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# Biotechnology for Biofuels

## An accurate description of *Aspergillus niger* organic acid batch fermentation through dynamic metabolic modelling --Manuscript Draft--

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|--|--|-----------------|
| <b>Manuscript Number:</b>                            | BBIO-D-17-00324  |                 |
| <b>Full Title:</b>                                   | An accurate description of <i>Aspergillus niger</i> organic acid batch fermentation through dynamic metabolic modelling  |                 |
| <b>Article Type:</b>                                 | Research   |                 |
| <b>Section/Category:</b>                             | Fungal/yeast genetics, physiology and metabolic engineering  |                 |
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| <b>Abstract:</b>                                     | <p><b>Background</b></p> <p><i>Aspergillus niger</i> fermentation has provided the chief source of industrial citric acid for over 50 years. Traditional strain development of this organism was achieved through random mutagenesis, but advances in genomics have enabled development of genome-scale metabolic modelling that can be used to make predictive improvements in fermentation performance. The parent citric acid producing strain of <i>A. niger</i>, ATCC 1015, has been described previously by a genome-scale metabolic model that encapsulates its response to ambient pH. Here, we report the development of a novel double optimisation modelling approach that generates time-dependent citric acid fermentation using dynamic flux balance analysis.</p> <p><b>Results</b></p> <p>The output from this model shows a good match with empirical fermentation data. Our studies suggest that citric acid production commences upon a switch to phosphate-limited growth and this is validated by fitting to empirical data, which confirms the diauxic growth behaviour and the role of phosphate storage as polyphosphate.</p> <p><b>Conclusions</b></p> <p>The calibrated time-course model reflects observed metabolic events and generates reliable in silico data for industrially relevant fermentative time series, and for the behaviour of engineered strains suggesting that our approach can be used as a powerful tool for predictive metabolic engineering.</p> |                 |
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1           **An accurate description of *Aspergillus niger* organic acid batch**  
2           **fermentation through dynamic metabolic modelling**

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## 24 **Abstract**

### 25 **Background**

26 *Aspergillus niger* fermentation has provided the chief source of industrial citric acid  
27 for over 50 years. Traditional strain development of this organism was achieved  
28 through random mutagenesis, but advances in genomics have enabled development  
29 of genome-scale metabolic modelling that can be used to make predictive  
30 improvements in fermentation performance. The parent citric acid producing strain of  
31 *A. niger*, ATCC 1015, has been described previously by a genome-scale metabolic  
32 model that encapsulates its response to ambient pH. Here, we report the  
33 development of a novel double optimisation modelling approach that generates time-  
34 dependent citric acid fermentation using dynamic flux balance analysis.

### 35 **Results**

36 The output from this model shows a good match with empirical fermentation data.  
37 Our studies suggest that citric acid production commences upon a switch to  
38 phosphate-limited growth and this is validated by fitting to empirical data, which  
39 confirms the diauxic growth behaviour and the role of phosphate storage as  
40 polyphosphate.

### 41 **Conclusions**

42 The calibrated time-course model reflects observed metabolic events and generates  
43 reliable *in silico* data for industrially relevant fermentative time series, and for the  
44 behaviour of engineered strains suggesting that our approach can be used as a  
45 powerful tool for predictive metabolic engineering.

46 **Keywords:** *Aspergillus niger*; citric acid; dFBA; metabolic modelling; polyphosphate

## 47 **Background**

48 Due to its natural ability to secrete organic acids and proteins, the filamentous  
49 fungus *Aspergillus niger* is an established organism for the industrial production of  
50 citric acid and enzymes. *A. niger* is metabolically highly versatile, a feature that has  
51 made it useful for a wide range of biotechnological biotransformations [1]. *A. niger*  
52 also produces a wide range of secondary metabolites, with over 100 reported to date  
53 [2]. *A. niger* is a saprotroph and its natural habitat is soil, although it can be found in  
54 wide-ranging habitats, such as rotting fruit, plant debris, and indoor environments.  
55 This fast-growing fungus is both acid- and thermo-tolerant, able to grow in the pH  
56 range 1.4-9.8 and in the temperature range 6-47°C [3]. This versatility and its ease of  
57 culture has helped it become an established industrial organism. Its haploid genome  
58 is around 35 Mb in size with 8 chromosomes which contain about 12,000 genes,  
59 57% of which have functional assignments [4]. Aspergilli are an important and  
60 diverse group, which in addition to *A. niger*, include well-studied species such as the  
61 model genetic organism *A. nidulans*, the pathogen *A. fumigatus* and the  
62 domesticated *A. oryzae*. Full genome sequences are currently available for 18  
63 species of the Aspergilli group [5] and some of these have been subject to extensive  
64 systems biology studies [6].

65  
66 With global production of 2 million tonnes a year, citric acid is an industrial chemical  
67 with many applications [7]. Its main use is in the food and drinks industry, but is also  
68 used in cleaning agents, pharmaceuticals, animal feed, and metal cleaning [8].  
69 Industries using *A. niger* fermentation are dependent on sucrose-based feedstocks,

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70 but with rising costs and increasing concerns over food security, a switch to more  
71 sustainable and lower cost feedstocks is desirable [9]. *A. niger* can assimilate a wide  
72 range of carbon sources, and therefore has great potential for exploiting underused  
73 resource streams such as pentose sugars from lignocellulose.

74

75 The best industrial strains are capable of producing over 70% of the theoretical yield  
76 of citric acid [10]. Such strains have been developed over many decades by time-  
77 consuming random mutagenesis. The genotype of resulting strains remains  
78 unknown, and random mutagenesis can lead to genetic instability of developed  
79 strains. Rational engineering of *A. niger* is now feasible, particularly with advances in  
80 genomics over recent years that have paved the way for genome-scale metabolic  
81 modelling [5, 11]. Industrially, *A. niger* is utilised via large-scale batch fermentations  
82 rather than continuous culture methods, typically in reactors in excess of 100,000  
83 litres [12]. In order for genome-scale models to accurately capture the behaviour of  
84 these cultures, techniques which model the batch growth, rather than simple  
85 chemostat-like cultures, are required.

86

87 The genome of the parent citric acid producing strain of *A. niger*, ATCC 1015, has  
88 been sequenced [4]. This enabled development of the genome-scale metabolic  
89 model for *A. niger*, MA871, which reflects ATCC 1015 metabolism [13]. The model  
90 was further developed to reflect the well-known behaviour of *A. niger* to acidify its  
91 surroundings in response to ambient pH [14]. This was achieved by incorporating  
92 acid-dissociation reactions for seven organic acids reportedly secreted by *A. niger*.

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93 Each reaction gives the number of protons released by a particular acid as a function  
94 of ambient pH. Citric acid production was modelled statically using flux balance  
95 analysis (FBA). The objective function was either set to proton production at a fixed  
96 growth rate or proton production was incorporated into the biomass equation. The  
97 nature of organic acid production in response to ambient pH is, however, a dynamic  
98 one, with acid-dissociation reactions changing as protons are produced.

99

100 In this article, we further develop the *A. niger* metabolic model to take into account  
101 the dynamic nature of organic acid production. By designing a novel modelling  
102 approach that employs dynamic flux balance analysis (dFBA), we demonstrate a  
103 model that gives time-course fermentative series of citric acid production. We  
104 validate the new model by fitting to empirical data from ATCC 1015 citric acid  
105 fermentations, and demonstrate how the resultant time-course calibrated model can  
106 be used as a powerful platform for metabolic engineering of *A. niger*.

107

## 108 **Results**

### 109 **Citric acid fermentation occurs as part of a diauxic growth response**

110 To investigate citric acid production by the parent citric acid producing ATCC 1015  
111 strain, empirical time course data were obtained from fermentation performed in  
112 shake flasks. Biomass and citric acid production were monitored with samples taken  
113 at 24 hour time-points. Diauxic growth behaviour was observed, with a drop in  
114 growth rate at day 3 (Fig 1A). Citric acid production commenced at day 3, coinciding  
115 with the diauxic growth shift (Fig 1B). 60 g/L citric acid was produced.

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2 117 In order to better understand the basis of this growth behaviour we developed a  
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5 118 dynamic flux balance analysis (dFBA) model based on the previously published FBA  
6  
7 119 model [13, 14]. To validate the model and further investigate the diauxic growth  
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10 120 behaviour, empirical data were obtained for citric acid fermentation under a range of  
11  
12 121 phosphate levels (0.05, 0.09 and 0.17 g/L). Samples of the cultures were taken  
13  
14 122 every 24 hours to produce a time-course of biomass dry weight, phosphate  
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17 123 depletion, citric acid production, and glucose consumption (Fig 2). Phosphate was  
18  
19 124 rapidly taken up and depleted by day 2 (Fig 2B), yet growth continued (Fig 2A).  
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22 125 Phosphate was therefore clearly stored internally to enable growth during absence of  
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24 126 external phosphate. Diauxic growth was observed, with growth becoming phosphate-  
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27 127 limited. The diauxic growth shift was synchronous with depletion of external  
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29 128 phosphate. The phosphate-limited growth rate was a function of the initial phosphate  
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32 129 concentration, with increased growth rate at higher phosphate. The timing of citric  
33  
34 130 acid production was observed to coincide with the onset of phosphate-limited growth  
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37 131 and external phosphate depletion (Fig 2C). Up to 50 g/L citric acid was produced,  
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39 132 with the culture at 0.17 g/L phosphate producing the most. Glucose uptake was  
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42 133 relatively slow for the lower phosphate cultures and a limiting factor in citric acid  
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44 134 production (Fig 2D).

135

47  
48 136 From these observations, we hypothesised that the diauxic growth shift is caused by  
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51 137 a switch to phosphate-limited growth, resulting in citric acid production. This  
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54 138 hypothesis was motivated by examination of our data, existing knowledge of *A. niger*



139 [10] and also the ecological evidence that organic acids are released extracellularly  
140 in order to facilitate the mobilisation of phosphate, especially in soil [15]. We decided  
141 to examine the plausibility of this hypothesis using dFBA modelling.

