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Workflows for optimization of enzyme cascades and whole cell catalysis based on enzyme kinetic characterization and pathway modelling

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To move towards a circular bioeconomy, sustainable strategies for the utilization of renewable, non-food biomass wastes such as lignocellulose, are needed. To this end, an efficient bioconversion of D-xylose – after D-glucose the most abundant sugar in lignocellulose – is highly desirable. Most standard organisms used in biotechnology are limited in metabolising D-xylose, and also *in vitro* enzymatic strategies for its conversion have not been very successful. We herein discuss that bioconversion of D-xylose is mostly hampered by missing knowledge on the kinetic properties of the enzymes involved in its metabolism. We propose a combination of classical enzyme characterizations and mathematical modelling approaches as a workflow for rational, model-based design to optimize enzyme cascades and/or whole cell biocatalysts for efficient D-xylose metabolism.

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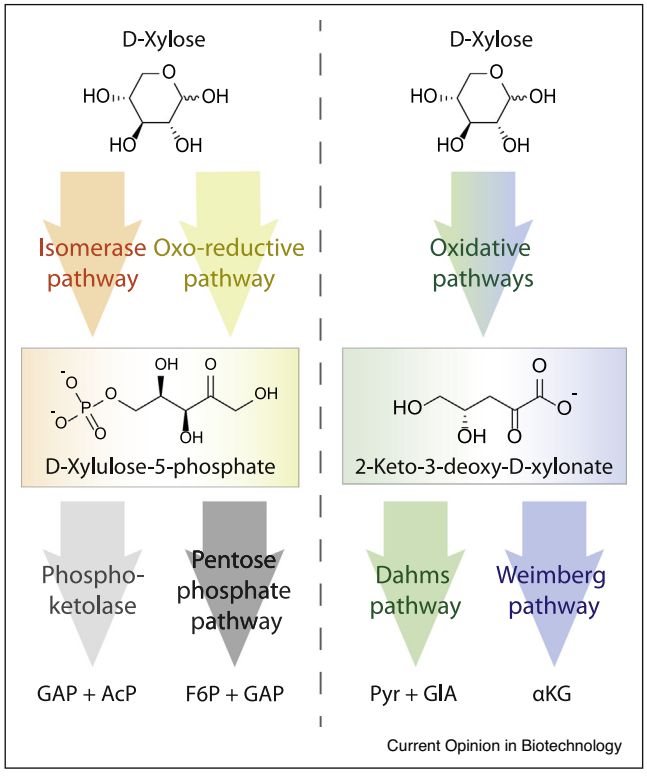
Introduction

The depletion of fossil resources and emission of greenhouse gases is driving the search for sustainable, environmentally friendly processes using renewable, non-food biomass. Lignocellulose is a promising resource for the production of 'second generation' biofuels and added-value chemicals/products as it does not compete with food supply. But the recalcitrant structure and complexity of lignocellulose poses a challenge to biotransformation requiring physicochemical and/or chemical pretreatment [1–4]. The gained hydrolysates comprise D-glucose and D-xylose as main fermentable carbon sources. Many of the current platform organisms used in biotechnology such as *Escherichia coli*, or yeast are able to utilize D-glucose whereas the conversion of D-xylose alone, or the ability for hexose/pentose co-fermentation is rather limited. Therefore, many metabolic engineering and synthetic biology approaches aim to achieve an efficient conversion and co-utilization of D-xylose [5,6,7•]. Here we give an overview of the different pathways for D-xylose degradation and describe the advantages of the Weimberg pathway. Furthermore, we discuss the advantages and disadvantages of whole-cell catalysis and enzyme cascades and highlight the potential of combined experimental modelling approaches.

Pathways for D-xylose degradation

For D-xylose degradation two main strategies have been reported in microorganisms (Figure 1) (for review and literature see Refs. [6,7•,8–11]). The first strategy relies on the formation of the key intermediate xylulose 5-phosphate which proceeds either via isomerases and kinases mainly in bacteria (isomerase pathway) or via reductases, dehydrogenases and kinases in fungi (xylose reductase/xylitol dehydrogenase pathway). The formed xylulose 5-phosphate is channelled into the pentose phosphate pathway or is cleaved to glyceraldehyde 3-phosphate and acetyl-phosphate (phosphoketolase pathway) as known for heterolactic acid bacteria, some Clostridia, and also some fungi. The second strategy for xylose degradation proceeds via the key intermediate 2-keto-3-deoxy-D-xylonate (KDX). This intermediate is formed through direct oxidation of the pentose mediated by xylose dehydrogenase (XDH) to the xylonolactone which is subsequently hydrolysed non-enzymatically or by a lactonase to D-xylonate. D-xylonate is then dehydrated to form KDX by xylonate dehydratase (XAD). The KDX is either cleaved by KDX aldolase to pyruvate and glycolaldehyde in the Dahms pathway and channelled into the glyoxylate bypass and finally as malate into the tricarboxylic acid (TCA) cycle. Alternatively, KDX is further dehydrated to α-ketoglutarate semialdehyde by KDX dehydratase (KDXD) and oxidized via α-ketoglutarate semialdehyde dehydrogenase (KGSADH) to yield

