Model based biotechnological potential analysis of *Kluyveromyces marxianus* central metabolism

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Abstract

The non-conventional yeast *Kluyveromyces marxianus* is an emerging industrial producer for many biotechnological processes. Here we show the application of a biomass-linked stoichiometric model of central metabolism that is experimentally validated, and mass and charge balanced for assessing the carbon conversion efficiency of wild type and modified *K. marxianus*. Pairs of substrates (lactose, glucose, inulin, xylose) and products (ethanol, acetate, lactate, glycerol, ethyl acetate, succinate, glutamate, phenylethanol and phenylalanine) are examined by various modeling and optimisation methods.

Our model reveals the organism's potential for industrial application and metabolic engineering. Modeling results imply that the aeration regime can be used as a tool to optimise product yield and flux distribution in *K. marxianus*. Also rebalancing NADH and NADPH utilisation can be used to improve the efficiency of substrate conversion. Xylose is identified as a biotechnologically promising substrate for *K. marxianus*.

Keywords *Kluyveromyces marxianus*. Modelling. Central metabolism. Metabolic engineering. Essentiality analysis.

Introduction

Kluyveromyces marxianus is an ascomycotous yeast with enormous biotechnological potential for multiple industrial applications. There are a number of characteristics of *K. marxianus* that are industrially useful, including: fast growth, broad substrate spectrum, thermotolerance, limited fermentation at sugar excess, and secretion of extracellular glycolytic enzymes. In addition, *K. marxianus* enjoys GRAS (Generally Regarded as Safe) status and therefore is useful in food or pharma related applications [30, 46].

K. marxianus can grow on glucose, fructose, xylose, galactose, lactose and inulin as the sole carbon sources [24]. Many of these carbon sources are of particular interest since they are waste products of forestry (xylose) or dairy (lactose) industries. Xylose is a pentose and the main sugar of plant hemicellulose; its content in hard wood wastes can be up to 30 % [50]. *K. marxianus* has been engineered for xylitol production from xylose [42]. Cheese whey is a lactose rich by-product of the dairy industry produced in an approximate 10 to 1 (v/w) ratio to cheese. Currently whey is considered as a potential substrate for future microbial fermentations [25, 65]. Inulin is one of the widely available plant polysaccharides common in many taxonomic groups (*Asteracea* family, wheat, onion, banana, etc.). Some of those (e.g. Jerusalem artischoke, chicory) accumulate inulin in their underground tubers in vast amounts [11, 18]. These plants might serve as “niche” substrates for fermentations by yeasts including *K. marxianus*, [11] if not deprecated on account of competition with food use.

K. marxianus is a prospective producer for a range of important food additives and chemicals: phenylethanol, phenylalanine [60], hexanoic acid [10], xylitol [107, 108] and ethylacetate [52]. Due to its protein excretion, *K. marxianus* is suitable for extracellular protein production (galactosidase, inulinase, etc.) [26, 98].

Stoichiometric models and reconstructions significantly facilitate analysis of metabolic effects and limitations of microorganism metabolism, as well as predicting the phenotype of recombinant strains [39, 40]. Modeling attempts on *K. marxianus* to date have been concentrated on particular problems:

e.g. kinetic models of ethanol batch fermentation [77], and of growth on cheese whey [51]. The first attempt at a genome scale metabolic reconstruction [47] is patented in unreadable form and cannot be used for metabolic flux calculations. A genome-scale metabolic model for the related species *K. lactis* has been published [16]. Analysing medium scale stoichiometric models of central metabolism, where the most significant metabolic fluxes are, has been successful for biotechnological applications. Examples include assessment and selection of productive routes in *Escherichia coli* [88–90] and *Zymomonas mobilis* [68]. Medium scale modelling also proved to be a successful strategy for describing the uncharacterised central metabolism of the non-conventional yeast *Pichia pastoris* on the basis of limited wet experimentation [87]. Recent extensive attempts at *K. marxianus* metabolic engineering [33, 42, 43, 105, 109] underline the immediate need for modelling of limits of its metabolic potential.

The aim of this study was to assess the biotechnological potential of *K. marxianus* by a constraint based stoichiometric [79] modeling approach. A biomass-coupled model of central metabolism was developed to be a basis for design of metabolic engineering and to assess *in silico* the production of ethanol, acetate, lactate, glycerol, ethylacetate, succinate, glutamate phenylethanol, phenylalanine. As well as being useful products in their own right, they are also representatives of other products that could be derived from the same precursor metabolites.

Materials and methods

Modeling methodology and software

Two major strands of stoichiometric modelling are the constraint-based Flux Balance Analysis (FBA) [63, 94] and elementary modes analysis [80]. A constraint based model of central metabolism including biomass production of *K. marxianus* was created adapting and combining the high-quality genome-scale metabolic reconstructions protocol [79] and structural modelling approach for development of medium scale reconstruction and models [39].

Our medium scale *K. marxianus* central carbon metabolism model is based on the general mass balance equation:

$dX/dt = r_{met}-\mu X_{met}$

With respect to intermediate metabolite accumulation, a cell's metabolism is in pseudosteady state and can be described by following equation:

$0 = r_{met} - \mu X_{met}$ [85].

We also assume the following:

- the specific growth rate (μ, h^{-1}) during the exponential growth phase is constant,
- the cells are at pseudosteady state: substrate uptake, metabolite and product fluxes are constant when μ is constant.

For constraint based and structural analysis, the ScrumPy modelling package [71] was used. Flux balance analysis (FBA) was carried out by setting a constant rate of substrate uptake to 10 mM g^{-1} DW h^{-1}, and searching for the maximum yield of one of the following products: ethanol, acetate, lactate, glycerol, ethylacetate, succinate, glutamate, phenylethanol or phenylalanine. Solutions were further examined using Flux Variability Analysis (FVA) [55] to determine the ranges of internal fluxes that are consistent with the maximum if there were multiple equivalent FBA solutions. Inconsistencies in the model formulation were additionally detected through null space analysis [21] combined with determination of inconsistent enzyme subsets [69] using ScrumPy. The essentiality of genes and reactions was analysed using FBA to check whether biomass production was feasible after deleting the relevant reaction(s) from the model. The gene essentiality test took into account the gene – protein – reaction (GPR) associations [86] that were determined for the model (next subsection). FVA was also used to calculate the potential range in product production taking into account minimal and maximal oxygen respiration levels at a fixed substrate uptake value.

Reactions

The *K. lactis* genome scale reconstruction [16] was used as a starting point given the high degree of similarity between its metabolic networks and that of *K. marxianus*. The amino acid sequences of *K.*

lactis genes from the NIH genetic sequence database GenBank [3] were compared against fungal species using NCBI BLAST [38]. The corresponding *K. marxianus* genes were also checked for presence in the Uniprot database [54]. For each reaction, its Enzyme Comission number (E.C. number) and reaction directionality was checked and validated. The IntEnz [22] (available at http://www.ebi.ac.uk/intenz/) database was the main reference source for mass and charge balance validation. To represent the *K. marxianus* biomass growth reaction, we used the *S. cerevisiae* biomass composition as described by Gombert et al. [31].