142

### 143 **Simulating citric acid fermentation by dynamic flux balance analysis**

144 To create time-course simulations comparable to the citric acid fermentation  
145 empirical data, dynamic flux balance analysis (dFBA) was used with the *MA871*  
146 metabolic model [13]. Citric acid production was modelled by incorporating kinetic  
147 acid-dissociation reactions into the dFBA schema for the organic acids in *MA871*  
148 and setting the objective to proton production. This explicit inclusion leads to an acid  
149 hierarchy [14], which suggested that citric acid production was the most efficient  
150 means of acidification with oxalic acid production switched off.

151

152 In the standard setting for the metabolic model citric acid secretion is included as a  
153 part of the external constraints during growth [13]; however, this is not supported by  
154 our observations. Therefore, a novel modelling approach was designed to simulate  
155 the diauxic growth behaviour with citric acid production commencing upon a diauxic  
156 growth shift coupled to phosphate intake. To achieve this, a double optimisation  
157 dFBA setup was designed (Fig 3). The objective is first set to biomass production,  
158 with the maximised growth rate then used in the second optimisation. The second  
159 objective is dependent on the growth-limiting condition of the first optimisation. The  
160 decision process uses a boolean expression. If the external phosphate flux is lower  
161 than its flux constraint, the second objective is set to phosphate storage to store

162 excess phosphate not used for growth. Otherwise, external phosphate flux is equal  
163 to its flux constraint and the second objective is set to proton production to make use  
164 of the carbon not used for growth (phosphate-limited growth).

165

166 The dynamic modelling approach, dFBA, therefore includes a number of metabolite  
167 pools that are tracked outside of the FBA, including external glucose, external  
168 phosphate, external pH, organic acids as well as the hypothesised stored phosphate.  
169 These metabolite pools are linked to the FBA simulations at each step via first order  
170 differential equations describing transport processes. These differential equations  
171 are solved at each time-step to provide flux constraints for the FBA optimisations  
172 occurring in a tandem fashion and assuming the metabolic system remains at a  
173 steady-state despite the small changes in the external constraints. All equations  
174 used are detailed in the methods, but are essentially either linear diffusion or  
175 Michaelis-Menten transport equations across the membrane as described below and  
176 mathematically in the methods section. Literature sources were used to  
177 parameterise the model wherever available as described below.

178

179 Following previous studies [16, 17], glucose uptake was modelled as the sum of  
180 passive diffusion and facilitated diffusion, using empirical values from the literature  
181 [16, 17] for all transport-mediated kinetic parameters (Table 2). The calculated  
182 parameter for passive diffusion overestimated glucose uptake, and therefore was  
183 fitted to empirical data (Table 1). Transport-mediated glucose uptake in *A. niger* is  
184 inhibited by low pH and non-competitively inhibited by external citrate [17], and this

185 was therefore included in the modelled glucose uptake. *A. niger* has both low- and  
186 high-affinity glucose transport systems [17], both of which were included in the  
187 model. The low-affinity system is reported only active above 150 g/L glucose, [17]  
188 and so this system was only included in the model at high glucose (>150 g/L).

189

190 Phosphate uptake and release of stored phosphate were modelled according to  
191 Michaelis-Menten kinetics. As no characterised phosphate transporters could be  
192 found for *A. niger* in the literature, kinetic parameters were fitted to empirical data on  
193 phosphate uptake (Table 1).

194

#### 195 **Fitting of model parameters to empirical data and model validation**

196 The empirical data obtained from the experiment varying phosphate were used to fit  
197 model parameters and validate the model. A total of eight parameters were fitted to a  
198 data-set containing 84 data-points. As each data-point was in quadruplicate with very  
199 low error margins, we decided to use the data-set for both model training and  
200 validation. The trained model was later applied to independent data-sets (Fig 4),  
201 which gave further validation. Using the trained model, citric acid fermentation was  
202 simulated for each of the phosphate levels tested, and model predictions plotted  
203 alongside empirical data (Fig 2). The modelled diauxic growth behaviour gave good  
204 fits to empirical data, with external phosphate depletion being the trigger that results  
205 in phosphate-limited growth and citric acid production. All the model outputs showed  
206 a strong qualitative comparison to the empirical data with unfitted parameters taken  
207 directly from the literature. Notably the modelled glucose uptake fitted empirical data

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208 closely (Fig 2D) with unadjusted literature values for transport-mediated uptake rate  
209 and affinity.

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211 However, a number of adjustments were required for the model to fit the empirical

212 data more closely. In particular, the model underestimated biomass production

213 during phosphate-limited growth, suggesting a lower phosphate demand not

214 reflected in the MA871 biomass equation. These contrasting observations in the

215 different areas of growth suggest that the biomass equation for the MA871 model

216 represents an average biomass composition over different growth conditions and

217 that therefore the biomass equation needs to be altered. Differences in biomass

218 composition in different growth conditions have previously been reported in

219 *Escherichia coli* [18]. To reflect citric acid producing conditions, two new fitted

220 parameters were added to the model, the nucleic acid and phospholipid components

221 of the biomass equation (Supplementary Table S1). The ratios between the different

222 components of each, and the total mass of the biomass components were kept

223 constant. Change in mass was balanced by adjustment of the glycerol component,

224 which has been reported to increase during citric acid producing conditions [19]. The

225 additional parameters increase the complexity of the model, and the likelihood of

226 overfitting. Therefore, Akaike Information Criterion (AIC) [20] was used to measure

227 the quality of fit and assess improvement in the model (Table 4) (see Methods).

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229 Our model initially overestimated citric acid production. This may be due to the many

230 internal constraints imposed on the internal metabolism by the intracellular

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231 accumulation of, or simply high throughputs of citrate that are not accounted for by  
232 the steady-state methodology of flux balance analysis. For example, the citrate  
233 sensitivity of 6-phosphofructo-1-kinase is a target of attempts to increase citrate  
234 production [21] and the rates of mitochondrial citrate export [22] and citrate secretion  
235 may be limiting. To reflect these constraints a limit to the citric acid output rate,  $v_{CIT}$ ,  
236 was added and fitted as a parameter to more closely reflect empirical data (Table 1).  
237 Carbon uptake was decreased slightly as a result of the constraint on citric acid  
238 output, but still gave close fits to empirical data. The new model was assessed by  
239 calculating the AIC (see Methods), which showed a significant improvement (Table  
240 4).

241

#### 242 **Citric acid production on other carbon sources**

243 To further investigate the diauxic growth behaviour, we tested citric acid fermentation  
244 using D-xylose as a substrate at an initial concentration of 160 g/L. The same diauxic  
245 growth shift coupled citric acid response was seen with xylose (Fig 4) as seen with  
246 glucose. We applied our model, with previously fitted parameters unchanged. The  
247 empirical data from this experiment was not used in previous model training, and  
248 served to provide further validation with glucose as substrate and at a different  
249 phosphate level. The uptake rate of xylose was modelled similarly to glucose as the  
250 sum of passive and facilitated diffusion. The kinetic parameters for xylose uptake  
251 were fitted to our empirical data (Table 1). Close fits were achieved for biomass  
252 production and carbon source consumption, demonstrating the wide applicability of  
253 the dynamic model. Citric acid production was overestimated by the model, which

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254 may suggest a further limiting factor with xylose as the carbon source. The constraint  
255 applied to citric acid output rate,  $v_{CIT}$ , was the same as for glucose (Table 1). The  
256 discrepancy may be due to differing morphology as we observed decreased biomass  
257 pellet sizes and higher viscosity in cultures grown on xylose.

258

### 259 **Investigating the role of phosphate during citric acid fermentation**

260 As growth on glucose continued beyond external phosphate depletion (Fig 2B), it  
261 became clear that *A. niger* has a phosphate storage mechanism, possibly via  
262 accumulation of polyphosphate as previously reported [23]. To investigate this,  
263 polyphosphate was extracted from biomass grown under citric acid producing  
264 conditions and quantified. Polyphosphate levels were observed to rise early on in  
265 fermentation, peaking at day 2 at the point of external phosphate depletion (Fig 5).  
266 Polyphosphate levels dropped rapidly from day 2 to day 4, with a more gradual  
267 decrease later in fermentation coinciding with phosphate-limited growth and citric  
268 acid production.

269

270 To further investigate the importance of phosphate, we searched for the genes  
271 encoding phosphate transporters in *A. niger* ATCC 1015. A total of 8 putative genes  
272 were found (based on similarity to known transporters), suggesting *A. niger* has  
273 evolved a range of phosphate uptake mechanisms as adaptation to different  
274 environmental conditions (Supplementary Table S2). It may be that only a subset of  
275 these genes encode phosphate transporters while others encode phosphate  
276 sensors. One of the genes (accession number EHA22558) has clear homologues in

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5 277 other species (Fig S1), but none of these have been characterised or parameterised  
6  
7 278 at the level of protein activity. The other gene annotations are more speculative so  
8  
9 279 may not encode phosphate transporters [24].  
10

