**Biocatalysts in Synthesis of Microbial Polysaccharides: Properties and Development Trends**

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**Abstract:** Polysaccharides synthesized by microorganisms (bacterial cellulose, dextran, pullulan, xanthan, etc.) have a set of valuable properties, such as being antioxidants, detoxifying, structuring, being biodegradable, etc., which makes them suitable for a variety of applications. Biocatalysts are the key substances used in producing such polysaccharides; therefore, modern research is focused on the composition and properties of biocatalysts. Biocatalysts determine the possible range of renewable raw materials which can be used as substrates for such synthesis, as well as the biochemistry of the process and the rate of molecular transformations. New biocatalysts are being developed for participating in a widening range of stages of raw material processing. The functioning of biocatalysts can be optimized using the following main approaches of synthetic biology: the use of recombinant biocatalysts, the creation of artificial consortia, the combination of nano- and microbiocatalysts, and their immobilization. New biocatalysts can help expand the variety of the polysaccharides’ useful properties. This review presents recent results and achievements in this field of biocatalysis.

**Keywords:** polysaccharides; renewable biomass; wastes; biocatalysts; consortia; conversion

**1. Introduction**

Biocatalytic synthesis of polysaccharides (PSs) is one of the promising and topical areas of the development of modern biotechnology [1]. The variety of useful properties (the ability for gelation, the formation of viscous solutions, high adhesive ability, etc.) helps the PSs to find still newer applications in a plethora of fields. These include the medicine, pharmaceutical, food, chemical, textile, and oil and gas industries, as well as the immobilization of cells and enzymes, etc. [2,3]. Many PSs have antitumor, prebiotic, antiviral, anti-inflammatory, antioxidant, immunomodulatory effects, facilitating wound healing and tissue regeneration, eliminating pain syndrome, neutralizing the side effects of medications, and stimulating hematopoiesis [4–6]. Currently, PSs of plant origin are actively used in the industry; however, plant-based production is necessarily seasonal and depends on weather conditions. Therefore, the interest in PSs synthesized by biocatalysts (BTCs) in the form of cells of various microorganisms (bacteria, fungi, etc.) taken in a suspended or immobilized state is steadily growing.

Microbial PSs are more diverse in composition and properties than those of plant origin. Moreover, via controlling the BTCs’ properties and the conditions of biocatalytic processes of biopolymers’ synthesis, it is possible to obtain polymers with the desired features and in the required quantities [7]. The microbial PSs are characterized by the presence of a large number of functional groups (hydroxyl, carboxyl, carbonyl, acetate, etc.), which make it possible to modify such biopolymer molecules in order to give them valuable properties [7,8].

In microorganisms considered BTCs and capable of synthesizing PSs, these biopolymers perform a number of diverse functions. These include, in particular, protective, reserve, nutritional, stabilizing ones; besides, PSs determine the immunological properties.
and virulence of strains, participate in adhesion processes and are responsible for the formation of biofilms. Among the microbial PSs synthesized by various BTCs, intracellular and extracellular biopolymers usually differ in their localization. Intracellular PSs are accumulated inside of BTCs, whereas extracellular PSs, exopolysaccharides (EPSs), are usually secreted into the medium containing the cell and can be separated from BTCs, while the biopolymers themselves can assume the form of capsules, mucus, layers, etc. The interest in EPSs today is mainly due to their unique properties, including those that can benefit mankind, particularly in using EPSs as prebiotics and immunomodulators. It is toward the development of approaches to the biocatalytic production of EPSs and their derivatives that the attention of many researchers is currently directed [9–12]. Despite the successes achieved in the field of biotechnology of microbial polysaccharides, the number of them produced by industry is extremely limited, and the problem of finding new cost-effective ways to obtain them is still acute. This is largely due to the low yield and high cost of the resulting products. The main ways to reduce costs include using cheap substrates, increasing yields by creating more productive strains using genetic engineering methods, and optimizing cell culture processes. The rate or degree of conversion of a carbohydrate substrate into a polymer product can be increased by improvement of the specific activity of enzymes involved in synthesis and regulating the biosynthesis pathways of EPS precursors. Another problem in the process of EPSs biosynthesis is the change in the rheological properties of the medium at the stage of EPSs formation, which creates difficulties during mixing and difficulties for mass transfer processes. Despite numerous studies and the creation of productive strains, optimal ways have not yet been found that would allow the creation of mutant strains that fully meet the requirements of industrial production. In addition, the use of genetically modified microorganisms on an industrial scale always has a number of significant limitations, primarily related to ecology. There is also a need to introduce expensive inducers into nutrient media for the biosynthesis of necessary enzymes in such cells and antibiotics to suppress native microflora. Undoubtedly, the development of new highly productive stable biocatalysts, providing, among other things, fundamentally new materials, will remove a number of restrictions on the use of EPSs.

The range of the most significant PSs obtained microbiologically includes pullulan, dextran, bacterial cellulose (BC), alginate, xanthan, levan, curdlan, succinoglycan, and others. The interest in them is primarily due to the variety of their possible practical applications. BTCs play an important role in EPS synthesis, ensuring the flow of interrelated enzymatic biochemical transformations from the initial substrate to the final product. The range of the substrates that can be successfully used for bioconversion and the characteristics of the products thus obtained depend on the BTCs. The latter also determines the possible biochemical transformations and thus influences the choice of methods and conditions for the synthesis of PSs, as well as the speed of the process and the yield of the target product.

The screening of natural BTCs and the construction of recombinant ones with enhanced productivity as well as the optimization of producer cultivation processes are among the most promising approaches available to date for developing novel BTCs. New PS-producing strains with modified or introduced enzymatic activity can be created with genetic engineering methods. These approaches can help expand the range of low-cost substrates available for PS production and increase the degree of their transformation into PSs. Today, it seems very attractive to obtain single-stage nanocomposite materials based on PSs by introducing various additives directly into reaction media with BTCs during the synthesis of PSs or organizing the synthesis process using a combination of several BTCs.

To date, the mechanisms of synthesis of individual PSs have been studied and are well known, whereas the knowledge about the mechanisms of intercellular interactions when combining several BTCs for PS synthesis is just beginning to emerge. On the other hand, the accumulated and systemized information concerning individual BTCs is certainly a solid base for understanding the general laws of the functioning of more complex biocatalytic
systems with multifunctionality and multiple enzymatic activities. Such complex microbial BTCs are the subject of the most recent research in this area. This review is devoted both to summarizing the information on the currently known BCs used for the synthesis of PSs, and to discussing the prospective development of modern research in this field of biocatalysis.

2. Immobilized Biocatalysts for the Synthesis of Exopolysaccharides

There are both prokaryotic cells and eukaryotes that prefer aerobic or anaerobic processes among the biocatalysts synthesizing PSs, and this generally determines the diversity of the biocatalytic processes synthesizing various EPSs. The common trait of all these catalysts in such processes is the increase in the concentration of cells and their transition to a quorum-sensing (QS) state. QS ensures the activation of the synthesis of PSs \([13,14]\) as stabilizing, protective, and reserve substances for highly concentrated microbial populations. Therefore, creating biosystems with a high content of cellular biomass producing PSs and supporting the microbial BTCs in such a concentrated and metabolically active state is one of the nature-like approaches to improving the efficiency and stability of BTCs (Figure 1).

![Figure 1. Main variants of BTCs used for producing microbial EPSs from various sources.](image)

It is known that immobilized cells, being in the QS state, can withstand significantly higher concentrations of toxic substances than free cells. Immobilized cells have a higher period of semi-inactivation and can be stored for a long time without a loss of metabolic activity. The immobilization of the cells leads to a change in their genetic and biochemical status, launching various cascade regulatory systems in these cells and the intensification of biochemical processes of basic metabolism. All these factors lead to an increase in the overall productivity, viability, and resistance of these cells \([15,16]\). This ensures a huge interest in the use of immobilized microbial cells as BTCs for the synthesis of EPSs.

For example, *Lactobacillus rhamnosus* RW-9595M cells immobilized on a solid insoluble carrier (ImmobaSil), which is a silicone polymer, when cultured in a medium containing
a serum permeate at a concentration of 5–8 wt.%, were able to synthesize EPS during 4 working cycles with the accumulation of EPSs in concentration of 1.7 g/L [17]. However, this EPS concentration was lower than that accumulated in the medium with free cells (2.35 g/L), which was due to the problems for mass transfer processes created by the carrier used. At the same time, a high concentration of immobilized cells (8.5 × 10^11 cells/g of carrier) led to an increase in EPS productivity (250 mg/L/h), which was almost 2.5 times higher than in the case of free cells (110 mg/L/h).

The continuous process of EPS production, organized using the same immobilized *L. rhamnosus* RW-9595 M cells [18], revealed morphological and physiological changes in the cells, leading to the formation of very large aggregates consisting of cells and EPS themselves, which reduced the level of accumulation of the latter (0.138 g/L). Therefore, the synthesized PS should be removed from the cells to provoke them for further synthesis.

