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Biotechnology of Lactulose Production: Progress, Challenges, and Prospects

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Abstract.
Lactulose is a prebiotic that has found a wide application in medicine and food industry. Commercial lactulose is usually synthesized by isomerization in alkaline media at high temperatures. Enzymatic methods offer a more sustainable alternative and require more moderate processing conditions.
This review covers 44 years of scientific publications (1978–2022) on the enzymatic synthesis and purification of lactulose. The materials were retrieved from Scopus, Web of Science, PubMed, and Elibrary databases.
The enzymatic approach to lactose-to-lactulose conversion has two methods: isomerization (direct) and transgalactosylation (via hydrolysis). Isomerization exploits cellulose-2-epimerases, but their safety status is still rather vague. As a result, cellulose-2-epimerases are not commercial. Epilactose is a by-product of isomerization. Transgalactosylation involves *β*-galactosidases with an official international safety status (GRAS). It is available on the market, and its action mechanism is well understood. This article systematizes various data on the conditions for obtaining the maximal yields of lactulose by different enzymes. The *Kluyveromyces lactis* yeast and the *Aspergillus oryzae* mold are the main sources of *β*-galactosidases in lactulose production. The yield can reach 30% if the processing conditions are optimal. Fructose remains the main problem in the production process. No scientific publications revealed a direct relationship between the maximal yields of lactulose and the molar fructose-to-lactose ratios. Cellobiose epimerases make it possible to achieve high yields of lactulose (70–80%). However, these enzymes are associated with genetic engineering and mutagenesis, which challenges their safety status. The most promising trends in lactulose biotechnology include secondary dairy raw materials, immobilized enzymes, membrane reactors, complex production processes, lactose-to-lactulose conversion, and purification of final product.

Keywords. Lactulose, lactose, bioconversion, enzymes, yield, *β*-galactosidase, *Kluyveromyces*, *Aspergillus*, cellobiose-2-epimerase, trends

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Биотехнология лактулозы: современные достижения, проблемы и перспективы

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Аннотация.
Лактулоза является пребиотиком, который широко применяется в медицине и пищевой промышленности. Производство лактулозы основано на изомеризации лактозы в щелочных средах при высоких температурах. Альтернативой являются ферментативные способы, которые считаются более экологически чистыми и позволяют проводить процессы в более мягких условиях.

Объектом исследования стали научные публикации по вопросам синтеза и очистки лактулозы с использованием ферментов. Для поиска информации были использованы базы данных Scopus, Web of Science, PubMed и Elibrary за период с 1978 по 2022 гг.

Рассмотрели два основных пути ферментативного превращения лактозы в лактулозу: изомеризация (прямой) и трансгалактозилирование (с промежуточным гидролизом). Для первого применяют целлюлозо-2-эпимеразы, которые пока не имеют статуса безопасности и их не производят в промышленных масштабах, а побочным продуктом их реакции является эпилактоза. Для трансгалактозилирования применяют *β*-галактозидазы, которые имеют международный статус безопасности (GRAS) и доступны на рынке, с хорошо изученным механизмом действия. Систематизировали данные об условиях получения максимальных выходов лактулозы при использовании разных ферментов.

Основными продуцентами *β*-галактозидаз для получения лактулозы являются дрожжи *Kluyveromyces lactis* и плесени *Aspergillus oryzae*. Выход лактулозы достигает 30 % в оптимальных условиях. Основной проблемой является необходимость внесения фруктозы. Прямой зависимости между максимальными выходами лактулозы и молярными соотношениями фруктоза:лактоза не выявлено. Применение целлобиозо-эпимераз позволяет достигать высоких выходов лактулозы (70–80 %), но для получения этих ферментов используют методы генной инженерии и мутагенеза, что ставит под сомнение их безопасность. К наиболее перспективным тенденциям развития биотехнологии лактулозы можно отнести использование вторичного молочного сырья и иммобилизованных ферментов, применение разных моделей реакторов, в т. ч. мембранных, и разработку комплексных взаимосвязанных процессов получения ферментов, конверсии лактозы в лактулозу и очистки готовых продуктов.

Ключевые слова. Лактулоза, лактоза, биоконверсия, ферменты, выход, *β*-галактозидаза, *Kluyveromyces*, *Aspergillus*, целлобиозо-2-эпимераза, тенденции

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Introduction

Lactulose (*β*-D-galactopyranosyl-(1,4)-*β*-D-fructofuranose) is the best known indigestible prebiotic disaccharide. It can selectively stimulate the growth and/or metabolism of beneficial intestinal microflora, especially bifidobacteria and lactobacilli. Its positive effect on human health has been confirmed by numerous studies. Lactulose-based pharmaceutical preparations have been used for more than 50 years to treat chronic constipation and hepatic encephalopathy. Nowadays, lactulose is also used to treat osteoporosis, food poisoning, and various infections [1, 2]. This unique carbohydrate has a potential to strengthen immune system, reduce inflammation, and prevent carcinogenesis [3–5].

Medicine employs lactulose in fairly high concentrations, e.g., ≥ 10–15 g/day. However, even 2 g of lactulose per day has a positive effect. This dose boosts useful metabolites, improves absorption of minerals in the intestine, and has no side effects [6]. Small doses of lactulose turn various foods into functional products and render them a number of additional economic and technological advantages, such as a shorter fermentation time, more viable starter cultures, a longer shelf life, a better sensory profile, etc. In addition, lactulose results in a lower caloric value of the finished product because it allows for partial sugar replacement. Naturally, lactulose is popular in the dairy, confectionery, and bakery food industries. Lactulose can be incorporated in the shells of micro- and nanocapsules that deliver probiotics, vitamins, and other beneficial substances to the lower intestine [1, 7].

Lactulose is one of the most valuable derivatives of lactose, which is the main carbohydrate of milk. Lactose makes up a significant proportion of solids in by-products of cheese, curd, casein, and butter production. Therefore, lactulose is a processed food raw material with a high added value and has a good potential for sustainable economy: it can provide a sustainable and green processing cycle [8].

The modern industrial production of lactulose relies mainly on isomerization in alkaline media (pH = 11) at high temperatures (70–80°C). This reaction produces by-products of caramelization and saccharic acids, as well as melanoidins that appear in the presence of proteins and peptides. The maximal degree of isomerization is 30% if sodium or calcium hydroxides serve as catalysts. Hydroxides are removed by electrodialysis, and the remaining lactose is usually crystallized. However, this process is slow because the resulting mix has a complex composition. The yield of lactulose can be brought up to 70–80% using complexing catalysts, e.g., aluminates or borates. Unfortunately, they are toxic, and their complete removal requires a selective ion exchange treatment. The resulting lactulose syrups contain residues of lactose, glucose, galactose, fructose, epilactose, and other carbohydrates, which have to be purified by chromatography [9, 10].

Some isomerization methods use electroactivated solutions (catholytes), where an alkaline medium appear without new reagents [9, 10]. A recent study reported 37% isomerization of lactose to lactulose as a result of optimization and electroactivation of ultrafiltrated whey permeate [11]. However, the same study also reported that electroactivation foamed secondary dairy raw materials, and the unreacted lactose, other sugars, and by-products had to be removed.

Enzymatic processing is an alternative to chemical production and purification. Enzymes are proteins that catalyze metabolic reactions in a living cell. Industrial enzymes of microbial origin are common in pharmaceutical and food biotechnology. Enzymatic processing is environmentally friendly, because enzymes are reusable, biodegradable, and produced from renewable resources. Enzymes are able to catalyze reactions under close-to-physiological conditions, which is beneficial in terms of energy consumption [10, 12].

Another advantage of lactulose biosynthesis is that lactose can be obtained from secondary dairy raw materials, e.g., cheese whey and/or curd whey, ultrafiltration permeates of skim milk and whey, etc. In additions, enzymes are selective in action; they require no extreme temperatures or pH, and, as a result, produce fewer by-products. Thus, lactulose purification is cheap and meets modern requirements for environmentally friendly technologies and natural products [13, 14].

Experts believe that lactulose production will increase, driven by bad dietary habits, as well as by the needs of athletes and senior population. High R&D investments in the food industry will sustain the growth of the global lactulose market through 2022–2028 [15]. In Russia, the growing demand for lactulose depends on foreign suppliers, and the development of domestic lactulose industry remains an urgent task [1].

The research objective was to systematize and analyze the data on enzymatic lactulose production in order to determine the promising research areas.

Study objects and methods

The research featured English and Russian scientific publications on the enzymatic production of lactulose. All the articles under analysis were registered in Scopus, Web of Science, PubMed, and Elibrary in the period between 1978, when the earliest publication appeared, and August 1, 2022. The pool included available review and research articles on the enzymatic lactulose synthesis, as well as individual articles that substantiated the relevance of the topic, described the properties and mechanisms of enzymes, identified prospective research areas, etc. The focus action was on articles published in scientific peer-reviewed journals with a high citation index over the past five years. The review did not include conference proceedings and book chapters.

Results and discussion

1. Processes, enzymes, and mechanisms of lactulose formation. The biotechnology of lactulose, like that of other functional carbohydrates, includes three interrelated processes: enzyme production, enzymatic synthesis, and subsequent processing [16]. Most publications featured ready-made commercially purified enzyme preparations, free or immobilized, while some of them described the preparation of the necessary enzymes with varying purification degrees.

Theoretically, the bioconversion of lactose to lactulose has two main routes: the direct one and the one with intermediate hydrolysis. The direct route requires isomerase, which transforms the glucose residue of lactose into fructose. A similar ability was discovered about ten years ago in cellulose-2-epimerases (hereinafter referred to as epimerases) of various origins. However, these enzymes have no safety status and are not produced on an industrial scale. This reaction also yields epilactose as a by-product.

The second and a more complex route looks as follows. Enzymes break down lactose into galactose and glucose, and then add galactose residue to fructose, i.e., transgalactosylation. Some glycosidases possess all the required properties, as well as an international safety status and market availability [10, 12, 14]. The enzymatic synthesis of lactulose via hydrolysis has attracted scientific attention for more than 20 years and still remains relevant, as evidenced by the growing number of publications on this topic. As for the prospects of epimerase-related bioconversion, they remain rather vague (Fig. 1).

According to the international classification of enzymes, glycosidases are marked as EC 3.2.1, i.e., they belong to the class of *Hydrolases*, the subclass of *Glycosylases*, and the subgroup of proteins that

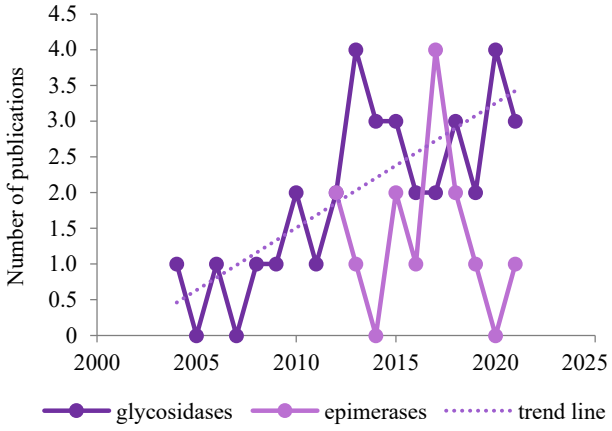


Figure 1. Publications on the biosynthesis of lactulose, by years

Рисунок 1. Распределение количества публикаций о биосинтезе лактулозы по годам

catalyze the hydrolysis of O- and S-glycosyl compounds. The biosynthesis of lactulose usually involves beta-galactosidases (*β*-galactosidases, lactases, EC 3.2.1.23), which can hydrolyze the terminal non-reducing residues of beta-D-galactose in beta-D-galactosides. These enzymes are common in nature. In fact, 593 species are capable of producing them, including various types of bacteria, fungi, plants, and animals [17].