Metabolites

Metabolite names, their neutral and charged formulas and InChI (International Chemical Identifier) strings [22] were taken from the CheBi database [13] (available at http://www.ebi.ac.uk/chebi/), and the yeast-specific Metacyc [7]. The PubChem database [96] (available at http://pubchem.ncbi.nlm.nih.gov/) was used to get additional information about metabolites [22].

***K. marxianus* strains and cultivation conditions**

The results of original experiments carried out by us to provide data for model development are marked in Table 1 as "this study". *K. marxianus* strain DSM 5422 was cultivated in semi synthetic medium containing (g/ l) $KHPO_4$ (1.0), $CaCl_2$ (0.1), $MgSO_4*7H_2O$ (0.5), NaCl (0.5), $(NH_4)_2SO_4$ (5.0) KH_2PO_4 (0.1) yeast extract (*Biolife*) (0.5). Different carbon sources (lactose or inulin) were added at concentrations of 5 or 10 % w/v. All fermentations were carried out in 1 litre *Infors* 2HT or 0.4 litre *Sartorius Biostat Qplus* 6-fold system fermenters at 35°C and 400 rpm.

Metabolite and biomass analyses

Extracellular lactose, ethanol, acetate and glycerol content were measured simultaneously using an *Agilent 1100 HPLC* system with a *Shodex Asahipak SH1011* column. Metabolites were quantitated with a refractive index detector (RI detector *RID G1362A*). The flow of the mobile phase (0.01 N H_2SO_4) was 0.6 ml min^{-1}, the sample injection volume was 5 µL.

Biomass growth was estimated by absorbance measurements at 600 nm (OD600). The conversion coefficient of *K. marxianus* DSM 5422 strain OD600 to culture dry weight was determined gravimetrically: OD600 1.0 was equivalent to 0.3 g dw.L^{-1}.

Results and Discussion

Model construction and properties

The model is shown diagrammatically in Fig.1 and is supplied in SBML [34] (Online Resource 1) format and in the form of a COBRA [79] MS Excel input file (Online Resource 2). Our *K. marxianus* metabolism model contains 113 reactions and 101 metabolites organized in 3 compartments: extracellular, cytoplasm and mitochondria. There are 72 cytosolic reactions (central metabolism pathways), 28 transmembrane transport reactions, 11 mitochondrial reactions, one extracellular and one biomass reaction (24 components).

Specific assumptions for our *K. marxianus* FBA model included:

- ammonium sulfate was the sole nitrogen and sulfur source and was available in excess;
- extracellular product accumulation had no effect on intracellular reactions;
- inorganic phosphate was available in excess;
- NADH and NADPH were assumed not to freely exchange between mitochondria and cytoplasm. Instead, redox equivalents could be translocated across the mitochondrial membrane by specific transport systems (shuttles). A malate – aspartate shuttle [17] and a 2-oxo glutarate – citrate carrier [8] were included to model NAD and NADP dependent redox exchange between cytosol and mitochondria.

To allow for succinate exchange across the mitochondrial membrane, a succinate - malate carrier was introduced [1, 64]. An electron transport chain was included in the model as a lumped reaction with the P/O ratio set to 1.2 [32].

AcetylCoA transport across the inner mitochondrial membrane occurs via a carnitine shuttle that is related to fatty acid metabolism [102]. Since the main fluxes for many biotechnologically important products stem directly from short chain carbon metabolites, we decided not to include a represention of fatty acid metabolism and described AcetylCoA transport across the mitochondrial membrane as a simple transport reaction (model reaction ACCOA_DIFF).

K. marxianus is an example of Crabtree negative yeast. Its physiology is believed to be closely related to its sister species *K. lactis* [95]. It is reported that ethanol production in *K. lactis* coincides with decreased oxygen supply [41]. It is assumed, that flux regulation around pyruvate bypass is the reason for Crabtree negative yeasts to choose between fermentation or oxidative growth. The cytoplasmic pyruvate bypass in *K. lactis* consists of pyruvate decarboxylase, NADP-dependent acetaldehyde dehydrogenase and acetyl-CoA synthetase. The first step of the pyruvate bypass in *K. lactis* is strongly upregulated during fermentative growth thus increasing the cytoplasmic production of AcetylCoA [41]. In the case of disturbed functioning of the mitochondrial pyruvate dehydrogenase complex, oxygen limitation or blockage of respiration chain, this bypass can supply enough cytoplasmic acetylCoA to support growth [103, 104].

Acetyl-CoA production in *K. marxianus* mitochondria occurs via the pyruvate dehydrogenase complex (model reaction ed5). In the model, cytoplasmic AcetylCoA synthesis was catalysed by acetyl-CoA synthetase (model reaction ACS). To model anaerobic or semi anaerobic fermentations, an AcetylCoA (reaction ACCOA_DIFF) transport reaction from cytoplasm to mitochondria was included.

Model validation

Data sources for validation/calibration.

The main carbon fluxes for model validation in *K. marxianus* were: substrate uptake, CO_2, ethanol, glycerol, acetate and biomass production. For a *K. marxianus* batch cultivation with limited oxygen supply these fluxes can account for up to 100 % of total carbon [77]. Therefore this set of fluxes is sufficient to validate this medium scale model. A similar set of fluxes has been successfully applied to validate the medium scale carbon metabolism model of *Pichia pastoris* [87].

Here the model outputs were compared with previously published and original experimental data. Metabolite and biomass data from the exponential growth phase were extracted from numerous published studies involving *K. marxianus* batch cultivations on various substrates (Table 1).

Model validation on lactose as substrate

Lactose is the main carbohydrate in cheese whey. In *K. marxianus*, lactose is split by the enzyme β-galactosidase into glucose and galactose, then each of these monosaccharides enters glycolysis at different levels: glucose is converted to glucose-6P, but galactose is converted to glucose-1 phosphate by the Leloir pathway. Strains of *K. marxianus* differ with respect to the first steps of lactose metabolism – some strains have intracellular and some - extracellular β-galactosidase [6]. We modelled *K. marxianus* lactose uptake with transport reaction lactD (lactose permease) and breakdown by reaction GALSID (β-galactosidase). The model was able to achieve a steady state solution for all the experimentally measured flux distributions (Table 1).

In addition to the published studies, we performed aerobic fermentations (semisynthetic broth with 7 % or 10 % lactose, aeration 0.2 or 1 vol vol^{-1} min^{-1} of fermentation volume). The measured fluxes of the extracellular metabolites are presented in Table 1 as ”this study”; ethanol was the major product with glycerol and acetate as the main byproducts.

