Simple and Practical Multigram Synthesis of D-Xylonate Using a Recombinant Xylose Dehydrogenase

Israel Sánchez-Moreno,* Raúl Benito-Arenas,† Pilar Montero-Calle,‡§ Carmen Hermida,† Eduardo García-Junceda,*‡ and Alfonso Fernández-Mayoralas*‡

†Venter Pharma S.L., Azalea 1, 28109 Alcobendas (Madrid), Spain
‡Departamento de Química Bioorgánica, Instituto de Química Orgánica General (IQOG-CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

ABSTRACT: An efficient multienzyme system for the preparative synthesis of D-xylonate, a chemical with versatile industrial applications, is described. The multienzyme system is based on D-xylose oxidation catalyzed by the xylose dehydrogenase from Calulobacter crescentus and the use of catalytic amounts of NAD+. The cofactor is regenerated in situ by coupling the reduction of acetaldehyde into ethanol catalyzed by alcohol dehydrogenase from Clostridium kluyveri.

Excellent conversions (>95%) were obtained in a process that allows easy product isolation by simple evaporation of the volatile buffer and byproducts.

INTRODUCTION

In recent years, increasing energy costs and environmental concerns have led to increased awareness about plant biomass waste being a valuable feedstock for biorefining processes that give rise to platform chemicals.1 Carbohydrates account for 75 wt % of the plant biomass and can be harvested from a wide variety of biomass wastes and crops.2 The first platform chemicals in the biorefinery can be sugars composed of different five- and six-carbon monosaccharides.3 Glucose is the most widely used monosaccharide, which at present is industrially produced by the enzymatic hydrolysis of corn.4 On the other hand, sugar acids are useful chemical intermediates usually derived from the oxidation of common aldoses. The most well-known sugar acid is D-gluconic acid, and up to 87 000 tons of it are produced annually worldwide.5 The increased demand for plant biomass feedstock has resulted in competition with food, higher prices, questionable land-use practices, and environmental concerns associated with their production.6 Therefore, numerous research efforts have focused on developing novel bioprocesses based on non-food renewable feedstock like hemicelluloses.7 A major plant hemicellulose of interest is xylan, a branched plant polysaccharide built of β-1,4-linked D-xylopyranosyl (Xylp) residues. In this sense, D-xylane could serve as a substitute for D-glucosyl with the advantage that D-xylose is a non-food sugar. D-Xylane has found applications as a cement dispersant,8 for decreasing acrylamide contained in the food after cooking,9 in the production of co-polyamides,10 and as a precursor for 1,2,4-butatriene synthesis.11 Recently, D-xylonic acid has also been used as a catalyst in a three-component reaction for the synthesis of pyrrole and xanthene derivatives with potential pharmaceutical properties.12

Conversion of D-xylose into D-xylonic acid can be achieved by chemical or biological methods.14 In biological systems, the enzyme responsible for the oxidation is D-xylose dehydrogenase (D-XDH), which converts D-xylose to D-xylonolactone. This reaction is followed by the hydrolysis of xylonolactone to xylonic acid, either spontaneously or catalyzed by a lactonase enzyme (Scheme 1).

The most commonly used method for the production of D-xylonic acid from D-xylose is microbial fermentation.15 Many of the examples use genetically modified microorganisms. Thus, the metabolic D-xylose pathway has been modified in Saccharomyces cerevisiae and Kluyveromyces lactis with the D-
XDH from *Hypocrea jecorina* to produce and accumulate D-xylonate. The same strategy was carried out employing the D-XDH from *Caulobacter crescentus* (XylB). As this enzyme is more efficient for D-xylose oxidation, the production of D-xylonate was greatly improved in the organisms modified with this gene. Further improvements of D-xylonate production have been accomplished by addition of the lactonase XylC also from *C. crescentus*. The use of isolated enzymes can reduce some of the problems associated with whole-cell catalysts, such as the diffusion limitations or the isolation and purification of final products. The first attempt within this strategy was the use of commercial glucose oxidase for the synthesis of D-xylonate. However, the reaction requires a large amount of enzyme due to its low selectivity for D-xylose and the addition of catalase to decompose the hydrogen peroxide formed as a byproduct. In this way, the use of the XylB isolated from *C. crescentus* has been recently explored as a promising method to obtain D-xylonate. This enzyme was employed in an artificial four-enzyme complex, which allowed the formation of D-xylonate from hemicelluloses at a micromolar scale. This work proved that the XylB enzyme could be a good candidate for the preparative synthesis of D-xylonate.

