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Glucose/Furfural Substrate Mixtures in Non-Engineered Yeast: Potential for Massive Rerouting of Fermentation to C–C Bond Formation on Furfural

Francesca Sannelli[+],[a] Sanni Gao[+],[a] Pernille Rose Jensen,[b] and Sebastian Meier*[a]

Suitable mixtures of glucose and furfural may provide novel strategies for C–C bond formation on furfural due to the versatility of low-cost biological catalysts. We use in-cell NMR with non-engineered commercial yeast as the catalyst to determine the interplay between furfural and glucose metabolism in non-engineered yeast. The presence of furfural is shown to modulate kinetic barriers in glucose conversion and to favor the accumulation of acetaldehyde in situ. As a result, glucose carbons are remarkably strongly redirected towards C–C bond formation between furfural and a glucose-derived C2 unit. In the presence of suitable glucose/furfural substrate mixtures in non-engineered yeasts, glucose carbons can achieve relative influxes of at least 80% into the C–C bond formation on furfural, compared to only 20% into ethanol. Chain-elongation of furfural by yeast thus seems a viable strategy for the upgrading of lignocellulosic biomass through concurrent conversion of furfural and glucose. The product is related to chemicals that already have found value in the fine chemical and pharmaceutical industries.

Introduction

In the transition to a sustainable production of carbonaceous chemicals, which are currently almost exclusively sourced from fossil resources,[1] biological and chemical catalysis are both poised to play important roles.[2,3] Enzymes and enzyme systems are increasingly routinely detected, catalogued, evolved,[4] and designed[5,6] de novo. Biocatalysis is most often employed for its substrate- and stereo-selectivity.[7–9] Recently, it has become clear that enzymes often have non-natural functionality that expands the substrate scope of enzyme-catalyzed conversions.[10–13] Arguably, the potential of biocatalysis has thus only been sparsely tapped, as non-evolved functions acting on non-natural substrates under non-natural conditions provide ample room for surprises.[14,15] Beyond the catalytic prowess of enzymes, biocatalysis is attractive due to its favourable metrics in terms of innocuous solvents, typical use of renewable bio-sourced reactants and low-temperature (low-energy) operation.

In addition to the use of purified and immobilized enzyme preparations, biocatalysis can be pursued with intact cells.[3,16–18] In the case of yeast, the cells may be available commercially at near-negligible costs. The yeast *Saccharomyces cerevisiae* is among the best-established agents for the conversion of abundant, bio-sourced sugars, and its use has been tightly knit to the advancement of society. Beyond its use in the food industry, *S. cerevisiae* has emerged as one of the most useful catalysts for biological hexose upgrading to fuels and materials.[19–21] Thus, *S. cerevisiae* can be used to produce fuel ethanol fermentatively after chemocatalytic hydrolysis of lignocellulosic biomass. By-products in this chemocatalytic hydrolysis of lignocellulosic biomass are furan chemicals such as the C5 compound furfural[22,23] and the C6 compound hydroxymethylfurfural,[24] which are under investigation as promising precursors for fuels and polymer building blocks. The formation of furan chemicals in the heat- and acid treatment of pentose- and hexose containing polysaccharides has been considered inevitable.[25]

Furan chemicals contribute to the inhibition of fermentative biofuel formation and *S. cerevisiae* itself is known to convert furfural (**1**) to furfurylalcohol (**2**), furoic acid (**3**) and dimeric species such as furil and furoin (Scheme 1).[20,25–28] In addition, an acyloin (α- hydroxyketone) adduct between pyruvate and furfural (**4** in Scheme 1) was tentatively inferred with mass spectrometry, despite the presence of a species lacking a mass of 44 amu (CO_2) from this tentative adduct.[20,29] Although furfural is known to detrimentally affect cell viability, growth rate and fermentation rate in yeast at concentrations above approximately 0.1% (v/v),[29,30] the mechanism of interference between furfural metabolism and glucose metabolism has neither been directly observed, nor has this interference

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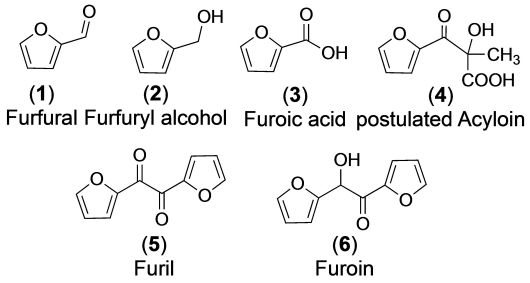


Scheme 1. Furfural and its previously described metabolites.

inspired exploitation for biosynthesis of furfural derivatives through concurrent conversion of glucose and furfural using commercial yeast.

Here, we report an in-cell study investigating the interplay between furfural and glucose conversion in non-engineered commercial yeast. Using in-cell NMR spectroscopy, we find that furfural inhibits glycolysis to a degree that can lead to an approximately 15-fold accumulation of intermediates in upper glycolysis. At the same time, furfural efficiently intercepts carbon flux from glucose. Under suitable conditions, furfural can redirect more than 80% of the central metabolite pyruvate away from alcoholic fermentation and towards the formation of a C7 adduct with furfural. The study shows that hybrid approaches using chemo-catalytically produced furfural, in situ produced fermentation products (here pyruvate/acetaldehyde), and whole-cell catalysis can provide an atom-efficient and facile strategy towards bio-sourced chemicals. C–C bond formation is achieved in this process at ambient temperatures in water using a suitable substrate mixture composed of glucose and furfural.