Depending on the oxygen supply, *K. marxianus* lactose fermentation is biomass (aerobic) or ethanol (anaerobic) orientated. Sansonetti et al [77] demonstrated results for *K. marxianus* DSM 5422 strain

232 lactose fermentation in “self anaerobic” mode reaching 3.33 units of ethanol per unit of lactose. In this
233 case biomass growth was slow ($\mu = 0.07\ h^{-1}$) and glycerol was produced as the main byproduct. On the
234 other hand, rapid biomass production by *K. marxianus* strain CBS 6556 from lactose has been
235 described under fully aerobic mode with comparatively low ethanol flux [51].
236
237 Interestingly, none of above mentioned cases reported acetate accumulation, which seems to be related
238 either to slow cytoplasmic consumption of AcetylCoA (as in the case of low μ), or sufficient
239 AcetylCoA supply by mitochondria (in the aerobic case). Longhi et al [51] reported possible
240 accumulation of acetate during fermentation, albeit they did not report exact concentrations.
241
242 **Model validation on glucose, sucrose and inulin as substrates**
243
244 Glucose, fructose and their derived glucose and fructose oligo- and polysaccharides form an important
245 group of substrates for industrial applications. Sugarcane or sugar beet molasses, starch, sucrose and
246 inulin are typical examples [66].
247 *K. marxianus* is able to hydrolyze inulin directly due to its extracellular inulinase activity [75]. We
248 performed fermentations with strain DSM 5422 in semisynthetic broth with inulin as a sole carbon
249 source; results are depicted in table 1.
250 For strain DSM 5422, extracellular inulinase activity by far exceeded the uptake of released
251 monosaccharides (data not shown). An ample amount of free fructose in the media due to extracellular
252 inulinase activity was also demonstrated by other authors [101]. In the model we assumed, that only
253 fructose is produced after inulin hydrolysis, glucose is released in negligible amount and has no effect
254 on fructose uptake. Similarly, when simulating the data of Etchmann et al. [20], we assumed that
255 sucrose is split outside the cell and invertase activity exceeds the rate of monosaccharide uptake [75].
256 Subsequent simultaneous consumption of glucose and fructose happens when sucrose is hydrolyzed by
257 invertase [23]. Fructose uptake (model reaction inulin_t) followed by fructose kinase (model reaction
258 onoHLK) was considered as a starting point for inulin consumption. All results from inulin, glucose
259 and sucrose fermentations described in table 1 were replicated by the model.
260
261 **Model validation on xylose as substrate**

K. marxianus is able to ferment xylose. As for many yeasts and fungi, in *K. marxianus* xylose is taken up and converted to xylulose-5 phosphate (pentose phosphate pathway intermediate) via three sequential reactions: xylose reductase (reaction XYL1) xylitol dehydrogenase (reaction XDH) and xylulose kinase (reaction pengluc3). Moreover, xylose reductase in *K. marxianus* is exclusively NADPH dependent [106]. Xylose reductase reaction in our model was represented as exclusively NADPH dependent.

There are many reports of xylose fermentation by *K. marxianus*. We chose three example fermentations [14, 56, 82] to extract data for model validation. All three xylose fermentations yielded slow biomass growth with μ varying from 0.007 to 0.08 h^{-1}. Interestingly enough, the experimental μ values correlated with oxygen supply: increased oxygen supply led to increased μ [82].

Reaction and gene essentiality

In this study we linked gene (or reaction) essentiality to the inability to form biomass (maximal biomass flux = 0) on deletion of all reactions catalysed by that gene product. Reaction deletion was performed by setting a zero flux for the reaction in model. Mostly there were one-to one relations between genes and reactions but in some cases there were 1) redundant genes when each of alternative genes encoded enzyme (OR relationship), 2) two or more genes encoded polypeptides that form functional enzyme (AND relationship) and 3) one gene encoded more than one reaction. According to the analysis results (Online Resource 3), the model contained 38 essential reactions. (Fig. 2). The 26 reactions (23%) essential for all analysed substrates belonged to central carbon metabolism. Due to the small model size (113 reactions), there were not many redundant or parallel pathways included. Large scale experimental deletion studies with *S. cerevisiae* report 17% [99] and 19% [29] essential proteins for viability in rich medium which is close to our medium scale model based prediction. For comparison, 2 - 66% of reactions are essential across different eukaryotes [9].

Model optimisation

291 The model was optimised by FBA for production of ethanol, acetate, lactate, glycerol, ethylacetate,
292 succinate, glutamate phenylethanol and phenylalanine at fixed substrate uptake rate (glucose/inulin,
293 lactose and xylose) at 10 mM g^{-1} DW h^{-1} and μ at 0.4 h^{-1} as a compromise between different substrate
294 consumption and growth rates. The substrate uptake flux was set high to make flux distribution and
295 yield calculations more practical. The maximal percentage of substrate carbon atoms converted into
296 product (Fig.3 A) always was below the maximal theoretical yield (Fig. 3B).
297
298 *K. marxianus* metabolism is sensitive to the oxygen consumption rate. To assess the opportunities of
299 metabolic control by variable oxygen supply, optimisation (FBA) and variability analysis (FVA) were
300 performed for two extreme respiration cases – low (necessary for biomass production) (Fig. 3 C) and
301 high (Fig. 3 D) oxygen consumption rates at fixed $\mu = 0.4\ h^{-1}$ in the following steps:
302 1) maximal and minimal oxygen consumption was determined minimised/maximised by FVA at $\mu =$
303 0.4 h^{-1} for each substrate;
304 2) 90 and 100% of maximum oxygen consumption rate (determined in step 1) were set as lower and
305 upper oxygen consumption rate bounds FBA analysis at high oxygen consumption;
306 3) minimal and three minimal oxygen consumption rates (determined in step 1) were set as lower and
307 upper oxygen consumption rate bounds FBA analysis at low oxygen consumption;
308 3) maximal product rate at low (Fig. 3C)/high (Fig. 3D) oxygen consumption was determined by FBA;
309
310 In the case of no constraints on oxygen consumption, high values of carbon flux to product (Fig. 3A)
311 were predicted for lactate, glutamate and phenylalanine. Ethanol, acetate and ethyl acetate yields were
312 identical and close to their theoretical maxima (Fig. 3B). The lowest fractions of carbon flux to product
313 were in the cases of succinate, phenylethanol and glycerol. All other cases attained at least 75% of their
314 theoretical yields. Succinate was the only product that had higher yields on xylose as substrate
315 compared to other substrates (Fig. 3A). At minimal oxygen consumption (Fig. 3C) for most products
316 the carbon flux to products was lower in the case of xylose as substrate. With lactose, inulin and
317 glucose as substrates, ethanol and lactate yields were very close to the theoretical maxima. Carbon flux
318 to products at maximal oxygen (Fig. 3D) consumption still was relatively close to maximum in case of
319 ethanol and acetate with lactose, inulin and glucose as substrates while lactate production had become
320 very low.

