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Review

Xylose Metabolism in Bacteria—Opportunities and Challenges towards Efficient Lignocellulosic Biomass-Based Biorefineries

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Abstract: In a sustainable society based on circular economy, the use of waste lignocellulosic biomass (LB) as feedstock for biorefineries is a promising solution, since LB is the world's most abundant renewable and non-edible raw material. LB is available as a by-product from agricultural and forestry processes, and its main components are cellulose, hemicellulose, and lignin. Following suitable physical, enzymatic, and chemical steps, the different fractions can be processed and/or converted to value-added products such as fuels and biochemicals used in several branches of industry through the implementation of the biorefinery concept. Upon hydrolysis, the carbohydrate-rich fraction may comprise several simple sugars (e.g., glucose, xylose, arabinose, and mannose) that can then be fed to fermentation units. Unlike pentoses, glucose and other hexoses are readily processed by microorganisms. Some wild-type and genetically modified bacteria can metabolize xylose through three different main pathways of metabolism: xylose isomerase pathway, oxidoreductase pathway, and non-phosphorylative pathway (including Weimberg and Dahms pathways). Two of the commercially interesting intermediates of these pathways are xylitol and xylonic acid, which can accumulate in the medium either through manipulation of the culture conditions or through genetic modification of the bacteria. This paper provides a state-of-the art perspective regarding the current knowledge on xylose transport and metabolism in bacteria as well as envisaged strategies to further increase xylose conversion into valuable products.

Keywords: D-xylose; biorefinery; circular economy; sustainable processes; xylose metabolism in bacteria

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1. Introduction

The human population has become an undisputable force that has a deteriorating effect on both human and environmental health [1]. Population growth, the rapid development of industries for economic growth, rapid urbanization, and the rise in living standards lead to a continuous increase in global resource consumption, depleting the amount of natural resources available on the planet [2,3]. Moreover, the evolution of production processes has transformed them into complex systems that often use composite and hazardous materials, producing waste from mixed sources, which hampers sustainability management [4].

In the past few years, society has been trying to embrace new measures in order to become more sustainable and to tackle the waste generation problem. In this context, new and sustainable raw resources for food, materials, and energy production are being

sought. The zero waste (ZW) concept has been highly embraced to stimulate sustainability regarding production and consumption as well as optimal recycling and resource recovery, while restricting mass incineration and landfilling [5]. Within this concept, the material flow is circular, which means no materials are wasted. If, at their end of life, products cannot be reused or repaired, they can instead be recycled or recovered from the waste stream and used as new raw materials, substituting the demand for the extraction of natural resources [2]. One solution that has been developed is to use biowaste as a new resource to produce valuable products. This concept can be included under the "biorefinery" umbrella, where biomass-based wastes are transformed into useful bio-compounds [6]. This biorefinery vision contributes to sustainability not only due to its inherent dependence on renewable bioresources but also by recycling wastes. These compounds can range from bulk products (e.g., bioenergy) to specialty chemicals and biodegradable polymers [6–8].

While industries face significant challenges, such as limitations of conventional processing technologies, feedstock logistics, and uncertain market economics, ambitious policies from all over the world are being implemented in order to support bioindustries to achieve climate and bioenergy goals. In fact, worldwide concerns prompted world leaders to act [9,10].

2. Biorefinery Concept

A great fraction of worldwide energy and material products come from fossil fuel refineries. The environmental concerns raised by the exploitation of fossil resources due to excessive pollution and consequent global warming, their ongoing price increase, uncertain availability, and non-sustainability are seen as reasons to invest in alternative solutions able to mitigate climate change and to reduce the consumption of fossil fuels.

The replacement of oil with biomass as raw material for fuel and chemical production is an interesting option and the driving force for the development of biorefinery plants, which operate in a similar way to oil refineries. In fact, the biorefinery concept can be explained as the processing of renewable biomass into a spectrum of fuels and valuable products [6,8,11,12]. This strategy needs a large investment to achieve the sustainability goals of our society.

One challenge that is common to all industries and crucial to the success of the business is to ensure abundant and inexpensive raw materials in order to manufacture products with a competitive market price. In the case of bioprocesses, the raw material in question is carbon sources. Biomass and biomass-derived materials have been pointed out as one of the most promising alternatives. Biomass, in this context, refers to a rich carbon renewable raw material that can substitute fossil-based raw materials in the energy and chemical products industries. In biorefinery, almost all types of biomass that come from forestry residues, marine plants, waste food crops, food processing, animal farming, or human wastes can be converted to different classes of biofuels and biochemicals through jointly applied conversion technologies [6,13]. These products can be intermediates and/or final compounds in food, feed, materials, chemicals, and energy production (fuels, power, and heat) [12]. Therefore, biomass is considered the only sustainable source of organic carbon and the perfect equivalent to petroleum for the production of fuels and chemicals.

2.1. Lignocellulosic Biomass as Raw Material

Lignocellulosic forestry, agricultural, and agro-industrial wastes accumulate every year in large quantities, often feeding forest fires and causing serious environmental problems when deposited on soil or landfill. Instead, it can be utilized for the production of several value-added products [14]. Lignocellulosic wastes are an abundant, non-edible, and low-cost raw material [15,16]. The use of these renewable resources as a carbon platform reduces the production costs of bioprocesses and has a positive environmental impact, supporting sustainability ideals.

Lignocellulosic biomass (LB) comprises different fractions that can be converted or extracted, through the implementation of the biorefinery concept, to obtain value-added products such as fuels, chemicals, and biomaterials [17–20]. Besides wood, LB includes corn

stover, straw, wheat stover, grass, and others. The primary components of lignocellulosic-based wastes are cellulose, hemicellulose, and lignin (structural components), together with small amounts of other constituents (nonstructural components) such as extractives, ash, and proteins [20]. Depending on the type of LB, the structural components are organized into complex nonuniform three-dimensional structures to different degrees and varying relative compositions [14]. For LB valorization, Romaní et al. (2020) highlighted polysaccharides, lignin, and extractives as the most important fractions [20]. Lignin is considered the main renewable source of aromatics. A recent work described benzene production using lignin as feedstock through chemical catalysis, claiming that a further scale-up of the process can be a sustainable alternative to the current production from fossil resources [21]. Besides carbohydrates and lignin, Romaní et al. (2020) provided an extensive and detailed description on chemical and biological properties, sources, methods of extraction, and applications of the most important extractable compounds (fatty acids, lipids, proteins, terpenes, terpenoids, steroids, and polysaccharides) with a particular focus on phenolic compounds [20]. Xylochemicals (a designation for wood-derived building blocks) are in the forefront of replacing oil-based precursors for polymer high-scale production, although research is still needed to design the steps that make them suitable for specific polymerization-type reactions [18,19]. The production and characterization of biobased polyesters from bisguaiacol (a bisphenol analogue) derived from lignin and the synthesis of a verbanone-based lactone from (−)-α-pinene by a chemoenzymatic route are recent examples of this trend [18,19].