Results and Discussion

Kinetic changes and emergence of a non-natural ketone-containing compound in the presence of furfural

Furfural chemistry in the living cell may be expected to be largely governed by the presence of a reactive aldehyde group attached to the heteroaromatic furan ring. Reduction or oxidation of the aldehyde group interferes with the cellular pool of oxidants or reductants. In addition, it had previously been suggested that furfural produces covalent adducts through reactions that are catalyzed by the purified pyruvate decarboxylase enzyme.[31–33] Hence, we tried to reconcile these findings and investigate both the mechanistic effects of furfural on yeast glycolysis and the ensuing effects on glucose conversion. NMR spectroscopy was employed due to its prowess in detecting and quantifying new chemicals in the transition to sustainable chemistries.[34–36] We hypothesized that adduct formation between furfural and metabolites of glucose degradation should be directly detectable with in-cell observations by NMR spectroscopy.

Mechanistic effects of furfural on glucose metabolism were initially explored using dissolution DNP (D-DNP) NMR spectroscopy. This method is a hyperpolarization technique that temporarily redistributes nuclear magnetism and enhances the NMR detectable spin polarization by approximately four orders of magnitude.[37] Through this enhancement, a tracer can be derived to trace the influx of suitably isotope labelled [U-^{2}H,^{13}C] glucose into glycolytic intermediates on the seconds time scale through NMR detection.[38–40] In this manner, kinetic bottlenecks and their responses to effectors can be observed in central metabolism.

Figure 1 shows that furfural significantly alters the kinetic barriers for the influx of glucose into various pathways. In the absence of added furfural, pyruvate is a major bottleneck, while triose phosphates (especially dihydroxyacetonephosphate; DHAP) and the oxidation product of triose phosphates (3-phosphoglycerate) are major bottlenecks upstream of pyruvate (Scheme 1). In the presence of 0.5% (v/v) added furfural, the conversion of 3-phosphoglycerate rather than DHAP formed a main kinetic barrier. This change is consistent with an increased oxidation of triose phosphates in the presence of furfural, which replenishes cellular oxidant (NAD^{+}) through its preferred reduction to furfuryl alcohol in the absence of aeration.[29] Importantly, reactive acetaldehyde rather than pyruvate accumulated in lower glycolysis. In addition, a new ketone with a ^{13}C NMR signal near 212 ppm emerges after the accumulation of acetaldehyde. The ^{13}C NMR signal near 212 ppm does not

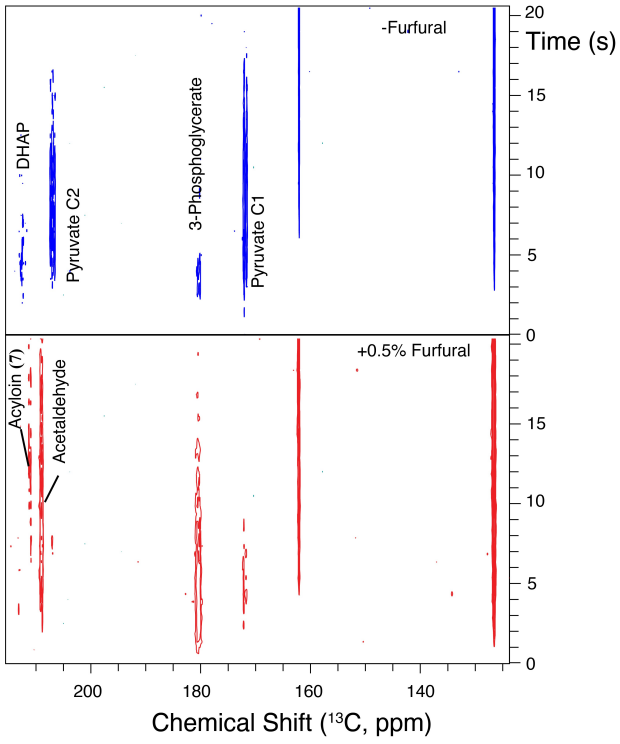
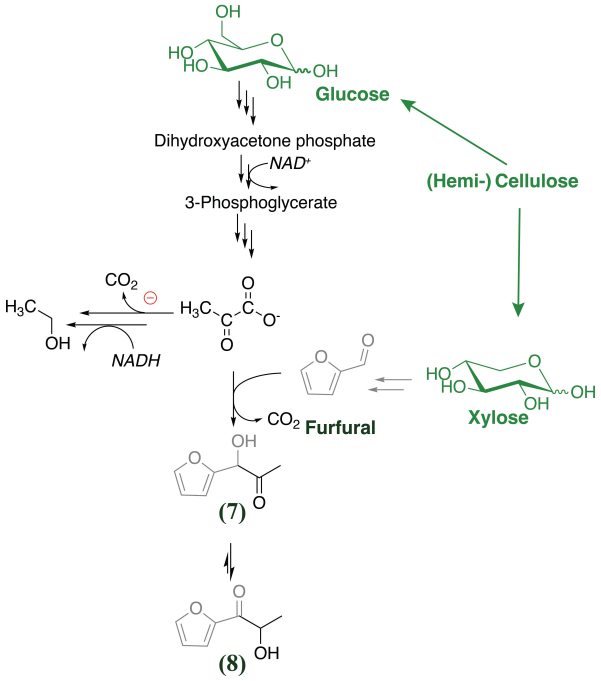


