Study of concentration of lactic acid obtained in the process of lactic fermentation of lactose contained in the spent whey using Lactobacillus

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Introduction

Whey as waste product of dairy industry is a complex mixture of many valuable ingredients: proteins, lactose, calcium and phosphorus compounds, organic acids and vitamins. At the same time it is a perishable material, as it quickly spoils because of the present microflora. Due to its specific properties, whey may serve as an excellent medium for culturing bacteria under laboratory conditions. Depending on the technological methods of manufacturing dairy and cheese products, dairy plants produce three types of the whey: rennet (so-called sweet), acid (so-called sour) and mixed whey. Rennet whey is produced in the process of ripened cheese (hard cheese), while the acid whey is obtained in the production of quark (cottage cheese). These wheys have different chemical composition and physico-chemical properties. Acid whey (pH 3.8 – 4.6) has higher lactic acid content (do 0.7%) and lower protein content than rennet whey (pH 5.2 – 6.7) [1, 2]. The annual whey production in Poland is approx. from 2 to 3 mln m³. It is estimated that from the total milk volume used for manufacturing cheese and quark, around 65 90% leaves the manufacturing process as whey [3]. One of the biotechnological directions of whey processing is production of lactic acid in the process of lactic fermentation. The high content of lactose (up to 6%) gives economic grounds to extract it from this product. Lactose is used in lactic fermentation process, during which it is converted to lactic acid using lactic acid bacteria (LAB) [4, 5].

Production of lactic acid in the process of lactic fermentation using LAB has this advantage, that in comparison with chemical synthesis, one can obtain specific isomer of lactic acid if the proper bacterial strain is chosen [6].

Lactic acid bacteria (LAB characteristics)

LAB form a very broad group of micro-organisms, which common property is ability for lactic fermentation. This group of bacteria includes Gram-positive cocci: Lactococcus, Streptococcus, Leuconostoc, Enterococcus, Oenococcus and Pediococcus Gram-positive, non-sporulating bacilli of the genus Lactobacillus and Carnobacterium. These micro-organisms differ in their nutritional requirements, type of produced metabolites, pH of the culture medium and culture temperature. Among them are thermophilic (growth at temp.: 37–45°C) mesophilic species (growth at temp.: 20–28°C). The majority belongs to relative anaerobes, however there are some absolute anaerobic species. Among LAB used as starter cultures in dairy industry, only bacteria Lactobacillus (L. helveticus, L. delbrueckii ssp. bulgaricus) prefer environment with pH 5.5–5.8. During the growth and fermentation, medium pH decreases as a result of accumulation of organic acids, including lactic acid [7–10].

Biotechnological production of lactic acid

The modern approach to question of biotechnological production of lactic acid assumes use of waste products generated by various industry branches, mainly food industry, as well as agricultural production. Among renewable raw materials used in protection of lactic acid, the following can be distinguished:

- starch materials: potatoes, wheat, corn, cassava, rice, sorghum, rye, oats, barley
- industrial waste products: molasses and whey
- cellulose products: rice, wheat and corn straw, as well as alfalfa fibers, waste wood, wastepaper [11–13].

As mentioned above, micro-organisms able to produce lactic acid are mainly LAB and they are dominating species used on large-scale by lactic acid manufacturers. However, it must be noted, that these micro-organisms have high nutritional requirements, due to the fact that LAB are unable to synthesis group B vitamins and amino acids. They require enriching production medium with organic easily absorbable nitrogen sources, vitamins and microelements [11–13]. The efficiency of lactic acid production for individual LAB species tend to vary, and type of substrate used in culture medium has an additional effect on the efficiency (Tab. 1).

As a rich carbon source for production of lactic acid whey is usually used. It is a by-product obtained in dairy industry. Next to carbon source, i.e. lactose, it contains also proteins, as well as mineral salts and fats. The complete use of lactose is only possible if the culture medium is supplemented with additional nitrogen sources. There are developed technologies that as additive to production medium use yeast extract, peptone, powdered milk, soy flour and corn steep liquor. Bacteria used for production of lactic acid from whey are the following strains L. delbrueckii subsp. bulgaricus, L. helveticus, as well as L. casei, L. lactis and S. thermophilus [11, 13]. The mentioned above LAB strains conduct lactic fermentation lactose using different metabolic pathways [36].

