrolysis by β-galactosidase and glucose isomerase / *2.6. att. Divpakāpju enzīmātiskā hidrolīze ar β-galaktozidāzi un glikozes izomerāzi*

2.7.1 Materials / *Materiāli*

The materials used in Stage IV are listed in Table 2.11 and the equipment in Table 2.12.

Table 2.11. / *2.11. tabula*

List of materials and chemicals used in the study/
Pētījumā izmantoto materiālu un ķīmisko vielu saraksts

Material or chemical / *Materiāls vai ķīmikālija*	**Brand / *Ražotājs***	**Country / *Valsts***
KOH (≥85%, pellets) / *KOH (≥85%, granulas)*	Sigma	Germany / *Vācija*
H_2SO_4 (Sulfuric acid / *Sērskābe*)		
H_2O_2 (Hydrogen peroxide / *Ūdeņraža peroksīds*)		
Kjeldehl tablets / *Kjeldāla tabletes*		
NaOH (Sodium hydroxide / *Nātrija hidroksīds*)		
Material or chemical / *Materiāls vai ķīmikālija*	**Brand / *Ražotājs***	**Country / *Valsts***
H_3BO_3 (Boric acid / *Borskābe*)	Sigma	Germany / *Vācija*
HCl (Hydrochloric acid / *Sālskābe*)		
α-Lactose monohydrate / *α-Laktozes monohidrāts*		
D-Glucose / *D-Glikoze* (≥98%)		
D-Galactose / *D-Galaktoze* (≥98%)		
Glucose isomerase / *Glikozes izomerāze*		
D-Fructose / *D-Fruktoze*		
Lactulose / *Laktuloze*		
Acetonitrile / *Acetonitrils* (≥99.93%)		
1.5 mL eppendorf tubes / *1,5 ml Ependorfa mēģenes*		
1.5 mL vial / *1.5 mL pudelīte*		
Column / *Kolonna* SUPELCOSILTM LC-NH2, (250 mm × 4.6 mm × 5 µm)		
Filter paper / *Filtrpapīrs*	ООО "МЕЛИОР XXI"	Russia / *Krievija*
Column / *Kolonna* Shodex KS-802 (300 mm × 8 mm)	Shodex	USA / *ASV*
Column / *Kolonna* YMC polyamine II (250 mm × 4.6 mm)	YMC	Japan / *Japāna*
50 mL tube / *50 mL mēģene*	Sarstedt	Germany / *Vācija*
15 mL tube / *15 mL mēģene*		

Table 2.12. / *2.12. tabula*

List of equipment used in the study/ *Pētījumā izmantoto iekārtu saraksts*

Equipment / *Iekārta*	**Brand / *Ražotājs***	**Country / *Valsts***
Evaporator / *Ietvaicētājs*	Armfield FT22	United Kingdom / *Apvienotā Karaliste*
Rotary vacuum evaporator / *Rotācijas vakuuma ietvaicētājs*	Laborota 4000 efficient	Germany / *Vācija*
Refractometer / *Refraktometrs*	30GS Mettler Toledo	Switzerland / *Šveice*
HPLC / *AEŠH*	Shimadzu LC-20	USA / *ASV*
HPLC / *AEŠH*	Agilent 1100	USA / *ASV*
Refractive index detector / *Refrakcijas indeksa detektors*	RID-10A	USA / *ASV*
Thermostat / *Termostats*	Memmert IN55	Germany / *Vācija*
Kjeltec Distillation unit / *Kjeltec destilācijas iekārta*	FOSS	Denmark / *Vācija*

2.7.2 Lactose hydrolysis and isomerisation / *Laktozes hidrolīze un izomerizācija*

The hydrolysis of lactose in sweet and acid whey permeates was carried out by adding 2.5 U mL^{-1} of commercial enzymes (Ha-Lactase 5200, NOLA^{TM}Fit5500 and GODO-YNL2) to 100 mL of permeate. The permeate samples were prepared and hydrolysed as described previously see Section 2.6.3.

After hydrolysis, the permeates were further prepared for glucose conversion into fructose using a modified version of a method described by Gaily *et al.* (2010). The permeates pH was adjusted to 7.5 by 10% (100 g L^{-1}) KOH solution, and 1 g of glucose isomerase was added to each permeate sample. The samples were put into the thermostat at 70 °C for 12 h. After isomerisation, the samples were filtered.

2.7.3 Kjeldahl method / *Kjeldāla metode*

Kjeldahl method was applied in accordance with ISO 8968-1:2002. The total protein equivalent including nitrogen from both protein and non-protein sources was determined in 5% and 20% permeates before hydrolysis and then after hydrolysis and isomerisation of each sample in both permeates. The determination was performed with 2±0.1 g of sample and in duplicate.

The total protein (p%) calculation was done by the following equation:

$$p\% = \frac{1.4007\times(V_S - V_b)\times M\times 6.38\times 100}{W}, \text{ where:} \quad (9)$$

V_S –HCl titrant used for sample, mL;

V_b – HCl titrant used for blank, mL;

M – molarity of HCl solution (0.1);

6.38 – factor which express percent nitrogen on a protein basis;

W – test portion weight, mg (AOAC 991.20., 2000).

2.7.4 Synthesis of lactulose / *Laktulozes sintēze*

The synthesis was performed using 100 mL of sweet and acid whey permeate with solids concentration 20% (Brix) where certain amount of fructose was added 1, 2.5, 5, 10, 20 g. Permeates were prepared according to Seok *et al.* (2013) method. Afterwards, 2.5±0.1 U mL^{-1} of commercial enzymes were added to each sample and they were placed into the thermostat for 4 hours at a temperature of 42.5 °C.

2.7.5 Determination of lactose, glucose, galactose, fructose, and lactulose / *Laktozes, glikozes, galaktozes, fruktozes un laktulozes noteikšana*

Permeate preparation was described in Ssection 2.4.5. Sugar calibration was prepared by a mixture which contained lactose, galactose, glucose, fructose and lactulose at concentration 8 g L^{-1} of each.

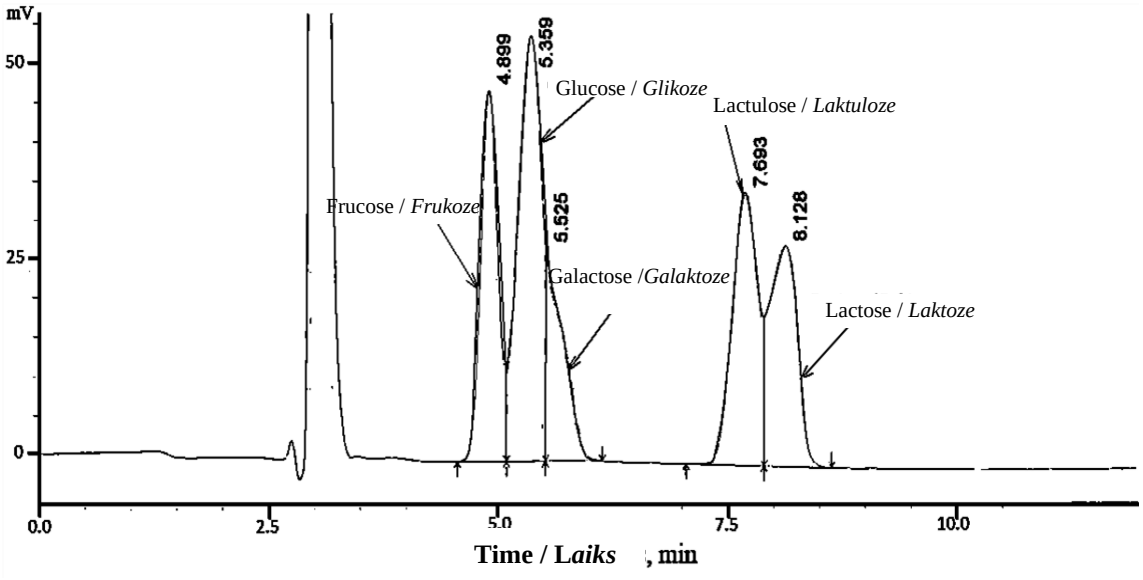


Fig. 2.7. Sugar calibration chromatogram (create by author)
2.7. att. Cukuru kalibrācijas hromatogramma (autora veidota)

2.7.6 Determination of galacto-oligosaccharides, glucose, galactose, and lactose / *Galakto-oligosagarīdu, glikozes, galaktozes un laktozes noteikšana*

GOS, glucose and galactose were determined by HPLC method (Lee *et al.*, 2014) using the Agilent 1100 chromatography system. Column: Shodex KS-802 (lenght 300 mm, ID 8 mm; refractive index detector; mobile phase – H_2O, flow rate 0.5 ml min^{-1}).

GOS was calculated by subtracting lactose concentration from the total disaccharide concentration, determined by Shodex KS-802.

Lactose was determined by HPLC method with the Agilent 1100 chromatography system (column: YMC polyamine II (lenght, 250 mm, ID 4.6 mm), refractive index detector; mobile phase – Acetonitrile/Water 65:35, flow rate 1.0 ml min^{-1}) and concentration was calculated by equation (3).

2.8 Stage V of research / *Piektais pētījuma posms*

Sensory evaluation was conducted in two stages:

1) sensory evaluation of hydrolysed sweet and acid whey permeates.

The hydrolysed sample preparation was done as describe in Section 2.7.2. In total 7 samples were evaluated – 1 commercial glucose-galactose syrup, 3 hydrolysed sweet whey permeate samples and 3 hydrolysed acid whey permeate samples. Commercial glucose-galactose syrup was used as a control, the solids concentration was reduced to 20% by distilled water. The hydrolysed sweet and acid whey permeates were evaluated by 36 trained panellists (students and staff members of the Faculty of Food Technology, Latvia University of Life Sciences and Technologies).

2) sensory evaluation of two-stage fermented syrups.

Sensory evaluation of two-stage fermented syrups (with solids concentration of 70%) was carried out after two-stage hydrolysis (β-galactosidase and glucose isomerase, see section 2.7.2). Commercial glucose-galactose syrup was used as a control in both stages. Isomerised syrups were evaluated by 30 trained panellists (students and staff members of the Faculty of Food Technology, Latvia University of Life Sciences and Technologies).

The assessment of sensory attributes was conducted according to the sensory standard ISO 4121:2003. The intensity of sweet, sour, salty and aftertaste attributes was evaluated using 12-point unstructured line scale where 0 - not detected and 12 - extremely strong. The format

of evaluation sheet and data collection were processed with FIZZ software (Biosystemes, France). Warm tea without sugar was used for taste neutralisation between samples.

The solids concentration for isomerised syrups was 70%. Solids concentration was measured with refractometer.

Sample preparation: each sample was placed into a glass beaker and placed on a tray in two lines, but glucose-galactose syrup (control) was placed at the beginning of each line, all samples were coded.

2.9 Statistical analysis of data / *Datu statistiskā analīze*

Results were expressed as mean±standard deviation (SD) of three replicates for composition and analytical measurements. Statistical analyses were carried out using Two-Way ANOVA, One-Way ANOVA and Tukey tests. The level of significance of the obtained data is characterized by the value of p (if $p>0.05$, the results do not differ significantly, if $p<0.05$, the results differ significantly). All data were calculated in MS Excel 2019 software.

Sensory data were analysed using a principal component analysis (PCA), calculated with Dimension Reduction (Factor Analysis), where the variables are sensory attributes and enzymes, as well as the data were also assessed by a Two-Way ANOVA. These statistical analyses were calculated with IBM SPSS 22.0 (SPSS Inc.).

3. RESULTS AND DISCUSSION / *REZULTĀTI UN DISKUSIJA*

3.1 The study of commercial β-galactosidase properties / *Komerciālās β-galaktozidāzes īpašību izpēte*

The aim of this study was to investigate the physical and chemical properties of the commercial β-galactosidases which were selected in the doctoral thesis. The results allow the researchers to evaluate the capability and potential of each enzyme when acid whey and sweet whey permeates will be used as a substrate.

3.1.1 Determination of β-galactosidase activity / *β-Galaktozidāzes aktivitātes noteikšana*

In this research the relationship between the activity and concentration of β-galactosidases was measured, and the results are shown in Figures 3.1., 3.2. and 3.3.

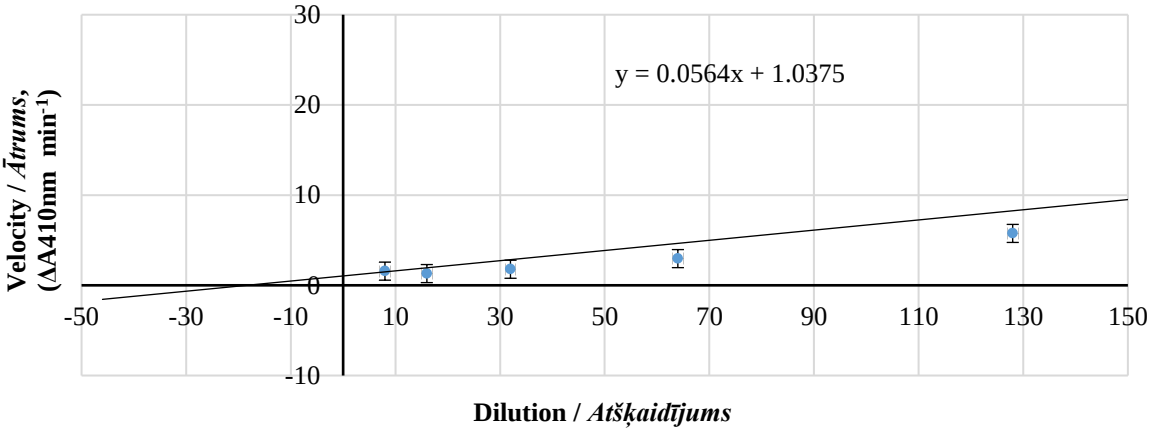


Fig. 3.1. *Bacillus licheniformis* β-galactosidase (NOLA™Fit5500) activity at different dilutions / *3.1. att. Bacillus licheniformis β-galaktozidāzes (NOLA™Fit5500) aktivitāte dažādos atšķaidījumos*

Fig. 3.1. shows the effect of NOLA™Fit5500 dilution on the reaction velocity. According to the obtained results, the effective ratio between substrate and NOLA™Fit5500 β-galactosidase is 1:16. This shows that up to a given dilution, the enzyme is able to provide a rapid reaction rate, but then the reaction rate slows down, indicating that the given substrate is consumed in the mixture (Robinson, 2019).

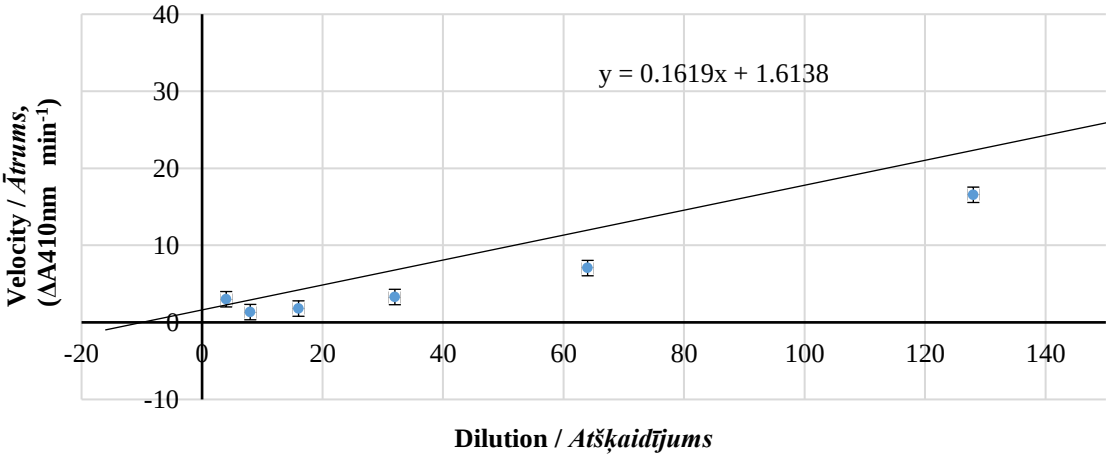


Fig. 3.2. *Kluyveromyces lactis* β-galactosidase (GODO-YNL2) activity at different dillutions / *3.2. att. Kluyveromyces lactis β-galaktozidāzes (GODO-YNL2) aktivitāte dažādos atšķaidījumos*

As the enzyme dilution ratio was increased above 1:8, the rate of reaction also increased progressively. In hydrolysis at higher enzyme concentrations, the reaction will take place very quickly, where it is less possible to analyse the reaction dynamics (Punekar, 2018). The results (Fig. 3.2.) indicate that the highest GODO-YNL2 β-galactosidase activity was observed in dilution of 1:8.

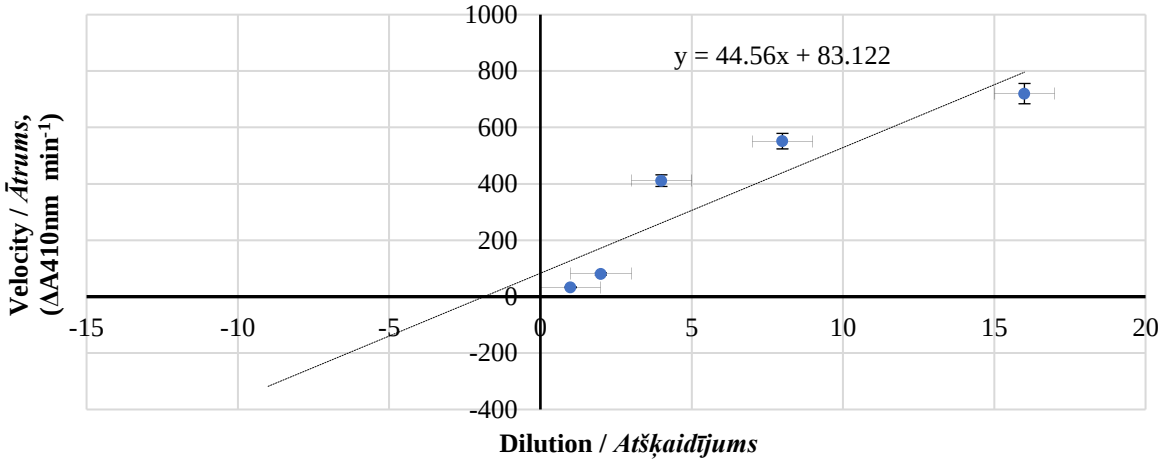


Fig. 3.3. *Kluyveromyces lactis* β-galactosidase (Ha-Lactase 5200) activity at different dilutions / *3.3. att. Kluyveromyces lactis iegūtā β-galaktozidāzes (Ha-Lactase 5200) aktivitāte dažādos atšķaidījumos*

The results in Fig. 3.3. show that the highest Ha-Lactase 5200 β-galactosidase activity was observed in 1:2.

The relationship between activity and concentration is influenced by many factors such as temperature, pH, substrate concentration, etc. The enzyme research should be designed in such a way that the activity observed is proportional to the concentration of the enzyme (Worthington *et al.*, 2019). The results of each enzyme showed the most suitable dilution ratio which will be used to study the ions effect on enzyme activity.

3.1.2 The analysis of the impact of calcium, sodium, potassium and magnesium ions on β-galactosidase activity / *Kalcija, nātrija, kālija un magnija jonu ietekme uz β-galaktozidāzes aktivitāti*

After determination of the most effective dilution ratios of the studied β-galactosidases, further we investigated the impact of several metal ions on enzyme activity.

