1 **Minimizing acetate formation in *E. coli* fermentations**

2 Marjan De Mey[*], Sofie De Maeseneire, Wim Soetaert and Erick Vandamme

3 Ghent University, Laboratory of Industrial Microbiology and Biocatalysis, Department of

4 Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Coupure links

5 653, B-9000 Ghent, Belgium

6

7 [*] **Correspondence address**: Marjan De Mey

8 Laboratory of Industrial Microbiology and Biocatalysis

9 Department of Biochemical and Microbial Technology

10 Faculty of Bioscience Engineering

11 Ghent University

12 Coupure links 653

13 B-9000 Gent

14 Tel: +32-9-264-60-28

15 Fax: +32-9-264-62-31

16 Marjan.DeMey@UGent.be

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18 **Abstract**

19 *Escherichia coli* remains the best established production organisms in industrial

20 biotechnology. However, during aerobic fermentation runs at high growth rates, considerable

21 amounts of acetate are accumulated as by-product. This by-product has negative effects on

22 growth and protein production. Over the last 20 years, substantial research efforts have been

23 spent to reduce acetate accumulation during aerobic growth of *E. coli* on glucose. From the

24 onset it was clear that this quest should not be a simple nor uncomplicated one. Simple

25 deletion of the acetate pathway, reduced the acetate accumulation, but instead other by-

26 products were formed. This minireview gives a clear outline of these research efforts and the

27 outcome of them, including bioprocess level approaches and genetic approaches. Recently,

28 the latter seems to have some promising results.

29

30 **1   Introduction**

31 *Escherichia coli* was the first and is still one of the most commonly used production

32 organisms in industrial biotechnology. Aerobic high cell density cultures of *E. coli* are most

33 frequently used to arrive at high biomass yields and high metabolite/protein concentrations.

34 Normally, glucose is fed as a carbon source in these high cell density fed-batch cultures.

35 Glucose is a cheap and simple molecule which enters the glycolysis (flow from glucose to

36 acetyl CoA) and the central metabolism through a minimum of steps. Furthermore, in a

37 medium with several carbon sources, glucose is first preferred one as a result of catabolite

38 repression. The glycolyse is the trunk routes of intermediary sugar metabolism in enteric

39 bacteria, which canalize 72% of the carbon supply. However, during aerobic fermentation

40 runs at high growth rates, considerable amounts of acetate are accumulated, as described by

41 Akkeson et al. (1999) [1] and references therein. In addition to a loss of carbon and therefore

42 an economic sink, acetate is also detrimental to recombinant protein production and inhibits

43 cell growth [2]. For more information about overcoming acetate in *E. coli* recombinant

44 protein fermentations see reference [3].

45 Formation of acetate in *E. coli* cultures under fully aerobic conditions can be caused by two

46 phenomena. On the one hand, a (local) lack of dissolved oxygen activates the fermentation

47 pathways, causing acetate excretion. This is referred to as mixed-acid fermentation. On the

48 other hand, this acetate excretion is also due to a metabolic overflow mechanism, caused by

49 an imbalance between the rapid uptake of glucose and its conversion into biomass and

50 products, diverting acetyl-CoA from the TCA-cycle towards acetate [1].

51

52 The two major aerobically active acetate producing pathways in *E. coli* are pyruvate oxidase

53 (*poxB*) and acetate kinase/phosphotransacetylase (*ackA-pta*). Two enzymes comprise the

54 *ackA-pta* pathway: phosphotransacetylase [EC 2.3.1.8] reversibly converts acetyl-CoA and

55 inorganic phosphate to acetyl phosphate and CoA, and acetate kinase [EC 2.7.2.1] reversibly

56 converts acetyl phosphate and ADP into acetate and ATP [4]. The two genes include one

57 operon [5] and are considered to be important for balanced carbon flux within the cell during

58 exponential growth both, aerobically and anaerobically [6, 7]. *E. coli* uses the *ackA-pta*

59 pathway as an ATP production source under anaerobic and even aerobic conditions [7].

60 The second enzyme, pyruvate oxidase [EC 1.2.2.2], is a pheripheral membrane protein that

61 converts pyruvate, ubiquinone and H_2O to acetate, ubiquinol and CO_2. It has been reported to

62 be a nonessential aerobic enzyme active in the early stationary phase [8, 9]. However, more

63 recent studies has shown that pyruvate oxidase plays an important role in the aerobic growth

64 efficiency of *E. coli* [10], perhaps to preserve the pool of free CoA-SH. Dittrich et al. (2005)

65 [2] confirm that the *poxB* pathway is more active during the late exponential and stationary

66 phases, whereas the *ackA-pta* pathway is more active in the exponential stage of the cell

67 growth. They also report that the two acetate producing pathways are affected by culture

68 conditions such as pH. Acidic environments repress the *ackA-pta* pathway, but activate the

69 poxB pathway. In addition, acetate itselfs has a strong negative effect on the two pathways.

70

71 Acetate formation has several disadvantages:

72 • acetate concentrations above ca. 1 g/l are damaging for both the biomass production

73   and the production of recombinant proteins [11]

74 • besides the inhibition on recombinant protein production, acetate has a negative effect

75   on the stability of intracellular proteins [12]

76 • organic acids already show negative effects at concentrations much lower than those

77   for mineral acids. The non-dissociated form of acetate can move freely through the

78   cell membrane and thus accumulates in the medium. A part of this extracellular, non-

79   dissociated form will re-enter the cell and dissociate due to the higher internal pH.