In view of methods of hexoses metabolism, LAB can be divided into two basic groups:

- homofermentative, i.e. ones that produce exclusively or almost exclusively (90%) lactic acid,
- heterofermentative, that produce next to lactic acid also carbon dioxide, acetic acid, formic acid and/or ethanol,
- and an additional group can be distinguished, facultatively heterofermentative LAB [4, 8, 11, 12].

Criteria of selection of proper production bacterial strains depend on desired properties of final fermentation product. Usually thermophilic strains producing lactic acid in a short time, that produce small amounts of by-products as well strongly dominate in the environment. The advantage of using thermophilic strains, i.e. bacteria of the genus Streptococcus or Enterococcus, is increased temperature of the process (40–50°C), which prevents growth of unfavorable microflora that could disrupt the lactic fermentation. Additionally, one of the selective factors in selection of appropriate strains is a form of produced lactic acid...
Unquestionable advantages of LAB in production of lactic acid as there is demand especially for products that are derivatives of this acid. Individual LAB species synthesise it in three different forms: dextrorotatory L(+), levorotatory D(−) and their racemic mixture. Lactic acid produced as a result of chemical synthesis is in form of only dextrorotatory L(+), levorotatory D(−) and their racemic mixture.

The optical purity of lactic acid is also important for proper course of lactate racemase (that converts form L(+) into D(−) or vice versa). Lactic acid produced as a result of chemical synthesis is in form of only dextrorotatory L(+), levorotatory D(−) and their racemic mixture. Lactic acid produced as a result of chemical synthesis is in form of only dextrorotatory L(+), levorotatory D(−) and their racemic mixture.

Table 1: Physiological characteristics of selected bacteria of Lactobacillus strain and their lactic acid production in culture media with different substrates

<table>
<thead>
<tr>
<th>Medium</th>
<th>Lactic acid bacterial strain</th>
<th>Optimum temperature °C</th>
<th>Type of lactic acid fermentation</th>
<th>Lactic acid content, g/L</th>
<th>Production efficiency of lactic acid, g/L h¹</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>Lactobacillus amylovorus</td>
<td>32 - 45</td>
<td>Heterofermentation</td>
<td>10.1</td>
<td>0.8</td>
<td>[16]</td>
</tr>
<tr>
<td>Cassava flour</td>
<td>Lactobacillus amylovorus</td>
<td>32 - 45</td>
<td>Heterofermentation</td>
<td>4.8</td>
<td>0.2</td>
<td>[16]</td>
</tr>
<tr>
<td>Potatoes</td>
<td>Lactobacillus amylovorus</td>
<td>32 - 45</td>
<td>Heterofermentation</td>
<td>4.2</td>
<td>0.1</td>
<td>[17]</td>
</tr>
<tr>
<td>Barley</td>
<td>Lactobacillus amylovorus</td>
<td>37 - 45</td>
<td>Heterofermentation</td>
<td>23.7</td>
<td>0.3</td>
<td>[18]</td>
</tr>
<tr>
<td>Wheat</td>
<td>Lactobacillus amylovorus</td>
<td>37 - 45</td>
<td>Heterofermentation</td>
<td>66.0</td>
<td>1.4</td>
<td>[20]</td>
</tr>
<tr>
<td>Rye</td>
<td>Lactobacillus amylovorus</td>
<td>10 - 37</td>
<td>Heterofermentation</td>
<td>84.5</td>
<td>2.4</td>
<td>[26]</td>
</tr>
<tr>
<td>Sorghum</td>
<td>Lactobacillus amylovorus</td>
<td>10 - 37</td>
<td>Heterofermentation</td>
<td>81.5</td>
<td>2.7</td>
<td>[26]</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Lactobacillus amylovorus</td>
<td>10 - 37</td>
<td>Heterofermentation</td>
<td>106.0</td>
<td>3.5</td>
<td>[27]</td>
</tr>
<tr>
<td>Goat milk</td>
<td>Lactobacillus amylovorus</td>
<td>32 - 45</td>
<td>Heterofermentation</td>
<td>129.0</td>
<td>2.9</td>
<td>[28]</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Lactobacillus amylovorus</td>
<td>37 - 45</td>
<td>Heterofermentation</td>
<td>106.0</td>
<td>1.0</td>
<td>[25]</td>
</tr>
<tr>
<td>Barley</td>
<td>Lactobacillus plantarum</td>
<td>28 - 32</td>
<td>Heterofermentation</td>
<td>41.0</td>
<td>1.0</td>
<td>[30]</td>
</tr>
<tr>
<td>Wheat</td>
<td>Lactobacillus plantarum</td>
<td>28 - 32</td>
<td>Heterofermentation</td>
<td>−15.0</td>
<td>−</td>
<td>[35]</td>
</tr>
<tr>
<td>Rye</td>
<td>Lactobacillus plantarum</td>
<td>28 - 32</td>
<td>Heterofermentation</td>
<td>−15.0</td>
<td>−</td>
<td>[35]</td>
</tr>
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<td>Lactobacillus plantarum</td>
<td>28 - 32</td>
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<td>Lactobacillus plantarum</td>
<td>28 - 32</td>
<td>Heterofermentation</td>
<td>−20.0</td>
<td>−</td>
<td>[35]</td>
</tr>
<tr>
<td>Paper industry</td>
<td>Lactobacillus plantarum</td>
<td>28 - 32</td>
<td>Heterofermentation</td>
<td>−30.0</td>
<td>−</td>
<td>[35]</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Lactobacillus rhamnosus</td>
<td>45 - 52</td>
<td>Heterofermentation</td>
<td>73.0</td>
<td>2.9</td>
<td>[33, 34]</td>
</tr>
</tbody>
</table>

