**Supplementary file 1.**

**1.1. Stoichiometric model for *P. pastoris growing in mixed glycerol-methanol substrates.***

**Methanol Metabolism**

1. MET => FORM
2. FORM => FOR + NADH
3. FOR + NAD+ => NADH + CO2
4. Xul5P + FORM + ATP => ADP + GAP + DHA
5. GA3Pper => GA3P
6. DHA => GA3P

**Glycerol assimilation**

1. GOL + NAD+ + Pi + ATP=> DHAP + NADH + ADP

**Glycolysis and Gluconeogenesis Pathways**

1. Fru6P <=> Glc6P
2. 2 GA3P => Fru6P +Pi
3. GA3P + ADP + Pi + NAD+ => PG3 + ATP + NADH
4. PG3 <=> Pep
5. Pep + ADP => Pyr + ATP
6. Pyr + NAD+ => ACCoAmit + CO2 + NADH
7. Pyr NAD + => ACCoAcyt +CO2 + NADH
8. Pyr + CO2 + ADP => Oaacyt + ATP

**Pentose Phosphate Pathway**

1. Glc6P + 2 NADP+ => Rul5P + 2 NADPH + CO2
2. Rul5P <=> R5P
3. Rul5P <=> Xul5P
4. R5P + Xul5P <=> Sed7P + GA3P
5. Sed7P + GA3P <=> Fru6P + E4P
6. Xul5P + E4P <=> Fru6P + GA3P
7. Xul5P + NADH => Ara + NAD+

**TCA cycle**

1. ACCoAmit + Oaamit => CIT
2. CIT => ICIT
3. ICIT + NAD+ => KG + CO2 + NADH
4. KG + NAD+ => SUCCoA + CO2 + NADH
5. SUCCoA + Pi + ADP => SUC + ATP
6. SUC + ATP => SUCCoA + ADP + Pi
7. SUC => FUM + FADH2
8. FUM <=> MAL
9. MAL + NAD + => Oaamit + NADH

**Biosynthesis of amino acids**

**Serine Family**

1. PG3 + Glu + NAD+ => Ser + KG + NADH +Pi
2. Ser + THF => Gly + MetTHF
3. Ser + ACCoA + H2S => Cys

**Alanine Family**

1. Pyr + NADPH => Ala + NADP**+**
2. Pyr + Glu => KG
3. 2 Pyr + NADPH => Kval + CO2 NADP+
4. Kval + Glu => Val + KG
5. Kval + ACCoAmit + Glu + NAD+=> Leu + KG + NADH + CO2

**Histidine Family**

1. R5P + ATP => PRPP + AMP
2. PRPP + ATP + Gln + 2NAD+ => His + KG + Pi + 2 NADH

**Aspartic Family**

1. OAA + Glu => Asp + KG
2. Asp + Gln + ATP => Asn + Glu + AMP
3. Asp + ATP + 2 NADPH => Ser + ADP + Pi + 2NADP+
4. Thr + NADPH + Glu + Pyr => Ile + KG + NH4 + CO2 + NADP+
5. ACCoA + Ser + H2S + MTHF => Met + THF

**Aromatic Family**

1. 2 Pep + E4P + ATP + NADPH => CHOR + ADP + 4 Pi
2. CHOR + Glu => Phe + KG + CO2
3. CHOR + Glu => Tyr + KG + NADH + CO2
4. CHOR + Gln + PRPP + Ser => Trp + Glu + Pyr + GA3P + CO2

**Glutamic Family**

1. KG +NH4 + NADPH => Glu
2. Glu + ATP + NH4 => Gln + ADP + Pi
3. Glu + ATP + 2 NADPH => Pro + ADP + Pi
4. Gln + CO2 + 2 ATP => CaP + Glu + 2 ADP + Pi
5. Glu + ACCoA + 4 ATP + NADPH + CaP + Asp => Arg + KG + 4 ADP + FUM + 5 Pi
6. 2 Glu + ACCoA + 3 ATP + 2 NADPH+ 2 NAD+ => Lys + KG + CO2 + 2 NADH + 2 NADP+