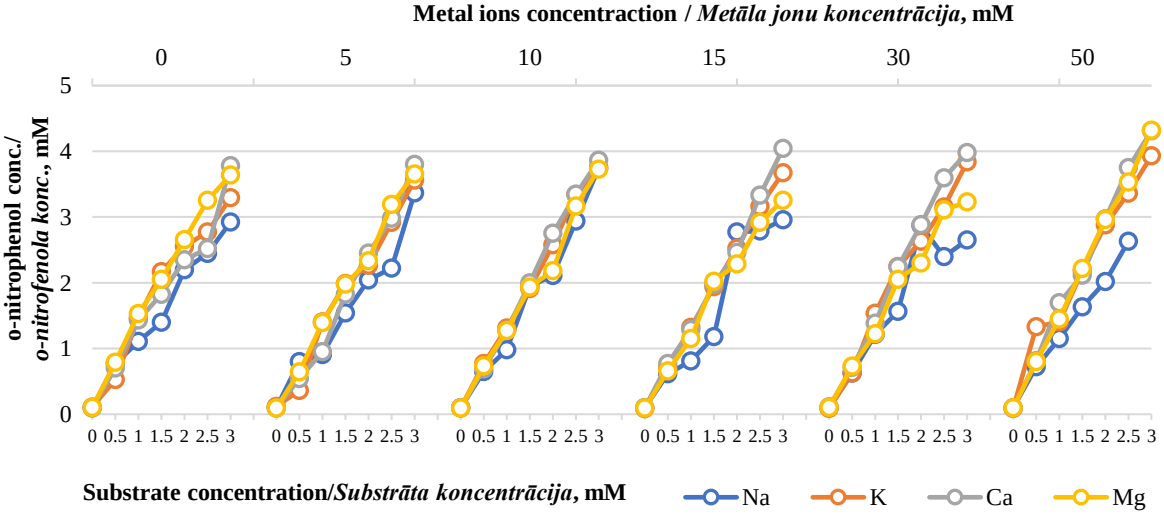


Fig. 3.4. The impact of metal ions on NOLA™Fit5500 activity / *3.4. att. Metāla jonu ietekme uz NOLA™Fit5500 aktivitāti*

The effect of ions on NOLA ™ Fit5500 β-galactosidase activity is shown in Figure 3.4. NOLA™Fit5500 β-galactosidase was stable until the concentration of metal ions did not exceed 10 mM. Starting from the concentration of 15 mM, changes in the activity of enzyme were observed. Enzyme started to become less active at the sodium (Na^+) concentration range of 15 to 50 mM and magnesium (Mg^{2+}) concentration range of 15 to 30 mM. Potassium (K^+) and calcium (Ca^{2+}) at the concentration range of 0 to 50 mM did not have a significant effect on enzyme activity. Similar research was done by Juajun *et al.* (2011) where it was established that monovalent ions, such as Na^+ and K^+, showed activator effect on the hydrolysis of ONPG by *Bacillus licheniformis* β-galactosidase. In contrast, the addition of Ca^{2+} ions in the concentration range of 1 to 10 mM slightly activated the enzyme and this finding can be beneficial for lactose hydrolysis in milk and whey.

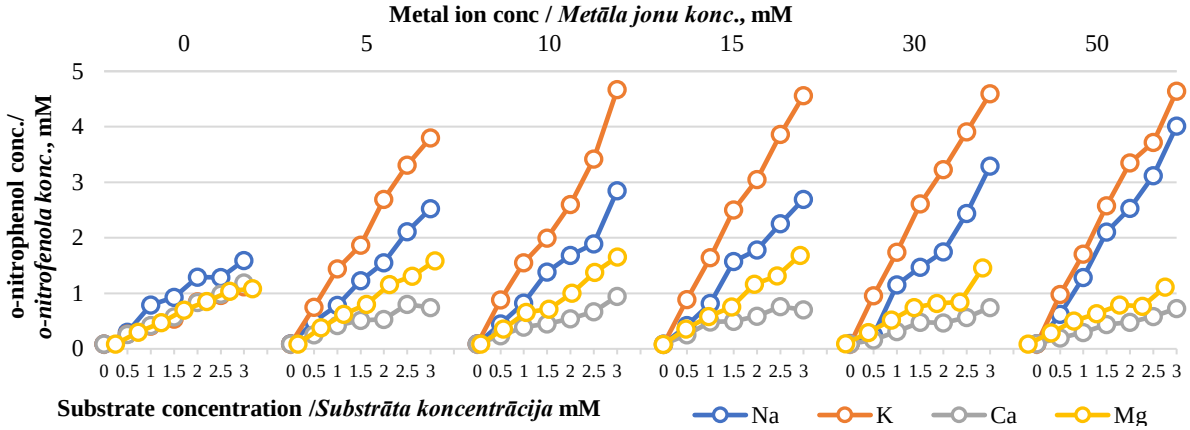


Fig. 3.5. The impact of metal ions on GODO-YNL2 activity /
3.5. att. Metāla jonu ietekme uz GODO-YNL2 aktivitāti

Metal ions affected GODO-YNL2 β-galactosidase (originated from *Kluyveromyces lactis)* activity differently, see in Figure 3.5. Potassium at the concentration range of 5 to 50 mM showed the highest results and was considered as an enzyme activator. In turn, Na^+ ions influence on enzyme activity was gradual, the enzyme activity had increased with an increased Na^+ ions concentration. In all measurements, Ca^{2+} ions showed the lowest results which is considered as an inactivator. The Mg^{2+} ions also worked as an enzyme inactivator at the concentration range of 30 to 50 mM.

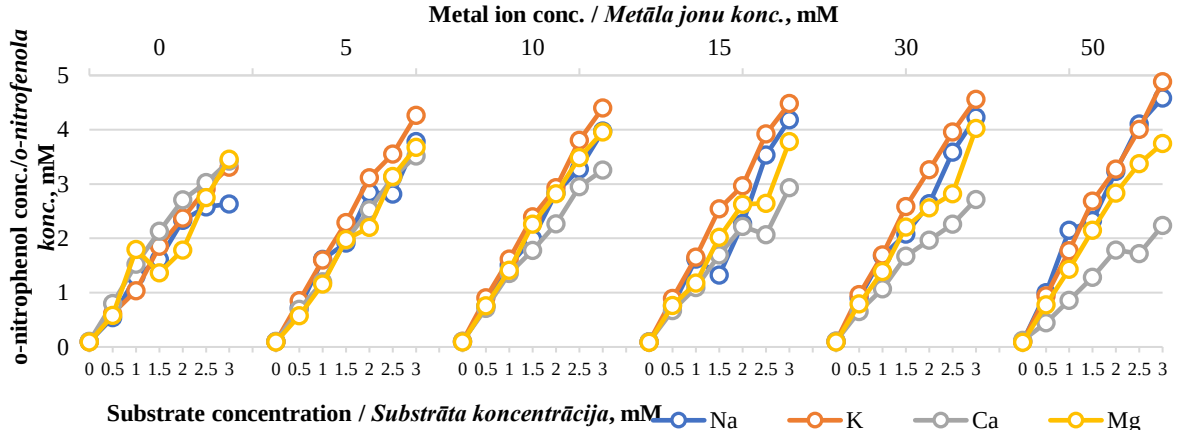


Fig. 3.6 The impact of metal ions on Ha-Lactase 5200 activity /
3.6 att. Metāla jonu ietekme uz Ha-Lactase 5200 aktivitāti

Ha-Lactase 5200 β-galactosidase (originated from *Kluyveromyces lactis)* activity is differently affected by each metal ions. As shown in Figure 3.6., K^+, Mg^{2+}, and Na^+ ions in the concentration range of 5 to 50 mM increased enzyme activity and mentioned ions were considered as activators, whereas Ca^{2+} concentration from 10 mM decreased enzyme activity and started to work as an inactivator.

Several researches indicate that K^+ and Na^+ ions are known as activators which increase the hydrolysis level by *Kluyveromyces lactis* β-galactosidase (Adalberto *et al.,* 2010; Jurado *et al.,* 2004; Wojciechowska *et al.*, 2018). However, there is little information on the effect and impact of these ions on β-galactosidase activity (Rico-Rodríguez *et al.,* 2020).

Table 3.1. / *3.1. tabula*

Content (mg kg^{-1}) of macroelements and phosphates in different solids permeates / *Makroelementu un fosfātu saturs (mg kg^{-1}) dažāda sausnas satura ultrafiltrātos*

Permeates / Ultrafiltrāts	Macroelements and phosphates / *Makroelementi un fosfāti*				
	Ca^{2+}	Na^+	K^+	Mg^{2+}	PO_4^{3-}
Sweet whey/ *Siera sūkalu*, 5%	349±14	429±11	1480±100	57±9	275±61
Acid whey/ *Biezpiena sūkalu*, 5%	1322±83	469±67	1636±189	123±16	736±34
Sweet whey/ *Siera sūkalu*, 20%	1071±99	660±57	2710±55	260±13	1265±50
Acid whey/ *Biezpiena sūkalu*, 20%	3400±86	1100±55	5600±140	340±18	2200±56

Table 3.1 demonstrates the amount of macroroelements and phosphates at different solids concentrations (5% and 20%) in sweet and acid whey permeates. The profile of macroelements in permeates gives a better understanding and knowledge about their effect on enzyme activity in the permeates based on the results described in Section 3.1.2. Potassium predominates in all samples, confirming that the presence of other elements (which could act as inactivators) would not affect β-galactosidase activity and that the degree of lactose hydrolysis would remain high. Lactose hydrolysis will be discussed in more detail in Chapter 3.3.

In general, salts in milk exist in such forms as inorganic compounds, or as a part of proteins, fats and nucleic acids (Zamberlin *et al.,* 2012). Almost all macro- and microelements in milk after protein coagulation are transferred to whey or whey permeate, thereby this is one of the reasons why whey is considered as a nutritionally valuable product (Kobukowski *et al.,* 2006; Bologa *et al.*, 2013; Wronkowska *et al.,* 2015). From sensory properties that can be used to describe whey, one is salty. The profile of macroelements presented in Table 3.1 shows that the amount of sodium and potassium is different. Both elements in combination with chlorides give a salty and bitter taste which is also described in the research of Chandrapala *et al.,* (2015), and Frankowski *et al.,* (2014).

3.1.3 Alternative methods for the determination of β-galactosidase activity / *Alternatīvās metodes β-galaktozidāzes aktivitātes noteikšanai*

The aim of this study was to experiment with four different analytical methods to measure β-galactosidase activity and lactose concentration during hydrolysis.

Table 3.2. / *3.2. tabula*

Determination of β-galactosidase activity by glucose strip test / *β-Galaktozidāze aktivitātes noteikšana ar glikozimetru*

Time / *Laiks*, min	GODO-YNL2, U g^{-1*}	NOLA^{TM}Fit5500, U g^{-1*}
0	0	0
1	3723±56	12103±181
2	2653±40	8586±34
4	1485±22	4737±71
8	1051±20	3382±50

* U = amount of enzyme required to release 1 micromole of reducing sugars min^{-1} / *Enzīma daudzums, kas nepieciešams, lai atbrīvotu 1 mikromolu reducējošo cukuru min^{-1}*

The focus of this experiment was to evaluate the sensitivity of glucose strip test, so a short measurement time was chosen. The results summarised in Table 3.2 showed that it was

possible to determine the glucose concentration and β-galactosidase activity by glucose strip test. This method showed that it is possible to determine the activity of β-galactosidase and to use in food science. This method is easy to carry out, it is fast and cheap. These observations are comparable to the research data by Hardee *et al.*, 2011 and Heinzerling *et al.*, 2012, where it was suggested that the glucose strip test should be calibrated for the use with aqueous solutions and for the measurement of β-galactosidase activity.

Table 3.3. / *3.3. tabula*

Determination of β-galactosidase activity by spectrophotometric method /
β-Galaktozidāzes aktivitātes noteikšana ar spektrafotometrisko metodi

O-NPG concentration / *O-NPG koncentrācija*, mM	GODO-YNL2, U g^{-1*}	NOLA^{TM}Fit5500, U g^{-1*}
0	0	7±1
0.5	130±3	857±13
1	271±6	1335±20
2.5	301±15	2656±19
3	393±20	2822±16

* U = amount of enzyme required to release 1 micromole of reducing sugars min^{-1} / *Enzīma daudzums, kas nepieciešams, lai atbrīvotu 1 mikromolu reducējošo cukuru* min^{-1}

Spectrophotometric method is used to measure the amount of o-nitrophenol which is necessary for the determination of β-galactosidase activity, see Table 3.3. This method is well known and primarily used in research and food sector. It was also described in Official Methods of Analysis of AOAC "Method 998.04 Neutral Lactase (β-Galactosidase) Activity in Industrial Enzyme Preparations" (AOAC 998.04, 2000). To make the method faster and easier to use, there is a β-Galactosidase Assay Kit available (www.eurogentec.com). The kit contains a 96-well plate with chemicals, and only a certain amount of β-galactosidase needs to be added for the analysis.

Table 3.4 / *3.4 tabula*

Determination of β-galactosidase activity by HPLC method /
β-Galaktozidāzes aktivitātes noteikšana ar AEŠH metodi

Time / *Laiks*, min	GODO-YNL2, U g^{-1*}	NOLA^{TM}Fit5500, U g^{-1*}
0	0	0
1	3341±22	1883±24
2	1704±14	1478±18
4	1049±29	1024±10
8	572±13	937±13

* U = amount of enzyme required to release 1 micromole of reducing sugars min^{-1} / *Enzīma daudzums, kas nepieciešams, lai atbrīvotu 1 mikromolu reducējošo cukuru* min^{-1}

The HPLC method was used to determinate lactose concentration during hydrolysis, from which the activity of the β-galactosidase was calculated, see Table 3.4. The results showed that the enzymes were capable to hydrolyse lactose, when reaction started GODO-YNL2 β-galactosidase activity after 8 min reduced more than 6 times but for NOLA^{TM}Fit5500 β-galactosidase only 2 times. However, this is one of the most expensive methods in terms of solvent, column, time consumed, and energy costs. On the other hand, this method is sensitive, provides useful information, as well as the possibility to determine the sugar profile and to calculate β-galactosidase activity. This observation is also supported by Yue *et al.* (2009) research results.

Cryoscopy is considered to be an alternative method to control the solids concentration of whey permeate and the hydrolysis of lactose.

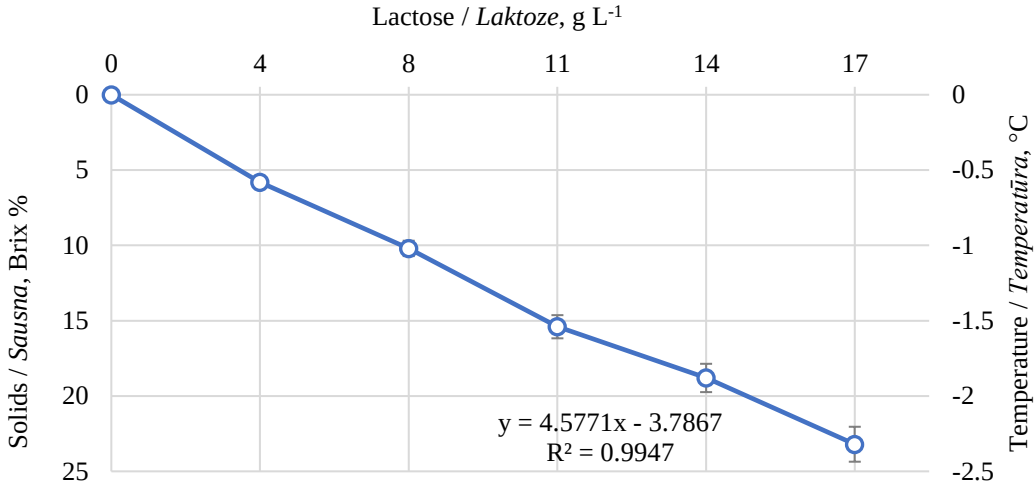


Fig. 3.7. Freezing point at certain whey permeate solids concentration /
3.7 att. Sasalšanas temperatūra noteiktā ultrafiltrāta sausnas saturā

The results presented in Fig. 3.7 demonstrate a linear relationship between the freezing point, solids and lactose concentration. The experiment was started with an evaporated whey permeate with an initial solids concentration of 23.2±1.2%, lactose concentration of 17±1 g L^{-1} and a freezing point of -2.20 °C. During the experiment, the solids concentration was purposefully reduced, and in total five concentrations were taken for evaluation. In general, the range of solids concentration was from 23.2±1.2% to 5.8±0.3%, the amount of lactose from 17±1 g L^{-1} to 4.2±0.2 g L^{-1} and freezing point was from -2.2±0.1 ºC to -0.5±0.1 °C. This shows a very significant linear relationship between results, i.e., freezing point changes due to the decreasing of solids concentration. Based on these findings, it can be concluded that lactose and solids concentration strongly affect the freezing point. The increase in salt and sugar concentration decreases water content and thereby lowers the freezing point in the permeate (Baer & Keating, 1987).

Nowadays, food manufacturers and laboratories are interested in using fast and inexpensive methods to determine a parameter in a food sample. The cryoscopy method may be one of the methods which could help determine the rate of lactose hydrolysis.

By modelling the appropriate calibration curve for each parameter required for the calculation, it is possible to determine the amount of lactose in the evaporated whey permeate by cryoscopy.

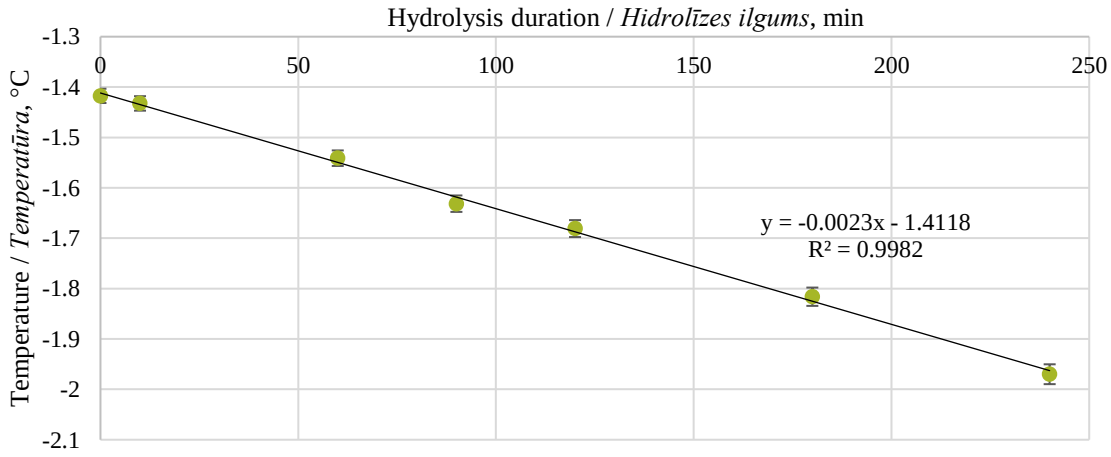


Fig. 3.8. Freezing point changes during lactose hydrolysis /
3.8 att. Sasalšanas temperatūras izmaiņas laktozes hidrolīzes laikā

The results showed that lactose hydrolysis in whey permeate started at the freezing point of -1.42±0.03 ºC and after four hours it had changed and dropped to -1.97±0.04 ºC. There are various factors which affect the freezing point measurement. Firstly, it is the composition of

the initial whey permeate. The concentration of lactose has an effect on the freezing point where low molecular salts and sugars cause the highest impact. For that reason, if the volume of the solution remain invariable the freezing point will be inversely related to the molecular weight of the soluble solids (Abbasi & Saeedabadian, 2013). Secondly, enzymatic hydrolysis of lactose increases the concentration of glucose and galactose, thus increasing the total molar concentration of sugars in permeate, and reducing the freezing point linearly (Churakova *et al.*, 2019).

The obtained results indicate that the cryoscopy is a fast and suitable method for the monitoring of lactose hydrolysis or solids concentration of whey before hydrolysis because components are known and they are easy to calibrate.

Four methods gave comparable, highly variable results, showing the possibility with each of them determinate hydrolyse yield and enzyme activity. Price calculation (Appendix 1 and Appendix 3) shows that it is possible to save money on analyses where it is necessary to use expensive laboratory instruments. Glucose strip test is a profitable method for small dairies which cannot afford large investments for modern and expensive laboratory equipment. The results are based on the material prices used in the experiments.

3.1.4 The study of β-galactosidase stability in GIT model *in vitro* / *Komerciālās β-galaktozidāzes stabilitātes izpēte KZT modeļvidē*

The experiment was performed under *in vitro* digestion conditions to determine a relative stability of the commercial β-galactosidase used in this regard.

The importance of this study is due to the limited information available in literature on *in vitro* studies of β-galactosidase, so it is important to evaluate the properties of this enzyme in such environment.