Ethanol production

K. marxianus is able to convert lactose, inulin (fructose), glucose and xylose into ethanol. Maximum theoretical ethanol to substrate ratios are: for lactose 4, for inulin/glucose 2, but for xylose 1.6. The model predicted the maximum ratio of lactose conversion into ethanol to be 3.93, inulin/ glucose 1.91 and xylose 0.96. While the model predictions for lactose and glucose/ inulin were close to the theoretical maximum, the low ethanol: xylose ratio came as a surprise. This most probably relates to the carbon flux re-routing through the glucose-6P dehydrogenase reaction to generate the NADPH needed for xylose reduction. Additionally, according to the model, a notable portion (0.5 units per unit of xylose) was directed to acetate production.

Many authors have noted the need for an oxygen supply for *K. marxianus* growth on respiration when fermenting xylose as a sole carbon source [82]. *K. marxianus* respration mutants are not able to ferment xylose [49]. This dependence might be related to different reasons/ factors.

Firstly, to metabolise xylose, a *K. marxianus* cell needs enough resources of cytoplasmic NAD+ which is consumed by xylitol dehydrogenase (reaction XDH). The cytoplasmic demand for NAD+ is fulfilled by the alcohol dehydrogenase reaction (reaction Alcohol4) producing ethanol and NAD+ [95] or through the activity of the mitochondrial malate – aspartate shuttle [17, 36]. The model predicted that the latter is not active in the case of a poor oxygen supply.

Secondly, the model predicted acetate accumulation, up to 50 % of the ethanol flux, if oxygen consumption was kept low. A similar effect was demonstrated in xylose fermentation by *S. cerevisiae* with engineered XYLT, XDH and XK (reaction pengluc3) reactions [44, 70]. According to our model, a decrease in acetate production was observed if the xylose reductase (XYLT) cofactor specificity was changed from NADPH to NADH; in this case the ethanol to xylose ratio reaches 1.6. This *in silico* result complements *in vivo* results from various authors, who engineered the xylose reductase cofactor (enzyme preference to use NADH or NADPH) specificity in *S. cerevisiae* [44] or explored cofactor specifity of wild type xylose reductases of various yeast species [5].

A third reason why respiration activity is crucial for xylose utilisation, is because of increased ATP consumption to maintain cytoplasmic pH. Stambuk et al. [83] found that xylose uptake in K. marxianus is symport. Each xylose is imported together with a proton. In this process cytoplasmic pH drops. To maintain cytoplasmic pH unchanged, cell membrane ATP ases exports protons at the expense of ATP hydrolysis.

The model predicted the maximum ratio of fructose conversion into ethanol to be 1.9 units of ethanol per unit fructose or glucose. Flux variability analyses revealed that if the maximum ethanol flux changes by 10 %, then 4 – 17 % of incoming carbon flux is always routed to glycerol production. Within the limits of error, our *in vivo* data on inulin fermentation were in good correlation with this model prediction. We observed ethanol to monosaccharide flux ratio to be 1.72 +/- 10 % (comprising 90 % of maximum) while the rest of the carbon was distributed between glycerol and acetate.

Acetate

Acetic acid (ethanoic acid) is among the first chemicals to have been industrially produced by microorganisms. Traditionally bacterial producers (*Clostridium* sp. or *Acetobacter* sp.) are used. Potentially *K. marxianus* can produce acetic acid, though its commercial value is low. Acetic acid accumulation during exponential growth phase is typical for *K. marxianus* fermentations. This has been demonstrated by us as well as other authors [53, 82]. Acetate, as with glycerol, is perceived as an unwanted fermentation side product. In our model, acetate and acetylCoA reactions in our model were cofactor “entangled”, so when there was need for acetylCoA, extra acetate was produced; in parallel, there was a risk of cytoplasmic redox imbalance since acetate is produced along with NADH.

Maximum theoretical acetate/substrate ratios were calculated: for lactose 4, for inulin or glucose to acetate 2, but for xylose 1.6. The model predicted the maximum ratio of lactose conversion into acetate to be 3.94, inulin or glucose 1.94 and the maximum ratio of xylose conversion into ethanol to be 1.44 units of acetate per unit of xylose.

Acetate production can occur without formation of significant byproducts, but strong aeration must be supplied (optimal oxygen consumption up to 2 units of O_2 per unit of substrate). Based on our model

results, cytoplasmic acetate accumulation occurred in two situations – if there was need for extra NADH (as in succinate production) or need for cytoplasmic acetylCoA (in the case of ethyl acetate or biomass production).

Lactate

Lactic acid is widely used acid in the food industry and has potential application as a monomer for biodegradable plastics. In both of those applications L-lactic acid is used. Microbial (bacterial, yeast or fungi) fermentation is one of the options for L-lactic acid isomer synthesis in industrial amounts [35]. *Kluyveromyces sp.* has been proposed as a prospective lactic acid producer due to its fast production rates and GRAS status [15]. Yeasts do not have lactate dehydrogenase, therefore for lactate production recombinant strains harbouring LDH of eukaryotic origin (mammals, moulds) are used.

The theoretical molar yield of L-lactate from mole of glucose was 2, from lactose 4, but from xylose 1.6. Our model prediced L-lactate formation with the following molar ratios: 3.9 from lactose, 1.9 from glucose and 1.6 from xylose.

Introduction of heterologous lactate dehydrogenase alone does not lead to maximal L-lactate production *in vivo*. Carbon flow towards lactate or ethanol was divided at the level of pyruvate by pyruvate decarboxylase (reaction in the model PDC) or the pyruvate dehydrogenase complex in mitochondria. If the pyruvate gets decarboxylated, direct lactate production from pyruvate was not possible, instead carbon was routed to acetaldehyde and ethanol or acetate formation. Flux variability analyses revealed that this was the case – when simulating a decrease in lactate flux, an equimolar increase in CO_2 and ethanol fluxes was observed.

Kluyveromyces sp., unlike *Saccharomyces*, contain just one PDC gene, therefore preparation of *pdc* functional knockouts is comparatively easy. Lactate dehydrogenase overexpression in a *K. lactis pdc* strain has proven to be an efficient strategy yielding a lactic acid: consumed glucose ratio up to 0.5 [72]. Lactate production close to the theoretical maximum was achieved when both pyruvate consuming branches (pyruvate decarboxylase and dehydrogenase) were inactivated. A molar lactate/ glucose ratio close to 2 in *K. lactis pdc pdh* knockouts was obtained by Bianchi and colleagues [4].

