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

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## 13 14 **Abstract**

15  
16 The non-conventional yeast *Kluyveromyces marxianus* is an emerging industrial producer for many  
17 biotechnological processes. Here we show the application of a biomass-linked stoichiometric model of  
18 central metabolism that is experimentally validated, and mass and charge balanced for assessing the  
19 carbon conversion efficiency of wild type and modified *K. marxianus*. Pairs of substrates (lactose,  
20 glucose, inulin, xylose) and products (ethanol, acetate, lactate, glycerol, ethyl acetate, succinate,  
21 glutamate, phenylethanol and phenylalanine) are examined by various modeling and optimisation  
22 methods.

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

28  
29 **Keywords** *Kluyveromyces marxianus*. Modelling. Central metabolism. Metabolic engineering.  
30 Essentiality analysis.

## 31 **Introduction**

32

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

39

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

51

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

56

57 Stoichiometric models and reconstructions significantly facilitate analysis of metabolic effects and  
58 limitations of microorganism metabolism, as well as predicting the phenotype of recombinant strains  
59 [39, 40]. Modeling attempts on *K. marxianus* to date have been concentrated on particular problems:

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

71

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

78

## 79 **Materials and methods**

80

### 81 **Modeling methodology and software**

82

83 Two major strands of stoichiometric modelling are the constraint-based Flux Balance Analysis (FBA)  
84 [63, 94] and elementary modes analysis [80]. A constraint based model of central metabolism including  
85 biomass production of *K. marxianus* was created adapting and combining the high-quality genome-  
86 scale metabolic reconstructions protocol [79] and structural modelling approach for development of  
87 medium scale reconstruction and models [39].

88

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

$$91 \quad dX/dt = r_{\text{met}} - \mu X_{\text{met}}$$

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

$$94 \quad 0 = r_{\text{met}} - \mu X_{\text{met}} \text{ [85].}$$

95

96 We also assume the following:

97 - the specific growth rate ( $\mu$ ,  $\text{h}^{-1}$ ) during the exponential growth phase is constant,

98 - the cells are at pseudosteady state: substrate uptake, metabolite and product fluxes are constant when  
99  $\mu$  is constant.

100

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

114

## 115 **Reactions**

116

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

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

126

### 127 **Metabolites**

128

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

133

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

135

136 The results of original experiments carried out by us to provide data for model development are marked  
137 in Table 1 as “this study”. *K. marxianus* strain DSM 5422 was cultivated in semi synthetic medium  
138 containing (g/l) KHPO<sub>4</sub> (1.0), CaCl<sub>2</sub> (0.1), MgSO<sub>4</sub>\*7H<sub>2</sub>O (0.5), NaCl (0.5), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (5.0) KH<sub>2</sub>PO<sub>4</sub>  
139 (0.1) yeast extract (*Biolife*) (0.5). Different carbon sources (lactose or inulin) were added at  
140 concentrations of 5 or 10 % w/v. All fermentations were carried out in 1 litre *Infors* 2HT or 0.4 litre  
141 *Sartorius Biostat Qplus* 6-fold system fermenters at 35°C and 400 rpm.

142

### 143 **Metabolite and biomass analyses**

144 Extracellular lactose, ethanol, acetate and glycerol content were measured simultaneously using an  
145 *Agilent 1100 HPLC* system with a *Shodex Asahipak SH1011* column. Metabolites were quantitated  
146 with a refractive index detector (RI detector *RID G1362A*). The flow of the mobile phase (0.01 N  
147 H<sub>2</sub>SO<sub>4</sub>) was 0.6 ml min<sup>-1</sup>, the sample injection volume was 5 µL.

148 Biomass growth was estimated by absorbance measurements at 600 nm (OD600). The conversion  
149 coefficient of *K. marxianus* DSM 5422 strain OD600 to culture dry weight was determined  
150 gravimetrically: OD600 1.0 was equivalent to 0.3 g dw.L<sup>-1</sup>.

151

## 152 **Results and Discussion**

153

### 154 **Model construction and properties**

155

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

162

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

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

172

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

176

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

182

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

193

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

198

199

## 200 **Model validation**

201



202 **Data sources for validation/calibration.**

203

204 The main carbon fluxes for model validation in *K. marxianus* were: substrate uptake, CO<sub>2</sub>, ethanol,  
205 glycerol, acetate and biomass production. For a *K. marxianus* batch cultivation with limited oxygen  
206 supply these fluxes can account for up to 100 % of total carbon [77]. Therefore this set of fluxes is  
207 sufficient to validate this medium scale model. A similar set of fluxes has been successfully applied to  
208 validate the medium scale carbon metabolism model of *Pichia pastoris* [87].