The conversion of organic solid matter through lignocellulosic gasification into a gaseous mixture of H_2, CO, CO_2, and CH_4 is another approach for LB valorization. The obtained synthetic—"syngas"—can be directly used as combustible for energy generation or as feedstock for chemical or biological processes [22]. Strict anaerobes, mostly acetogens, are used for syngas conversion to organic acids, alcohols, and other chemicals by "indirect fermentation"(as in this process, the biomass is previously converted to syngas and only then fed to microorganisms) [22,23].

Raw LB is considered unfermentable because most microbes cannot degrade it. In fact, in LB, the sugars are locked in a recalcitrant structure, and the degradation of carbohydrate polymers into fermentable sugars often involves two steps: a pre-treatment, after which most of the hemicelluloses are degraded into mono- and oligosaccharides, followed by hydrolysis of the cellulose and the remaining oligosaccharides, either by weak acids or enzymatic catalysis [24]. Nevertheless, LB raw materials are still most probably the most feasible alternative to biorefineries as a carbon source thanks to their unique eco-friendly nature, since they are biosynthesized from available atmospheric CO_2, water, and sunlight through biological photosynthesis [14].

Currently, more than 40 lignocellulosic biorefineries operate across Europe, increasing the turnover of the total bioeconomy in the last few years [25,26]. Examples of potential bio-based products include biofuels (e.g., bioethanol, biodiesel, and biogas), biochemicals (e.g., industrial enzymes and nutraceuticals), and biomaterials (e.g., biodegradable plastics). However, supported by specific EU policies, bioenergy, and biofuels have received greater attention. By the year 2030, the EU aims to replace 30% of oil-based chemicals with bio-based chemicals and to supplant nondegradable materials with degradable ones [25].

2.2. Lignocellulosic Biomass Treatments

The composition of lignocellulosic biomass varies with the type of biomass, with cellulose generally being its main component [27]. Cellulose is a highly crystalline, water-insoluble structure, consisting of linear polymeric chains of β(1→4) linked β-D-glucopyranose units [28,29]. Concerning hemicelluloses, they are amorphous polymers, mainly arabinoxylans composed of D-xylose and L-arabinose, D-glucose, or other sugars that are embedded in the plant cell walls to form a complex network of bonds. They provide structural strength by linking cellulose fibers into microfibrils and by cross-linking with lignin [30]. Thanks to the high resistance to enzymatic and chemical degradation offered by lignin, LB pre-treatments

can involve aggressive treatments and expensive procedures, which greatly increases the cost of these raw materials and, consequently, the price of the final products. Different types of processes, of physical, chemical, physicochemical, and biological nature, are currently employed. The idea is to improve the digestibility of LB carbohydrates, trying to avoid the generation of inhibitory compounds that may result from further degradation (e.g., aldehydes, phenolic acids, and furfural) when harsh chemical conditions are used [31]. These pre-treatments include, among others, mechanical size reduction, steam, ammonia or supercritical CO_2 explosion, acid, alkali or biological processes, ionic liquid extraction, or various combinations of these [28–36]. Through research and development, the optimization of an LB pretreatment has great potential to improve the efficiency and to reduce of the production cost [33]. A recent review highlights the nanobiotechnology contribution to this preliminary step [31]. Further hydrolysis to obtain monomeric sugars is usually performed using enzymatic catalysis, although other methods (such as chemical hydrolysis) can be employed. The sugar monomers obtained from hemicelluloses are mainly D-xylose, D-mannose, D-glucose, D-arabinose, D-galactose, and D-galacturonic acid, and their amounts depend on the LB source [37,38]. The integration of various biomass treatment methods with other processes, such as enzymatic saccharification, detoxification, fermentation of hydrolysates, and product recovery greatly reduce the overall cost of using lignocellulose for practical purposes [39,40].

3. Major Routes of Xylose Transport and Metabolism in Bacteria

Xylose is a very common sugar in residual lignocellulosic biomass, being the second major sugar found in most lignocellulosic hydrolysates and the major sugar in hemicellulosic hydrolysates. For that reason, xylose is a very promising carbon source, and it makes sense to understand the fundamentals of the mechanism used by bacteria to metabolize xylose into high-value by-products.

This review focuses on the metabolism of xylose to xylitol and xylonic acid by native xylose-consuming bacteria. Other valuable compounds, such as 1,2,4-butanetriol, butanediol, glutaric acid, γ-aminobutyric acid, 3,4-dihydroxybutyric acid, glycolic acid, ethylene glycol, and acetoin have also been produced from xylose using genetically engineered bacteria (see Section 3.2) [41]. Biodegradable polyesters such as polyhydroxyalkanoates (PHAs) have also been synthesized from xylose by native xylose-consumers such as *Paraburkholderia* sacchari (formerly classified as *Burkholderia sacchari*), *Pseudomonas pseudoflava*, and *Burkholderia cepacia*. In this case, PHA synthesis is promoted by manipulating culture conditions, i.e., by limiting the concentration of N or P nutrients and by supplying excess carbon [17,42]. Xylitol and xylonic acid have been ranked within the top 30 high-value chemicals from biomass by the U.S. Department of Energy [43]. In spite of its biotechnological importance, xylose metabolic pathways are far less studied than the ones of other fermentable sugars, namely glucose. This section aims to present a review of the major pathways involved in xylose transport and metabolism in bacteria and its conversion into xylitol and xylonic acid. Additionally, the main metabolic engineering efforts to increase titers and productivity of these metabolites using genetically modified bacteria are described.