β-Galactosidases are used in the dairy industry to hydrolyze lactose in order to obtain low-lactose and lactose-free foods for people with lactase deficiency. They can also prevent lactose crystallization in the production of condensed foods, sweet glucose-galactose syrups, and prebiotic galactooligosaccharides. The most popular commercial *β*-galactosidases come from such safe sources as *Kluyveromyces* yeasts (*Kluyveromyces lactis*, *Kluyveromyces fragilis*) and *Aspergillus* molds (*Aspergillus oryzae*, *Aspergillus niger*). Galactooligosaccharide production also uses such commercial preparations as *β*-galactosidases from *Bacillus circulans* and *Escherichia coli*. The synthesis can employ *Rhodotorula minuta* yeasts, lactic cocci and lactobacillus, bifidobacteria, and other microorganisms [18–21].

The technology of beta-galactosidase production includes the following processes. A pure culture is cultivated in elective media under optimal conditions, followed by isolation and purification of the enzyme. The cultivation process consists of three stages. First, the inoculum is obtained and activated, i.e., inoculation. The second stage involves the cultivating and growth of the inoculum biomass. The third stage is the main fermentation, which covers both the growth of microorganisms and the biosynthesis of the enzyme. The enzyme isolation process consists of pre-treatment, solid-liquid separation, filtration, concentration, stabilization, and final purification. If the enzyme accumulates inside cells, the cells of the producer are lysed. If the enzyme is released into the fermentation medium, it is separated from the cells of the producer during the separation of the solid and liquid phases. The cellular material is usually separated from the enzymes by a membrane filtration, e.g., ultrafiltration. To obtain a liquid enzyme preparation, it is stabilized, e.g., with glycerol. When the dry enzyme preparation is obtained, the ultrafiltration concentrate is spray-dried and granulated, e.g., with maltodextrin or flour. The finished product falls under the general specifications for commercial enzyme preparations [20].

The action mechanism of *β*-galactosidase includes three stages. During the first stage, it forms a complex with lactose. The second stage yields a galactosyl complex with the enzyme, and glucose is released. During the third stage, the galactose is transferred to a nucleophilic acceptor that contains a hydroxyl group. Galactose is formed if water serves as an acceptor. If it is the carbohydrate, the reaction results in transoligosaccharides. *β*-Galactosidase is able to accept nucleophiles other than

water in the active site. Lactulose may be formed in the presence of fructose, or the process may yield more complex transoligosaccharides. This ability depends on several factors: (1) the tertiary structure of the enzyme and the amino acids that form the active center, (2) the concentration of lactose, which is the donor for the formation of the galactosyl-enzyme complex, and (3) the concentration of fructose, which is the acceptor of galactosyl [22].

Processes where hydrolase enzymes serve as pseudotransferases are a kinetically controlled synthesis. Such reactions give short-term maximal yields that depend on the ratio of the synthesis rate of the target product and the hydrolysis rate of the activated donor. The result totally depends on the properties of the biocatalyst; that is why the choice of the enzyme and the form is critical. Immobilization can simplify the recovery of its application and makes it possible to reuse the enzyme, as well as increase its stability, activity, and specificity [23].

β-Glucosidase (EC 3.2.1.21) is another glycosidase enzyme that can facilitate the biosynthesis of lactulose [24, 25]. It is rather common in nature and targets beta-D-glucosides. In fact, 467 species can produce *β*-glucosidase. It is a key enzyme for lignocellulose degradation that participates in commercial synthesis of glycoconjugates and oligosaccharides [17].

Epimerases are fundamentally different enzymes. They belong to the class of isomerases. Cellobiose-2-epimerase (EC 5.1.3.11) belongs to a subgroup of enzymes that catalyze the interconversion between D-glucose and D-mannose. It residues at the reducing end of *β*-1,4-linked disaccharides by epimerization of the hydroxyl group in the C-2 position of the glucose residue [17]. Cellobiose-2-epimerase was first discovered in the culture fluid of the *Ruminococcus albus* anaerobic cicatricial bacteria. It owes its name to its ability to catalyze the epimerization of cellobiose with the formation of 4-O-*β*-D-glucopyranosyl-D-mannose. Cellobiose-2-epimerase can catalyze the mutual transformations of monosaccharides, disaccharides, and trisaccharides. In addition, it is the only epimerase known that affects unsubstituted disaccharides [26].

Cellobiose epimerases have recently entered the focus of scientific attention. So far, they have been found only in anaerobic and aerobic bacteria. Most cellobiose epimerases exhibit nothing but epimerization activity. However, those that come from thermophilic microorganisms, e.g., *Caldicellulosiruptor saccharolyticus*, *Caldicellulosiruptor obsidiansis*, *Dictyoglomus turgidum*, or *Spirochaeta thermophila*, possess isomerization activity. They can convert glucose or mannose disaccharides into fructose, and, therefore, can be used to synthesize lactulose from lactose [27]. Some recent studies report that mesophilic epimerases also exhibit isomerization activity and can be used for lactulose bioconversion [28]. The exact mechanism of this reaction remains unknown. However, the isomerization of most cellobiose epimerases is much lower than their epimerization (≤ 10%), and lactose is first converted to epilactose and only then to lactulose. Cellobiose epimerases seem to be the only exceptions: their site-directed mutagenesis reduces the yield of epilactose or completely blocks its formation [29–31].

Cellobiose epimerase producers are bacteria with a dubious safety status. Their production usually involves genetic engineering and includes the following steps. First, the genomic DNA of the enzyme is isolated from producer cells. Then, the gene enters host cells, e.g., *E. coli* or *Bacillus subtilis*. The resulting culture grows in elective media under optimal conditions. After that, the enzyme is isolated and purified to obtain the enzyme preparation. The cultivation of recombinant *E. coli* and *B. subtilis* cells occurs in an elective medium. Kanamycin or ampicillin are added until the required optical density (OD_{600} = 0.6–0.8), after which isopropyl-*β*-D-thiogalactopyranoside (IPTG) enters the reaction to inactivate the lac repressor and induce the cellobiose epimerase synthesis. Cellobiose epimerase is an intracellular enzyme. At the end of fermentation, the cells are destroyed by ultrasound or lysozyme. After that, the solid and liquid phases are separated. Affinity chromatography purifies the enzymes, but sometimes ultrafiltration may be used instead. Purification of the enzyme increases the costs and complicates the technology. Therefore, unpurified enzymes or whole bacterial cells have more commercial prospects. The finished enzyme preparation is tested for purity by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to determine its activity [28, 29, 31–37].

Publications on epimerazes in lactulose production occupied less than a quarter of all application identified publications on the topic, and all of them featured epimerazes of a bacterial origin (Fig. 2). More than 60% of publications focused on *β*-galactosidase

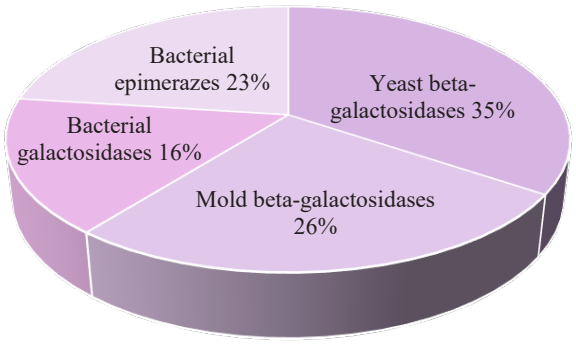


Figure 2. Publications on the biosynthesis of lactulose by enzymes and their producers

Рисунок 2. Распределение количества публикаций о биосинтезе лактулозы по ферментам и их продуцентам

of fungal origin as glycosidase producers. Enzymes from unicellular budding micromitzet (yeast) turned out to be a more popular research subject than from mycelial fungi (molds). Of all the works on bacterial glycosidase, eight articles described *β*-galactosidase, and only two featured beta-glucosidase.

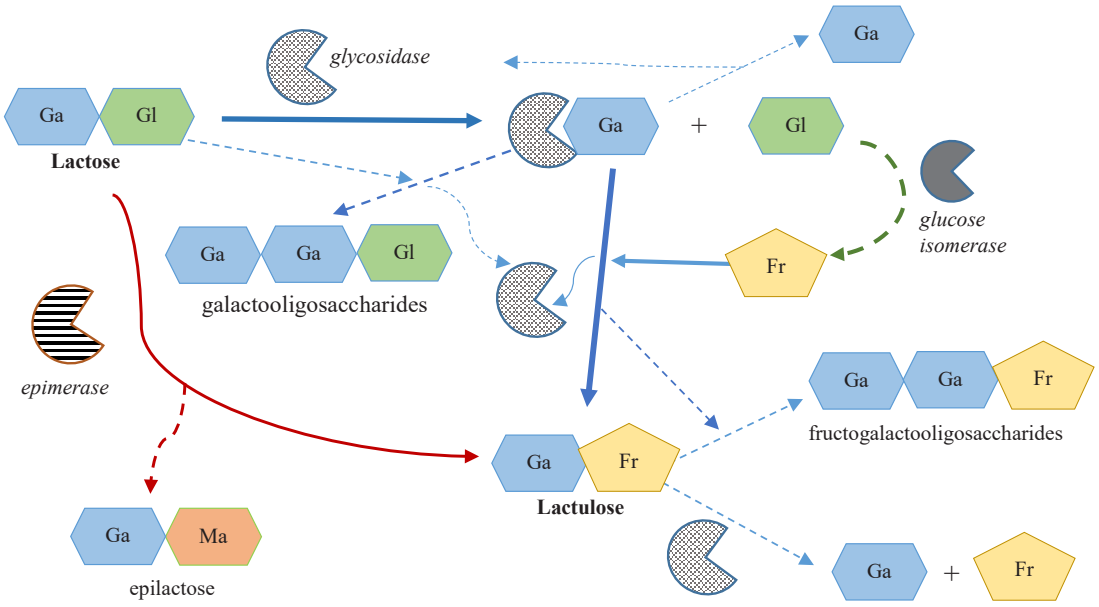
Lactulose biosynthesis is a complex process that can be accompanied by other enzymatic reactions that produce various carbohydrates. Under certain conditions, some glycosidases can also catalyze reactions that transfer galactose residue into lactose and produce galactooligosaccharides and/or transfer galactose residue into lactulose and produce fructogalactooligosaccharides. The resulting lactulose can be hydrolyzed to galactose and fructose. The amount of fructose necessary for biosynthesis can be introduced into the reaction mix as a separate substance or obtained by using glucoisomerases to isomerize glucose formed during the lactose hydrolysis [10, 12, 14].

Figure 3 illustrates the main pathways for the enzymatic production of lactulose with possible side reactions.

The final process of lactulose biotechnology is its isolation from the reaction solution which contains the enzyme (protein) and a lactose residue. Depending on the raw materials and the reaction conditions, the solution may also contain minerals, nitrogenous substances, monosaccharides (glucose, galactose, fructose), oligosaccharides (epilactose, galactooligosaccharides, fructogalactooligosaccharides), and other by-products. In the biosynthesis of functional oligosaccharides, the downstream processing accounts for 50–70% of the total production costs. High-purity products are even more expensive [16].

The lactulose yield affects the cost of purification and depends on a lot of factors, e.g., the properties of the substrates and enzymes, the reaction conditions, etc. Different publications use different units of measurement to describe the conditions of lactulose biosynthesis, as well as various indicators to evaluate the reaction results, which makes their comparison a complicated task. Some publications that attempt to generalize the available data on lactulose biotechnology report incomplete, fragmentary, and/or contradictory information [7, 10, 12, 13, 38, 39]. This situation required additional efforts to clarify the conditions for the maximal yield of lactulose and to identify the most important factors associated with different enzymes.

2. *β*-Galactosidases of micromycetes (yeasts and molds). The earliest publication that introduced biosynthesis of lactulose using yeast *β*-galactosidase appeared in 1978 [40]. In 2004, Lee *et al*. reported the potential of commercial *β*-galactosidases of various origins for lactulose synthesis [41]. They tested yeasts (*K. lactis*, *K. fragilis*), mold (*A. oryzae*), and bacteria (*E. coli*). The enzyme obtained from *K. lactis* gave the highest yield of lactulose. Since that time, more than 30 articles have been published on the processes of lactulose biosynthesis using micromycetic *β*-galactosidases. Most of these studies featured *Kluyveromyces* and *Aspergillus*.