80   Acetate thus acts as a proton conductor and the process causes a reduction in proton

81   motive force [13]

82 • accumulation of acetate in the medium will acidify the medium. When the pH is below

83   5.0, cell lysis will appear due to the irreversible denaturation of proteins and DNA

84   [14].

85 The level of acetate produced during aerobic fermentations is depending on the *E. coli* strain,

86 the growth conditions, the actual glucose concentration in the medium and the overall

87 composition of the fermentation medium. For more detailed information about why, when and

88 how bacterial cells excrete acetate, see reference [15].

89

90 Researchers have tried a wide variety of strategies to reduce acetate accumulation in high cell

91 density fed-batch *E. coli* fermentations. These strategies are situated at two levels: the

92 bioprocess level and the genetic level.

4

93

2 Bioprocess level approaches to minimize acetate formation

These methods mostly intervene in the medium composition and/or the cultivation conditions. The fermentation run can be optimized by controlling a range of parameters such as temperature, agitation regime, volume, foaming, dissolved oxygen tension (DOT), pH, optical density, (limiting) substrate concentration, etc.

99

A culture of *E. coli* will generate acetate when the cells surpass a threshold specific rate of glucose consumption, regardless of the availability of oxygen to the culture [3]. In the literature, several specific fermentation strategies are mentioned to reduce acetate production [1, 16-20]. levels were developed. These methods are based on mathematical models that describe growth patterns and the expected demand for nutrients. These strategies include various glucose feeding approaches [21-27], limitations of growth rate by substrate-limited fed batch schemes [16, 18, 26, 28-30] and utilization of alternative feeds such as glycerol [26, 31], mannose [32] or fructose [33]. For example, reduced acetate and an increase in protein yield have been reported when fructose was used as carbon source instead of glucose [33]. Also supplementing the medium has proven to be positive on reducing acetate [34]. Recently, the combination of glucose pulses with an amino-acid containing feed stream has been demonstrated to be successful to minimize acetate production [35]. Another approach to hold the growth rate below the threshold for acetate production, is the pH-stat, where a nutrient feed is activated when the pH increases and variants, where the culture is dosed with more nutrient than necessary. However a fundamental drawback of the pH-stat is that it detects starvation rather than the acetate threshold directly [3]. In general, a consequence of limiting the growth rate is that biomass is generated at a slower rate than the cells are capable of achieving.

118

119   Instead of lowering the growth rate, the produced acetate can be removed from the culture

120   during the fermentation process to reduce the inhibitory effects of acetate. In literature, the

121   use of a dialyse-fermentors is reported to remove acetate from the culture [36, 37]. Dialysis is

122   defined as the separation of solute molecules by their unequal diffusion through a semi-

123   permeable membrane based on a concentration gradient. Recently, another method to remove

124   acetate from the fermentor was reported via the use of macroporous ion-exchange resins [38].

125   However, methods to remove acetate from the culture tend to remove also nutrients. In

126   addition, this strategy do not deal with the fact that carbon is diverted to a by-product and thus

127   the economic sink.

128

129   Although, these methods are extensively used in the industry, they are not the best solutions

130   because they undermine the maximum growth and production capacity. Therefore, we will

131   emphasize genetic approaches to minimize acetate formation.

132

133   **3   Genetic approaches to minimize acetate formation**

134   Several strategies which intervene with acetate formation on the genetic level have also been

135   reported. These strategies are based on the alteration of the central metabolism of *E. coli* (see

136   figure 1, table 1). First, the effect of alterations in the glucose uptake mechanism and in the

137   TCA cycle will be discussed. The pathway from glycolysis to the TCA cycle is very

138   important because of the many reactions which play a role in the pyruvate branch point. From

139   this branch point, the carbon flux can be directed to acetate production via the genes coding

140   for acetate kinase (*ackA*), phosphotransacetylase (*pta*), acetyl-CoA synthase (*acs*) and

141   pyruvate oxidase (*poxB*). The flux can also be directed to the TCA-cycle (citrate synthase,

142   *gltA*) where the glyoxylate bypass plays an important regulation control (isocitrate lyase,

143   *ackA*; malate synthase, *aceB* and isocitrate dehydrogenase; *icd*). In a final paragraph, the

144   influence of alterations in coenzyme pools on the acetate metabolism will be discussed.

145

146   Since *E. coli* is a facultative anaerobic strain, part of the glucose (even under aerobic

147   conditions) will be catabolized via fermentation (consuming no oxygen). Besides a lower

148   energy yield per mol glucose obtained by anaerobic fermentation, this causes a faster

149   utilization of glucose by the cells, as compared with aerobic respiration. [39]. Because of the

150   occurrence of reactions which run normally anaerobically in aerobic conditions, some

151   enzymes which are active under anaerobic growth conditions will be discussed. Strictly

152   anaerobic culture strategies and strategies based on involvement of non-*E. coli* pathways (e.g.

153   *pyc* pathway) will not be discussed.

154

155   3.1   The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase systems (PTSs)

156   The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase systems, which are both

157   transport and sensing systems, are an example of group translocation enzymes. PTS (figure 2)

158   is involved in the transport and the phosphorylation of a large number of carbohydrates (PTS

159   carbohydrates), in the movement of cells towards these carbon sources (chemotaxis), and in

160   the regulation of a number of metabolic pathways. The PTS catalyzes the following overall

161   process:

162

163 $$PEP_{(in)} + carbohydrate_{(out)} \xrightarrow{PTS} pyruvate_{(in)} + carbohydrate - P_{(in)}$$

164

165   Carbohydrate phosphorylation is coupled to carbohydrate translocation across the cytoplasmic

166   membrane, the energy for these processes being provided by the glycolytic intermediate PEP.

