Harvest 2.0, Final Report
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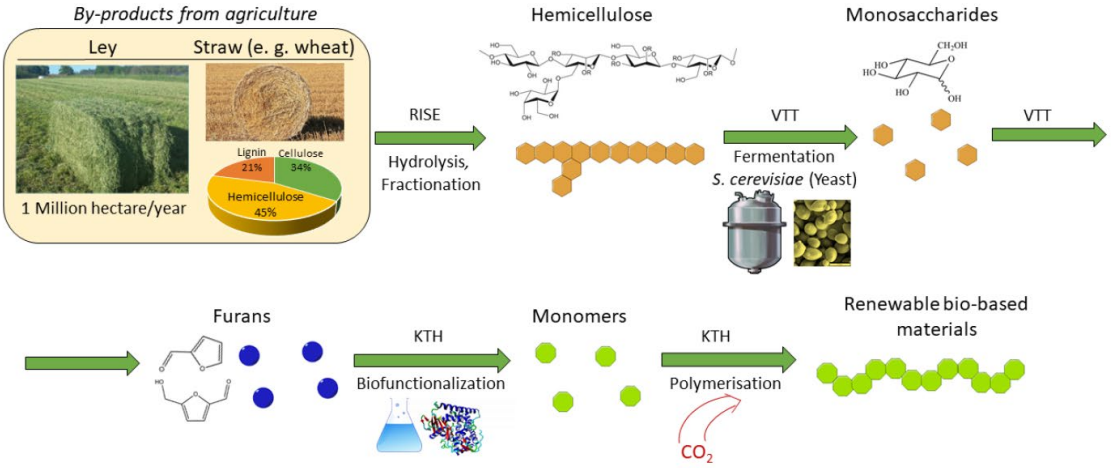
Summary
The main goal of this interdisciplinary project was to generate renewable advanced materials from biomass through innovative green technologies.



The specific goals of **Harvest 2.0** - and how they were addressed throughout the project- are given below:

- *Evaluate technologies for fractionation of hemicellulose from straw and ley* (**RISE**, year 1-2)

  This was successfully delivered as disseminated in detail in the report RISE Bioeconomy Report No: 110. Due to the structural complexity of hemicelluloses from ley, the project focused initially on wheat straw as raw material, with a more uniform sugar composition.

- *Design of cellfactories for generation of monosaccharides from hemicellulose by biotechnologies* (**VTT**, year 1-2)

  VTT delivered an engineered yeast strain that was shown to efficiently depolymerize commercial sugar substrate into xylose. Depolymerization of fractionated hemicellulose from ley, generated in the project, was demonstrated in lab scale.

- *Delivery of at least two furan-based building blocks from straw and ley* (**VTT**, year 1-3)

  The project undertook the strategy to demonstrate that furoic acid is a versatile platform chemical for material synthesis. This was emphasized in the following published journal article:

  W. Farhat, A. Biundo, A. Stamm, E. Malmström, **P.-O. Syrén***. *Lactone monomers obtained by enzyme catalysis and their use in reversible thermoresponsive networks*. *J. Appl. Polym. Sci.* **2020,** *137*, 48949.

  The work was featured on the journal cover.

  One furan-based building block was delivered, namely the butyl ester of furoic acid in preparative scale (100 g) according to the project plan. The synthesis route started from commercial sugar substrate which was upgraded to xylonic acid. Process challenges *en route* to furan product, e.g. reduced solubility of furoic acid under reaction conditions, precluded direct biosynthesis of furoic acid from fractionated hemicellulose.

Delivery of biocatalytic systems for green functionalization of furans to tailor-made monomers in laboratory scale (**KTH**, year 1-2)

We have successfully delivered a biocatalytic system for valorization of furoic acid into 2,5-furandicarboxylic acid (FDCA) by enzymatic CO_2 sequestration in water under mild conditions. This green chemical step provided a key direct link between C5 furans and functionalized C6 monomer units amenable for polymer synthesis.

- *Generation of at least two bio-based polymers among which at least one contains CO_2 in its backbone* (**KTH**, year 1-3)

  The project resulted in three novel biomaterials originating from hemicellulose, all of which had CO_2 incorporated in their backbone according to the project plan. This part of the project has resulted in two manuscripts, the first of which will be submitted shortly and the other after the summer.