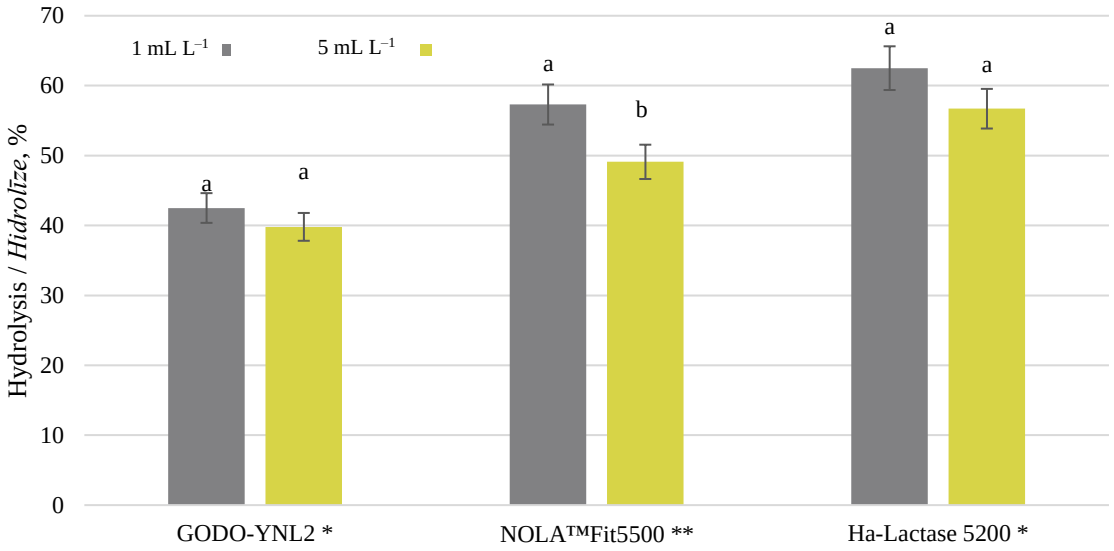


Fig. 3.9. Lactose hydrolysis (%) after GIT withstand β-galactosidases /
3.9 att. KZT pakļautās β-galaktozidāzes laktozes hidrolīzes (%) pakāpe

The values marked with the same letter for each enzyme at different concentrations do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katram enzīmam dažādās koncentrācijās būtiski neatšķiras (p>0.05)*

A significant difference was observed in the result of hydrolysis, both between enzymes ($p<0.05$) and between different concentrations of the same enzyme ($p<0.05$).

As it was shown in Fig. 3.9, Ha-Lactase 5200 (62.5±3.9 %) and NOLA™Fit5500 (57.3±4.3%) β-galactosidases at concentration 1 mL L^{-1} showed higher yield of lactose hydrolysis. However, GODO-YNL2 β-galactosidase showed low hydrolysis yield at enzyme concentration 1 and 5 mL L^{-1}. These results can be explained by the temperature and pH variation during the gastric-intestinal track. The temperature for experiment was 37 °C which

was less effective for the GODO-YNL2 β-galactosidase (40 °C), but for NOLA™Fit5500 (35–50 °C) and Ha-Lactase 5200 (35–45 °C) β-galactosidases were within the optimal interval. At gastric phase, the pH of the medium was 2 but at the intestinal phase pH was 7. Enzymes of *Kluyveromyces lactis* origin (GODO-YNL2 and Ha-Lactase 5200) are active at pH range of 6.5 to 8.0, while the NOLA™Fit5500 β-galactosidase of *Bacillus licheniformis* origin is active under slightly more acid conditions in the range of 5 to 7 pH. The research results of several articles (Dagbagli & Goksungur, 2008; Vidya *et al.*, 2014) showed that β-galactosidase activity and stability are influenced by the enzyme origin, growth conditions (temperature, pH, aeration, agitation, incubation time) and components present in the growth medium. Moreover, Vrese *et al.* (2001) concluded that the β-galactosidase combined with probiotic β-galactosidase is able to pass through gastric phase, as well as, slower gastric process and intestinal transportation of the product. Therefore, based on the results of the experiment, the ability of the enzymes to retain their activity during *in vitro* was shown.

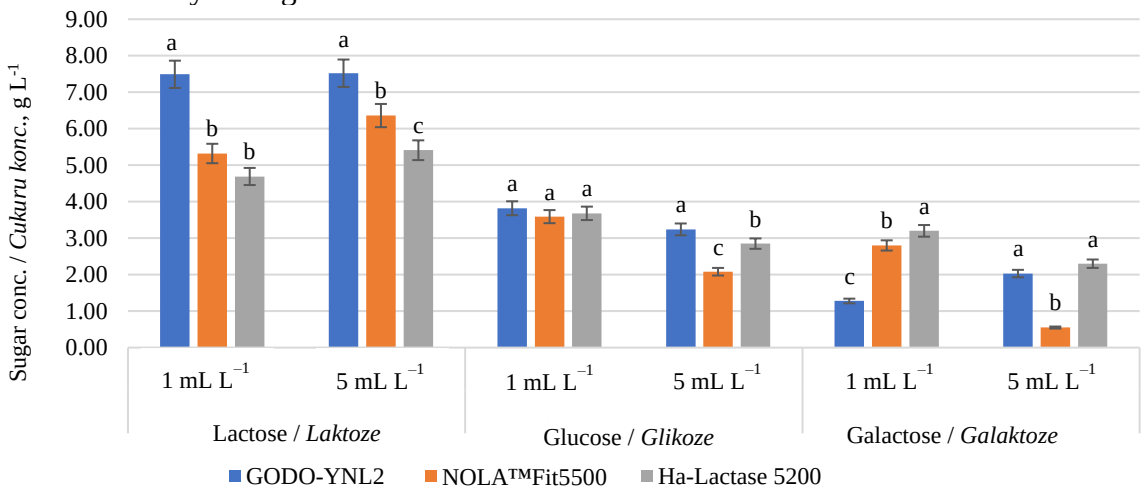


Fig. 3.10. Evaluation of lactose hydrolysis products by β-galactosidase subjected to GIT / *3.10 att. KZT pakļautās β-galaktozidāzes laktozes hidrolīzes produktu novērtējums*

The marked values with the same letter within each enzyme and sugar concentration do not differ significantly ($p>0.05$) / *Vērtības apzīmētas ar vienādu burtu katra enzīma un cukura ietvaros būtiski neatšķiras ($p>0.05$)*

The hydrolysis of lactose by *Bacillus licheniformis* and *Kluyveromyces lactis* β-galactosidases was significantly different ($p<0.05$). A comparatively high hydrolysis yield was achieved with the NOLA ™Fit5500 and Ha-Lactase 5200 β-galactosidases at a concentration of 1 mL L^{-1}. It should be emphasised that one of the factors that could affect the degree of hydrolysis is the concentration of enzyme used.

As shown in our results in Fig. 3.10, the highest hydrolysis effect was achieved using 1 mL L^{-1} of β-galactosidase concentration instead of 5 mL L^{-1}. It should be noted that the study method was based on the ratio among food, digestive enzyme and stock solution intake. The findings show that low enzyme concentration is more likely to retain activity in the GIT environment. It should be noted that various enzymes were added in *in vitro* experiments, different environmental parameters and substance concentrations were provided, which also have the greatest influence on the activity and behaviour of β-galactosidase.

Kotz *et al.* (1994) had compared β-galactosidase activity in commercial unflavoured yoghurt and in high lactase yoghurt which was hydrolysed for 60 minutes at 37 °C and pH 3.5. β-Galactosidase in high lactase yoghurt showed lower activity in acidic media than β-galactosidase in commercial yoghurt, which might be explained by the potential inactivation of β-galactosidase in GIT. The authors concluded that the high enzyme concentration and low media pH had a significant impact on β-galactosidase sensitivity and promoted its denaturation.

Summary of Chapter 3.1 / *3.1. Nodaļas kopsavilkums*

The concentration of salts in whey permeates is different and results implies that each ion affects β-galactosidase differently. That gives information of the impact of acid whey and sweet whey permeates composition on enzyme action and lactose hydrolysis yield. The most appropriate solution to ensure an optimal substrate pH for β-galactosidase activity would comprise Na^+ and/or K^+ ions. It is possible to use the glucose strip test for the determination of glucose amount during lactose hydrolysis.

β-Galactosidases from *Kluyveromyces lactis* and *Bacillus licheniformis* were effective under *in vitro* digestion as a strategy for improving lactose intolerance. The concentration of β-galactosidase is one of the factors which can affect the degree of lactose hydrolysis in the human gastrointestinal tract. The research demonstrated that β-galactosidase at high concentrations and low pH is more sensitive to denaturation. Under *in vitro* conditions, the highest yield of lactose hydrolysis showed NOLA™Fit5500 and Ha-Lactase 5200 enzymes, which would be the most effective at mitigating lactose intolerance.

Minerālvielu saturs sūkalu ultrafiltrātā ir dažāds un pētījuma rezultāti pierādīja, ka katrs jons atšķirīgi ietekmē β-galaktozidāzes aktivitāti . Pētījums sniedz informāciju par biezpiena un siera sūkalu satāva iespējamo ietekmi uz enzīma darbību un laktozes hidrolīzes iznākumu. Vides pH pielāgošanai būtu ieteicams izmantot šķīdumus, kas satur Na^+ un / vai K^+ jonus. Glikozes satura noteikšanai laktozes hidrolīzē ir iespējams izmantot glikozimetru.

β-Galaktozidāze, kas iegūta no Kluyveromyces lactis un no Bacillus licheniformis, apstiprina stabilitāti KZT modeļvidē un spēju īstenot laktozes hidrolīzi. Viens no svarīgākajiem faktoriem, kas var ietekmēt laktozes hidrolīzes reakciju ir pievienotās β-galaktozidāzes daudzums. Iegūtie rezultāti parādīja, ka, izmantojot komerciālās β-galaktozidāzes, ir iespējams samazināt laktozes nepanesības simtomus patērētājiem. Enzīmi ar augstu koncentrāciju un darbības optimimu zemākā pH ir jutīgāki pret denaturāciju. In vitro apstākļos augstākie hidrolīzes rezultāti bija NOLA™Fit5500 un Ha-Lactase 5200 enzīmiem, tos varētu uzskatīt par sefektīvākajiem laktozes nepanesības simptomu mazināšanai.

3.2 Physical properties of dehydrated whey permeate and lactose / *Dehidrēta sūkalu ultrafiltrāta un laktozes fizikālās īpašības*

In this study was investigated the origin of permeates, which are present in different whey streams, the affects on their surface and structural properties during spray drying. Ultrafiltration was used in the study to obtain whey permeate. Fig. 3.11 represents the amount of main components and pH of sweet and acid whey permeates.

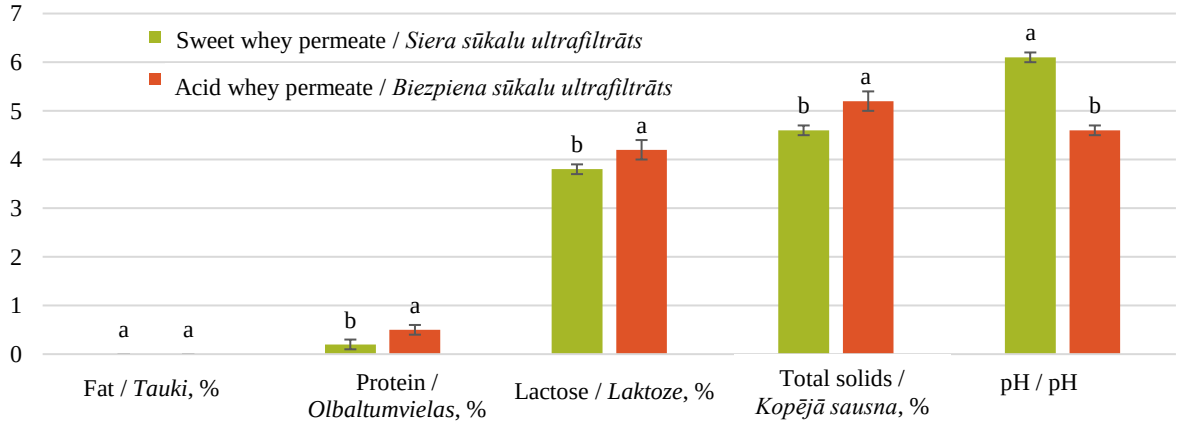


Fig. 3.11 Composition of permeates and pH before drying /
3.11 att. Ultrafiltrātu sastāvs un vides pH pirms kaltēšanas

The values marked with the same letter within each component and pH do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katras sastāvdaļas un pH ietvaros būtiski neatšķiras (p>0.05)*

The composition of the permeate depends on the whey origin, storage conditions and time, as well as ultrafiltration parameters (Barile *et al.*, 2009). Overall, Fig. 3.11. shows that permeates are high in lactose, the presence of protein is very low, and may contain salts, which are included in the total solids matter. Permeates were stored at 4 °C temperature for no longer than 24 h prior the experiment. This condition was sufficiently suitable for the lactose crystalisation.

Salts play an important role where attraction to lactose affects the formation of anhydrous lactose. The effect of salts on the crystallisation of lactose varies according to their type and amount (Islam & Langrish, 2008). This should be taken into consideration if dehydrated lactose and permeate powder are intended to be used in enzymatic lactose hydrolysis. Based on the results provided in Chapter 3.1, the ionic environment affects the enzymatic activity and lactose hydrolysis reaction (Demirhan *et al.*, 2008). It is important to point out that the salts and proteins in dehydrated whey permeate promote hygroscopicity of the powder (Ibach & Kind, 2007).

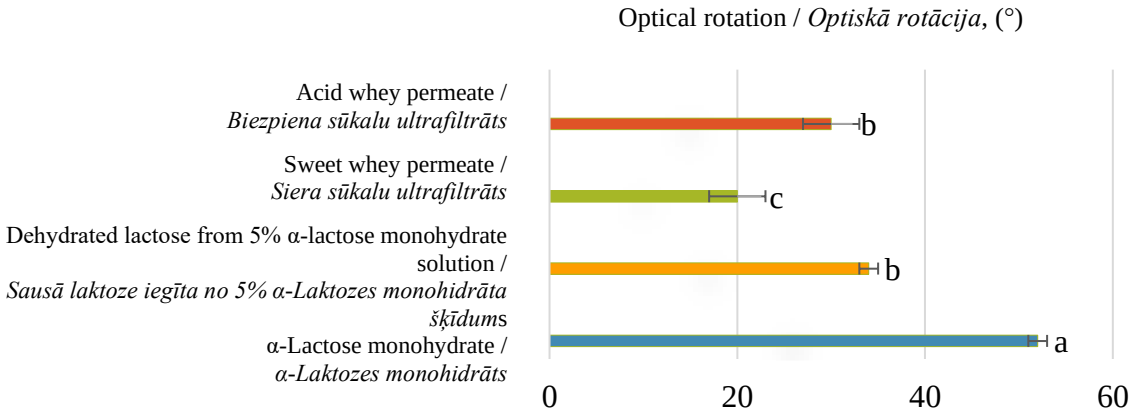


Fig. 3.12 Optical rotation of the α-lactose monohydrate (control) and dehydrated lactose / *3.12. att. Sausā α-laktozes monohidrāta (kontrole) un sausās laktozes optiskā rotācija*

The values marked within the same letter do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu būtiski neatšķiras (p>0.05)*

Optical rotation analysis was performed to compare permeate samples with the control and for further studies it would provides a better understanding of the mechanism of lactose hydrolysis. Dehydrated samples showed low optical rotation measurements comparing to α-lactose monohydrate 52±0.1° which was used as a control. Lactose in dehydrated permeates can be observed in several forms, such as, an amorphous glassy state or in the α and/or β crystalline, mainly depending on the conditions of the drying and storage for no longer than 24 hours before processing (Chandrapala & Vasiljevic, 2017). Significant difference was not observed between dehydrated acid whey permeate 30±3° and powder obtained from 5% α-lactose monohydrate solution 34±1°. The optical rotation of dehydrated sweet whey permeate was 20±3° which is the lowest result and could be affected by the concentration of solids in the initial whey. During spray drying high inlet temperature was used which caused initial forming of the Maillard reaction products. These products have the main influence on powder characteristics including optical rotation (Nishanthi *et al.*, 2017). The use of ultrafiltration for whey was to remove all molecules except sugars and salts. It is important to note that the pore size of the ultrafiltration membranes significantly affects the amount and types of proteins that can be transferred to the permeate as shown in Fig. 3.11. and acknowledge by the researchers' findings (Butylina *et al.*, 2006; Szpendowski *et al.*, 2006; Ma & Amamcharla, 2019). Based on the authors' reports, permeates have a high potential to comprise aldehydes or ketones and free primary amine group of amino acids, peptides, proteins or any nitrogenous substances that react with reducing sugars and can cause the Maillard reaction (Tu *et al.*, 2015).

The powders were prepared for surface visualization and examination by microscopy.

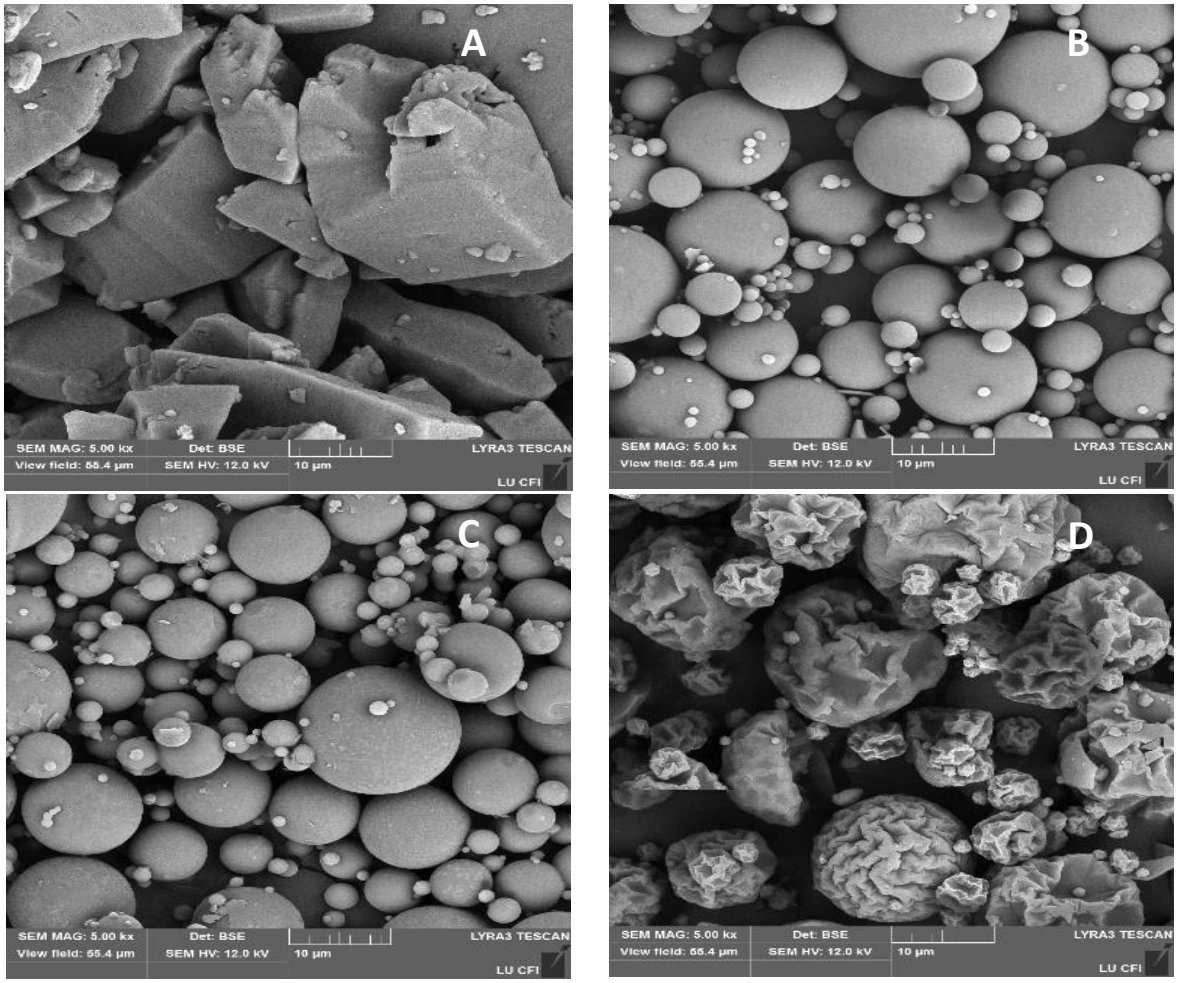


Fig. 3.13 Surface morphology of lactose crystals /
3.13. att. Laktozes kristālu virsmas morfoloģija /

α-lactose monohydrate crystals (A) and dehydrated lactose obtained from 5% α-lactose monohydrate solution (B); dehydrated sweet whey permeate lactose (C); dehydrated acid whey permeate lactose (D). All micrographs are shown at 5000× magnification / *α-Laktozes monohidrāta kristāli (A) un izsmidzināšanas kaltē iegūtās laktozes kristāli (iegūti no 5% α-laktozes monohidrāts šķīduma (B)); sausā siera sūkalu ultrafiltrāta laktoze (C); sausā biezpiena sūkalu ultrafiltrāta laktoze (D). Visi attēli doti 5000×palielinājumā.*

Surface morphology of the powders from 5% α-lactose monohydrate solution, sweet whey, and acid whey permeates, as well as α-lactose monohydrate crystals have been investigated using scanning electron microscopy, seen in Fig. 3.13. As it can be seen in Fig. 3.13. B–D dehydrated lactose and permeates have an amorphous form of lactose. Gänzle *et al.* (2008) reported that during the spray–drying, evaporation of water occurs very rapidly and the viscosity of the solution increases as well, that is the reason why crystals fail to form and lactose is remained in an amorphous form. Fig. 3.13 A shows standard crystalline form of α-lactose monohydrate crystals which have Tomahawk crystals showing faceted structure (Wong & Hartel, 2014). However, the micrographs of permeate powders were different. Dehydrated lactose obtained from 5% α-lactose monohydrate solution (Fig. 3.13. B) and sweet whey permeate (Fig. 3.13. C) had droplet surface of spherical shapes with a smooth surface, and the size of particles were relatively similar in both images, while the morphology of dehydrated acid whey permeate droplets in Fig. 3.13 D showed that one part of the droplets surface had a shrunken appearance, another part has cracks or fractures and the other part was smooth. As shown in Fig. 3.11, acid whey had pH below 6 which could be the reason for the appearance of the droplet surface to be strongly affected. The solids concentration (fat, protein, salts, lactose) and pH of the original product are responsible for the formation of the surface of the droplets (Ebrahimi *et al.*, 2015).