411 Alternatively, additional heterologous expression of lactate dehydrogenase by increasing gene copy
412 numbers can be a strategy to increase lactate production [67].
413
414 **Glycerol production**
415
416 Glycerol is a typical byproduct of yeast ethanol fermentation that forms in response to the need to
417 balance cytoplasmic NADH oxidation. Glycerol formation as an NADH sink becomes crucial when
418 NADH oxidation via the electron transport chain is not possible (limited oxygen supply). Even though
419 glycerol synthesis by microbial producers *per se* has no applications in biotechnology, we included this
420 metabolite in our analyses since this is one of the major carbon and redox sinks in the *K. marxianus*
421 metabolism.
422 Theoretical maximal glycerol production from different substrates in molar ratios were: from lactose 4,
423 from glucose 2, from xylose 1.66. Our medium scale *K. marxianus* metabolic model predicted
424 maximum molar yields from lactose 2.4 from glucose 1.2 and from xylose 1.2. The model predicted the
425 need for a certain respiratory activity (up to 0.5 units of O_2 per unit of substrate) for glycerol
426 production to reach a maximum, hence CO_2 was the only byproduct in the case of optimal glycerol
427 production.
428 We and other researchers have observed similar effects *in vivo* in inulin and lactose fermentations with
429 *K. marxianus* – higher aeration leads to smaller ethanol and glycerol flux and *vice versa* [82]. Severe
430 fermentation dependence on oxygen supply has also been demonstrated in the physiology of *K.*
431 *marxianus'* sister species *K. lactis* [58].
432
433 **Ethyl acetate production**
434
435 Ethyl acetate is a volatile, slightly polar molecule, used as an organic solvent. Nowadays it has many
436 applications in cosmetics (nail polish remover), electronics (cleaning circuit boards, etc.), and has a
437 potential future application as an environmentally friendly acyl acceptor in biodiesel production instead
438 of methanol. Currently ethyl acetate is produced from petrochemical sources, but it can be produced
439 through biotechnological synthesis by many yeasts. Currently *K. marxianus* is regarded as the most
440 productive ethyl acetate producer [52].

For ethyl acetate, the theoretical molar product/substrate yield when considering pyruvate decarboxylation, was 2 for lactose, 1 for glucose and 1 for xylose. Our model predicted the maximum ethyl acetate to substrate ratio from lactose to be 1.97, 0.72 from xylose, and 0.97 for inulin or glucose. FVA results revealed strong effects of aeration on ethyl acetate formation. Most ethyl acetate was produced at increased aeration. However, the most effective ethyl acetate formation was not during growth with maximal respiration (Fig 3C). Additionally, FVA revealed a notable increase in glycerol production during oxygen limitation, which indicated the necessity of cytoplasmic NADH reoxidation to support acetate production. In the case of respiration, cytoplasmic NADH could be reoxidised through the electron transport chain and mitochondrial shuttle activity.

Careful fine-tuning of oxygen consumption might be a strategy for maximum ethyl acetate production. A similar strategy was applied when limiting *K. marxianus* access to metal ions [91, 92]. Metal ion (Fe, Cu, Zn) limitation was found to affect ethyl acetate production. Amongst them, Fe limitation had the most effect. *K. marxianus* culture starving for Fe produced ethyl acetate at close to 50% of theoretical maximum. Fe limitation lowered the activities of Fe-dependent mitochondrial aconitase and succinate dehydrogenase; this subsequently led to accumulation of acetylCoA, which was used to increase ethyl acetate production [92].

Succinate production

Succinate is one of the 12 most recognised sugar-derived chemical precursors. There is biotechnological potential for succinate due to its wide application spectrum, since it can serve as a precursor for tetrahydrofuran, butanediol, succinonitrile etc. Cheap microbial production of succinate has huge market potential [97]. There are already several examples of microbial succinate production at industrial scale (Reverdia, Myriant, BioAmber, BASFPurac, etc.). At least one of the processes is yeast-based (Reverdia, *S. cerevisiae*) [12].Even though succinate yields close to the theoretical stoichiometric maximum are reached by bacterial cells, yeast offer several advantages over bacteria: they are not obligately anaerobic, they are robust, acid and osmotically tolerant, and non pathogenic organisms [73].

The theoretical maximal molar ratio for succinate production from lactose was 3, from glucose 1.5 and xylose 1.25. Our *K. marxianus* carbon metabolism model predicted the maximum succinate production ratio from glucose to be 0.55, from lactose 1.1 but from xylose 0.78. From here, it seems, that xylose might be the most potent substrate for succinate production; however, there are not many *in vivo* results on succinate production by *Kluyveromyces sp.* from xylose. Interestingly, a xylose / ethanol mixture is suggested as a prospective substrate for glyoxylate production along with succinate (isocitrate lyase reaction) in *S. cerevisiae* and *K. lactis* isocitrate lyase overexpressed strains [45].

Succinate can be produced via the tricarboxylic acid cycle or the glyoxylate shunt. It is not a redox neutral product with respect to carbohydrate substrates – theoretically, 2 NAD+ are consumed per each molecule of succinate. Reduced cofactors can be oxidised in the electron transport chain or by production of glycerol or ethanol – NAD regenerating pathways. The model predicted accumulation of at least one byproduct when optimised for succinate production. FVA results demonstrated that, depending on oxygen supply, many byproducts were formed. Interestingly, the model predicted glycerol formation in the case of poor aeration, independent of substrate. The compensatory NADH reoxidation through increased glycerol production in *S. cerevisiae* strains, optimised for succinic acid production, was demonstrated *in vivo* [73].

Based on our medium scale model, phenylalanine can also be formed as a byproduct in rather large amounts (0.3 to 0.6 units of phenylalanine per unit of lactose) if oxygen is supplied in surplus (3.7 units of oxygen per unit of lactose). In this case, production of a relatively large amount of phenylalanine is possible, since our medium scale model is not nitrogen (ammonia) restricted (see model assumptions). In real applications, however, nitrogen bioavailability might prevent such high levels of phenylalanine production being reached.

Deletion of the genes for succinate dehydrogenase subunits is a popular strategy for yeast-based succinate production [73]. Succinate accumulation in the case of KlSDH1 (succinate dehydrogenase subunit) deletion was observed in the case of *Kluyveromyces lactis* [76]. Our model predicted that inactivation of the aspartate malate shuttle in combination with increased oxygen consumption (up to

501 1.3 per unit of glucose) would give maximum succinate yield, while inactivation of succinate
502 dehydrogenase together with inactivated glyoxylate shunt would be preferable in the case of
503 fermentation of xylose.
504
505 **Glutamate production**
506
507 In our central metabolism model, the *K. marxianus* biomass reaction consisted of 24 metabolites,
508 excluding the amino acids, although phenylalanine and glutamate are included in the model as desired
509 products. The amino acid content of yeast biomass is of particular industrial interest, since some of
510 them (like glutamic acid) are responsible for developing of umami taste [37]. Random mutations is a
511 typical method for generating yeast strain with increased glutamic acid content [61]. Here we provide
512 model-based theoretical analysis of possible scenarios for increasing glutamic acid yield from substrate
513 in *K. marxianus.*
514
515 The theoretical maximal glutamate production from different substrates in molar ratios would be: from
516 lactose 2.4, from glucose 1.2, from xylose 1. Our medium scale *K. marxianus* metabolic model
517 predicted maximum molar yields from lactose 1.97, from glucose 0.97, and from xylose 0.8. Glutamate
518 in *K. lactis* and, most probably also in *K. marxianus,* can be produced by either of two reactions:
519 NADP dependent glutamate dehydrogenase (EC 1.4.1.4 reaction GLUDE_nadp) or by GOGAT (EC
520 1.4.1.13, reaction Glude_NAD) [74]. Our model predicted the larger carbon flux to be routed through
521 NADPH-dependent glutamate dehydrogenase. To recover enough cytoplasmic oxoglutarate an NADP
522 – oxoglutarate – citrate shuttle was used (see Fig. 1). At the same time, a high respiration rate was
523 needed to reach maximum glutamate production if glucose or lactose were used as substrates (approx.
524 1.5 O_2 / glucose). Interestingly, a higher fractional of molar yield of glutamate was achieved by xylose
525 fermentation (80 % from theoretical) and less oxygen needed to be supplied per substrate moiety (1.2).
526 In addition, the main glutamate synthesis flux in *K. marxianus* consuming xylose was predominantly,
527 through the GOGAT reaction, unlike when lactose or glucose were consumed (see above).
528
529 In *K. marxianus* glutamate synthesis is tightly product-regulated by feedback inhibition. As in *S.*
530 *cerevisiae,* glutamate synthesis via NADPH dependent glutamate dehydrogenase is subject to nitrogen