Fractionation and purification of hemicellulose (Leader: RISE)

Two technologies have been initially investigated for extraction and fractionation of hemicelluloses (mainly xylan), obtained from dried straw delivered to the project from Lantmännen. The first one was alkaline extraction as a method for high recovery yield and the second one is acidolysis followed by alkaline extraction for simultaneous xylan fractionation into branched (i.e. with a high arabinose/xylose ratio) and unbranched structures (i.e. without arabinose).
Technology investigation continued in a parallel track and connection to a practical ethanol process. In that procedure, xylan extraction was set up as a pretreatment before the process setup to convert cellulose into ethanol. It was conducted by cooking wheat straw (WS) with circulating water at an elevated temperature. It has been shown that a practical autohydrolysis at 170°C for 65 min of 10-15 cm wheat straw in kg-scale resulted in a removal of ca. 60% of available xylan. According to our data, yield in our process setup was up to 20% (Table 1).

Table 1. Summary of fractionated xylan samples from Wheat straw.

Xylan sample	Yield	Analytical data
Solution xylan	6.7L/kg	Table 3
Solid Xylan IV	18.8%	Table 3
Solid Xylan V	3.85%	Table 4

The starting wheat straw was hydrolyzed by acid which degraded all carbohydrates into monomer sugars for ion chromatography (IC) quantification. The insoluble part was weighed as the quantity of lignin (Klason lignin). The total carbohydrate content is 67.9% and the relative xylose content among all sugars is 37.1%. This reveals a native xylan quantity of ca. 25% based on the starting mass. It should be noted that this xylan quantity is calculated as a pure xylo polysaccharide backbone structure, excluding any branches of either arabinose or glucuronic acids, neither the acetyl groups that are also present in the native xylan structure. If these structures will be included, the native xylan content is higher. As reported in the literature, the xylan content in wheat straw is up to 33%. Obviously, the valorization of WS can be largely realized through value-added utilizations of the xylan. The data also shows that if all xylans will be extracted out as one product the arabinose/xylose (ara/xyl) ratio will be 0.10.

Table 2. Structures and compositions of starting wheat straw, different xylan samples obtained, and extracted residues after acidolysis and alkaline extractions

Extraction	Sample	Molecular size	Construction (%)		Carbohydrate composition* (relative %)					Branching degree*
		Mp (Da)	Lignin (Klason lignin)	Carbo-hydrate	ara	gal	glu	xyl	man	Ara/Xyl ratio
-	Starting WS	-	21.2	67.9	3.8	0.80	57.9	37.1	0.38	0.10
Acidolysis	Xylan I	Not-seen**	69.4	9.5	20.8	0	0	79.2	0	0.26
	Xylan II	~3100	20.5	51.0	0	0	4.45	95.5	0	0.00
	Residue I	-	2.1	86.2	0.1	0	79.1	20.8	0	0.005
Alkaline plus H_2O_2	Xylan III	~2200	5.1	62.8	14.5	2.48	4.26	78.7	0	0.18
	Residue II	-	3.5	72.7	2.8	0.14	66.7	30.4	0	0.09

* ara: arabinose, gal: galactose, glu: glucose, xyl: xylose, man: mannose

** not-seen: a very broad range of peaks were seen but no peak could be identified as representing molecular structure for the xylan-lignin complex structures

Autohydrolysis is a more practical extraction method than the acidolysis or alkaline extraction mentioned above. In this setup, also explored in the project, Willey milling was avoided aiming at energy saving and preserving the structure of the extracted residue for further ethanol or pulp production. One of the best autohydrolysis extractions reported was using 190°C with a so called severity factor of 3.81 (Sipponen et al. 2014). In our experiment, however, 170°C is the highest temperature limit of our cooking facility. Therefore, 170°C for 65 min was applied as the cooking conditions, with a severity factor of 3.86 that is very close to this optimal severity factor of 3.81.

Table 3. Structures and compositions of Solution xylan and Xylan IV obtained after autohydrolysis extraction at 170°C for 65 min.

Sample	Liquid property		Solid Construction (%)		Free monomer sugar in Solution Xylan (mg/L) or Carbohydrate composition in Xylan IV (relative %)					Branching degree	Toxic component	
	pH	Solid content	Lignin (Klason lignin)	Carbo-hydrate	ara	gal	glu	xyl	man	Ara/Xyl ratio	furfural	HAc
Solution Xylan	3.9	28g/L	-	-	839	154	126	1103	0	-	2.6g/L	7.0g/L
Xylan IV	-	-	13.4	56.8	6.9	3.4	9.1	78.7	2.0	0.087	~0	12% (W/W)

Furfural and HAc are known toxins towards microorganisms (Felipe 2004). Since both structures are expectedly being the exclusive volatiles in the solution, the Solution xylan sample was detoxified by freeze-drying. As a result, the obtained Xylan IV contains all other components from the solution without any furfural. The content of HAc is largely reduced to 12% (W/W) from the starting value of ca. 25% (weight over the dry mass) in the solution.