167   PTS consists of three kinds of proteins: enzyme I and histidine protein (HPr), which

168   participate in the phosphorylation of all PTS carbohydrates and thus have been called the

169   general PTS proteins, and enzyme II, which is carbohydrate specific.

170   Chou *et al.* (1994)[40], tried to reduce the acetate excretion by knocking out the *ptsG* gene,

171   coding for the glucose specific enzyme II of PTS [EC 2.7.1.69]. This method did not totally

172   prevent the uptake of glucose, but its uptake rate was reduced. Consequently, the flux through

173   the glycolysis was decreased, causing a reduced acetyl-CoA accumulation. Chou *et al.* (1994)

174   [40] observed no acetate excretion in cultures of this mutant. Similar results where found by

175   [41-43]. Moreover, Han *et al.* (2004)[41] found an increase in biomass and recombinant

176   protein production as result of knocking out *ptsG*. Another way to intervene in the PTS is to

177   influence the regulation of *ptsG*. It was found that the regulator ArcA binds to the promoter of

178   *ptsG*. Deletion of the *arcA* gene caused about a 2-fould increase in the *ptsG* expression.

179   Overexpression of *arcA* significantly decreased glucose consumption and hence decreased the

180   acetate accumulation [44]. Knocking out *ptsG* and overexpressing *arcA*, however, are a

181   genetic variant of limiting the glucose feed rate. Moreover, mutation of a PTS gene causes an

182   efficiency reduction in the energy metabolism [17].

183   However, recently it is been reported that the deletion of the ArcA gene in combination with

184   the overexpression of a heterologous NADH oxidase increased the glycolytic flux and

185   reduced acetate production [45, 46].

186

187   3.2   Pyruvate branch point

188   Many strategies of metabolic engineering are focussing on the enzymes around the pyruvate

189   branch point since the intracellular level of pyruvate has an immediate influence on acetate

190   excretion. Pyruvate is the substrate or end product of many reactions and thus an interesting

191   target for manipulation. The enzymes of the main reactions around pyruvate are: pyruvate

192   kinase (*pyk*), pyruvate dehydrogenase (*pdh*), pyruvate formate lyase (*pfl*), lactate

193   dehydrogenase (*ldh*), PEP-carboxylase (*ppc*) and PEP-carboxykinase (*pck*).

194   Lowering the pyruvate pool has been many times described in the literature as a way to

195   reduce acetate production [47, 48].

196   *Pyruvate kinase (pyk)*

197   Pyruvate kinase [PYK, EC 2.7.1.40] is one of the key enzymes of glycolysis. It catalyzes the

198   conversion of PEP into pyruvate and simultaneously converts ADP to ATP:

199 $$PEP + ADP \xrightarrow{\;PYK\;} pyruvate + ATP$$

200   In almost each cell type, the flux through this reaction controls the global flux through the

201   glycolysis. There are two isoenzymes of pyruvate kinase, encoded by *pyk-I* (or *pyk-F*) and

202   *pyk-II* (or *pyk-A*). The enzyme PYK-F is activated by fructose-1,6-bisphosphate while PYK-A

203   is activated by intermediary products of the pentose phosphate pathway, such as ribose-5-

204   phosphate [49].

205   Inactivation of one or both pyruvate kinase isoenzymes has already been tried several times to

206   reduce acetate production [50-52]. Several studies have reported that the glycolysis was down

207   regulated in *E. coli pykF* mutants under aerobic conditions [50, 53]. It was found that the flux

208   through phosphoenol pyruvate carboxylase and malic enzyme were up-regulated in the *pykF*⁻

209   mutant as compared with the wild type, and acetate formation was significantly reduced in the

210   mutant. Inactivation of one *pyk*-enzyme caused a slight decrease of the maximum growth rate.

211   This indicates that the other *pyk*-enzyme can compensate for the supply of the pyruvate pool,

212   together with the PTS. Inactivation of both *pyk*-enzymes causes a major decrease in the

213   growth rate and the acetate production [51]. Emmerling *et al*. (2002) [50] reported that

214   relatively more oxaloacetate is derived from PEP and more pyruvate from malate in

215   comparison with the wild type. This mechanism is probably activated to compensate to some

216   extent for the pyruvate kinase knock-out.

217 *Pyruvate dehydrogenase (pdh), expressed under aerobic culture conditions, in E. coli*

218 Pyruvate dehydrogenase [PDH, EC 1.2.4.1] complex catalyzes the conversion of pyruvate

219 into acetyl-CoA with the formation of one molecule CO_2.

220 $$pyruvate + CoA - SH + NAD^+ \xrightarrow{PDH} acetyl - CoA + NADH + H^+ + CO_2$$
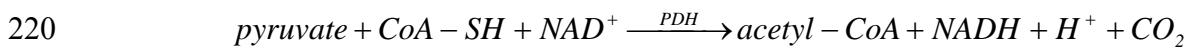
221 A too large pool of acetyl-CoA contributes to a large extent to the overflow of acetate.

222 Elmansi & Holms (1989) [54] tried to reduce the acetate excretion by decreasing the flux

223 from pyruvate to acetyl-CoA. This was achieved by adding 3-bromopyruvate, an inhibitor

224 which directly acts on the active domain of pyruvate dehydrogenase. They reported no acetate

225 production at all. However, there was still lactate produced and a decrease of the growth rate

226 with respect to the wild type was observed.

