Analysis of experimental data of citric acid production by *Candida lipolytica*

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Background

A paper regarding an experiment of a strain of *Candida lipolytica*, and its ability of producing citrate during nitrogen limited conditions, from 1979 performed by scientists Aiba and Matsuoka (Villadsen et al., 2011). Experiments were performed in a chemostat at different dilution rates. The rate of sugar uptake, carbon dioxide production, citrate production, protein production, isocitrate production as well as for carbohydrates were measured during the trials. Experimental data were evaluated using both through black box analysis and with metabolic flux analysis. Problems 1 and 2 were solved using Excel and problems 3 and 4 were solved using MATLAB.

1. Black box analysis

The stoichiometry for the overall reaction was found to be according to Reaction 1. The calculated yield coefficients for the reaction are shown in Table 1, for each one of the four different dilution rates. Protein and carbohydrate yields were assumed to be part of biomass growth, which is why protein and carbohydrates are not included in Reaction 1.

$$CH_2O + Y_{SO}O_2 + Y_{SN}(NH_4)SO_4 \rightarrow Y_{SX}CH_{1.8}O_{0.5}N_{0.1} + Y_{SCit}CH_{8/6}O_{7/6} + Y_{SCit}CH_{8/6}O_{7/6} + Y_{SC}CO_2 \quad \text{(Reaction 1)}$$

The yields can be calculated from the rates of formation of each product and the substrate using equation 1. All rates were first standardized to c-mole L-1 h-1 by, dependending on the measurement given, multiplying by dilution rate or biomass concentration, or by dividing by c-molar mass.

$$Y_{s,product} = r_{product} / r_s \quad \text{(Eq 1)}$$

Table 1. Shows the calculated yields of the black box reaction from Reaction 1.

	D=0.0122 h-1	**D=0.0300 h-1**	**D=0.048 h-1**	**D=0.0769 h-1**
Y_{sp}	0.044	0.056	0.087	0.123
Y_{sCarb}	0.088	0.112	0.183	0.199
Y_{sc}	0.360	0.272	0.308	0.341
Y_{sx}	0.357	0.417	0.493	0.734
Y_{sCIT}	0.296	0.312	0.132	0.004
Y_{sICT}	0.074	0.065	0.041	0.002
Y_{sn}	0.036	0.042	0.049	0.073
Y_{so}	0.321	0.248	0.315	0.171
RQ	1.122	1.096	0.977	1.999

The yield coefficients were found to vary across the 4 dilution rates, indicating that the macroscopic reaction changes based on dilution rate. The yield for biomass increases proportionally with dilution rate, implying that the wash-out point has not been reached. Citrate and isocitrate yields decrease with dilution rate but drop sharply for the last dilution rate. This could mean that the TCA cycle decreases in activity and so the cell keeps citrate and isocitrate in the cycle rather than excrete them. This is supported by the RQ value for the last dilution rate, which is almost double the previous dilution rate's RQ value. There is less oxygen consumption in comparison with CO_2 formation, which could mean the cells are using more fermentative metabolism rather than the TCA cycle.

2. Respiratory quotient

The respiratory quotients (RQ), shown in Table 1, were calculated for the different dilution rates, by usage of Equation 2. The oxygen yield was obtained from a degree of reduction balance on Reaction 1.

$$RQ = Y_{SC}/Y_{SO} \quad \text{(Eq 2)}$$

In order to increase the production of citrate it is more favourable to maintain a value under 1 for RQ, which is the case for dilution rate 0.048. A RQ-value lower than 1 indicates that the respiratory metabolism is favoured and by that, the citrate formation would be enhanced through the amplification of the TCA cycle. However, a value equal or larger than 1 for RQ is an indication of a fermentative metabolism is favoured instead. By that, the TCA cycle is reduced.

3. Metabolic flux analysis

The results of the metabolic flux analysis when the pathway of reaction 11 was removed are shown in Table 3. The fluxes were found by creating matrix Ω from the stoichiometric coefficients, shown as Table 2. Removing unmeasured, non-steady-state variables from Ω and transposing the matrix gives T_2. The fluxes were found from Equation 3, where v is a vector of fluxes for each metabolic pathway and r is the corresponding vector of rates of production.

$$T_2 \cdot \nu = r \Leftrightarrow (T_2)^{-1} \cdot T_2 \cdot \nu = (T_2)^{-1} \cdot r \Leftrightarrow \nu = (T_2)^{-1} \cdot r \quad \text{(Eq 3)}$$

Table 2. Shows the matrix Ω which holds the stoichiometric coefficients of the reactions.