Another study of EPS production in various media with BTCs in the form of immobilized *Lactobacillus delbrueckii* subsp. *bulgaricus* using Ca-alginate, k-carrageenan, and a number of other carriers was performed [19]. This study showed that the maximum concentration of EPS can be obtained by culturing cells immobilized in Ca-alginate gel in an Elliker nutrient medium with the addition of sucrose (5 wt.%). The process duration was 18 h at 37 °C and pH 5.5. The productivity of such cells exceeded that of free cells by 46%.

Immovilized BTCs were also used in low-fat cheese production technology, and it was shown that the maximum amount of EPSs (5.7 mg/g of cheese) was formed after 22 days of the process. A study of EPS production using *L. plantarum* MK O2 bacterial cells immobilized in agar and alginate gels [20] showed a EPS yield of 225 mg/L, which was only slightly higher than that in the case of free cells. On the other hand, another study noted an almost fivefold increase in EPS synthesis (0.9 ± 0.1 g/L) by *L. plantarum* MTCC 9510 cells immobilized in Ca-alginate gel when the cells were cultured in a medium containing 40 g/L of lactose for 72 h [21]. Such an increase in the synthesis of EPS by immobilized cells was ascribed to an increase in the cell density per unit volume, as well as to the separation of cells from EPS caused by the carrier.

Until recently, the possibility of BTC immobilization in gel matrices was practically not considered as an acceptable option. It was supposed that such methods could work only in the case of both the substrate and the synthesis product having a low molecular weight [22].

The production of such high-molecular substances as BC by cells immobilized in polymer gels was considered impossible. It was assumed that BC synthesized inside the granules of the gel carrier should block its own secretion into the medium. However, relatively recently, the possibility of efficiently using *Acetobacter xylinum* cells immobilized in Ca-alginate gel for producing a food product based on a BC layer was demonstrated [23]. Moreover, the immobilized cells were found to be capable of being reused in batch mode. The duration of one working cycle was 264 h with an average thickness of the formed BC layer of 0.8 cm. After two working cycles, the viability of the immobilized cells was still high enough. However, Ca-alginate gels are known to have relatively low mechanical strength, which depends on the pH of the reaction medium and the ionic strength of the solutions in which the BTCs are functioning. In some cases, the carrier gels can be destroyed due to cell growth inside the gel matrices. The metabolic activity of the cells was also found to reduce the operational life of such carriers and, consequently, that of the BTCs [24].

Poly(vinyl alcohol) (PVA) cryogels have high mechanical and thermal resistance, a rigid macroporous structure with variable pore size, and chemical stability in various environments. They have long been successfully used for the immobilization of various microorganisms and their use in environments with a complex chemical composition, under various conditions, pH values and buffering of the medium, and thus can serve as an alternative to alginate gels [25–31]. Thus, bacterial *Komagataeibacter xylinum* B-12429 cells immobilized in PVA cryogel easily synthesized and “pushed” the formed BC filaments through the pores of the polymer carrier, which eventually merged into a dense gel film without covering the cells.
The latter, thus, were deprived of the possibility of transition to a state of rest, and the synthesis of BC by the cells even became steadily more active [32]. When \textit{K. xylinum} B-12429 cells immobilized in PVA cryogel were cultivated in a medium containing 20 g/L of glucose, the mass of the synthesized BC was 1.6 times higher than that obtained in a suspension culture. Glucose was completely consumed by immobilized cells in 120 h of cultivation. The accumulation of free cells in the medium during the cultivation of the immobilized BTCs was six times less than the concentration of cells in the suspension culture. A more dramatic decrease in pH was also observed in the environment with immobilized cells during the first 70 h of cultivation, which indicated a more intensive formation of metabolites reducing the pH of the medium.

The above-mentioned role of QS in PS synthesis was also confirmed by the following experiment. An increase in the concentration of the producer cells in the composition of immobilized BTCs (from 20 to 40 g dry cell/kg) led to a noticeable increase in the accumulation of BC in the reaction medium [32]. Note that the specific form of BTCs used in the process (granules or layers) had virtually no effect on the level of BC accumulation. The possibility of reuse of immobilized cells that retain 100% of their metabolic activity for at least 10 working cycles was demonstrated both in media containing pure glucose and those with Jerusalem artichoke hydrolysates. The study of the BC samples synthesized by \textit{K. xylinum} B-12429 cells in free and immobilized form under identical conditions showed that polysaccharide films produced by immobilized BTCs had a greater tensile strength, 30% greater thickness, and a higher degree of polymerization.

Although only a few studies of the use of immobilized cells for PSs biosynthesis have been performed, the high efficiency of such an approach has been proven beyond doubt. The immobilized cells in most cases were in the state of highly concentrated populations and had therefore significantly higher metabolic rates, thus ensuring a higher yield of many target PSs compared to the free cells. The possibility of their long-term functioning (Table 1) is yet another advantage of the BTCs’ immobilization, allowing an essential increase in the overall efficiency of the BC production. The approach based on the use of immobilized cells makes it possible to obtain PSs from various types of renewable non-food raw materials and biomass, providing an essential advantage over the free cell case in terms of both the process efficiency and the characteristics of the produced PSs (Table 1).

| Biocatalyst [Reference]; Features of Cells | Substrate Specificity of BTCs and Main Product (PS) | Conditions for BTCs’ Use | Rate of PS Synthesis |
|--------------------------------------------|--------------------------------------------------|---------------------------|----------------------|
| \textit{Leuconostoc mesenteroides} SF3 [33] | Sucrose-100.0 g/L, Dextran-22.5 g/L | pH 6.5, 30 °C, 20 h | 1.13 g/L/h |
| \textit{L. mesenteroides} SF3 [34] | Sucrose-100.0 g/L, Dextran-20.8 g/L | pH 6.0, 28 °C, 24 h | 0.87 g/L/h |
| \textit{Weissella confusa} R003 [35] | Sucrose-100.0 g/L, Dextran-25.0 g/L | pH 7.5, 30 °C, 24 h, 125 rpm | 1.04 g/L/h |
| \textit{L. mesenteroides} N7 [36] | Sucrose 100.0 g/L, Dextran-13.2 g/L | 30 °C, 24 h, 100 rpm | 0.55 g/L/h |
| \textit{L. citreum} B-2 [37] | Sucrose 75.0 g/L, Dextran-28.3 g/L | 30 °C, 48 h, 80 rpm | 0.59 g/L/h |
| \textit{L. pseudomesenteroides} XG5 [38] | Sucrose-125.0 g/L, Dextran-35.5 g/L | 30 °C, 48 h | 0.74 g/L/h |
| \textit{L. pseudomesenteroides} G29 [39] | Sucrose-101.4 g/L, Dextran-38.4 g/L | pH 5.5, 30 °C, 10 h, 200 rpm | 3.84 g/L/h |
| \textit{W. cibaria} 27 [40] | Sucrose-60.0 g/L, Dextran-24.8 | pH 6.2, 22 °C, 24 h | 1.03 g/L/h |
| Biocatalyst [Reference]; Features of Cells | Substrate Specificity of BTCs and Main Product (PS) | Conditions for BTCs’ Use | Rate of PS Synthesis |
|-------------------------------------------|--------------------------------------------------|--------------------------|-----------------------|
| *W. confusa Ck15 [41]*                    | Sucrose-20.0 g/L                                | pH 6.3, 30 °C, 24 h      | 0.62 g/L/h            |
|                                           | Chickpea flour-280.0 g/L                        |                          |                       |
|                                           | Dextran-14.9 g/L                               |                          |                       |
| *L. mesenteroides NRRL B-512F [42]*       | Sucrose-200.0 g/L                              | 30 °C, 24 h              | 1.79 g/L/h            |
|                                           | Milk permeate powder-150.0 g/L                  |                          |                       |
|                                           | Dextran-42.9 g/L                               |                          |                       |
| *L. mesenteroides MTCC 7337 [43]*         | Sugars in sugarcane juice-50.0 g/L              | pH 7.0, 30 °C, 72 h, 150 rpm | 0.20 g/L/h |
|                                           | Dextran-14.3 g/L                               |                          |                       |
| *L. pseudomesenteroides DSM20193 [44]*    | Sucrose-40.0 g/L                              | pH 6.4, 25 °C, 24 h, 200 rpm | 0.46 g/L/h |
|                                           | Brewers’ spent grain-100.0 g/L                  |                          |                       |
|                                           | Dextran-11.1 g/L                               |                          |                       |
| *Lactobacillus mali CUPV271 [45]*         | Sucrose-20 g/L/                              | pH 5.5; 28 °C; 48 h      | 0.24 g/L/h            |
|                                           | Dextran-11.7 g/L                               |                          |                       |
| *Weissella cibaria 10 M; mutant strain [46]* | Sucrose-171.0 g/L                              | pH 5.4–6.2, 25 °C, 24 h | 0.58 g/L/h            |
|                                           | Dextran-14.0 g/L                               |                          |                       |
| *L. mesenteroides KIBGE-IB22M20; mutant strain [47]* | Sucrose-250.0 g/L                              | pH 7.5, 25 °C, 12 h     | 0.88 g/L/h            |
|                                           | Dextran-10.5 g/L                               |                          |                       |
| *L. mesenteroides subsp. dextranicum B-5481; immobilized in PVA cryogel [48]* | Sucrose-200.0 g/L                              | pH 7.0, 28 °C, 15 h, 200 rpm | 4.20 g/L/h; reuse in 5 cycles |
|                                           | Dextran-63.0 g/L                               |                          |                       |
| *L. mesenteroides KIBGE HA1 [49]; immobilized in Ca-alginate gel* | Sucrose-100.0 g/L                              | pH 5.0, 30 °C, 24 h 200 rpm | 0.33 g/L/h; reuse in 12 cycles |
|                                           | Dextran-8.0 g/L                               |                          |                       |
| *Bacillus subtilis MTCC 441 [50]*         | Sucrose-100.0 g/L                              | pH 7.0, 37 °C, 20 h, 150 rpm | 1.52 g/L/h            |
|                                           | Levan-30.4 g/L                                 |                          |                       |
| *Z. mobilis PTCC 1718 [51]*               | Sucrose-300.0 g/L                              | 28 °C, 48 h              | 1.19 g/L/h            |
|                                           | Levan-57.0 g/L                                 |                          |                       |
| *Bacillus sp. MTCC 1434 [52]*             | Sucrose-250.0 g/L                              | pH 6.0, 30 °C, 30 h, 100 rpm | 2.03 g/L/h            |
|                                           | Levan-61.0 g/L                                 |                          |                       |
| *Brachybacterium phenoliresistens*         | Sucrose-300.0 g/L                              | pH 7.8, 30 °C, 72 h, 150 rpm | 0.12 g/L/h            |
|                                           | Levan-8.6 g/L                                  |                          |                       |
| *B. subtilis (NCIM 5021) [50]*             | Fresh coconut inflorescence sap (sugars g/L: sucrose–172.3 glucose-16.2, fructose-6.2) | pH 6.5, 35 °C, 17 h, 150 rpm | 3.65 g/L/h            |
|                                           | Levan-62.1 g/L                                 |                          |                       |
| *Zymomonas mobilis CCT4494; immobilized in PVA cryogel [54]* | Sucrose-300.0 g/L                              | pH 7.0, 30 °C, 12 h      | 6.77 g/L/h            |
|                                           | Levan-81.2 g/L                                 |                          |                       |
| *Z. mobilis CCT4494; immobilized in Ca-alginate gel [55]* | Sucrose-350.0 g/L                              | pH 4.0, 30 °C, 24 h, 200 rpm | 0.88 g/L/h; reuse in 12 cycles |
|                                           | Levan-21.1 g/L                                 |                          |                       |
| *Z. mobilis CCT4494; immobilized on sugarcane bagasse [56]* | Sucrose-350.0 g/L                              | pH 4.0, 30 °C, 24 h      | 1.34 g/L/h; reuse in 12 cycles |
|                                           | Levan-32.1 g/L                                 |                          |                       |
### Table 1. Cont.