Figure 1. Dissolution DNP NMR with hyperpolarized [U-^{13}C, U-^{2}H] glucose to compare kinetic barriers in yeast glycolysis in the absence and in the presence of added furfural. A non-natural ketone was observed in the presence of furfural. Reaction conditions: 31 mg dry yeast, 530 µl phosphate buffer (90 mM phosphate, 10 mM KCl, 1 mM $MgSO_4$, pH 6), without (top) or with 0.5% (v/v) added furfural (bottom), 6.9 mM hyperpolarized [U-^{13}C, U-^{2}H] glucose, 303 K.

coincide with expected signals for any natural metabolite of central glucose metabolism. Hence the new species was hypothesized to derive from an adduct with furfural (Scheme 2). As D-DNP operates on the seconds timescale, the influx of glucose carbon into this species can be deemed very rapid.

Formation of a C7 acyloin from glucose/furfural in non-engineered yeast

Subsequently, reactions were tracked by conventional NMR spectroscopy in the presence of glucose/furfural substrate mixtures of different compositions. Time series following the conversion of 90 mM uniformly ^{13}C enriched glucose by commercial *S. cerevisiae* at pH 6 were acquired for 150 minutes with a time resolution of 0.59 min. The direct observation showed various effects of furfural on fermentation. In the absence of added furfural, ethanol and glycerol were the principal products formed from glucose, as expected. Evidently, a new product emerged with increasing furfural content in the substrate mixture (Figure 2). The by-product exhibited ^{13}C NMR signals near 28.1 and 211.8 ppm, consistent with the observation of the signal near 212 ppm by D-DNP NMR. The identical time profile (correlated intensities) of these signals and their correlation with the furfural concentration strongly suggested that both carbon atoms derive from the same species. Rather notably, no carboxylic acid signal emerged in parallel to the signals at 28.1 and 211.8 ppm, which are characteristic for methyl and ketone groups, respectively. Thus, furfural appears to primarily accumulate a C7 rather than a C8 adduct in the fermenting *S. cerevisiae* cells. The primary product is then



Scheme 2. Schematic overview of the effect of furfural on glucose metabolism.

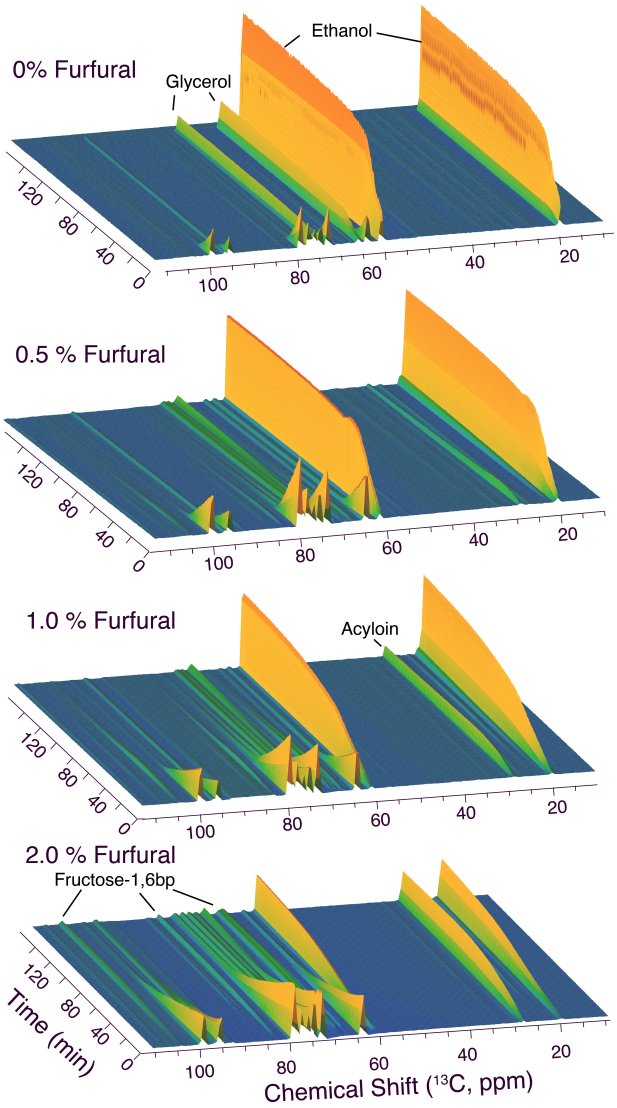


Figure 2. Time series of one-dimensional ^{13}C NMR spectra assaying the conversion of glucose in substrate mixtures with varying content (v/v) of furfural. Some metabolites responding significantly to furfural are labelled, decaying sets of signals correspond to the glucose reactant. Reaction conditions: 100 mg dry yeast, 550 µl phosphate buffer (90 mM phosphate, pH 6), 50 µl D_2O, 90 mM [U-^{13}C] glucose, 303 K, furfural added to the given % (v/v) values.

consistent with the molar mass previously observed (140 amu).[20,29]