209

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

213

214 **Model validation on lactose as substrate**

215

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

224

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

229

230 Depending on the oxygen supply, *K. marxianus* lactose fermentation is biomass (aerobic) or ethanol  
231 (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 ( $\mu = 0.07 \text{ h}^{-1}$ ) 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  $\mu$ ), 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**

262

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

269

270 There are many reports of xylose fermentation by *K. marxianus*. We chose three example  
271 fermentations [14, 56, 82] to extract data for model validation. All three xylose fermentations yielded  
272 slow biomass growth with  $\mu$  varying from 0.007 to 0.08 h<sup>-1</sup>. Interestingly enough, the experimental  $\mu$   
273 values correlated with oxygen supply: increased oxygen supply led to increased  $\mu$  [82].

274

## 275 **Reaction and gene essentiality**

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

288

## 289 **Model optimisation**

290

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  $10 \text{ mM g}^{-1} \text{ DW h}^{-1}$  and  $\mu$  at  $0.4 \text{ h}^{-1}$  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  $\mu = 0.4 \text{ h}^{-1}$  in the following steps:

302 1) maximal and minimal oxygen consumption was determined minimised/maximised by FVA at  $\mu =$   
303  $0.4 \text{ h}^{-1}$  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.

321

322 **Ethanol production**

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

331

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

335

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

341

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

350

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

356

357 The model predicted the maximum ratio of fructose conversion into ethanol to be 1.9 units of ethanol  
358 per unit fructose or glucose. Flux variability analyses revealed that if the maximum ethanol flux  
359 changes by 10 %, then 4 – 17 % of incoming carbon flux is always routed to glycerol production.  
360 Within the limits of error, our *in vivo* data on inulin fermentation were in good correlation with this  
361 model prediction. We observed ethanol to monosaccharide flux ratio to be 1.72 +/- 10 % (comprising  
362 90 % of maximum) while the rest of the carbon was distributed between glycerol and acetate.

363

#### 364 **Acetate**

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

373

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

378

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

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

384

### 385 **Lactate**

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

392

393 The theoretical molar yield of L-lactate from mole of glucose was 2, from lactose 4, but from xylose  
394 1.6. Our model predicted L-lactate formation with the following molar ratios: 3.9 from lactose, 1.9 from  
395 glucose and 1.6 from xylose.

396

397 Introduction of heterologous lactate dehydrogenase alone does not lead to maximal L-lactate  
398 production *in vivo*. Carbon flow towards lactate or ethanol was divided at the level of pyruvate by  
399 pyruvate decarboxylase (reaction in the model PDC) or the pyruvate dehydrogenase complex in  
400 mitochondria. If the pyruvate gets decarboxylated, direct lactate production from pyruvate was not  
401 possible, instead carbon was routed to acetaldehyde and ethanol or acetate formation. Flux variability  
402 analyses revealed that this was the case – when simulating a decrease in lactate flux, an equimolar  
403 increase in CO<sub>2</sub> and ethanol fluxes was observed.

404

405 *Kluyveromyces sp.*, unlike *Saccharomyces*, contain just one PDC gene, therefore preparation of *pdc*  
406 functional knockouts is comparatively easy. Lactate dehydrogenase overexpression in a *K. lactis pdc*  
407 strain has proven to be an efficient strategy yielding a lactic acid: consumed glucose ratio up to 0.5  
408 [72]. Lactate production close to the theoretical maximum was achieved when both pyruvate  
409 consuming branches (pyruvate decarboxylase and dehydrogenase) were inactivated. A molar lactate/  
410 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<sub>2</sub> per unit of substrate) for glycerol  
426 production to reach a maximum, hence CO<sub>2</sub> 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].



441

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

451

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

459

#### 460 **Succinate production**

461

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

471

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

479

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

489

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

496

497 Deletion of the genes for succinate dehydrogenase subunits is a popular strategy for yeast-based  
498 succinate production [73]. Succinate accumulation in the case of KISDH1 (succinate dehydrogenase  
499 subunit) deletion was observed in the case of *Kluyveromyces lactis* [76]. Our model predicted that  
500 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<sub>2</sub> / 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, though there have been attempts to use other  
542 yeasts, including *K. marxianus* [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<sup>+</sup>-ATPase  
580 system.