**Biosynthesis and Interconversion of One-carbon Units**

1. DHF + NADPH => THF + NADP+
2. Gly + THF + NAD+ => MetTHF + NH4+ + NADH + CO2
3. MetTHF + NADH => THF + NAD+

**Transport Reactions**

1. Oaacyt <=> Oaamit
2. Glycerolext => Glycerolcyt
3. NH4+ext + ATP => NH4+cyt + ADP + Pi
4. SO4-2 ext + ATP => SO4-2cyt + ADP + Pi
5. Metalext + ATP => Metalcyt + ADP + Pi

**Respiratory Chain**

1. NADH + 0.5 O2 => NAD+ + H2O
2. ADP + Pi => ATP

**Biomass Synthesis**

**1. Protein synthesis** (Composition derived from the measured biomass amino acid content at each experimental condition. The energy needed to biosynthesize 1 C-mol of protein was derived from the synthesis of each amino acid and the protein polymerization value taken from [1]

***- X-33 ROL at chemostat conditions of  =0.05 h-1***

1. 0.139 Pyr + 0.080 NAD+ + 0.436 NADPH + 1.21 ATP + 0.006 R5P + 0.014 E4P + 0.068 KG + 0.009 Oaamit + 0.014 ACCoAcyt => 1 C-mol Protein + 0.002 GA3P + 1.21 ADP + 1.21 Pi + 0.436 NADP+ + 0.08 NADH + 0.045 CO2

***- X-33 ROL at chemostat conditions of  =0.16 h-1***

1. 0.135 Pyr + 0.083 NAD+ + 0.0423 NADPH + 1.21 ATP + 0.006 R5P + 0.013 E4P + 0.009 Oaamit + 0.073 KG + 0.014 ACCoAcyt => 1 C-mol Protein + 0.002 GA3P + 0.083 NADH + 0.0423 NADP+ + 1.21 ADP + 1.21 Pi + 0.043 CO2

**2. Carbohydrate Synthesis** (Composition derived from [2]**)**

1. 0.113 Glc6P + 0.053 Fru6P + 0.167 ATP => 1 C-mol Carbohydrate + 0.167 ADP

**3. Lipids Synthesis** (derived from the mean lipid composition from [3])

1. 0.0002 Glc6P + 0.0055 Pyr + 0.011 GA3P + 0.005 CO2 + 0.0292 ACCoAmit + 0.441 ACCoAcyt + 0.07 NADH + 0.599 NADPH + 0.42 ATP + 0.057 O2 => 1 C-mol Lipid + 0.07 NAD+ + 0.599 NADP+ + 0.42 ADP + 0.42 Pi

**4. RNA Synthesis** (derived from the RNA composition proposed by [1])

1. 0.06 Pyr + 0.1136 CO2 + 0.111 R5P + 0.104 NAD+ + 0.075 NADPH + 1.1 ATP + 0.0479 Oaamit => 1 C-mol RNA + 1.1 Pi + 1.1 ADP + 0.075 NADP+ + 0.104 NADH

**5. DNA Synthesis** ( Composition derived from [4] and the DNA polymerization from [1]

1. 0.054 Pyr +0.102 NAD+ + 0.273 NADPH + 1.146 ATP+ 0.132 CO2 + 0.108 R5P + 0.051 Oaamit => 1 C-mol DNA + 1.146 Pi + 1.146 ADP + 0.102 NADH + 0.273 NADP+