Other effects of furfural on reaction progress were directly evident from the spectra of Figure 2. The conversion of glucose was increasingly inhibited by the presence of furfural as expected, leading to slower conversion (Figure S1). Due to inhibited glucose conversion and the formation of the non-natural C7 product, conversion of glucose to ethanol declines. Formation of glycerol declines even stronger than the formation of ethanol, by a factor of 20 and more in the presence of 0.5% (v/v) and more furfural (Figure S2). Formation of glycerol is a means of restoring the oxidant NAD^+ if alcoholic fermentation is inhibited. These observations are consistent with a predom-

inant function of furfural as an oxidant in non-aerated yeast cells, disturbing the conversion of glucose to glycerol ($C_3H_8O_3$) in a reduction reaction and the conversion to ethanol and CO_2 in redox neutral reaction. The effects of furfural on central metabolism in yeast also affected the accumulation of glycolytic intermediates on the minutes time scale. Metabolites of upper glycolysis, especially fructose 1,6-bisphoshphate, accumulated 15 times more strongly in the presence of furfural (Figure 2) than in its absence. This accumulation of metabolites from upper glycolysis parallels the slower glycolytic flux in the presence of furfural.

Following the reaction, yeast cells were removed by centrifugation and the supernatant was analyzed. The supernatant contained the compound with ^{13}C signals at 28.1 and 211.8 ppm, and a full structure determination was pursued with heteronuclear 2D NMR. The combination of ^{1}H-^{13}C HSQC, its multiplicity edited version and ^{1}H-^{13}C HMBC allowed to identify the structure with a terminal methyl group adjacent to a carbonyl group, which was in turn adjacent to a secondary alcohol group that was directly bonded to the heteroaromatic furan ring (Figure S3). Hence, the predominant adduct was identified as the C7 acyloin **7** shown in Scheme 2. The fully assigned chemical shifts determined in a post reaction mixture are consistent with previously reported chemical shifts for a purified compound with the same structure.[31]

Fermentation can be massively redirected towards C7 acyloin formation from glucose/furfural

The experiments of Figure 2 indicated that sizeable amounts of the C7 acyloin can be formed from glucose/furfural substrate mixtures. Hence, metabolic inhibitors from biomass pre-treatment emerge as viable substrates for conversion by yeast. The flux towards the acyloin **7** was further optimized and a content of 3% (v/v) furfural (360 mM) was found to be acceptable in phosphate buffer of pH 6 and in the presence of 90 mM glucose. To our surprise, the resultant time series of Figure 3 indicates that carbon flux towards ethanol by fermentation can be massively rerouted to the formation of acyloin **7**. Specifically, a molar selectivity of up to approximately 4:1 in favour of formation of acyloin **7** relative to formation of ethanol could be achieved, corresponding to a distribution of carbons between non-natural C7 product and natural C2 product of approximately 14:1. Due to the inhibitory effect of furfural on alcoholic fermentation, the conversion of glucose at initially 3% (v/v) furfural dropped to 61% after 150 min reaction time at 303 K, and no significant conversion at all was detected at 5% (v/v) furfural (Figure S1).

The observation of adduct formation at the levels that are evident from Figure 3 show that furfural efficiently incorporates two carbons from glucose in yeast cells by establishing a new pathway that operates in competition to alcoholic fermentation and glycerol formation (Scheme 2). The time series of Figure 3 indicates that ^{13}C from isotope enriched glucose enters the acyloin **7** under concurrent release of CO_2. This experiment thus underlines that no stable adduct with pyruvate (**7** in Scheme 2) appears to be formed. A possible interpretation would be that furfural intercepts acetaldehyde, whose accumulation in the presence of furfural was evident from D-DNP NMR observations as described above. Notably, the formation of the C7 acyloin **7** was equally observed when using pyruvate/furfural substrate mixtures, thus validating that the glucose serves as a precursor for pyruvate and its downstream products in the glucose/furfural substrate mixture.

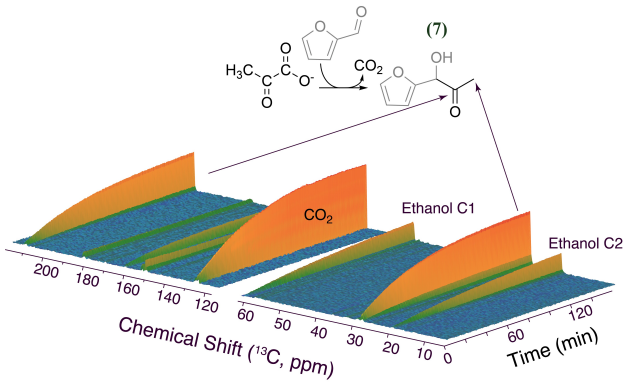


Figure 3. Time series of the conversion of glucose under reaction conditions as in Figure 2 except for the presence of 3% (v/v) furfural.Under these conditions, glucose conversion is massively rerouted in the presence of 3% (v/v) furfural towards formation of the shown acyloin 7.