| Biocatalyst [Reference]; Features of Cells | Substrate Specificity of BTCs and Main Product (PS) | Conditions for BTCs’ Use | Rate of PS Synthesis |
|-------------------------------------------|---------------------------------------------------|--------------------------|----------------------|
| **Xanthan**                              |                                                   |                          |                      |
| *Xanthomonas campestris* AM001 [57]       | Maltose-70.0 g/L                                 | pH 7.0, 32 °C, 80 h, 600 rpm | 0.51 g/L/h           |
| *X. campestris* ATCC 13951 [58]          | Winery wastewater (sugars g/L-30.7)              | pH 7.0, 29 °C, 96 h, 475 rpm | 0.25 g/L/h           |
| *X. campestris* pv. campestris 1866 and 1867 [59] | Coconut shells or cocoa husks hydrolysates-20.0 g/L (25.0 g/L of sugars) Xanthan-3.6 g/L (coconut shells) Xanthan-4.5 g/L (cocoa husks) | 28 °C, 96 h, 250 rpm | 0.04-0.05 g/L/h |
| *X. campestris* WXLB-006; mutant strain [60] | Glycerol-40.0 g/L (+fed batch 1–3 g/L/h) Xanthan-33.9 g/L | pH 7.0, 30 °C, 60 h, 200 rpm | 0.57 g/L/h           |
| *X. campestris* ATCC 13951; immobilized in polyurethane foam [61] | Sucrose-50.0 g/L/ Xanthan-59.9 g/L | 28 °C, 96 h, 180 rpm | 0.62 g/L/h; reuse in 12 cycles |
| *X. campestris* PTCC 1473; self-immobilized cells on stainless-steel support [62] | Glucose-20.0 g/L/ Xanthan-3.5 g/L | pH 6.9, 30 °C, 47 h, 180 rpm | 0.08 g/L/h           |
| *X. campestris* PTCC1473; immobilized in calcium alginate–polyvinyl alcohol-boric acid gel [63] | Hydrolyzed starch-20.0 g/L Xanthan-9.2 g/L | pH 6.6, 28 °C, 48 h, 180 rpm | 0.19 g/L/h; reuse in 3 cycles |
| *X. campestris* PTCC 1473; immobilized on plastic (polyethylene) support [64] | Glucose-20.0 g/L/ Xanthan-8.0 g/L | pH 7.2, 30 °C, 48 h, 180 rpm | 0.17 g/L/h           |
| **Alginate**                             |                                                   |                          |                      |
| *Pseudomonas stutzeri* [65]               | Sucrose-20.0 g/L/ Alginate-5.0 g/L                | pH 7.0, 30 °C, 600 h, 200 rpm | 0.008 g/L/h          |
| *Azotobacter vinelandii* 12 [66]         | Sucrose-35.0 g/L/ Alginate-2.7 g/L                | pH 7.2, 28 °C, 72 h, 210 rpm | 0.04 g/L/h           |
| *A. vinelandii*, NRRL-14641 [67]         | Apple peels-10.0 g/ Alginate-180.6 mg/g          | pH 7.5, 38 °C, 48 h       | 3.76 mg/g/h          |
| *A. vinelandii* AT9; mutant strain [68]   | Sucrose-20.0 g/L/ Alginate-3.8 g/L                | pH 7.2, 29 °C, 72 h, 200 rpm | 0.05 g/L/h           |
| *A. vinelandii* B10436; immobilized in PVA cryogel [22] | Sucrose-30.0 g/L/ Alginate-2.5 g/L | pH 7.0, 29 °C, 72 h, 200 rpm | 0.035 g/L/h; reuse in 5 cycles |
| **Pullulan**                             |                                                   |                          |                      |
| *Aureobasidium melanogenum* TN1-2 [69]   | Sucrose-140.0 g/L/ Pullulan-114.0 g/L             | 28 °C, 132 h, 250 rpm | 0.86 g/L/h           |
| *Rhodospirillum paludigenum* PUPY-06 [70] | Sucrose-50.0 g/L/ Pullulan-21.0 g/L               | pH 6.0, 25 °C, 168 h, 150 rpm | 0.125 g/L/h          |
| *A. melanogenum* A4 [71]                 | Maltose-303.0 g/L/ Pullulan-122.3 g/L             | pH 7.0, 30 °C, 120 h, 180 rpm | 1.02 g/L/h           |
### Table 1. Cont.

| Biocatalyst [Reference]; Features of Cells | Substrate Specificity of BTCs and Main Product (PS) | Conditions for BTCs’ Use | Rate of PS Synthesis |
|------------------------------------------|--------------------------------------------------|--------------------------|----------------------|
| *A. pullulans* Y-4137; immobilized in PVA cryogel [15] | Hydrolysate of Jerusalem artichoke tubers, hydrolysate of potato pulp, hydrolysate of *Chlorella vulgaris* biomass (glucose-15.0–25.0 g/L) Pullulan-3.5–16.8 g/L | pH 5.5, 26 °C, 50 h, 200 rpm | 0.07–0.33 g/L/h reuse in 15 cycles |

#### Bacterial cellulose (BC)

| Biocatalyst; Reference | Substrate Specificity | Conditions | Rate of PS Synthesis |
|------------------------|----------------------|------------|----------------------|
| *Lactobacillus hilgardii* IITRKH159 [72] | Fructose-50.0 g/L BC-7.2 g/L | pH 6.0, 30 °C, 134 h | 0.05 g/L/h |
| *Komagataeibacter maltaceti* [73] | Dextrin-8.0 g/L BC-6.5 g/L | pH 6.0, 30 °C, 134 h | 0.04 g/L/h |
| *K. nataicola* [73] | Maltose-10.0 g/L BC-5.4 g/L | pH 6.0, 30 °C, 134 h | 0.008 g/L/h |
| *K. rhaeticus* [74] | Acerola waste hydrolysate-50.0 g/L + glucose 20.0 g/L BC-2.3 g/L | pH 3.6, 30 °C, 288 h | 0.004 g/L/h |
| *Medusomyces gisevii* Sa-12 [75] | Miscanthus biomass hydrolysate (sugars-20.1–21.2 g/L) BC-1.24 g/L | pH 4.0–4.6, 27 °C, 288 h | 0.004 g/L/h |
| *Gluconacetobacter xylinus* CGMCC 2955; mutant strain [76] | Glucose-25.0 g/L BC-4.3 g/L | pH 6.0, 30 °C, 360 h | 0.012 g/L/h |
| *K. xylinum*; immobilized in PVA cryogel [32] | Glycerol-20.0 g/L BC-2.8 g/L | | 0.017 g/L/h |
| *Chlorella vulgaris* C1 biomass hydrolysate (sugars-45.1 g/L) BC-2.6 g/L | | | 0.015 g/L/h |
| *Laminaria saccharina* biomass hydrolysate (sugars-36.6 g/L) BC-0.07 g/L | | | 0.0004 g/L/h |
| *Acanthophora muscoide* biomass hydrolysate (sugars-56.0 g/L) BC-0.4 g/L | | | 0.002 g/L/h |
| *Ulva lactuca* biomass hydrolysate (sugars-24.1 g/L) BC-0.08 g/L | | | 0.0005 g/L/h reuse in 10 cycles |
### Table 1. Cont.