3.1. Mechanisms of Xylose Transport

The transport of nutrients to the cells is the first step in their metabolic utilization. In order to transport sugars and other nutrients in and out of the cells, various organisms can use different types of transport mechanisms. Regarding D-xylose, yeast and fungi can use facilitated diffusion or active transport, while bacteria tend to use active transport mechanisms [41]. These types of mechanisms are mediated by carrier proteins and, hence, exhibit the properties of specific inhibition, substrate specificity, and saturability. These processes enable sugar transportation against a concentration gradient at the expense of metabolic energy.

Bacteria species such as *Bacillus, Clostridium, Escherichia coli*, and *Lactococcus* use active transport for the uptake of xylose into the cell [41,44]. Usually, there are high- and low-affinity transporter routes. In *E. coli*, the most studied species, the high-affinity transporter (XylFGH) belongs to the ATP-binding cassette (ABC) family, while the low-affinity transporter (XlyE), a proton-coupled symporter, belongs to the major facilitator superfamily (MFS), with xylose transport being driven by a proton motive force [45–48]. The low-affinity transport mechanism is also present in *Bacillus megaterium*, *Bacillus subtilis*, *Lactobacillus brevis*, *Salmonella typhimurium*, *Tetragenococcus halophila*, and some ruminal bacteria [47,49–55]. The ABC transporter is more efficient concerning xylose uptake and comprises a D-xylose-binding protein XylF, the membrane permease XylH, and the ATP-binding protein XylG [48,56]. This system is also present in bacteria such as *Clostridium*, *Geobacillus*, or *Thermoanaerobacter species* [41,57–59]. However, this transport can be inhibited when other readily fermentable substrates, such as glucose, are present. Many microbial strains have a regulatory mechanism, carbon catabolite repression (CCR), mainly mediated by components of the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS), which prevents the expression of genes needed for catabolism of other carbon sources, namely pentoses, while the substrate that enables the fastest growth (normally glucose) is present [60,61]. Concerning *E. coli*, at least two mechanisms of xylose transport and metabolism repression were reported, including the XylR regulator and cyclic AMP (cAMP) receptor protein (CRP)-dependent control of Xyl genes and the presence of arabinose, since the transporters allow for the transportation of this sugar at lower efficiencies [62]. The presence of high levels of glucose leads to the dephosphorylation of the component EIIA of PTS, which becomes unable to activate the enzyme adenylate cyclase, originating low levels of cAMP. In contrast, when glucose levels drop, cAMP increases, activating CRP that, together with XylR (activated when bound by xylose), stimulate the operons xylFGH and xylAB, involved in xylose transport and metabolism [53]. These repressive mechanisms could bring difficulties in the utilization of lignocellulosic hydrolysates, where both sugars are present. In this kind of media, a diauxic growth is observed and the preferential substrate, usually glucose, is consumed first. When glucose is depleted in the culture medium and another non-repressive substrate such as xylose is present, there is a temporary cessation of growth and catabolic repression is then relieved.

As previously said, these repression mechanisms are found in *E. coli*, where several exponential phases separated by lag phases can be observed when a sugar mix is supplied as a carbon source to the cultivation, indicating a sequential sugar consumption [63]. A repression behavior is also present in other bacteria strains. In *P. sacchari*, when the cells are grown in a medium containing glucose, xylose, and citric acid, the uptake of glucose and xylose only takes place when the citric acid has been completely consumed. *P. sacchari* can co-metabolize xylose and glucose, but the rate of xylose consumption decreases substantially in the presence of high glucose concentrations [42,60,64]. Concerning *Clostridium* species, a specific repressor XylR and a pleiotropic regulator CcpA have been found [65]. In the strain *C. acetobutylicum*, a mutation in the 14-nucleotide catabolite responsive element (CRE) sequence relieved CCR and increased 7.5-fold xylose consumption in the presence of glucose [66]. Different strategies, such as the inactivation of PTS components and mutagenesis of *CRP* or of *XylR*, were used in *E. coli* with the aim of relieving CCR, allowing for the co-utilization of xylose and glucose [53,67–69].

3.2. Xylose Metabolic Network in Bacteria

Xylose can be naturally metabolized by several microorganisms, including bacteria belonging to Archaea domain, Clostridia, proteobacteria, yeast, and filamentous fungi [70,71]. In order to optimize pentose utilization, genetic manipulation based on metabolic engineering has also been used to obtain yeast and bacterial recombinant strains with improved capacity to transport and metabolize xylose [72–74].

Xylose is consumed mainly through three different metabolic pathways: (i) the isomerase pathway; (ii) the oxidoreductase pathway; and (iii) the oxidative pathway, also

recognized as the non-phosphorylative pathway. The xylose metabolic network scheme is represented in Figure 1. The isomerase pathway converts xylose into xylulose, which is then phosphorylated to xylulose phosphate entering the pentose phosphate pathway (PPP). This is typically used by prokaryotes, namely by *E.coli*, *Bacillus* sp., and *Lactobacillus* sp. [75–78]; the oxidoredutase pathway is mostly present in eukaryotic microorganisms. Here, xylose is first converted to xylitol, which is then dehydrogenated and phosphorylated to xylulose phosphate, entering the PPP. The oxidative pathway has a common trunk that divides in two branches: the Weimberg and the Dahms pathways. This oxidative pathway is mainly carried out by bacteria.