Recently, we have reported the overexpression in *Escherichia coli* of the XylB from *C. crescentus* NA 1000 and its isolation. The purified recombinant enzyme showed a strong preference for D-xylose against other assayed mono- and disaccharides as well as strong stability when stored as a freeze-dried powder with a half-life of at least 250 days both at 4 °C and at room temperature. In addition, more than 80% of the initial activity of the rehydrated freeze-dried enzyme remained after 150 days of incubation at 4 °C. Considering these features, XylB is an excellent candidate for large-scale biocatalytic methods. For this reason, we decided to study the synthetic potential of this D-xylose dehydrogenase for the preparative synthesis of D-xylonate in a direct and simple way, as outlined in Scheme 2. A major drawback of this approach for large-scale production is that the enzyme requires an expensive cofactor, NAD+. To make practical and cheap the preparative enzymatic synthesis of D-xylonate, the development of a cofactor-regenerating system was explored.

For the in situ regeneration of NAD⁺, we employed an orthogonal cascade composed of the recombinant XylB from *C. crescentus* for xylose oxidation and the alcohol dehydrogenase (ADH) from *Clostridium kluyveri* for NAD⁺ recycling by the reduction of acetaldehyde to ethanol (Scheme 2). Here, we report our results on the synthesis of D-xylonate by XylB-catalyzed oxidation of D-xylose.

## RESULTS AND DISCUSSION

The reactions were performed in NH₄HCO₃ buffer (10 mM, pH 8.0). The D-xylonic acid formed during the oxidation was continuously neutralized with NaOH, and the pH of the medium was maintained constant during the reaction. The conversion rate in the enzymatic reaction is defined as the amount of D-xylose that is oxidized under the given reaction conditions per unit of time. The amount of substrate conversion to product was measured using a variant of the XylB activity assay, as described in the Experimental Section. This method has been previously optimized and described by us. The percentage of conversion was calculated with respect to the initial amount of D-xylose, considering the limiting substrates in the optimization reactions. The efficiency of the enzymatic reactions was evaluated through their substrate conversion rates: elevated conversion rates indicate that the amount of accumulated product per unit of time is higher in a given reaction. Thus, the efficiency of different reaction conditions could be quantified and compared.

The incubation of D-xylose (33 mM) with XylB (6 U), acetaldehyde (16 mol equiv), and alcohol dehydrogenase (35 U) in 3 mL of buffer proceeded with complete conversion (entry 1, Table 1).

| entry | D-xylose/CH₃CHO ratio | NAD⁺ (mM) | conversion (%) |
|-------|------------------------|-----------|----------------|
| 1     | 1:16                   | 2.3       | 100            |
| 2     | 1:5                    | 2.3       | 100            |
| 3     | 1:3                    | 2.3       | 99             |
| 4     | 1:1.5                  | 2.3       | 95             |
| 5     | 1:3                    | 1.1       | 99             |
| 6     | 1:3                    | 0.83      | 99             |
| 7     | 1:3                    | 0.33      | 99             |
| 8     | 1:3                    | 0.16      | 98             |

The system efficiency was so high that the D-xylose/CH₃CHO ratio could be reduced from 1:16 to 1:1.5 without significant loss in the percentage of conversion (entries 1–4, Table 1). The ratios 1:3 and 1:1.5 were chosen as optimal since the conversion was nearly quantitative. Interestingly, the NAD⁺ concentration needed to feed the system could be reduced to 0.16 mM, just 2 times the *Kₐ* value of recombinant XylB for NAD⁺, which corresponds to only 2% mol equiv (entry 8, Table 1). The high efficiency of the NAD⁺ recycling system, which works properly even when very low concentrations of the cofactor were employed to feed the system, is remarkable.