531 catabolite repression [59]. To analyse these type of product – substrate interactions, kinetic modelling
532 would need to be applied.
533
534 **Phenylethanol and phenylalanine as products**
535
536 Phenylalanine is the precursor metabolite for many industrial fragrances as well as an ingredient of the
537 artificial sweetener aspartame. Phenylethanol is a rose flavour used in food and pharmaceutical
538 industries. It is usually extracted from rose petals. The global demand for phenylethanol continues to
539 increase, and it cannot be fulfilled by traditional extraction methods. Bacterial synthesis might be an
540 sustainable alternative for phenylethanol production [84]. Traditionally the yeast *S. cerevisiae* is
541 considered as a vehicle for phenylethanol production, thought there have been attempts to use other
542 yeasts, including *K. maxianus* [19].
543
544 Central *K. marxianus* carbon metabolism was extended to phenylethanol production. Phenylethanol
545 production in the model was introduced as a linear, 11 reaction chain from PEP and E4P. This chain
546 consumed additional NADPH, NADH, ATP and PEP.
547
548 The final steps of phenylethanol production were introduced according to Uzunov et al. [93]. The
549 model supported two instances of phenylethanol production by *K. marxianus:* from sucrose and
550 glucose It predicted the maximum phenylethanol to substrate ratio from lactose to be 1.1 and around
551 0.5 from xylose, inulin, sucrose or glucose. That is far above *in vivo* experiments where phenylethanol
552 to substrate ratio ranges around 0.02-0.1 were typically observed [28, 100].
553

554 Conclusions

555
556 We developed a mass and charge balanced stoichiometric model of central *Kluyveromyces marxianus*
557 carbon metabolism including biomass production. The model is published in forms ready for
558 simulation (SBML and COBRA toolbox formats). Most of the model information was based on recent
559 *K. marxianus* genome annotations [48]. Mitochondrial shuttles were included to describe proton and
560 molecule allocation between mitochondria and cytoplasm. The model is able to reproduce the

561 experimentally observed mix of industrially valuable products, as well as explaining formation of
562 unwanted side products (acetate and glycerol).
563
564 Our modeling results imply that oxygen control can be used to influence product yield and flux
565 distributions. Also cofactor swapping (NADPH to NADH and/or vice versa) can significantly improve
566 xylose conversion to products. Interestingly, xylose turned out to be a biotechnologically promising
567 substrate for *K. marxianus* with yet unused potential linked to fine-tuned redox engineering. Succinate
568 is a commercially appealing product that could potentially be produced from xylose in a more efficient
569 way compared to other substrates analysed.
570
571 The model predicted that ethanol, acetate, L-lactate and ethyl acetate can be produced at close to their
572 theoretical yield and without the need for genetic engineering in the cases of lactose, glucose and inulin
573 as substrates. At the same time, the model predicted that a high fraction of the phenylethanol and
574 succinate theoretical yields could not be achieved without metabolic engineering, for which the
575 proposed model is a powerful tool.
576
577 Our model can be used for analysis of *K. marxianus* and its metabolic engineering as well as basis of
578 larger scale models. It would be valuable to extend the model to include precise characteristics of
579 transport reactions (proton symport and antiport), together with the plasma membrane H^{+}-ATPase
580 system.
581
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585

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Fig. 1 The scheme of *Kluyveromyces marxianus* central carbon metabolism model

Fig 2. Model reaction essentiality for biomass production depending on substrate. Analyses revealed 26 essential reactions for all substrates, 5 reactions exclusively essential for xylose, and 4 for lactose. Glucose and inulin are shown as one substrate. Fructose kinase and inulinase were essential reactions for inulin consumption, while hexokinase was essential for both lactose and glucose consumption.

Fig 3 A. Maximal percentage of substrate carbon atoms converted into product at biomass growth μ=0.4 h^{-1}

Fig 3 B. Difference between theoretical yield and maximal carbon flux to product at biomass growth μ=0.4 h^{-1}

Fig. 3 C. Maximal percentage of substrate carbon atoms converted into product at low oxygen consumption and biomass growth fixed at μ=0.4 h^{-1}

Fig. 3 D Maximal percentage of substrate carbon atoms converted into product at high oxygen consumption and biomass growth fixed at μ=0.4 h^{-1}