In order to obtain pure polymeric xylan, on the other hand, the solution sample was treated by addition of EtOH to reach 60% EtOH concentration. This precipitated only the xylo oligosaccharides (XOS) and a pure xylan sample termed Xylan V was obtained. By analysis, it contains 4.6% lignin and 83.7% carbohydrates with a comparative xylose percentage of 73.4% (Table 4). Its *M*p is ca. 2000 Da. The branch degree of ara/xyl ratio is 0.023. As revealed by HPLC, it is indeed a pure XOS eluting as a single fraction at 37.5 min. The yield of this purified xylan is 3.85% based on the starting WS.

Table 4. Structures and compositions of Xylan V and Xylan VI obtained and their corresponding residues (Residues III and IV) after autohydrolysis extraction at 170°C for 65 min and for 80 min respectively

Extraction	Sample	Molecular size	Construction (%)		Carbohydrate composition (relative %)					Branching degree
		Mp	Lignin (Klason lignin)	Carbo-hydrate	ara	gal	glu	xyl	man	Ara/Xyl ratio
Autohydrolysis (170°C, 65 min)	Xylan V	2000	4.6	83.7	1.7	3.5	19.3	73.4	2.1	0.023
	Residue III	-	30.1	60.5	0.5	0.3	78.6	19.7	0.8	0.027
Autohydrolysis (170°C, 80 min)	Xylan VI	1300	2.5	89.1	1.0	2.9	22.8	71.0	2.4	0.014
	Residue IV	-	30.7	64.5	0.4	0.3	80.3	17.8	1.3	0.020

The autohydrolysis could expectedly be improved by starting with smaller size WS, using higher temperature and shorter cooking time, e.g. at 190°C for 5 min like in Carvalheiro et al. 2004. More practically, the detoxification step could be replaced by less expensive ordinary thermal drying. The purification step could be conducted by ultrafiltration. Since ca. 40% of all available xylan still remained in the residue, the xylan extraction could include a further extraction from the residue. Alternatively, this residual xylan could be processed together with the cellulose to e.g. chemical pulp for an additional value for improved pulp strength.

Generation of furans (Leader: VTT)

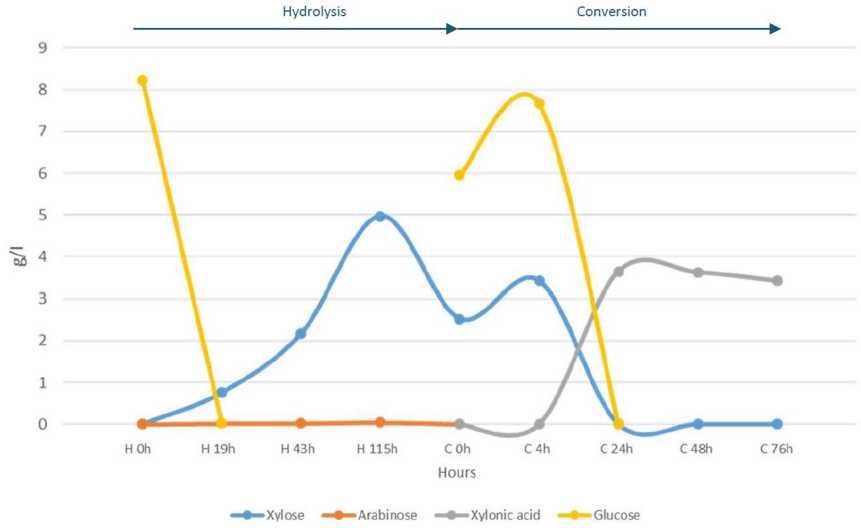
S. cerevisiae was engineered to secrete endoxylanase, β-xylosidase and arabinofuranosidase individually and in different combinations, to hydrolyse xylan to xylose and arabinose. Ca 10% of the theoretical xylose and arabinose in the Megazyme (reference sugar) substrate was released when all three activities were present in one microorganism. In comparison, ca 5% of the theoretical xylose was released from the RISE substrate (fractionated hemicellulose from wheat straw), representing a two-fold reduction in xylan-degrading activity. The enzymes retain their activity at least for 170 hours from the start of the hydrolysis.

As a proof of principle, generation of xylonic acid in laboratory scale was demonstrated by two yeast strains acting in tandem during hydrolysis, to generate xylose, and subsequent biosynthesis of xylonic acid. As substrate, xylan originating from wheat straw hemicellulose was used.

Hydrolysis phase in 50ml:

The cultivation medium was SC-glucose supplemented with WSI xylan. The theoretical amount of xylose in the medium is 25g/l and arabinose 0.5g/l. *Saccharomyces* strains were inoculated into OD (optical density) of 1. 20% of the theoretical amount of xylose in WSI xylan was released in 115h.