The synthesis of D-xylonate was optimized with respect to the xylose concentration (Table 2). Complete conversion was achieved at 33, 66, and 100 mM although longer reaction times were required in a concentration-dependent manner. At 130 mM, the transformation was not complete after 19 h, probably because some enzyme inactivation took place.

It is especially remarkable that the D-xylose oxidation was also highly effective using a cell-free extract (CFE) from the recombinant strain of *E. coli*, which contains the overexpressed xylose dehydrogenase (entries 5–8, Table 2). Thus, similar
the chemical structure of a dixylonate monoester (Scheme 3).

The ion at high-resolution mass spectra of the reaction mixtures contained the catalyst.
im, acetaldehyde (3 equiv with respect to D-xylose).

The substrate (entry 4, Table 2) could be due to the accumulation of the reaction rate and the final conversion at 130 mM, with additional enzyme and a long reaction time also.

250 mM, with additional enzyme and a long reaction time also, the conversion was excellent (entry 8, Table 2). The decrease of ethanol (see Experimental Section).

We found that pH control is critical to avoid the formation of byproducts, particularly when the reactions were carried out at concentrations above 100 mM. In these cases, the appearance of new signals at 3–5 ppm in the NMR spectra, which cannot be assigned to D-xylonate, were observed. The high-resolution mass spectra of the reaction mixtures contained the ion at m/z 313.08 (Figure S3) which is compatible with the chemical structure of a dixylate monoester (Scheme 3).

Scheme 3. Chemical Structure of Dixylonate Monoester, which Derives from the Ring-Opening Reaction between a Molecule of D-Xylonolactone and a Hydroxyl of Xylonate

This compound must derive from the ring-opening reaction between xylonolactone and a hydroxyl of xylonate. Its formation was ascribed to a deficient basification during the progress of the reaction, thus leaving a high proportion of xylonolactone without being hydrolyzed and susceptible to being attacked by a hydroxyl nucleophile of another molecule, an intermolecular reaction that is favored at the highest concentrations. Considering these observations, the reactions were performed strictly maintaining the pH at 8.0. Under these conditions, the formation of byproducts was avoided and the reactions afforded pure D-xylonate.

Regarding the large-scale synthesis of D-xylonate, this approach has two important advantages: (i) it involves a volatile buffer, and both the substrate and the product of the ADH-catalyzed reaction can be removed by evaporation, and (ii) the use of CFE containing XylB makes unnecessary the expensive purification of the enzyme. Optimization of the large-scale synthesis was carried out in three reactions where different amounts of D-xylose and CFE with overexpressed XylB were employed (Figure 1).

When 0.25 mM of D-xylose was used, reactions were not totally completed after 5 h of incubation: the highest achieved conversion of the substrate was 96%, even when XylB was increased from 400 to 610 U. However, a small decrease in the D-xylose concentration from 0.25 to 0.22 mM resulted in a quantitative oxidation after 3 h (Figure 1).

We carried out a gram-scale preparation of D-xylonate under these optimized reaction conditions. Since we obtained a total conversion of the substrate D-xylose (Figure S4), we decided to increase the scale of the reaction to 10 g. Also, at this scale, the conversion of D-xylose to D-xylonate was practically quantitative. After the filtration of proteins and evaporation of the solvents, D-xylonate was obtained in pure form as indicated by its $^1$H NMR spectrum (Figure 2). Therefore, the present two-enzyme system allowed us to quantitatively obtain D-xylonate without the need of purification and using catalytic amounts of NAD$^+$. The concentration of xylose in the reaction was 33.3 g/L, which is similar to, or even higher than, that described in some fermentative processes. It is true that this substrate concentration is far from that of the most efficient fermentative methods (ref 18, 160–190 g/L), but the reaction time considerably decreases in the biocatalytical process: 2 h of reaction is enough to carry out a near quantitative reaction. Therefore, the conversion rate was much higher than that of any fermentative process described in the literature to date: 15.66 g/(L h). This is more than 6 times better than that of the best of fermentation processes.