11 280

### 12 281 **The dFBA model provides a platform for predictive metabolic engineering**

13 282 A prediction of the model is that oxalic acid production is the most efficient means of  
14 283 acidification at initial pH 7, followed by citric acid. It is well known that *A. niger*  
15 284 predominantly secretes oxalic and gluconic acid at higher initial pH and that by  
16 285 imposing a low initial pH during fermentation, production of these competing organic  
17 286 acids is prevented and citric acid production is increased [14]. Our model suggests  
18 287 that by switching off oxalic acid production by deletion of oxaloacetate hydrolase  
19 288 (oah), citric acid will solely be produced. The model does not predict gluconic acid  
20 289 production suggesting this may be decoupled from proton production and is instead  
21 290 a means of quickly sequestering glucose, through the action of extracellular glucose  
22 291 oxidase, early in fermentation.  
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38 293 To investigate this phenomenon, we engineered the ATCC 1015 strain by targeted  
39 294 gene deletion strategies to knockout oah and the gene encoding glucose oxidase  
40 295 (gox) responsible for gluconic acid production. We created two single knockouts  
41 296 ( $\Delta$ oah and  $\Delta$ gox) and a double knockout ( $\Delta$ oah  $\Delta$ gox), and characterised citric acid  
42 297 fermentation by these knockout strains at initial pH 7 (Fig 6). Citric acid yield was  
43 298 significantly increased in the  $\Delta$ oah strain with a further marginal improvement in  
44 299  $\Delta$ oah  $\Delta$ gox. This was not the case for the  $\Delta$ gox strain suggesting gluconic acid  
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1 300 production occurs independently of proton production without impacting citric acid  
2 301 fermentation. Gluconic acid was produced early in fermentation while oxalic and  
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4 302 citric acid production occurred later. The synchronicity of oxalic and citric acid  
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7 303 production suggests they are part of the same proton production response. In this  
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10 304 experiment, the  $Mn^{2+}$  concentration was increased to 1000 ppb. Citric acid  
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12 305 production usually requires  $Mn^{2+}$ -deficient media, though was previously reported  
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14 306 insensitive to  $Mn^{2+}$  in an *oah* and *gox* double negative mutant strain at pH 5 [25]. The  
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16 307 presence of  $Mn^{2+}$  did not prevent citric acid production at initial pH 7, suggesting that  
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18 308 its effect is limited to low pH conditions.  
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24 310 We applied our dFBA model to the  $\Delta oah$  and  $\Delta gox$  knockouts at initial pH 7, which  
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26 311 gave close fits for oxalic and gluconic production. The differences in predicted citric  
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28 312 acid production between the knockout strains showed a qualitative fit with empirical  
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30 313 data (Fig 6). However, constraints on oxalic and citric acid output rates,  $v_{OXAL}$  and  
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32 314  $v_{CIT}$  respectively (Table 1), were required to achieve the close fits. The constraint on  
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34 315 citric acid output rate,  $v_{CIT}$ , was different to that applied at initial pH 2. This may be  
35  
36 316 due to morphological differences as we observed increased biomass pellet sizes  
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38 317 when *A. niger* was grown at higher initial pH. The impact of differing morphology on  
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40 318 transport processes and on anaerobicity within pellets requires further investigation.  
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42 319 The widely reported absence of oxalic acid production below pH 2 [10, 25] was  
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44 320 implemented in the model to reflect empirical data. To simulate gluconic acid  
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46 321 production, the flux of the extracellular GOX and gluconic acid dissociation reactions  
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48 322 were forced dependent on GOX kinetic parameters and the ambient pH. The kinetic  
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323 parameters  $V_{\max}$  and  $K_M$  of GOX (Table 2) were taken from the literature [26, 27].

324 The concentration of GOX per gram biomass dry weight is unknown for these  
325 experimental conditions so was fitted to empirical data (Table 1). The proportion of  
326 active GOX was based on empirical data of GOX activity at varying pH [28].

327

## 328 **Discussion**

329 We have developed a novel dynamic model of *A. niger* citric acid fermentation that  
330 employs dFBA, to give time-course simulations of batch fermentation relevant to the  
331 industrial and experimental modes of *A. niger* fermentation. Our physiologically  
332 motivated double optimisation approach is a novel use of dFBA. Previous work  
333 incorporated proton production into the *MA871* metabolic model and used FBA in a  
334 static manner to give predictions on organic acid production [13, 14] at fixed values  
335 of pH. Since acid dissociation reactions are dependent on the dynamic ambient pH,  
336 the application of dFBA with the dynamic tracking of pH enables more accurate  
337 predictions on organic acid production. The dynamic model was also expanded to  
338 include alternative feedstocks. Xylose was chosen as it is a pentose sugar abundant  
339 in hemicellulose in plant biomass and readily metabolised by *A. niger*. This new  
340 dynamic model is therefore a valuable addition to the *A. niger* metabolic modelling  
341 toolbox and a powerful demonstration of the promise of dFBA for applications in  
342 industrial biotechnology.

343

344 We tested the ability of the model to predict the impact of genetic modifications on  
345 organic acid fermentation at higher initial pH. We deleted genes encoding

1 346 oxaloacetate acetylhydrolase (oah) and glucose oxidase (gox) to eliminate oxalic  
2 347 and gluconic production respectively. Deletion of oah significantly increased citric  
3  
4 348 acid production, and this was also observed in model predictions, though less  
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6  
7 349 pronounced. This suggests that the presence of oxalic acid in the cytosol in the oah  
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10 350 positive strains may have negating effects on citric acid production not reflected by  
11  
12 351 the model. It is expected that cytosolic organic acid accumulation may occur as a  
13  
14 352 result of constrained transport, which is likely to have regulatory effects on organic  
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17 353 acid production as a safeguard mechanism.

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22 355 *A. niger* has been an industrial workhorse for decades and is essential to the world's  
23  
24 356 citric acid production. This is achieved through batch or fed-batch fermentation and  
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27 357 the new model enables simulation of the dynamic process for the first time. The  
28  
29 358 underlying causes of the naturally evolved property of organic acid production are  
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31  
32 359 still unclear. It was previously reported through static FBA predictions [14] that this  
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34 360 may be driven by the biological objective of proton production. In line with empirical  
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37 361 findings, oxalic acid production was revealed as the most efficient means of proton  
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39 362 production at wide ranging pH with citric acid second at low pH. We have now shown  
40  
41 363 this in a dynamic manner with variable external pH taken into account. Empirical  
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44 364 data revealed that oxalic and citric acid production are synchronous upon a switch to  
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47 365 phosphate-limited growth. This suggests they are coupled and part of the same  
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49 366 proton production response. This is further supported by the significant increase in  
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51 367 citric acid production in  $\Delta$ oah.

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369 The role of phosphate is striking as organic acid secretion has been reported in *A.*  
370 *niger* and other organisms as a phosphate mobilisation strategy [15, 29, 30]. The  
371 observed phosphate-limited growth results from the ability of *A. niger* to rapidly take  
372 up phosphate and store it as polyphosphate. The constraint on polyphosphate  
373 hydrolysis then limits growth, enabling flux of carbon to organic acid production.  
374 While *A. niger* has sufficient stored phosphate for growth, it does not use it and  
375 keeps it reserved. This behaviour may be due to the energy storage value of  
376 polyphosphate. We have observed a release of phosphate late in fermentation upon  
377 carbon depletion, which suggests *A. niger* is capable of rapid polyphosphate  
378 hydrolysis as a means to create ATP when other energy sources are limiting. The  
379 control mechanisms that exist in *A. niger* to regulate polyphosphate hydrolysis and  
380 their relation to organic acid production warrant further investigation.

381

382 Our modelling approach has further demonstrated the potential of dFBA; the  
383 augmentation of static steady state FBA by dynamic transport processes and time  
384 varying pools of metabolites. It has also revealed some fundamental issues with the  
385 application of these techniques to real applications. The objective function – the  
386 biomass equation – is fundamental to FBA and is typically constructed with evidence  
387 from mass spectrometry. Our work suggests that this function is strongly dependent  
388 on the fermentation context and may even be variable over the growth process.  
389 Biologically this is highly plausible, but dramatically increases the complexity of  
390 model implementation and fitting. In addition, it is clear that important regulatory  
391 constraints on the metabolic process, in this case citrate accumulation, need to be

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392 included. In this manner, we have created an augmented dFBA model in a  
393 potentially grey area between a complete kinetic model and the genetically based  
394 simplicity of an FBA model. Further work is required to fully understand validity of  
395 such models.

396

## 397 **Conclusions**

398 Our findings reveal a naturally evolved behaviour that has been exploited by industry  
399 for decades to produce citric acid. Our work, encapsulated in a dynamic model,  
400 further elucidates the causative factors in organic acid fermentation by *A. niger*  
401 exploited by industrial processes. The model provides a means to further probe this  
402 behaviour and accurately explore the effects of genetic changes on organic acid  
403 fermentation in a dynamic manner. This new addition to the *A. niger* systems biology  
404 toolbox paves the way for metabolic engineering efforts to create new strains  
405 capable of enhanced citric acid production on low-cost feedstocks.

406

## 407 **Methods**

### 408 **Shake flask experiments**

409 Citric acid fermentation experiments were performed in 250 ml DeLong neck baffled  
410 shake flasks (Bellco Glass Inc.; Vineland, NJ, USA) with 30 ml medium. Flasks were  
411 siliconized with 2% (v/v) dimethyldichlorosilane. Cultures were incubated at 30°C  
412 with shaking at 250 rpm. The following medium was used: glucose (160 g/L), urea  
413 (3.6 g/L), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.52 g/L), K<sub>2</sub>HPO<sub>4</sub> (0.5 g/L), CaCO<sub>3</sub> (0.03125 g/L),  
414 MgSO<sub>4</sub>·7H<sub>2</sub>O (0.275 g/L), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.00225 g/L), FeSO<sub>4</sub>·7H<sub>2</sub>O (0.0095 g/L),

1 415 CuSO<sub>4</sub>·5H<sub>2</sub>O (0.0117 g/L), MnCl<sub>2</sub>·(H<sub>2</sub>O)<sub>4</sub> (0.0000108 g/L), citric acid monohydrate  
2 416 (3.3 g/L), Tween 80 (0.0094%). The Mn<sup>2+</sup> concentration was confirmed as 7 ppb by  
3  
4 417 ICP-MS (Biorenewables Development Centre, York, UK). The medium was  
5  
6 418 autoclaved (121°C 15 minutes) excluding glucose which was filter sterilised (0.22  
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8 419 µm). The pH of the medium was adjusted after autoclaving by addition of sterile 2 M  
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11 420 H<sub>2</sub>SO<sub>4</sub>. The medium included 10 mM uridine in experiments using pyrG negative  
12  
13 421 strains. The medium was inoculated with 1×10<sup>6</sup> spores/ml. Spores were harvested  
14  
15 422 from potato dextrose agar slants incubated for 2 days at 37°C. 2 ml saline Tween  
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17 423 (0.1% Tween 80, 9 g/L NaCl) was added per slant and shaken to disperse spores.  
18  
19 424 Spores were washed 3 times in saline Tween. 500 µl samples of cultures were taken  
20  
21 425 every 24 hours for determination of biomass, metabolites and phosphate. Samples  
22  
23 426 were collected in pre-dried, pre-weighed 1.5 ml Eppendorf tubes and centrifuged at  
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25 427 9000 g for 5 minutes. The supernatant was retained for metabolite analysis and  
26  
27 428 phosphate determination and stored at -20°C.  
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### 36 430 **Biomass dry weight determination**

37 431 Mycelia were washed 4 times in 1 ml dH<sub>2</sub>O and centrifuged at 9000 g for 5 minutes.  
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39 432 Biomass was dried at 70°C to constant weight. Biomass dry weight was determined  
40  
41 433 by subtracting weight of the pre-dried 1.5 ml Eppendorf tube.  
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### 46 434

### 47 435 **Metabolite analysis**

48 436 Enzymatic assay kits were used to determine the level of metabolites. Glucose, citric  
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50 437 acid, xylose, glycerol and gluconic acid were determined using Megazyme assay kits  
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438 (K-GLUC, K-CITR, K-XYLOSE, K-GCROLGK, and K-GATE respectively)  
439 (Megazyme International Ireland Ltd., Wicklow, Ireland). Oxalic acid was determined  
440 using the LIBIOS oxalate assay kit (Oxalate-100; LIBIOS, France).