Conversion phase 40ml:
Saccharomyces cells were removed and 35ml of the cultivation medium was supplemented with amino acids and glucose, pH was adjusted to 5. *Pichia kudriavzevii* was inoculated into OD of 1. *P. kudriavzevii* converts all of the xylose in the medium into xylonic acid in 24 h (as shown in Figure 1).



The *Saccharomyces* strains:
Harvest 56: gre3Δ::*T.reesei xyn2* loxP,fur1Δ::KanMX, plasmid B10647 (*URA3*)
Harvest 61: gre3Δ::*T.reesei xyn2* loxP,fur1Δ::KanMX, plasmid B10652 (*T.reesei bxl1,A.pullulans abfB,URA3*)
Harvest 64: gre3Δ::*T.reesei xyn2* loxP,fur1Δ::KanMX, plasmid B10536 (*T.reesei bxl1,URA3*)

Pichia kudriavzevii (VTT-C-12903): pdc1Δ::*C.c* xylB- loxP *S.c* MEL5 loxP/PDC1

Figure 1. Engineered yeast consortia converts WS xylan to xylonic acid, a key intermediate to manufacture butyl ester of furoic acid.

Due to limited availability of RISE xylan, pure xylose was used to produce xylonic acid in a fermentation process based on an engineered *Pichia kudriavzevii* strain. A fed-batch bioreactor cultivation (20 L) was carried out in a medium containing xylose and a small amount of glucose. At the end cells were removed and the final 18 litre sample contained 15% xylonic acid and 2% xylitol. The culture broth was concentrated by freeze-drying. The dried broth was solid below 0°C and viscous liquid in RT. To synthesize butyl ester of furoic acid, an established process at VTT was used (WO2019043300) to generate product at 100 g scale from xylonic acid ester.

The project also attempted direct synthesis of furoic acid from xylonic acid generated. For this purpose, hydrodeoxygenation experiments (five parallel) were done in Parr parallel pressure reactor in 6 bar pressure: the xylonic acid + solid acid catalyst (three different were tested) + butanol as a solvent. The reaction yields were low (about 10%). No furoic acid were seen with GC-MS chromatography. The solid fractions from the experiments were extracted with ethanol and analyzed by using NMR spectroscopy. The spectra showed clearly that furoic acid was formed in the reaction, albeit at low yields. Si-Tosic acid was the most effective solid catalysts in these reaction conditions. The original process (WO2019043300) was developed for xylonic acid butyl ester, which is soluble in higher alcohols. Now the starting material and the product were not soluble in the reaction solvent. If xylonic acid is used for the reactions instead of xylonic acid ester, the process needs to be optimized for hydrophilic compounds to improve the yield. For that reason, the project focused on production of the butyl ester of furoic acid.

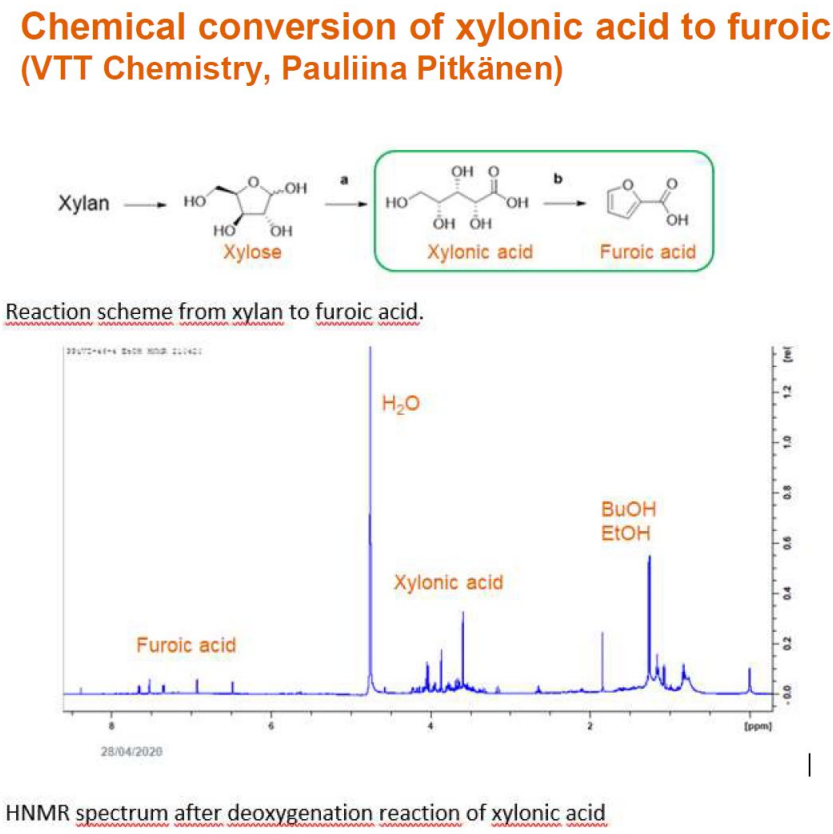


Figure 2. Generation of furoic acid directly from xylonic acid is possible as shown here by ^{1}H-NMR, but results in low yields.