Figure 1



Overview of metabolic pathways for D-xylose degradation. Abbreviations: GAP, glyceraldehyde 3-phosphate; AcP, acetyl-phosphate; F6P, fructose 6-phosphate, Pyr, pyruvate, GlA, glycolaldehyde; αKG, α-ketoglutarate.

the final product α-ketoglutarate (αKG) in the Weimberg pathway which again enters the TCA cycle. These pathways are also called the non-phosphorylative routes or xylose oxidative pathways.

Weimberg pathway and its advantages

In general, the suitability of a pathway for an application depends on the desired product and therefore each of the D-xylose degradation pathways may be preferable for a specific biotechnological approach [5,11,25] depending on the primary D-xylose degradation product of the pathway and the required precursor for the desired product formation (Figure 2). And even the simultaneous use of two or more of the pathways can have synergistic effects.

The Weimberg pathway has initially been reported in *Pseudomonas* spp. and was identified in different mainly aerobic Bacteria and Archaea [6,7•,9,10,12–17]). The pathway is meanwhile best studied from *Caulobacter crescentus* [18,19,20••]. The oxidative Weimberg pathway offers several advantages [20••]: Especially for *in vitro* processes it is beneficial that no ATP is required (e.g. for sugar phosphorylation) and only one cosubstrate (NAD^+) is involved for which regeneration methods are well established via for example, NADH oxidase [21] or lactate dehydrogenase and pyruvate [20••]. Furthermore, all individual steps and thus also the pathway as a whole are thermodynamically favourable and run to completion which is advantageous for product purification and yield. Also important for product yield, no carbon loss occurs up to αKG formation. And particularly useful for whole-cell biocatalysis, the pathway is well separated from central metabolic routes making it less sensitive to metabolic interference. Finally, the pathway product αKG itself is an attractive target compound for the nutritional, cosmetic, pharmaceutical and also the medical sector, and it also represents a key intermediate in the cellular metabolism and serves as precursor for synthesis of various valuable products [21,22]. In addition, chemical synthesis of αKG is rather complicated and includes the use of toxic chemicals and wastes [23,24].

Whole cell catalysis and enzyme cascades (advantages and disadvantages)

Because of these advantages the Weimberg pathway or parts thereof have been used for metabolic engineering in several model organisms for the production of value-added products from D-xylose including *E. coli*, *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* [11,20••,26–30]. However, the outcome was in most cases unsatisfactory and suffered for example, from intermediate accumulation or low expression levels of the heterologous proteins and additional time consuming non-targeted, non-rational approaches like laboratory evolution had to be applied for pathway optimization. This underscores the general problems of whole-cell systems, that is, missing or insufficient control mechanisms of expression, narrow range of reaction conditions, as well as interferences with the intrinsic metabolism [31••,32]. Notably, the respective bottle-necks of such *in vivo* metabolic engineering approaches remained mostly unresolved likely because of the elevated complexity of whole cells.

In vitro enzyme cascades can bypass most of these issues of whole-cell biocatalysis and are often regarded as simpler and thus more promising tools for bioconversions. They usually provide higher yields/productivities, do not suffer from metabolic interferences, can be run under a wider range of conditions like for example, pH, osmolarities, temperatures, solvent and metabolite concentrations, and can also make use of side activities of enzymes [31••,32]. However, despite the above-mentioned advantages, *in vitro* enzymatic approaches also bear major drawbacks including the requirement of elaborate enzyme production and purification, supply of cofactors, and costly scale-up for industrial production [31••]. Additionally, also enzyme systems show a certain degree of complexity and are often subject to regulation by feed-forward or feed-back loops and/or allosteric control by (co) substrates, intermediates, or products. And this becomes