227 *PEP carboxylase (ppc) and PEP carboxykinase (pck)*

228 PEP carboxylase [PPC, EC 4.1.1.31] converts PEP into oxaloacetate and is referred as PPC

229 shunt by Noronha et al (2000) [55]:

230 $$PEP + CO_2 \xrightarrow{PPC} oxaloacetate + P_i$$

231 This reaction is activated by acetyl-CoA, guanosinetriphosphate and fructose-1,6-biphosphate,

232 and inhibited by aspartate and malate [56].

233 PEP-carboxykinase [PCK, EC 4.1.1.49] catalyzes the reverse reaction with consumption of 1

234 molecule ATP:

235 $$oxaloacetate + ATP \xrightarrow{PCK} PEP + ADP + CO_2$$
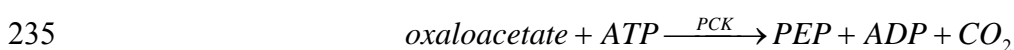
236 PCK is inhibited by high levels of PEP and nucleotides [56]. The purpose of PCK is to

237 maintain the PEP:oxaloacetate ratio and stabilize the pool of intermediate products of the

238 Krebs cycle. The net reaction of the cycle formed by both reactions consumes one molecule

239 of ATP. In *E. coli* wild type strains, this futile cycle is strongly regulated. Inactivation of the

240 *pck* gene causes a decrease of PEP carboxylation and a stimulation of the glyoxylate cycle.

241 Yang et al. (2003) [57] reported that *pck* deletion mutants are able to grow on high

242 concentrations of glucose without acetate production.

243   Chao & Liao (1994) [58] decided that overexpression of both enzymes gives an increased

244   activation of the futile cycle with a higher production of fermentative products as a

245   consequence. The double overexpression was also responsible for less growth and a higher

246   consumption of glucose and oxygen. All these are consequences of the leakage of energy via

247   the futile cycle.

248   Simple overexpression of the *pck* causes a slight increase in acetate production [59]. On the

249   other hand, overexpression of *ppc* can completely eliminate acetate production [59, 60].

250   According to Chao & Liao (1993) [59], overexpression of *ppc* decreases the glucose

251   consumption rate and organic acid excretion, while growth and respiration rate remain

252   unchanged. Farmer (1997) [17] described the effect of overexpressing PPC in *E. coli* VJS632

253   aerobic cultures and conclude that the final acetate concentration is reduced by 60%. A

254   reduction of 60% of the acetate excretion by overexpression of PPC was also obtained for *E.*

255   *coli* ML308 by Holms (1996) [61]. Knocking out *ppc* has a negative effect on the overall cell

256   metabolism: growth rate is impaired and the excretion of undesirable metabolites increases

257   [60, 62 , 63].

258   Noronha et al. (2000) [55] showed that the TCA cycle/PPC shunt flux ratio is differing

259   between a low acetate producer, *E. coli* BL21 and a high acetate producer, JM109. The PPC

260   shunt is active in BL21 and inactive in JM109. This was confirmed by Yang et al. (2003)

261   [57]. In contrast to the wild type, *ppc* overexpression mutants show more activity of the

262   glyoxylate bypass [57] making a higher flux through the Krebs cycle possible. This means

263   that the ratio PPC bypass:Krebscycle will decrease strongly. According to Yang et al (2003)

264   [57], this ratio is very important for the production of acetate.

265   It is generally accepted that PPC activity strongly regulates the PCK activity. In the wild type,

266   PPC is more active than PCK, firstly, to compensate for the activity of PCK and secondly, to

267   supply the Krebs cycle with sufficient intermediates. Inactivation of *pck* leads immediately to

268   a decrease in PPC activity, since compensation of PCK is no longer needed [57].

269   Overexpression of *pck* deregulates this system, causing an increase of the acetate production.

270   3.3   Phosphotransacetylase (*pta*) and acetate kinase (*ackA*)

271   Phosphotransacetylase [PTA, EC 2.3.1.8] and acetate kinase [ACKA, EC 2.7.2.1] are the

272   enzymes that accomplish the production of acetate from acetyl-CoA. Phosphotransacetylase

273   catalyzes the conversion of acetyl-CoA to acetylphosphate with production of CoA-SH.

274   Phosphotransacetylase is activated by pyruvate and inhibited by $NADH+H^+$ [64]

275    $$acetyl - CoA + P_i \xrightarrow{PTA} acetylphosphate + CoA - SH$$
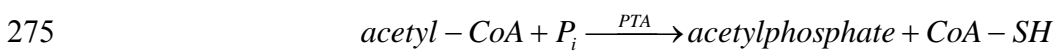
276   Acetate kinase uses the product formed by phosphotransacetylase as substrate; this is the last

277   step of the acetate pathway.

278    $$acetylphosphate + ADP \xrightarrow{ACKA} acetate + ATP$$
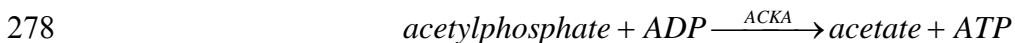
279   Both reactions are reversible. As such, the cell can convert acetate to acetyl-CoA and

280   subsequently use it for biosynthesis reactions.