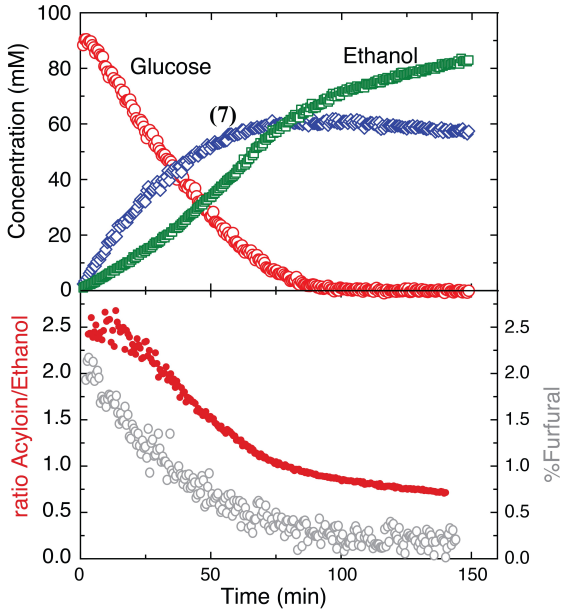


Figure 4. Concentration of glucose and product signals (top) and integrals of natural abundance furfural signal (bottom, right ordinate, open symbols) as well as ratio between the products (bottom, left ordinate, filled symbols). Reaction conditions: 100 mg dry yeast, 550 µl phosphate buffer (90 mM phosphate; pH 6), 50 µl D_2O, 90 mM [U-^{13}C] glucose, 303 K, 2% (v/v) furfural.

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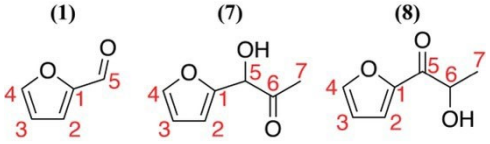
Table 1. Assignment of furfural metabolites relative to DSS at 303 K according to numbering scheme given below.[a].



	1	2	3	4	5	6	7
furfural (**1**)							
δ^1H	–	7.52	6.71	7.87	9.43	–	–
δ^{13}C	154.6	128.5	116.0	152.7	183.6	–	–
furfuryl alcohol (**2**)							
δ^1H	–	6.46	6.50	7.56	4.62	-	–
δ^{13}C	156.2	110.9	113.2	145.9	58.4	–	–
furoic acid (**3**)							
δ^1H	–	7.60	6.76	7.90	–	–	–
δ^{13}C	146.8	124.6	115.5	151.9	167.1	–	–
1-(furan-2-yl)-1-hydroxypropan-2-one (**7**)							
δ^1H	–	6.63	6.57	8.04	5.51	–	2.21
δ^{13}C	152.2	113.1	113.6	146.7	75.3	211.8	28.1
1-(furan-2-yl)-2-hydroxypropan-1-one (**8**)							
δ^1H	–	–	n.d.	n.d.	–	5.07	1.42
δ^{13}C	n.d.	n.d.	n.d.	n.d.	195.2	72.0	22.9
ethanol							
δ^1H	3.64	1.17					
δ^{13}C	60.1	19.51					

[a] Chemical shifts in ppm.

Reaction progress in batch reduces the selectivity for C–C formation due to the consumption of furfural

The experiments of Figure 2 and Figure 3 show that the selectivity towards the C7 acyloin **7** declined during reaction progress. This decline is expected with declining furfural concentration in the batch reaction. Figure 4 displays the integrals of glucose and furfural substrates alongside ethanol and acyloin products following a start concentration of 2% (v/v) furfural. At this furfural content, close to full conversion of 90 mM glucose could be achieved. Glycolytic activity and furfural conversion appear to be correlated both at 2% (v/v) initial content of furfural (Figure 4 top and bottom), and at other initial contents of furfural (Figure S1). The selectivity towards acyloin was compared to the residual furfural content in the reaction mixture by plotting the time-dependent acyloin/ethanol ratio (Figure 4, bottom) and the time-dependent furfural content. This comparison shows that the preference for acyloin formation is initially high but decreases in parallel with the decline in furfural. The data corroborate that the selectivity towards the formation of acyloin and the content of furfural are positively correlated. Initial selectivity in the presence of 2% (v/v) furfural is still vastly in favour of the acyloin product with an initial molar distribution of 73% C7 acyloin (**7**) and 27% ethanol.

The rerouting of glucose metabolism to acyloin (**7**) thus is not only a nuisance. Xylose-derived furfural and glucose can effectively be combined to the furanic acyloin (**7**) using whole cell catalysts with negligible cost, without the need for catalysis by purified enzyme. The declining selectivity over time in batch indicates that flow processes with immobilized yeast[18,41] and constant glucose/furfural substrate concentrations may provide attractive metrics for this and similar reactions. The time course in Figure 4 shows that the concentration of the acyloin reached a maximum and that the acyloin was not the thermodynamic