**1.2) Reactions and atom transitions network used in 13C flux analysis, following the notation by Wiechert [5] .**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Name** |  | **Reaction** | | | |
|  |  |  |  |  |  |
| **feedGlyA:** |  | FullyGly > Gly\_ext | | |  |
|  |  | #abcdef > #abcdef | | |  |
|  |  |  |  |  |  |
| **feedGlyB:** |  | CGly > Gly\_ext | | |  |
|  |  | #abcdef > #abcdef | | |  |
|  |  |  |  |  |  |
| **uptGly:** |  | Gly\_ext > Gly\_int | | |  |
|  |  | #abcdef > #abcdef | | |  |
|  |  |  |  |  |  |
| **feedMeOHA:** |  | MeOHL > MeOH\_ext | | |  |
|  |  | #a > #a | | |  |
|  |  |  |  |  |  |  |
| **feedMeOHB:** |  | MeOHN > MeOH\_ext | | |  |
|  |  | #a > #a | | |  |  |
|  |  |  |  |  |  |  |
| **uptMeOH:** |  | MeOH\_ext > MeOH\_int | | |  |
|  |  | #a > #a | | |  |
|  |  |  |  |  |  |
| **upt1:** |  | Gly\_int > DHAP | | |  |
|  |  | #abcdef > #abcdef | | |  |
|  |  |  |  |  |  |
| **upt2:** |  | MeOH\_int > Form | | |  |
|  |  | #a > #a | | |  |
|  |  |  |  |  |  |
| **emp1:** |  | Fru6P <> Glc6P | | |  |
|  |  | #abcdef > #abcdef | | |  |
|  |  |  |  |  |  |
| **emp2:** |  | FBP <> Fru6P | | |  |
|  |  | #abcdef > #abcdef | | |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |
| **emp3:** |  | DHAP + GA3P <> FBP | | |  |
|  |  | #abc + #def > #cbadef | | |  |
|  |  |  |  |  |  |
| **emp4:** |  | DHAP <> GA3P | | |  |
|  |  | #abc > #abc | | |  |
|  |  |  | | |  |
| **emp5:** |  | GA3P <> PG3 | | |  |
|  |  | #abc > #abc | | |  |
|  |  |  |  |  |  |
| **emp5B:** |  | PG3 <> PEP | | |  |
|  |  | #abc > # abc | | |  |
|  |  |  |  |  |  |
| **emp5C:** |  | Pep > Pyr | | |  |
|  |  | #abc > #abc | | |  |
|  |  |  |  |  |  |
| **emp6:** |  | Pyr > ACCOcyt + CO2 | | |  |
|  |  | #abc > #bc + #a | | |  |
|  |  |  |  |  |  |
| **emp7:** |  | Pyr + CO2 > OAAcit | | |  |
|  |  | #abc + #d > #abcd | | |  |
|  |  |  |  |  |  |
| **emp9:** |  | OAAcit > OAAmit | | |  |  |
|  |  | #abcd > #abcd | | |  |  |
|  |  |  |  |  |  |  |
| **emp8:** |  | Pyr > Pyrmit | | |  |
|  |  | #abc > #abc | | |  |
|  |  |  |  |  |  |
| **emp11:** |  | ACCOcyt > ACCOmit | | |  |
|  |  | #AB > #AB | | |  |
|  |  |  |  |  |  |
| **ppp1:** |  | Glc6P > CO2 + Rul5P | | |  |
|  |  | #abcdef > #a + #bcdef | | |  |
|  |  |  | | |  |
| **ppp2:** |  | Rul5P <> Xul5P | | |  |
|  |  | #abcde > #abcde | | |  |
|  |  |  | | |  |
| **ppp3:** |  | Rul5P <> Rib5P | | |  |
|  |  | #abcde > #abcde | | |  |
|  |  |  |  |  |  |
| **ppp4:** |  | Xul5P + Ery4P <> GA3P + Fru6P | | |  |
|  |  | #ABCDE + #abcd > #CDE + #ABabcd | | |  |
|  |  |  |  |  |  |
| **ppp5:** |  | Xul5P + Rib5P <> Sed7P + GA3P | | |  |
|  |  | #abcde + #ABCDE > #ABabcde + #CDE | | |  |
|  |  |  |  |  |  |
| **ppp6:** |  | GA3P + Sed7P <> Ery4P + Fru6P | | |  |
|  |  | #ABC + #abcdefg > #defg + #abcABC | | |  |
|  |  |  |  |  |  |
| **TCA1:** |  | Pyrmit > ACCOmit + CO2 | | |  |
|  |  | #ABC > #BC + #A | | |  |
|  |  |  | | |  |
| **TCA2:** |  | ACCOmit + OAAmit > CIT | | |  |
|  |  | #AB + #abcd > #dcbaBA | | |  |