acid. The type of synthesised optical isomer is determined by stereospecificity of lactate dehydrogenases (LDHs) and activity of lactate racemase (that converts form L(+) into D(−) or vice versa). The used media can be divided due to consistency: solid (solidified with nutrient agar or nutrient gelatin) containing 1.5 – 2% of agar used for growth of bacteria and differentiation, semi-liquid, containing 0.1–0.7% of agar and used in cultures of microorganisms with smaller oxygen demand, and liquid (normal nutrient broth) that are used mainly for growth of bacteria nutrient agar [3–5].

due to their origin we can distinguish natural, semi-synthetic and synthetic media.

Natural media are media of not fully defined chemical composition containing extracts from plant or animal tissues, while synthetic media are media of strictly defined and known chemical composition. In addition there are also semi-synthetic media partially known in respect of chemical composition, e.g. M9 medium (Adams) enriched with glucose [42–44].

Culture media can be also divided due to contents of nutrients. The following are distinguished: minimal media, containing only these ingredients that are necessary to sustain vital functions of defined group of microorganisms and enriched media, that contain in addition to basic addition, additional ones such as: sheep blood, glucose and vitamins. Growth media that are used for obtaining large biomass of studied strain; they are usually liquid media; differential media, where few or several bacteria species can grow and selective media with addition of substance that allow only growth of specified bacteria species, while inhibiting growth of other species. Growth-selective media are media where limited number of bacteria species. An example of this kind of

General properties of culture and nutrient (microbiological) media

Nutrient media for bacterial culture shall have the following features [40, 41]:

- shall contain biogenic elements (C, O, H, N, P, S), mineral salts with cations (Na, Ca, K, Mg), microelements (Mn, Zn, Mo, Cu, Co, Ni) and growth substances,
- shall exhibit optimum pH and redox potential,
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- shall have appropriate value of osmotic pressure,
- shall be transparent (exception: media containing insoluble compounds, such as CaCO₃, fats),
- must be sterile.