| Biocatalyst [Reference]; Features of Cells | Substrate Specificity of BTCs and Main Product (PS) | Conditions for BTCs’ Use | Rate of PS Synthesis |
|------------------------------------------|-----------------------------------------------------|--------------------------|----------------------|
| Fructo-oligosaccharides                   |                                                     |                          |                      |
| *A. pullulans* FRR 5284 [77]              | Substrate: sucrose-500.0 g/L Fructo-oligosaccharides-306.3 g/L | pH 5.5, 55 °C, 3 h       | 102.1 g/L/h          |
| *A. pullulans* CCY 27-1-94; immobilized on reticulated polyurethane foam [78] | Substrate: sucrose-200.0 g/L Fructo-oligosaccharides-108.2 g/L | pH 5.5, 28 °C, 25 h, 150 rpm | 4.33 g/L/h          |
| *A. pullulans* CCY 27-1-94; immobilized on walnut shell [78] | Substrate: sucrose-200.0 g/L Fructo-oligosaccharides-126.5 g/L | pH 5.5; 28 °C; 36 h, 150 rpm | 3.51 g/L/h          |
| Curdlan                                   |                                                     |                          |                      |
| *Bacillus cereus* PR3 [79]                | Substrate: starch-100.0 g/L Curdlan-40.9 g/L          | pH 7.0, 35 °C, 84 h, 200 rpm | 0.22 g/L/h          |
| *Paenibacillus* sp. NBR-10 [80]           | Substrate: sucrose-50.0 g/L Curdlan-4.8 g/L           | pH 7.0, 30 °C, 168 h, 200 rpm | 0.06 g/L/h          |
| *Agrobacterium* sp. ATCC 13140 [81]       | Substrate: juice of discarded asparagus–100 g/L (Sucrose-50.0 g/L) Curdlan-40.2 g/L | pH 5.5, 30 °C, 168 h, 200 rpm | 0.24 g/L/h          |
| *Agrobacterium* sp. DH-2 [82]             | Substrate: cassava starch hydrolysate (sugars-90 g/L) Curdlan-28.4 g/L | pH 5.5, 30 °C, 96 h, 250 rpm | 0.30 g/L/h          |
| *Agrobacterium* sp. CGMCC 11546; mutant strain [83] | Substrate: sucrose-60.0 g/L Curdlan-48.0 g/L | pH 5.0, 30 °C, 96 h, 250 rpm | 0.50 g/L/h          |
| *Agrobacterium* sp. IFO 13140; immobilized on loofa sponge [84]; | Substrate: glucose-50.0 g/L Curdlan-17.8 g/L | pH 6.5, 30 °C, 240 h, 150 rpm | 0.07 g/L/h; reuse in 5 cycles |
| Succinoglycan                             |                                                     |                          |                      |
| *Rhizobium radiobacter* ATCC4720 [85]     | Substrate: rice husk hydrolysate-100.0 g/L Succinoglycan-69.0 g/L | pH 7.0, 30 °C, 72 h, 100 rpm | 0.96 g/L/h          |
| *R. radiobacter* ATCC4720 [86]            | Substrate: deproteinized whey-50.0 g/L Succinoglycan-13.7 g/L | pH 7.0, 30 °C, 192 h, 180 rpm | 0.07 g/L/h          |
| *R. radiobacter* 18052 N-11; mutant strain [87] | Substrate: sucrose-70.0 g/L Succinoglycan-32.5 g/L | pH 7.2, 30 °C, 72 h, 250 rpm | 0.45 g/L/h          |
| *Agrobacterium radiobacter* NBRC 12665; immobilized on loofa sponge [88] | Substrate: sugarcane molasses-75.0 g/L Succinoglycan-14.1 g/L | pH 7.0, 30 °C, 192 h, 180 rpm | 0.07 g/L/h; reuse in 5 cycles |

* Parameter was estimated by the authors of the review based on the data in the corresponding publications or taken from the references.

### 3. New Natural, Mutated and Genetically Modified Strains as BTCs for PS Synthesis

Following the initial success in the use of immobilized cells for EPS biosynthesis, research continues for finding ways of controlling the biocatalytic process and for improving the characteristics of BTCs themselves. The latter is achieved via both searching for new producer strains among various microbial cells [69–73] or by producing mutant strains [46,47,60,68,83,87,89] (Table 1, Figure 1). Note that great success has already been achieved in these studies, and the prospects for further development are feasible, especially in the joint use of the advantages of new strains and cell immobilization in concentrated forms. For example, new strains producing pullulan (*Aureobasidium melanogenum* TN1-2 (from natural honey), *Rhodosporidium paludigenum* PUPPY-06 (from fresh and rotting plant...
leaves), *Aureobasidium melanogenum* A4 (from soil) and BC (*Lactobacillus hilgardii* IITRKH159 (from sapodilla), strains of *Komagataeibacter maltaceti* (from grape and apple cider vinegar) and *Komagataeibacter nataicola* (from sloe apple cider vinegar)) were recently isolated and studied [69–73]. It was found that the high efficiency of pullulan biosynthesis (0.86 g/L/h) in *A. melanogenum* TN1-2 is due to the high activity of such enzymes as glucosyltransferase and phosphofructo-2-kinase.

The rate and degree of conversion of carbohydrate-containing substrates into a polymer product can be improved by increasing the specific activity of enzymes involved in PSs synthesis and by regulating the biosynthesis pathways of the EPS precursors. It was found, however, that the overexpression of genes of enzymes involved in the synthesis of EPSs can have a negative impact as well as a positive one. The former can be due to the distortion of the multi-domain protein complex responsible for polymerization and polymer secretion [90].

The main directions of research aimed at improving the characteristics of recombinant BTCs are the following: inducing the EPS-producing cells to increase the synthesis of enzymes responsible for the key reactions producing the target polymers [83,87]; and transformation of the cells by plasmids carrying genes that increase the sustainable functioning of cellular BTCs under PSs synthesis conditions [76].

Note that, despite numerous studies aimed at creating productive strains, none of them have led so far to the development of mutant strains that fully meet the requirements for large-scale biotechnological production. The available inducers required for the biosynthesis of the necessary recombinant enzymes in the nutrient media are still too expensive for industrial-scale application. Antibiotics, which are generally expensive, are needed to maintain the conditions for the selective cultivation of the genetically modified PSs producers. Therefore, in addition to creating BTCs using various genetic constructs, many studies have been made for developing natural BTCs for the biosynthesis of EPSs.

4. BTCs as Native or Artificial (Co-Cultured) Consortia for EPS Production

The use of natural and synthetic consortia is one of the most popular approaches to the development of BTCs for EPS synthesis (Table 2, Figure 1). Various native consortia composed of different cells including bacteria and fungi (yeasts) are widely known as *Kombucha* and applied for producing BC with high enough crystallinity [91–93]. The native symbiotic consortia of bacteria and yeast cells (SCOBY), however, can ensure only a relatively low rate of BC synthesis compared to bacterial monocultures producing BC [91–93]. There are several reasons for this: the concentration of the producing cells in such consortia is less than that in pure cultures; additionally, the degradation of substrates and accumulation of metabolites by BC producers occur in competition with other cells in the consortium.

*Kombucha* consortia require media with a very simple composition, namely black tea or herbal extracts with the addition of sucrose (100 g/L), for synthesizing BC, which sets them at an advantage compared to other BC-producing BTCs. It was found that the plant polyphenols present in these media impart antioxidant activity with respect to free radicals to the produced BC biopolymers, which expands the prospects for their use. It has been shown that nitrogen-rich components of the media (peptone or green tea) contribute to an increase in the efficiency of polymer synthesis with SCOBY [92] (Table 2).

The highest rate of BC synthesis was recorded in the joint presence of *Brettanomyces bruxellensis* MH393498 and *Brettanomyces anomalus* KY103303 yeast cells, as well as *Komagataeibacter saccharivorans* LN886705 bacteria in the *Kombucha* consortium [91] (Table 2). In the medium with an optimized composition containing 1% black tea and 6% glucose at a pH 6 and 30 °C for 10 days under conditions of static cultivation, the *Kombucha* consortium produced 0.31 g BC/g glucose. This is 82% higher than in the case of using individual genetically modified BC producers [76,91] (Tables 1 and 2).

