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琥珀酸生物合成过程中CO_2代谢调控的研究进展

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摘要 作为重要的四碳平台化合物, 琥珀酸被美国能源部列为12种最具潜力的大宗生物基化学品之首. CO_2作为琥珀酸合成的必需底物, 是决定琥珀酸合成效率的关键因素. 本文从CO_2溶解、转运及转化、固定和组合调控4个方面系统评述了琥珀酸生物合成过程中CO_2代谢调控的研究进展, 并探讨了CO_2调控策略的未来发展方向.

关键词 琥珀酸, CO_2, 生物合成, 代谢调控

作为一种重要的绿色化工四碳平台化合物, 琥珀酸是生产表面活性剂、食品添加剂、药物中间体及离子螯合剂等的原料, 在洗涤、食品、医药和电镀等行业得到广泛应用[1~3], 因此美国能源部将其列为12种最具潜力的大宗生物基化学品之首[4,5]. 目前琥珀酸每年的市场容量约在3~5万吨, 市场价格2400~3000美元/吨[6], 到2020年市场容量有望突破70万吨[7], 其中生物基琥珀酸的市场销售额有望达到8.4亿美元(http://www.transparencymarketresearch.com/succinic-acid.html).

琥珀酸生产方式有传统的石油化工技术路线和日益兴起的生物制造, 前者以不可再生的战略资源石油产品作为原料, 原料供给不可持续、生产过程环境污染严重, 影响了琥珀酸的发展. 随着经济的发展, 全球化学品市场日益增加, 面对日益枯竭的化石资源和其所产生的严重环境污染, 为了经济和社会的可持续发展, 必须以可再生的生物质资源替代不可再生的石化资源, 以清洁高效的生物炼制加工方式替代污染低效的石油化工技术路线, 即利用微生物发酵生产琥珀酸, 实现资源利用和物质加工方式的根本改变, 从而推动生物基琥珀酸的大规模生产, 同时利用琥珀酸发酵过程耗CO_2这一特性, 促进CO_2减排, 缓解温室效应.

琥珀酸作为三羧酸循环的中间产物, 可以通过众多微生物如*Actinobacillus succinogenes*[8,9], *Mannheimia succiniciproducens*[10,11], *Anaerobiospirillum succiniciproducens*[12,13], *Escherichia coli*[14,15], *Saccharomyces cerevisiae*[16,17]和*Corynebacterium glutamicum*[18,19]等合成, 其中产琥珀酸放线杆菌(*A. succinogenes*)和大肠杆菌(*E. coli*)的研究最为广泛. *A. succinogenes*是一种兼性厌氧

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菌, 底物利用范围广, 以琥珀酸为主要发酵产物, 能够耐受高浓度的葡萄糖和琥珀酸盐, 但需要复杂的营养条件[20,21], 而*E. coli*在厌氧条件下通常进行混合酸发酵, 主要合成甲酸、乙酸和乳酸等, 琥珀酸仅微量合成, 但因为其培养条件相对简单、遗传背景及代谢途径清楚, 便于采用各种分子生物学技术对菌种进行改造, 所以被广泛应用于琥珀酸生物合成的研究[22]. 大肠杆菌主要通过好氧到厌氧的两阶段发酵来合成琥珀酸[14,23], 但改造后在好氧条件下琥珀酸也能够超量合成[24,25].

琥珀酸作为C4化合物, 在经过C3化合物磷酸烯醇式丙酮酸(phosphoenolpyruvate, PEP)进入三羧酸(three carboxylic acid, TCA)循环还原臂时, 需要固定一分子的CO_2生成C4化合物草酰乙酸(oxaloacetate, OAA), 进而转化为目标产物琥珀酸, 因而CO_2是琥珀酸生物合成过程中的必需代谢底物, 但CO_2被微生物细胞利用需经历从气相溶解到液相, 然后从胞外转运到胞内及转化, 再参与胞内代谢这3个过程(图1), 是决定琥珀酸合成效率的关键因素.

1 CO_2溶解过程

CO_2溶解浓度的提高可以有效促进琥珀酸的生成, 也可降低杂酸产量, 提高琥珀酸/杂酸生成比例. 根据亨利定律, CO_2溶解浓度与CO_2分压直接相关, 在恒定温度下分压越高, 溶解浓度越高, 直到达到饱和, 但实际发酵过程中, 由于发酵液中复杂的溶液成分与溶解环境, CO_2的溶解度还与溶质种类、pH及温度等因素有关. 德国布伦瑞克大学的Schumpe研究组[26,27]在1996年针对CO_2等15种常见气体, 建立了一套可适用于62种有机物质(如葡萄糖、玉米浆等)与50种无机离