Biocatalysis and material generation (Leader: KTH)

Throughout the project, we explored the synthesis of novel polyesters from furoic acid delivered from VTT (formally the butyl ester). To activate the substrate for material synthesis, we demonstrated efficient upcycling of butyl ester of furoic acid into the acid form, and then further into FDCA by CO_2 assimilation via carboxylation, utilizing both enzymatic and chemical routes. The first step involved acid hydrolysis of the butyl ester furan derived from hemicellulose to generate furoic acid. For chemical carboxylation of the former, FDCA was synthesized from 2-furoic acid using LDA as a catalyst in presence of CO_2, briefly as follow; In a pre-cold (-78 °C) round bottomed flask, LDA catalyst was dissolved in THF. Under Ar-atmosphere, a solution of 2-furoic acid in THF was added dropwise into the flask over a period of 20 min and followed by stirring for 1h. The mixture was, subsequently, bubbled with CO_2 for 30 min at -78 °C and next 30 min at 0 °C. The resulting slurry was diluted with aqueous ammonium chloride and the aqueous phase was separated and acidified to pH 1. The FDCA was then precipitated at 80 °C for 2 h, filtered and oven dried. The conversion was 100% as shown by NMR analysis.

For enzymatic CO_2 fixation we identified the carboxylase PtHmfF from *P. thermopropionicum* as a potent biocatalyst. The 2-furoic acid was prepared in bicarbonate buffer (pH 6.8). One mL of the substrate solution was then transferred into a 2mL glass tube. The carboxylation reaction was started by adding 500 µg of the PtHmfF enzyme to the substrate solution and incubated at 30°C, 40°C and 50°C overnight. The reaction tubes were covered in thin foil to avoid any direct light contact to the enzyme, leading to inactivation. Following the overnight incubation 50 µl of the reaction mixture was

removed and was directly mixed with 50 µl of methanol to stop the reaction. Analysis of reaction conditions investigated was performed by HPLC.

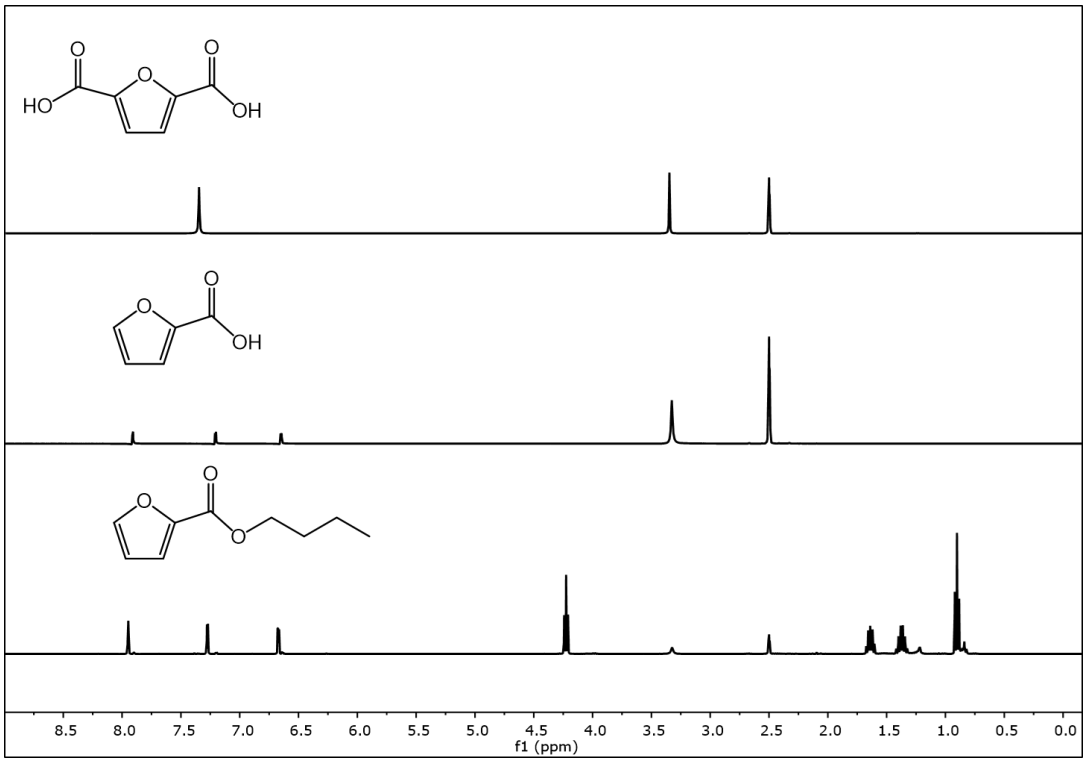


Figure 3. Hydrolysis of butyl ester of furoic acid followed by its chemical carboxylation into FDCA. NMR spectra of substrates and products are shown.