|  |  |  |  |  |  |
| **TCA3:** |  | CIT > KG + CO2 | | |  |
|  |  | #ABCDEF > #ABCEF + #D | | |  |
|  |  |  |  |  |  |
| **TCA4:** |  | KG > SUCC + CO2 | | |  |
|  |  | #ABCDE > #BCDE + #A | | |  |
|  |  |  |  |  |  |
| **TCA4B:** |  | KG > SUCC + CO2 | | |  |
|  |  | #ABCDE > #EDCB + #A | | |  |
|  |  |  |  |  |  |
| **TCA5:** |  | SUCC <> FUM | | |  |
|  |  | #ABCD > #ABCD | | |  |
|  |  |  |  |  |  |
| **TCA5B:** |  | SUCC <> FUM | | |  |
|  |  | #ABCD > #DCBA | | |  |
|  |  |  |  |  |  |
| **TCA6:** |  | FUM <> MAL | | |  |
|  |  | #ABCD > #ABCD | | |  |
|  |  |  |  |  |  |
| **TCA7:** |  | MAL <> OAAmit | | |  |
|  |  | #ABCD > #ABCD | | |  |
|  |  |  |  |  |  |
| **Met1:** |  | Form > CO2 | | |  |
|  |  | #A > #A | | |  |
|  |  |  |  |  |  |
| **Met2:** |  | Xul5P + Form > DHA + GA3Pper | | |  |
|  |  | #ABCDE + #F > #FAB + #CDE | | |  |
|  |  |  |  |  |  |
| **Met2B:** |  | Xul5P + Form > DHA + GA3Pper | | |  |
|  |  | #ABCDE + #F > #ABF + #CDE | | |  |
|  |  |  |  |  |  |
| **Met3:** |  | DHA > DHAP | | |  |
|  |  | #ABC > #ABC | | |  |
|  |  |  |  |  |  |
| **Met4:** |  | GA3Pper > GA3P | | |  |
|  |  | #ABC > #ABC | | |  |
|  |  |  |  |  |  |
| **BIO1:** |  | Glc6P > Glc6Pbio | | |  |
|  |  | #ABCDEF > #ABCDEF | | |  |
|  |  |  |  |  |  |
| **BIO2:** |  | Fru6P > Fru6Pbio | | |  |
|  |  | #ABCDEF > #ABCDEF | | |  |
|  |  |  | | |  |
| **BIO3:** |  | ACCOcyt > ACCObio | | |  |
|  |  | #BC > #BC | | |  |
|  |  |  |  |  |  |
| **BIO4:** |  | OAAmit > OAAbio | | |  |
|  |  | #ABCD > #ABCD | | |  |
|  |  |  |  |  |  |
| **BIO5:** |  | Ery4P > Ery4Pbio | | |  |
|  |  | #ABCD > #ABCD | | |  |
|  |  |  |  |  |  |
| **BIO6:** |  | Rib5P > Rib5Pbio | | |  |
|  |  | #ABCDE > #ABCDE | | |  |
|  |  |  |  |  |  |
| **BIO7:** |  | GA3P > GA3Pbio | | |  |
|  |  | #ABC > #ABC | | |  |
|  |  |  |  |  |  |
| **BIO8:** |  | KG > KGbio | | |  |
|  |  | #ABCDE > #ABCDE | | |  |
|  |  |  |  |  |  |
| **BIO9:** |  | Pyrmit > Pyrbio | | |  |
|  |  | #ABC > #ABC | | |  |
|  |  |  |  |  |  |
| **CO2out:** |  | CO2 > CO2ext | | |  |
|  |  | #A > #A | | |  |

[1]. Stephanopoulos G, Aristidou A, Nielsen J. Review of cellular metabolism. Metabolic Engineering: Principles and Methodologies. 1998. page 22–76.

[2]. Pitkanen J. Metabolic flux analysis of xylose metabolism in recombinant Saccharomyces cerevisiae using continuous culture. Metabolic Engineering. 2003;5(1):16–31.

[3]. Carnicer M, Baumann K, Töplitz I, Sánchez-Ferrando F, Mattanovich D, Ferrer P, et al. Macromolecular and elemental composition analysis and extracellular metabolite balances of Pichia pastoris growing at different oxygen levels. Microbial Cell Factories. 2009;8:65.

[4]. Schutter K De, Lin Y, Tiels P, Hecke A Van, Glinka S, Peer Y Van De, et al. Genome sequence of the recombinant protein production host Pichia pastoris. Nature Biotechnology. 2009;27(May):561–6.

[5]. Wiechert W, De Graaf a a. In vivo stationary flux analysis by 13C labeling experiments.. Advances in biochemical engineering/biotechnology. 1996. page 109–54.