441

#### 442 **Phosphate determination**

443 Phosphate was determined by the ammonium molybdate method, using an assay kit  
444 (ab65622; Abcam, Cambridge, UK).

445

#### 446 **Polyphosphate extraction and quantification**

447 Mycelia were grown up in shake flasks using the same method as previously  
448 described. Mycelia were harvested at 8 time-points (days 1 to 8) in triplicate. To  
449 obtain sufficient biomass, one flask was harvested per sample. Day 1 samples  
450 required the pooling of 4 flasks per replicate. Mycelia were harvested using a double  
451 layer of Miracloth (Calbiochem) and washed in 300 ml ice-cold 100 mM Tris.HCl pH  
452 7 followed by 600 ml ice-cold dH<sub>2</sub>O. Washed mycelia were transferred to 15 ml  
453 Falcon tubes, flash frozen in liquid nitrogen, freeze dried, and stored at -80°C.  
454 Freeze dried mycelia were weighed out in 2 ml vials, approximately 50 mg per vial.  
455 Biomass was ground using the TissueLyser II (QIAGEN; Crawley, UK) at 30 Hz for  
456 90 seconds 3 times. Each vial contained 2 beads. Powdered mycelia were lysed by  
457 adding 2 ml 10% (w/v) lysing enzymes from *Trichoderma harzianum* (Sigma, Dorset,  
458 UK) and incubating at 30°C with shaking for 3 hours. Samples were centrifuged and  
459 supernatant discarded. Polyphosphate was extracted following a previously  
460 described protocol [23]. All centrifuge steps were done at 13,000 rpm for 10 minutes

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461 at 4°C and all shaking was done at 30 rpm. The polyphosphate fraction was dried in  
462 a Savant SPD131DDA SpeedVac Concentrator (Thermo Fisher Scientific).  
463 Polyphosphate was quantified by measuring free phosphate before and after acid  
464 hydrolysis using the previously described phosphate determination method. Acid  
465 hydrolysis was performed by adding 2 ml 0.5 M H<sub>2</sub>SO<sub>4</sub> to the dry pellet and boiling at  
466 100°C for 3 hours.

467

### 468 **Dynamic modelling of organic acid fermentation**

469 Modelling was performed using the MA871 metabolic model [13] as the model for  
470 the flux balance analysis. During this project, a more complete model of *A. niger*  
471 metabolism was published [31] but as this retains the core of MA871 and is not  
472 specific to ATCC 1015 we have not adopted this model. The FBA calculations were  
473 performed using bespoke Java code which implements the GLPK toolkit (GNU).  
474 dFBA routines were written directly into the Java code with the differential equations  
475 solved by simple time-stepping (Euler method) with small values for the time-step.  
476 The ODEs (ordinary differential equations) were solved according to

$$477 \quad C_{n+1} = C_n + t f_n B_n, \quad (1)$$

478 where  $C_{n+1}$  is the mmol of compound at time-point n+1,  $C_n$  is the mmol of compound  
479 at time-point n,  $t$  is the time-step (1/60 h),  $f_n$  is the flux (mmol gDW<sup>-1</sup> h<sup>-1</sup>) at time-  
480 point n, and  $B_n$  is the biomass (gDW) at time-point n. The flux constraints at time-  
481 point n+1 were calculated by the following kinetic equations. External phosphate  
482 input (P<sub>le</sub>==>) was constrained according to

$$54 \quad v_{Pe} = \frac{v_{Pe,max} P_e}{K_{Pe} + P_e}, \quad (2)$$

484 where  $v_{pe}$  is the external phosphate uptake rate ( $\text{mmol gDW}^{-1} \text{h}^{-1}$ ) and  $P_e$  is the  
 485 external phosphate concentration (mM).

486 Internal phosphate input (PI $\rightleftharpoons$ ) was constrained according to

$$487 \quad v_P = \frac{v_{P,max}P}{K_P+P}, \quad (3)$$

488 where  $v_P$  is the internal phosphate input rate ( $\text{mmol gDW}^{-1} \text{h}^{-1}$ ) and  $P$  is the  
 489 concentration of stored phosphate (mM).

490

491 If external glucose was below 150 g/L, external glucose uptake (DGLCe $\rightleftharpoons$ DGLC)  
 492 was constrained according to

$$493 \quad v_G = v_{G1}G + \frac{v_{G2,max}G}{K_{G2}\left(1+\frac{C}{K_{i2}}\right)+G\left(1+\frac{C}{K_{i2}}\right)}, \quad (4)$$

494 where  $v_G$  is the external glucose uptake rate ( $\text{mmol gDW}^{-1} \text{h}^{-1}$ ),  $G$  is the external  
 495 glucose concentration (mM), and  $C$  is the external citrate concentration (mM).

496 If external glucose was greater than or equal to 150 g/L, external glucose uptake  
 497 was constrained according to

$$498 \quad v_G = v_{G1}G + \frac{v_{G2,max}G}{K_{G2}\left(1+\frac{C}{K_{i2}}\right)+G\left(1+\frac{C}{K_{i2}}\right)} + \frac{v_{G3,max}G}{K_{G3}\left(1+\frac{C}{K_{i3}}\right)+G\left(1+\frac{C}{K_{i3}}\right)}, \quad (5)$$

499 where  $v_G$  is the external glucose uptake rate ( $\text{mmol gDW}^{-1} \text{h}^{-1}$ ),  $G$  is the external  
 500 glucose concentration (mM), and  $C$  is the external citrate concentration (mM).

501 If external xylose was below 150 g/L, external xylose uptake (XYLe $\rightleftharpoons$ ) was  
 502 constrained according to

$$503 \quad v_X = v_{X1}X + \frac{v_{X2,max}X}{K_{X2}+X}, \quad (6)$$

504



1 505 where  $v_X$  is the external xylose uptake rate ( $\text{mmol gDW}^{-1} \text{h}^{-1}$ ), and  $X$  is the external  
2 506 xylose concentration (mM).  
3

4 507 If external xylose was greater than or equal to 150 g/L, external xylose uptake was  
5 508 constrained according to  
6

$$7 \quad v_X = v_{X1}X + \frac{v_{X2,max}X}{K_{X2}+X} + \frac{v_{X3,max}X}{K_{X3}+X}, \quad (7)$$

8 509  
9 510 where  $v_X$  is the external xylose uptake rate ( $\text{mmol gDW}^{-1} \text{h}^{-1}$ ), and  $X$  is the external  
10 511 xylose concentration (mM).  
11

12 512 The extracellular GOX (glucose oxidase) reaction rate was calculated according to,  
13  
14

$$15 \quad v_{GOX} = p_{GOX} \frac{v_{GOX,max}G}{K_{GOX}+G}, \quad (8)$$

16 513  
17 514 where  $v_{GOX}$  is the GOX reaction rate,  $p_{GOX}$  is the proportion of active GOX, and  $G$  is  
18 515 the external glucose concentration (mM).  
19

20 516 The proportion of active GOX,  $p_{GOX}$ , as a function of pH was determined according  
21 517 to,  
22

$$23 \quad p_{GOX} = -0.102pH^2 + 1.082pH - 1.95 \quad (9)$$

24 518  
25 519 The kinetic parameters were either fitted to our empirical data (Table 1) or set to  
26 520 empirical values from the literature if available (Table 2).  
27

28 521  
29 522 The MA871 model was adapted to include proton production as an objective  
30 523 function and acid-dissociation reactions for seven acids (citric, oxalic, gluconic,  
31 524 acetic, malic, succinic, lactic) but as a function of a dynamic external pH rather than  
32 525 a fixed pH [14]. The number of protons released in each acid-dissociation reaction  
33 526 was calculated at each time-step according to the following equation based on  
34 527 ambient pH and pKa values.  
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$$H = \frac{K_1(H_e)^{-1} + 2K_1K_2(H_e)^{-2} + 3K_1K_2K_3(H_e)^{-3}}{1 + K_1(H_e)^{-1} + K_1K_2(H_e)^{-2} + K_1K_2K_3(H_e)^{-3}}, \quad (10)$$

528 where  $K_1$ ,  $K_2$ , and  $K_3$  are constants calculated from pKa values of each acid species  
 529 (Table 3), and  $H_e$  is the external molar concentration of protons that is tracked in the  
 530 dFBA as a dynamic pool.  
 531

532  
 533 An output reaction was added for external protons ( $H_{pe} \rightleftharpoons$ ), which was set as the  
 534 objective when maximising proton production. An explicit phosphate storage reaction  
 535 was also included in the dFBA. An input reaction for internal phosphate ( $PI \rightleftharpoons$ )  
 536 was added to the metabolic model, and the dynamic pool of internal phosphate was  
 537 tracked in the dFBA. This new reaction was set as the objective when maximising  
 538 phosphate storage.  
 539

540 When plotting alongside empirical data, the dFBA start time was taken as the spore  
 541 germination time, 18 hours after inoculation. The initial biomass dry weight was set  
 542 to 0.3125 g/L following empirical data.  
 543

#### 544 **Model parameterisation**

545 Glucose transport-mediated uptake [16, 17] and glucose oxidase [26, 27] kinetic  
 546 parameters were calculated from empirical data in the literature (Table 2). The  
 547 concentration of active GOX enzyme [GOX] was fitted to empirical data (Table 1).  
 548 The other kinetic parameters in the model were fitted to empirical data via a manual  
 549 fitting routine (Table 1).  
 550

551 **Quality of fit assessment and model selection**

552 Akaike Information Criterion (AIC) [20] was used to measure the quality of fit and  
553 assess improvement in the model. The AIC was calculated according to

$$554 \quad AIC = 2k + n \ln \left( \frac{RSS}{n} \right), \quad (11)$$

555 where  $k$  is the number of fitted parameters,  $n$  is the number of data-points, and  $RSS$   
556 is the residual sum of squares.