Figure 1

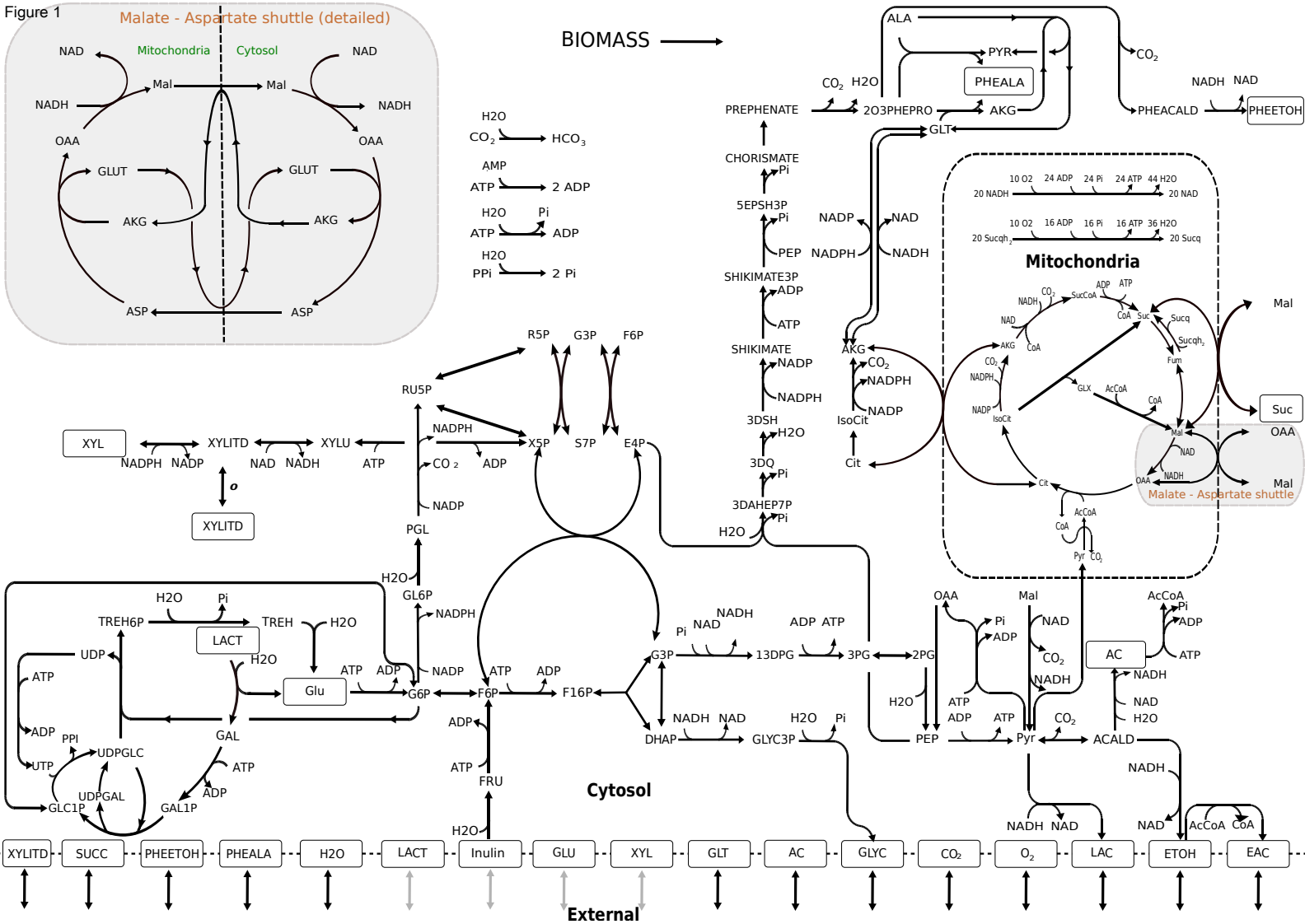
Malate - Aspartate shuttle (detailed)
Mitochondria
Cytosol
NAD
NADH
Mal
OAA
GLUT
AKG
ASP
BIOMASS
ALA
PYR
PHEALA
CO2
H2O
PREPHENATE
2O3PHEPRO
AKG
GLT
PHEACALD
PHEETOH
CHORISMATE
5EPSH3P
PEP
SHIKIMATE3P
SHIKIMATE
3DSH
3DQ
3DAHEP7P
NADP
NADPH
ADP
ATP
Pi
PPi
HCO3
2 ADP
2 Pi
Mitochondria
Malate - Aspartate shuttle
Suc
Sucq
Fum
Cit
IsoCit
AcCoA
CoA
GLX
R5P
G3P
F6P
RU5P
X5P
S7P
E4P
XYL
XYLITD
XYLU
PGL
GL6P
G6P
F6P
F16P
TREH6P
TREH
LACT
Glu
UDP
UDPGLC
GAL
GAL1P
UDPGAL
GLC1P
FRU
Inulin
G3P
13DPG
3PG
2PG
PEP
DHAP
GLYC3P
Pyr
ACALD
AC
OAA
Mal
Cytosol
External
XYLITD
SUCC
PHEETOH
PHEALA
H2O
LACT
Inulin
GLU
XYL
GLT
AC
GLYC
CO2
O2
LAC
ETOH
EAC
AcCoA
CoA

Figure 2

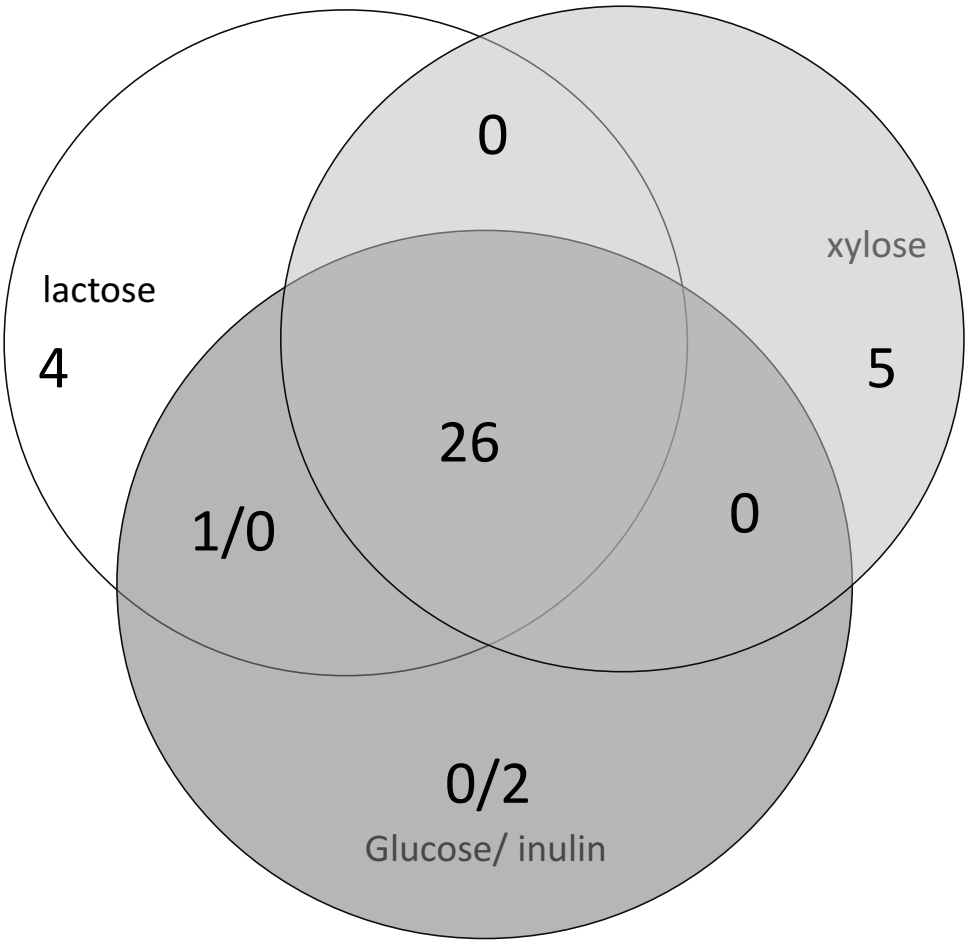

Figure 3

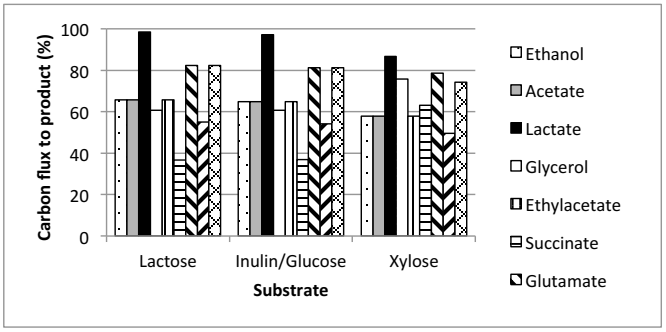

Fig 3 A. Maximal percentage of substrate carbon atoms converted into product at biomass growth μ=0,4 h^{-1}