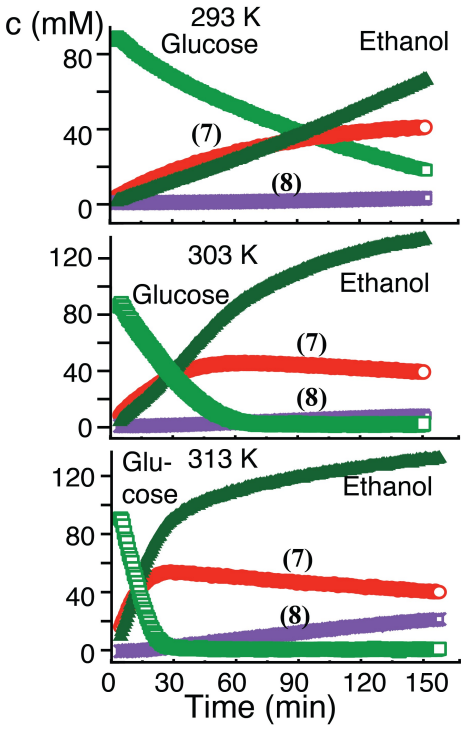


Figure 5. Effect of temperature on the kinetics of acyloin formation. Reaction conditions: 100 mg dry yeast, 550 µl phosphate buffer (90 mM phosphate; pH 6), 50 µl D_2O, 90 mM [U-^{13}C] glucose, 1.5% (v/v) furfural, variable temperature (293 K, 303 K, or 313 K).

product. Analysis of reaction progress curves and of the product mixtures showed the presence of an isomeric acyloin **8** (1-(furan-2-yl)-2-hydroxypropan-1-one) with a conjugated double bond system. This product (Scheme 2) has previously been proposed to form as the thermodynamic product in enzyme-catalyzed conversions of furfural with pyruvate.[31] Two dimensional NMR showed that this compound contains ^{13}C enrichment in a methyl group and a secondary alcohol group, while ^{1}H-^{13}C HMBC correlations showed the attachment to a ketone group with a chemical shift below 200 ppm due to conjugation to the furan ring (Figure S4). Highly resolved HSQC spectra in Figure S5 further corroborated the assignment by showing that the methyl protons of acyloins **7** and **8** are singlets and doublets, respectively, due to the respective presence of no and one hydrogen on the adjacent carbon. The chemical shift assignments of the relevant adducts are compiled in Table 1. A full assignment of **8** was not pursued, as it remained the minor product under all relevant conditions used herein.

The isomerization between acyloins **7** and **8** lead to an overall stable product pool of acyloins under conditions, where also ethanol had reached a stable level (Figure S6). This isomerization hence did not appear to be dependent on active glycolysis in yeast.

Effects of temperature and catalyst loading

A comparison of temperature effects on kinetic profiles in the conversion of glucose/furfural substrate was subsequently pursued at 293, 303 and 313 K. Maximum acyloin concentrations were comparable in the presence of 1.5% (v/v) furfural at all three temperatures. Slightly reduced furfural concentration was used as this level was well tolerated by the cells at 303 K. Unsurprisingly, the rate of glucose conversion and of acyloin formation doubled approximately with temperature increases of 10 K. The formation of the conjugated acyloin from the primary acyloin product proceeded approximately an order of magnitude slower than the formation of the initial acyloin adduct at all temperatures (Figure 5). The experiments of Figure 5 indicate that the selectivity between formation of acyloins **7** and **8** and ethanol is not strongly dependent on the reaction temperature.

Finally, a screening approach was applied with various amounts of yeast cells and varying furfural/glucose substrate ratios in 1.5 mL reaction tubes using a ThermoMixer. For simplicity, the glucose concentration was kept constant at 90 mM. Reaction mixtures were analyzed after 150 minutes reaction time at 303 K to describe the product distribution in dependence on furfural concentration in the substrate mixture and furfural content per cell. The resultant product distributions are shown in Figure 6. As expected, the fraction of acyloin products increases with the content of furfural in the substrate mixture. This increase is faster than linear in the beginning, possibly due to the multiple effects of furfural, which serves both as an effector of glycolysis and as a substrate.

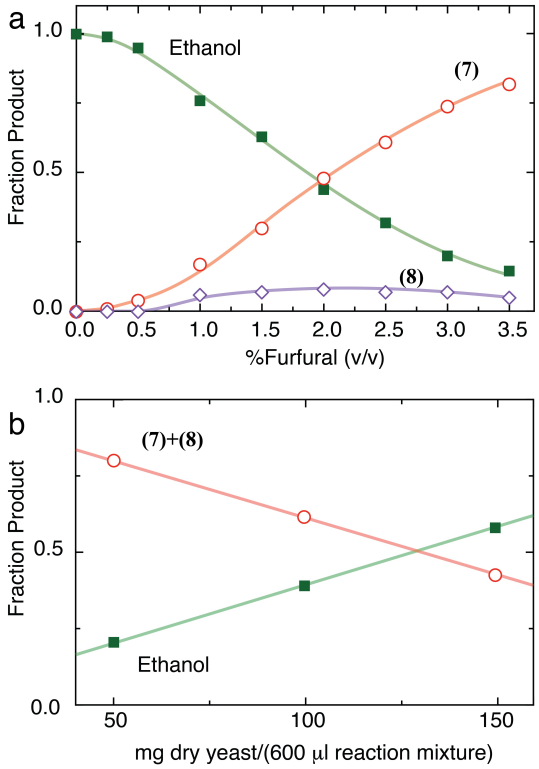


Figure 6. Product distribution from glucose/furfural in dependence on furfural concentration in the substrate mixture (a) and furfural content per cell (b). Reaction conditions: (a) 100 mg dry yeast, 550 µl phosphate buffer (90 mM phosphate; pH 6), 50 µl D_2O, 90 mM natural abundance glucose, 303 K, variable amount of furfural as indicated (% (v/v)), 2.5 hours of incubation under shaking. (b) 50 mg, 100 mg, or 150 mg dry yeast, 550 µl phosphate buffer (90 mM phosphate; pH 6), 50 µl D_2O, 90 mM natural abundance glucose, 303 K, 2.5% (v/v) furfural, 2.5 hours of incubation under shaking.