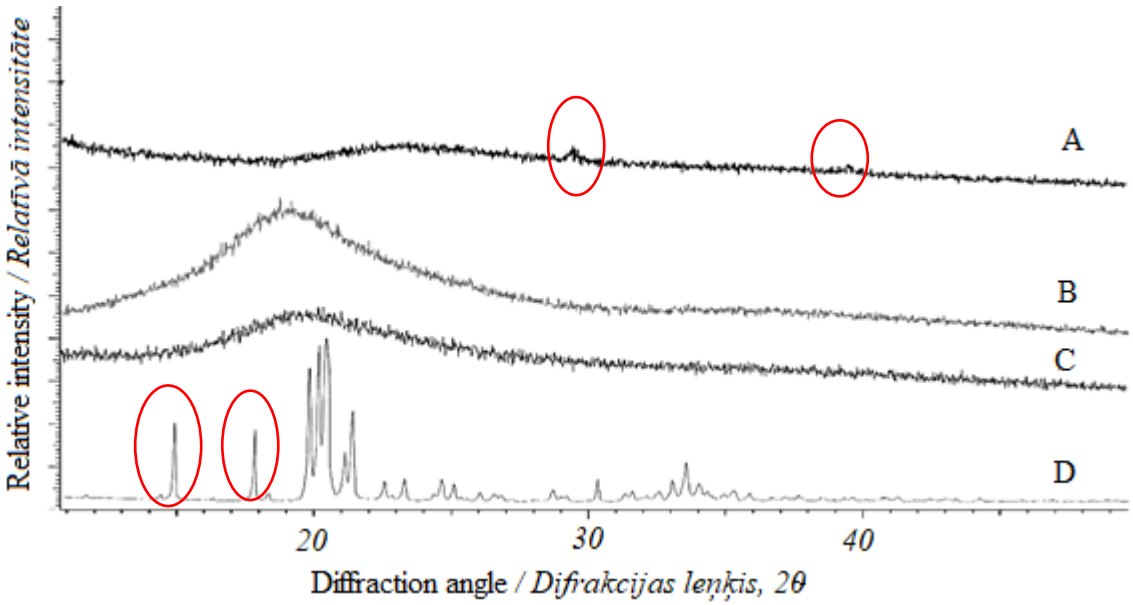


Fig. 3.14 Properties of lactose crystals by X-ray diffraction /
3.14 att. Laktozes kristālu īpašību noteikšana ar rentgenstaru difrakciju

α-lactose monohydrate crystals (D) and dehydrated lactose obtained from 5% α-lactose monohydrate solution (B); hehydrated sweet whey permeate (A); dehydrated acid whey permeate (C) / *α-laktozes monohidrāta kristāliem (D) un sausai laktozei (iegūtai no 5% α-laktozes monohidrāta šķīduma (B)), siera sūkalu ultrafiltrāta (A), biezpiena sūkalu ultrafiltrātam (C) paraugiem*

The glass transition of samples is shown in Fig. 3.14. The α-lactose monohydrate contains water molecule which plays an important role in structural cohesion, but in this particular treatment, the change of forms (Garnier *et al.*, 2002). The form in which lactose crystallises depends on the relative humidity, water activity, and temperature conditions during crystallisation, drying and storage (Allan *et al.*, 2020). All dehydrated powder patterns showed that lactose gained an amorphous form using certain spray-drying parameters. The results were analysed by database ICDD PDF2 where the presence of potassium chloride at a diffraction angle of 28±0.5 ° and 40±0.5 ° 2θ degrees in a sample of dehydrated sweet whey permeate was identified. This could be due to the interaction between lactose and this particular salt or organic acids and their salts under certain conditions, such as ultrafiltration, cheese processing, the composition and amount of ingredients in substrate, and average pH (Chen *et al.*, 2015). The diffraction peak of α-lactose monohydrate (Fig. 3.14 D) showed that it was possible to determine and identify the types and forms of lactose crystals. The peak that best describes the lactose crystals is within the relative range of 10 to 19°. The presence of α-lactose monohydrate was found at diffraction angles 14.9° and 17.8°. This value was also reported by Nijdam *et al.*, (2007) for pure α-lactose monohydrate powder. Based on the specification of α-lactose monohydrate, the concentration of β-lactose was low and this could be a cause for detection difficulties.

To better understand the thermal processes of dehyderated powder polymorphs, such as dehydration and melting, DSC thermogram was performed.

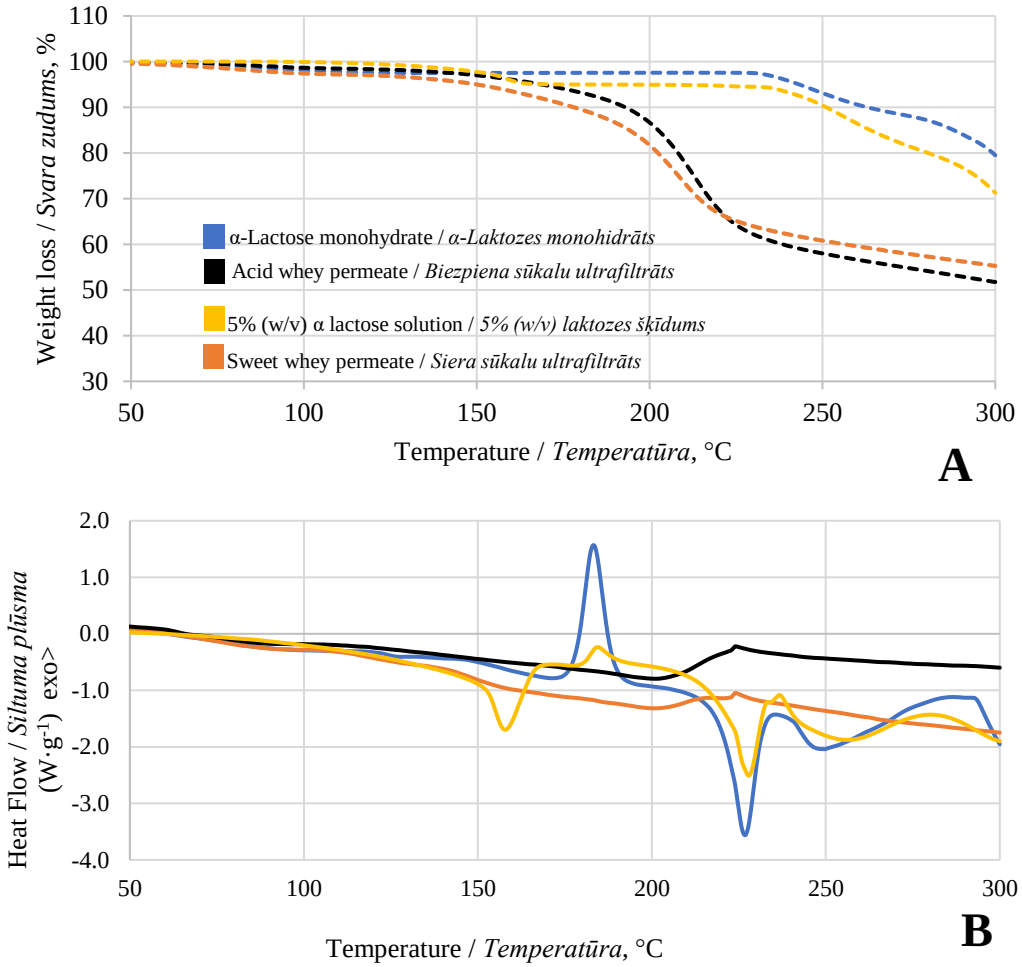


Fig. 3.15. Analysis of lactose crystal properties with DSK/TGA /
3.15 att. Laktozes kristālu īpašību analīze ar DSK/TGA
DSC thermogram (B) and TGA weight loss (A) / *DSK termogramma (B) un TGA masas zuduma (A) profils*

Both profiles show the physical properties of each sample and the differences among permeate powders and pure α-lactose monohydrate in Fig. 3.15. at the temperature range of 25 to 300°C. Fig. 3.15. B shows the two endothermic processes of the α-lactose monohydrate crystals. The first process is at approximately 158±0.5°C indicating dehydration of the crystallized water and 226±0.5°C is the melting point of α-lactose monohydrate before decomposition, the total weight loss of the sample was 18±0.5%, see Fig. 3.15. A. The dehydrated lactose thermogram in Fig. 3.15. B shows that the glass transition was started at temperature 58±1°C where at that particular moment the weight loss was 2±0.5%. Afterwards, recrystallisation appears at 183.5±0.5°C and melting at 227±0.5°C where total weight loss of the sample was 27.5±1%, see Fig. 3.15. A. This indicates that the crystal of α-lactose monohydrate contains about 5% water, which is less tightly bounded. The recrystallisation of α-lactose monohydrate and dehydrated lactose (Fig. 3.15. B) was observed at 184±1°C and, according to the reports of Badal Tejedor *et al.* (2018), the samples were transformed in the same physical form that was caused by the same melting point. During heat treatment, there is a possibility that lactose converts into α and β forms. The thermograms of dehydrated permeate powders (Fig. 3.15. B) showed similar behaviour. The first thermal process for permeate powder samples started in the range of 85–95°C then melting of samples took place at 202±2°C. During the DSC analysis, the weight loss of permeate powder samples, see Fig. 3.15 A, was observed in the interval of 40–50%.

The results indicate that the presence of other substances such as fats, proteins, salts, lactic acid decrease the thermal resistance and hydrolyse process of lactose.

Summary of Capter 3.2 / *3.2. Nodaļas kopsavilkums*

In this chapter the properties of dehydrated whey permeate were investigated in more detail and compared with commercially available pure lactose. According to literature, lactose is a sensitive sugar and can be easily affected by environmental conditions. The physical properties of dehydrated permeate powders are strongly influenced by the presence of substances in solids. The experiments showed that all the data of permeate powders were lower or considerably different from the results of commercial α-lactose monohydrate. According to the research findings, permeate powders do not contain highly pure α-lactose monohydrate, also the presence of other substances has been established, which affects the physical properties of the powder. On the other hand, the presence of salts in the permeate powder may be considered beneficial, if they are used as an additive to the substrate for lactose hydrolysis.

Šajā nodaļā sīkāk izpētītas sauso ultrafiltrātu īpašības un salīdzinātas ar tirdzniecībā pieejamo α-laktozes monohidrātu. Kopumā laktoze ir ļoti jutīgs cukurs un vides apstākļi to viegli ietekmē. Siera un biezpiena sūkalu ultrafiltrātu laktozes fizikālās īpašības galvenokārt ietekmē citu vielu klātbūtne. Visi eksperimenta rezultāti apstiprināja, ka sauso ultrafiltrātu laktozes īpašības ievērojami atšķirās no komerciālās α-laktozes monohidrāta īpašībām. Pētījumi pierādīja, ka sauso ultrafiltrātu laktoze nav ar augstu tīrības pakāpi. Turklāt sāļu klātbūtne sausajos ultrafiltrātos var būt efektīva, ja to izmantotu kā piedevu laktozes hidrolīzei noteiktā substrātā.

3.3 Yield of hydrolysis at various substrate solids concentrations / *Hidrolīzes iznākums dažādās substrāta sausnas koncentrācijās*

The aim of this study was to investigate β-galactosidases capability to hydrolyse lactose at different solids concentration of sweet and acid whey permeates and in the presence of different amounts of enzyme units. These results provide more accurate information about the substrate in which the commercial β-galactosidase is able to hydrolyse lactose to a maximal extent. Comparing the results of hydrolysis yield, it is shown that all enzyme unit amounts have a high perspective for hydrolysis at all permeate solids concentrations.

The results of hydrolysis yield (%) in both Tables 3.5. and 3.6. is also converted into g L^{-1}, see in Appendixe 2.

Table 3.5 illustrates the degree of hydrolysis in acid whey permeate, which was calculated as the percentage of lactose that is converted into galactose and glucose or used in the transgalactosylation reactions. The experiment was carried out using acid whey permeate with the initial lactose concentration 195±5 g L^{-1}, 274±17 g L^{-1} and 391±12 g L^{-1}.

Table 3.5. / *3.5. tabula*

Comparison of hydrolysis yield (%) in <u>acid whey permeate</u> at different concentrations of solids and enzyme units / *Hidrolīzes iznākuma salīdzinājums dažāda sausnas satura <u>biezpiena sūkalu ultrafiltrātā</u>, pievienojot dažādas enzīma aktivitātes vienības*

Enzyme units / *Enzīma vienības*, U mL^{-1}	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2
0.5	30±1^{c}	62±4^{a}	33±1^{b}	30±1^{b}	37±3^{a}	34±1^{a}	14±1^{b}	25±1^{a}	26±1^{a}
2.5	68±2^{c}	94±2^{a}	74±2^{b}	81±2^{b}	91±2^{a}	55±1^{c}	47±4^{b}	93±2^{a}	43±4^{b}
5	97±3^{a}	97±1^{a}	82±3^{b}	81±9^{a}	87±3^{a}	88±2^{a}	51±10^{b}	73±9^{a}	48±9^{b}
	20% solids / *20% sausna*			30% solids / *30% sausna*			40% solids / *40% sausna*		

The values marked with the same letter within each enzyme and dry matter concentration do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra enzīma un sausnas koncentrācijas ietvaros būtiski neatšķiras (p>0.05)*

Table 3.6. / *3.6. tabula*

Comparison of lactose hydrolysis (%) in <u>sweet whey permeate</u> at different concentrations of solids and enzyme units / *Laktozes hidrolīzes salīdzinājums dažāda sausnas satura <u>siera sūkalu ultrafiltrātā</u>, pievienojot dažādas enzīma aktivitātes vienības*

Enzyme units / *Enzīma vienības,* U mL $^{-1}$	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2
0.5	17±1^{b}	47±5^{a}	42±1^{a}	12±1^{b}	24±3^{a}	13±1^{b}	9±1^{b}	16±2^{a}	10±1^{b}
2.5	47±5^{c}	92±1^{a}	79±2^{b}	31±2^{c}	71±4^{a}	68±2^{a}	12±3^{c}	79±1^{a}	47±2^{b}
5	73±7^{b}	96±2^{a}	76±8^{b}	47±6^{b}	85±5^{a}	52±5^{b}	39±6^{b}	66±8^{a}	34±8^{b}
	20% solids / *20% sausna*			30% solids / *30% sausna*			40% solids / *40% sausna*		

The values marked with the same letter within each enzyme and dry matter concentration do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra enzīma un sausnas koncentrācijas ietvaros būtiski neatšķiras (p>0.05)*

After 4 hours of hydrolysis it was found that the lactose conversion was 97±3% and 81±9%, for permeate solids concentration 20% and 30%, respectively, using 5 U mL^{-1} of NOLA^{TM}Fit5500 β-galactosidase. In addition, using the same enzyme 2.5 U mL^{-1} for all solids concentrations of permeate, lactose hydrolysis was within an interval from 47±4% to 81±2%. Similar results were obtained with Ha-Lactase 5200 β-galactosidase where the degree of lactose hydrolysis was 91±2% and 94±2% at 20% and 30% solids concentration, respectively. Moreover, using 0.5 U mL^{-1} of Ha-Lactase 5200 the highest hydrolysis degree at all permeate concentrations was reached compared to other enzymes, within an interval from 25±1% to 62±4%. When using 2.5 and 5 U mL^{-1} of GODO-YNL2 β-galactosidase for all solids concentrations, degree of hydrolysis was within the interval of 43±4 to 88±2%, respectively. No significant ($p>0.05$) differences were observed between all enzymes using 5 U mL^{-1} and permeate solids concentration 30%.

The experiment was also carried out using sweet whey permeate with the initial lactose concentration 201±8 g L^{-1}, 304±27 g L^{-1} and 400±17 g L^{-1}. Analysing Table 3.6 data, the tendency can be observed that the percentage of lactose hydrolysis decreases by increasing the solids concentration of sweet whey permeate. The highest lactose hydrolysis using 2.5 and 5 U mL^{-1} of Ha-Lactase 5200 β-galactosidase for all permeate solids concentrations, was within the interval from 66±8% to 96±2% and for GODO-YNL2 from 34±8% to 79±2%, respectively.

The results of the study were analysed taking into consideration the concentration of final sugars. The results indicate that the highest lactose hydrolysis rate was observed at solids concentration of 20% using 2.5 and 5 U mL^{-1} of enzyme for both permeates. However, the results of Ha-Lactase 5200, which showed a similar feature at all solids concentrations using 2.5 U mL^{-1} of enzyme should be highlighted.

Martínez - Villaluenga *et al.* (2008) reported that after 120 min lactose hydrolysis was close to 80% using an initial lactose concentration 150 and 250 mg mL^{-1} and 3 U mL^{-1} of Lactozym 3000 L HP G (*Kluyveromyces lactis*) β-galactosidase. Another study from Rodriguez-Colinas *et. al.* (2014) showed that lactose hydrolysis of skimmed milk using *Kluyveromyces lactis* β-galactosidase (Lactozym pure 6500 L) was able to gain high hydrolysis results approximately 95% comparing to *Aspergillus oryzae* β-galactosidase (Lactase F) where hydrolysis percentage was only 43% and *Bacillus circulans* β-galactosidase (Biolactase NTL-CONC) - 50%.

Moreover, the solution of 10% KOH has an important role in lactose hydrolysis reaction. Jurado *et al.* (2004) reported that metal ions have an impact on β-galactosidase stability and activity. It has been found that monovalent K^+ ions serve as an activator for *Kluyveromyces lactis* β-galactosidase, but K^+ and Na^+ ions for *Bacillus licheniformis* β-galactosidase (Juajun *et al.*, 2011; Jurado *et al.*, 2004). During hydrolysis 10% KOH solution was used for pH control. It should be noted that the added volume of 10% KOH was different for each sample, but for GODO-YNL2 β-galactosidase to adjust an optimal condition in all permeate samples more 10% KOH was added compared to other enzymes. In the case of hydrolysis by NOLA^{TM}Fit5500 β-galactosidase the addition of 10% KOH solution promotes high results in all solids concentrations. Therefore, it was approved that K^+ ions activate *Bacillus licheniformis* β-galactosidase. According to European Regulation No. 231/2012 potassium hydroxide (KOH) has also been used as a food additive E525, which is safe and can be used for lactose hydrolysis. This implies that the composion of the permeate and the addition of the solution for pH adjustment stimulated the enzyme of the specific species to be more active (section 3.1.).

In addition, a comparison of the hydrolysis yield (%) in permeates showed that by using acid whey, a higher percentage of hydrolysis can be gained. Therefore, the most productive solids concentration for lactose hydrolysis is 20% and 2.5 U mL^{-1} of enzyme.

Tables 3.7 and 3.8 show glucose and galactose amount at different concentrations of permeate solids and β-galactosidase units.