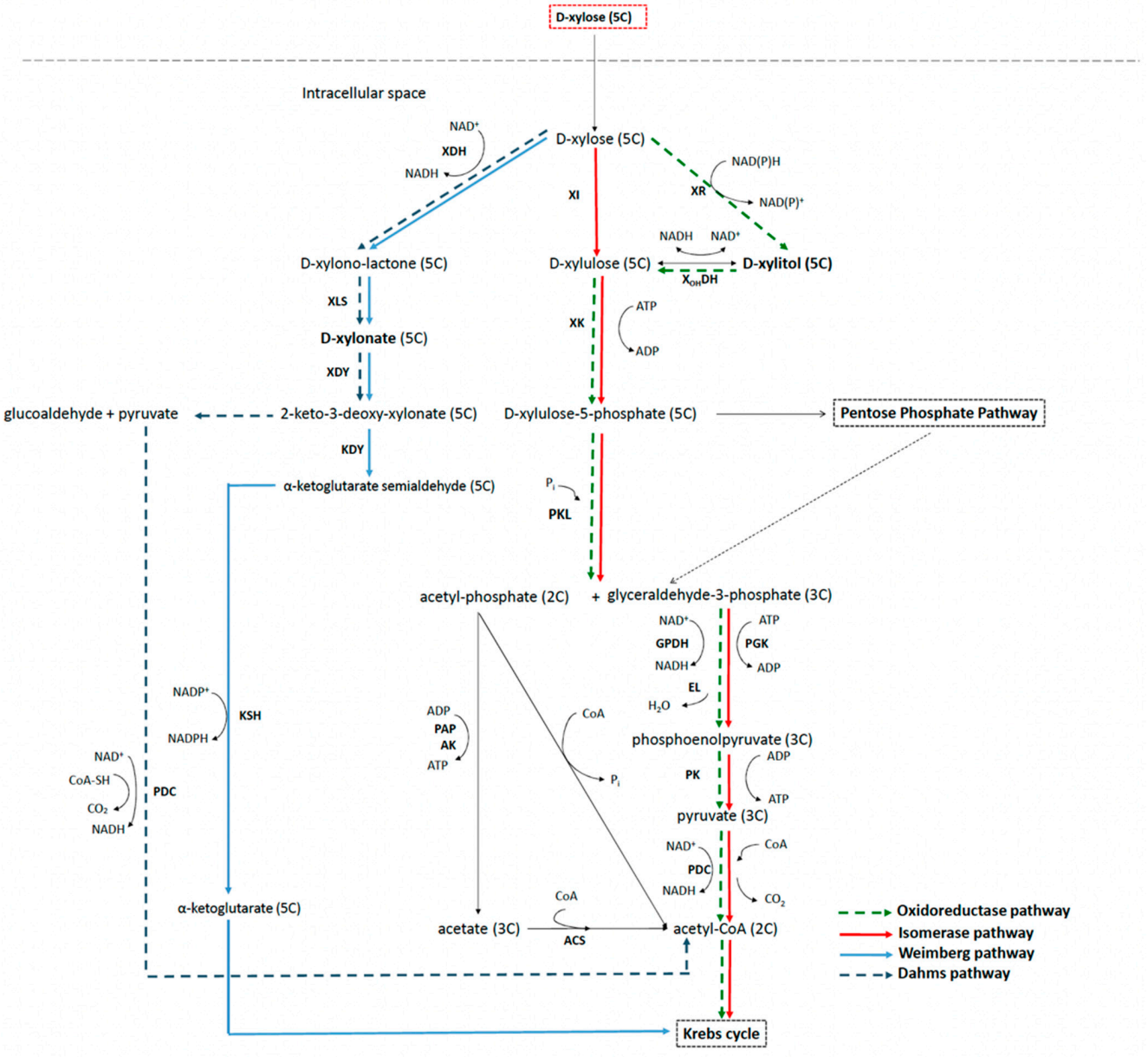

Figure 1. Three pathways of xylose metabolization by microorganisms: solid light blue, Weinberg pathway; dashed dark blue, Dahms pathway; dashed green, oxidoreductase pathway; and solid red, isomerase pathway. The enzymes are abbreviated as follows: acetyl-CoA synthase (ACS); acetate kinase (AK); enolase (EL); glyceraldehyde-3-phosphate dehydrogenase (GPDH); 2-keto-3-deoxyxylonate dehydratase (KDY); α-ketoglutarate semialdehyde dehydrogenase (KSH); pyrophosphate-acetate phosphotransferase (PAP); pyruvate dehydrogenase complex (PDC); 3-phosphoglycerate kinase (PGK); phosphoketolase (PK); phosphoketolase (PKL); xylonate dehydratase (XDY); xylose dehydrogenase (XDH); xylitol dehydrogenase ($X_{OH}DH$); xylose isomerase (XI); xylulokinase (XK); xylonolactonase (XLS); and xylose reductase (XR). Adapted from [79,80].

Regarding the isomerase pathway, xylose is first isomerized to xylulose by the enzyme xylose isomerase (Figure 1). Xylulose is then phosphorylated to xylulose-5-phosphate by XK (Figure 1). Genes coding for both enzymes are induced by xylose and repressed by glucose and other more readily usable substrates, according to the CCR mechanism, previously reported [62,81]. Xylose isomerase may be activated in the presence of divalent ions such as Mg^{2+}, Co^{2+}, or Mn^{2+}, whereas others such as Ca^{2+} act as inhibitors [41]. This enzyme may also be strongly inhibited by polyol xylitol, especially at a pH below 6 [82,83].

The metabolism of xylulose-5-phosphate continues generally via the PPP pathway, entering the central carbon metabolism. The PPP consists of several reversible transketolase and transaldolase reactions, of which the main function is to produce NAD(P)H, thus providing the reducing power for biosynthetic reactions [84]. Some bacteria, however (e.g., *Clostridium acetobutylicum, Clostridium beijerinckii*, and *Lactococcus lactis*), present an alternative mechanism, cleaving xylulose-5-P into acetylphosphate and glyceraldehyde-3-phosphate in a reaction catalyzed by phosphoketolase [85–87].

The oxidoreductase pathway is more common in yeast and fungi and uses two steps for the conversion of xylose in xylulose, employing the enzymes XR and XDH (Figure 1), with xylitol being an intermediate in this conversion [88]. Xylulose is then further metabolized via XK and PPP (Figure 1). Either via the isomerase or the oxidoredutase pathways, carbon exits the sugar-phosphate pool by various routes, with one of them being responsible for the formation of pyruvate. The pyruvate undergoes an oxidative decarboxylation to form acetyl-coenzyme A (acetyl-CoA), which is further oxidized via the Krebs cycle (KC) to generate energy.