PSs production by synthetic consortia relies on the efficient joint functioning of various microorganisms. Controlling such interaction is a promising direction for the rapid production of PSs with improved characteristics. So, it is known that the biological activ-
ity of curdlan increases with a decrease in its molecular weight, and the low-molecular
curdlan is suitable for a variety of applications in the food industry and the agricultural
sector. During the synthesis, the accumulated curdlan covers the cell surface of the main
producer (Agrobacterium sp.) and has a negative effect on the mass transfer and aerobic
respiration of the cells, thus inhibiting the PS synthesis [94]. β-1,3-endoglucanase from
Trichoderma harzianum GIM 3.442 can cleave β-bonds at random sites of the PS chain and
exhibits high activity and specificity for the hydrolysis of curdlan. Thus, an efficient BTC
can be formed by combining Agrobacterium sp. ATCC 31749 and Trichoderma harzianum
GIM 3442 cells, which are producers of curdlan and β-1,3-endoglucanase, respectively [94]
(Table 2). By using this synthetic consortium, the molecular weight of the curdlan was
reduced by 34.01% (from 110.85 kDa to 73.15 kDa), and the curdlan yield (47.9 g/L) and
the conversion efficiency of glucose to curdlan (0.60 g/g) increased by 119.93% and 36.36%,
respectively [94].

Table 2. Natural and synthetic consortia as BTCs for PS production.

| Biocatalysts [Reference]                                    | Substrate Product Concentrations | Conditions of BTCs Use                           | * Rate of Biocatalysis Provided by BTCs |
|-------------------------------------------------------------|----------------------------------|-------------------------------------------------|----------------------------------------|
| **Consortia Kombucha** (Komagataeibacter saccharivorans LN886705, Brettanomyces bruxellensis MH393498, Brettanomyces anomalus KY103303) [91] | Glucose-60 g/L BC-18.7 g/L       | pH 6, 30 °C, 240 h                              | 0.078 g/L/h                           |
| **Symbiotic community of bacteria and yeast cells (SCOBY) [92]** | Sucrose-10 g/L BC-22 g/L         | 26 °C, 336 h                                   | 0.065 g/L/h                           |
| **Consortia Kombucha [93]**                                 | Sucrose-110 g/L BC-10.6 g/L      | 22 °C, 504 h                                   | 0.021 g/L/h                           |
| **Agrobacterium sp. ATCC 31749 and Trichoderma harzianum GIM 3.442 [94]** | Glucose-50.0 g/L Curdlan-47.9 g/L | pH 7.5, 37 °C, 96 h                            | 0.33 g/L/h                            |
| **“Step-by-step” culturing**                                | Molasses-300 g/L Fructo-oligosaccharides-169.5 g/L | pH 5.5, 55 °C, 1 h                            | 169.5 g/L/h                           |

* Parameter was estimated by the authors of the review based on the data in the corresponding publications or taken from the references.

Kefiran is a well-known EPS, which is used as a thickener, stabilizer, emulsifier, fat
substitute, gelling agent, etc. When the rate of lactate production of L. kefiranofaciens exceeds
that of lactate consumption by S. cerevisiae yeast contained in the kefir starter culture, the
rate of kefiran synthesis decreases. The presence of lactate inhibits the growth of lactic acid
bacteria, even if the pH of the medium is regulated by the addition of alkali. Therefore, to
increase the productivity of BTCs, lactate should be removed from the reaction medium.
To optimize the synthesis of kefiran, the conditions of joint cultivation of kefiran-producing
lactic acid bacteria Lactobacillus kefiranofaciens with lactate-assimilating yeast Saccharomyces
cerevisiae were controlled as described below (Table 2). When the pH decreased to 4.95
due to the accumulation of lactate produced by L. kefiranofaciens, the reaction medium
was aerated to allow S.cerevisiae yeast to consume a part of the lactate. As soon as the pH
reached the value of 5.05, the reaction system was transferred to anaerobic conditions due to bubbling $N_2$.

Fructo-oligosaccharides (FOS) function as prebiotics and improve mineral absorption in the large intestine, enhance immunity, and reduce total cholesterol. Due to the sequential combination of two catalysts, molasses could be successfully used as a substrate for the production of FOS [77] (Table 2). Incubation of sugarcane molasses with an invertase-free *Saccharomyces cerevisiae* prior to the addition of *A. pullulans* as BTCs eliminated the inhibition of the PS producer by glucose and increased FOS accumulation from 44% to 56% in 1 h. This study demonstrated that various approaches can be used for controlling the interactions in cell combinations in order to optimize the production of EPSs from various renewable resources and waste.

### 5. Features of BTCs Determine the Palette of Substrates (Types of Biomass and Methods of Its Pretreatment) for EPS Synthesis

The choice of BTCs determines the variety of substrates and their possible concentrations for conversion to EPS. Most microbial cells used as BTCs are capable of converting simple sugars (mono- and disaccharides) into PS. These sugars can be obtained by hydrolysis of various renewable raw materials and biomass sources. Hydrolysis methods can be different, and various chemical agents and/or enzymes, including enzymatic complexes, can be used for these purposes [15,32,59,63,82,85].

Such processes, which include the conversion of any type of industrial or agricultural waste into a final biotechnological product through successive stages of chemical and biocatalytic transformation, which individually cannot give a similar product, have recently been termed “hybrid” ones [26,95,96]. The hydrolysates of raw materials containing target sugars for conversion to EPS are complex media which can contain various natural inhibitors of PS-producing cellular enzymes. Some products of the hydrolytic pretreatment of raw materials can also have an inhibiting effect on the PS producers. Therefore, it is more appropriate to use stabilized forms of BTCs in such hybrid processes. The complexity of biochemical transformations in such processes imposes additional limitations on the choice of raw materials. Thus, the nature of the raw material itself, the source of the desired substrate and its chemical composition undoubtedly affect the degree of conversion, the rate of the process, and the yield of the desired product (Table 1).

For example, the maximum concentration of BC (4.5 g/L) and the productivity of the process (0.75 g/L/day) were achieved by cultivating immobilized *K. xylinum* B-12429 in a medium where enzymatic hydrolysates of Jerusalem artichoke tubers were used as a substrate. These parameters were higher than in the case of pure glucose being used as a substrate for the same cells [32,97]. During the transformation of sugars contained in micro- and macroalgae biomass hydrolysates under the action of the same biocatalyst, the maximum concentration of BC (2.6 g/L) was obtained using enzymatic hydrolysates of the *Chlorella* biomass. At the same time, the yield of BC in this medium was significantly lower since the hydrolysates of this raw material had high viscosity and low concentration of sugars suitable for conversion into BC [32]. The thickest and most durable BC film was formed when using immobilized BTCs in the medium with the Jerusalem artichoke tuber hydrolysate. The other characteristics of the obtained BC films were almost identical in these experiments.

Hydrolysis of biomass to obtain carbohydrate substrates and their subsequent conversion into PS is often performed in different reactors, and these two processes thus occur successively. It is possible, however, to shorten the total duration of the EPS production process via using a single reactor instead of two so that both stages are running simultaneously. A similar approach used for obtaining ethanol or organic acids from renewable raw materials is usually called “simultaneous saccharification and fermentation” [25]. For such an organization of the EPS production process, it is important to find a compromise between the optimal conditions for the existence of several biocatalysts (hydrolytic enzymes and producer cells), which allows to achieve maximum PS yield (Figure 1).
The same process option can also be implemented if the polysaccharide producer itself is able to synthesize hydrolytic enzymes, such as the pullulan producer *Aureobasidium pullulans* [98,99]. However, even in this case, the accumulation period of the maximum concentration of active hydrolases and the final product can be strongly separated in time, which means that until the enzymes are synthesized by cells, no significant accumulation of the target product will occur. In addition, part of the substrate in the medium will be consumed by the producing cells for the synthesis of the necessary enzymes. This means that the percentage of conversion of the initial substrate into the target product (EPS) and the level of its accumulation will be reduced compared to the usual two-stage “hybrid” process.

Note that PS production can involve BTCs, which yield completely different products. However, the byproducts of the functioning of these BTCs can be used as substrates for PS-producing cells. Yeast cells are an example of such BTCs: the media after their cultivation in the form of distillery stillage (waste from production of ethanol via fermentation and consequent distillation) are often used as a basis of nutrient media for the PS-producing BTCs because stillage is rich in carbohydrates and organic nitrogen [44,58].

Other types of waste from biotechnological industries can also provide the nutrient media for PS accumulation. Thus, in the case of biocatalytic wastewater treatment with phototrophic microbial cells involving the accumulation of suspended biomass, which is subsequently used for the production of biodiesel, the waste is glycerin and biomass residues of microalgae and cyanobacteria. Both of these substances can be used to obtain polysaccharides [32,60]. When developing BTCs for such combined biotechnological solutions, it is necessary to ensure the biological compatibility of the processes involved. Namely, the waste produced after the first stage should contain the lowest possible concentration of substances that are toxic for BTCs used at the next stage. At the same time, the second-stage BTCs based on PS producers should have appropriate substrate specificity and increased stability during operation.