Successive evaporations with water and ethanol were carried out to eliminate all of the solvents present in the sample and derive an accurate yield value of the xylonate obtained. However, a single evaporation would be enough to obtain a wet but still very pure xylonate, which could contain some ammonium salt. We would like to highlight that this is the first multigram synthesis of xylonate, where the final yield is

Table 2. Optimization of the D-Xylose Concentration$^a$

| entry | XylB (units) | D-xylose (mM) | reaction time (h) | conversion (%) |
|-------|--------------|----------------|-------------------|---------------|
| 1     | 6            | 33             | 1                 | 99            |
| 2     | 6            | 66             | 3.5               | 100           |
| 3     | 6            | 100            | 5                 | 99            |
| 4     | 6            | 130            | 19                | 73            |
| 5$^a$ | 6            | 33             | 1                 | 100           |
| 6$^a$ | 6            | 66             | 3.5               | 100           |
| 7$^b$ | 13           | 100            | 2                 | 100           |
| 8$^b$ | 27           | 250            | 4                 | 97            |

$^a$Reactions were carried out in 3 mL of NH$_4$HCO$_3$ buffer (10 mM, pH 8.0) containing ADH (35 U), NAD$^+$ (0.33 mM), and acetaldehyde (3 equiv with respect to D-xylose).

$^b$Cell-free extract (CFE) containing overexpressed xylose dehydrogenase was used as the catalyst.

Figure 1. Optimization of the scaled-up reactions: (●), 0.25 mM D-xylose and 400 U of XylB; (○), 0.25 mM D-xylose and 610 U of XylB; (▲), 0.22 mM D-xylose and 610 U of XylB.
calculated as the mass of the pure product and not on basis of xylose conversion in the fermentation process. In addition, fermentation processes usually quantify both intra- and extracellular accumulated xylonate.

■ CONCLUSIONS

In this work, we present a biocatalytic cascade for producing D-xylonate based on the use of xylose dehydrogenase from *Caulobacter crescentus* and an efficient cofactor regeneration method employing the enzyme ADH from *Clostridium kluyveri*. One important advantage of this system is that the buffer and both the substrate and the product of the ADH-catalyzed reaction are volatile, allowing, after complete conversion, an easy work-up and isolation of the product. In addition, the use of CFE containing XylB makes unnecessary the expensive purification of the enzyme. This process has been easily scaled up to 10 gram-size reactions. We believe that this biocatalytic procedure provides a new path to D-xylonic acid production, competitive with the fermentative processes currently used. We believe that this biocatalytic procedure provides a new path to D-xylonic acid production, competitive with the fermentative processes currently used.

The optimized methodology for oxidation and cofactor regeneration presented in this work could also be applied to the synthesis of different aldonic sugars, if XylB were replaced by other dehydrogenases with alternative substrate specificities and scopes.

■ EXPERIMENTAL SECTION

General Information. Xylose dehydrogenase from *C. crescentus* NA 1000 (XylB) was produced in our lab as previously described. Alcohol dehydrogenase from *C. kluyveri* was purchased from Sigma-Aldrich. All other chemicals were purchased from commercial sources as reagent grade. IR spectra were obtained in FT-IR Spectrum One. The frequency of absorption maxima ($\nu$) is expressed in cm$^{-1}$ and the samples were analyzed in a KBr tablet. UV spectra were acquired on Molecular Devices Spectramax PLUS 384. High-resolution mass spectra (HRMS) were recorded on an Agilent 6520 Accurate Mass Q-TOF spectrometer with an ESI source. $^1$H and $^{13}$C NMR spectra, using D$_2$O as the solvent, were recorded on a Varian System 500 spectrometer equipped with a 5 mm HCN cold probe with field z-gradient, operating at 500.13 and 125.76 MHz for $^1$H and $^{13}$C, respectively. The sample temperature was maintained constant at 298 K.