559 **Targeted gene deletion of oah and gox**

560 Targeted gene deletion was performed using a previously reported strategy [32]. As  
561 this technique requires a pyrG negative strain, the pyrG gene first had to be deleted  
562 from ATCC 1015. This was achieved using homologous recombination. ATCC 1015  
563 was transformed with linear DNA containing 2 kb up- and 1.5 kb down-stream  
564 flanking regions of the pyrG gene (accession number EHA25155), kindly given by M  
565 Kokolski (University of Nottingham). Polyethylene glycol (PEG)-mediated  
566 transformation of protoplasts was used [32]. Successful deletions were selected by  
567 resistance to 5-fluoroorotic acid (5-FOA) (Fluorochem; Derbyshire, UK) and uridine  
568 auxotrophy, and confirmed by PCR and DNA sequencing using primers external to  
569 the gene (pyrG\_ex\_fw and pyrG\_ex\_rv). The oah and gox genes were identified in  
570 the ATCC 1015 genome as accession numbers EHA22250 and EHA27180,  
571 respectively. 1.5 kb up- and down-stream flanking regions were cloned from ATCC  
572 1015 gDNA using Phusion HF DNA polymerase (Thermo Fisher Scientific), and the  
573 following primers: oah\_up\_fw, oah\_up\_rv, oah\_down\_fw, oah\_down\_rv, gox\_up\_fw,

574 gox\_up\_rv, gox\_down\_fw, gox\_down\_rv. 15-bp tails (underlined) were added to  
575 outermost primers for In-Fusion® HD cloning (Clontech; France) into the pc3 vector  
576 between the NotI and SpeI restriction sites. To join up- and down-stream fragments  
577 together, overlap extension PCR was used with 30-bp overlapping tails (underlined)  
578 added to innermost primers. Overlapping fragments were first annealed as follows:  
579 50 µl reaction containing 200 ng each fragment, 400 µM dNTPs, HF buffer, and 1 U  
580 Phusion HF DNA polymerase run on SOE1 programme (94°C 5 minutes, then 94°C  
581 30 seconds, 60°C 90 seconds, 72 °C 90 seconds 10 times, then 10 °C forever). The  
582 annealed product was then amplified using outermost primers as follows: 100 µl  
583 reaction containing 50 µl first reaction, 1 µM each primer, 400 µM dNTPs, HF buffer  
584 and 1 U Phusion HF DNA polymerase run on SOE2 programme (94°C 2 minutes,  
585 then 94°C 30 seconds, 60°C 30 seconds, 72°C 90 seconds 35 times, then 72°C 10  
586 minutes, 10°C forever). The annealed product was gel purified using the QIAquick  
587 gel extraction kit (QIAGEN; Crawley, UK). Transformation was performed using  
588 XL10-Gold Ultracompetent cells according to the manufacturer's instructions (Agilent  
589 Technologies; Cheshire, UK). Plasmid was isolated using the Wizard® Plus SV  
590 minipreps DNA purification kit (Promega; Southampton, UK). Plasmid integrity was  
591 confirmed by DNA sequencing. ATCC 1015 ΔpyrG was transformed with the pc3-  
592 oah and pc3-gox deletion vectors using the previously reported PEG-mediated  
593 protoplast transformation protocol [32]. The gene deletion procedure previously  
594 outlined [32] was then followed with minor modifications. 1.5 g/L 5-FOA was used to  
595 select for pyrG negative colonies with incubation at 37°C for 3 days. oah and gox  
596 knockouts were identified by PCR screening with primers external and internal to the

1 597 deletion site (oah\_ex\_fw, oah\_ex\_rv, oah\_int\_fw, oah\_int\_rv, gox\_ex\_fw, gox\_ev\_rv,  
2 598 gox\_int\_fw, gox\_int\_rv). Gene deletion was further confirmed by DNA sequencing of  
3  
4 599 the region external to the deletion site. To create the  $\Delta$ oah  $\Delta$ gox double knockout,  
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7 600 the deletion procedure for gox was applied to ATCC 1015  $\Delta$ pyrG  $\Delta$ oah.  
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## 11 12 602 **Declarations**

### 13 14 15 603 **Ethics approval and consent to participate**

16  
17  
18 604 Not applicable.

### 19 20 605 **Consent for publication**

21  
22 606 Not applicable.

### 23 24 25 607 **Availability of data and material**

26  
27 608 The datasets used and/or analysed during the current study are available from the  
28  
29 609 corresponding author on reasonable request.  
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### 32 33 610 **Competing interests**

34  
35  
36 611 The authors declare that they have no competing interests.  
37

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### 43 44 614 **Authors' contributions**

45 615 DJU, AJW and SMM wrote the manuscript. All authors read and approved the final  
46  
47 616 manuscript.  
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52  
53 618 Not applicable.  
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706 **Table 1.** Parameters fitted to our empirical data.

| Parameter  | Description  | Value                              |
|--|--|------------------------------------|
| $v_{Pe,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> ) | External phosphate maximum input rate <sup>a</sup>         | 0.08                               |
| $K_{Pe}$ (mM)  | External phosphate Michaelis constant                      | 0.0333                             |
| $v_{P,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )  | Internal phosphate maximum input rate                      | 0.0008                             |
| $K_P$ (mM)   | Internal phosphate Michaelis constant                      | 0.0833                             |
| $v_{G1}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )     | External glucose passive uptake rate                       | 0.00031419<br>× [GLC] <sup>b</sup> |
| $v_{X1}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )     | External xylose passive uptake rate                        | 0.00033 ×<br>[XYL] <sup>c</sup>    |
| $v_{X2,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> ) | External xylose high-affinity transport maximum rate       | 0.2                                |
| $K_{X2}$ (mM)  | External xylose high-affinity transport Michaelis constant | 3.33                               |
| $v_{X3,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> ) | External xylose low-affinity transport maximum rate        | 2.5                                |
| $K_{X3}$ (mM)  | External xylose low-affinity transport Michaelis constant  | 3.33                               |
| [GOX] (mg gDW <sup>-1</sup> )                          | Concentration of external glucose oxidase enzyme           | 0.1                                |
| $v_{CIT}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )    | Citric acid output rate constraint <sup>d</sup>            | 0.12                               |
| $v_{OXAL}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )   | Oxalic acid output rate constraint                         | 0.01                               |

<sup>a</sup>External phosphate input rate changed 8 hours after the dFBA start time to 0.015 mmol gDW<sup>-1</sup> h<sup>-1</sup> if initial pH 2 or 0.004 mmol gDW<sup>-1</sup> h<sup>-1</sup> if initial pH 7. <sup>b</sup>[GLC] is concentration of external glucose in mM. <sup>c</sup>[XYL] is concentration of external xylose in mM. <sup>d</sup>Citric acid output rate constraint changed to 0.016 mmol gDW<sup>-1</sup> h<sup>-1</sup> if initial pH above 2.

712 **Table 2.** Parameters set to empirical values from the literature.

| Parameter   | Description  | Value                  | References |
|---|--|------------------------|------------|
| $v_{G2,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )  | External glucose high-affinity transport maximum rate                | 0.186                  | [16, 17]   |
| $K_{G2}$ (mM)   | External glucose high-affinity transport Michaelis constant          | 0.26                   | [16, 17]   |
| $K_{i2}$ (mM)   | External glucose high-affinity transport citrate inhibition constant | 933                    | [16, 17]   |
| $v_{G3,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> )  | External glucose low-affinity transport maximum rate                 | 2.706                  | [16, 17]   |
| $K_{G3}$ (mM)   | External glucose low-affinity transport Michaelis constant           | 3.67                   | [16, 17]   |
| $K_{i3}$ (mM)   | External glucose low-affinity transport citrate inhibition constant  | 233.21                 | [16, 17]   |
| $v_{GOX,max}$ (mmol gDW <sup>-1</sup> h <sup>-1</sup> ) | Glucose oxidase (GOX) maximum reaction rate                          | $27.48 \times [GOX]^a$ | [26]       |
| $K_{GOX}$ (mM)  | Glucose oxidase (GOX) Michaelis constant                             | 33                     | [26, 27]   |

713 <sup>a</sup>[GOX] is concentration of external glucose oxidase enzyme in mg gDW<sup>-1</sup> and was fitted to  
714 empirical data (Table 1).

715 **Table 3.** Acid constants for Equation 10.

716

| Acid species  | $K_1$         | $K_2$         | $K_3$         |
|---------------|---------------|---------------|---------------|
| Citric acid   | $10^{-3.128}$ | $10^{-4.761}$ | $10^{-6.396}$ |
| Gluconic acid | $10^{-3.7}$   | 0             | 0             |
| Acetic acid   | $10^{-4.757}$ | 0             | 0             |
| Malic acid    | $10^{-3.459}$ | $10^{-5.097}$ | 0             |
| Succinic acid | $10^{-4.207}$ | $10^{-5.636}$ | 0             |
| Lactic acid   | $10^{-3.86}$  | 0             | 0             |
| Oxalic acid   | $10^{-1.252}$ | $10^{-4.266}$ | 0             |

717

718 **Table 4.** AIC scores for model selection.