Table 3.7. / *3.7. tabula*

Amount of glucose and galactose (g L^{-1}) after lactose hydrolysis using different sweet whey permeate solids concentration and enzyme units / *Glikozes un galaktozes saturs (g L^{-1}) pēc laktozes hidrolīzes, izmantojot dažāda sausnas satura siera sūkalu ultrafiltrātu un enzīmu vienības*

	20% solids / *20% sausna*			**30% solids / *30% sausna***			**40% solids / *40% sausna***		
Enzyme units / *Enzīma vienības*, U mL^{-1}	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2
Glucose / *Glikoze*, g L^{-1}									
0.5	17 ± 3^{a}	43 ± 3^{b}	38 ± 1^{b}	11 ± 2^{a}	25 ± 5^{b}	13 ± 3^{b}	6 ± 2^{a}	20 ± 1^{b}	7 ± 1^{a}
2.5	62 ± 2^{a}	105 ± 2^{b}	92 ± 3^{c}	34 ± 2^{a}	92 ± 9^{b}	86 ± 8^{b}	22 ± 2^{a}	152 ± 2^{b}	63 ± 7^{c}
5	100 ± 17^{ab}	137 ± 15^{a}	92 ± 4^{b}	85 ± 8^{a}	107 ± 23^{a}	89 ± 6^{a}	83 ± 7^{a}	192 ± 6^{b}	77 ± 4^{c}
Galactose / *Galaktoze*, g L^{-1}									
0.5	15 ± 1^{a}	26 ± 1^{b}	20 ± 1^{c}	11 ± 1^{a}	33 ± 2^{b}	13 ± 9^{a}	13 ± 2^{a}	29 ± 1^{b}	12 ± 1^{a}
2.5	19 ± 5^{a}	33 ± 3^{b}	25 ± 1^{c}	39 ± 4^{a}	30 ± 3^{b}	25 ± 5^{b}	15 ± 1^{a}	40 ± 2^{b}	25 ± 4^{a}
5	41 ± 4^{a}	44 ± 13^{ab}	28 ± 3^{b}	49 ± 15^{a}	35 ± 16^{a}	32 ± 13^{a}	28 ± 6^{a}	49 ± 9^{a}	51 ± 8^{a}

The values marked with the same letter within each enzyme and dry matter concentration do not differ significantly ($p>0.05$) / *Vērtības apzīmētas ar vienādu burtu katra enzīma un sausnas koncentrācijas ietvaros būtiski neatšķiras ($p>0.05$)*

Table 3.8. / *3.8. tabula*

Amount of glucose and galactose (g L^{-1}) after lactose hydrolysis using different acid whey permeate solids concentration and enzyme units / *Glikozes un galaktozes saturs (g L^{-1}) pēc laktozes hidrolīzes, izmantojot dažāda sausnas satura biezpiena sūkalu ultrafiltrātu un enzīmu vienības*

	20% solids / *20% sausna*			**30% solids / *30% sausna***			**40% solids / *40% sausna***		
Enzyme units / Enzīma vienības, U mL^{-1}	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2
Glucose / *Glikoze*, g L^{-1}									
0.5	15 ± 3^{a}	35 ± 3^{b}	19 ± 1^{c}	27 ± 4^{a}	42 ± 3^{b}	25 ± 3^{a}	12 ± 1^{a}	35 ± 2^{b}	19 ± 2^{c}
2.5	67 ± 3^{a}	88 ± 2^{b}	56 ± 3^{c}	62 ± 4^{a}	112 ± 5^{a}	92 ± 8	109 ± 9^{a}	208 ± 9^{b}	63 ± 6^{c}
5	87 ± 9^{a}	118 ± 6^{a}	70 ± 6^{a}	73 ± 12^{ab}	161 ± 5^{a}	117 ± 3^{b}	119 ± 7^{a}	122 ± 6^{a}	92 ± 6^{b}
Galactose / *Galaktoze*, g L^{-1}									
0.5	19 ± 11^{ab}	23 ± 1^{b}	11 ± 1^{a}	17 ± 1^{a}	30 ± 2^{b}	27 ± 1^{b}	17 ± 2^{a}	45 ± 3^{b}	32 ± 3^{c}
2.5	32 ± 2^{a}	35 ± 2^{a}	20 ± 2^{b}	40 ± 4^{a}	55 ± 2^{b}	28 ± 2^{c}	74 ± 6^{a}	93 ± 6^{b}	39 ± 3^{c}
5	93 ± 12^{a}	43 ± 4^{b}	38 ± 1^{b}	112 ± 6^{a}	68 ± 10^{b}	54 ± 8^{b}	57 ± 19^{b}	99 ± 6^{a}	58 ± 9^{b}

The values with the same letter within each enzyme and dry matter concentration do not differ significantly (p>0.05) / *Vērtības ar vienādu burtu katra enzīma un sausnas koncentrācijas ietvaros būtiski neatšķiras (p>0.05)*

In general, the hydrolysis of lactose and the formation of new sugars require the cleavage of the glycosidic bond (Paterson & Kellam, 2009). The concentration and profile of carbohydrates change over time and vary noticeably depending on the different permeate solids and salts concentrations (Warmerdam *et al.*, 2013). The results showed that the addition of 0.5 U mL^{-1} of enzyme to both permeates resulted in a low monosaccharide formation. Therefore, in sweet whey permeate glucose concentration was in the range of 6±2 to 43±3 g L^{-1} and galactose from 11±1 to 33±2 g L^{-1} but in acid whey permeate glucose from 12±1 to 42±3 g L^{-1} and galactose from 11±1 to 45±3 g L^{-1}. It should be emphasized that for both permeates with solids concentration of 40% using all three-enzyme unit amounts the concentration of galactose was higher than glucose. In many cases GODO-YNL2 β-galactosidase showed the lowest concentration of monosaccharides that leads to assume that at these particular conditions the hydrolysis should be prolonged. Ha-Lactase 5200 β-galactosidase showed the highest amount of monosaccharide production almost at all concentrations in both permeates. Moreover, comparing glucose and galactose concentrations between permeates it can be seen that by using a sweet whey permeate, the concentration of monosaccharides is higher than in acid whey permeate. In the results reported by Bozanic *et al.* (2014), acid whey has higher concentration of calcium, phosphate, lactic acid and lactate than in sweet whey which could be indicated as the main factor that strongly affects β-galactosidase activity and ability to hydrolyse lactose in acid whey. According to Demirhan *et al.* (2008), glucose and galactose at concentrations 10 g L^{-1} and 13 g L^{-1}, respectively, start acting as an inhibitor slowing down lactose hydrolysis reaction. For a complete lactose hydrolysis of 20% solids concentration permeate, time extension for the reaction would be an option.

Comparing the results and assuming the optimal conditions to obtain the glucose-galactose syrup, the highest percentage of hydrolysis was obtained at 20% solids concentration using 2.5 and 5 U mL^{-1} amount of enzymes. In the further work, 2.5 U mL^{-1} of enzyme was selected based on the profitable perspective and results from other research articles (Dutra Rosolen *et al.*, 2015; Martínez-Villaluenga *et al.*, 2008; Song *et al.*, 2013).

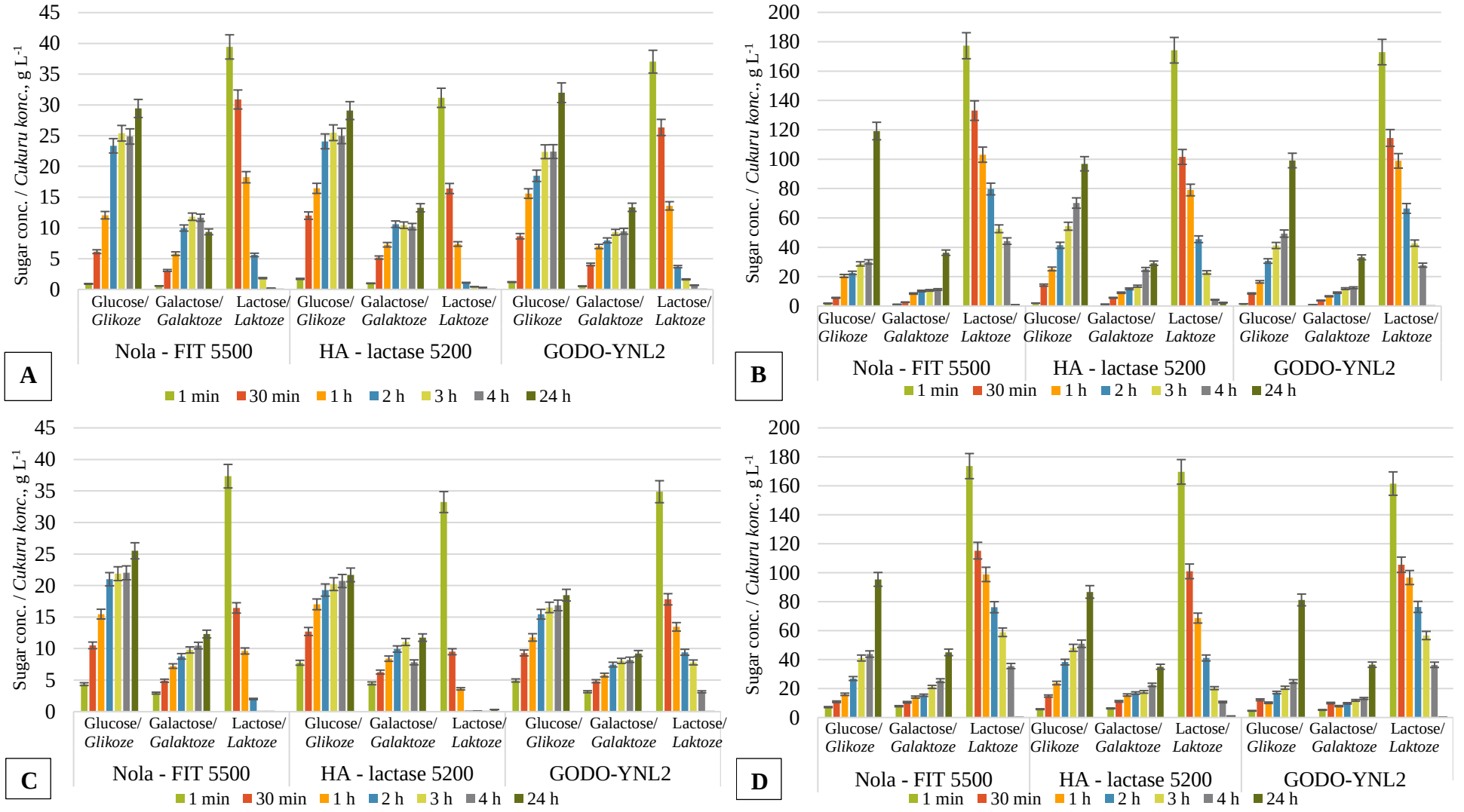


Fig 3.16 Sugar formation during lactose hydrolysis / *3.16. att. Cukuru veidošanās laktozes hidrolizē*

The experiment was performed for 24 hours using sweet whey permeate with initial solids concentration A - 5%; B – 20% and acid whey permeate C - 5%; D – 20%, enzyme units 2.5 U mL^{-1}, at 42.5 °C temperature, to see the differences in sugar concentrations. Similarity was observed at each permeate solids concentration. Using permeates with solids concentration 5% and 2.5 U mL^{-1} of enzyme, the concentration of lactose decreases rapid for up to 2 hours but for 20% solids permeates – 4 hours. The slowest reaction rate was observed for GODO-YNL2 and NOLA™Fit5500 β-galactosidases, where 24 hours should be considered for hydrolysis of permeate. Further decrease of lactose hydrolysis was not attained or it continued to decrease - it just became slower for the rest of the time. Complete hydrolysis of lactose by β-galactosidase is difficult to achieve, because the presence of the components of the products – galactose, glucose, permeate composition (salts, acids, proteins) inhibits β-galactosidase activity. Inhibition reduces the rate of hydrolysis and slows it down, which, in turn, prolongs the hydrolysis reaction to achieve at least 80% lactose hydrolysis (Mahoney, 1997). The hydrolysis properties of *Bacillus licheniformis* enzyme have been less investigated, and therefore information is limited to better understand the obtained results. In addition, lactose hydrolysis in sweet whey permeate resulted in higher concentration of monosaccharides compared with acid whey permeate samples.

The results of three enzymes Fig. 3.16. B and D showed that glucose concentration was higher than galactose during hydrolysis. After some period of time the amount of glucose and galactose in each substrate changes and is no longer equimolar (1:1). The conversion factor for the forming lactose into glucose and galactose ranged from 1.05 to 1.11 (Samadov *et al.*, 2019). Which means that the β-galactosidase starts to produce galacto-oligosaccharides under certain conditions (Mariyani *et al.,* 2015). This activity begins when the sugar concentration changes and the water activity becomes more optimal for the β-galactosidase side reactions (Suárez *et al.,* 2018). It should be noted that the conversion factor for the conversion of lactose into glucose and galactose ranged from 1.05 to 1.11. As the fermentation continued, glucose level decreased due to the formation of a new oligosaccharide

Summary of chapter 3.3 / *3.3. Nodaļas kopsavilkums*

The data obtained from this study revealed that the highest hydrolysis yield can be obtained at total solids concentration 20% and 2.5 U mL^{-1} of enzymes. Both substrates at certain concentration have different physical and chemical properties which influence the profile of final sugar outcome. Therefore, the high concentration of calcium, phosphate, lactic acid and lactate could be indicated as the main factors which strongly affect enzyme activity and capability of lactose hydrolyses in acid whey. The use of KOH for pH adjustement can be evaluated positively because it works as an activator for β-galactosidase. Almost all results showed that the dominant monosaccharide after hydrolysis of lactose is glucose. That could be explained based on the conversion factor and activation of the side reactions. Permeates with solids concentration of 5% absolute lactose hydrolysis can be achieved in up to 2 hours, and for a permeate with solids concentration of 20%, the optimal hydrolysis time would be 4 hours, but a complete process of lactose hydrolysis takes 24 hours.

Šī pētījuma rezultāti pierādīja, ka efektīvākā ultrafiltrāta sausnas koncentrācija laktozes hidrolīzei ir 20%, izmantojot 2.5 U mL^{-1} enzīma. Katram ultrafiltrātam ir atšķirīgas fizikālās un ķīmiskās īpašības, kas ietekmē β-galaktozidāzes aktivitāti. Augsta kalcija, fosfātu, pienskābes un laktātu koncentrācija ir faktori, kas ietekmē enzīma aktivitāti un to spēju hidrolizēt laktozi biezpiena sūkalu ultrafiltrātā. Izmantojot KOH vides pH kontrolei, var novērot, ka tas darbojas kā enzīma aktivātors. Gandrīz visi rezultāti parādīja, ka dominējošais monosaharīds laktozes hidrolīzē ir glikoze. To varētu izskaidrot, pamatojoties uz konversijas koeficientu un blakus reakciju aktivizēšanos. Ultrafiltrātā ar sausnas saturu 5% laktozes hidrolīze notiek 2 stundās, ultrafiltrātā ar sausnas saturu 20% 4 stundās, bet pilnīgai laktozes hidrolīzei ir nepieciešamas 24 stundas.

3.4 Glucose isomerisation for incresing the sweetness of syrup / *Glikozes izomerizācija sīrupa salduma palielināšanai*

To produce a sweeter syrup from whey permeates, first hydrolysis was performed and second, the hydrolysed permeates were used as a feedstock for glucose isomerisation to fructose by glucose isomerase. Following the supplier's recommendations and ensuring all parameters for a successful reaction, the permeate's pH was adjusted to 7.5 using 10% KOH. In general, the glucose isomerisation to fructose is a selective reaction which requires less energy where a smaller amount of by-products is produced and better taste was achieved comparing to chemical methods (Wang *et al.*, 2012).

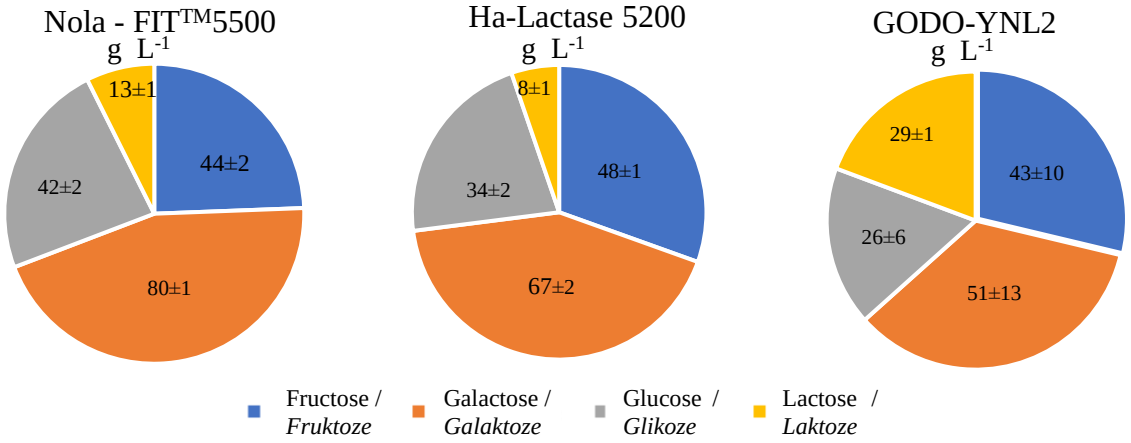


Fig. 3.17 Sugar concentration in sweet whey permeate using glucose isomerase / *3.17. att. Cukuru saturs siera sūkalu ultrafiltrātā, izmantojot glikozes izomerāzi*

The data presented in Fig. 3.17 shows the composition of isomerised products in sweet whey permeate samples where fructose was obtained within the range of 43±10 to 48±1 g L^{-1}.

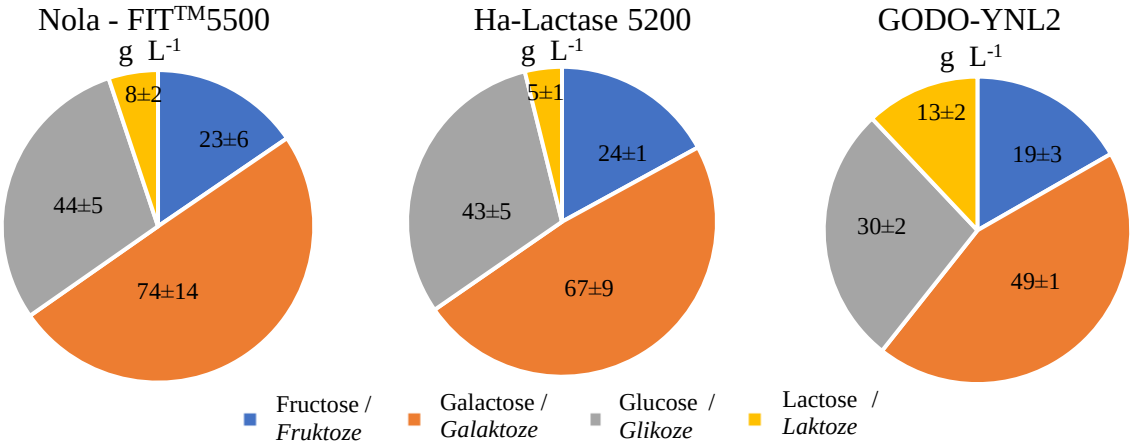


Fig. 3.18 Sugar concentration in acid whey permeate using glucose isomerase / *3.18. att. Cukuru saturs biezpena sūkalu ultrafiltrātā, izmantojot glikozes izomerāzi*

The experiment was carried out using sweet and acid whey permeate with the initial lactose concentration 205±3 g L^{-1} and 180±10 g L^{-1}, respectively. The data presented in Fig. 3.18 shows the concentrationt of fructose after isomerisation in acid whey permeate samples was within the range of 19±3 to 24±1 g L^{-1}. The use of β-galactosidase in this study was limited because it is difficult to achieve a complete hydrolysis of lactose due to the side reactions (Andrade *et al.*, 2020). Whey is a complex mixture of many ingredients, each of which has a significant effect on the hydrolysis of lactose. There are many studies (Elnashar & Yassin, 2009; Fernandes *et al.*, 2002; Fischer & Kleinschmidt, 2015; Pessela *et al.*, 2003; Rico-Rodríguez *et al.*, 2020; Van De Voorde *et al.*, 2014) where the rate of lactose hydrolysis was compared between pure lactose solution and whey permeate. Studies have shown that hydrolysis of lactose using soluble enzymes and taking into account the reaction parameters (temperature, pH,

substrate and enzyme concentration), the substrate composition and the inhibition factors that occur during the reaction would result in a hydrolysis range of 60 to 90%. It is reflected in our current experiments, see Fig 3.17 and Fig. 3.18. To improve and be able to achieve almost 100% hydrolysis of lactose, an appropriate immobilised enzyme and substrate pre-treatment can be used as an option to reduce the development of inhibition factor.

Comparing the amount of fructose between the permeates, the results indicate that fructose formation was observed higher in sweet whey permeate than in acid whey permeate. The highest fructose amounts 48±1 (Fig. 3.17) and 24±1 (Fig. 3.18) g L^{-1} were obtained in the samples where permeates were isomerised using Ha-Lactase 5200 β-galactosidase, and 44±2 (Fig. 3.17) and 23±6 (Fig. 3.18) g L^{-1} NOLA^{TM}Fit5500 β-galactosidases. Moreover, permeate samples with GODO-YNL2 β-galactosidase showed the lowest amounts of monosaccharides but the highest of lactose.