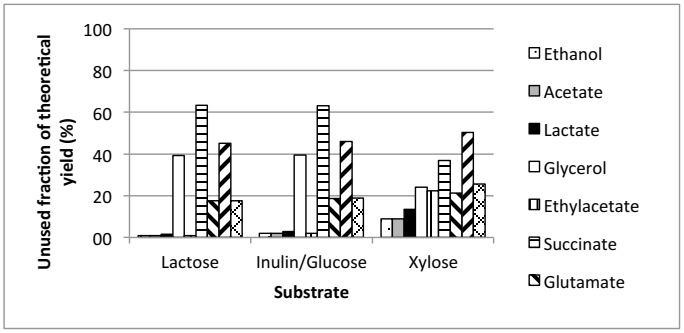

Fig 3 B. Difference between theoretical yield and maximal carbon flux to product at biomass growth μ=0,4 h^{-1}

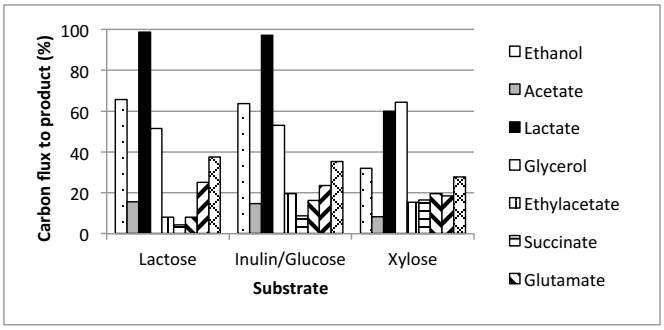

Fig. 3 C. Maximal percentage of substrate carbon atoms converted into product at low oxygen consumption and biomass growth fixed at μ=0,4 h^{-1}

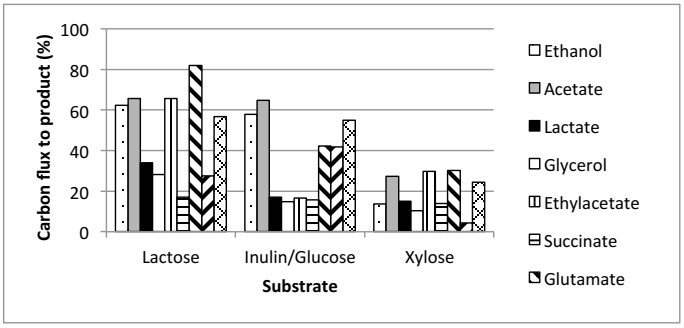

Fig. 3 D Maximal percentage of substrate carbon atoms converted into product at high oxygen consumption and biomass growth fixed at μ=0,4 h^{-1}

Table 1

Table 1. Model validation data. Substrate uptake, biomass growth and product formation fluxes (mM g DW^{-1} h^{-1}) were calculated from exponential phase of batch fermentations. Data was extracted from other author publications or obtained from our fermentations (denoted as "this study"). Means and standard deviation are calculated from 3 technical replicates where applicable.

Lactose as substrate	Substrate consumption	Biomass umax h^{-1}	Ethanol	Glycerol	Acetate	Ethylacetate	Aeration Vol/vol *min
This study, lactose	4.4 +/- 0.4	0.39 +/- 0.06	19 +/- 2	0.72 +/- 0.2	0.24 +/- 0.05	ND	0.2
This study, lactose	5.0 +/- 0.4	0.30 +/- 0.05	9.0 +/- 0.5	0.57 +/- 0.16	0.17 +/- 0.017	ND	1
[57]	13.04	0.31	30.04	0.86	0.19	ND	1
[77]	3.6	0.07	12	0.34	ND	ND	Self anaerobic
[51]	2.3	0.48	2.73	ND	ND	ND	3
[51]	2.1	0.40	7.2	ND	ND	ND	3
[53]	2.4	3.6	1.0	ND	3.0	0.97	1.32
Inulin, glucose and sucrose as substrates	Summary sugar / glucose consumption	Biomass umax h^{-1}	Ethanol	Glycerol	Acetate	Phenyl ethanol	Aeration Vol/vol *min
this study, glucose,	15.20	0.48	26.69	1.33	0.013	ND	0.25
this study, glucose,	13.71	0.54	24.25	0.46	0.008	ND	2.5
this study, inulin	5.8 +/- 0.5	0.25 +/- 0.2	10 +/- 1.0	0.16 +/- 0.02	0.04 +/- 0.002	ND	1.5

[43], substrate glucose	0.47	0.02	ND	ND	ND	0.7	ND
[78], substrate inulin	2.60	0.2	ND	ND	ND	ND	Shake flasks
[27], substrate inulin	42.6	0.26	72.4	ND	ND	ND	Self anaerobic
[101], substrate inulin	16	0.14	18	ND	ND	ND	Shake flasks
[28], substrate grape must	2.3	0.40	1.1	ND	ND	0.068	1 and 2
[20], substrate sucrose	4.8	0.26	10	ND	ND	0.33	Shake flasks
[100], substrate glucose	5.0	0.081	1.7	0.34	ND	0.14	1
Xylose as substrate	Substrate consumption	Biomass umax h^{-1}	Ethanol	Acetate	Xylitol		Aeration Vol/vol *min
This study,	1.06	0.11	0.18	0.04	ND	ND	2.5
This study,	1.38	0.089	0.085	0.00007	ND	ND	0.25
[82]	0.55	0.014	0	0.4	ND	ND	1
[14]	0.43	0.007	0.28	ND	ND	ND	1
[42]	0.84	0.023	ND	ND	0.66	ND	Shake flasks
[62]	5.78	0.09	3.02	ND	0.04	ND	Shake flasks, +30
[62]	7.54	0.10	2.55	ND	2.44	ND	Shake flasks +37
[81]	1.20	0.13	ND	ND	ND	ND	Shake flasks
[56]	1.83	0.08	1.54	ND	ND	ND	Shake flasks

[2]	0.69	0.12	ND	ND	ND	ND	Shake flasks
Simultaneous uptake of xylose and glucose	Glucose/xylose consumption	Biomass umax h^{-1}	Ethanol	Acetate	Glycerol	Xylitol	Aeration Vol/vol *min
[105]	4.94/ 6.67	0.17	4.83	ND	ND	4.02	anaerobic