![1H NMR spectrum of the concentrated reaction mixture containing D-xylose (10 g, 220 mM), NAD$^+$ (0.33 mM), acetaldehyde (330 mM), CFE containing overexpressed XylB (6500 U), and ADH (3600 U) in NH$_4$HCO$_3$ buffer (10 mM, pH 8.0).](image-url)
Enzyme Activity Assays. XylB enzyme activity was monitored by spectrophotometric measurement of the NADH formed from cofactor reduction. Variations of absorbance at 340 nm are proportional to the D-xylose concentration consumed in the reaction ($\varepsilon_{NADH} = 6220 \text{ cm}^{-1} \text{ M}^{-1} \text{ cm}^{-1}$). Reaction mixtures of 1 mL contained NaH$_2$PO$_4$ (50 mM, pH 8.0), NAD$^+$ (0.5 μmol), D-xylose (5 μmol), and the enzyme sample (0.2–0.5 μg protein). Activity assays were performed at 25 °C. One unit (U) of XylB activity was defined as the amount that produces 1 μmol of D-xyloganolate per minute under the above conditions.

Tracking of the Reaction Progress. The amount of D-xylose consumed in the reaction was followed by the enzymatic quantification assay described above, with slight modifications. Aliquots of 20 μL from the reaction mixture were collected over time and quenched with 7% HClO$_4$ (1:5 v/v) and 10% NaOH (1:5 v/v). Detection of D-xylose was performed in 1 mL reactions containing NaH$_2$PO$_4$ buffer (50 mM, pH 8.0), NAD$^+$ (0.5 μmol, 0.5 mM), XylB (0.45 U, 15 μg of protein), and a quenched aliquot (between 1 and 2.5 μL). Variation of absorbance at 340 nm ($\Delta$Abs$_{340\text{nm}}$) before and after XylB addition was used to calculate the final D-xylose concentration (Figure S1).

Optimization of the D-Xylonate Synthesis. Small-scale reactions for optimization of the synthesis parameters were carried out in 3 mL of NH$_4$HCO$_3$ buffer (10 mM, pH 8.0) containing the enzymes XylB (6 U) and ADH (35 U) and different final concentrations and ratios of the reagents D-xylose, NAD$^+$, and CH$_3$CHO. Initially, the effects of four different D-xylose/CH$_3$CHO ratios (1:16, 1:5, 1:3, and 1:1.5) on the reaction efficiencies were assessed (Table 1). The effect of decreasing the cofactor concentration was examined in five reactions carried out at different NAD$^+$ concentrations (2.3, 1.1, 0.83, 0.33, and 0.16 mM) using a D-xylose/CH$_3$CHO ratio of 1:3 (0.33 and 0.99 mM, respectively) (Table 1). The optimization of the final D-xylose concentration was tested in five reactions conducted at increasing concentrations of this substrate (33, 66, 100, 130, and 250 mM) and 3 equiv of CH$_3$CHO, employing a NAD$^+$ concentration of 0.33 mM (Table 2). The biotransformation using CFE as the source of the XylB enzyme was examined by a repetition of two of the D-xylose optimization reactions (carried out with 33 and 66 mM), but employing CFE instead of pure XylB, to compare the obtained results (Table 2). In addition, two larger-scale reactions were carried out under similar conditions using both CFE containing XylB and pure enzyme (Figure S2). Finally, the optimal D-xylose/XylB ratio in reactions employing CFE as the catalyst was studied in four reactions with increasing amounts of D-xylose (33, 66, 100, and 250 mM) and XylB (6, 6, 13, and 27 U, respectively) (Table 2). A more detailed description of the procedures used in every optimization step can be found in the Supporting Information.