The schemes in Figure 1 summarize the known approaches to developing various BTCs, and also serve as a kind of guide for creating new BTCs and developing “hybrid” processes with their participation. The “hybrid” processes can extend the range of possible raw materials to include non-traditional ones, help reduce the number of bioconversion stages and the total duration of the process and cause an increase in the conversion rate.

At the same time, the main perspectives in the development of BTCs, in our opinion, lie primarily in the field of synthetic biology based on the use of nature-like solutions [77,94]. The systems of this type are strongly influenced by such phenomena as the QS and interactions between different cultures of microorganisms, including those realized via signaling molecules. At the same time, in developing such BTCs, it is important to take into account the possibility of the hydrolysis of these molecules under the action of hydrolytic enzymes that can be used in the EPS production processes that are combined into one stage.

### 6. Factors Affecting the Properties of BTCs Used for PS Synthesis

The nature of the substrate significantly affects the characteristics of the PS obtained using BTCs. In general, the factors influencing the synthesis of PS encompass the characteristics of the reaction medium itself, including the type of the carbon source used and those of the accumulated metabolic products of the cells comprising the BTCs, as well as the conditions of PS biosynthesis (temperature, pH, aeration of the medium, mixing rate, etc.). Changes in the components of the medium affect the growth of new generations of cells introduced to the process as BTCs, their metabolic activity and the orientation of the metabolism (synthesis and the level of synthesis of enzymes for a certain biochemical pathway), predetermining the formation of the target biopolymer directly or indirectly. The release of EPSs is usually most noticeable when the process with BTCs is carried out in media with a high concentration of carbon and a low (limiting) concentration of the nitrogen source.
For example, BC samples obtained using *Gluconacetobacter intermedius* cells as BTCs and glycerol or xylose as the main carbon sources had a reduced degree of polymerization compared to those obtained in a medium with glucose [100]. This was due to the lower activity of enzymes involved in the synthesis of BC when using xylose and glycerol as substrates.

BC synthesized by *A. xylinum AJ3* cells had the highest porosity (80%) in the case of using sucrose as a substrate and lower ones in the case of other substrates: glucose (70%), fructose (66%), and glycerol (65%) [101]. The introduction of additives in the form of lignosulfonates (1 wt.%) in a reaction medium with *G. xylinus* cells as BTCs led to the synthesis of BC samples with an increased degree of crystallinity [102].

Among the various carbon sources, glucose, fructose, and sucrose are most often used to produce EPS (Table 1). However, metabolites (such as gluconic, acetic, and lactic acids) reducing the pH of the reaction medium often inhibit PS formation when glucose is used as the only carbon source. A higher yield of BC was observed when using a mixture of various sugars, for example, sucrose and fructose, whereas the presence of glucose in combination with other sugars led to a decrease in the yield of EPS [102].

Polyatomic alcohols, such as arabite and manite, can also be used as the only carbon source for BC synthesis, and an increase in synthesis efficiency compared to the case of glucose has been shown for these substrates [103]. Glycerol was successfully tested as a substrate (20 g/L) for the production of BC, and it was found that during the synthesis of the biopolymer without mixing in the medium with the mentioned concentration, the yield of the BC exceeded the level achieved with 50 g/L of glucose [104].

When cultivating *Komagataeibacter sp. PAP1* cells in a medium containing soy whey hydrolysate, the produced BC samples had 108% greater tensile strength and 841% greater Young’s modulus than those synthesized in a standard medium based on glucose [105]. BC samples produced by culturing *G. xylinus* cells in a medium containing corn stalks hydrolysate had a higher degree of crystallinity compared to those obtained in media with pure glucose [106]. Note that different microorganisms used as BTCs react differently to the changes in the composition of the medium [107]. Therefore, the choice of BTCs essentially determines the influence of the composition of the medium on the structure and the properties of the synthesized BC. Cultivation of cells synthesizing PS can be carried out in reactors that provide static conditions for the process or in those that allow mixing (Table 1). Static cultivation is a relatively simple and widely used method of obtaining EPS. As a rule, samples of biopolymers with a more regular structure and increased crystallinity are formed in this case [108,109]. The reactors with active mixing tend to produce biopolymers with a wider spread of molecular weight distribution, reduced crystallinity and, accordingly, decreased mechanical strength compared to those obtained under static conditions. This was observed, e.g., in the production of expanded PSs with BTCs [108,109].

Introduction of various additives into the reaction medium as well as changes in the process conditions (temperature, concentration of oxygen, etc.) can have a great impact on the properties of the polymers [110]. It is known, for example, that the synthesis of BC is predetermined by the appearance in the medium of such a substance as c-di-GMP. However, its artificial introduction into the medium leads to the activation of phosphodiesterases in the cells of BTCs, which catalyze the degradation of c-di-GMP. Therefore, only the appearance of this substance as a result of its synthesis by the cells themselves is beneficial for PS production [111]. Thus, in order to increase the synthesis of BC, it is necessary to stimulate the production of this substance by cells. However, c-di-GMP is a QS factor, that is, a substance that is synthesized by cells and is necessary for their transition to a quorum state. QS is a feature of highly concentrated populations which can be stabilized by the expression of “silent genes” and the synthesis of EPS with a simultaneous decrease in the rate of active cell growth. Therefore, it is necessary to stimulate the cells producing PS to transition to the QS, that is, to the programmed genetic response of cells, to increase their concentration per unit volume and their synthesis of c-di-GMF molecules for this purpose [32,112]. Thus, individual additives in the medium may not have the desired effect on the synthesis of PS.
The concentration of oxygen in the reaction medium is an important factor affecting the synthesis of EPS. An excess of dissolved oxygen in the medium can lead to cell proliferation and growth, which can be counterproductive because part of the substrate is spent on this, and not on PS synthesis. The growth can, in turn, be accompanied by the accumulation of metabolites in the form of organic acids and ultimately lead to a decrease in the pH of the medium, which can cause a further decrease in the level of BC synthesis. Conversely, a too-low oxygen concentration in the medium can lead to a significant decrease in the rate of cell metabolism, including a decrease in the intensity of oxidative phosphorylation and ATP formation. Thus, the effect of aeration on BC synthesis by *Gluconacetobacter xylinus* DSM46604 cells was studied when varying this parameter in the range of 1.5–7.5 L/min at constant stirring of 150 rpm. The efficiency of the BC synthesis had a maximum at 5 L/min, and then decreased by 10% with a further increase in aeration rate [104].

Studies of optimal conditions for obtaining xanthan have shown that the temperature and pH at which the BTCs are applied have a notable influence on the yield of the final product. First of all, this is due to the activation of enzymes that are involved in the biosynthesis process. The optimal conditions for the growth of cells producing the EPS and the synthesis of xanthan are the temperature in the range of 28–30 °C and the pH of 7–8 [113]. With a temperature beyond the optimal range, the molecular weight of xanthan decreases, and pyruvate and acetyl residues appear in the biopolymer, which reduces the viscosity of the target solution. During the biosynthetic process, the pH of the medium decreases to values below 5 due to the formation of organic acids that leads to a sharp decrease in the accumulation of xanthan. However, the study of the effect of pH showed that the control of this parameter affects only the growth of bacterial cells but has no impact on the accumulation of PSs [114].

To obtain xanthan, it is necessary to have trace amounts of potassium, calcium, iron and magnesium salts in the medium. A small amount of organic acids, such as citric and succinic, introduced into the reactor increases the synthesis of PSs. However, the presence of fumaric acid residues leads to the formation of xanthan with a highly branched structure [114].

Note that among the many factors that can affect the functioning of BTCs and the process of obtaining a specific PS, there is often the one that makes the greatest contribution. For example, it is the aeration of the reaction medium in the case of curdlan synthesis. It is shown that aeration and mixing of the medium have a significant effect on the synthesis of curdlan since the synthesized polymer surrounding the cells prevents the transfer of oxygen from the medium to the cells. It was also found that the productivity of the curdlan process decreases with an increase in the volume of the nutrient medium in the reactor. Controlling the mixing rate and the filling factor of the reactor for curdlan accumulation allows maintaining the concentration of dissolved oxygen in the medium in the range of 45–60%, which is optimal for the synthesis of this EPS [115].

Sometimes there are several key conditions at once that must be fulfilled to optimize the PS synthesis. Thus, for most BTCs synthesizing succinoglycan, a temperature of 28–30 °C and neutral pH are the optimal conditions. A decrease in the pH of the reaction medium leads to a decrease in the synthesis of the EPSs [116,117]. Aeration of the medium is also necessary for the synthesis of succinoglycan since the accumulated polymer greatly changes the viscosity of the medium, limiting oxygen access to cells. It has been shown that the maximum concentration of the PSs is achieved during the cultivation of *Agrobacterium radiobacter* 1654 cells in a medium with sucrose at a mixing speed of 250 rpm and with molasses at a mixing speed of 300 rpm [116]. So, a variety of factors should be taken into account for such PS production processes.