Figure 2

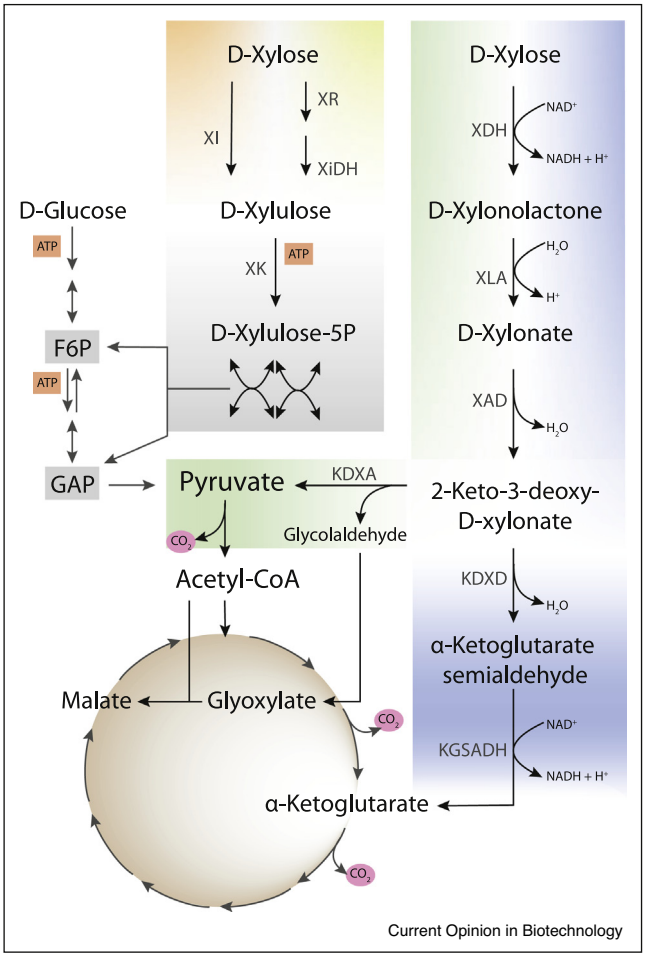


Illustration of the linear, oxidative Weimberg pathway (blue) as a 'parallel metabolic route'. The different pathways for pentose degradation are shown according to the colour code in Figure 1, that is, isomerase pathway (orange), oxo-reductive pathway (yellow), Dahms pathway (green) and Weimberg pathway (blue). In addition, the Embden-Meyerhof-Parnas (EMP) pathway for D-glucose degradation and the tricarboxylic acid (TCA) cycle with the glyoxylate bypass are depicted. The representation highlights the advantages of the Weimberg pathway for biotechnological application, particularly the separation and thus non-interference with the rest of the metabolism with the only entry point at the level of αKG. The absence of ATP usage and CO_2 production distinguish the Weimberg pathway from the other routes. Abbreviation: XI, xylose isomerase; XR, xylose reductase, XiDH, xylitol dehydrogenase; XK, xylulose kinase; XDH, xylose dehydrogenases; XLA, xylonolactonase; XAD, xylonate dehydratase; KDXD, 2-keto-3-deoxy-xylonate dehydratase; KGSADH, α-ketoglutarate semialdehyde dehydrogenase; KDXA, 2-keto-3-deoxy-xylonate aldolase; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate.

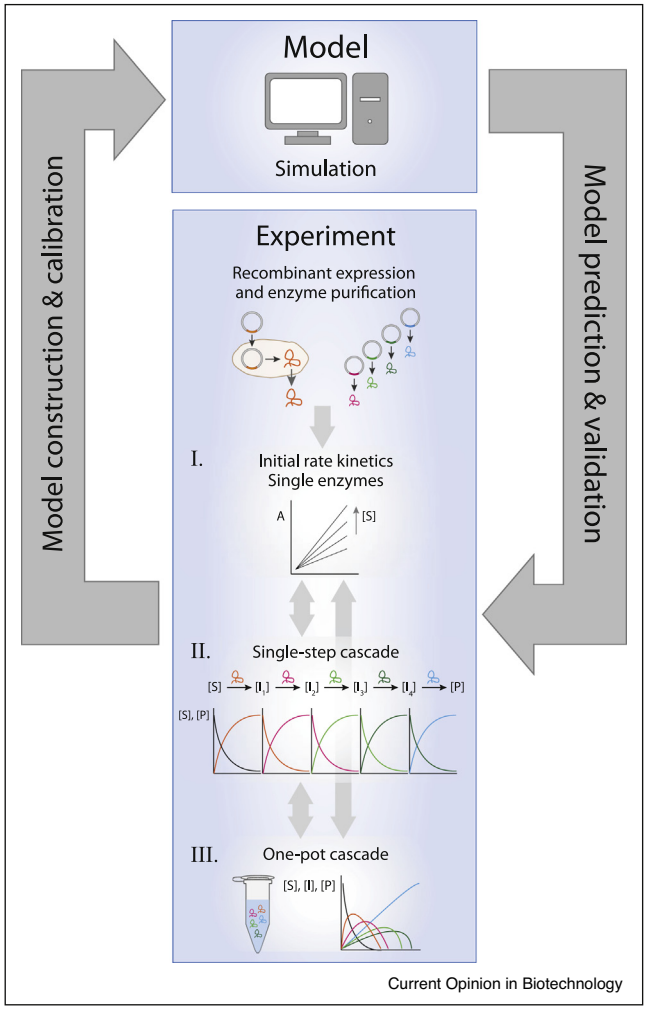
more and more pronounced the more complex the cascade gets [33,34].