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media is MRS agar. It is used for detecting presence and determining number of LAB of genera Lactobacillus in whey and food products. This medium is used for detecting all the Lactobacillus including slow-growing (L. brevis and L. fermenti) and Bifidobacterium spp. Selective factors in MRS medium are ammonium citrate and sodium acetate that inhibit growth of Gram-negative bacteria. This medium can be used for surface and deep inoculations. Lactobacillus spp. are microaerophilic bacteria and for incubation atmosphere enriched with 5% of CO₂ is recommended [42–46].

Analysis methods for lactic acid content in fermentation broth

The literature presents a lot of information regarding qualitative and quantitative analysis methods for lactic acid. However, among the usually used analytical standards, special attention shall be paid to ones that apply to determination of lactic acid in diary products, that is ones that methods of potentiometric acid-base titration or spectrophotometry. After their modifications, the new In-house Standards were developed for the purposes of the project and implemented as a part of own research on spent whey and fermentation brothes.

The first own standard (In-house Standard ZN-G 2:2013) regarding direct determination of lactic acid content using potentiometric titration can be applied to fermentation broth, whey, milk and solutions thereof. It was developed based on the Polish Standard PN-ISO 6091:2012 [48]. The method involves titration up to pH 8.40 of test sample with standard 0.1 M solution of sodium hydroxide in the presence of 1% phenolpthalein solution as an auxiliary indicator.

The subject of the next own standard (In-house Standard ZN-G 5:2012) is colorimetric determination of lactic acid content by means of La-Fe (III) complex method in milk products and solutions of lactic acid not exceeding concentration of 1% [49]. This standard was developed on the basis of previous norms, including not only Polish, but also European standards: PN-EN ISO 8069:2008 [50], PN-A-86060:1994 [51] and PN-A-86062:1994 [52]. Complex of lactic acid with iron (III) is formed as a result of reaction with acid with iron (III) chloride, and the measurement of its absorbance is performed at 410 nm wavelength using spectrophotometer. Lactic acid content for testes sample expressed as for 100 m is read from previously prepared calibration curve.

The enzymatic method of determination of lactic acid and lactates content is a third analytical method used in in-house laboratory practices and is based on the international standard PN-EN ISO 8069:2008 [50]. This method is the most accurate among the three mentioned analytical techniques, as it allows to determine contents of individual lactic acid isomers. In this case, as standard solutions are used solutions of lithium D-lactate or L-lactate of concentration 50 mg/L, depending on which isomer content is being determined. This method is used for fermentation broth, whey, milk and their solutions, that were not only previously deproteinized, but also fat content was removed. In this method, the titrate of test sample is subject to activity of enzymes: L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH) in the presence of Nicotinamide adenine dinucleotide (NAD) in order to oxidize lactate to pyruvate and convert NAD to its reduced form – NADH. Amount of formed NADH is then determined by means of spectrophotometry at 340 nm wavelength and is proportional to contents of lactic acid and lactates in the test sample.

Analysis methods for lactose content in fermentation broth

Whey is a rich natural source of lactose. Thanks to that it can be used for a process of lactic acid fermentation. In this process lactose, as a main substrate of lactic acid fermentation using LAB is converted to lactic acid. During the lactic acid fermentation, lactose content in microbiological culture decreases. In order to correctly monitor process of lactic acid fermentation, on each stage it is necessary to determine among other lactose content in fermentation reactor [47, 53, 54].