One of the factors which could cause less formation of fructose in acid whey permeate might be the presence of metal ions. Li et. al. (2017) study showed that the major divalent metal ions that inhibit glucose isomerase activity even at a small concentration was Ca^{2+}. Table 3.1 demonstrates Ca^{2+} concentration in both permeates at solids concentration of 20% and it shows that acid whey permeate contains three times more 3400±86 mg kg^{-1} Ca^{2+} ions than sweet whey permeate 1071±99 mg kg^{-1}. Acid whey is a by-product obtained from acid coagulated milk products, including fresh cheese, Greek style yoghurt and casein. This type of whey contains more salts because colloidal calcium and inorganic phosphate are dissolved during acidification of milk (Tanguy *et al.,* 2019). In addition, during processing, variations in pH and temperature and different starting cultures can cause fluctuations in the concentration of calcium, phosphate and lactic acid in whey (Chandrapala *et al.,* 2015).

Another factor might be the reaction mechanism which displays that glucose-fructose enzymatic isomerisation is reversible (Dehkordi *et al.,* 2009). Under certain conditions fructose can be transformed back to glucose. This shows that glucose isomerase is sensitive and can be easily impacted and the whey substrate composition and reaction conditions are very important for fructose formation.

Glucose isomerase from *Streptomyces murinus* is described as hyperthermophilic where the optimal conditions for the reaction are pH 7.0 – 7.5 and temperature 60 – 70 °C (Bandlish *et. al.,* 2002). Comparing the amount of glucose and galactose obtained before glucose isomerisation Table 3.7 and 3.8 and after Figure 3.17 and 3.18, there are noticeable changes. There are several reasons why it had happened and the first one is that the β-galactosidases were not inactivated. The permeate pH and temperature were changed immediately after 4 hours of hydrolysis. During the increase in pH and temperature β-galactosidase had time to continue reaction. The second reason, the glucose isomerase reaction conditions can not fully inactivate β-galactosidase activity, for example NOLA^{TM}Fit5500, therefore the enzyme was still capable to be active. The third reason, 10% KOH addition increases the permeate's pH till 7.5 and plays an important role in enzyme productivity. Foda & El-Rahman (2000) showed that monovalent ions such as Na^+ and K^+ work as activators for glucose isomerase (Sweetzyme type-T originated from *Streptomyces murinus)*. The highest β-galactosidase activity 86.48% was reached at 0.04 M concentration of K^+ ions. Unfortunately, due to the increase in K^+ ions concentration to 0.07 M, the enzyme activity of lactose hydrolysis was less effective -70.05%, and this is in the agreement with our results. The added volume of 10% KOH solution for glucose isomerase reactions to acid whey permeate was higher than for sweet whey permeate, see Table 3.9.

Hydrolysis of lactose to glucose and galactose would ultimately increase the sweetness of the syrup, but the taste would not be very noticeable. Isomerisation of glucose to fructose would create glucose-galactose-fructose syrup and significantly increase the sweet taste. The two-stage fermentation of permeate have shown that reducing lactose and replacing it with other sugars can significantly improve the sweetness and overall sensory properties of the final product. There are several authors who consider (Araya *et al.,* 2019; Cervantes *et al.,* 2020; Cheng *et al.,* 2020; Luzzi *et al.,* 2020) that fermentation technology allows better use of

resources and milk waste to be transformed into a valuable food sweetener with many possible uses.

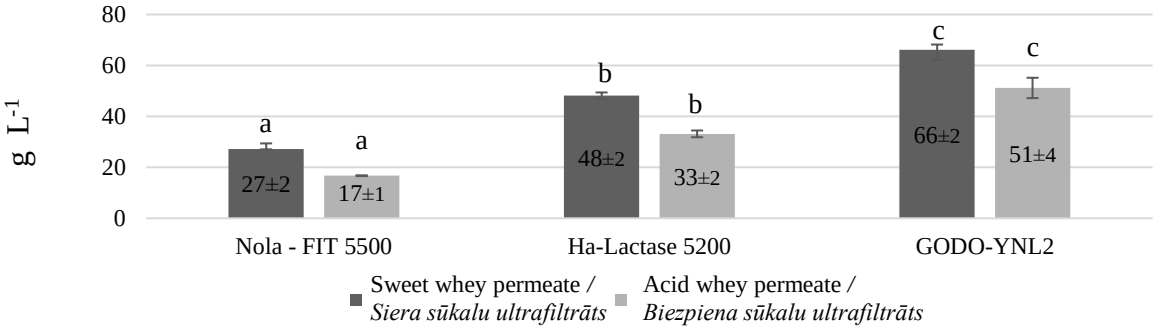


Fig. 3.19 Concentration of total galacto-oligosaccharides (g L^{-1}) in samples / *3.19. att. Kopējais galakto-oligosaharīdu saturs (g L^{-1}) paraugos*

The values marked with the same letter within each enzyme do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra enzīma ietvaros būtiski neatšķiras (p>0.05)*

Overall, the obtained GOS amount in hydrolysed acid whey permeate varied in the interval of 17±1 to 51±4 g L^{-1} but in sweet whey permeate from 27±2 to 66±2 g L^{-1}. GOS synthesis was considerably lower in acid whey permeate. Our experiment showed that GODO-YNL2 β-galactosidase has the highest activity of GOS production, but NOLA^{TM}Fit5500 β-galactosidase the least activity. The yield of GOS is highly dependent on temperature, pH, reaction time, source of β-galactosidase, and the initial concentration of lactose. In addition, the transgalactosylation reaction may be intensified due to low water activity (Botvynko *et al.*, 2019). Luzzi *et al.* (2020) used four commercial β-galactosidase preparations for lactose hydrolysis as well as for GOS production, and their results indicated that the lowest GOS yield 71 g L^{-1} was obtained using NOLA^{TM}Fit5500 β-galactosidase which is also in agreement with our results. In turn, Andrade et al. (2020) findings showed that, using GODO-YNL2 β-galactosidase for GOS synthesis in goat's milk, 5.9±1.0% GOS was obtained, which is close to our results. Overall, these results emphasise the ability of each β-galactosidase to form GOS with different structures and the possibility to produce GOS with potentially different prebiotic properties (Corzo-Martínez *et al.*, 2013). Based on the origin of β-galactosidase, the enzyme which is obtained from *Kluyveromyces lactis* produces β-(1 →6) galactooligosaccharides mainly 6′-galactosyllactose, allolactose and 1-6-β-D-galactobiose but from *Bifidobacterium bifidus* primarily GOS is produced with a β-(1→3) glycosidic bond (Gänzle, 2012).

To determine the most productive β-galactosidase preparation for GOS synthesis in sweet and acid whey permeates, each GOS amount was expressed as percentage of total amount of GOS in sample.

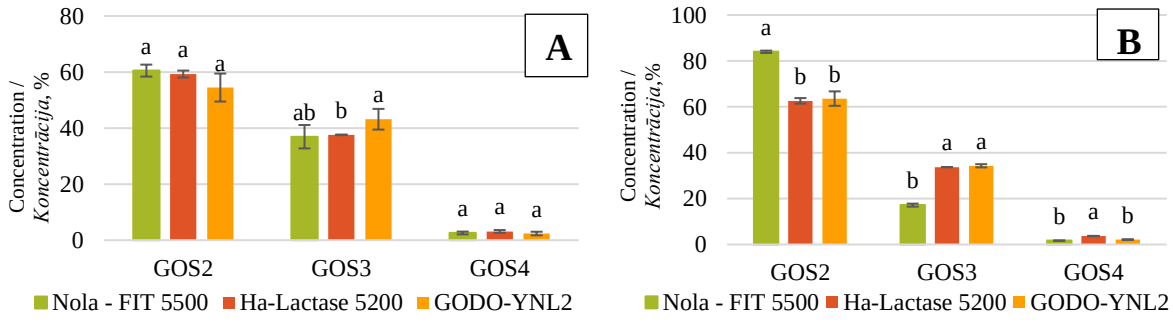


Fig. 3.20 Galacto-oligosaccharides concentration (%) in A – sweet whey permeate and B-acid whey permeate samples / *3.20. att. Galakto-oligosaharīdu saturs (%) A – siera sūkalu ultrafiltrāta un B – biezpiena sūkalu ultrafiltrāta paraugos*

The values marked with the same letter within each galacto-oligosaccharide (GOS) do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra galakto-oligosaharīda (GOS) ietvaros būtiski neatšķiras (p>0.05)*

As can be seen in Fig. 3.20, each enzyme has a specific transgalactolytic activity. The GOSs are the short-chain carbohydrates that are composed of 2—20 molecules of galactose and one molecule of glucose (Tokošová *et al.*, 2015). All β-galactosidases produce mainly disaccharides (GOS2) (which could be galactobiose, allolactose) and trisaccharide (GOS3) (which could be 6′ galactosyl lactose). A small concentration of tetrasaccharides (GOS4) can also be detected. All enzymes showed that in sweet whey permeate more GOS can be produced. The maximum yield of GOS2 was obtained in acid whey permeate by NOLA^{TM}Fit5500 β-galactosidase 84±1% and GOS3 by GODO-YNL2 and Ha-Lactase 5200 β-galactosidases 34±1 and 37±4%, respectively. Comparing the GOS types, enzymes showed the tendency to produce large amount of GOS2 in each whey permeate. The lactose isomer, allolactose, is a disaccharide that is similar to lactose, where instead of β-1→6 glycosidic bond is β-1→4 bond. The study revealed that the β-galactosidase has an additional role in bond modification (Otieno, 2010; Osman, 2016).

Fischer & Kleinschmidt (2015) reported that the transgalactosylation behaviour of β-galactosidase originated from *Klyveromyces lactis* strongly depends on the concentration of salts which initially is in whey and their ratio. A significant effect on enzyme activity can be observed in case of the addition of a 10% KOH solution, which was used to adjust the pH of the substrate, thus increasing K^+ concentration. The effect of metal ions on β-galactosidase activity is based mainly on ionic radius and the enzyme structure is also affected (Rajakala & Selvi, 2006).

The effect of added fructose on acid whey permeate hydrolysis was investigated.

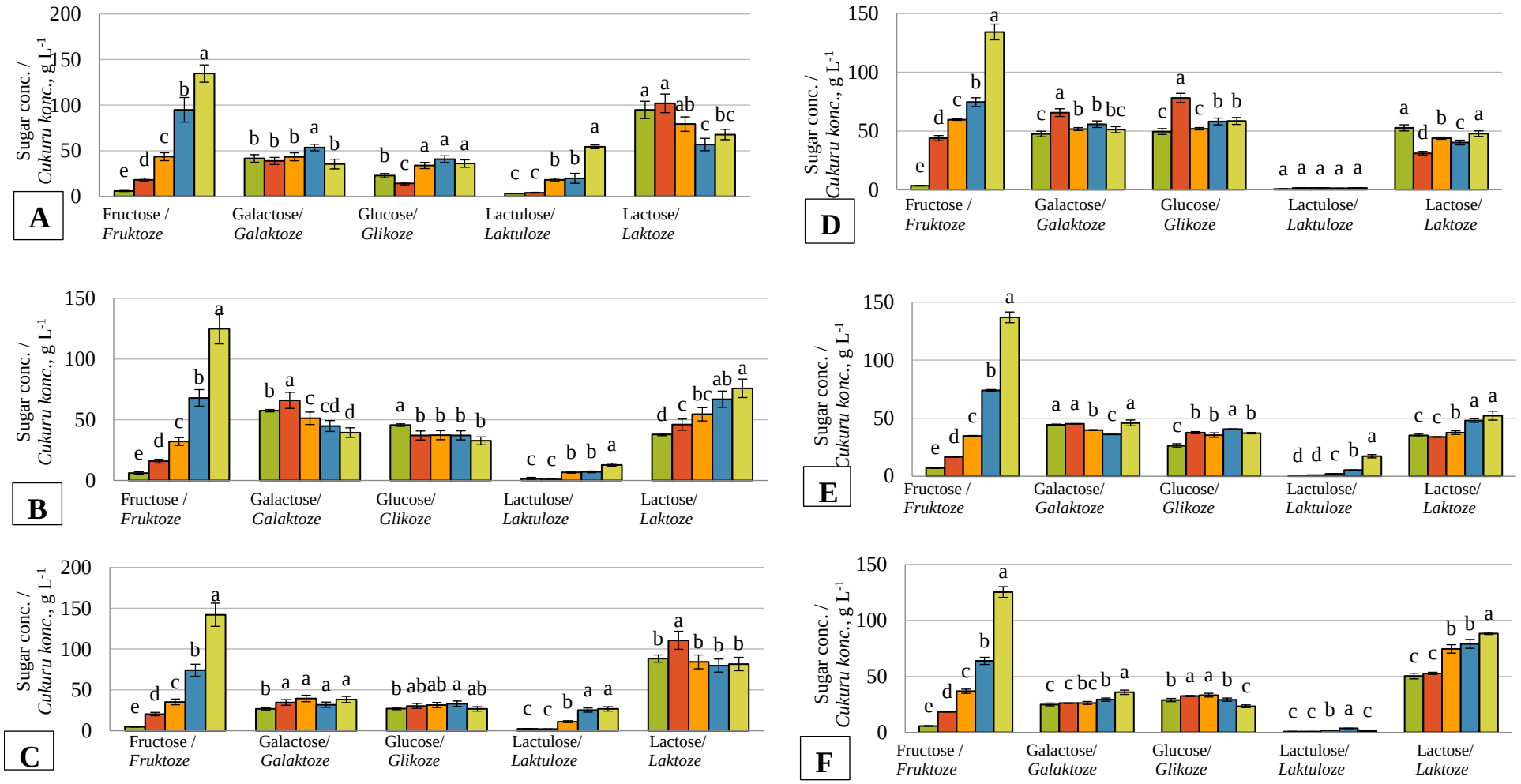


Fig. 3.21. Sugars content (g L^{-1}) after sweet and acid whey permeate hydrolysis with addition of different fructose amounts / 3.21. att. *Cukuru saturs (g L^{-1}), hidrolizējot siera un biezpiena sūkalu ultrafiltrātu ar dažādu pievienoto fruktozes saturu*

The values marked with the same letter within each sugar do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra cukura ietvaros būtiski neatšķiras (p>0.05).* Sweet whey permeate / *Siera sūkalu ultrafiltrāts* (A- NOLA™Fit5500; B- Ha-Lactase 5200; C- GODO-YNL2). Acid whey permeate / *Biezpiena sūkalu ultrafiltrāts* (D- NOLA™Fit5500; E- Ha-Lactase 5200; F - Ha-Lactase 5200).

The experiment was carried out using acid whey permeate with the initial lactose concentration 166±7 g L^{-1}. Fig.3.21. (D; E; F) summarizes the amount of sugars (g L^{-1}) after 4 hours of permeate hydrolysis with solids concentration of 20%. All enzymes showed the following trend – the higher amount of fructose was added, the higher amount of lactulose was formed. The synthesis of lactulose by NOLA^{TM}Fit5500 β-galactosidase (D) was within the range of 1±0.1 to 2±0.1 g L^{-1}, Ha-Lactase 5200 (E) within the range of 1±0.2 to 17±1 g L^{-1} and GODO-YNL2 (F) within the range of 1±0.1 to 3±0.1 g L^{-1}. It was observed that in almost every sample there was no significant difference between the amount of glucose and galactose. The concentration of these two monosaccharides is quite close. This study suggests that the presence of fructose affects β-galactosidase, making it more active and stable during the lactose hydrolysis to lactulose than the transition to the transgalactosylation reactions. In general, the results show that *Kluyveromyces lactis* β-galactosidase is capable to synthesize higher amounts of lactulose in acid whey permeate compared to *Bacillus licheniformis* β-galactosidase.

Fig. 3.21. also summarises the amount of sugars (g L^{-1}) after 4 hours of sweet whey permeate hydrolysis (A; B; C) with solids concentration of 20%. The experiment was carried out using permeate with the initial lactose concentration 172±5 g L^{-1}. The results showed that synthesis of lactulose by NOLA^{TM}Fit5500 (A) was within the range of 3±0.3 to 54±2 g L^{-1}, Ha-Lactase 5200 (B) within the range of 1±0.2 to 13±1 g L^{-1} and GODO-YNL2 (C) within the range of 2.57±0.26 to 26.82±2.68 g L^{-1}. This indicates that it is possible to synthesize more lactulose using sweet whey permeate than acid whey permeate. The amounts of galactose and glucose differ from acid whey permeate. Almost all samples had higher levelsof galactose than glucose, indicating that sweet whey permeate is a valuable substrate for higher lactulose synthesis.

Both experiments showed that the concentration of lactulose depends on the amount of added fructose. This observation is consistent with Seok *et al.* (2013) a report where it was found that fructose concentration strongly influenced lactulose synthesis. In addition, the authors pointed out that glucose and galactose concentrations serve as β-galactosidase inhibitors and small amounts of lactulose can be obtained. Fig. 3.21. (A; B; C) shows that glucose and galactose concentrations are higher than it was presented in the results shown in Fig. 3.21. (D; E; F), but lactulose concentration was lower in acid whey permeate samples (D; E; F) (Vera *et al.*, 2011) indicated that galactose is a competitive inhibitor and inhibits the activity of enzyme.

3.4.1 The effect of the pH on permeate properties / *Vides pH ietekme uz ultrafiltrāta īpašībām*

During the two-stage fermentation, it was important to observe any changes in whey permeates. Permeate is a medium that contains several components, each of which affects the hydrolysis and isomerisation reactions.

Fig. 3.22 Hydrolysed sweet – A and acid – B whey permeates /
3.22. att. Hidrolizētie siera – A un biezpiena – B sūkalu ultrafiltrāti

It was observed that increasing the 10% KOH amount in permeates, they began to lose their clarity obtaining higher turbidity (Fig. 3.22), the cause of that being higher electrostatic repulsive forces between protein particles (Rasouli *et al.*, 2020). The samples showed that the

more 10% KOH is added the whiter the permeate becomes and it also showed higher stickiness. Similar observations were made by Vargas-Díaz *et al.* (2019) where authors concluded that if there was variation with sweetened condensed milk pH, samples with different texture was obtained. The variation with pH spread the electric charge of the proteins and improved the interaction between them. The degree of ionization of amino groups (-NH_2) and carboxyl groups (-COOH) of the protein molecules depend on the pH and ionic strength of the surrounding aqueous phase (Sriprablom *et al.*, 2019). The volume of added 10% KOH significantly affects the characteristics of permeates.

Table 3.9 / *3.9. tabula*

Summary of added 10% KOH volume (mL) for the adjustment of sample pH / *Kopsavilkums par pievienotā 10% KOH daudzumu (mL), parauga vides pH standartizēšanai*

Permeate / *Ultrafiltrāts*	**Enzyme / Enzīms**	**Added 10% KOH, mL to 100 mLpermeate / *Pievienotais 10% KOH, mL 100 mL^{-1} ultrafiltrāta***	**K (potassium) / *K (kālijs)*, g**
For hydrolysis / *Hidrolīzei*			
Sweet / *Siera*	Nola^{TM}FIT 5500	0.0±0.0	0.0±0.0
	Ha-Lactase 5200	3.3±0.1	0.3±0.1
	GODO-YNL2	5.1±0.7	0.4±0.1
Acid / *Biezpiena*	Nola^{TM}FIT 5500	0.0±0.0	0.0±0.0
	Ha-Lactase 5200	6.6±0.2	0.5±0.1
	GODO-YNL2	8.3±1.1	0.6±0.1
For isomerisation / *Izomerizācijai*			
Sweet / *Siera*	Nola^{TM}FIT 5500	4.7±0.3	0.3±0.2
	Ha-Lactase 5200	1.9±0.2	0.1±0.1
	GODO-YNL2	0.0±0.0	0.0±0.0
Acid / *Biezpiena*	Nola^{TM}FIT 5500	7.8±0.2	0.6±0.1
	Ha-Lactase 5200	3.1±0.1	0.2±0.1
	GODO-YNL2	0.0±0.0	0.0±0.0

Table 3.9 represents the volume (mL) of 10% KOH and as well as added potassium (K) amount (g) was calculated which was added to samples before reaction. One of the main reasons for adding specific volumes of 10% KOH was the buffering capacity of the whey. Whey permeate contains some proteins with good buffering capacity that can capture free hydroxyl ions in positively charged amino groups (Aider & Gimenez-Vidal, 2012). Another reason is the presence of lactic acid, where it is more noticeable for acid permeate than for sweet permeate (Panesar *et al.*, 2007).