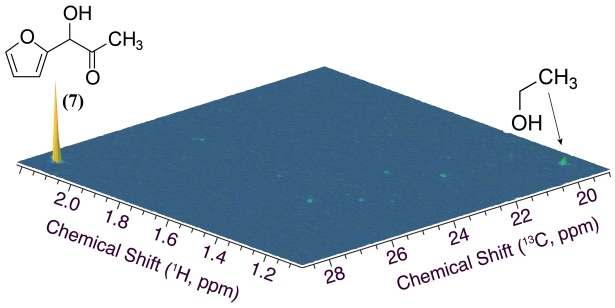


Figure 7. Surface plot of ^{1}H-^{13}C HSQC spectrum of the product mixture obtained from 90 mM natural abundance glucose in the presence of 2.5% (v/v) furfural and 50 mg dry yeast. Product ratio between acyloin and ethanol is 80:20. Reaction conditions: 50 mg dry yeast, 550 µl phosphate buffer (90 mM phosphate; pH 6), 50 µl D_2O, 90 mM natural abundance glucose, 303 K, 2.5% (v/v) furfural, 2.5 hours of incubation under shaking.

When repeating the experiment at a constant furfural concentration, but variable loading of yeast cells, a surprisingly strong dependence on the yeast cell loading was observed (Figure 6): a higher fraction of acyloin product was observed for a smaller yeast cell loading after 150 min reaction time,

consistent with a lower consumption of furfural in the presence of less catalyst and with a higher number of furfural molecules per catalyst. The results of reactions conducted in reaction vials under shaking corroborated the in-cell NMR data acquired on the running reaction. Product selectivities of 80% acyloin relative to 20% ethanol were obtained from glucose in suitable substrate mixtures converted by non-engineered commercial yeast (Figure 6 and 7).

Conclusion

In concluding, we find that substrate mixtures of furfural and glucose can be used in whole cell catalysis with commercial yeast of near-negligible cost to provide C–C bond extensions on furfural. Chemocatalytic production of xylose and biological conversion of resultant furfural with glucose can thus be combined[42] to provide new chemicals in aqueous solvent at very mild temperatures. Changes to in-cell kinetic barriers in the presence of furfural, the accumulation of acetaldehyde and the formation of a non-natural ketone were identified through D-DNP NMR. The accumulation of acyloin products was validated and correlated to furfural levels by ^{13}C NMR reaction tracking. Screening for conditions that provide high selectivity towards the C7 acyloin adducts was finally conducted with quantitative 2D NMR. The composition of the substrate mixture and the number of yeast cells had a strong impact on product distribution.

Although non-engineered yeast is traditionally used to form ethanol due to its preference for alcoholic fermentation, 80% of glucose flux can readily be redirected from ethanol to acyloin formation. The correlation of furfural concentration and adduct formation indicates that flow-processes with optimized glucose/furfural substrate concentrations and immobilized yeast may be particularly promising for C–C bond formation on furfural or similar bio-sourced aldehydes.[11,18] Related acyloins have shown utility in the fine chemical and pharmaceutical industries.[11]

Experimental Section

Materials

All chemicals including isotope-enriched [U-^{13}C] glucose, [U-^{13}C, U-^{2}H] glucose and D_2O were ordered from Sigma Aldrich (Andover, MA, USA) and were used without further purification. Commercial dry yeast (Seitenbacher, Buchen, Germany) was obtained through online retail.

Dynamic Nuclear Polarization

Hyperpolarization of glucose isotopologues by dynamic nuclear polarization (DNP) and subsequent dissolution (D-DNP) was performed as previously established: briefly, solid state dynamic nuclear polarization of [U-^{13}C, U-^{2}H] glucose was conducted using trityl radical OX063 (27 mM; Oxford Instruments, Abingdon, UK) and gadoteridol (1.5 mM; Bracco Imaging, Italy). The final substrate samples contained 50 μmol [U-^{13}C, U-^{2}H] glucose in 19 mg of aqueous polarization medium including trityl radical OX063 and gadoteridol. These preparations were placed in a sample cup for hyperpolarization, where the [U-^{13}C, U-^{2}H] glucose preparations first were flash-frozen in liquid helium. Polarization transfer occured by DNP at 1.2 K, using microwave irradiation at 93.89 GHz with 100 mW in a magnetic field of 3.35 T for one hour to yield solid-state polarizations above 30%. Upon dissolution with heated phosphate buffer containing 10 mM KCl, 1 mM $MgSO_4$ (5 mL of 90 mM buffer, pH 6.0), hyperpolarized samples with a final [U-^{13}C, U-^{2}H] glucose concentration of 11 mM hexose were obtained. Of these hyperpolarized samples, 0.33 mL were manually injected into 0.2 mL of yeast cell suspension (31 mg dry yeast) containing furfural to a final concentration of either 0.0% or 0.5% (v/v), equilibrated to 303 K inside a 500 MHz Bruker spectrometer that was equipped with a 5 mm DCH CryoProbe and a 11.7 T UltraShield magnet. This procedure yielded a final concentration of 6.9 mM hyperpolarized [U-^{13}C, U-^{2}H] glucose. Reactions were followed by a series of ^{13}C NMR spectra acquired with an excitation pulse of approximately 12°. Pseudo-2D spectra using a receiver gain of 10 were used to acquire the ^{13}C FID for 344.7 ms every 0.5 s, sampling 11264 complex data points for acquisition. Data acquisition was started prior to substrate injection to minimize experimental dead-time. The data were processed with ample zero filling in Bruker Topspin 4.1.3.