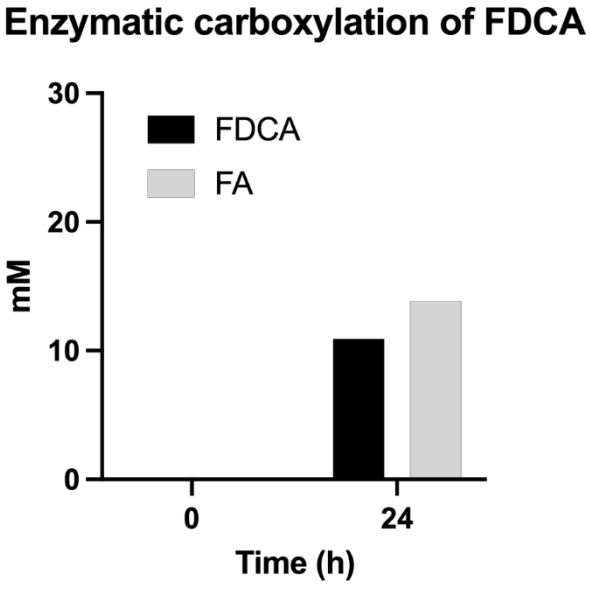


Figure 4. Enzymatic carboxylation of furoic acid (FA) by the carboxylase enzyme from *Pelotomaculum thermopropionicum* (PtHmfF) at 50°C.

For polyester generation from furans, different materials were targeted as shown in figure 5 below. FDCA, generated as described above, was copolymerized with other biomass-derived synthons, namely the terpene-based diol, 1-(1'-hydroxyethyl)-3-(2''-hydroxyethyl)-2,2-dimethylcyclobutane (HHDC) made by us, using $Ti(BuO)_4$ as a catalyst at different monomers feed ratios. The composition of the formed polyester revealed the successful formation of poly ((2,5-furandicarboxylate)-*co*-(1-(1'-hydroxyethyl)-3-(2''-hydroxyethyl)-2,2-dimethyl cyclobutane) P(F-HHDC) with molecular weights in the range of 5000-8000 g mol^{-1}. We also investigated another green monomer, dimethyl carbonate (DMC), as a co-monomer with FDCA to produce polyester in the presence of 1,10-decandiol (DD). Materials obtained were analyzed by proton nuclear magnetic resonance (^{1}H-NMR), size exclusion chromatography (SEC) and differential scanning calorimetry (DSC). The molecular weight of the produced poly ((2,5-furandicarboxylate)-*co*-dimethyl carbonate-(1,10-decandiol)) P(F-DMC-DD) was found to be 3400 g mol^{-1} and with a polydispersity (Đ) of 1.1. The thermal properties revealed that the T_g of P(F-HHDC) and P(F-DMC-DD) were 50 °C and -60 °C, respectively.