In the non-phosphorylative pathway, xylose can be converted either to α-ketoglutarate (Weimberg route) or to pyruvate and glucoaldehyde (Dahms route) via a 2-keto-3-deoxy-xylonate intermediate. *Burkholderia xenovorans*, *Caulobacter crescentus*, *Gluconobacter oxydans*, *Paraburkholderia sacchari*, *Pseudomonas fragi*, and *Pseudomonas taiwanensis* are examples of native bacteria that use this pathway to metabolize xylose, with xylose being converted into D-xylonolactone via XDH (Figure 1) [78,89–91]. D-xylonolactone is in turn converted to D-xylonate, which is further converted to 2-keto-3-deoxy-xylonate, the common intermediate to both routes. In the Weimberg pathway (first discovered in *P. fragi*), 2-keto-3-deoxy-xylonate can be metabolized to α-ketoglutarate semialdehyde and then to α-ketoglutarate, a KC intermediate [90]. Alternatively, in the Dahms route, 2-keto-3-deoxy-xylonate can be cleaved to pyruvate and glucoaldehyde [92]. While pyruvate is then directly converted to acetyl-CoA and enters the central carbon metabolism (CCM), glucoaldehyde is incorporated into CCM at the level of glyoxylate, which requires two reactions generating two reduced equivalents. This has been observed in the hyperthermophilic archaea *Sulfolobus solfataricus* [93].

In shake flask assays with recombinant *Saccharomyces cerevisiae*, Borgström et al. (2019) observed a low consumption of D-xylose because of the acidification caused by the excretion of D-xylonate to the medium. Furthermore, a deficient NAD^+ recycling mechanism caused by a low oxygen titer was pointed out as the reason for the low efficiency of the oxidative Weimberg pathway. In bioreactor experiments, the accumulation of α-ketoglutarate semialdehyde was detected. This together with the accumulation of D-xylonate suggest the activity of KSH and XDH as rate-controlling steps caused by a deficient regeneration of the needed coenzymes NAD^+ and $NADP^+$ [94]. These results are in line with the observations of Shen et al. 2020, who, based on a quantitative model for the Weimberg pathway, have proposed these two reactions to be sensitive steps. Those authors, based on metabolic control analyses, found the dehydrogenases to control at high NADH levels while the dehydratase KDY controls at lower NADH levels [95].

Enzyme activity assays in *Caulobacter crescentus* showed that the Weimberg route is active in bacterial cells grown on xylose but not in the presence of glucose, indicating that the CCR mechanism is also present in this pathway [96].

The non-phosphorylative pathway has several advantages compared with the isomerase and oxidoredutase pathways because it directly converts D-xylose towards pyruvate and α-ketoglutarate (a KC cycle intermediate) by-passing the PPP, thus minimizing the number of enzymatic steps and the usage of ATP. In addition to saving P, the Weimberg pathway also conserves carbon during xylose metabolism and is thus a very efficient route. The positive aspects of the oxidative pathway promoted the development of recombinant strains using this pathway for the production of different valuable compounds as reviewed by Bañares et al. [97]. Target products such as 1,2,4-butanetriol, butanediol, glutaric acid, γ-aminobutyric acid, and 3,4-dihydroxybutyric acid have been produced from the Weimberg pathway, while glycolic acid, ethylene glycol, and acetoin were obtained using the Dahms pathway.

Particularly for glycolic acid or ethylene glycol, there are no known natural microbial pathways to directly produce these metabolites from carbohydrates. For this reason, attempts to attain high product yields and productivities are still being assessed by several authors through the establishment of synthetic pathways from pentoses, hexoses, or ethanol and using industrial microorganisms such as *E. coli, C. glutamicum, Saccharomyces cerevisiae*, or *Kluyveromyces lactis* as hosts [98]. Additionally, native ethylene glycol and glycolic acid producers such as *Enterobacter cloacae* have been used. This strain is not able to produce these two metabolites from xylose; however, it can use xylonic acid as a substrate for ethylene glycol and glycolate production [99].

A genome analysis of 492 bacteria strains of *Bacillus*, *Bifidobacterium*, *Caulobacter*, *Corynebacterium*, *Escherichia*, *Lactobacillus*, and *Xanthomonas* and the search for genes involved in xylose metabolism have been performed. The analysis revealed that the pathways of XI and Weimberg were the prevalent routes, with the XI pathway being the best preserved (Figure 1) [100].

3.3. Metabolic Pathways to Xylitol and Xylonic Acid

Intermediates of xylose metabolism with biotechnological high-value, such as xylitol or xylonic acid, may accumulate in the medium for several reasons such as redox imbalance, insufficient availability of cofactors, enzyme inhibition, and a lack or overexpression of genes encoding for intermediate enzymes [97]. The conversion of xylose into these metabolites and the strategies used to enhance their productivity in bacteria will be further discussed.

3.3.1. Xylitol

The large-scale production of xylitol is generally carried out by a chemical route based on the catalytic hydrogenation of highly pure D-xylose in solution (obtained from hardwood hydrolysates) submitted to high temperature (140–200 °C) and pressure (50–60 bar) conditions, and in the presence of the toxic Raney nickel catalyst [101]. The chemical process requires several purification steps because only pure xylose can be used in this chemical reduction. The conversion efficiency of the chemical process can be estimated as 8–15% from the initial raw material (hardwood hydrolysates), 50–60% from the hemicellulose in the raw material, and almost 98% from pure xylose [102,103].

Biotechnological methods are a promising choice that has been studied as alternatives to the conventional method, since they involve much milder conditions and can use a mixture of sugars as raw materials. This alleviates the purification step of the lignocellulosic hydrolysates used as carbon source, resulting in a less energy-demanding and cheaper process [104].

According to Dasgupta et al. [104], yeasts are preferred for xylitol production primarily due to their high pentose assimilation rates and xylitol productivity, as result of stable expression levels of XR and X_{OH}DH. Currently, there are a few studies on xylitol productions by yeast (namely different species of *Candida*) with impressive results, achieving conversion efficiencies of nearly 86%, with a volumetric productivity of 4.88 g L^{-1} h^{-1}, which is relatively high when compared with other reported microorganisms [104,105]. However, most of these yeasts are not considered as GRAS (Generally Recognized as Safe) by the

FDA. Furthermore, filamentous fungi were also reported to be xylitol producers; however, their xylitol production is too low to be competitive. Thus, there is a need to look for other microorganisms capable of producing xylitol with high yields, namely bacterial species. Several studies have shown the ability of some bacterial strains, namely *Cellulomonas*, *Corynebacterium* sp., *Enterobacter liquefaciens*, *Gluconobacter oxydans*, *Mycobacterium smegmatis*, and *Serratia*, to produce xylitol [106–110].