Another factor that can affect the functioning of PS producers in the case of immobilized cells is the carrier used (Table 1). In particular, the screening and selection of carriers for the immobilization of *Aureobasidium pullulans* cells (synthetic materials, waste from the agro-industrial complex, and mineral materials) was performed. The successful immobilization of cells on all the studied carriers was shown, and their ability to produce fructooligosaccharides (FOS) was investigated [78]. Polyurethane foam was one of the
most effective synthetic carriers, providing immobilization of up to 75% of injected cells for obtaining BTCs. This made it possible to increase the output of FOS by 12% (w/w) compared to free cells. Although the walnut shell had a much lower immobilization efficiency (less than 20% (by weight), the highest FOS performance was unpredictably found for this material with an increase in yield by 25% (by weight). Thus, according to the results obtained, the use of walnut shells as a recyclable and inexpensive carrier, which is actually a waste of processing natural raw materials, for the immobilization of \textit{A. pullulans} for the production of FOS turned out to be an interesting alternative to synthetic porous carriers.

7. Preparation of Nanocomposites in the Process of Biocatalytic PS Synthesis

A separate direction in the application of BTCs for PS synthesis is the production of nanocomposites (NCs) with various target properties. Particular attention in this research is paid to such characteristics of the final product as modulus of elasticity, elongation, and tensile strength [118]. Most often, such NC are produced after PSs have already been synthesized using BTCs. For example, to obtain composite fibrous materials with antibacterial properties, BC samples were modified with poly-\(\varepsilon\)-caprolactone or poly(hydroxybutyrate), and then functionalized with an enzyme-polyelectrolyte complex of quorum-quenching enzymes, such as hexahistidine-tagged organophosphorus hydrolase stabilized by poly(glutamic acid) or by suspension of tantalum nanoparticles [119]. It was proposed to obtain a new multifunctional three-dimensional material for bone tissue engineering by coelectrospinning using a combination of poly(hydroxybutyrate-co-hydroxyvalerate), poly(\(\varepsilon\)-caprolactone) fibers with an antibiotic and pullulan with diatomite inclusions [120].

It was found that the usually obtained chaotically oriented spider-like shape of BC nanofibers prevents the realization of their full potential for some applications, because molecules of such shapes are difficult to modify to produce nanocomposites. This increases the interest in the processes of obtaining nanocomposite materials directly within the synthesis of PS under the action of BTCs (Table 3). Producing NCs in the single-stage synthesis of PS is possible via introducing various additives directly into the reaction medium during the functioning of BTCs.

For example, a nanostructured Fe-containing polysaccharide hydrogel can be obtained by using Fe-reducing strain of \textit{K. oxytoca} as BTCs and ferric citrate as the main substrate. Such NCs are considered promising for use in the field of medicine in tissue engineering, diagnostic magnetic resonance imaging, DNA detection, intracellular tags, etc. [121].

**Table 3.** BTCs for obtaining nanocomposites in the process of PS synthesis.

| Biocatalysts [Reference] | Substrate for BTCs; Additives for NCs Formation; NCs as Products | Conditions for BTCs Use; * Rate of PS Synthesis | Possible Applications |
|-------------------------|---------------------------------------------------------------|-------------------------------------------------|----------------------|
| \textit{Klebsiella oxytoca} SK01 [121] | Ferric citrate-50 Mm; Composite of EPS with Ferrihydrite (5Fe₂O₃ × 9H₂O) as nanoparticles (1.8 nm); EPS attached to the cell surface and containing galactose, glucuronic acid, and rhamnose that display metal-binding properties. | pH 7.4  
25 °C  
336 h | Biomedical and farming industries: tissue engineering, diagnostic magnetic resonance imaging, DNA detection, intracellular labeling, magnetic transfections, anti-proliferative hyperthermia therapy, targeted drug delivery systems |
Table 3. Cont.

| Biocatalysts [Reference] | Substrate for BTCs; Additives for NCs Formation; NCs as Products | Conditions for BTCs Use; * Rate of PS Synthesis | Possible Applications |
|--------------------------|---------------------------------------------------------------|-----------------------------------------------|----------------------|
| Natural consortium from apple biomass fermentation [122] | Sucrose-110 g/L Graphene oxide (GO)-50 mg/L; Composite of BC with GO with high water content (~400%), cellulose nanofiber ~100 nm. Composite BC/GO with high sorption capacity (60 mg paracetamol/g BC) | pH 3.5 25 °C 240 h | Composites for the creation of dressings with prolonged release of medicinal substances |
| Komagataeibacter xylinus ATCC 11142 [123] | Glucose from sugar cane straw fermentolysate-20 g/L Reduced graphene oxide (RGO)-2 wt% Nanocomposite of BC with RGO The partial oxidation of GO in presence of peroxides resulted in incorporation of hydroxyl and epoxy groups in RGO. | pH 6.5 30 °C 360 h 0.0125 g/L/h | Electrodes/supercapacitors, adsorbents, scaffolds for tissue regeneration |
| G. xylinus KCCM 40,216 [124] | Mannitol-2.5% w/w Multi-walled CNT-dispersed carbon tubes-0.001% (w/v) Composite of BC with Carbon nanotubes (CNTs) with pore size-85.4 ± 2.8 µm. | pH 6.0 28 °C 336 h | Functional nanomaterials for bone regeneration with high efficiency in vivo, promoting osteogenesis of mesenchymal stem cells, bone calcification |
| G. xylinus CGMCC 2955 [125] | Glucose-20 g/L Oriented glass fiber-20 wt% Composite of BC-glass fiber (with a diameter between 7-15 µm) | pH 6.0 30 °C 72 h electric field of 10 mA | Materials for cell growth and subsequent tissue organization |
| Acetobacter xylinum ATCC 53582 [126] | Glucose-5.5 g/L, PVA-6% Composite of BC with PVA with further modification by genipin-crosslinked silk sericin (SS) and azithromycin (AZM) | pH 5.0 30 °C 192 h | Materials for biomedical and farming industries with inhibition and disruption of biofilms due to AZM action |
| Komagataeibacter hansenii ATCC 23769 (previously known as Gluconacetobacter hansenii) [127] | Glucose-50 g/L Pullulan-15 g/L Composite of BC with pullulan | pH 5.0 30 °C 200 rpm 168 h 0.005 dry g/L/h | Materials for food packaging, transparent organic electronic carriers, biomedical material |

Consolidated bioprocess

| Co-culture of Gluconacetobacter hansenii ATCC 23769 with Escherichia coli ATCC 700728 [128] | Glucose-20 g/L Composite of BC with EPS | pH 5.5 30 °C 120 h 0.013 dry g/L/h | Materials for medicine: artificial skin, vessels, grafts, cardiovascular implants |
| Co-culture of Komagataeibacter hansenii ATCC 23769 (previously known as Gluconacetobacter hansenii) with Aureobasidium pullulans ATCC 201,253 [118,127] | Glucose-50 g/L Composite of BC with pullulan | pH 5.0 30 °C 200 rpm 168 h 0.002 dry g/L/h | Materials for food packaging, transparent organic electronic carriers, biomedical material |
Table 3. Cont.

| Biocatalysts [Reference] | Substrate for BTCs; Additives for NCs Formation; NCs as Products | Conditions for BTCs Use; * Rate of PS Synthesis | Possible Applications |
|--------------------------|------------------------------------------------------------------|-----------------------------------------------|----------------------|
| Komagataeibacter xylinus C3 and Aureobasidium pullulans C7 [129] | Molasses–20 g/L Composite of BC with pullulan-16.8 g/L | pH 6, 30 °C, 168 h 0.1 g/L/h | Matrix for obtaining a “synthetic synbiotic” consortia for testing in vitro and in vivo, an effective cell delivery system to the host body; the basis for the creation of symbiotic dairy products with a preventive effect; matrix for immobilized probiotic cultures of microorganisms |

* Parameter was estimated by the authors of the review based on the data in corresponding publications or taken from the references.

A simple method for producing BC-based composites involves adding graphene oxide additives to a medium with BTCs [122,123,130]. Graphene oxide was bound to BC via hydrogen bonds in the resulting NCs when a natural consortium isolated during the acetate fermentation of Golden Delicious apples and capable of producing BC was used as BTCs in the presence of graphene oxide. The obtained BC/GO composites look attractive for use as dressing materials with prolonged drug release. BC/GO composite membranes were characterized by sorption of paracetamol (60 mg/g BC) with large desorption time of the pharmaceutical substance: 80% of paracetamol was released within 24 h [122]. Other NCs based on BC and graphene oxide were characterized by a low rate of ibuprofen release [130].

In media with other BTCs (e.g., Komagataeibacter xylinus ATCC 11142), the presence of reduced graphene oxide (RGO) in various concentrations significantly changed the characteristics of BC synthesis. Higher yields of graphene oxide NCs with BC were achieved. Strongly integrated graphene nanoparticle layers between BC nanofibers were obtained via introduction of RGO at a concentration of 2 wt.% in the reaction medium of BTCs. Note that a gradual decrease in the content of RGO was observed during the synthesis of BC with the formation of thick BC/RGO hydrogels at the air–water interface. As expected, hydroxyl functional groups present in BC (which are formed during in situ biosynthesis) created conditions that had a significant impact on the RGO incorporation into the NCs. This resulted in an improved distribution of RGO nanoparticles between BC nanofibers, forming a percolation mesh structure, which led to an improvement in the electrical conductivity and mechanical properties of the resulting nanocomposite. BC/RGO films formed after in situ biosynthesis were light, very flexible, and had a high tensile strength [123]. The strategy of including graphene into NCs within the PS synthesis process led to the production of NCs with properties comparable to those of BC chemically modified ex situ [131,132].