Ga – galactose, Gl – glucose, Fr – fructose, Ma – mannose; target reactions are shown by solid lines, additional and side reactions are dotted.

Figure 3. Bioconversion pathways from lactose to lactulose

Рисунок 3. Основные пути биоконверсии лактозы в лактулозу

Table 1. Conditions for the maximal yields of lactulose using micromycetic *β*-galactosidases

Таблица 1. Условия получения максимальных выходов лактулозы с использованием *β*-галактозидаз микромицет

Producer	Enzyme (preparation, form)	Substrate composition, % weight/volume	Processing conditions	Y_{ly}, %*	Source
		***Kluyveromyces* yeasts**			
K. fragilis	Free	Lactose solution 12% and fructose 20%	pH = 7.2, 37°C Periodic**	7.5	[40]
K. lactis	Novo Nordisk Free	Lactose solution 15% and fructose 5%	pH = 6.5, 37°C, 2 h Periodic	6.1	[41]
K. fragilis	Sigma Free	Lactose solution 15% and fructose 5%	pH = 7.3, 30°C, 5 h Periodic	3.5	[41]
K. lactis ATCC 8585	Free, from untreated cells; from cells permeabilized with 50% (v/v) ethanol	Lactose solution 40% and fructose 20%	pH = 7.0, 60°C, 3 h Periodic	4 5	[41]
K. lactis	Maxilact 2000 Free	Lactose solution 20% and fructose 15%	pH = 6.5, 40°C, 1 h Periodic	16.5	[42]
K. fragilis	Lactozym 2000L Free	Lactose solution 20% and fructose 15%	pH = 6.5, 40°C, 45 min Periodic	18.5	[42]
K. lactis	Lactozym 3000L Free	Lactose solution 10% and fructose 30%	pH = 6.7, 40°C, 2 h Periodic	12.4	[43]
K. lactis	Produced by Novozyme Free	Lactose solution 80% and fructose 50% in a two-phase medium cyclohexane:water 95:5	pH = 7, 30°C, 2 h Periodic	13	[44]
K. lactis	Lactozym 3000L HP G Free	Lactose solution 33% and fructose 17%	pH = 6.5, 40°C Periodic	15.3	[45]
K. lactis	Maxilact 5000 Free	Lactose solution 20% and fructose 20%	pH = 6.8, 38°C Periodic	7.7	[46]
K. lactis	Sigma, immobilized on carbon nanotubes	Lactose whey 20% and fructose 20%	pH = 7.5, 47°C Continuous**	7.1	[47]
K. lactis	Sigma, immobilized on silica gel	Lactose solution 40% and fructose 20%	pH = 7.5, 47°C Periodic	3.9	[48]
K. lactis	Sigma, immobilized on silica gel	Lactose whey 20% and fructose 20%	pH = 7.5, 47°C Periodic Continuous	5.4 9.6	[49]
K. lactis	Maxilact 5000 Free	Lactose solution 25% and fructose 10%	pH = 7.5, 40°C Periodic	12	[50]
K. lactis	Lactozym 3000L HP G Free	Lactose solution 12% and fructose 36%	pH = 6.7, 40°C Periodic	8.7	[51]
K. lactis	Lactozym 2600L Free	Lactose solution 24.5% and fructose 25.5%	pH = 6.8, 40°C Periodic Continuous	6.9 5.2	[52, 53]
K. lactis	Lactozym 2600L Free	Lactose solution 12% and fructose 36%	pH = 6.8, 40°C Continuous	6.2	[54]
K. lactis	Biolactase-NL Enzeco Lactase NL Maxilact L200 Lactozym Pure 2600L All free	Lactose solution 1.68% and fructose 38.2%	pH = 7.0, 40°C Periodic	 19 23 24 20	[55]
K. lactis	Maxilact 5000 Free Free + Mg^{2+} immobilized on chitosan with glutaraldehyde	Lactose solution 15% and fructose 3% Cheese whey and fructose 30%	pH = 7.5, 40°C Periodic	 6.9 15.1 11.5	[56]
K. lactis	Biolactase Free Lactozym Free	Ultrafiltrated permeate, lactose 5.2% and fructose 9%	pH = 6.0, 6°C, 72 h Periodic	 21 22	[57]
K. lactis	Lactozym Free Biolactase Free	Ultrafiltrated permeate, lactose 5.5% and fructose 11.7% Lactose solution 4.4% + and fructose 9%	pH = 6.6, 6°C, 48 h Periodic pH = 6.6, 6°C, 72 h	 17.5 21	[58]

Continuation of Table 1.

Producer	Enzyme (preparation, form)	Substrate composition, % weight/ volume	Processing conditions	Y_{ly}, %*	Source
		***Kluyveromyces* yeasts**			
K. lactis	Crude, immobilized on chitosan with glutaraldehyde	Cheese whey lactose 6.5% and fructose 30%	pH = 7, 50°C Periodic.	26.7	[59]
K. lactis	Lactozym, immobilized on chitosan with agarose and polyethyleneimine	Lactose solution 10% and fructose 10% Cheese whey lactose 10% and fructose 10%	pH = 7, 50°C Periodic	13.8 28	[23]
		***Aspergillus* molds**			
A. oryzae	Sigma Free	Lactose solution 15% and fructose 5%	pH = 4.5, 30°C, 6 h Periodic	1.7	[41]
A. oryzae	Sigma-Aldrich Free	Lactose solution 3.4% and fructose 27%	pH = 5, 37°C Periodic	30.3	[24]
A. oryzae	Ha-Lactase Free	Ultrafiltrated permeate, lactose 20% and fructose 15%	pH = 6.5, 40°C, 50 min Periodic	32.8	[42]
A. oryzae	Enzeco® fungal lactase Free	Lactose solution 3.1% and fructose 46.9%	pH = 4.5, 40°C Periodic	28.2	[45]
A. oryzae	Enzeco® fungal lactase, Fungal lactase for alternative strains, Tolerase All free	Lactose solution 1.68% and fructose 38.2%	pH = 4.5, 40°C Periodic	25 25	[55]
A. niger	Klerzyme 150 (DSM) Free Rapidase (DSM) Free	Lactose solution 1.68% and fructose 38.2%	pH = 3.5, 40°C Periodic	20 23	[55]
A.aculeatus	Pectinex Ultra (Novozymes) Free	Lactose solution 1.68% and fructose 38.2%	pH = 3.5, 40°C Periodic	20	[55]
A. oryzae	Enzeco® fungal lactase Free immobilized with glutaraldehyde	Lactose solution 2.1% and fructose 47.9%	pH = 4.5, 50°C Periodic	31 25.4	[60]
A. oryzae	Sigma-Aldrich Free	Lactose solution 16% and fructose 34%	pH = 4.6, 40°C Periodic	5.5	[61]
A. oryzae	Enzeco® fungal lactase Free immobilized on: glyoxyl-agarose, amino-glyoxyl-agarose, chelate-glyoxyl-agarose	Lactose solution 4% and fructose 46% (1:20)	pH = 4.5, 50°C Periodic	34 30 28 32	[62]
A. oryzae	Enzeco® fungal lactase Free immobilized on glyoxyl-agarose	Lactose solution 5.3% and fructose 44.7% (1:16)	pH = 4.5, 50°C Periodic	32 34	[63]
A. oryzae	Enzeco® fungal lactase immobilized on glyoxyl-agarose	Lactose solution 7% and fructose 43% (1:12)	pH = 4.5, 50°C Periodic	60	[64]
A. oryzae	Enzeco® fungal lactase Free	Ultrafiltrated permeate, lactose 5.2% and fructose 9%	pH = 6, 6°C, 48 h Periodic	27	[57]
A. niger	Maxilact A4 Free	Ultrafiltrated permeate, lactose 5.2% and fructose 9%	pH = 4.5, 6°C, 48 h Periodic	13	[57]
A. oryzae	Enzeco® fungal lactase Free	Ultrafiltrated permeate, lactose 5.8% and fructose 23%	pH = 4.6, 6°C, 48 h Periodic	17.8	[58]
A. oryzae	Enzeco® fungal lactaseimmobilized on glyoxyl-agarose	Lactose solution 9.6% and fructose 40.4% (1:8)	pH = 4.5, 50°C Periodic	54	[65]

Continuation of Table 1.

Producer	Enzyme (preparation, form)	Substrate composition, % weight/volume	Processing conditions	Y_{ly}, %*	Source
		***Aspergillus* molds**			
A. oryzae	Enzeco® fungal lactase immobilized by aggregation and cross-linking using ethanol and propanol	Lactose solution 9.6% and fructose 40.4% (1:8)	pH = 4.5, 50°C Periodic	24	[66]
A. oryzae	Enzeco® fungal lactase immobilized on quaternary ammonium agarose	Lactose solution 7% and fructose 43% (1:12)	pH = 6, 50°C Periodic	24	[67]
A. oryzae	Enzeco® fungal lactase Free	Lactose solution 9.6% and fructose 40.4% (1:8)	pH = 4.5, 50°C Periodic	21	[68]

* lactulose yield, % of initial lactulose, calculated value is based on published data;

** Periodic – periodic method, Continuous – continuous fermentation.

* выход лактулозы, % от исходной лактозы, расчетное значение по данным публикации;

** Periodic – периодический способ, Continuous – непрерывный способ ферментации.

The result of enzyme-catalyzed reactions depends both on their properties, e.g., origin and form, and the reaction conditions, e.g., composition and concentration of the initial substrate, temperature and pH, fermentation time, etc. Table 1 summarizes the data on the processing conditions that yield the maximal concentrations of lactulose. Hereinafter, the presentation in the tables is structured according to the year of publication.

Table 1 shows that the main producers of *β*-galactosidases are the *K. lactis* yeast and the *A. oryzae* mold. Although the yields of lactulose vary considerably for enzymes from different producers, mold-produced *β*-galactosidases usually give higher lactulose yields.

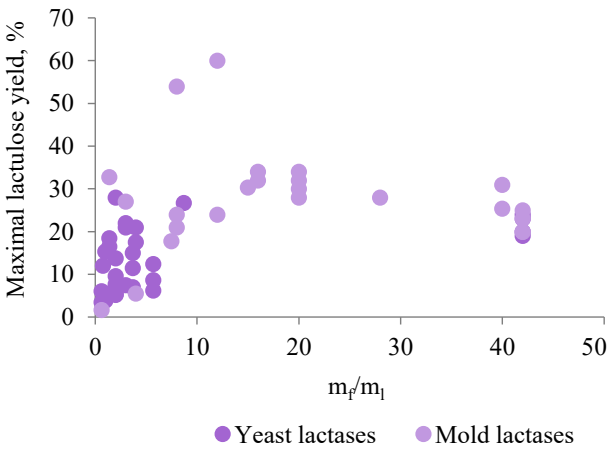


Figure 4. Maximal lactulose yield and the ratio of molar concentrations (m_f/m_l) when using micromycetic *β*-galactosidases

Рисунок 4. Зависимость максимального выхода лактулозы от отношения молярных концентраций фруктозы и лактозы ($m_ф/m_л$) при использовании *β*-галактозидаз из микромицет

Their difference may be associated with the differences in the structure of the enzymes. Lactulose synthesis mainly relies on commercial preparations of *β*-galactosidase, since most of them have a safety status issued by GRAS or its equivalent and are produced on an industrial scale in the USA, the EU, Brazil, or China.

The maximal yield of lactulose with yeast lactases of 28% is a relatively new achievement [23]. Only *K. lactis β*-galactosidase provided the best enzymatic synthesis (50.3%), which even exceeded the results of chemical synthesis. The maximal lactulose yield catalyzed by sulfite and sodium hydroxide was 10.3%, by boric acid and triethylamine – 4.7% [69]. Unfortunately, the publication provided no exact conditions that provided such high rates of biosynthesis, so it was not included in Table 1. All other publications agree that enzymatic methods remain less effective than those based on lactose isomerization in alkaline media with complexing reagents, e.g., aluminates and borates.