Scale up of the synthesis of D-Xylonate to 10 g. The optimized preparative reaction was carried out in 300 mL of NH$_4$HCO$_3$ buffer (10 mM, pH 8.0) containing 10 g of D-xylose (66.6 mmol, 220 mM), 1.5 equiv of CH$_3$CHO (100 mmol, 330 mM), 40 mL of CFE with overexpressed XylB (162.5 U/mL; total activity 6500 U), alcohol dehydrogenase (12 mg; 3600 U), and NAD$^+$ (100 μmol, 0.33 mM). The reaction was initiated by the addition of NAD$^+$. The pH of the reaction mixture was continuously adjusted to 8.0 by the addition of a concentrated solution of NaOH (8 M) and maintained until no progressive acidification was observed. The protein present in the reaction mixture was precipitated by the addition of ethanol (300 mL, 1 vol) and removed by centrifugation (9000 x g, 5 min at 4 °C). The supernatant was transferred to a 500 mL round-bottom flask and completely evaporated (rotary evaporator). To ensure that all of the volatile compounds were removed, successive additions and evaporations of Milli-Q water (four times, 30 mL each) were performed. The residue was suspended in 5 mL of Milli-Q water and filtered to eliminate residues of the precipitated protein (filter Whatman 0.2 μm, Milipore). The filtrate was freeze-dried to obtain D-xylonate as a hygroscopic white foam (16.150 g). The product obtained was dissolved in hot EtOH (50 mL), and later the solvent was removed under reduced pressure (three times). Additionally, the product was dried at 50 °C under reduced pressure over 5 h to obtain D-xylonate as a yellowish solid (11.710 g). The amount of D-xylonate obtained corresponds to a yield of 94%, considering that the sodium salt of the acid coordinated with 0.45 water molecules (see below the elemental analysis). $[\alpha]_{D}^{25} = +15.0^\circ$ (c 1.0, water). IR (KBr): 3420, 2936, 1606, 1415, 1126, 1089, 720 cm$^{-1}$. $^1$H NMR (300 MHz, D$_2$O): δ 4.01 (d, $J = 2.5$ Hz, 1H), 3.82 (dd, $J = 6.3, 2.5$ Hz, 1H), 3.75 (td, $J = 6.4, 3.7$ Hz, 1H), 3.69 (dd, $J = 11.9, 3.7$ Hz, 1H), 3.58 (dd, $J = 11.9, 6.5$ Hz, 1H). $^{13}$C NMR (100 MHz, D$_2$O): δ 178.49, 72.76, 72.74, 72.41, 62.43. HRMS (ESI$^+$) $m/z$ (calcld 165.0399): 165.0401 [M – H]$. Anal. calcld for C$_3$H$_5$O$_2$Na·0.45H$_2$O: C, 30.61; H, 5.09; found: C, 30.13; H, 4.79.

**ASSOCIATED CONTENT**

 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01090.

Optimization of D-xylonate synthesis; materials, experimental descriptions, and analytical methods (PDF)

**AUTHOR INFORMATION**

Corresponding Authors

*E-mail: isanchez@venterpharma.com (I.S.-M.).

*E-mail: eduardo.junceda@csic.es (E.G.-J.).

*E-mail: alfonso.mayoralas@csic.es (A.F.-M.).

ORCID

Eduardo García-Junceda: 0000-0002-2344-8743

Present Address

Centro de investigación Médica Aplicada (CIMA), Instituto de Investigación Sanitaria de Navarra, Universidad de Navarra, Avenida Pio XII 55, 31008 Pamplona, Spain (P.M.-C.).

**Author Contributions**

I.S.-M. and A.F.-M. conceived and designed the study. I.S.-M., R.B.-A., and P.M.-C. performed the experiments. I.S.-M., E.G.-J., and A.F.-M. supervised the work and analyzed and interpreted the results. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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REFERENCES

(1) Kwapiński, W.; Byrne, C. M. P.; Kryachko, E.; Wolfram, P.; Adley, C.; Leahy, J. J.; Novotny, E. H.; Hayes, M. H. B. Biochar from biomass and waste. Waste Biomass Valorization 2010, 1, 177–189.

(2) Chatterjee, C.; Pong, F.; Sen, A. Chemical conversion pathways for carbohydrates. Green Chem. 2015, 17, 40–71.

(3) Isikgor, F. H.; Becker, C. R. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym. Chem. 2015, 6, 4497–4559.

(4) Koutinas, A.; Du, C.; Wang, R. H.; Webb, C. In Introduction to Chemicals from Biomass; Clark, J. H., Deswarte, F., Eds.; John Wiley & Sons Inc.: Hoboken, 2008; Chapter 4.

(5) Sauer, M.; Porro, D.; Mattanovich, D.; Branduardi, P. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 2008, 26, 100–108.

(6) Hill, J.; Nelson, E.; Tilman, D.; Polasky, S.; Tiffany, D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl. Acad. Sci. U.S.A. 2006, 103, 11206–11210.