719

| Additional parameters   | Number of fitted parameters | AIC score |
|---|-----------------------------|-----------|
| None  | 5                           | 438       |
| Nucleic acid component of biomass equation  | 6                           | 416       |
| Phospholipid component of biomass equation  | 6                           | 422       |
| Nucleic acid and phospholipid components of biomass equation                                    | 7                           | 393       |
| Nucleic acid and phospholipid components of biomass equation, and citric acid output constraint | 8                           | 300       |

720

721 **Table 5.** Primers used in this work.

| Primer      | Nucleotide sequence (5' to 3')                                   |
|-------------|--|
| pyrG_ex_fw  | CTTTGCAGGTGTGGCTGAAC   |
| pyrG_ex_rv  | ACAGCAGTGCTTATCTGCGA   |
| oah_up_fw   | <u>ACCGCGGTGGCGGCCGCGCTGTGTCCATACCATCAATCC</u>                   |
| oah_up_rv   | <u>GAATGTTGCAGACAGACAGAAAGCAAAGAGCAGGCAGTAGTAAGCAA</u><br>GAAT   |
| oah_down_fw | <u>TCTTTCTTATTCTTGCTTACTACTGCCTGCTCTTTTGCTTTCTGTCTGTC</u><br>TGC |
| oah_down_rv | <u>CGGGGGATCCACTAGTTCTCCTCTTCCCCTGCCTTT</u>                      |
| gox_up_fw   | <u>ACCGCGGTGGCGGCCGCGAGATGGCAATTTCCGCGAC</u>                     |
| gox_up_rv   | <u>GAATATTCGAGGATTGTGGGAGAGACAGCGCGTGCAAACCTCACCACC</u><br>AAG   |
| gox_down_fw | <u>CTGTCTTGACCTTGGTGGTGAGTTTGCACGCGCTGTCTCTCCCACAAT</u><br>CC    |
| gox_down_rv | <u>CGGGGGATCCACTAGTCTACGCTCATGTCCTGGTCC</u>                      |
| oah_ex_fw   | TAAGGCTACCCAACCCACCC   |
| oah_ex_rv   | GCTTATCTAGGCCCTGCTG  |
| oah_int_fw  | ACCCAACCACACCATCCTTC   |
| oah_int_rv  | ACCCAGTTCCCCACTAACAC   |
| gox_ex_fw   | CACTATCGCCAAGCAGGGAT   |
| gox_ex_rv   | AAGGTCTCGTTGAAGGTGGC   |
| gox_int_fw  | AGCAACCAGCCTTTCCTCTC   |
| gox_int_rv  | CCCAGTTCCAGCCCTCATTT   |

722

723

724 **Figure legends**

725

726 **Figure 1.** Citric acid production commences upon a diauxic growth switch. Empirical  
727 data plotted is the mean average of 4 biological replicates and error bars represent  
728 standard deviation. Citric acid data are normalised to reflect the amount produced.

729 **A)** Change in biomass dry weight (g/L) over time. **B)** Change in external citric acid  
730 concentration (g/L) over time.

731

732 **Figure 2.** Comparing empirical and *in silico* data in response to varying phosphate.

733 Markers represent empirical data and lines represent *in silico* data. Green circles and  
734 dashed-dotted lines correspond to 0.05 g/L phosphate. Purple triangles and dashed  
735 lines correspond to 0.09 g/L phosphate. Brown squares and solid lines correspond to

736 0.17 g/L phosphate. Empirical data plotted is the mean average of 4 biological  
737 replicates and error bars represent standard deviation. Citric acid data are

738 normalised to reflect the amount produced. *In silico* data-points are one per minute.

739 **A)** Change in biomass dry weight (g/L) over time. **B)** Change in external phosphate  
740 concentration (g/L) over time. **C)** Change in external citric acid concentration (g/L)

741 over time. **D)** Change in external glucose concentration (g/L) over time.

742

743 **Figure 3.** Simulating citric acid fermentation by dynamic flux balance analysis. A

744 schematic showing the decision process implemented in the dFBA model.

745

746 **Figure 4.** Comparing empirical and *in silico* data in response to different carbon

747 sources. Markers represent empirical data and lines represent *in silico* data. Green

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748 circles and solid lines correspond to glucose. Purple triangles and dashed lines  
749 correspond to xylose. Empirical data plotted is the mean average of 4 biological  
750 replicates and error bars represent standard deviation. Citric acid data are  
751 normalised to reflect the amount produced. *In silico* data-points are one per minute.  
752 **A)** Change in biomass dry weight (g/L) over time. **B)** Change in external phosphate  
753 concentration (g/L) over time. **C)** Change in external citric acid concentration (g/L)  
754 over time. **D)** Change in external carbon source concentration (g/L) over time.

755  
756 **Figure 5.** Change in polyphosphate levels during citric acid fermentation. Empirical  
757 data plotted is the mean average of 3 biological replicates and error bars represent  
758 standard deviation.

759  
760 **Figure 6.** Comparing empirical and *in silico* data in response to  $\Delta oah$  and  $\Delta gox$   
761 knockouts. Markers represent empirical data and lines represent *in silico* data. Green  
762 circles and solid lines correspond to  $\Delta oah \Delta gox$ . Purple triangles and dashed-dotted  
763 lines correspond to  $\Delta oah$ . Brown squares and dashed lines correspond to  $\Delta gox$ .  
764 Blue diamonds and dotted lines correspond to  $\Delta pyrG$  control. Empirical data plotted  
765 is the mean average of 4 biological replicates and error bars represent standard  
766 deviation. Citric acid data are normalised to reflect the amount produced. *In silico*  
767 data-points are one per minute. **A)** Change in external citric acid concentration (g/L)  
768 over time. **B)** Change in external oxalic acid concentration (g/L) over time. **C)**  
769 Change in external gluconic acid concentration (g/L) over time. **D)** Change in  
770 external phosphate concentration (g/L) over time.

771 **Table S1.** Biomass equation parameters altered to fit empirical data.

| Compound | Before fitting (mmol gDW <sup>-1</sup> h <sup>-1</sup> ) | After fitting (mmol gDW <sup>-1</sup> h <sup>-1</sup> ) |
|----------|--|---|
| AMP      | -0.01402222  | -0.0046740733   |
| GMP      | -0.01688834  | -0.0056294467   |
| CMP      | -0.01402222  | -0.0046740733   |
| UMP      | -0.01117424  | -0.0037247467   |
| DAMP     | -0.00193736  | -0.0006457867   |
| DCMP     | -0.00201544  | -0.0006718133   |
| DTMP     | -0.00193736  | -0.0006457867   |
| DGMP     | -0.00201544  | -0.0006718133   |
| PC       | -0.015312  | -0.005104   |
| PS       | -0.000359  | -0.0001196667   |
| PE       | -0.034807  | -0.0116023333   |
| GL       | -0.46  | -0.9030928715   |
| ADP      | 71.60986992  | 71.5025795733   |
| PI       | 71.60986992  | 71.5025795733   |
| ATP      | -71.60986992   | -71.5025735267  |
| H2O      | -69.08036756   | -69.0511151733  |

772



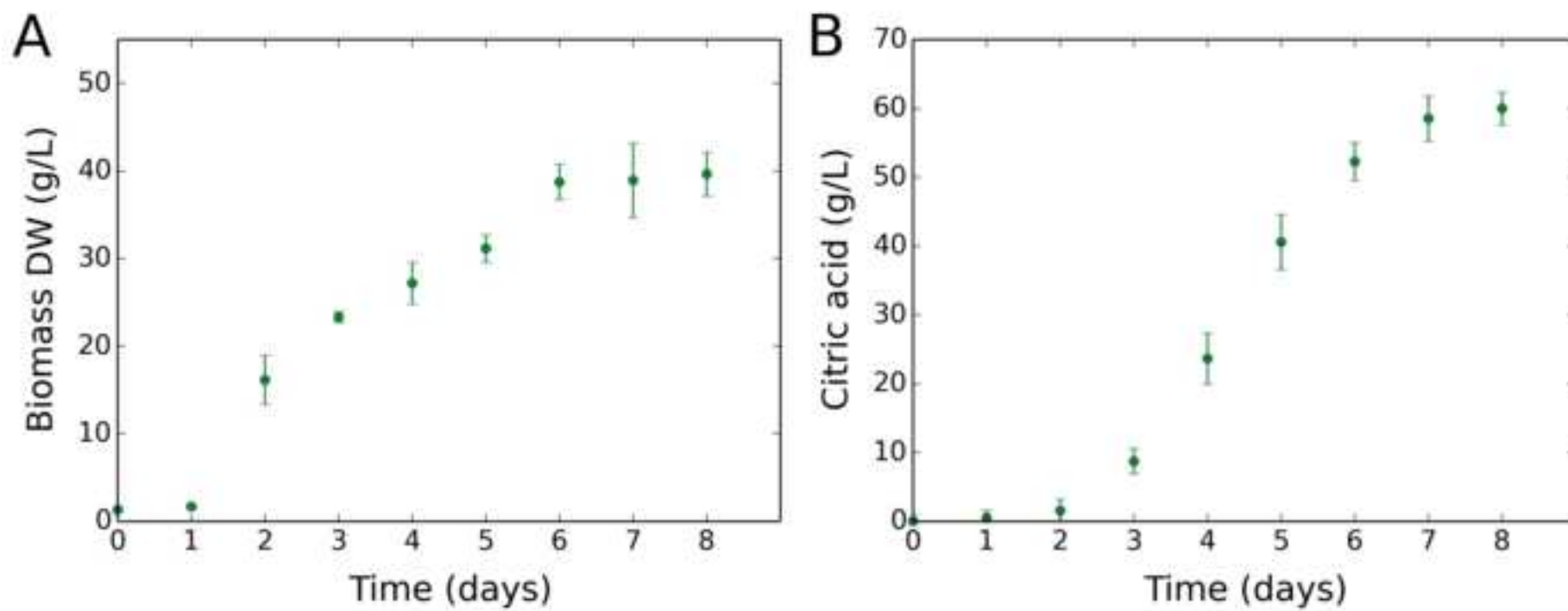
773 **Table S2.** Putative phosphate transporters in ATCC 1015. Top BLASTP hits with  
 774 phosphate transporters in SwissProt database are given.