281

282   Mutations in both *pta* and *ackA* have frequently been investigated [2, 11, 54, 65]. All data

283   report a strong reduction of acetate production, when *ackA* and/or *pta* are eliminated. This is

284   at the expense of the growth rate and is accompanied by an increase in the production of other

285   fermentation products such as lactate and formate. Though lactate and formate are less toxic

286   to the cells, they are still disadvantageous for cell growth.

287

288   3.4   Acetyl-CoA synthetase (*acs*)

289   When glucose is used in high cell density cultures, acetate is produced and excreted in the

290   medium. Acetyl-CoA synthethase [ACS, EC 6.2.1.1] can use the re-absorbed acetate and

291   convert it to acetyl-CoA via a two step reaction scheme. These reactions are irreversible and

292   thus they are only responsible for acetate consumption and not for acetate production.

293 $$acetate + ATP + CoA - SH \xrightarrow{ACS} acetyl - CoA + AMP + PP_i$$

294   The enzyme has a strong affinity for acetate (Km of 200μM), which allows it to function at

295   low acetate concentrations, but it is inhibited by glucose [66, 67]. On the other hand, the

296   reversible *ackA-pta* pathway can assimilate acetate only at high acetate concentrations [15].

297

298   Contiero *et al*. (2000) [11] investigated the effect of the deletion of *acs* on the growth on

299   glucose at high cell densities in fed-batch fermentations. No clear conclusion could be drawn

300   from their research. It only indicated that acetyl-CoA plays a key role in accomplishing high

301   cell densities and has no or few importance during normal growth.

302   The overexpression of *acs* in *E. coli* resulted in a significant reduction in acetate formation

303   during glucose metabolism. It also enhanced the assimilation of acetate when used as the sole

304   carbon source. These characteristics guarantee *acs* overexpression as a positive approach to

305   coping with acetate in *E. coli* fermentations [68].

306

307   3.5   Pyruvate oxidase (*poxB*)

308   Pyruvate oxidase [POXB, EC 1.2.2.2] catalyses the oxidative carboxylation of pyruvate to

309   acetate and CO_2 [69]. This 'non-essential' enzyme is a part of the respiratory chain.

310 $$pyruvate + H_2O + ubiquinone - 8 \xrightarrow{POXB} acetate + CO_2 + ubiquinol - 8$$
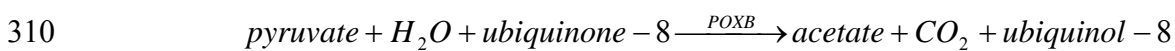
311   An elevated intracellular concentration of pyruvate activates this enzyme, suggesting that

312   POXB regulates the flux partitioning of pyruvate, presumable to reduce the carbon flux

313   towards acetyl-CoA in order to maintain the intracellular pool of CoA for other metabolic

314   functions [10]. Abdel-Hamid et al. (2001) [10] investigated the function of *poxB* in *E. coli* by

315   knock-out mutants. Inactivation of *poxB* results in a decrease of 24% of the carbon converted

316   into biomass. The amount of carbon necessary for energy production increased with 23%.

317   They concluded that puruvate oxidase is essential for a good functioning of the overall

318   metabolism. They advised not to use *poxB* as target to decrease acetate production. However,

319   Causey et al. (2004), have reported the beneficial effect of $poxB^-$ mutation on pyruvate

320   production and cell growth. The relationship between POXB and acetate formation has been

321   studied. Vemuri et al. (2005) [70] studied the physiological response of *Escherichia coli*

322   central metabolism to the expression of heterologous pyruvate carboxylase (PYC) in the

323   presence or absence of pyruvate oxidase. The presence of PYC activity in *E. coli* substantially

324   increases the cell yield from glucose, particularly for a *poxB* mutant, biomass which appears

325   to be derived directly or indirectly from acetate [70]. Recently, a *poxB* knockout strain (and

326   also knockouts in *ldhA* and *pflB* genes) demonstrated significantly reduced acetate formation

327   when the strain was subjected to oscillatory oxygenation [71].

328   3.6   Citrate synthase (*gltA*)

329   Citrate synthase [CS, EC 2.3.1.1] is the first enzyme of the Krebs cycle. It delivers acetyl-

330   CoA in the cycle via binding with oxaloacetate. During this reaction one molecule of citrate is

331   formed.

332 $$oxaloacetate + acetyl - CoA \xrightarrow{CS} citrate + CoA - SH$$
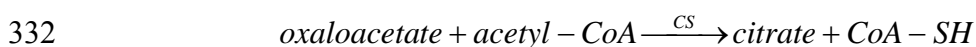
333   Citrate synthase is inhibited by α-ketoglutarate and activated by $NADH+H^+$ [56]

334   This reaction is often indicated as the rate limiting step of the Krebs cycle. Overexpression of

335   *gltA* showed a decrease of acetate production but no real elimination of it [60]. Knocking out

336   *gltA* caused a strong increase of the acetate production, accompanied by an increase in

337   formate and pyruvate excretion [72].

338

339   3.7   Isocitrate lyase (*aceA*), malate synthase (*aceB*) and isocitrate dehydrogenase (*icd*)

340   Isocitrate lyase [ICL, EC 4.1.3.1] and malate synthase [MS, EC 2.3.3.9] catalyse the reactions

341   of the glyoxylate bypass. Isocitrate lyase converses isocitrate in one molecule succinate and

342   one molecule glyoxylate.

343 $$isocitrate \xrightarrow{ICL} glyoxylate + succinate$$
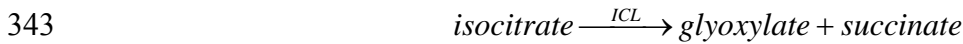
344   Malate synthase convert glyoxylate and acetyl-CoA into one molecule malate.