As for mold-based enzymes, *A. oryzae* achieved the maximal yield of 60% [64]. However, the result was short-term, and the yield did not exceed the standard 30% for mold enzymes during the remaining reaction time.

The carbohydrate composition of the initial substrate is an important factor that affects the yield of lactulose, especially the ratio of lactose and fructose in the solution. Theoretically, as the concentration of fructose increases, so does the chance that fructose, and not water or lactose, will act as the galactosyl acceptor. The yield of lactulose was reported to increase three times as the molar ratio of fructose to lactose (m_f/m_l) rose from 1 to 8 [45]. The highest yields of lactulose (≥ 30%) were obtained when the ratio of m_f/m_l reached 8, 12, 16, and 20 [27, 62, 64, 65, 67]. However, no direct relationship was detected between the maximal yields of lactulose in [23, 24, 40–68] and the molar ratios of fructose and

lactose (Fig. 4). These data can be represented as a single diagram, despite the fact that the experiments had different reaction conditions, e.g., pH, temperature, total carbohydrate concentration, etc. In fact, the nature of the kinetically controlled reaction means that the main factor is the origin of the enzyme, not the conditions [23, 45, 55].

Figure 4 shows that when the values of m_f/m_l reach ≥ 20, the lactulose yield does not increase. Moreover, low m_f/m_l ratios (≤ 5) are more financially promising. A larger amount of fructose increases the cost of raw materials, while most of the fructose does not become involved in the synthesis of lactulose and remains in the solution, which complicates the purification. The publications on yeast enzymes mostly involved lower m_f/m_l values than those that used mold enzymes, which characterizes the former as more practical.

One of the earliest works on yeast *β*-galactosidases revealed that when m_f/m_l rose from 0.5 to 1, the maximal yield of lactulose increased by 1.6 times. But when m_f/m_l reached 2, no further increase in yield followed [41]. An increase in the total concentration of carbohydrates from 37.5 to 60% led to a 1.5-fold increase in the yield of lactulose and protected the enzyme from denaturation at 60°C. However, it reduced the reaction rate because the viscosity of the solution also increased. The same publication was the first to report the idea that the ratio of lactulose and galactooligosaccharides could be regulated [41]. This possibility is important, since lactulose and galactooligosaccharides can have a synergistic effect on the prebiotic index [1].

Table 1 shows that the maximal yields of lactulose were obtained at different pH values that did not always coincide with the optimal values for this type of enzyme. It ranged 6.0–7.5 in the case of *K. lactis β*-galactosidases, the optimal pH being 7.0 [55]. For *A. oryzae* enzymes, it was 3.5–6.6, the optimal pH being 4.5 [55]. The reaction temperatures also varied. For *K. lactis β*-galactosidases, it ranged from 37 to 60°C, the optimal temperature being 50°C [55]. For *A. oryzae* enzymes, it was between 30 and 50°C with 65°C as the optimal temperature [55]. Some experiments were carried out at 6°C in order to increase the microbiological purity of the products and gave an unexpectedly high yield of lactulose (≤ 27%) [57, 58].

The enzyme concentration also affects the selectivity of the lactose bioconversion reaction. When the concentration of the enzyme was high (≥ 15 U/mL), both primary (lactose hydrolysis) and secondary hydrolysis (lactulose degradation) were faster [61]. As a kinetically controlled reaction, secondary hydrolysis does not stop after it reaches the maximal lactulose concentration. This phenomenon has often been described in publications that featured free (native) or immobilized enzymes [41, 44, 49, 52].

Continuous process might solve the problem of lactulose hydrolysis, e.g., in an enzymatic membrane reactor, a microchannel, or a packed-bed reactor [25, 47, 52–54, 61]. For instance, an enzymatic membrane system made it possible to remove the new lactulose, thus limiting its breakdown. The specific productivity remained constant at 0.7 mg of lactulose per enzyme unit per hour [52]. However, the enzymatic membrane reactor eventually reduced the enzyme activity as a result of electrostatic interaction between the enzyme and the membrane surface, as well as due to mechanical and thermal inactivation. On day 7, the concentration of lactulose decreased by 31% in an experiment with *K. lactis β*-galactosidase [54].

An automated control of the enzyme feed may be a way out. Researchers added new portions of *A. oryzae β*-galactosidase, i.e., 10% of the initial amount every 48 h, and the output lactulose concentration maintained constant at 8.8 g/L (5.5% yield) for 28 days [61]. The lactulose concentration increased 3.3 times when the packed-bed reactor operated in the continuous mode compared to the multiple batch operation mode, although in the latter case the catalyst could be reused as many as 10 times [48].

Some publications compare different enzymes. Guerrero *et al*. compared eleven different commercial *β*-galactosidases of various origins [55]. They tested four yeasts and six molds for catalytic potential for hydrolysis and transgalactosylation, as well as their affinity for galactose donors and acceptors. All the *β*-galactosidases were able to use lactose and lactulose as galactose acceptors and donors. However, the yeast lactases showed a higher hydrolytic than transgalactosylating activity, which was greater for lactose than for lactulose. When the concentrations of carbohydrates in the solution exceeded 10%, transgalactosylation prevailed over hydrolysis. When the concentration exceeded 40%, the hydrolysis of lactose and lactulose was more prominent. The maximal yields of lactulose obtained from *A. oryzae β*-galactosidases were by 5% higher than those obtained from *A. niger* and *Aspergillus aculeatus β*-galactosidases and by 1–6% higher than those obtained with *K. lactis β*-galactosidases. The concentration and composition of the obtained galactooligosaccharides depended on the type of enzyme. *A. oryzae* enzymes yielded mainly tri- and tetrasaccharides, while *A. niger* and *K. lactis* yielded di- and trisaccharides. These data confirmed that the origin of the enzyme determines its transgalactosylation potential and the ability to use various molecules as acceptors of the galactose residue. In addition, the origin of the enzyme determines the molecular structure of transgalactosylated oligosaccharides, e.g., composition, hexose units, types of bonds, etc.

Schmidt *et al*. tested seven different *β*-galactosidases for their ability to produce lactulose at 6°C [57]. Three enzyme preparations of micromycetic origin (Biolactase, Lactozym, Enzeco) produced high yields of lactulose (≥ 20%) within 24 h. Enzeco proved highly active at pH 4.5–6.5, while Lactozym and Biolactase were effec-

tive at pH 6.0–6.5. Low-temperature synthesis guarantees the microbiological purity of the resulting products and a long interval of high lactulose concentration, which provides an industrial-scale inactivation.

Other studies described various forms and types of *β*-galactosidase immobilization for lactulose biosynthesis: silica gel, nanotubes, chitosan with glutaraldehyde, agarose and polyethyleneamine, glutaraldehyde, and various agarose derivatives [23, 48, 49, 56, 59, 60, 62–65, 67, 71].

Neto *et al*. immobilized different commercial *K. lactis* *β*-galactosidases using different methods [23]. The immobilization affected the properties of *β*-galactosidases in kinetically controlled synthesis. The enzyme sample that was immobilized on glyoxylagarose appeared inactive in the production of lactulose at 25, 37, and 50°C. The sample immobilized on agarose coated with polyethyleneimine produced lactulose only at 50°C. The sample immobilized on chitosan activated with glutaraldehyde was effective at all three temperature modes. When they applied whey instead of pure lactose and fructose, the yield increased in the agarose sample and decreased in the chitosan sample.

Serey *et al*. immobilized the enzyme obtained from *A. oryzae* in quaternary ammonium agarose (QAA) [67]. The sample with repeated batch operation provided better results for total lactulose yield and cumulative specific productivity than the sample with the free enzyme. They were able to reduce the economic costs by reusing the quaternary ammonium agarose, which neither change its maximal enzymatic capacity nor reduced the productivity and selectivity of lactulose synthesis. Ramírez *et al*. increased the efficiency of biocatalysts by repeated periodic feeding [68].

Guerrero *et al*. used cross-linked aggregates of *A. oryzae* *β*-galactosidase for lactulose synthesis [60]. Although they recorded a 17% reduction in the yield relative to free enzyme, they managed to reuse the catalyst 100 times, so that the accumulated mass of product per mass unit of the used catalyst increased by a factor of twelve.

Lactulose technology is more economical if microorganism cells serve as sources of biocatalysts, not purified enzymes. Lee *et al*. synthesized lactulose in solutions of lactose and fructose using a cellular suspension of *K. lactis* permeabilized with ethanol [41]. They cultivated *K. lactis* on a thick nutrient medium and activated it in an elective liquid nutrient medium at 28°C for 24 h. After that, they transferred 1% of the inoculum to a fermentation medium and cultivated it there under the same conditions. The resulting inoculum was centrifuged at 8000 rp for 5 min. The separated cells were resuspended in a potassium phosphate buffer (pH 7.0). To permeabilize the cells, they were resuspended in 50% (v/v) ethanol, stirred for 15 min at 4°C, rinsed twice with distilled water, and resuspended in the potassium phosphate buffer with lactose and fructose to produce lactulose. The reaction was stopped by boiling. The maximal lactulose concentration of 14.8 g/L occurred when the concentration of permeabilized cells was 8 g/L. However, the productivity (1.64 g/L·h) was lower than when the cell concentration was 10.4 g/L (2.0 g/L·h), which was eventually assessed as optimal. The samples with untreated cells demonstrated the maximal concentration of synthesized lactulose of 17 g/L, which was comparable to the results of the permeabilized samples (19 g/L). However, the control sample needed 5 h to reach this concentration, whereas the experimental sample needed only 3 h. Therefore, the productivity of the sample with permeabilized cells was higher [41].

Some scientists tried using secondary dairy raw materials in an attempt to reduce the production costs. As a rule, they turned to whey and ultrafiltrated permeates [23, 42, 47, 49, 56–59]. The optimal process conditions turned out to be 48 h at 6°C. For acid whey, the process involved Enzeco enzyme from *A. oryzae* with an activity of 2.0 μkat/kg at pH 4.4, 1.28 mol/kg fructose, and 0.17 mol/kg lactose. For sweet whey, it was Lactozym from *K. lactis* with an activity of 2.8 μkat/kg at pH 6.6, 0.74 mol/kg fructose, and 0.19 mol/kg lactose [58].

De Freitas *et al*. used *K. lactis* *β*-galactosidase using cheese whey and immobilization on 2.0% (w/v) glutaraldehyde-activated chitosan [59]. They obtained a highly active and stable biocatalyst capable of simultaneously hydrolyzing lactose (42.8% conversion rate) and produced lactulose at a level of 17.3 g/L. They used clarified whey to increase the *β*-galactosidase activity of yeast and for the main cultivation. The processes occurred at 30°C and presupposed 24 h of stirring at 180 rp. The cell mass was separated by centrifugation, washed, and suspended in a potassium phosphate buffer with $MnCl_2$. Mg^{2+} and Mn^{2+} ($\leq 5\times10^{-3}$ mM) activated the resulting *β*-galactosidase, while Ca^{2+} ($\geq$ 1 mg/L) and Zn^{2+} ($\geq$ 0.011 mg/L) inhibited it. As an intracellular enzyme, yeast *β*-galactosidase can be extracted by various methods. When the cells were destroyed by shaking with glass beads for 30 min at 2000 rpm, the process was 87% more effective than when the yeast cells were destroyed with ultrasonic waves for 40 min at 25°C.