Although more common in yeasts or fungi, XR (Figure 1) is not totally specific of these microorganisms, as this enzyme was also present in some bacterial extracts. For example, *Corynebacterium* sp. NRRL B 4247 exhibited both NADH- and NADPH-dependent XR activity in enzymatic assays using cell-free extracts. In these bacteria, the highest yield obtained was of 0.57 g g^{-1} xylose, when an initial D-xylose concentration of 75 g L^{-1} was used. This yield was however improved when potassium gluconate was added to the medium [108]. For *Corynebacterium* sp. NRRL B 4247, xylitol production appears to be growth-associated [108].

XR (Figure 1) enzymatic activity is present in *Enterobacter liquefaciens*, with NADPH as co-factor, and a productivity of 0.35 g L^{-1} h^{-1} has been reported in this case [110]. The XR route has also evolved in enteric bacteria such as *E. coli*; however, the efficiency of the pathway is low.

P. sacchari is also able to accumulate xylitol. In this case, it was hypothesized that the metabolic pathway used by *P. sacchari* is the isomerase pathway, since genes present in its genome include those that encode for XI, XK (Figure 1), and ABC xylose transporter, whereas the genes of the oxidoreductase route were not found, suggesting that *P. sacchari* cannot assimilate xylose by this pathway [111]. In xylose-richer mixtures, *P. sacchari* was able to accumulate both xylitol and xylonic acid, but the mechanism that leads to this production was not exploited [60].

As referred, the most common way used by microorganisms to produce xylitol is through a single-step mechanism, where xylose is reduced to xylitol by xylose reductase while one of the cofactors NADH/NADPH (depending on the species preference) undergoes oxidation. If the cofactor NAD^+ is abundant in the medium, the enzyme $X_{OH}DH$ (Figure 1) converts xylitol into xylulose, which enters in the PPP pathway after being phosphorylated. The accumulation of xylitol in the cultivation media is believed to be a consequence of co-factor imbalance. This can be attributed to the co-factor preference of xylose reductase towards NADPH and xylitol dehydrogenase towards NAD (Figure 1). Furthermore, it has been reported that NADH accumulation inhibits the NAD linked xylitol dehydrogenase [107].

Many pentose-utilizing microorganisms suffer from bottlenecks, resulting in low xylitol productivity. For this reason, the genetic manipulation of wild species by adaptation, mutation, and recombinant techniques has been proposed. Selective gene manipulation to improve productivity has been targeted after gradual enrichment of the genetic database and increased knowledge about the metabolic mapping of many of these microorganisms. With this tool, it is possible to optimize selective xylose transporter systems to increase the xylose uptake rate, as previously referred. Improvements for xylitol production have included the overexpression of XR to maximize the reduction in xylose into xylitol or deletion of the $X_{OH}DH$ gene to stop the oxidation of xylitol into xylulose, besides the optimization of the supply of cofactors involved in xylitol production (as NADH or NADPH) [104,112–114]. XR genes from yeasts such as *C. tenuis* or *C. tropicalis* were successfully expressed in *E. coli* and the conversion of xylose into xylitol was then achieved [115–117]. It is also reported that xylitol production from xylose can be improved through the heterologous expression of xylose transporters [116].

Currently, most studies on xylitol production in bioreactors focus on yeast-based processes [105,118]. To improve productivities, fed batch or continuous reactor operation modes as well as high cell density strategies that include cell immobilization or retention inside membranes should be preferred. The use of immobilized cells during a continuous fermentation mode is often attractive. This option allows for cell re-use, ensuring high productivities during an extended period, compared with freely suspended cells. However,

most bioreactor studies regarding xylitol production are reported to be operated under batch mode. For xylitol production, non-growing cells in a viable state ("resting cells") require an additional co-substrate to carry out the reduction reaction. Hence, a fed-batch bioreactor system with intermediate co-substrate addition at repeated intervals may be a solution to improving the productivity and therefore the xylitol yield [104].

Fine-tuning concerning operational parameters control is paramount [60]. Regarding microbial xylitol production, a number of cultivation parameters need to be considered, such as the pH of the culture, initial substrate concentration, medium composition, inoculum level, temperature, and aeration conditions [119]. With this many variables at play, the search for the optimal conditions for each microorganism has been an area extensively addressed.

As reported by Winkelhausen and Kuzmanova (1998), both XR and X_{OH}DH (Figure 1) enzyme activities are dependent on the concentration of xylose in the culture medium, since xylitol formation does not occur in the absence of this pentose [120]. Moreover, the xylose concentration in the lignocellulosic hydrolysate should be as high as possible to ensure an economically viable process, although it should be borne in mind that, when concentrating the hydrolysates, the inhibitors present (e.g., furfural and hydroxymethylfurfural) may reach levels that adversely affect the behavior of the micro-organisms [119,121].

Another key factor on the xylitol production is the aeration rate in the culture since this is directly related to the regeneration of the cofactors NAD^+/NADH or $NADP^+$/NADPH. Under aerobic conditions, the NADH formed during xylose metabolism can be reoxidized into NAD^+ in the electron transport system, and consequently, xylitol is not produced, since the high NAD^+/NADH ratio favors xylitol oxidation to xylulose. Under anaerobic conditions, microorganisms are unable to metabolize D-xylose because of the redox imbalance between NAD^+ and NADH. Under oxygen-limited conditions, the electron transport system is unable to oxidize intracellular NADH completely, increasing the NADH concentrations, which subsequently favors the xylulose conversion into xylitol by the X_{OH}DH enzyme or the reduction of xylose into xylitol by the XR enzyme (Figure 1) [107]. However, other authors claim that an increase in the amount of dissolved oxygen (DO) also increases the quantity of xylitol produced, since the activity of the enzymes depends not only on the regeneration of cofactors but also on oxygen availability [38].

Table 1 summarizes the contents of this section regarding a selection of bacterial strains able to convert xylose into xylitol.

Table 1. List of bacteria (wild type and genetically modified) with potential for D-xylitol production from lignocellulosic biomass (n.a.—information not available).