The advantages and drawbacks of the cell-based and cell-free approaches were recently reviewed by Claassens *et al.* [31••] and they proposed to combine the strengths of both approaches to overcome the drawbacks. One of the major advantages of *in vitro* enzymatic reconstructions is that the cascade composition like for example, the concentrations of enzymes and (co)substrates can accurately be controlled and thus allow for computational pathway modelling and characterization which in turn enables the rational design of a pathway. Hence, a combined strategy should involve an initial design, testing and optimization of the pathway in cell-free systems and a subsequent transfer into platform organisms with controlled expression systems that allow for model assisted pathway optimization. However, such modelling assisted pathway characterization and optimization approaches are only rarely applied presumably because they are elaborate, particular when moving to the whole-cell biocatalysis. Instead, for optimization of pathways mostly non-targeted, combinatorial approaches with non-biased combinations of enzymes and/or reaction conditions are used to identify the rate-limiting steps and to enhance pathway performance [35–37] (for review Ref. [38]). If kinetic parameters are used at all, they are often retrieved from databases such as BRENDA or SABIO-RK, which are well curated and useful in their own right for isolated reactions, but seldomly meet the conditions required for a specific production process with respect to physicochemical conditions, enzyme expression levels, substrate concentrations and so on (see e.g. Ref. [33]). Thus, whereas the know-how for characterizing the individual processes, that is, enzyme kinetics, intact cell analysis, and mathematical modelling exist, a poor integration of these processes in a coherent and validated mathematical model limits the development of sufficient *in vitro* production systems as well as of high-performance whole cell biocatalysts and the need for the development of generic strategies to overcome these limitations have been stressed [39•,40•,41•].

Potential of a combined experimental modelling approach

Recently, a novel combined modelling and experimental validation approach for the *C. crescentus* Weimberg pathway was published [20••] which substantiated and concretized the idea proposed by Claassens *et al.* [31••] and potentially represents a generic strategy for systematic pathway setup and analysis (for a schematic illustration see Figure 3). This workflow includes firstly (recombinant) enzyme production and purification, followed by a kinetic characterization of each of the pathway enzymes, typically using initial rate kinetics. Importantly, all enzymes must be characterized under the same assay conditions, which should reflect the production conditions. Depending on the kinetics (e.g. hyperbolic or sigmoidal saturation curves) a suitable rate equation is selected (or derived if the kinetic mechanism of the enzyme is known) and an initial parameterization for substrate dependencies, and maximal catalytic rate is

Figure 3



Schematic representation of the iterative experimental and modelling approach. The result of the first round (I) are the parameterized rate equations which are in the second round combined to a model-based prediction of the single-step cascade with subsequent experimental validation followed by model optimization. In the third round (III) this model is used to predict the one-pot-cascade again followed by experimental validation and model optimization finally leading to the reference-state model which can then further be applied.

made. In a first validation step these initial rate equations are integrated and used to predict the step by step conversion of substrate to product after adding one enzyme after the other along the pathway ('single-step cascade'). Usually the model predicts a conversion rate that is higher than the one that is experimentally observed due to product inhibition, which accumulates to considerable levels in these assays. In a subsequent model construction step, if possible the product inhibition is tested in initial rate kinetics or a product inhibition constant is fitted using the conversion data. In the next validation round, model predictions for a one-pot cascade conversion of substrate to product with all enzymes present at the start of the assay (third step) are compared to the experimental data for the conversion. Typically, at this step, allosteric regulation can cause significant differences between the model prediction and experimental observation. Model analysis can reveal what metabolites act as allosteric regulators and this can be tested again in initial rate kinetic experiments. Finally, this leads to a reference state model which can be further validated in its capacity to predict specific perturbations experiments, for example, alterations in relative abundances of the pathway enzymes, or omitting the recycling system for cofactors.