Carbohydrates determination methods can be in general divided into: physical (densimetry, refractometry, polarimetry), physico-chemical (anthrone, resorcinol, ferricyanide method) and biological methods. However, the most important in quantitative analysis of carbohydrates are chemical methods based on reduction of copper (II) salts in alkaline conditions (Bertrand’s, Fehling’s, Lane-Eynon and Luff-Schoorl method). Chemical methods can be successfully used for determination of lactose in samples from lactic acid fermentation. Reducing properties, apart from carbohydrates, are also exhibited by amino acids, proteins, organic acids, aldehydes and purine bases. Therefore, before starting analysis it is necessary to remove all types of contaminants from test sample in order not to overestimate the results. One of the most often used method to hit end is the Carezz’s method that involves using the same volume of potassium hexacyanoferrate (II) and zinc (II) sulphate (VI) and formation of colloidal solution of zinc (II) hexacyanoferrate (II). The obtain solution contains micelles that absorb macromolecular substances, thus purifying test sample from contaminants. After purification of the sample the actual analysis can begin. In the laboratory practice, one of the most often used method for reducing sugars (i.e. lactose) in fermentation broth is Bertrand method (PN-67/A-86430) [55]. Lactose determination is performed using indirect method based on quantity of potassium manganate (VII) used for titration of Fe²⁺ ions that correspond to quantitative reduction of Cu²⁺ to Cu²⁺ ions with lactose from the tested whey. The reaction occurs in alkaline conditions (pH approx. 12) and at increased temperature. After adding Bertrand’s I and II reagents (containing copper (II) sulphate (VI) pentahydrate, sodium-potassium tartrate and sodium hydroxide) and after heating, lactose from the sample reduces quantitatively complex of copper (II) that is formed in the solution to copper (I) oxide. Precipitated copper (I) ions undergo oxidation reaction with a third Bertrand liquid (acid solution of iron (III) sulphate (VII)) to Cu²⁺ ions, while Fe²⁺ are reduced to Fe³⁺ ions. Quantity of Fe³⁺ ions is determined by titration with standard solution of potassium manganate (VII). The amount of determined in such a manner Cu₂O is then converted to amount of sugar, using relevant tables [47, 53]. After modifying of conversion of lactose content, the In-house Standard (ZN-G 1:2013) was developed for the purposes of the project, where lactose content can be calculated using the following formula:

\[
C_{lactose} = V_{(0.02 \text{ mol/KMnO}_4)} \cdot 0.604 \left[ \text{g/l} \right]
\]

where:

\( V \) – volume of used for titration KMnO₄ of 0.02 mol/dm³ concentration

0.604 – conversion for lactose in 10 mL.

Own research

Within the project of the Innovative Economy Operational Programme 2007 – 2013, realized by Glokor Sp. z o.o. an attempt was made to develop large-scale (industrial) installation for processing diary industry waste, while obtaining biodegradable biopolymer. The own research was broken down into several phase. The first research phase focused on obtaining lactic acid in the process of lactic acid fermentation of lactose contained in the acid whey. The following LAB strains were used in studies of lactic acid fermentation: Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei and Lactobacillus fermentum. LAB strains were from the collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław. LAB were incubated on different solid media (ACA, YGA, MRS, agar) and then they were moved...
to fermentation reactor containing liquid medium. In order to optimize lactic acid fermentation, the following factors were studied: various strains of LAB, medium composition, temperature effect, pH and process duration. Additionally, resistance of production environment to presence of harmful microflora was studied. On each stage of lactic acid fermentation, the lactose content was determined using modified Bertrand’s method and acidity using potentiometry.

LAB bacilli were identified under optical microscope (DELTA Optical) at optical magnification x400 and x600. The preparations were made based on the In-House standard using Gram staining method [46]. In the final stage of lactic acid fermentation, lactic acid of concentration in range of 3–4% was found depending on the applied conditions. Lactic acid was purified from fermentation broth, and then concentrated using membrane process. In the final stage the obtained lactic acid was polymerized to PLA, as described previously in the monthly CHEMIK 2014, 68, 8 [37].

Summary

This publication is a literature review on lactic acid fermentation process and includes description of own research on analytical methods for determination of lactic acid and lactose, as well as on optimization of lactic acid fermentation of lactose from spent whey to lactic acid. Utilizing environmentally burdensome waste, which is an acid whey, is an important economic and economical problem for dairy industry. Utilization of this waste is a significant financial burden for dairy plans. It seems reasonable that using whey as a substrate of fermentation medium is an innovative solution of waste management. The article discusses processes of utilization of whey in the process of lactic acid fermentation, which are still quite a new issue that requires further research, especially regarding large-scale industrial installations.

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Literature


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