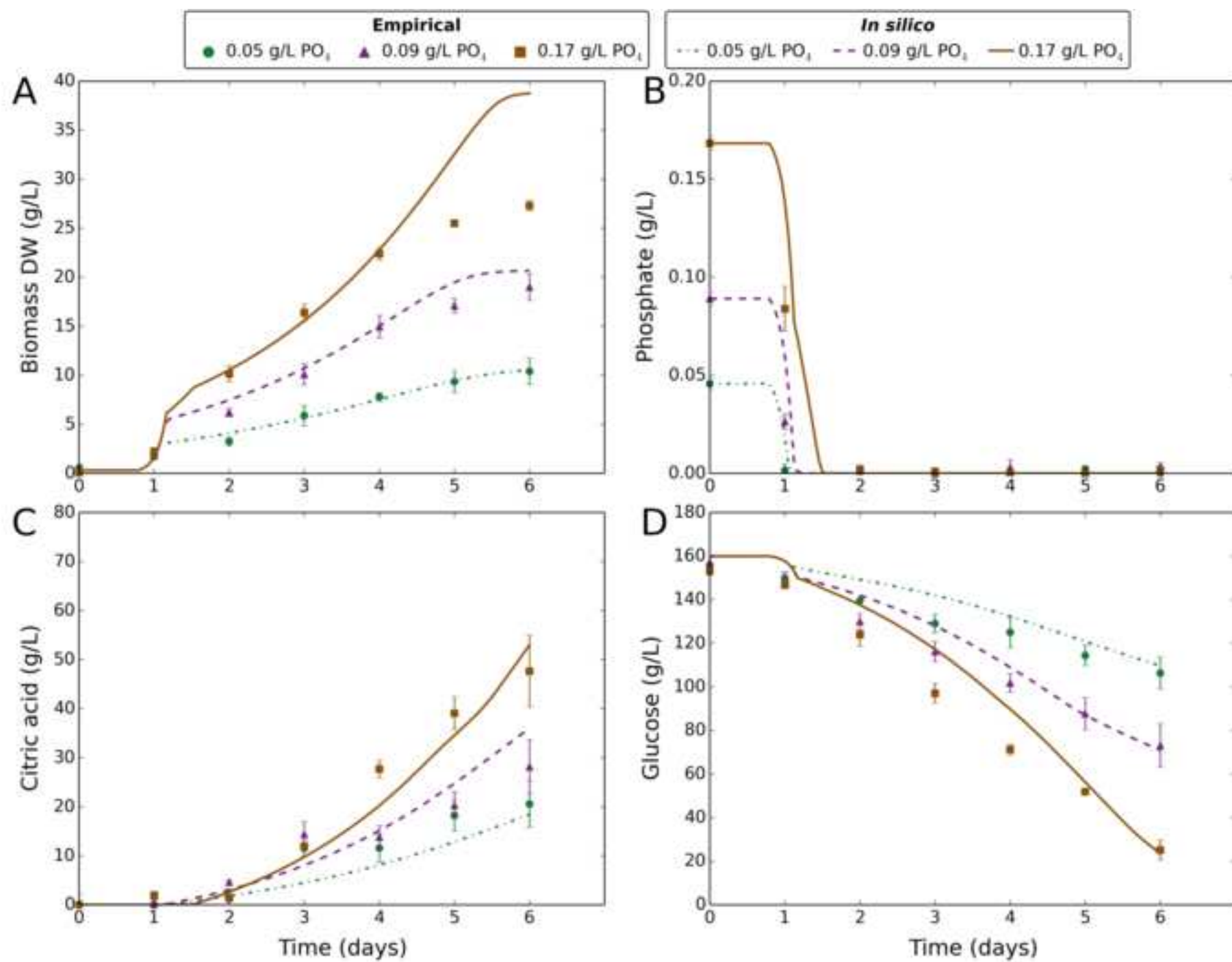
| ATCC 1015 locus tag | GenBank accession | Top BLASTP hit (SwissProt) | Identity (%) | E-value |
|---------------------|-------------------|----------------------------|--------------|---------|
| ASPNIDRAFT_173247   | EHA22558          | P15710.1                   | 37           | 4e-130  |
| ASPNIDRAFT_190334   | EHA20653          | P25297.2                   | 34           | 2e-90   |
| ASPNIDRAFT_121846   | EHA27663          | Q7RVX9.2                   | 61           | 0.0     |
| ASPNIDRAFT_52154    | EHA22720          | O42885.2                   | 29           | 2e-42   |
| ASPNIDRAFT_42307    | EHA25335          | Q9S735.1                   | 27           | 6e-12   |
| ASPNIDRAFT_175394   | EHA23128          | Q8H074.1                   | 25           | 2e-26   |
| ASPNIDRAFT_206238   | EHA26306          | P27514.2                   | 41           | 0.0     |
| ASPNIDRAFT_35379    | EHA27197          | Q8H074.1                   | 24           | 2e-22   |

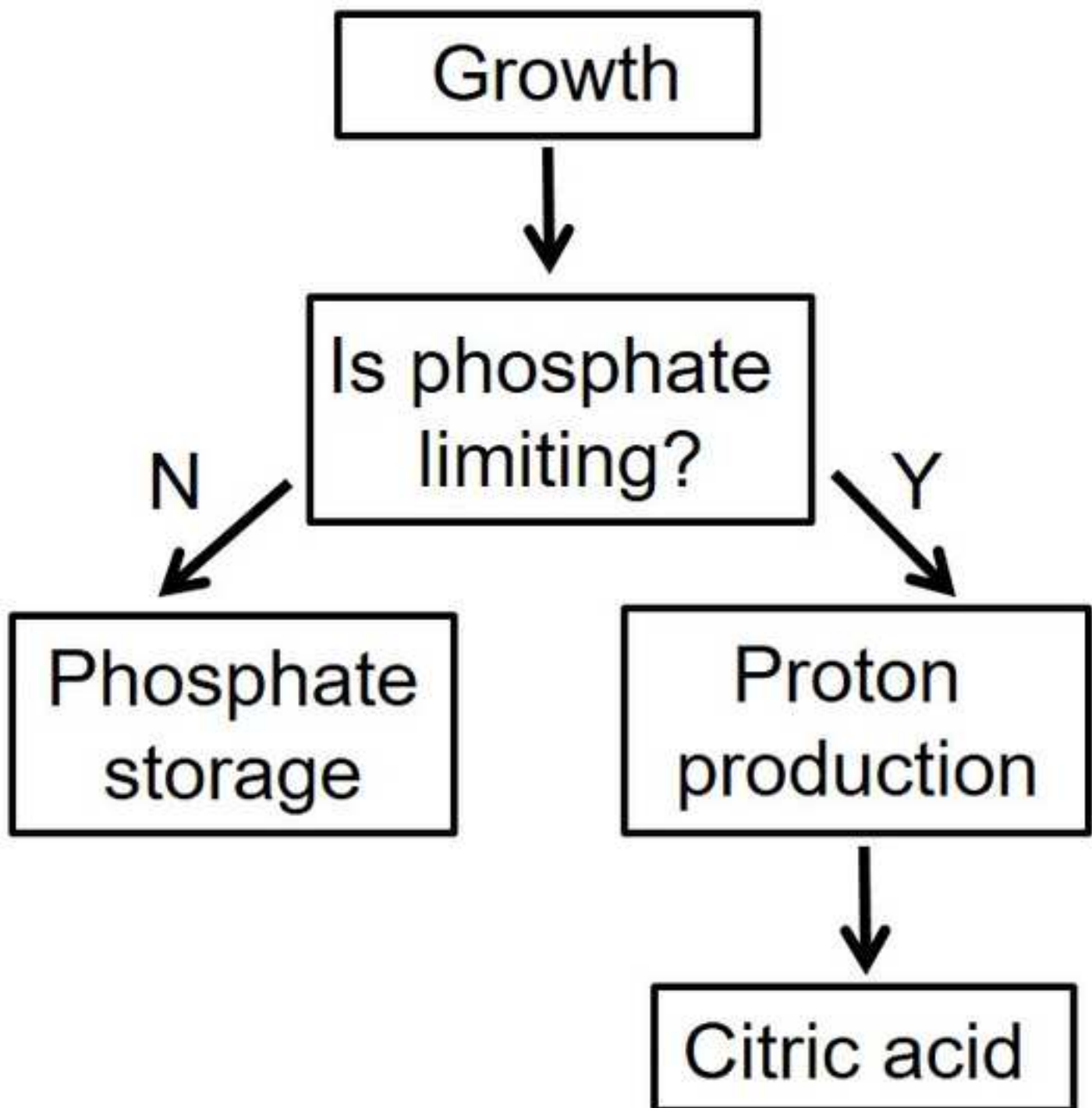
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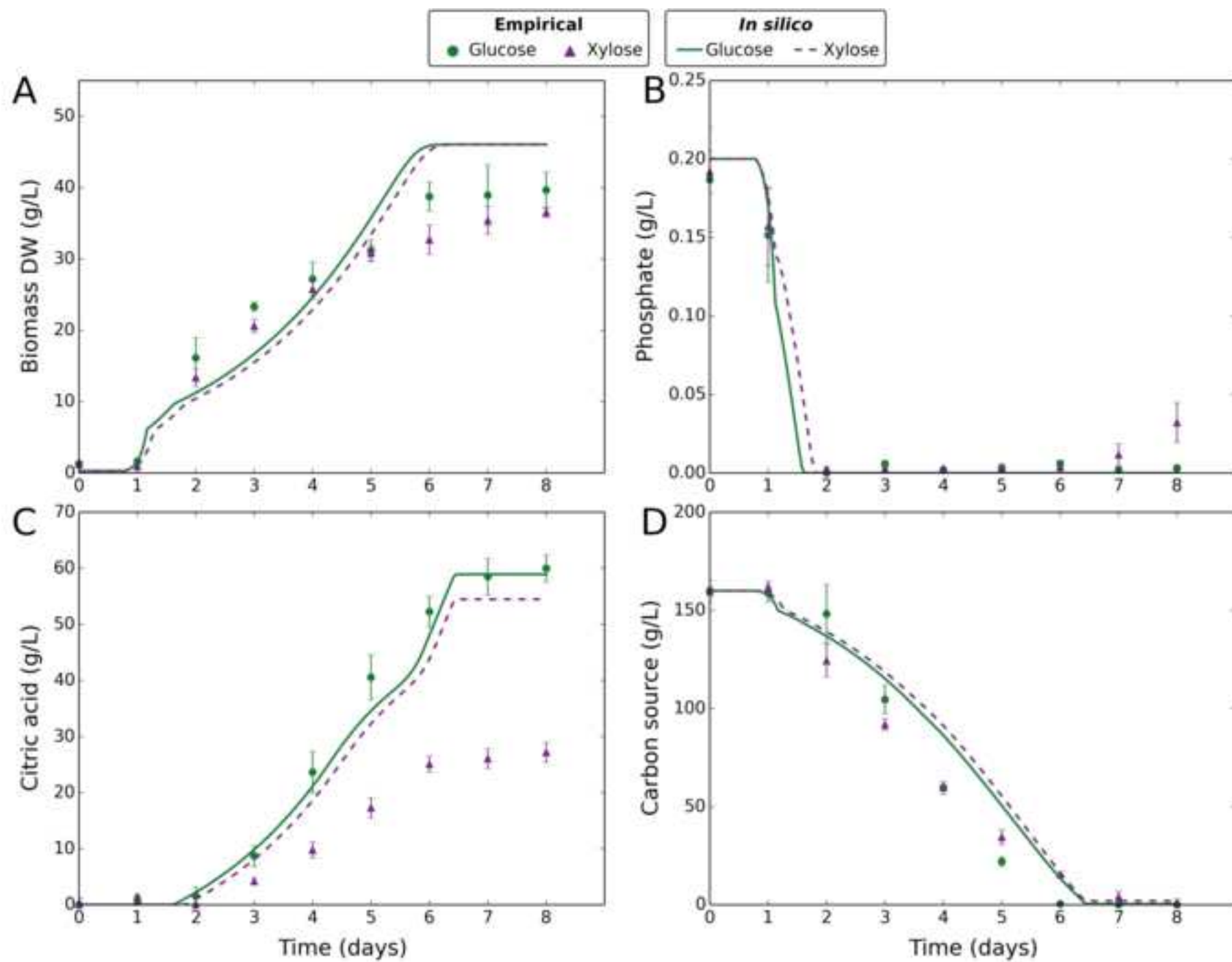
776 **Supplementary Figure legends**