Strains	C-Source	Genetic Modification	Growth Conditions	Xylitol ($g\ L^{-1}$) *	$Y_{xylitol/xylose}$ ($g\ g^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$) *	Ref.
Corynebacterium glutamicum Cg-ax3	arabinose glucose xylose	Yes	Batch shake flask	6.7	n.a.	n.a.	[122]
			Fed-batch shake flask	31	n.a.	0.28 $g\ g_{cdw}^{-1}\ h^{-1}$	
	acid pre-treated liquor of sorghum stover		Fed-batch shake flask	27	n.a.	0.22 $g\ g^{-1}_{cdw}\ h^{-1}$	
Corynebacterium sp. NRRL B 4247	xylose	No	Shake flask	1.7	0.57	0.071	[108]
			6-phosphogluconate (source of NADPH) added to the medium Shake flask	10	n.a.	0.067	
Corynebacterium sp. *no. 208*	xylose	No	6-phosphogluconate (source of NADPH) was added to the medium Shake flask	69	n.a.	0.21	[123]
Enterobacter liquefaciens 553	xylose	No	Shake flask	33	n.a.	0.35	[110]
E. coli BL21(DE3)	xylose	Yes	Shake flask	202	1.0	6.37	[113]

Table 1. *Cont.*

Strains	C-Source	Genetic Modification	Growth Conditions	Xylitol (g L^{-1}) *	$Y_{xylitol/xylose}$ (g g^{-1})	Productivity (g L^{-1} h^{-1}) *	Ref.
Escherichia coli IS5-d	xylose and glucose	Yes	5 L Batch STR	110	n.a.	3.06	[112]
Escherichia coli IS5-M	corncob hemicellulosic hydrolysate and 24 g L^{-1} corn steep liquor	Yes	15 L Fed-batch STR	144	n.a.	1.84	[112]
Escherichia coli HK402	xylose and glucose	Yes	15 L Fed-batch STR	172	>0.95	1.57	[112]
	detoxified hemicellulosic hydrolysate and glucose			150	>0.95	1.40	
Escherichia coli WZ51	detoxified hemicellulosic hydrolysate	Yes	15 L Fed-batch STR	132	0.95	2.09	[114]
Mycobacterium smegmatis	xylose	No	immobilized D-xylose isomerase from *Bacillus coagulans* and immobilized *M. smegmatis* Shake flask	5 g	0.80	n.a.	[106]
Paraburkholderia sacchari DSM 17165	xylose	No	2 L Fed-batch STR	17	n.a.	0.39	[60]
Paraburkholderia sacchari DSM 17165	xylose	No	2 L Fed-batch STR	70	0.39	0.50	[80]

* unless otherwise indicated.

3.3.2. Xylonic Acid

Although xylonic acid is a valuable platform chemical that can substitute gluconic acid in a range of applications (from pharmaceuticals to paints, solvents, and adhesives), large-scale production has not yet been developed [124].

In contrast with xylitol, xylonic acid is mainly produced from xylose by bacteria by the unconventional non-phosphorylative pathway (Figure 1). *B. xenovorans*, *C. crescentus*, *G. oxydans*, *P. sacchari*, *P. fragi*, *Pseudoduganella danionis*, and *Pseudomonas putida* are examples of bacterial native producers, while *Corynebacterium glutamicum*, *E. coli*, and *Klebsiella pneumoniae* have been engineered to produce xylonic acid [78,80,122,125–131]. The enzymes that catalyze the conversion of xylose to D-xylonolactone may belong to three different groups based on the cofactor used. Xylose dehydrogenase catalyzes the reaction in *K. pneumoniae* and is located in the inner membrane of the periplasmic space. It uses pyrroloquinoline quinine (PQQ) as the cofactor [129]. D-xylose dehydrogenase from *Trichoderma reesei* uses $NADP^+$ as the cofactor, whereas NAD^+ is the cofactor for D-xylose dehydrogenase from *C. crescentus* [78,132] D-xylose dehydrogenases are located in the cytosol. The efficiency of D-xylonolactone production differs greatly depending on the cofactor needed and the location of the dehydrogenase enzymes. D-xylonolactone is then further metabolized into D-xylonate either spontaneously or via xylonolactonase, with the activity of this enzyme being induced by the substrate D-xylonolactone [128].

E. coli is an attractive model to produce compounds with biotechnological value, such as xylonic acid, as its physiology and genetics are deeply known and there is a vast amount of genetic and bioinformatic tools available. However, this species does not naturally produce xylonic acid from xylose, as its main metabolic pathway to metabolize xylose is the XI pathway [133,134]. Nevertheless, this limitation can be circumvented through the use of metabolic engineering. In fact, the heterologous expression of the genes coding for XDH (*xdh*) and xylonolactonase (*xylC*) from *Caulobacter crescentus* together with the

disruption of the genes encoding XI and XK to block xylose utilization via the xylose isomerase pathway as well as of two genes coding for XDY (Figure 1) to prevent further xylonic acid metabolism resulted in xylonic acid accumulation by the genetically modified strain, and up to 27.3 g L^{-1} xylonate from 30 g L^{-1} xylose was achieved [134,135].

Besides *E. coli*, *C. glutamicum* was also modified to enhance xylonic acid production [136]. In this strain, the deletion of the transcriptional repressor gene *iolR*, together with the expression of the xylose uptake IolT transporter, improved xylose uptake, cell growth, and the sugar conversion into xylonate [137]. The use of recombinant bacteria strains is thus promising and can be a good alternative for xylonic acid production.

Bioprocess engineering strategies have also been assessed to attain higher xylonic acid productivities. *G. oxydans* is a native xylonic acid producer and has been intensively investigated for the production of this metabolite [126,127,138–140]. High volumetric production rates of 4.7 g L^{-1} h^{-1} with concentrations of 586 g L^{-1} have been attained. This species nevertheless has a low biomass production, extremely slow growth, complex nutritional requirements, and thus a high cost in terms of biocatalyst preparation. Moreover, due to the need for high aeration rates, extreme foam production during xylonic acid occurs when air is used for aeration. To circumvent this, pressurized bioreactors and pure oxygen have been used, thus increasing the cost of the production process. Besides being a good producer, *Gluconobacter* has a higher resistance to the inhibitors present in lignocellulosic biomass hydrolysates, being a good candidate to use these residual materials as a source of xylose.