The presence of brown colour Fig. 3.23 B and C indicated that during the isomerisation the Mailard reaction was activated, where the sugar reacted with the amine group as a result of a feedback reaction to form Amadori compounds, which are responsible for food flavor and aroma (Coté *et al.*, 2004). The peculiarity of glucose isomerase reaction was the temperature of 70° C where there is a high possibility that monosaccharides would start to caramelise and it is also an impact factor of sample browning (Ajandouz *et al.*, 2008).

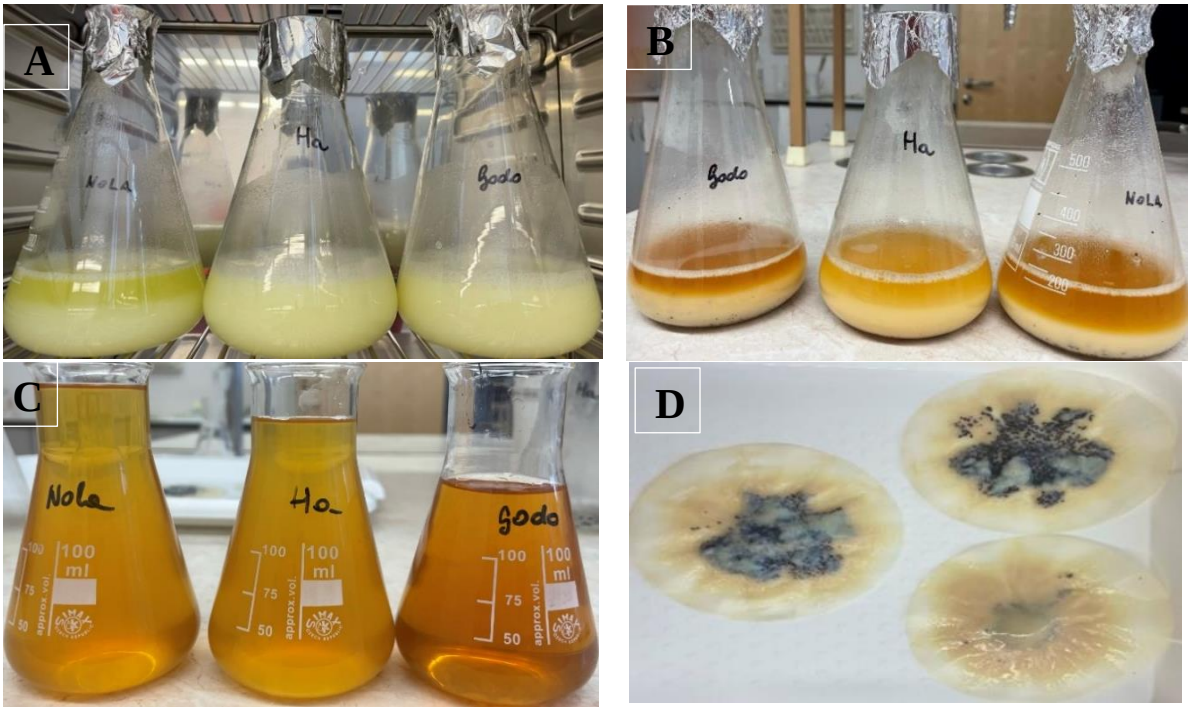


Fig. 3.23 Acid whey permeate processing into GGS; A – after hydrolysis; B – after isomerisation; C – after filtration; D - sediments in filtration / *3.23. att. Biezpiena ultrafiltrāta pārstrāde GGS; A - pēc hidrolīzes; B - pēc izomerizācijas; C - pēc filtrēšanas; D – nogulsnes*

In Fig 3.23 D the sediments are shown after isomerisation, where the black spots are glucose isomerases and light brown spots are β-galactosidase biomass and denatured whey proteins.

The total protein concentration increased during the two-stage fermentation. Whey permeates are chemically complex compounds and even during the ultrafiltration process most of the proteins were removed, some nitrogen, typically 0.7%, wt wt^{-1}, on a solid basis, is present as protein and non protein nitrogen compounds (Coté *et al.*, 2004). Suitable ultrafiltration membranes with a smaller pore sizes or nanofiltration should be used to maximally remove protein and non proterin nitrogen compounds.

It is important to notice these changes during the two-stage fermentation, as this shows that it is possible to increase the nutritional value and sweetness of GGS.

Summary of Chapter 3.4 / *3.4. Nodaļas kopsavilkums*

The data from this study showed that comparing the amount of fructose between permeates, the results indicate that by using sweet whey permeate, higher fructose yield can be obtained than using acid whey permeate. The glucose isomerase is sensitive and can be easily affected by the conditions of the reaction, so the substrate composition and the conditions of the reaction are very important for the formation of fructose. In terms of GOS production, all enzymes showed that higher amounts of GOSs could be produced in sweet whey permeate, and GODO-YNL2 has the highest GOS formation activity but NOLA^{TM}Fit5500 β-galactosidase the lowest. The origin of β-galactosidases plays a key role in the production of GOSs, since it affects the reaction conditions and the mechanism of the reaction. The results of lactulose production showed that sweet whey permeate is a valuable substrate for higher lactulose synthesis. In order to increase the purity of GGS, suitable ultrafiltration or nanofiltration membranes should be used to avoid the turbidity and browning of the substrate.

Salīdzinot fruktozes saturu starp ultrafiltrātiem, rezultāti norāda, ka, izmantojot siera sūkalu ultrafiltrātu, fruktozi var iegūt vairāk nekā no biezpiena sūkalu ultrafiltrāta. Glikozes izomerāze ir jutīga un reakcijas apstākļi to var viegli ietekmēt, tādējādi substrāta sastāvs ir ļoti nozīmīgs fruktozes veidošanai. Sintezējot GOS, visi enzīmi parādīja, ka, izmantojot siera sūkalu

ultrafiltrātu, var iegūt ievērojamu GOS saturu. Visaugstākā GOS veidošanās aktivitāte ir GODO-YNL2, bet mazāka aktivitāte ir NOLA™Fit5500 β-galaktozidāzei. GOS sintēzē galvenā loma ir β-galaktozidāzes izcelsmes avotam, jo tas ietekmē reakcijas apstākļus un reakcijas mehānismu. Siera sūkalu ultrafiltrāts ir vērtīgs substrāts, lai iegūtu lielāku laktulozes saturu. Lai palielinātu GGS tīrību, jāizmanto piemērotas ultrafiltrācijas vai nanofiltrācijas tehnoloģijas, kas palīdzētu izvairīties no substrāta duļķainības un brūnēšanās iespējas.

3.5 The sensory evaluation of syrups / *Sīrupu sensorā novērtēšana*

The intensity of sensory attributes of hydrolysed sweet whey permeate samples and control sample are given in Table 3.10.

Table 3.10 / *3.10 tabula*

Sensory attributes intensity of hydrolysed sweet whey permeate samples /
Hidrolizēto siera sūkalu ultrafiltrāta paraugu sensoro īpašību intensitāte

Attribute / *Īpašība*	**Control**	**NOLA™Fit5500**	**Ha-Lactase 5200**	**GODO-YNL2**
Sweet taste / *Saldā garša*	7.2^{b}	8.6^{a}	8.6^{a}	7.5^{b}
Sour taste / *Skābā garša*	1.0^{a}	1.9^{b}	1.2^{a}	1.1^{a}
Salty taste / *Sāļā garša*	1.2^{b}	1.9^{a}	1.8^{a}	1.7^{a}
Aftertaste / *Pēcgarša*	2.8^{b}	7.2^{a}	6.6^{a}	7.0^{a}

The values marked with the same letter not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu būtiski neatšķiras (p>0.05)*

Sweet whey permeate samples with NOLA™Fit5500 and Ha-Lactase 5200 β-galactosidases were evaluated similarly at the score 8.6 (out of 12), which were significantly ($p<0.05$) sweeter compared to the control and GODO-YNL2 samples, see Table 3.10. This indicates, that based on the obtained results the concentration of solids used and enzyme units were effective to increase sweetness. Chromatographic results showed that the control sample contains 73±3 g L^{-1} glucose, 59±1 g L^{-1} galactose, 15±4 g L^{-1} lactose and 27±5 g L^{-1} GOS. In Tables 3.7 and 3.8 it can be found that the amount of glucose in hydrolysed permeate samples ranged from 62 to 105 g L^{-1} and galactose amount 19 to 33 g L^{-1}. Moreover, the sour taste intensity differed significantly ($p<0.05$) for the NOLA™Fit5500 sample and was rated at 1.9 (out of 12), while the other samples ranged from 1.0 to 1.2. Salty taste intensity did not differ significantly between hydrolysed samples ($p>0.05$) except for NOLA™Fit5500 sample. Overall, the sour and salty taste intensity was scored below 2 (out of 12), indicating a weak presence of this taste compounds in the samples. The concentration of lactic acid in sweet whey is significantly lower compared to acid whey and the range is 0.5 to 2.0 g L^{-1} (Panesar *et al.*, 2007; Fischer, Kleinschmidt, 2015; Merkel *et al.*, 2021). The difference in aftertaste was significant ($p<0.05$) and all hydrolysed samples were scored from 6.6 to 7.2 but the control 2.8.

The mean scores of hydrolysed sweet whey permeate samples and commercial syrup are shown in Table 3.11.

Table 3.11 / *3.11 tabula*

Sensory attributes intensity of hydrolysed acid whey permeate samples /
Hidrolizēto biezpiena sūkalu ultrafiltrāta paraugu sensoro īpašību intensitāte

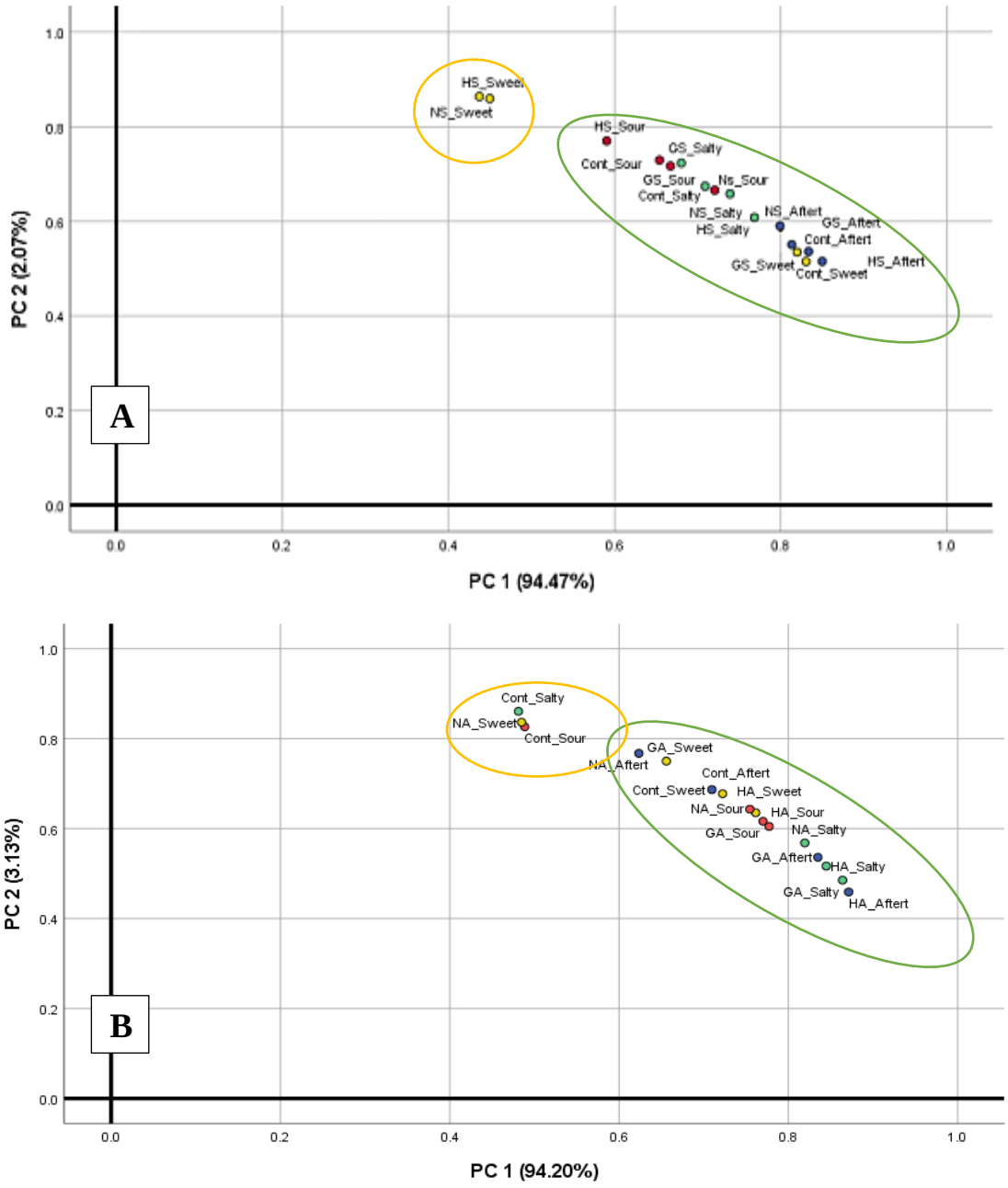
Attribute / *Īpašība*	**Control**	**NOLA^{TM}Fit5500**	**Ha-Lactase 5200**	**GODO-YNL2**
Sweet taste / *Saldā garša*	7.2^{a}	2.5^{c}	4.3^{b}	3.9^{bc}
Sour taste / *Skābā garša*	1.0^{c}	6.6^{a}	4.2^{b}	4.3^{b}
Salty taste / *Sāļā garša*	1.2^{b}	6.2^{a}	6.5^{a}	6.1^{a}
Aftertaste / *Pēcgarša*	2.8^{b}	3.3^{b}	5.6^{a}	6.0^{a}

The values marked with the same letter not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu būtiski neatšķiras (p>0.05)*

All samples showed significant difference (p<0.05) in sweet taste intensity where hydrolysed samples have low scores within the range of 2.5 to 4.3 but the control has the highest score 7.2 (out of 12). It should be indicated that NOLA^{TM}Fit5500 sample has the lowest score (2.5) in sweet taste intensity and the highest score in sour taste intensity (6.6). The salty taste intensity of all hydrolysed samples was not significantly different (p>0.05) and scored from 6.1 to 6.5 compared to the control 1.2 (out of 12). Aftertaste intensity showed that the control sample and NOLA^{TM}Fit5500 sample were 2.8 and 3.3, respectively, and did not differ significantly (p>0.05) but Ha-Lactase 5200 and GODO-YNL2 samples were 5.6 and 6.0, respectively, and did not differ significantly as well (p>0.05).

One of the main differences between the hydrolyzed permeate samples Tables 3.10 and 3.11 is that the samples obtained from acid whey have a high salty and low sweet taste intensity. The factors which need to be specified are – the source of whey permeate, pH and the volume of added 10% KOH. Mainly acid whey permeate can be obtained from fermented milk products (cottage cheese, Greek yoghurt) and after ultrafiltration, protein and fat fractions are removed but lactic acid and salts are untouched which is the reason of low pH and salty and acidic taste (Merkel *et al.*, 2021; Talebi *et al.*, 2020). The data in Table 3.5 and 3.6 show that hydrolysis can be reached above 60% and monosaccharide level is considerable, however, the panellists scored sweet taste intensity lower 2.5 to 4.3 than sour intensity 4.2 to 6.6 despite the fact that the pH of the medium was close to neutral. Optimal pH for each enzyme is different. The experiment with NOLA^{TM}Fit5500 β-galactosidase was carried out at pH 5.6±0.1 which could explain the lowest mean score in sweet taste and the highest in sour taste intensity. Wen's *et al.* (2020) report has a summary of several researches where K^{+} ions were described as a contributor to the salty taste. The findings of the report explain the results of the evaluation of salty taste of sweet and acid whey permeates.

The results of the principal component analysis (PCA) are presented in Figure 3.24 A where the hydrolysed sweet whey permeate samples are shown, and Figure 3.24 B represents the hydrolysed acid whey permeate samples. The KMO sampling adequacy measure for the PCA in Figure 3.24 A was 0.9 and for PCA in Figure 3.24 B 0.8. All correlated variables showed a direct relationship.



Cont - Control /; **NA** - NOLA^{TM}Fit5500; **HA** - Ha-Lactase 5200; **GA** - GODO-YNL2
Kontrole **NS** - NOLA^{TM}Fit5500; **HS** - Ha-Lactase 5200; **GS** - GODO-YNL2

Fig. 3.24 Principal component analysis of the sensory attributes (● sweet, ● sour, ● salty and ● aftertaste) intensity of hydrolysed A – sweet and B – acid whey permeates samples (n = 36) / *3.24. att. Galveno komponentu analīze, analizējot hidrolizēto A - siera un B - biezpiena sūkalu ultrafiltrāta paraugu sensoro īpašību intensitāti (● salda, ● skāba, ● sāļa un ● pēcgarša) (n = 36)*

Principal Component Analysis (PCA) in Fig. 3.24. A showed that the first two components of the PCA analysis (PC1 and PC2) explained the 96% variation in sensory evaluation between control and sweet whey permeate samples. The first component (PC1) explained 94% of the data variation, showing that green-lined attribute scores of samples were quite similar. While the second component (PC2) explained only 2%, indicating that yellow-lined NOLA^{TM}Fit5500 and Ha-Lactase 5200 samples had the highest intensity of sweet taste.

In contrast Fig. 3.24 B results showed that the first two components of the PCA analysis (PC1 and PC2) explained the 97% variation in sensory evaluation between control and acid

whey permeate samples. The first component (PC1) explained 94% of the data variation, showing that the green-lined attribute scores of samples were similar. While the second component (PC2) explained only 3%, indicating that yellow-lined samples had the lowest intensity of sweet taste NOLA^{TM}Fit5500 sample and salty and sour taste intensity control sample.

After hydrolysis, samples were isomerised with glucose isomerase and evaporated till solids concentration of up to 70%. Panellists performed evaluation according to the method which was described in the Materials and Methods (see Section 2.8). Sensory attributes – sweet, sour, salty taste and aftertaste – intensity of isomerised sweet whey permeate syrups are given in Table 3.12.

Table 3.12 / *3.12 tabula*

The sensory properties of sweet whey permeate syrups made in two-stage fermentation / *Divpakāpju fermentācijā iegūto siera sūkalu ultrafiltrāta sīrupu sensorās īpašības*

Attribute / *Īpašība*	**Control**	**NOLA^{TM}Fit5500**	**Ha-Lactase 5200**	**GODO-YNL2**
Sweet taste / *Saldā garša**	7.4^{b}	9.2^{a}	9.3^{a}	8.4^{ab}
Sour taste / *Skābā garša**	1.2^{a}	1.9^{a}	1.5^{a}	1.4^{a}
Salty taste / *Sāļā garša**	2.4^{b}	2.2^{b}	2.9^{a}	3.1^{a}
Aftertaste / *Pēcgarša**	3.7^{b}	4.7^{a}	3.9^{b}	3.8^{b}

The values marked with the same letter do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu būtiski neatšķiras (p>0.05)*

The results in Table 3.12 showed that that there were significant differences (p<0.05) in sweet, salty taste and aftertaste intensity. The statistical difference was observed in sweet taste intensity where GODO-YNL2 with NOLA^{TM}Fit5500 and Ha-Lactase 5200 samples were not significantly different (p>0.05) and GODO-YNL2 with control samples were not significantly different (p>0.05) either. In the evaluation of aftertaste intensity, NOLA^{TM}Fit5500 sample differed significantly from all samples (p<0.05). In the evaluation of acid taste intensity, all samples showed that the evaluation did not differ significantly (p>0.05). Isomerisation reaction was used for the production of fructose from glucose and the final syrup was with an increased sweet taste intensity of which is reflected in Fig. 3.17.

Table 3.13 / *3.13 tabula*

Sugar sweetness / *Cukuru salduma pakāpe*

Sugars / *Cukuri*	**Sweetness degree / *Salduma pakāpe***	**References /*Atsauces***
Glucose / *Glikoze*	0.74-0.80	Evdokimov et. al, 2015; Rocha & Guerra, 2020
Galactose / *Galaktoze*	0.60	
Lactose / *Laktoze*	0.16	
Sucrose / *Saharoze*	1.00	
Fructose / *Fruktoze*	1.17-1.75	

Table 3.13 represents the sweetness degree of each saccharide showing that fructose is twicly sweeted than glucose approving that the developed syrups are sweeter than the control sample.

The mean sensory data of two-stage fermentation whey permeate syrup and control sample are presented in Table 3.14.