Another important aspect is the possibility of forming different conformations of lactulose. Enzymatic transgalactosylation may trigger such bonds as $\beta1 \rightarrow 4$ or $\beta1 \rightarrow 1$, $\beta1 \rightarrow 3$, $\beta1 \rightarrow 5$, and $\beta1 \rightarrow 6$ [39]. Two main isomers appear as a result of enzymatic synthesis of lactulose. Lactulose $\beta1\rightarrow4$ is formed during the chemical isomerization of lactose to lactulose. Lactulose $\beta1\rightarrow1$, also called 1-lactulose or allolactulose, appears exclusively during enzymatic transgalactosylation. 1-lactulose is the main product of this reaction. All yeast *β*-galactosidases form > 0.75 of this isomer while mold *β*-galactosidases form only

about 0.6. Shen *et al*. performed structural studies for a certain *K. lactis* β-galactosidase by high performance liquid chromatography [46]. They used the method of nuclear magnetic resonance to detect an additional peak generated by similar reactions that corresponded to 1-lactulose [52].

In general, the maximal yields of lactulose for biosynthesis with micromycetic β-galactosidases (20–30%) were comparable with the results of alkaline isomerization (30%), but much lower than in chemical synthesis with complexing borates (70%). Most studies used expensive highly-purified commercial preparations of β-galactosidases to obtain lactulose, which significantly increases the production cost. Crude enzyme preparations could solve this problem. Any culture of non-pathogenic lactose-fermenting yeast can serve as a source of β-galactosidases, not only *K. lactis*, but also *Kluyveromyces marxianus*. *K. marxianus* is a promising source of enzymes for the food industry and biotechnology because some of its strains can produce both endo- and exo-enzymes [72]. Lactase-producing yeasts have long been the favorites with Russian scientists [73]. Such strains of *K. marxianus* as VKM Y-453, VKM Y-459, VKM Y-460, VKM Y-1338, VKM Y-1341, and VKM Y-1342 have great prospects since their high β-galactosidase activity was confirmed by independent experiments [74]. Lactic acid microorganisms can also serve as producers of β-galactosidases, e.g., those that have long been used as starter cultures. A joint cultivation of yeast and lactic acid microorganisms is a promising method of obtaining complex enzyme preparations of β-galactosidases [75].

Another important and rather new trend is to use affordable and inexpensive secondary dairy raw materials. Cheese and curd whey have a good record for β-galactosidase production, while ultrafiltrates of skim milk and whey can be used in lactulose synthesis. The yields depend on the optimal pH level for the enzymes: sweet whey works best with *K. lactis* β-galactosidases, while acid whey requires *A. oryzae* β-galactosidases.

Fructose is necessary to obtain lactulose with β-galactosidases. This stage can be called the bottleneck of this method because:

– fructose is introduced in high concentrations (≥ lactose), which increases the costs;

– a significant part (≤ 90%) of fructose does not participate in the synthesis and remains in the solution; its high solubility makes it difficult to remove it from the reaction mix.

As a result, the reaction conditions should be such that require a minimum dose of fructose and provide a maximal yield of lactulose. Section 4 of this article introduces another prospective method: the biotransformation of glucose formed during the hydrolysis of lactose into fructose.

3. Applying bacterial glycosidases. Micromycetes are not the only ones with the ability to produce β-galactosidases. Many bacteria can do that, e.g., *Bacillus* (*Bacillus acidocaldarius*, *B. circulans*, *Bacillus coagulans*, *B. subtilis*, *Bacillus megaterum*, *Bacillus stearothermophilus*), *Lactobacillus* (*Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helviticus*, *Lactobacillus kefiranofaciens*, *Lactobacillus lactis*, *Lactobacillus sporogenes*, *Lactobacillus themophilus*, *Lactobacillus delbrueckii*), *Bifidobacterium* (*Bifidobacterium bifidum*, *Bifidobacterium infantis*), as well as *Arthrobacter*, *Bacteriodes*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Leuconostoc*, *Pediococcus*, *Propioionibacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Streptococcus*, *Sulfolobus*, *Thermoanaerobacter*, *Thermus*, *Trichoderma*, *Vibrio*, and *Xanthomonas* [17, 76].

Bacterial β-galactosidases are proteins of intracellular localization that differ in molecular weight, number of subunits, affinity for various substrates, optimal pH, and thermal stability. *E. coli* β-galactosidase is very well represented in scientific literature. It can be toxic and, therefore, cannot be used in the food industry: it usually serves as a model for understanding the catalytic mechanism of the enzyme action. Like yeast, bacterial β-galactosidases exhibit maximal activity at pH 6.5–7.5 [55].

Some bacterial β-galactosidases need high temperature and thermal stability. Only a few publications provide data on β-galactosidases of meso- and psychrophilic bacteria that are active at low temperatures. For example, mesophilic bacteria *Xanthomonas* sp. and *B. subtilis* demonstrated the catalytic activity at 10°C. Guerrero *et al*. registered the highest catalytic activity of the enzyme at 39.7% of the maximum detected at 40°C [45]. They explained it as a consequence of the fact *Bacillus* sp. produces at least two molecular forms of the that enzyme protein. β-Galactosidase obtained using *Pseudoalteromonas haloplanktis* TAE 79b had the optimal activity at 26°C and exhibited at least 33% activity at 4°C, while the *Pseudoalteromonas* sp. demonstrated only 20% of the maximum recorded at 40°C.

Although a lot of bacteria can produce β-galactosidases, *B. circulans* is the most popular strain in industrial production, while *Streptococcus thermophilus* and *B. stearothermophilus* are considered as potential sources [21, 76].

The method for obtaining bacterial glycosidase is similar to those described for isolating β-galactosidase from micromycetes. It includes cultivating in elective media under optimal conditions, isolating and purifying the enzyme, and obtaining the enzyme preparation. The main task in the production of enzymes associated with the cell wall is to extract them without losing their activity. The destruction of bacterial cells makes it possible to obtain a sufficiently high yield and activity of β-galactosidase. The most effective methods include ultrasonic treatment, high-speed bead mills, and high-pressure homogenizers. The most effective purification

methods applied to *β*-galactosidase are ultrafiltration, gel permeation, ion-exchange, and affinity chromatography [77, 78].

The earliest publication on lactulose biosynthesis with *E. coli* *β*-galactosidase appeared in 1987 [79]. The first research to feature *β*-glycosidase obtained from thermophilic archaea *Pyrococcus furiosus* was published in 2004 [24]. Other publications mentioned commercial *β*-galactosidases from *B. circulans* and some other bacterial species. Table 2 demonstrates the optimal processing conditions for lactulose production.

The glycosidases in Table 2 can be divided into two groups: (1) thermostable with an optimal temperature of 50–85°C and (2) mesophilic with maximal activity at 37–40°C. The first enzymes are very often used for lactulose biosynthesis because thermostable glycosidases have a lot of industrial advantages. They ensure good substrate solubility, high yield and reaction rate, low inhibition, and low contamination risk. These enzymes are often obtained with the help of genetic engineering [24, 25, 83]. For instance, thermostable *Caldivirga maquilingensis* glycoside hydrolase has 100% *β*-galactosidase activity (50% glycosidase and 25% xylosidase). According to Letsididi *et al*., it provided excellent lactose conversion at 85°C, and the yield of lactulose increased together with temperature [83]. However, further heating above 90°C led to a significant decrease in the activity of glycoside hydrolase, probably as a result of its inactivation. The resulting glycoside hydrolase remained highly active even at pH = 4–5. It was synthesized in a slightly acidic medium to reduce the chances of the Maillard reaction.

The genomic DNA of *Sulfolobus solfataricus* that encoded the synthesis of *β*-galactosidase was cloned and inserted into *E. coli* ER2566. The resulting *β*-galactosidase had an optimal pH that ranged from 5.0 to 7.0, while retaining ≥ 90% of its initial activity. The enzyme exhibited first order thermal inactivation kinetics; half-lives (t1/2) at 75, 80, 85, 90, and 95°C were 91, 48, 35, 2.6, and 0.72 h, respectively. The *β*-galactosidase was the most active at 80°C. At ≤ 80°C, the transgalactosylation reaction proceeded faster, and

Table 2. Biosynthesis of lactulose with bacterial glycosidases

Таблица 2. Биосинтез лактулозы с использованием бактериальных гликозидаз

Producer	Enzyme (preparation, form)	Substrate, % weight/volume	Processing conditions	Y_{ly}, %*	Source
β-galactosidases					
Escherichia coli	Immobilized on Eupergit	Lactose solution 10% and fructose 50%	pH = 6.8, 37°C Periodic**	6.3	[79]
Sulfolobus solfataricus	Recombinant in *Escherichia coli* cells Free	Lactose solution 40% and fructose 20%	pH = 6.0, 80°C Periodic	12.5	[80]
Bacillus circulans	Lactoles L3 Free	Lactose solution 32.8% and fructose 17.4%	pH = 5.5, 45°C Periodic	1.5	[45]
Lactobacillus acidophilus	Free	Lactose solution 40% and fructose 20%	pH = 7.0, 40°C Periodic	6.25	[81]
Bacillus circulans	Biocon NTL 3000 Free	Lactose solution 13.3% and fructose 26.6%	pH = 6.0, 60°C Periodic	9	[55]
Bacillus stearothermophilus	Spores immobilized on the surface of *Bacillus subtilis* spores	Lactose solution 20% and fructose 10%	pH = 6.0, 75°C Periodic	4.4	[82]
β-glucosidases					
Pyrococcus furiosus	Recombinant in *Escherichia coli* cells Free Immobilized on Eupergit C	Lactose solution 3.42% and fructose 27%	pH = 5.0, 75°C Periodic	44 45	[24]
Pyrococcus furiosus	Recombinant in *Escherichia coli* Free Immobilized on Eupergit C Immobilized on Amberlite IRA-93	Lactose solution 3.4% and fructose 27%	pH = 5.0, 75°C Continuous** Continuous Periodic Continuous	41 43 43 43	[25]
Glycoside hydrolase					
Caldivirga maquilingensis	Recombinant in *Escherichia coli* cells Free	Lactose solution 14% and fructose 56%	pH = 4.5, 85°C Periodic	77	[83]

* lactulose yield, % of initial lactulose, calculated value is based on published data;

** Periodic – periodic method, Continuous – continuous fermentation.

* выход лактулозы, % от исходной лактозы, расчетное значение по данным публикации;

** Periodic – периодический способ, Continuous – непрерывный способ ферментации.

the yield of lactulose increased. At ≥ 80°C, the amount of lactulose decreased because β-galactosidase grew less stable. Extra Fe^{2+} cations increased the amount of lactulose by 10%, while extra Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} had no effect on the yield. The composition of the substrate and the amount of the enzyme had a significant impact on the biosynthesis of lactulose. The yield of lactulose increased together with the substrate concentration. This effect required a greater activity of the introduced enzyme [80]. However, this β-galactosidase yielded little lactulose.

Such lactic acid microorganisms as *L. acidophilus*, *Lactobacillus reuteri*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus brevis*, *Lactobacillus plantarum*, and *S. thermophilus* are known to produce β-galactosidase [17]. However, few publications describe their use in the lactose-to-lactulose bioconversion. The transgalactolizing activity of *L. acidophilus* NRRL 4495 β-galactosidase was tested for the synthesis of lactulose and oligosaccharides under various conditions. The prebiotic yield depended on the reaction time, the concentration of the enzyme, and the composition of the substrate. The maximal amount of lactulose was obtained after 7 h (6.89 g/L); it decreased between 17–24 h. The content of other oligosaccharides after 7 and 10 h was 17.2 and 19.68 g/L, respectively, which exceeded the amount of lactulose obtained. The concentration of the introduced enzyme was another factor that affected the lactulose yield. When its content was raised from 2 to 8 U/mL, the amount of lactulose reached 25 g/L (3.6 times more than the control) and 15.4 g/L (2 times more than the control) after 7 and 10 h, respectively. A further increase in the enzyme concentration to 10 U/mL inhibited the production of lactulose. When the bioconversion occurred at pH 7.0 and the enzyme content was 2, 4, 6 U/mL, it increased the production of tetraoligosaccharides. At 8 and 10 U/mL, it was pentaoligosaccharides; at pH 5.5 and 8 U/mL, it was trisaccharides. The composition of the substrate was insignificant. The lowest yield of lactulose (5.05 g/L) was obtained from the reaction mix that contained 20% galactose, 20% lactose, and 20% fructose. However, these conditions provided a high yield of other oligosaccharides (17.25 g/L) [81].