581

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583

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585

586

587

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888

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

890

891 Fig 2. Model reaction essentiality for biomass production depending on substrate.

892 Analyses revealed 26 essential reactions for all substrates, 5 reactions exclusively essential for  
893 xylose, and 4 for lactose. Glucose and inulin are shown as one substrate. Fructose  
894 kinase and inulinase were essential reactions for inulin consumption, while hexokinase  
895 was essential for both lactose and glucose consumption.

896

897 Fig 3 A. Maximal percentage of substrate carbon atoms converted into product at biomass

898 growth  $\mu=0.4 \text{ h}^{-1}$

899

900

901 Fig 3 B. Difference between theoretical yield and maximal carbon flux to product at biomass

902 growth  $\mu=0.4 \text{ h}^{-1}$

903

904

905 Fig. 3 C. Maximal percentage of substrate carbon atoms converted into product at low oxygen

906 consumption and biomass growth fixed at  $\mu=0.4 \text{ h}^{-1}$

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908

909 Fig. 3 D Maximal percentage of substrate carbon atoms converted into product at high oxygen

910 consumption and biomass growth fixed at  $\mu=0.4 \text{ h}^{-1}$

911



Figure 2

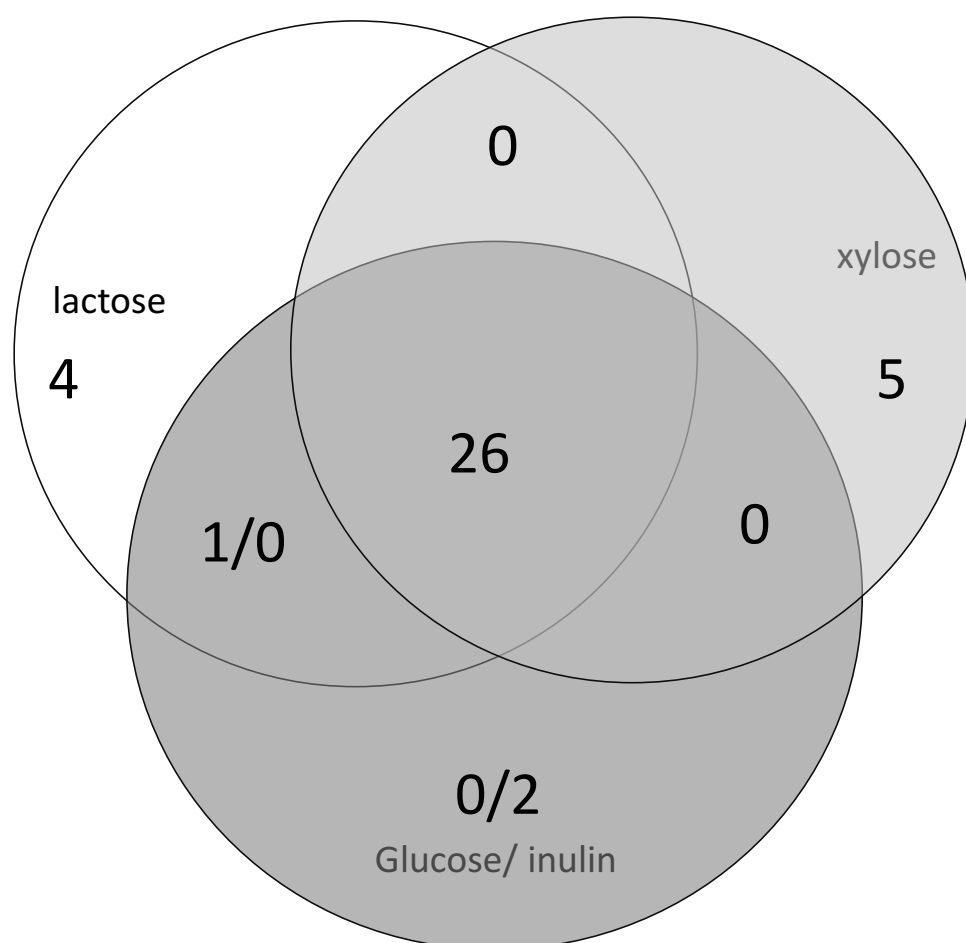


Figure 3

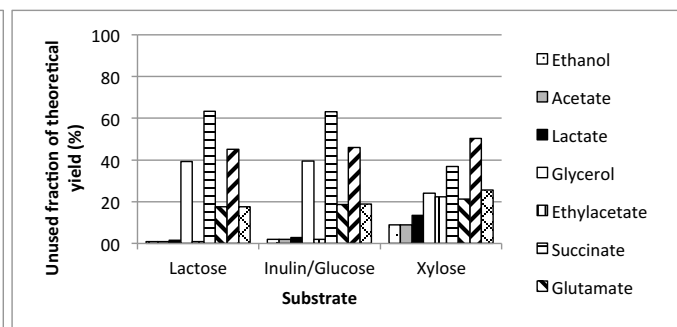
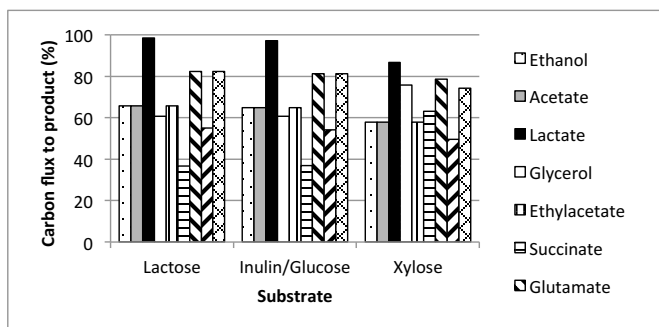