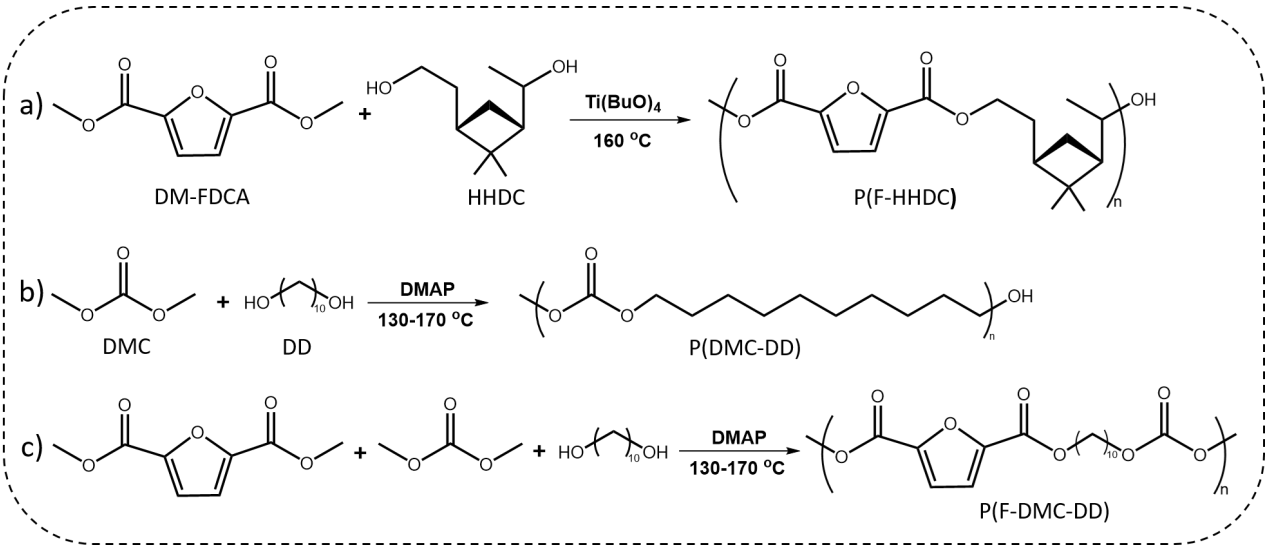


Figure 5. *Synthesis of bio-based polymers from green monomers, DM-FDCA and DMC.*

Table 5. *SEC measurements of P(F-HHDC) and P(F-HHDC-EG)*

Sample	M_n (g mol^{-1})	M_w (g mol^{-1})	Đ	Conversion%
P(F-HHDC)$_1$	8400	11500	1.35	86
P(F-HHDC)$_2$	4000	5800	1.47	70
P(F-HHDC)$_3$	4900	5700	1.15	72
P(F-HHDC)$_4$	5200	6000	1.15	
P(F-HHDC-EG)	1700	3400	2	

Table 6. *SEC measurements of P(DMC-DD) and P(F-DMC-DD)*

Sample	M_n (g mol^{-1})	M_w (g mol^{-1})	Đ	Conversion%
P(DMC-DD)	3200	3500	1.1	87
P(F-DMC-DD)	3400	3600	1.1	81

Finally, in the material platform at KTH in the project we also investigated generated furoic acid from hemicellulose as a reversible cross-linking agent in the creating of advanced functional materials.