345 $$glyoxylate + H_2O + acetyl - CoA \xrightarrow{MS} malate + CoA - SH$$
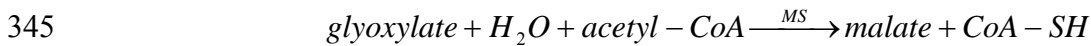
346   Besides their role in the Krebs cycle, oxaloacetate and α-ketoglutarate are also used for

347   further biosyntheses. This can cause an exhaustion of the Krebs cycle intermediates, because

348   of the continuous need for those essential intermediates. The glyoxylate bypass has as goal to

349   provide the Krebs cycle with additional oxaloacetate. The switch over from the Krebscycle to

350   the glyoxylate bypass occurs at the isocitrate dehydrogenase [IDH, EC 1.1.1.42] step.

351 $$isocitrate + NADP^+ \xrightarrow{IDH} \alpha - ketoglutarate + NADPH + H^+ + CO_2$$
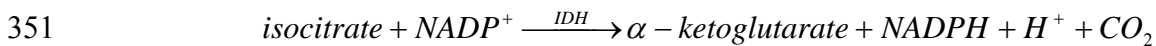
352   Isocitrate dehydrogenase has more affinity for isocitrate than isocitrate lyase. This regulation

353   takes place by reversible phosphorylation of isocitrate dehydrogenase under the influence of

354   the intracellular oxaloacetate level. At high levels of oxaloacetate, isocitrate dehydrogenase is

355   phosphorylated; this phosphorylated form of isocitrate dehydrogenase is not active and as a

356   result, the glyoxylate shunt is activated [57].

357

358   Aoshima et al. (2003) [73] found that knocking out isocitrate dehydrogenase results in an

359   increase of citrate. El-Mansi *et al*. (1994) [74] tried to delete the glyoxylate shunt by

360   overexpression of isocitrate dehydrogenase. Because of this, the flux through isocitrate lyase

361   decreased, but the intracellular pool of isocitrate became exhausted; they concluded that

362   isocitrate dehydrogenase is not the rate limiting step in the Krebs cycle. Farmer and Liao

363   (1997) [17] stimulated the flux through the glyoxylate shunt by inactivation of the *fadR*

364 operon. This operon negatively controls the expression of isocitrate lyase and malate

365 synthase. Acetate production decreased with 13% by stimulating the glyoxylate shunt.

366

367 Yang et al. (2003) [57] concluded that the glyoxylate bypass is of big importance in the

368 regulation of the ratio PPC-shunt/Krebs cycle. A high ratio should give a higher acetate

369 production. When the oxaloacetate concentration in the cell is too low, the balance between

370 the glycolyse and the Krebs cycle is deregulated, causing acetate production. It has also been

371 observed that the glyoxylate shunt is active in a low acetate producer, while it is inactive in a

372 high acetate producer [55].

373

374 3.8 Alterations in the coenzyme pools

375 Most current metabolic engineering studies have focused on enzyme levels and on the effect of

376 amplification, addition, or deletion of a particular pathway.

377 When enzyme levels are not limiting, the availability and occurrence of coenzymes can

378 become limiting. It is conceivable that in coenzyme-dependent production systems, coenzyme

379 availability and the proportion of coenzyme in the active form may play an important role in

380 dictating the overall process yield. Hence, the manipulation of these coenzyme levels may be

381 crucial in order to further increase production [75-78].

382 *$NADH+H^+/NAD^+$*

383 NAD^+ plays a significant role in primary metabolism. It is a coenzyme of more than 300

384 redox reactions. By using this coenzyme, the cell can maintain its redox state in balance.

385 NAD^+ and $NADH+H^+$ play a major role in catabolism. To catabolize glucose into precursors,

386 NAD^+ is used as coenzyme and converted to $NADH+H^+$. In anabolism, $NADP^+$ and

387 $NADPH+H^+$ occur more frequently, but with a similar function. The cell regenerates the

388 produced $NADH+H^+$ into NAD^+ by the reduction of oxygen (under aerobic conditions), or via

389 another oxidising product or via fermentation. It is also generally known that the ratio

390 NADH+H$^+$/NAD$^+$ regulates the expression of certain genes, such as *adhE*, coding for alcohol

391 dehydrogenase [EC 1.1.1.1], and the activity of certain enzymes, such as the enzymes of the

392 pyruvate dehydrogenase complex [75].

393 Berrios-Rivera et al. (2002) [75] investigated mainly alterations in the ratio

394 NADH+H$^+$/NAD$^+$. Under aerobic conditions, formate was added to activate pathways that

395 normally do not function. The results were depending on whether the formate dehydrogenase

396 was endogenous or not (originating from *Candida boidinii*). Adding formate to the strain with

397 the cell-own formate dehydrogenase under aerobic conditions resulted in a large increase in

398 the acetate production compared to the strain with the heterologous enzyme, were a small

399 increase of the acetate production was observed [75].