(7) Alonso, S.; Rendueles, M.; Díaz, M. Microbial production of specialty organic acids from renewable and waste materials. Crit. Rev. Biotechnol. 2015, 35, 497–513.

(8) Chun, B.-W.; Dair, B.; Macuch, P. J.; Wiebe, D.; Porteneuve, C.; Jeknavorian, A. The development of cement and concrete additive. Appl. Biochem. Biotechnol. 2006, 129–132, 645–658.

(9) Tornoda, Y.; Hanaoka, A.; Yasuda, T.; Takayama, T.; Hiwatashi, A. Method of Decreasing Acrylamide in Food Cooked under Heat. U.S. Patent US20040131737A1, 2004.

(10) Zamora, F.; Bueno, M.; Molina, I.; Iribarren, J. I.; Muñoz-Guerra, S.; Galbis, J. A. Stereoregular copolyamides derived from D-xylene and D-L-arabinose. Macromolecules 2000, 33, 2030–2038.

(11) Valdehuesa, K. N. G.; Liu, H.; Ramos, K. R. M.; Park, S. J.; Nisola, G. M.; Lee, W.-K.; Chung, W.-J. Direct bioconversion of D-xylene to 1,2,4-butanetriol in an engineered Escherichia coli. Process Biochem. 2014, 49, 25–32.

(12) Kim, W.; Molefe, M. N.; Frost, J. W. Microbial Synthesis of the Energetic Material Precursor 1,2,4-Butanetriol. J. Am. Chem. Soc. 2003, 125, 12998–12999.

(13) Ma, J.; Zhong, L.; Peng, X.; Sun, R. D-Xyonic acid: a solvent and an effective biocatalyst for a three-component reaction. Green Chem. 2016, 18, 1738–1750.

(14) Fuente-Hernández, A.; Corcos, P.-O.; Beauchet, R.; Lavoie, J.-M. In Liquid, Gaseous and Solid Biofuels - Conversion Techniques; Fang, Z., Ed.; InTech: Rijeka, 2013; Chapter 1.

(15) Toivari, M. H.; Nygård, Y.; Penttilä, M.; Ruohonen, L.; Wiebe, M. G. Microbial d-xylonate production. Appl. Microbiol. Biotechnol. 2012, 96, 1–8.

(16) Nygård, Y.; Toivari, M. H.; Penttilä, M.; Ruohonen, L.; Wiebe, M. G. Bioconversion of d-xylene to d-xylonate with Kluyveromyces lactis. Metab. Eng. 2011, 13, 383–391.

(17) Toivari, M. H.; Ruohonen, L.; Richard, P.; Penttilä, M.; Wiebe, M. G. Saccharomyces cerevisiae engineered to produce D-xylonate. Appl. Microbiol. Biotechnol. 2010, 88, 751–760.

(18) Zhang, Y.; Guo, S.; Wang, Y.; Liang, X.; Xu, P.; Gao, C.; Ma, C. Production of α-Xylonate from Corn Cob hydrolysate by a metabolically engineered Escherichia coli strain. ACS Sustainable Chem. Eng. 2019, 7, 2160–2168.

(19) Toivari, M. H.; Vehkomäki, M.-L.; Nygård, Y.; Penttilä, M.; Ruohonen, L.; Wiebe, M. G. Low pH d-xylonate production with Pichia kudriavzevii. Bioreour. Technol. 2013, 133, 555–562.

(20) Toivari, M.; Nygård, Y.; Kumpula, E. P.; Vehkomäki, M.-L.; Bencina, M.; Valkonen, M.; Maaheimo, H.; Andberg, M.; Koivula, A.; Ruohonen, L.; Penttilä, M.; Wiebe, M. G. Metabolic engineering of Saccharomyces cerevisiae for bioconversion of d-xylene to D-xylonate. Metab. Eng. 2012, 14, 427–436.

(21) Liu, H.; Valdehuesa, K. N.; Nisola, G. M.; Ramos, K. R. M.; Chung, W.-J. High yield production of d-xyloic acid from d-xylene using engineered Escherichia coli. Bioreour. Technol. 2012, 115, 244–248.