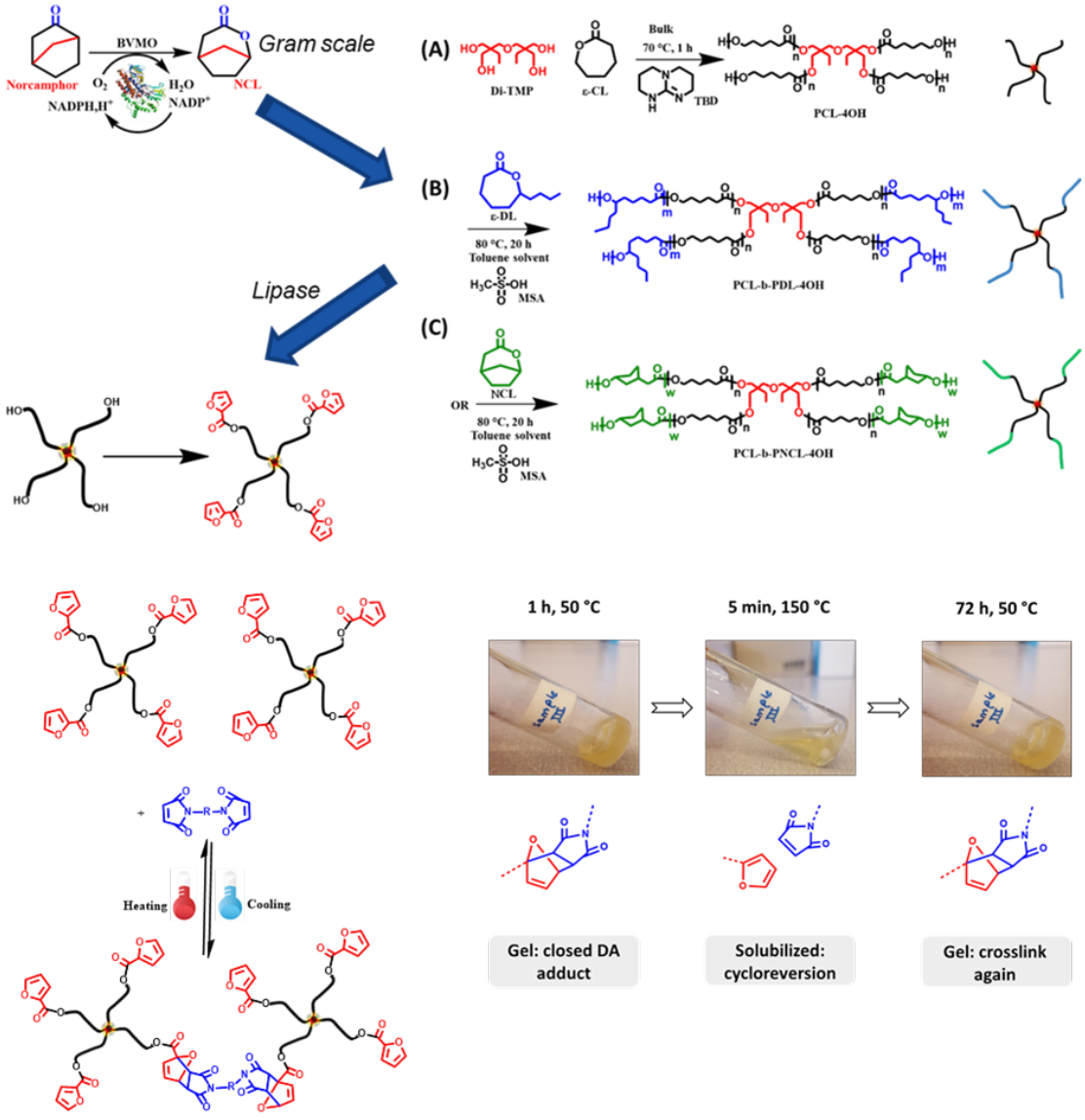


Figure 6. Synthesis of advanced functional biomaterials using generated furoic acid as versatile cross linker by reversible Diels Alder chemistry. Adapted from W. Farhat, A. Biundo, A. Stamm, E. Malmström, P.-O. Syrén. Lactone monomers obtained by enzyme catalysis and their use in reversible thermoresponsive networks. *J. Appl. Polym. Sci.* **2020,** *137*, 48949.

Project conclusions

Hydroxymethylfurfural (HMF) has been used as a key molecule for the production of a wide variety of valuable chemicals, such as 2,5-furandicarboxylic acid (FDCA). FDCA has gained great industrial interest and was found to be a promising bio-based alternative to replace oil derived terephthalic acid (TPA), an important plastic precursor. Until now, studies on polysaccharide utilization have mainly focused on C6-sugars from cellulose, from which HMF can be retrieved. Less attention has been paid to C5-sugars from hemicellulose which represent a large amount of biomass. This project has established furoic acid from hemicelluloses as a versatile green building block. According to our strategy, furoic acid is further functionalized into monomers by CO_2 assimilation, resulting in a carbon sink. Starting from furoic acid, we generated three novel biopolymers. We showed how both one and two molecules of CO_2 could be incorporated in the backbone of materials produced. The underlaying upcycling of furoic acid from hemicellulose to diacid monomer was performed both biotechnologically (50% yield) and chemically (in 100% yield).

Wheat straw is an excellent source for xylan and is very valuable for further valorization of the xylan to furan-based polymers. Its xylan component consists of two basic types of structures, one with arabinose branches and another without. It links chemically with lignin and is present in wheat straw fibers at different morphological locations, leading to different accessibilities towards different extraction methods. Autohydrolysis is a chemical-free and environmentally-friendly extraction method. Without any energy-intensive fibre milling, the overall xylan yield is substantial (ca 18%) by the autohydrolysis extraction. The obtained solution xylan sample in the project contains a mixture of different xylo oligosaccharides, xylobiose and free xylose together with other monomeric sugars. This solution can be detoxified to remove furfural and acetic acid. It can also be purified to obtain pure xylo oligosaccharide. Since all the xylan derived structures in the solution could eventually be converted, it could be proposed to directly use the solution for an integrated conversion to the furan intermediate needed towards the final furan-based polymers. The residual xylan in the extracted residue could either be post-extracted out and combined into the main furan route for application or be kept for further pulp production offering improved pulp strength.

Yeast is a versatile cell factory in enabling depolymerization of fractionated hemicellulose. Herein we used engineered *Saccharomyces* strains in concert with *Pichia kudriavzevii* harboring additional enzymes to make xylonic acid from xylan originating from wheat straw. We showed how the microbial consortia efficiently degrades xylan substrates. In lab scale (grams), we demonstrated yields of depolymerization of ca 5% starting from hemicellulose from ley, which was two-fold lower than that obtained with commercial reference sugar substrate. Due to the limited availability of substrate, commercial xylose was also converted to xylonic acid, and then finally to butyl ester of furoic acid at 100 gram scale. The generated substrate was used by KTH to manufacture three novel biomaterials in excellent yields (70-90%) and molecular weights up to 11500 g/mol. By choosing polymer composition and amount of CO_2 incorporated, the properties of the materials could be controlled. Overall this project has cemented the importance of agricultural byproducts in generation of furoic acid as a versatile green platform chemical.