Real time kinetic NMR assays

Commercial dry yeast (100 mg) was suspended in phosphate buffer (550 μl, 90 mM) of pH 6.0 containing 10 mM KCl, 1 mM $MgSO_4$ and desired amounts of furfural. To this cell suspension, 10 mg [U-^{13}C] glucose dissolved in D_2O was added to yield a final concentration of glucose of 90 mM. The sample was rapidly transferred to an 800 MHz Bruker Avance III instrument equipped with a 5 mm TCI cryoprobe and an Oxford magnet. A time series of ^{13}C NMR spectra using 30° flip angle excitation pulses and inverse gated decoupling of ^{1}H was acquired by accumulating 16 transients with an interscan relaxation delay of 1.5 seconds by sampling the FID for 0.68 s (16384 complex data points). The time series of ^{13}C NMR spectra was implemented as a pseudo-2D experiment acquiring 256 time points with a time resolution of 0.59 min. The pseudo-2D spectra were used to track reactions for 150 minutes and were acquired using Bruker Topspin 5pl6. The spectra for reaction tracking were processed with ample zero filling, integrated, and analyzed using Bruker Topspin 4.1.3. Integrals were plotted using pro Fit 7.0.18 (Quansoft, Zurich). Following the reaction, yeast cells were removed by centrifugation at 10,000 g and the supernatant was analyzed by 2D NMR experiments including ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC as described above. All 800 MHz NMR spectra were acquired using Bruker Topspin 3.5pl6. The data were processed with ample zero filling in all dimensions and analyzed using Bruker Topspin 4.1.3.

Ex situ NMR assays

Commercial dry yeast (100 mg) was suspended in phosphate buffer (550 mL, 90 mM) of pH 6.0 containing 10 mM KCl, 1 mM $MgSO_4$ and desired amounts of furfural. To this cell suspension, glucose dissolved in D_2O was added to a final concentration of 90 mM and the yeast cells in the presence of furanic/glucose substrate mixtures were incubated for 150 min under vigorous shaking (700 rpm) with a DITABIS Heating-ThermoMixer MHR 13. Subsequently, the yeast cells in the incubation were removed by centrifugation. The supernatant was transferred to an 800 MHz Bruker Avance III HD instrument equipped with a 5 mm TCI cryoprobe and a SampleJet sample changer. Two-dimensional NMR assignment spectra were

acquired at 298 K to identify non-natural products **7** and **8** in the reaction mixture. The 2D NMR experiments included ^{1}H-^{13}C HSQC spectra recorded by sampling 1024 complex data points for 107 ms in the ^{1}H dimension and 256 complex data points for 6 ms in the ^{13}C dimension. ^{1}H-^{13}C HMBC spectra were recorded by sampling 2048 complex data points for 200 ms in the ^{1}H dimension and 200 complex data points for 4 ms in the ^{13}C dimension. For quantifying the products in dependence on catalyst and furfural concentration, high-resolution ^{1}H-^{13}C HSQC spectra sampling 1024 complex data points for 160 ms in the ^{1}H dimension and 200 complex data points for 33 ms in the ^{13}C dimension were acquired with a carrier offset of 20 ppm and a spectral width of 30 ppm in the ^{13}C dimension. For relative quantifications, responses were determined through calibration relative to a fully relaxed high-resolution ^{1}H-^{13}C HSQC spectrum acquired with an inter-scan relaxation delay of 10 seconds. Ex situ NMR experiments were repeated with pyruvate (160 mM)/furfural substrate mixtures, yielding the same adducts as glucose/furfural mixtures (Figure S7).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: C–C bond formation · furfural · glucose · NMR spectroscopy · whole cell catalysis

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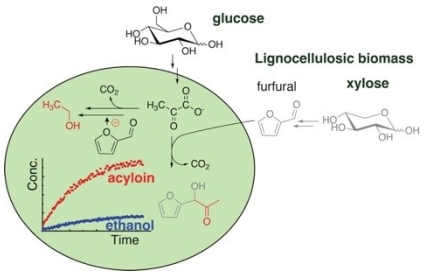
RESEARCH ARTICLE

Furfural and Glucose Conversion in Non-Engineered Yeast: Glucose/furfural substrate mixtures can be concurrently upgraded by whole cell catalysis. Through in situ production and interception of pyruvate/acetaldehyde in non-engineered yeast, such substrate mixtures can efficiently be used for C–C bond formation on furfural using whole-cell catalysis, while ethanol and glycerol formation can be vastly suppressed.



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Glucose/Furfural Substrate Mixtures in Non-Engineered Yeast: Potential for Massive Rerouting of Fermentation to C–C Bond Formation on Furfural