Table 3.14 / *3.14 tabula*

The sensory properties of acid whey permeate syrups made in two-stage fermentation /
Divpakāpju fermentācijā iegūto biezpiena sūkalu ultrafiltrāta sīrupu sensorās īpašības

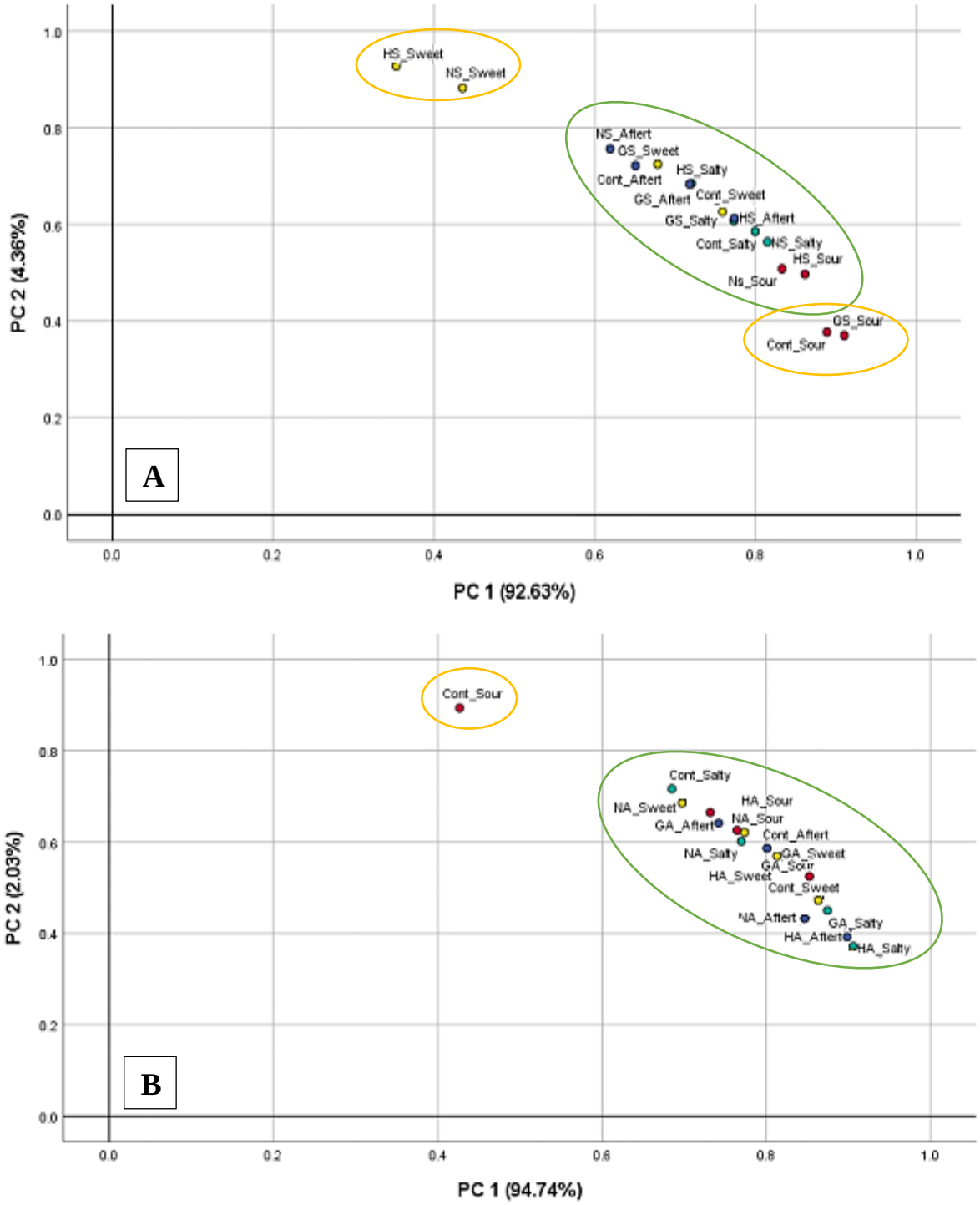
Attribute / *Īpašība*	**Control**	**NOLA^{TM}Fit5500**	**Ha-Lactase 5200**	**GODO-YNL2**
Sweet taste / *Saldā garša**	7.6^{a}	4.2^{b}	4.5^{b}	3.4^{c}
Sour taste / *Skābā garša**	1.5^{c}	3.2^{b}	3.9^{b}	5.1^{a}
Salty taste / *Sāļā garša**	2.4^{c}	5.5^{b}	8.7^{a}	8.1^{a}
Aftertaste / *Pēcgarša**	3.7^{c}	8.1^{a}	8.7^{a}	5.1^{b}

The values marked with the same letter do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu būtiski neatšķiras (p>0.05)*

The results showed that two-stage fermentation increases sweetness of samples. The sweet taste intensity of control sample was scored 7.60 (out of 12), which was the highest. The sweet taste intensity of NOLA^{TM}Fit5500 and Ha-Lactase 5200 samples was not significantly different (p>0.05) 4.2 and 4.5, respectively, and GODO-YNL2 sample was scored at least 3.4 (out of 12).

Glucose isomerise which was used in the study is active at pH 7.5, and this means that after hydrolysis 10% KOH solution needs to be added to the samples. Therefore, the increase in K^{+} ions concentration gives a stronger sour, salty taste, and aftertaste intensity. One of the main problems for acid whey is its high acidity and for many recycling processes whey should by neutralised. The common method is to use strong alkali solutions such as sodium hydroxide (NaOH) or potassium hydroxide (KOH). However, this approach affects the ionic composition of whey and neutralises desalination, making the technology, even less efficient (Kravtsov *et al.*, 2020). The increase in pH to 7.5 in acid whey permeate samples made a significant impact on syrup content and sensory properties. The use of strong alkali solutions affects the reactive groups of the amino acids, resulting in changes in the noncovalent forces that influence bounding, such as van der Waals forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonding (Onwulata *et al.*, 2006). This explains the appearance of white particles and increase in non protein nitrogen concentration in the syrup.

The KMO sampling adequacy measure for the PCA in Figure 3.25 A was 0.8 and for PCA in Figure 3.24 B 0.7. All correlated variables showed a direct relationship.



Cont - Control /; **NA** - NOLA^{TM}Fit5500; **HA** - Ha-Lactase 5200; **GA** - GODO-YNL2
Kontrole **NS** - NOLA^{TM}Fit5500; **HS** - Ha-Lactase 5200; **GS** - GODO-YNL2

Fig. 3.25. *Principal component analysis of the sensory attributes (○ sweet, ● sour, ● salty and ● aftertaste) intensity of syrups made in two-stage fermentation (A – sweet and B – acid whey permeates) (n = 30) / 3.25. att. Galveno komponentu analīze, analizējot divpakāpju fermentācijā iegūto sīrupu (A - siera un B - biezpiena ultrafiltrātiem) sensoro īpašību intensitāti (○ salda, ● skāba, ● sāļa un ● pēcgarša) (n = 30)*

The PCA in Figure 3.25. A showed that the first two components (PC1 and PC2) of the analysis explained 97% variation in the sensory properties between control and syrups obtained from sweet whey permeate. The PC1 explained 92% of the data variation, showing that green-lined attribute scores of samples are similar, while the PC2 explained only 4%, indicating that yellow-lined NOLA^{TM}Fit5500 and Ha-Lactase 5200 samples had the highest sweet taste

intensity, while the acid taste intensity of the control and GODO-YNL2 samples was evaluated the lowest, indicating that the acidity was practically not felt in these samples.

In contrast, PCA in Figure 3.25. B showed that the first two components (PC1 and PC2) of the analysis explained 97% variation in the sensory evaluation between control and syrups obtained from acid whey permeate. The PC1 explained approximate 95% of the data variation, showing that green-lined attribute scores of samples were quite similar. While the PC2 explained only 2%, indicating that yellow-lined control sample had the lowest score in the sour taste intensity. This shows that the permeate samples had a more notable sour taste after two-stage fermentation.

This research showed that the sensory profile of two-stage permeate fermentation is effective and is able to increase the sweetness of the syrup. Although isomerisation has some side effects, additional preparations are needed to improve the clarity of the syrup, especially in the case of acid whey syrup. The sweet taste scoring results of final syrups obtained from sweet whey permeate suggest that this product has the potential to enter the food market as a sweet additive. However, the sensory results of acid whey permeate syrups show that they would be less likely as a sweet additive. Although, the most expressive salty and sour taste makes it possible to include this syrup in culinary as one of the ingredients in sauces or as a dressing for salads.

Summary of Chapter 3.5 / *3.5. Nodaļas kopsavilkums*

The syrups obtained from both permeates showed significantly different results in sensory evaluation. The study demonstrated that two-stage fermentation can be successfully used to develop syrups with additional sugar concentration. Sensory analysis showed that the fructose significantly increased the sweetness of syrup and the performance was above commercial glucose-galactose syrup. The taste characteristics of acid whey permeate syrup have proved the importance of the results of the study and also how optimal conditions of enzyme have influence the medium of the acid whey. However, by using some additional technological solutions it could be possible to remove several compounds from the acid whey, so that the sweet taste of the syrup becomes more pronounced and perceivable.

Iegūtie sīrupi uzrādīja ievērojami atšķirīgus sensorā novērtējuma rezultātus. Divpakāpju fermentāciju var veiksmīgi izmantot, lai iegūtu sīrupus ar dažādu salduma pakāpi. Sensorais novērtējums parādīja, ka fruktozes sintēze ievērojami palielina saldo garšu sīrupam, kas iegūts no siera sūkalām. Sensorie vērtēšanas rezultāti bija augstāki nekā komerciālajam glikozes-galaktozes sīrupam. Pētījuma rezultāti ir pierādījuši, ka no biezpiena sūkalu ultrafiltrāta iegūtā sīrupa garšas īpašības nosaka sūkalu saastāvs un enzīma darbības efektivitāte sūkalu vidē. Izmantojot dažus papildu tehnoloģiskos risinājumus, ir iespējams no biezpiena sūkalām atdalīt savienojumus, iegūstot izteiktāku un patīkamāku sīrupa saldo garšu.

CONCLUSIONS

1. The results of the study confirm the proposed hypothesis: the two-stage fermentation increases the sweetness of glucose-galactose syrup.
2. Permeates do not contain lactose of high purity which, in turn, influences lactose hydrolysis reaction.
3. *Kluyveromyces lactis* β-galactosidase was more strongly inhibited by calcium ions than *Bacillus licheniformis* β-galactosidase. The main activators in the substrates were potassium and sodium ions for all enzymes.
4. The glucose test strip can be used as a routine device for the analysis of amount of hydrolysed lactose in a substrate.
5. The study indicates that β-galactosidases originated from *Kluyveromyces lactis* and *Bacillus licheniformis* were able to retain their activity after GIT *in vitro* simulation, showing a difference in lactose hydrolysis from 57±4% (NOLA™Fit5500) to 63±4% (Ha-Lactase 5200) with the addition of an *in vitro* treated β-galactosidase at concentration 1 mL L^{-1}.
6. Each permeate at a certain concentration has different physical and chemical properties which influence enzyme activity and the glucose, galactose and lactose concentration in the hydrolysed permeates.
7. The most convenient hydrolysis can be obtained at total solids concentration 20% of permeate and 2.5 U mL^{-1} of enzymes. Both substrates at a certain concentration have different physical and chemical properties which influence the enzyme activity and profile of final sugar outcome.
8. In two-stage fermentation, more fructose is formed in the sweet whey permeate. The highest fructose content (48±1 and 24±1 g L^{-1} in sweet and acid whey permeates, respectively) was obtained using Ha-Lactase 5200 β-galactosidase.
9. The experiments showed that the concentration of lactulose depends on the amount of added fructose. The amount of lactulose in sweet whey permeate was within the range of 1±0.1 to 54±2 g L^{-1} and in acid whey permeate it was 1±0.1 to 17 ±1 g L^{-1}.
10. The highest activity of GOS production was shown by GODO-YNL2 β-galactosidase, where the amount of GOS in isomerised acid whey permeate varied in the interval from 17±0.1 to 51±4 g L^{-1} but in sweet whey permeate from 27±2 to 66±2 g L^{-1}.
11. The results of the sensory evaluation showed that the sweet whey permeate samples had a higher sweet taste intensity.
12. The study revealed that using two-stage fermentation is possible to increase the sweetness intensity of glucose-galactose syrup obtained from sweet whey permeate.

SECINĀJUMI

1. Iegūtie pētījuma rezultāti apstiprina izvirzīto hipotēzi: divpakāpju fermentācija palielina glikozes-galaktozes sīrupa salduma pakāpi.
2. Ultrafiltrāts nesatur augstas tīrības laktozi, kas ietekmē laktozes hidrolīzes reakciju.
3. Kalcija joni inhibē *Kluyveromyces lactis* β-galaktozidāzes darbību, pretēji *Bacillus licheniformis* β-galaktozidāzei. Galvenie substrātu aktivatori analizētajiem enzīmiem ir kālija un nātrija joni.
4. Glikometru var izmantot kā rutīnas ierīci hidrolizētās laktozes satura analīzei substrātā.
5. *Kluyveromyces lactis* un *Bacillus licheniformis* β-galaktozidāze spēja saglabāt aktivitāti kuņģa-zarnu trakta modeļvidē, uzrādot atšķirīgu laktozes hidrolīzes spēju no 57±4% (NOLA ™ Fit5500) līdz 63±4 % (Ha-Lactase 5200), pievienojot *in vitro* apstrādātu β-galaktozidāzi saturošu šķīdumu 1 mL L^{-1}.
6. Katram substrātam noteiktā koncentrācijā ir atšķirīgas fizikālās un ķīmiskās īpašības, kas ietekmē enzīma aktivitāti un glikozes, galaktozes un laktozes saturu.
7. Pētījums pierādīja, ka veiksmīgāk hidrolīze īstenojama siera un biezpiena sūkalu ultrafiltrātam ar sausnu 20%, pievienojot 2.5 U ml^{-1} enzīma. Abiem substrātiem ir atšķirīgas fizikālās un ķīmiskās īpašības, kas ietekmē enzīma aktivitāti un radušos monosaharīdu saturu.
8. Divpakāpju fermentācijā fruktozes vairāk veidojas siera sūkalu ultrafiltrātā. Lielākais fruktozes saturs (48±1 un 24±1 g L^{-1} attiecīgi siera un biezpiena sūkalu ultrafiltrātos) tika iegūts, izmantojot Ha-Lactase 5200 β-galaktozidāzi.
9. Eksperimenti pierādīja, ka iegūtās laktulozes saturs ir atkarīgs no pievienotās fruktozes daudzuma. Noteiktais laktulozes saturs siera sūkalu ultrafiltrātā bija robežās no 1±0.2 līdz 54±2 g L^{-1} un biezpiena sūkalu ultrafiltrātā no 1±0.1 līdz 17±1 g L^{-1}.
10. Augstāko galakto-oligosaharīdu producēšanas spēju uzrāda GODO-YNL2 β-galaktozidāze. Biezpiena sūkalu ultrafiltrātā galakto-oligosaharīdu saturs svārstījās intervālā no17±1 līdz 51±4 g L^{-1}, bet siera sūkalu ultrafiltrātā no 27±2 līdz 66±2 g L^{-1}.
11. Sensorā novērtējuma rezultāti parādīja, ka siera sūkalu ultrafiltrāta paraugos ir augstāka saldās garšas intensitāte.
12. Pētījumā noskaidrots, ka, izmantojot divpakāpju fermentāciju, ir iespējams paaugstināt no siera sūkalu ultrafiltrāta iegūtā glikozes-galaktozes sīrupa salduma intensitāti.

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APPENDIXES / *PIELIKUMI*

Appendix 1 / *1. Pielikums*

Glucose strip test / *Glikometra tests*

	A	B
Material and chemical / *Materiāls un ķīmijas*	Original / *Orģinālais*	1 test / *Vienam testam*
Glucose / *Glikoze*	36.45/100g	0.03
Glucometer / *Glikometrs*	9.50	-
Test strip / *Testa plāksnīte*	10.43/50	0.21
Total / *Kopā*:		0.24

HPLC methode / *AEŠH metode*

	A	B
Material and chemical / *Materiāls un ķīmijas*	Original / *Orģinālais*	1 test / *Vienam testam*
Vial / *Pudelīte*	27.7	0.14
Lid / *Vāciņš*	22.2	2.20
Acetonitril / *Acetonitrils*	305/2,5L	2.44
α-Lactose monohydrate / *α-Laktozes monohidrāts*	49.7/1 kg	0.05
Total / *Kopā*:		4.58

Spectrophotometric methode / *Spektrofotometriskā metode*

	A	B
Material and chemical / *Materiāls un ķīmijas*	Original / *Orģinālais*	1 test / *Vienam testam*
96-well plate / *96-lauciņu mikroplate*	183/50	3.66
2-Nitrophenyl-galactoside / *2-Nitrofenil-galaktozīds* (o-NPG)	49.80/1	1.00
Total / *Kopā*:		4.66

Cryoscopic method / Krioskopijas metode

	A	B
Material and chemical / *Materiāls un ķīmijas*	Original / *Orģinālais*	1 test / *Vienam testam*
Test tube / *Testa mēģene*	16.5/12	1.40
α-Lactose monohydrate / *α-Laktozes monohidrāts*	49.70/100g	0.05
Total / *Kopā*:		1.45

Amount of hydrolysed lactose (g L ^{1}) in <u>acid whey permeate</u> at different concentrations of solids and enzyme units /
***Hidrolizētās laktozes satura (g L^{-1}), izmantojot <u>biezpiena sūkalu ultrafiltrātu</u> ar dažādu sausnas saturu un atšķirīgām pievienotām enzīma* vienībām**

Enzyme units / *Enzīma vienības*, U mL^{-1}	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2
0.5	61±1^{c}	121±7^{a}	64±1^{b}	81±2^{b}	102±6^{a}	94±2^{a}	56±3^{b}	99±2^{a}	101±1^{a}
2.5	129±4^{c}	184±4^{a}	144±4^{b}	222±7^{b}	249±10^{a}	151±3^{c}	182±8^{b}	362±4^{a}	167±8^{b}
5	190±5^{a}	190±3^{a}	159±6^{b}	221±17^{a}	238±6^{a}	241±5^{a}	198±20^{b}	284±1^{a}	188±1^{b}
	20% solids / *20% sausna*			30% solids / *30% sausna*			40% solids / *40% sausna*		

The values marked with the same letter within each enzyme and dry matter concentration do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra enzīma un sausnas koncentrācijas ietvaros būtiski neatšķiras (p>0.05)*

Comparison of hydrolysed lactose (g L^{-1}) in <u>sweet whey permeate</u> at different concentrations of solids and enzyme units /
Hidrolizētās laktozes saturs (g L^{-1}), salīdzinājums izmantojot <u>siera ultrafiltrātu</u> ar dažādām sausnas koncentrācijām un enzīmu vienībām

Enzyme units / *Enzīma vienības*, U mL^{-1}	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2	NOLA™Fit 5500	Ha-Lactase 5200	GODO-YNL2
0.5	34±2^{b}	92±6^{a}	84±3^{a}	35±1^{b}	73±6^{a}	38±3^{b}	35±3^{b}	63±4^{a}	39±1^{b}
2.5	94±8^{c}	183±2^{a}	157±3^{b}	88±3^{c}	212±6^{a}	197±4^{b}	49±4^{c}	316±3^{a}	187±6^{b}
5	146±12^{b}	192±4^{a}	149±12^{b}	140±10^{b}	255±9^{a}	152±6^{b}	155±10^{b}	263±12^{a}	135±12^{b}
	20% solids / *20% sausna*			30% solids / *30% sausna*			40% solids / *40% sausna*		

The values marked with the same letter within each enzyme and dry matter concentration do not differ significantly (p>0.05) / *Vērtības apzīmētas ar vienādu burtu katra enzīma un sausnas koncentrācijas ietvaros būtiski neatšķiras (p>0.05)*

Appendix 3 / *3. Pielikums*

Cost of one assay for determination of β-galactosidase activity /
Vienas analīzes izmaksas β-galaktozidāzes aktivitātes noteikšanai

Parameters / *Parametri*	HPLC method / *AEŠH metode*	Glucometer test / *Glikometra metode*	Spectrophotometric method / *Spektrafotometriskā metode*	Cryoscopic method / *Krioskopijas metode*
Chemicals / *Ķīmikālijas*	2.44	0.03	1.00	0.05
Consumables / *Palīgmateriāli*	2.39	0.21	3.66	1.40
Time / *Laiks*, min	20	5	10	5
Price / *Cena*, Euro	4.58	0.21	4.66	**1.45**

The price of the equipment is not included in the cost calculation / *Iekārtas cena nav iekļauta izmaksu aprēķinā*