Immobilization is another promising direction in the enzymatic synthesis of lactulose with bacterial β-galactosidases. Lactose releases monosaccharide D-galactose, which is a powerful inhibitor of many mesophilic β-galactosidases. Immobilization reduces this effect and facilitates further isolation of the enzyme from the reaction mix. Mayer *et al.* compared hyperthermostable *P. furiosus* β-glycosidase immobilized on Eupergit C(CelB) with industrial β-galactosidase *A. oryzae* (Sigma-Aldrich) [24]. β-Glycosidase yielded by 14% more lactulose than β-galactosidase. For both enzymes, a higher initial concentration of lactose in the reaction mix increased the absolute yield of the product, but the average yield went down. A higher initial concentration of lactose resulted in more oligosaccharides differ from lactulose. Extra fructose brought the average yield of lactulose up to 48%. However, the reaction was inhibited when the fructose content reached 36%. The immobilized β-glycosidase was more stable and active during biosynthesis.

Mayer *et al.* also tested a continuous enzymatic process to produce lactulose with trans-galactosylation using free and immobilized *P. furiosus* β-glycosidase [25]. They obtained free β-glycosidase (CelB) heterologously in *E. coli* BL21. Next, they immobilized the enzyme on an anion exchange resin (Amberlite IRA-93) or on Eupergit C. The efficiency of the immobilization appeared to depend on the pH level (7.5) and temperature (30°C). The activity of β-glycosidase immobilized on Eupergit C (1500 μkat/g) was higher than in the case of Amberlite IRA-93 (920 nkat/g). The final yield of lactulose was approximately the same in all three cases (41–43%), but the immobilization increased the stability and lifespan of the enzyme.

Most bacterial β-galactosidases are intracellular enzymes. Nevertheless, some microorganisms are known to release them into the medium as a result of cell autolysis after the end of the cultivation process. Montanari *et al*. tested 32 lactose-fermenting strains of *L. brevis* and 15 strains of *L. plantarum* obtained mainly from fermented horse's milk (koumiss) and other products, e.g., fermented silage or sausages [84]. *L. brevis* were able to release intracellular β-galactosidase into the medium at the end of the cultivation process. The release of the enzyme began immediately after the end of reproduction and was associated with the autolysis of cells and the destruction of their walls. *L. plantarum* did not release β-galactosidase, since their autolysis occurred in a different way. Therefore, the screening of microorganisms capable of releasing enzymes into the medium still remains an urgent task and can significantly simplify lactulose production.

Bacterial enzymes allow for a wide temperature range, from 20°C (*Arthrobacter*) to 75°C (*Pyrococcus*) and even 80°C (*Sulfolobus*) [24, 25, 80, 85]. However, most bacterial β-galactosidases give a low yield of lactulose. Genetically engineered *P. furiosus* glycosidases (45% lactulose) and *C. maquilingensis* glycoside hydrolases (77%) were an exception, but the synthesis was too complicated and expensive. *L. acidophilus* β-galactosidase gave an option of using safe lactic acid starter cultures, but this issue requires additional research. Some scientists [1, 85] used f *Arthrobacter* sp. Others [45, 55] proved that *B. circulans* β-galactosidase provided a low yield of lactulose, but a high yield of galactooligosaccharides and can be used for their biosynthesis.

Thus, the prospective directions in the biosynthesis of lactulose with bacterial glycosidases include: (1) enzymes that retain their stability at high (≥ 75°C)

and low (≤ 20°C) temperatures, as well as low-acidic environmental conditions (pH = 4–5); (2) *β*-galactosidases of safe lactic acid microorganisms and bacteria that release the enzyme in the cultivation medium; (3) available and cheap methods of enzyme immobilization that require no extra expenditures on their isolation and stability increase but allow for secondary use.

4. Combined use of *β*-galactosidases and glucoisomerases. The main disadvantage of glycosidase-based synthesis of lactulose is that it is impossible without fructose in the substrate. Only a part of fructose enters the bioconversion reaction as an acceptor of galactosyl. Its residues have to be removed from the mix after the end of the reaction, which complicates the process and increases its cost. However, the new glucose can be isomerized into fructose, e.g., with commercial glucose isomerase (EC 5.3.1.18), which is used to obtain fructose. Table 3 summarized the available data on the processing conditions that render the maximal concentrations of lactulose.

Hua *et al*. synthesized lactulose by a double enzymatic method in water-organic two-phase media from lactose and fructose [44]. They immobilized *β*-galactosidase by crosslinking it with magnetic microspheres (5–10 μm) that consisted of Fe_3O_4 and chitosan. Phosphate buffer served as the aqueous phase. The organo-aqueous media increased the transglycosylating activity of lactase and the lactulose yield. As the amount of water in the system increased, both the yield of lactulose and the rate of lactose conversion went down. No lactulose developed without water. At the onset of the reaction, the fructose concentration was low because the low glucose content was low, and extra fructose had to be added to the reaction mix. The lactulose yield reached its maximal value at 30°C. When the temperature reached 35°C, the yield dropped by half. The lactulose yield peaked for 2–4 h at pH 6.0–9.0: all this time, the degree of lactose conversion remained 65%. This methos has two main disadvantages. First, the concentrations of organic solvents to be removed are too high. Second, the immobilization process is too difficult to design.

Song *et al*. used whey to obtain lactose [87]. They produced fructose by glucose isomerization and did not add extra fructose to the substrate. They immobilized enzyme by adding activated silica gels to a pre-treated solution of *β*-galactosidase or glucose isomerase at 20°C for 12 h of constant stirring.

Song *et al*. assessed the effect of lactose concentration on lactulose synthesis using different concentrations of lactose in the reaction mix (w/v) [87]. The yield of lactulose increased as the concentration of lactose rose from 14 to 20%. At a higher concentration, the viscosity of the reaction mix increased sharply, which eventually inhibited the enzymes. The fructose concentration went up as the proportion of immobilized glucose isomerase increased. It reached its maximal concentration of 33.9 g/L after 2 h when the ratio of immobilized *β*-galactosidase and glucose isomerase was 1:6. The yield of lactulose increased in proportion to the fructose concentration until the enzyme ratio became 1:5. Probably, it happened because the mass transfer was limited when the viscosity of the reaction mix started to grow as the amount of the immobilized enzyme increased [87]. This method has the following disadvantages. First, purified enzyme preparations are expensive. Second, the immobilization process is too complex. Third, the yield of lactulose is too low.

Lorenzen *et al*. used the bi-enzyme system to treat condensed ultrafiltrated permeate of skimmed milk [86]. They introduced 0.5% $MgSO_4 \cdot 7H_2O$ and incubated the mix with *β*-galactosidase and glucose isomerase at pH 7.8 and 45°C. The formation rate of transoligosaccharides was higher during the first hour of fermen-

Table 3. Biosynthesis of lactulose based on *β*-galactosidases and glucoisomerases

Таблица 3. Биосинтез лактулозы с использованием *β*-галактозидаз и глюкоизомераз

Producer	Enzyme (preparation, form)	Substrate composition, % weight/volume	Processing conditions	Y_{ly}, %*	Source
Kluyveromyces lactis *Streptomyces murinus*	*β*-galactosidase Immobilized Glucoisomerase Immobilized (Novozyme)	Cyclohexane buffer system 95:5 (vol./vol.), lactose 80% an fructose 10%	pH = 8, 30°C, 2 h Periodic**	18.9	[44]
Kluyveromyces lactis *Streptomyces rubiginosus*	*β*-galactosidase Lactozym Free Glucoisomerase Gensweet Free	Condensed ultrafiltrated permeate of skimmed milk, lactose 40%	pH = 7.8, 45°C Periodic	1.1	[86]
Kluyveromyces lactis *Streptomyces rubiginosus*	*β*-galactosidase Immobilized Glucoisomerase Immobilized	Lactose solution 20%	pH = 7.5, 53,5°C Periodic	3.8	[87]

* lactulose yield, % of initial lactulose, calculated value is based on published data;

** Periodic – periodic method, Continuous – continuous fermentation.

* выход лактулозы, % от исходной лактозы, расчетное значение по данным публикации;

** Periodic – периодический способ, Continuous – непрерывный способ ферментации.

tation, when 16.4% galactooligosaccharides and 0.7% lactulose were obtained, and then decreased. After 4 h, the yield was 21.4 and 1.1%, respectively.

Thus, the combined use of *β*-galactosidases and glucoisomerases makes it possible to completely or partially solve the problem of introducing fructose into the substrate for lactulose synthesis. However, the process is complex and does not increase the yield of lactulose. Such methods require additional enzymes (glucoisomerases). The same problems arise with purified enzyme preparations, i.e., cost, economic sanctions, etc. Still, glucoisomerases can be produced by some types of yeast, in particular baker's yeast, e.g., *Saccharomyces cerevisiae*, and they might be introduced to obtain crude *β*-galactosidases. Another option is to use the natural symbiosis of kefir grains, which contains several types of lactic acid bacteria and yeast.

5. Applying bacterial cellobiose epimerases for lactulose production. Lactose turns into lactulose under the action of cellobiose epimerase. This reaction is based on isomerization, where the glucose fragment of lactose isomerizes into fructose [26]. The exact mechanism of lactose-to-lactulose transformation by using epimerases still remains unclear.

All known cellobiose epimerases have a near-neutral optimal pH. The optimal temperature depends on the source organism of the enzyme and ranges from 8 to 85°C [26]. However, Chen *et al*. reported that low temperatures in lactulose biosynthesis yielded epilactose as the main reaction product [33]. Epilactose has a beneficial biological effect on human health, promotes the development of bifidobacteria and lactobacilli, and improves the absorption of calcium and magnesium [26]. Several publications focused on epilactose as the main product of lactose conversion [33, 37, 88]. However, if the aim of the work was to increase the yield of lactulose, epilactose was considered undesirable as the final product of biosynthesis. Table 4 summarized the available data on the process conditions that render the maximal concentrations of lactulose.

Table 4 shows that the cellobiose-2-epimerase of *C. saccharolyticus* is the most popular raw material in lactulose synthesis. The yield of lactulose ranges from 43 to 88%. The highest value was obtained by adding boric acid to the reaction mix, probably, due to the formation of lactulose-borate complexes [36]. The binding affinity of borate for lactulose is 59 times higher than for lactose. By binding the new lactulose, borate contributes to its removal from the reaction mix, and the synthesis process continues. The greater the molar ratio of borate and lactose, the higher the degree of lactose isomerization. However, the ratio of 1:1 M results in a plateau, and the degree of lactose isomerization reaches its maximum. In addition, 10% of epilactose appeared as a by-product of the reaction after 1 h. After 3 h, it decreased and remained below 2%. This method provides a high yield of lactulose and is simpler and more environmentally friendly than alkaline synthesis. Unfortunately, borate has to be removed using ion-exchange resins, which significantly increases the production costs.

Quite often, works on cellobiose epimerase as a catalyst for lactose bioconversion used a controlled or random mutagenesis. Most often, its goal was to increase the thermal stability of the enzyme [28, 31, 32, 35, 36, 89, 92]. Thermostable enzymes have a number of industrial advantages. For instance, solubility of the substrate and the rate of the enzymatic reaction increase at high temperatures. The recombinant cellobiose-2-epimerase of the thermophilic *C. obsidiansis* demonstrated high thermal stability up to 86.7°C. Its half-life was 8.1, 2.8, and 0.6 h at 75, 80, and 85°C, respectively. With lactose as substrate, epilactose appeared very rapidly. The highest yield of epilactose (55 g) was achieved already after 10 min of reaction, with only 10 g of lactulose formed, i.e., the initial epimerization rate was significantly higher than the isomerization rate. After 10 min, the output of epilactose slowly went down, and lactulose production remained steady for 4 h [32].