Functional nanomaterials for bone tissue regeneration were proposed to be obtained during the one-stage synthesis of BC by introducing carbon nanotubes (CNTs) into a reaction medium with G. xylinus KCCM 40216 cells used as BTCs. At the same time, the pretreatment of CNTs with an amphiphilic comb-like polymer synthesized by free radical polymerization of methyl methacrylate, poly(ethylene glycol) methacrylate, poly (ethylene glycol), or methyl ether of methacrylate not only facilitated the dispersion of CNTs, but also induced the hybridization of CNTs and BC [124].

A simple method for orientation of nanofibers was developed due to the effect of an electric field (10 mA) on such BTCs as Gluconacetobacter xylinus CGMCC 2955 during PS synthesis, when 20% wt.-oriented glass fiber was injected into the reaction medium. The resulting nanocomposite “BC-glass fiber” demonstrated a significant increase in the tensile strength and thermal stability [125].

The possibilities of the direct inclusion of various PSs into the BC matrix during its synthesis have been experimentally established. Chitin, pectin, pullulan, agar, xyloglu-
can, alginate, carboxymethylcellulose, poly (vinyl alcohol), poly-ε-caprolactone, poly-3-hydroxybutyrate, etc., were added to the medium [126–128,133].

It was found that in the single-stage production of nanocomposites, additives introduced into the medium with BTCs can interact with the BTC cells, influence their productivity, and can also directly bind to the PS fibrils during their synthesis. Thus, additives can affect the yield, structure, morphology, and physical properties of the final nanocomposite, or can contribute to giving PSs new properties, such as optical, antibacterial, and catalytic activity, and so on.

Levan can bind to BC microfibrils, form hydrogen bonds, and thereby affect the size of BC microfibrils and the properties of the final NCs. Linear PSs, such as glucomannan and xylan, can also be incorporated into the BC matrix. The addition of other PSs, such as pectin, pullulan, agar, xanthan, etc., can help in improving the production activity of BTCs by increasing the viscosity of the reaction medium. PSs which increase the viscosity of the medium can protect bacterial cells from shear forces, thus preventing the formation of large clumps of BC, and help to enhance the BC production [127]. Additives of carboxymethyl cellulose can increase the production of the BC by a factor of 1.7.

The introduction of pullulan into the medium can have a double impact: an increase in the viscosity of the reaction medium can have a positive effect on the rate of BC synthesis by the BTCs, and the increase in the interactions between the BC fibrils can lead to an improvement in the mechanical properties of the resulting NCs [127]. It is known that BC production can be increased with the addition of pullulan. The BC production can be increased from 0.447 g/L to 0.814 g/L and 1.997 g/L in the presence of pure pullulan in concentrations of 1.5 and 2.0%, respectively [127].

Another interesting approach to obtaining modified PS directly in the process of their biosynthesis is the joint cultivation of several different BTCs producing PS [134,135]. Composites based on PS and chemically synthesized polymers (having no direct natural analogues) are usually called “ecocomposites” or “biocomposites”, and those based entirely on natural fibers and biopolymers are called either “green composites” or “green biocomposites” [136]. Note that the creation of synthetic consortia and the introduction of several BTCs into the joint biocatalytic process, known as consolidated bio-processing, is an interesting direction in the development of synthetic biology. This approach is widely used in industrial biotechnology, including the production of enzymes, food additives, antimicrobials, microbial fuel cells, etc. [135,137].

Thus, experiments were made for milk fermentation with the addition of probiotic strains of *Lactiplantibacillus plantarum* MWLp-12 and *Limosilactobacillus fermentum* MWLF-4 to the medium with bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* 6047, and *Streptococcus thermophilus* 6038, which can produce EPS. These tests have led to the production of fermented milk products with an increased content of EPS, and better characteristics in consistency, water retention capacity and acidity compared to using only the existing commercial fermentation bacteria [12].

The study of the effect of co-cultivation on the production of EPS by three strains of *Lactobacillus rhamnosus* (ATCC 9595, R0011, and RW-9595M) in combination with *Saccharomyces cerevisiae* cells showed that the yield of EPS in 48 h increased by 39%, 49%, and 42% when these microorganisms were co-cultured, compared with the case when the same strains were used individually [138]. Note also that, although lactic acid bacteria and yeasts are found in various fermented products, the molecular mechanisms associated with the microbial interactions and their effect on EPS biosynthesis are still insufficiently studied.

In general, especial progress in obtaining NCs in the synthesis of various PS should be noted specifically for BC [135]. The introduction of producers of other PS (alginate, pullulan, etc.) into the media with BTCs catalyzing the synthesis of BC and their joint use for single-stage production of “green biocomposites” provides a decrease in the cost of their production compared to the processes organized in several stages. This approach also allows obtaining materials with new or modified properties [134]. In addition, intercellular interactions of various cells used as BTCs in the process of joint PS synthesis
can have unexpected beneficial effects, for example, an increase in the synthesis rate of a particular PS.

The method of co-cultivation of *K. xylinus* with other bacteria “in one pot” has been successfully applied to the synthesis of composites BC/hyaluronic acid [139] and BC/polyhydroxybutyrate [140].

To obtain BC-based composites with improved mechanical properties, the joint cultivation of two BTCs based on *Glucosacetobacter hansenii* ATCC 23769 and *Escherichia coli* ATCC 700728 cells under static conditions was proposed. It was found that during the use of BTCs, mannose-rich EPSs are synthesized by *E. coli* cells, and the EPSs were included into the BC matrix without a significant change in the level of BC crystallinity. For BC films obtained by co-cultivation of different cultures, Young’s modulus and tensile stress increased by 81.9% and 79.3%, respectively, compared to those of the BC samples obtained in the absence of EPSs. When the two BTCs were used together, the synthesis rate and the output of both BC and EPSs were increased. The maximum concentration of EPSs during the co-cultivation of *E. coli* and *G. hansenii* was observed after 24 h and was 11.6 mg/L, whereas for the *E. coli* monoculture, it was more than twice lower (5.1 mg/L) [128].

Thus, there are studies in which EPSs are injected into the medium directly during BC synthesis by certain BTCs to obtain NCs based on BC, and similar NCs are obtained when BTCs producing similar EPSs are loaded directly into the medium for simultaneous biosynthesis of BC. Of course, it was interesting to compare the characteristics of similar PSs produced with these two approaches. Such studies and comparative analyses of BC-based NCs obtained during the implementation of two strategies were in fact performed: (1) co-fermentation of *Komagataeibacter hansenii*, a BC producer, with *Aureobasidium pullulans*, producing pullulan; and (2) synthesis of BC using a monoculture of *Komagataeibacter hansenii* with the addition of previously synthesized pullulan into the reaction medium [118,127]. It was found that in both cases, the obtained NCs had an increased Young’s modulus compared to BC without pullulan. However, when two BTCs were cultured together, the degree of glucose consumption for 168 h was lower than in the process with monoculture and the addition of ready-made pullulan. For co-cultivation, it was suggested that the growth of *A. pullulans* disrupted the growth of *K. hansenii*, since less glucose was consumed, and less BC was produced. After 7 days of the joint cultivation of two BTCs, a significant amount of glucose remained in the broth (~30 g/L). Thus, for joint cultivation, it is important to have positive contact between microorganisms used as fusion participants in co-cultivation processes. To achieve this, it is necessary to make a careful choice of BTCs among possible candidates, as well as selecting substrates that reduce the level of competition between the cells of combined BTCs in order to analyze the effect of media composition. This thesis was confirmed by the experiment which showed that during the joint cultivation of BTCs, the rate of PS synthesis and the mechanical properties of the NCs can vary depending on the composition of the medium, as well as the type of reactor used [118]. It was noted that various wastes, including molasses (sugar production waste), can be successfully used as a cost-effective substrate for one-stage synthesis of NCs, whereas substrates based on food sugars provided much worse results [129].

8. Conclusions

The reviewed studies demonstrate the key role that BTCs play in the synthesis of EPSs. On the one hand, BTCs impose certain restrictions on the implementation of these processes, in terms of the media composition, combinations with other BTCs, process conditions, etc. On the other hand, developing novel BTCs and especially combining BTCs in hybrid processes has huge potential for involving new classes of wastes into the production of EPSs suitable for a variety of practical applications. The EPSs with enhanced properties, including nanocomposites, can be obtained either via adding new functional components to the existing processes of PS production, or via combining several BTCs in a single process for supplying the initial components for the target composites in real time. The use of such BTCs ensures the production of new composite materials based on EPSs directly in
their synthesis. Such creation of materials with fundamentally different characteristics opens new areas of their application. Understanding the principles of “living” biocatalysts functioning in such processes allows purposefully controlling biocatalysis, improving the characteristics of the processes themselves, and obtaining new materials with a variety of functions.

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