400 Acetate overflow at high glucose consumption rates is believed to result from an enzymatic

401 limitation in the TCA cycle causing excess carbon from glycolysis to be shunted acetate or

402 from a saturation of the respiratory pathways used to reoxidize NADH [46]. Since the

403 glycolysis and TCA cycle generate NADH while acetate formation does not, saturation of

404 NADH oxidation at high glucose consumption rates could cause the cell to form acetate in

405 order to modulate the redox balance [45]. Recently a strong link was demonstrated between

406 redox ratio (in vivo molar concentration ratio NADH/NAD) and acetate overflow metabolism

407 in *E. coli* [45]. The authors revealed that the initiation of acetate overflow metabolism

408 occurred above a critical NADH/NAD ratio of 0.06. In addition, the acetate production could

409 be delayed by the expression of the heterologous NADH oxidase. Expression of the

410 heterologous NADH oxidase coupled with the deletion of the regulatory *arcA* gene in *E. coli*,

411 increased the glycolytic flux and reduced acetate production [46]. The presence of the

412 heterologous NADH oxidase or the absence of ArcA reduced acetate about 50% and

413 increased the recombinant protein production by 10-20%. The presence of the heterologous

414 NADH oxidase in the *arcA* knock-out strain eliminated acetate production entirely in batch

415 fermentations and resulted in a 120% increase in the recombinant protein production.

Coenzyme A (CoA-SH)

417 A second important type of coenzymes is coenzyme A and its derivates (acetyl-CoA,

418 succinyl-CoA,…). Acetyl-CoA is an essential intermediate in many energy yielding

419 processes. More than 100 different reactions of the central metabolism depend on this

420 substrate. It is the main source of activity of the Krebs cycle [78].

421 The intracellular pool contains mainly short chain CoA-thioesters such as acetyl-CoA and

422 succinyl-CoA. CoA A-thioesters of long chain fatty acids form the intermediates of the β-

423 oxidation route and in the production of phospholipids. Besides the major role of CoA-SH in

424 these pathways, it has a substantial regulatory effect. CoA-SH inhibits or activates reactions

425 of the central metabolism and of the fatty acid biosynthesis [79].

426 The ratio acetyl-CoA/free acetyl-CoA is constant in *E. coli* cells grown on glucose. This ratio

427 might regulate certain enzymes of the central metabolism [56].

428 San et al. (2002) [78] investigated the production and availability of CoA-SH by means of the

429 biosynthesis of isoamylacetate. This pathway does not occur in *E. coli* wild type cells. Acetyl-

430 CoA is used as substrate for the production of isoamylacetate and thus manipulation of the

431 CoA-SH pool influences the production of isoamyl acetate. Pantothenate kinase is the rate

432 limiting step in the CoA-SH production. This enzyme is inhibited by CoA-SH and acetyl-

433 CoA. San et al. (2002) [78] observed small differences in the central metabolism when *coaA*

434 is overexpressed. Acetate and ethanol concentration increased hardly with respect to the

435 isoamyl acetate level, which increased 3 times.

436   3.9   Alteration in ATP level

437   Metabolic control theory postulates that flux control can be shared by many enzymes in a

438   pathway and that control could also reside outside the pathway, for instance, in the process

439   that consumes the ATP generated in the glycolysis (ATP demand). Koebmann et al. (2002)

440   [80] investigated whether ATP consumption by cellular processes determines the steady-state

441   flux through glycolysis, by increasing the current ATP consumption rate. Therefore, they

442   introduced an ATP-consuming process that does not interfere with other aspects of

443   metabolism. The added ATP activity resulted in up to 70% increase in the rate of glycolysis

444   and they estimate that major control (>75%) resides outside the glycolysis, i.e., in enzymes

445   that consume ATP.

446

447   **4   Conclusion and perspectives**

448   Over the last 20 years, substantial research efforts have been spent to reduce acetate

449   accumulation during aerobic growth of *E. coli* on glucose. From the onset it was clear that this

450   quest should not be simple. Simple deletion of the acetate pathway, reduced the acetate

451   accumulation, but instead other by-products were formed. From the current state of the

452   literature, we can conclude that reduction of acetate requires a multigene action. In particular,

453   one has to pay attention to the regulation of futile cycles, anapleurotic pathways, coenzyme

454   levels, acetate producing pathways and ATP consuming pathways. The expression of the

455   heterologous NADH oxidase in an *arcA* knock-out strain seems promising. However, the

456   intuitive prediction of the manipulation consequences of several genes is difficult. In most

457   cases the construction of a producer strain did not turn out to be as straightforward as was

458   initially anticipated. Indeed, in complex metabolic networks, it is often a difficult task to ad

459   hoc predict the impact, both qualitatively and quantitatively, of a genetic intervention [81].

460   Moreover, as the focus in metabolic engineering is shifting from massive overexpression and

461   inactivation of genes towards the fine tuning of gene expression [82-90], the need for a

462   reliable, quantitative predictor, i.e. a model, is rapidly growing. The use of metabolic flux

463   analysis is vital here. Metabolic models allow a better prediction of genetic interventions and

464   can help to predict the construction of the ideal *E. coli* phenotype.