Fig 3 A. Maximal percentage of substrate carbon atoms converted into product at biomass growth  $\mu=0,4 \text{ h}^{-1}$

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

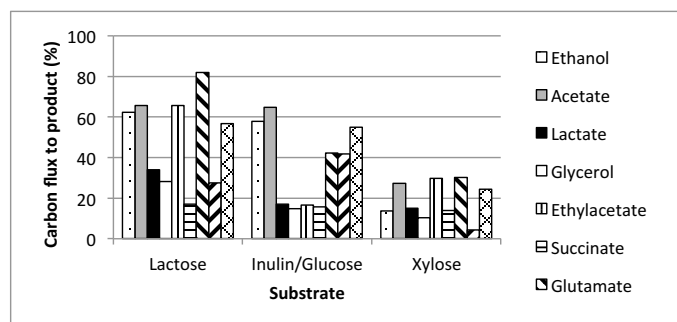
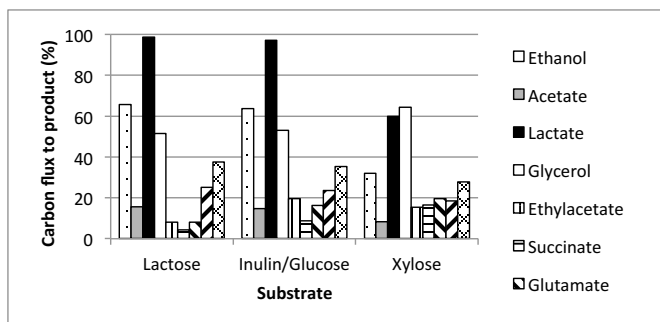


Fig. 3 C. Maximal percentage of substrate carbon atoms converted into product at low oxygen consumption and biomass growth fixed at  $\mu=0,4 \text{ h}^{-1}$

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

1 **Table 1.** Model validation data. Substrate uptake, biomass growth and product formation fluxes (mM  
 2 g DW<sup>-1</sup> h<sup>-1</sup>) were calculated from exponential phase of batch fermentations. Data was extracted from  
 3 other author publications or obtained from our fermentations (denoted as “this study”).  
 4 Means and standard deviation are calculated from 3 technical replicates where applicable.  
 5

<b>Lactose as substrate</b>	Substrate consumption	Biomass $\mu_{max}$ h <sup>-1</sup>	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
<b>Inulin, glucose and sucrose as substrates</b>	Summary sugar / glucose consumption	Biomass $\mu_{max}$ h <sup>-1</sup>	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
<b>Xylose as substrate</b>	Substrate consumption	Biomass $u_{max} h^{-1}$	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
<b>Simultaneous uptake of xylose and glucose</b>	Glucose/xylose consumption	Biomass $\mu_{max} h^{-1}$	Ethanol	Acetate	Glycerol	Xylitol	Aeration Vol/vol *min
[105]	4.94/ 6.67	0.17	4.83	ND	ND	4.02	anaerobic

6