In case of the *C. crescentus* Weimberg pathway this workflow led in the first round of model construction, that is, the single-step cascade, to the discovery of extended product inhibition of most pathway enzymes and in the second round of modelling and validation (one-pot cascade) to the discovery of allosteric regulation of some of the enzymes by pathway intermediates. The finally adjusted model could then fairly accurately predict the requirement of an NAD^+ recycling system for complete substrate to product conversion and the necessity of lactonase at high flux rates. The NAD^+ recycling was necessitated by a pronounced product inhibition of both, the XDH and the KGSADH, with NADH as well as by an allosteric inhibition of the XAD with NADH, discovered in the first and second round of modelling and validation, respectively, which impeded a complete substrate to product conversion. The model then further enabled a rational optimization of the pathway in terms of enzyme amounts added for the highest conversion efficiency to produce αKG. This model was useful in experimental design, for instance to set up enzyme cascades for the synthesis of (2S, 3R, 4S)-4-hydroxyisoleucine where the Weimberg enzyme cascade provides αKG as a cofactor of the dioxygenase catalysed reaction of isoleucine to hydroxyisoleucine.

The insights provided by the *in vitro* cascade analyses already suggested a couple of implications on *in vivo* whole cell systems [20••]. Particularly, the redox state appears to have a strong influence on the pathway performance implying that the carbon flux needs to be balanced with the respiratory capacity and electron acceptor availability. In addition, the balancing of hydratase activity (i.e. XAD) by the addition of divalent metal ions was crucial for pathway performance in the enzyme cascade as well as cell extract assays. Importantly, the mathematical model could accurately predict the *in vivo* pathway performance in *C. crescentus* cell-free extracts after inserting specific activities as measured in the extract. Metabolic control analyses based on the cell extract modelling further supported the findings that under high NADH concentrations (NADH/NAD^+ ratios) the dehydrogenases (especially the XDH) become rate

limiting. Conversely, at low NADH concentrations the dehydratases become limiting, mainly the XAD. Under *in vivo* conditions these limitations might lead to unwanted by-products and thus carbon losses for example, via xylonate or KGSA excretion, which have indeed been observed in metabolic engineering approaches [42]. Thus, the developed model can provide explanations for issues observed in whole cell biocatalysis using the *C. crescentus* Weimberg pathway underlining the potential and the usefulness of the *in vitro* analyses and modelling for *in vivo* pathway design.

Conclusions and future prospects

Application of modern -omics type of techniques has opened up many possibilities for large scale analysis and are important for genome scale flux-based modelling approaches. However, for detailed kinetic modelling of metabolic pathways, parameterization of the models on the basis of system-wide data is often problematic due to the limited perturbations that can be made to intact cells, leading to parameter identifiability issues. We propose to construct models using a bottom-up approach based on classic enzyme kinetic data, with special attention to enzyme-assay conditions, which should reflect the production conditions. In a number of iterations between experiment and model a full model calibration, including product inhibition, allosteric regulation and regulation via ATP/ADP, NAD^+/NADH cofactor couples is possible. The detailed validated model is an important tool for experimental design, to quantify flux control distribution over the pathway, and to optimize protein distribution over the enzymes in the pathway for maximal efficiency and conversion rates. We have shown on behalf of the Weimberg pathway how the model could be used for simulations of cell extracts, simply by inserting the specific activity measurements for the enzymes in the extract. The *C. crescentus* Weimberg model, together with all the enzyme kinetic data and parameterization scripts, are available on the FAIRDOMHub portal, https://fairdomhub.org/investigations/284 and may aid the community in metabolic engineering approaches converting D-xylose containing biomass into added value products regardless of the platform organism chosen. The next step will be to apply the model to heterologous expression systems and to optimize the conversion rates in production strains.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Laura Kuschmierz: Writing – original draft, Visualization. **Lu Shen:** Writing – original draft. **Christopher Bräsen:** Conceptualization, Writing – original draft, Visualization, Writing – review & editing. **Jacky Snoep:** Conceptualization, Writing – original draft, Writing – review & editing. **Bettina Siebers:** Conceptualization, Writing – original draft, Writing – review & editing.

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- •• of outstanding interest

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