Shen *et al.* developed eight mutants with the *C. saccharolyticus* cellobiose-2-epimerase gene, and five of them had a prolonged inactivation half-life at 80°C [71]. Genetic engineering increased the maximal activity temperature of the enzyme from 80 to 87.5°C. Some E161D/S180P/S351G and E161D/N365P mutants retained ≥ 50 and ≥ 70% of their activity, respectively, even at 95°C. In addition, the mutant enzymes were more resistant to chemical denaturation and exhibited a wider pH range. The authors believed that these mutants had good prospects for lactulose biosynthesis but gave no data on its yield.

Xiao *et al*. also confirmed that thermophilic bacterial cellobiose epimerases as catalysts produce the highest yield of lactulose [92]. They obtained cellobiose-2-epimerase from *Dictyoglomus thermophilum*, a thermophilic microorganism. The resulting enzyme had a significantly higher initial epimerization rate and a larger amount of epilactose produced in the first 10 min of the reaction. After that, the epilactose yield started to decrease, but the synthesis of lactulose went on, providing a sufficiently high yield even after 4 h. A greater amount of lactose in the substrate did not increase the degree of its conversion.

Kim & Oh worked with the *C. saccharolyticus* recombinant enzyme [89]. The experiment showed first order kinetics for thermal inactivation and half-lives (inactivation rate constants) of 74, 34, 16, and 5 h at 65, 70, 75, and 80°C, respectively. The yield of lactulose and epilactose grew together with the concentration of the enzyme until a plateau at 150 U/mL of cellobiose-2-epimerase. A higher concentration of lac-

Table 4. Biosynthesis of lactulose using cellobiose-2-epimerases

Таблица 4. Биосинтез лактулозы с использованием целлобиозо-2-эпимераз

Producer	Enzyme (preparation, form)	Substrate composition, % weight/volume	Processing conditions	Y_{ly}, %*	Source
Caldicellulosiruptor saccharolyticus	Recombinant in *Escherichia coli* Free	Lactose solution 70%	Рн = 7.5, 80°C, 2 h Periodic**	58	[89]
Dictyoglomus turgidum	Recombinant in *Escherichia coli* Free	Lactose solution 34.2%	pH = 7.0, 70°C, 3 h Periodic	54.3	[35]
Caldicellulosiruptor saccharolyticus	Recombinant in *Escherichia coli* Free	Lactose solution 70% + borate 12% Lactose solution 70%	pH = 7,5, 80°C, 3 h Periodic	88 58	[36]
Caldicellulosiruptor saccharolyticus	Recombinant in *Escherichia coli* Spores immobilized of *Bacillus subtilis*	Lactose solution 70%	pH = 7.0, 80°C, 4 h Periodic	56.4	[34]
Caldicellulosiruptor saccharolyticus	Recombinant in *Escherichia coli* Free	UHT milk: fat 1.5%, lactose 4.85%	pH = 7.5, 50°C, 24 h Periodic pH = 7.5, 8°C, 72 h Periodic	57.7 56.7	[37]
Caldicellulosiruptor saccharolyticus	Mutant in *Escherichia coli* Free	Lactose solution 50%	pH = 7.5, 80°C, 4 h Periodic	76	[30]
Flavobacterium johnsoniae	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 8.4, 25°C Periodic	< 3	[28]
Dyadobacter fermentans	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 7.7, 40°C Periodic	6–9	[28]
Ruminococcus albus	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 7.5, 20°C Periodic	6–9	[28]
Rhodothermus marinus	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 6.3, 70°C Periodic	19	[28]
Bacteroides fragilis	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 7.5, 35°C Periodic	< 3	[28]
Butyrivibrio sp. AE2015	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 7.0, 50°C Periodic	6–9	[28]
Firmicutes bacterium	Recombinant in *Escherichia coli* Free	Lactose solution 51%	pH = 7.5, 35°C Periodic	< 3	[28]
Caldicellulosiruptor saccharolyticus	Recombinant in *Bacillus subtilis WB800* Free	Cheese whey (lactose 20%)	pH = 7.0, 80°C Continuous**	58.5	[90]
Caldicellulosiruptor obsidiansis	Recombinant in *Escherichia coli* Free	Lactose solution 20%	pH = 7.5, 70°C, 4 h	55	[32]
Caldicellulosiruptor saccharolyticus	Mutant in *Escherichia coli* Free	Lactose solution 20%	pH = 7.5, 65°C, 2 h Periodic	43	[29]
Caldicellulosiruptor saccharolyticus	Recombinant in *Escherichia coli* Immobilized in the form of cross-linked aggregates (CLEAs)	Lactose solution 25%	pH = 7.5, 80°C Continuous	58.8–61.7	[91]
Dictyoglomus thermophilum	Recombinant in *Escherichia coli* Free	Lactose solution 20% Lactose solution 40%	pH = 7.0, 80°C Periodic	50.7 46.7	[92]
Caldicellulosiruptor saccharolyticus	Mutant in *Escherichia coli* Free	Lactose solution 27.4%	pH = 7.5, 70°C Periodic	75	[31]
Bacillus thermoamylovorans	Mutant in *Escherichia coli* Free	Lactose solution 27.4%	pH = 7.5, 70°C Periodic	69	[31]
Rhodothermus marinus	Mutant in *Escherichia coli* Free	Lactose solution 27.4%	pH = 7.5, 70°C Periodic	82	[31]
Ruminococcus albus	Mutant in *Escherichia coli* Free	Lactose solution 27.4%	pH = 7.5, 70°C Periodic	69	[31]
Spirochaeta thermophila	Mutant in *Escherichia coli* Free	Lactose solution 27.4%	pH = 7.5, 70°C Periodic	56	[31]

* lactulose yield, % of initial lactulose, calculated value is based on published data; ** Periodic – periodic method, Continuous – continuous fermentation.

* выход лактулозы, % от исходной лактозы, расчетное значение по данным публикации; ** Periodic – периодический способ, Continuous – непрерывный способ ферментации.

tose in the substrate led to a proportional increase in lactulose and epilactose. This phenomenon distinguishes cellobiose epimerase from *β*-galactosidase, because the transgalactosylating activity of *β*-galactosidase increases together with the substrate concentration. Cellobiose-2-epimerase can produce lactulose even at low lactose concentrations in the reaction mix, which makes it possible to use dairy products with a low lactose content as a substrate.

For example, Rentschler *et al*. used UHT milk as a substrate for lactulose biosynthesis [37]. To maintain microbiological purity, they chose 8 and 50°C because these temperatures produce almost no undesirable organoleptic taste changes. The study involved two bioconversions with different initial activity of *C. saccharolyticus* cellobiose-2-+epimerase: 4.16 ± 0.21 μkat $_{\text{epilactose, 50°C}}$ and 39.5 ± 1.4 μkat $_{\text{epilactose, 50°C}}$. In both cases, epilactose was the first rapidly formed lactose conversion product. Its maximal concentration was reached after 40 and 5 min, respectively. After that, its concentration decreased steadily for 6 h until the equilibrium. At a 9.5 times higher initial activity of the enzyme, the yield of epilactose (% wt./wt.) fell in relation to the initial milk lactose from 33.6 to 15.5%. Lactulose appeared with a delay. Another bioconversion was performed at 8°C with the initial activity of cellobiose-2-epimerase CsCE 27.8 ± 1.3 μkat $_{\text{epilactose, 50°C}}$. Epilactose and lactulose developed according to the same pattern as at 50°C, but slower than expected. The maximal amount of lactulose was reached after 72 h, and the concentration of epilactose was 13.6%. The synthesis conducted at 8°C resulted in a higher residual cellobiose-2-epimerase activity of 72%, which was almost 10 times greater than when the temperature was 50°C. These data can be used to produce dairy products and fortify them with prebiotic lactulose *in situ*. However, this method involved genetically modified microorganisms, which causes concern and controversy among both food producers and consumers.

Wang *et al*. used whey with > 65% lactose and > 11% protein as a substrate for lactulose biosynthesis in the EMR system [90]. The method required *B. subtilis* as host cells to express the genomic DNA of *C. saccharolyticus* cellobiose-2-epimerase. Unlike *E. coli*, *B. subtilis* produces no toxins during growth and development and is recognized as safe for the food industry and pharmacy. In addition, genetically modified *B. subtilis* produced extracellular cellobiose epimerase, which reduced the costs and simplifies the process. The continuous synthesis of lactulose in the EMR system reduced the loss of enzyme, which could be reused. The yield of lactulose increased as a result of its continuous removal from the reaction mix. The lactose conversion rate was high during the first 30 min. However, it started to decrease when the sugar conversion exceeded 50% and reached its maximum after 2 h. The short reaction time made it possible to reduce energy consumption and costs, which is of great importance for industrial production.

Another goal of cellobiose epimerase mutagenesis is to reduce its epimerization activity and, as a result, reduce the amount of epilactose, while simultaneously increasing the isomerization activity and the yield of lactulose. Shen *et al*. developed a G4-C5 mutant of cellobiose-2-epimerase obtained from *C. saccharolyticus* DSM 890 [30]. They used random mutagenesis, which had a 3.0-fold higher activity than the natural enzyme (WT) and did not compromise thermal stability. The G4-C5 mutant also retained higher activity at low temperatures and a wide range of pH activity compared to the natural WT enzyme. After 4 h, the yield of lactulose and epilactose, catalyzed by the natural WT enzyme, was approximately 57 and 16%, respectively. The lactulose concentration catalyzed by the G4-C5 mutant increased from 0 to 90 min and then gradually increased to a constant level of 76%. The G4-C5 mutant produced no epilactose.

Park *et al*. performed a rational genetic modification of *C. saccharolyticus* cellobiose-2-epimerase [29]. They used the superposition of three known structural models of this enzyme and identified two residues: Tyr114 and Asn184. Probably, these residues had an important role in epilactose binding. They modified these residues to prevent the epimerization of lactose to epilactose and compared the ability of the natural and mutant cellobiose-2-epimerases to synthesize lactulose. Enzyme Y114E made it possible to obtain 42% more lactulose and 80% less epilactose than the natural enzyme.

Wang *et al*. tried to increase the isomerization activity and thermal stability of *C. saccharolyticus* (CsCE) cellobiose-2-epimerase [31]. They designed four mutants by replacing flexible loops. They used CsCE enzyme as a protein scaffold and replaced the lower part of its flexible loops with the corresponding parts of four other enzymes, namely *Bacillus thermoamylovorans* (BtCE), *Rhodothermus marinus* (RmCE), *R. albus* (RaCE), and *S. thermophila* (StCE). All the cellobiose epimerase mutants except RmC showed low isomerization and epimerization activity. The isomerization activity of RmC (8.50 U/mg) increased by 2.2 times compared to CsCE (3.85 U/mg), while the epimerization activity of RmC (43.73 U/mg) approximated that of CsCE (44.22 U/mg). CsCE was the only variant with the maximal activity temperature to reach 75°C: in other mutants, this value dropped to 70°C. All enzymes exhibited first order kinetics for thermal inactivation. The half-life value (t1/2) of StC was 38.29 h, which exceeded that of CsCE (29.07 h). Conversely, the t1/2 values of RmC (19.22 h), RaC (17.08 h), and BtC (23.11 h) were lower than that of CsCE. Therefore, a better catalytic activity of an enzyme could lower its thermal

stability. All four mutants demonstrated lactose bioconversion similar to CsCE and yielded 56–82% lactulose and 7–10% epilactose.