	Lip	NH4	Gluc	CIT	ICT	Pro	Carb	CO2	G6P	Pyr	ACA	GOX	OGT	SUC	MAL	OAA
v1	0	0	**-1**	0	0	0	0	0	**1**	0	0	0	0	0	0	0
v2	0	0	0	0	0	0	0	0	**-1**	**1**	0	0	0	0	0	0
v3	0	0	0	0	0	0	0	**1/3**	0	**-1**	**2/3**	0	0	0	0	0
v4	0	0	0	0	0	0	0	**1/4**	0	**3/4**	0	0	0	0	0	**1**

v5	0	0	0	**3**	0	0	0	0	0	0	**-1**	0	0	0	0	**-2**
v6	0	0	0	**-1**	**1**	0	0	0	0	0	0	0	0	0	0	0
v7	0	0	0	0	**-1**	0	0	**1/6**	0	0	0	0	**5/6**	0	0	0
v8	0	0	0	0	0	0	0	**1/5**	0	0	0	0	**-1**	**4/5**	0	0
v9	0	0	0	0	0	0	0	0	0	0	0	0	0	**-1**	**1**	0
v10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**-1**	**1**
v11	0	0	0	0	**-1**	0	0	0	0	0	0	**1/3**	0	**2/3**	0	0
v12	0	0	0	0	0	0	0	0	0	0	**-1**	**-1**	0	0	**2**	0
v13	0	0	0	0	0	0	**1**	0	**-1**	0	0	0	0	0	0	0
v14	**1**	0	0	0	0	0	0	0	0	0	**-1**	0	0	0	0	0
v15	0	**-1**	0	0	0	**1**	0	0	0	0	0	0	**-1**	0	0	0

All production of glyoxylate has been stopped due to the cut off of pathway 11. Due to the fact that there was no glyoxylate, pathway 12 could no longer be used, so its flux is 0 for all dilutions. Malate can still be produced from succinate, so the TCA cycle can continue. Additionally, there is a general trend for fluxes to increase with higher dilution rates, see Table 3. The increase in flux of most pathways with increasing dilution rate is expected due to the fact that there is a higher amount of substrate available when the dilution rate increases. Oxoglutarate production in pathway 7 is increased here compared with removing reaction 4. This is reasonable due to the fact that the isocitrate can only be used for the production of oxoglutarate since the alternative conversion of isocitrate to glyoxylate has been inactivated.

Table 3. Shows the calculated metabolic fluxes with pathway 11 removed

	D=0.0122 h-1	**D=0.0300 h-1**	**D=0.048 h-1**	**D=0.0769 h-1**
v1	0.00147	0.00310	0.00419	0.00452
v2	0.00134	0.00275	0.00342	0.00362
v3	0.00103	0.00206	0.00284	0.00327
v4	0.00042	0.00092	0.00077	0.00046
v5	0.00048	0.00081	0.00085	0.00068
v6	0.00102	0.00146	0.00200	0.00204
v7	0.00091	0.00126	0.00183	0.00203
v8	0.00069	0.00087	0.00116	0.00113
v9	0.00055	0.00070	0.00093	0.00091
v10	0.00055	0.00070	0.00093	0.00091
v11	0	0	0	0
v12	0	0	0	0
v13	0.00013	0.00035	0.00077	0.00090
v14	0.00020	0.00057	0.00104	0.00150
v15	0.00007	0.00018	0.00036	0.00056

4. Metabolic flux analysis

The results of the metabolic flux analysis when pathway of reaction 4 was removed are shown in Table 4. Because pathway 11 is active, succinate is being produced in another way other than from oxoglutarate. Due to this, it is possible to reverse part of the TCA cycle and go from succinate to oxoglutarate and also further to isocitrate. This occurs for the second dilution rate. The fluxes indicate that succinate mostly goes via pathway 9 into malate but at the same time some succinate takes the reversible pathway of reaction 8 backwards to oxaloacetate (in the opposite direction from what has been defined in this study). When path 4 is removed, all pyruvate must produce acetyl Co-A through pathway 3, which therefore has a higher flux. Some of the extra acetyl Co-A goes to producing malate since the fluxes to citrate and lipids, which are the other options of acetyl Co-A, do not change. Additionally, oxoglutarate production decreases when path 11 is reintroduced because citrate can split between pathways 7 and 11. The flux through pathway 8 decreases as well, which is probably due to the fact that there is less oxoglutarate produced, but the flux through pathway 15 does not change.

Table 4. Shows the calculated metabolic fluxes with pathway 4 removed

	D=0.0122 h-1	**D=0.0300 h-1**	**D=0.048 h-1**	**D=0.0769 h-1**
v1	0.00147	0.00310	0.00419	0.00452
v2	0.00134	0.00275	0.00342	0.00362
v3	0.00134	0.00275	0.00342	0.00362
v4	0	0	0	0
v5	0.00048	0.00081	0.00085	0.00068
v6	0.00102	0.00146	0.00200	0.00204
v7	0.00029	-0.00012	0.00067	0.00134
v8	0.00017	-0.00028	0.00019	0.00056
v9	0.00055	0.00070	0.00093	0.00091
v10	0.00097	0.00162	0.00170	0.00137
v11	0.00062	0.00138	0.00116	0.00069
v12	0.00021	0.00046	0.00039	0.00023
v13	0.00013	0.00035	0.00077	0.00090
v14	0.00020	0.00057	0.00104	0.00150
v15	0.00007	0.00018	0.00036	0.00056