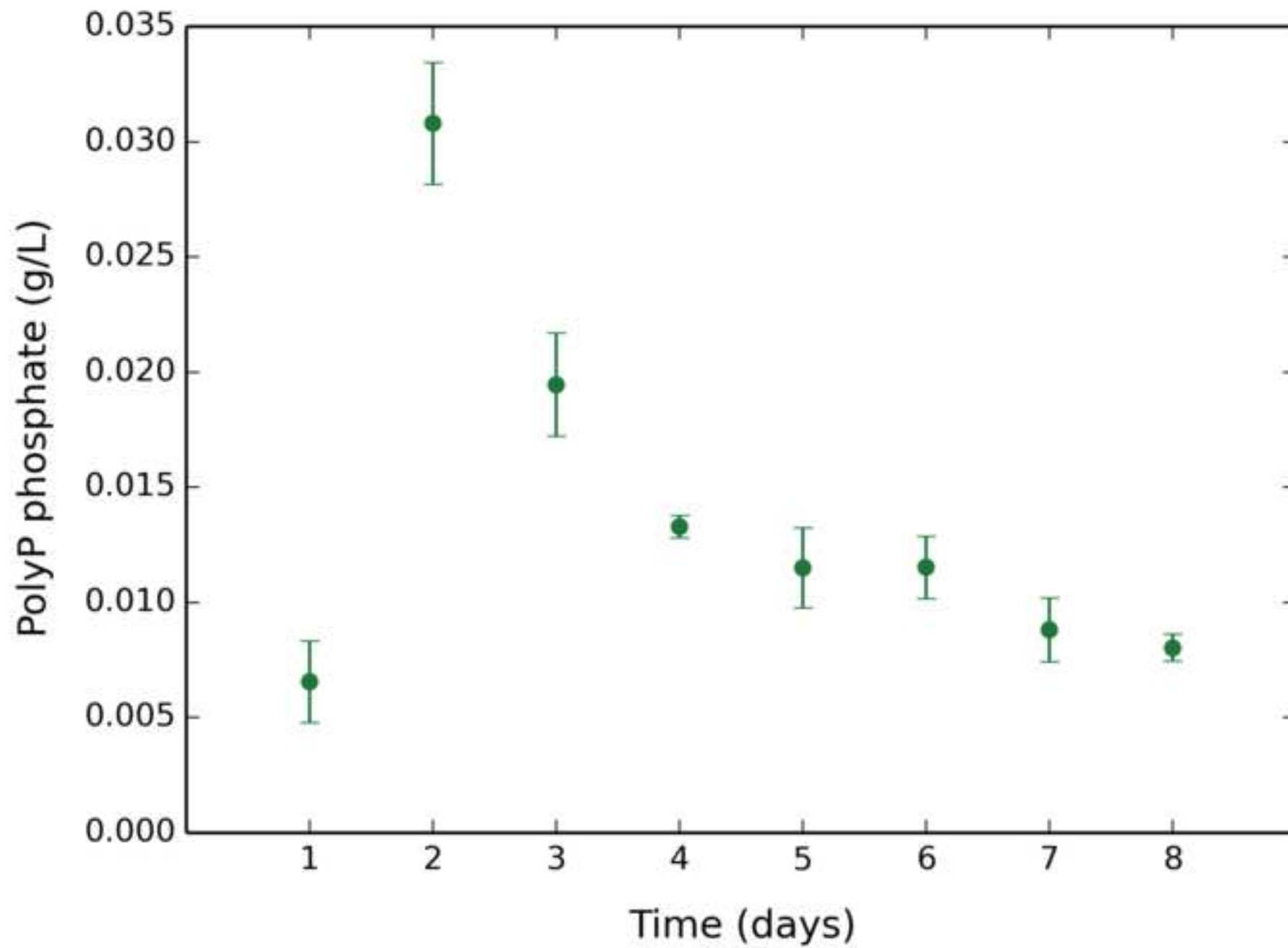
777 **Figure S1.** Phylogenetic tree of putative phosphate transporters in ATCC 1015.

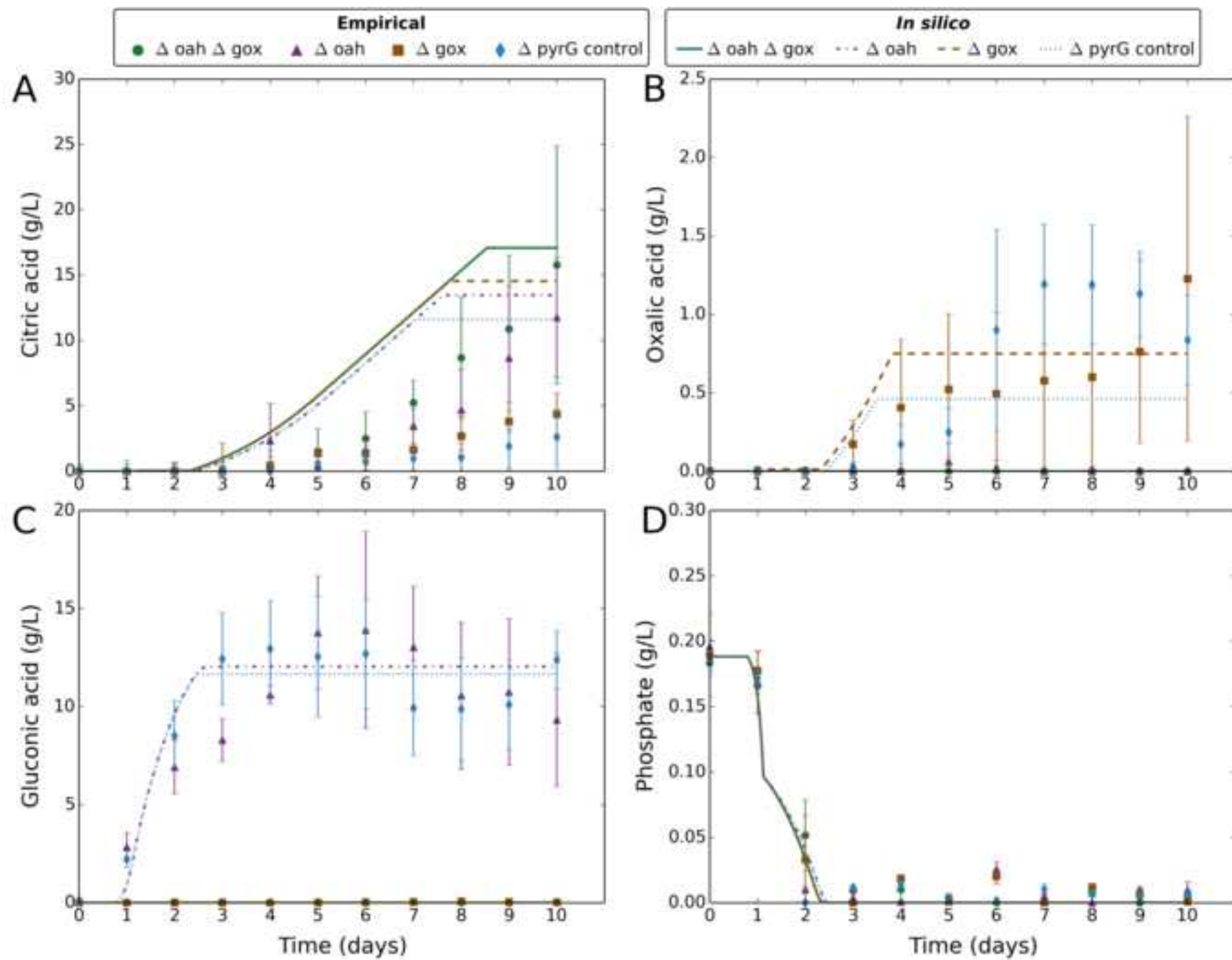


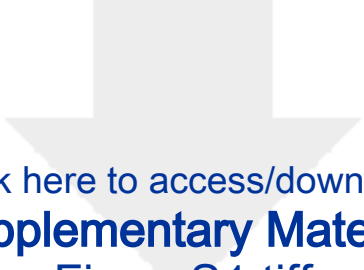












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FigureS1.tiff