Gu *et al*. described another promising direction in lactulose biosynthesis [34]. By immobilizing cellobiose epimerase, they increased the economic efficiency, simplified the process of isolation process, and increased its stability. In addition, they managed to make the enzyme highly reusable. They induced the *C. saccharolyticus* cellobiose-2-epimerase in *E. coli* BL21 and immobilized it on *B. subtilis* WB600 spores. The strength that bound the enzyme to the carrier was an important indicator. The amount of cellobiose epimerase adsorbed on spores was very sensitive to pH because the pH of the adsorption medium changed the ionization state of the enzyme and the spore surface. The highest ability of cellobiose-2-epimerase to bind to the spore surface was observed after 2 h of immobilization at pH 4.0–4.5 and 4°C. As a result, the enzyme remained active in a wider pH range. Its thermal stability increased, thus raising the stability of lactulose biosynthesis in general. As a result, the enzyme retained 70% of its activity when it was reused eight times. The initial biosynthesis stage had a higher rate of conversion under the action of the free enzyme than when the enzyme was immobilized, probably because the immobilized enzyme had a granular form and was less homogeneously distributed in the mix. After 4 h, the immobilized cellobiose epimerase yielded by 52% more lactulose than the sample with the free enzyme [34].

Wang *et al*. immobilized the cellobiose-2-epimerase of *C. saccharolyticus* using cross-linked enzyme aggregates (CLEA) [91]. They selected fructose-monosaccharide supported CLEA (Fru-CLEA) and sucrose-disaccharide supported CLEA (Suc-CLEA) as the most effective biocatalysts. CLEA showed no increase in the optimal operating temperature but had an extended temperature range (70–80°C) and retained higher activity at 50–90°C. A lower-temperature lactose conversion is more energy-efficient and preserves the final product. In this case, however, the process of lactulose biosynthesis with free cellobiose-2-epimerase and CLEA demonstrated no fundamental differences from other methods, and the epilactose content was 9.3–10.2%.

Cellobiose epimerases of mesophilic microorganisms are seldom used in lactulose bioconversion because the yield of the prebiotic is quite low. Kuschel *et al*. used mesophilic cellobiose-2-epimerase obtained from *Flavobacterium johnsoniae* (FjCE), *R. albus* (RaCE), *Dyadobacter fermentans* (DfCE), *Bacteroides fragilis* (BfCE), *R. marinus* (RmCE), *Firmicutes bacterium* CAG:534, and *Butyrivibrio* sp. AE2015 [28]. However, they failed to raise the lactulose yield. Moreover, the amount of epilactose was 30%, which exceeded that of lactulose. The isomerization activity of these cellobiose epimerases varied from 8.7 ± 0.1 to 1300 ± 37 μkat/mg.

In general, when lactulose synthesis was catalyzed by cellobiose epimerases, the yield was rather high (38–88%) and comparable with alkaline and borate isomerization. Such high results mean that the method can be used for industrial production.

The type of cellobiose-epimerase producer affects the final yield of lactulose, as does the amount and form of the enzyme. A number of publications reported a stable and high lactulose yield of 54–58% provided by native, not genetically modified, cellobiose-2-epimerase of thermophilic bacteria *C. saccharolyticus*, *D. turgidum*, and *C. obsidiansis* [30, 35–37, 89, 90]. However, directed or random mutation can increase the activity of cellobiose epimerases, improve their thermal stability, and raise the lactulose yield. Mutant cellobiose epimerase were reported to yield 56–82% lactulose [31, 71].

Cellobiose epimerases have a great advantage over other methods of lactulose synthesis: they need a single substrate, i.e., lactose, in the reaction medium. As a rule, a greater concentration of lactose in the substrate leads to a proportional increase in lactulose. Cellobiose epimerases can synthesize lactulose from a lactose-poor substrate, if whey enters the reaction.

Unfortunately, cellobiose epimerases are not commercial. The problem is that cellobiose epimerases are produced from *Arthrobacter*, *Dictyoglomus*, and *Calidicellulosriptor*, whose safety status remains vague. As a result, this method always involves genetic engineering of genes responsible for the production of cellobiose epimerases in the cells of other microorganisms, e.g., *B. subtilis* or *E. coli*. Genetically modified microorganisms not only complicate the production process but also raise public concerns.

Thus, the lactulose biosynthesis with cellobiose epimerases includes the following directions. First, genetic engineering and mutagenesis methods can be used to produce an enzyme with desired properties, e.g., a better thermal stability, a higher isomerization activity, etc. Second, immobilization can increase the stability and activity of the enzyme. This method allows for multiple use of the enzyme and simplifies the isolation process.

6. Purification of lactulose. Various chemical, physical, mechanical, and biological methods can be used to remove ballast substances from lactose-lactulose solutions. Some publications give a detailed description of physicochemical methods that isolate lactulose as a separate stage of purification [9, 10]. Electrodialysis and ion exchange are popular methods of demineralization. By-products of dark-colored melanoidin and caramelization are usually removed by refining, and lactose is removed by crystallization [9]. The choice of cleaning processes depends on the product requirements, production volume, and financial capabilities of the enterprise. Nutritional supplements with prebiotic oligosaccharides require rather cheap but promising methods, e.g., high-throughput cascade nanofiltration and/or activated carbon adsorp-

tion. These methods separate mono-, di-, and oligosaccharides because they have different hydrophobicity and spatial orientation of CH groups. Pharmaceutical preparations require expensive methods of highly selective chromatography. Complex and interrelated processes of conversion and purification make it possible to organize continuous work and reduce costs [16].

Botelho *et al*. used this approach to build a membrane bioreactor that provided simultaneous synthesis and fractionation of oligosaccharides [93]. They mixed *β*-galactosidase with a 15% lactose solution to decrease the permeability of ultrafiltration cellulose acetate membranes with respect to monosaccharides, disaccharides, and trisaccharides by 60, 20, and 75%, respectively. The ratio of synthesis and hydrolysis of galactooligosaccharides depended on the cross-flow rate and the operating pressure. The optimization increased the galactooligosaccharide yield by 60% compared to reactions without membrane processes.

Biological treatment methods have recently gained a lot of scientific attention. These methods rely on the fact that microorganisms ferment only certain carbohydrates, and at different rates. Once the cultures are selected, they can be used to remove particular mono- and/or disaccharides from oligosaccharide solutions. *S. cerevisiae*, *K. marxianus*, and *K. lactis* are the most popular yeast strains to be used. As a rule, biological treatment requires neither pretreatment of solutions nor special equipment. However, it may be expensive since it presupposes additional fermentation processes, as well as removal of utilized microorganisms and their metabolic products [16].

Biotreatment processes are popular in the field of galactooligosaccharide studies. Glucose removal methods can be used together with galactose removal or alone. They involve non-lactose-fermenting yeast species, as well as the removal of mono- and disaccharides. *Kluyveromyces* yeast strains with a high rate of glucose, galactose, and lactose metabolism make it possible to obtain 90% high-purity galactooligosaccharides [94].

Similar strategies can be applied in lactulose technology. Julio-Gonzalez used biological and physicochemical methods to purify commercial lactulose syrups. They used four commercial preparations of *β*-galactosidase for selective enzymatic hydrolysis of lactose and epilactose. The experiment revealed that *β*-galactosidases obtained from *B. bifidum* (Saphera® 2600 L) and *B. circulans* (Biolactas NTL*2) had the lowest activity towards lactulose and the highest activity towards lactose and epilactose. The samples with hydrolyzed lactose and epilactose were treated with activated carbon and an aqueous solution of ethanol to remove all monosaccharides. The research resulted in a high-purity product that contained > 94% lactulose [95].

Guerrero *et al*. used the *S. cerevisiae* and *K. marxianus* yeasts to purify a mix of transoligosaccharides obtained by enzymatic synthesis with the *A. oryzae β*-galactosidase [96]. The resulting product was monosaccharide-free and contained 28% lactulose and 20% oligosaccharides.

Co-fermentation is a promising trend in lactulose biotechnology. It can rely on a combined use of lactose-fermenting and non-fermenting enzymes from different types of yeast, as well as on the co-fermentation of yeast and lactic acid microorganisms. For example, Huang *et al*. used co-fermentation to improve the physicochemical and organoleptic properties of goat milk, while Ryabtseva *et al*. used it to obtain complex enzyme preparations of *β*-galactosidases [75, 97]. Co-immobilization of enzymes in lactulose production can also involve two or more enzymes, both at the stage of lactose conversion and purification. Wilson *et al*. produced lactofructose syrup from lactose based on simultaneous fermentation with coimmobilized *β*-galactosidases and glucose-(xylose)-isomerases [98].

Conclusion

Modern food science knows two ways of enzymatic lactose-to-lactulose conversion: isomerization (direct) and transgalactosylation (with intermediate hydrolysis). Isomerization requires cellulose-2-epimerases, which have no safety status and, as a result, cannot be produced on an industrial scale. The reaction produces epilactose as a by-product, and its exact mechanism remains unknown. Transgalactosylation involves glycosidases that break down lactose to galactose and glucose, as well as add a galactose residue to fructose. This method usually requires *β*-galactosidases, which are officially safe and market-available. Their mechanism of action is a highly researched matter. The publications dynamics indicates the high relevance of *β*-galactosidase-based lactulose biosynthesis.

β-Galactosidases are able to accept nucleophiles other than water in the active center. As a result, they can form not only lactulose, but also more complex oligosaccharides, including galactooligosaccharides of various structures. They owe this ability to the tertiary structure of the enzyme, as well as the concentration of lactose and fructose in the substrate. This reaction makes it possible to control the ratio of carbohydrates, which is important since lactulose and galactooligosaccharides are prebiotics and can have a synergistic effect. However, to obtain pure lactulose, other oligosaccharides have to be removed, which increases the production cost.

The *Kluyveromyces lactis* yeast and the *Aspergillus oryzae* mold are the main producers of *β*-galactosidases. Micromycetic enzymes produce a wide range of lactulose concentrations, but optimal conditions can result in a 30% lactulose yield, which makes it as effective as alkaline isomerization.

No publication has reported a direct relationship between the maximal yield of lactulose and the molar

ratio of fructose:lactose (m_f/m_l). The area of $m_f/m_l \leq 5$ proved to be the most cost-efficient one. Large amounts of fructose increased the cost of raw materials, while most of the fructose did not participate in the synthesis of lactulose and remains in solution, which makes purification processes difficult. Therefore, the optimal reaction conditions presuppose the minimal possible dose of fructose and the maximal yield of lactulose. The glucose formed during the lactose-to-fructose hydrolysis can be biologically transformed.

Cellobiose epimerases make it possible to raise the lactulose yields to 70–80%, which is the level of chemical catalysis with aluminates and borates. However, this method involves genetic engineering and mutagenesis, which improve the safety of enzymes but raise public concerns and complicate the technology. The chemical synthesis of lactose has the same probems. Cheap secondary dairy raw materials, e.g., whey, seem to be a more practical lactose production method.

The most promising methods of lactulose biotechnology are cheap and continuous. They include the following trends:

– secondary dairy raw materials can cultivate enzyme producers and synthesize lactulose;
– immobilization makes enzymes more stable, active, reusable, and easy to isolate from the reaction mix;
– different models of reactors, e.g., membrane installations, can provide simultaneous synthesis and fractionation of lactulose and galactooligosaccharides;
– complex and interrelated processes combine the production of enzymes, the conversion of lactose to lactulose, and the purification of finished products, including co-fermentation and co-immobilization.

Contribution

S.A. Ryabtseva developed the research idea, designed the study, collected publications, translated them, analyzed the data, and wrote the manuscript. A.G. Khramtsov analyzed the data and revised the article. M.A. Shpak, A.D. Lodygin, G.S. Anisimov, S.N. Sazanova, and Yu.A. Tabakova collected publications, translated them, analyzed the data, and wrote the manuscript.

Conflict of interest

The authors reported no conflict of interests regarding the publication of this article.

Критерии авторства

С. А. Рябцева – идея и структура работы, поиск, перевод и анализ информации, написание статьи. А. Г. Храмцов – анализ информации, существенная переработка научного и интеллектуального содержания статьи. М. А. Шпак, А. Д. Лодыгин, Г. С. Анисимов, С. Н. Сазанова и Ю. А. Табакова – поиск, перевод и анализ информации, написание статьи.

Конфликт интересов

Авторы заявляют об отсутствии конфликта интересов.

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