P. fragi also produces xylonic acid at similar volumetric rates to *G. oxydans* but at lower specific rates [124].

P. sacchari has recently been reported to be a good xylonic acid producer. In *P. sacchari*, the genes involved in the Weimberg pathway were found to have homologous genes. However, the gene encoding for 2-keto-3-desoxy-D-xylonate dehydratase (KDY), responsible for converting 2-keto-3-desoxy-D-xylonate into α-ketoglutarate semialdehyde was not found. This finding could explain the accumulation of xylonic acid by *P. sacchari* reported by Raposo et al. [60].

This bacterium has shown a promising capacity to produce xylonic acid from xylose, as productivities in lab-scale reactor reached 1.5 g L^{-1} h^{-1} using xylose as the sole carbon source and over 6 g L^{-1} h^{-1} in fed-batch cultivations using glucose for cell growth and xylose in the feed for xylonic acid production. Xylonic acid titers reached 390 g L^{-1} and yields of 1.1 $g_{xyl\ acid}$ g^{-1}_{xylose} [80]. In *P. sacchari*, the genes involved in the Weimberg pathway were found to have homologous genes.

The assays with *P. sacchari* showed that it is necessary to maintain a high dissolved oxygen concentration to promote a high xylonic acid productivity titer and yield on xylose. This is explained by the need to restore reduced cofactors (NADH/NADPH) through oxidation and to maintain a continuous bioconversion process. Particularly in the case of *P. sacchari*, a high oxygen concentration in the medium also discouraged the formation of other xylose-derived metabolites such as xylitol (see Section 3.3.1), thus increasing the yield of xylonic acid on xylose. These authors have also observed that, if cells were previously grown on glucose and then fed with xylose, xylonic acid productivity increased by a factor of 4 or 5 [80].

Table 2 gathers information on various examples of bacterial strains able to convert xylose into xylonic acid.

Table 2. List of bacteria (wild type and genetically modified) with potential for D-xylonic acid production from lignocellulosic biomass (n.a.—information not available).

Strains	C-Source	Genetic Modification	Growth Conditions	Xylonic Acid (g L^{-1}) *	$Y_{xylonic\ acid/xylose}$ (g g^{-1})	Productivity (g L^{-1} h^{-1}) *	Ref.
Corynebacterium glutamicum ATCC13032	xylose	Yes	Shake flask	50.7	0.76	0.42	[136]
Corynebacterium glutamicum ATCC31831	rice straw hydrolysate after dilute sulfuric acid pretreatment	Yes	Shake flask	42.9	1.1	0.37	[136]
	xylose			56.3	0.84	0.47	
Escherichia coli BL21	xylose	Yes	Shake flask	9.1	1.10	0.45	[141]
			2 L Batch STR	6.9	0.89	0.11	
Escherichia coli W3110	xylose and glucose	Yes	Shake flask	5.1	0.51	0.084	[134]
			5 L Fed-batch STR	39.2	0.98	1.09	
Escherichia coli BL21	xylose and glycerol	Yes	5 L Fed-batch STR	27.3	n.a.	1.8	[135]
Gluconobacter oxydans ATCC 621	xylose	No	3 L Batch STR	109	0.95	2.5	[127]
	steamed and enzymatically hydrolyzed birchwood			12.4	0.50	n.a.	
Gluconobacter oxydans DSM 2003	corn stover hydrolysate after dry dilute acid pretreatment	No	3 L Batch STR	38.9	0.9	n.a.	[139]
Gluconobacter oxydans DSM 2003	xylose	No	3 L Batch STR	66.4	n.a.	5.5	[142]
Gluconobacter oxydans NL71	xylose	No	Compressed oxygen-supplied sealed stirred tank reactor (COS-SSTR); pure oxygen supply	586.3	0.95	4.7	[138]
	corn stover diluted sulfuric acid hydrolysates without detoxification			143.9	0.97	1.0	
Gluconobacter oxydans NL71	xylose in distillation stillage of cellulosic ethanol fermentation broth	No	COS-SSTR; fed-batch addition of xylose with cell-recycling	1813 g in 6-fold cell recycling; 1 L culture medium	n.a.	16.8 g h^{-1} in 108 h	[140]
Gluconobacter oxydans NL71	corn stover hydrolysate after dry diluted acid pretreatment	No	Two-stage fermentation in a 3 L COS-SSTR bioreactor with cell recycling	167.4 g from 1 kg corn stover	0.97	3.7	[143]
Gluconobacter Oxydans ATCC 621	xylose	No	Fed-batch bioreactor; Immobilized whole-cells; pressurized pure oxygen supply followed by electrodialysis acid chamber (POA-SSB-OE)	329.2 g xylonic acid	n.a.	7.1 g h^{-1} in 48 h	[144]
Klebsiella pneumoniae (modified)	bamboo hydrolysate	Yes	Fed-batch cultivations	103 g L^{-1}	0.98	n.a.	[129]
Paraburkholderia sacchari DSM 17165	xylose	No	2 L Fed-batch STR xylose as carbon source; high dissolved oxygen concentration	150 g L^{-1}	0.85	1.5	[80]
	xylose and glucose		2 L Fed-batch STR high dissolved oxygen concentration	390 g L^{-1}	1.1	6.0	

* unless otherwise indicated.

4. Conclusions

Lignocellulosic waste is abundant and renewable; hence, biological processes using xylose-rich lignocellulosic hydrolysates as a carbon source can dramatically reduce the

share of raw material-related costs while helping to achieve process sustainability. The hydrolysis of the hemicellulose of lignocellulosic biomass gives rise to a mixture of oligo- and monosaccharides, of which xylose is usually the main component. Although hexoses are generally preferred by most microorganisms, xylose can also be metabolized into commercially relevant molecules by several microorganisms. This article reviews current research on both xylose metabolic pathways in different bacteria, and knowledge regarding cultivation parameters to achieve high yields of xylitol or xylonic acid. It is important to keep in mind that neither wild-type strains nor genetically modified bacteria cultivations are feasible on a large scale if there is no thorough knowledge and optimization of the cultivation parameters suitable for the production conditions of the target product. Some studies highlight oxygen supply, initial xylose concentration, and pH as being of utmost importance for process optimization.

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