465

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734 **Figure legends**

735 **Figure 1. The central metabolism of *Escherichia coli***

736

737 **Figure 2. PTS-system and regulation**

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739 **Tables**

740 **Tabel 1. Influence of genetic approaches to minimize acetate formation. Abbreviations: KO: knock-out; OE: overexpression**

pathway	gene	protein	KO/OE	result	reference
PTS	*ptsG*	glucose specific enzyme II	KO	glycolyse flux ↓, no acetate excretion	[40]
				energy metabolism ↓	[17]
				acetate ↓	[43]
				growth rate ↓, flux to TCA cycle ↑	[42]
				acetate ↓, recombinant protein ↑, biomass ↑	[41]
	arcA	regulator *ptsG*	KO	ptsG ↑	[44]

			OE	glucose consumption ↓	[44]
				acetate accumulation ↓	
			KO *ptsG*, *pykF* and *pykA*	acetate ↓	[43, 91]
Glycolyse	*pfk*	phophofructokinase	OE	ethanol ↓,lactate and acetate ↑	[92]
Pyruvate branchpoint	*pyk*	pyruvate kinase	KO	KO_1: growth rate and acetate ↓	[50]
				KO_2: growth rate and acetate ↓↓	[51]
			KO *PykF*		[53]
	pdh	pyruvate dehydrogenase	Inhibition PDH	no acetate production, growrh rate ↓, lactate ↑	[93]
	pfl	pyruvate formate lyase	KO	lactate ↑, small ↓ flux acetyl CoA to acetate	[94-96]
	ldh	lactate dehydrogenase	OE	acetate ↓	[97]
			KO *pfl* and *ldh*	acetate and lactate ↓, malate ↑	[98]

	ppc	PEP carboxylase	KO	acetate ↓, growth rate ↓, glyoxylate shunt ↑, glycolysis and PPpw ↓	[60, 62, 63]
			OE	no/↓ acetate , growth yield ↑,	[17, 59-61]
	pck	PEP carboxykinase	KO	PEP carboxylation ↓, glyoxylate ↑, no acetate on high glucose	[57]
			OE	acetate ↑	[59]
			OE *ppc* and *pck*	fermentative products ↑,growth yield ↓	[58]
			OE *ppc* or *pck*	PPC bypass:Krebscycle ↓↓	[55, 57]
	ackA *pta*	acetate kinase phosphotransacetylase	KO *ackA* or *pta*	acetate ↓↓, groxth rate ↓, formate and lactate ↑	[2, 11, 54, 97, 99]
	acs	acetyl-CoA synthetase	KO	no clear conclusions	[11]

			OE	acetate ↓↓	[68]
	poxB	pyruvate oxidase	KO	C-yield ↓↓	[10]
	gltA	citrate synthase	KO	acetate ↑↑, pyruvate and formate ↑	[72]
			OE	acetate ↓	[60]
	aceA	isocitrate lyase	stimulation shunt	acetate ↓	[17]
	aceB	malate synthase			
	icd	isocitrate dehydrogenase	KO	accumulation citrate	[73]
			OE	no rate limiting step Krebs	[74]

741

Metabolic pathway map.

Metabolites and cofactors (diagram labels):

Dihydroxyaceton-P, Glyceraldehyde-3-P, Fructose-1,6-bis P, Fructose-6-P, Glucose-6-P, Glucose, Methylglyoxal, Lactate, Acetoïne + CO_2, Formate, CO_2, Acetyl-CoA, Glycerate-1,3-bisP, Glycerate-3-P, Glycerate-2-P, PEP, Pyruvate, Acetylphosphate, Acetate, Ubiquinone-8, Ubiquinol-8, Acetaldehyde, Ethanol, Oxalacetate, Citrate, Malate, Isocitrate, Glyoxylate, α-Ketoglutarate, Fumarate, Succinate, Succinyl-CoA.

Cofactor labels: NAD^+, NADH, ATP, ADP, P_i, AMP, CoA, H_2O, CO_2, $NADP^+$, NADPH, FAD^+, $FADH_2$, NAD$^+$/NADP$^+$, NADH/NADPH.

Enzyme labels on arrows: mgsA, fba, tpi, pgi, pyfA, pyfB, glk, pyk, pps, pdh, ldhA, ilvBG₂HIMN, pflB tdcB, poxB, ackA, pta, acs, aldH, adhE, adhCEP, gapA, pgk, gpm, eno, ppc, pck, gltA, acn, mdh, maeA maeB, fumA, aceB, aceA, icd, sucAB lpd, sucCD, sdhABCD.

Enzyme legend:

Abbreviation	Enzyme
aceA	Isocitratelyase
aceB	Malate synthase
ackA	Acetate kinase
acn	Aconitase
acs	AcetylCo synthase
adhCEP	Ethanol dehydrogenase
adhE	Acetyldehydrogenase
aldH	Aldehyde dehydrogenase
aspA	Aspartate ammonia-lyase
aspC	Aspartate aminotransferase
eno	Enolase
fba	Fructosebisphosphate aldolase
fumA	Fumarase
gapA	Glyceraldehyde-3-phosphate dehydrogenase
glk	PTS, glucokinase
gltA	Citrate synthase
gpm	Phophoglyceratemutase
icd	Isocitrate dehydrogenase
ilvBG₂HIMN	Acetolacetate decarboxylase
ldhA	Lactate dehydrogenase
maeA,B	Malic enzyme
mdh	Malate dehydrogenase
mgsA	Methylglyoxal synthase
pck	Phosphoenolpyruvatecarboxykinase
pdh	Pyruvate dehydrogenase
pfkB, tdcE	Pyruvateformatelyase
pgi	Phosphogluco-isomerase
pgk	Phophoglyceratekinase
poxB	Pyruvate oxidase
ppc	Phosphoenolpyruvatecarboxylase
pps	Phosphoenolpyruvate synthase
pta	Acetylphosphotransferase
pyfA,B	6-phophofructokinase
pyk	Pyruvate kinase
sdhABCD	Succinate dehydrogenase
sucAB, lpd	α-ketoglutarate dehydrogenase
sucCD	Succinate thiokinase
tpi	Triosephosphateisomerase