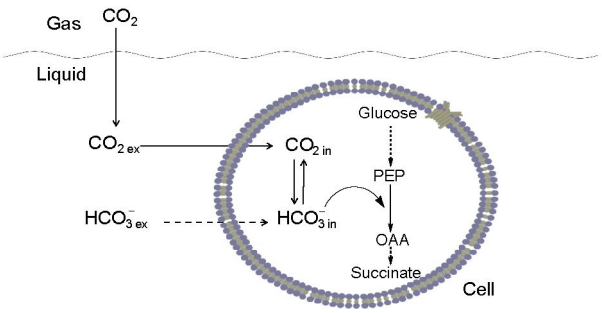

图 1　CO_2从溶解、转运及转化到固定的过程

子的亨利系数模型, 并将该模型的应用范围推广至含有多种有机物与无机离子的体系. 在此基础上, Lee研究组[28]建立了适用于复杂混合溶液的CO_2溶解模型, 并将其应用于指导CO_2溶解度的调控. 当CO_2分压从38.0 kPa上升至101.3 kPa时, 发酵液中CO_2溶解浓度由8.7 mmol/L上升至23.0 mmol/L, 提高165.8%, 琥珀酸产量由7.4 g/L上升至8.3 g/L, 提高11.7%.

在琥珀酸发酵过程中, 为了增加CO_2的溶解度, 会外源添加碳酸盐或碳酸氢盐作为CO_2供体. Zou等人[29]发现, $MgCO_3$添加量由2.9 g/L提升至11.7 g/L时, CO_2溶解浓度由55.0 mmol/L提升至159.2 mmol/L, 对应的琥珀酸产量由15.3 g/L提高至25.9 g/L. 这些碳供体不但可以提高CO_2溶解浓度, 还能在一定程度调控发酵液pH[30,31]. 此外, 多种金属离子的引入, 还对发酵过程产生其他影响, 例如, Liu等人[31]和Bazaes等人[32]发现, Na_2CO_3, $MgCO_3$和$CaCO_3$分别添加后, Na^+会导致细胞凝聚, Ca^{2+}对细胞通透性影响显著, Mg^{2+}可能作为PEP羧化激酶辅助因子而对该酶有激活作用.

CO_2在气相和液相间的溶解和释放是一个动态平衡的过程, 因而溶解的CO_2如果不能尽快利用, 就会转为气态释放出去. 针对这个问题, Ye研究组[33]设计了带有自吸型搅拌桨的生物反应器, 搅拌桨是中空结构, 桨顶端带有空腔, 开口位于液面上方, 当搅拌桨转速达到300 r/min, 搅拌桨顶端空腔形成的低压就会使液面上方的CO_2通过搅拌桨再次进入发酵液中, 使CO_2能够循环利用, 从而维持较高的CO_2气相到液相的转换速率, 每生成1 g琥珀酸所需提供的CO_2量从文献报道的9.4~21.9 g降低到约0.3 g, 有效提高了CO_2的利用率.

2 CO_2跨膜转运及胞内转化过程

CO_2分子在发酵液中主要以CO_2, CO_3^{2-}和HCO_3^- 3种形式存在[34], 且均能够以被动扩散的形式从细胞外进入细胞内被利用. 其中CO_2分子较容易透过细胞膜扩散到胞内[35], 也能够轻易地由胞内扩散到胞外, 因此很难维持胞内高浓度CO_2. 同时由于微生物细胞中进行CO_2固定的PEP羧化酶(phosphoenolpyruvate carboxylase, PPC)或羧化激酶(phosphoenolpyruvate carboxylase kinase, PCK), 均以HCO_3^-为直接底物[36,37], 因而需要CO_2在胞内进行转化, 以HCO_3^-形式参与代谢. 由于大肠杆菌属于混酸发酵, 为了提高琥珀酸产量, 通常会

对副产物合成编码基因进行敲除, 减少PEP到丙酮酸的转化, 而积累的PEP同时需要更多的HCO_3^-共同作为底物进行羧化, 因而更高浓度的HCO_3^-对于琥珀酸合成十分重要. Wang等人[38]通过过表达来自*Anabaena* sp. 7120的碳酸酐酶编码基因(carbonic anhydrase), 使大肠杆菌中碳酸酐酶活性从检测不到上升至21.8 U (mg protein)$^{-1}$, 有效提高细胞内CO_2分子与HCO_3^-离子之间的转化速率, 增强HCO_3^-的供给, 从而使琥珀酸的对糖得率从0.2 mol/mol提高到0.4 mol/mol, 可见高浓度的HCO_3^-是促进琥珀酸合成的有力保证.

HCO_3^-作为CO_2在发酵液中溶解的另一种形式, 其对大肠杆菌细胞膜的渗透性相对较差[39], 只有小部分能扩散到细胞内部, 导致胞内HCO_3^-浓度较低, 可见单纯依靠被动扩散的摄取方式使大肠杆菌内部CO_2和HCO_3^-的浓度都处于低水平, 限制了底物的摄入效率, 直接制约琥珀酸产量的提高.

蓝藻细胞作为一种自养原核生物, 具备积累高浓度胞内HCO_3^-的能力. 在蓝藻中至少发现5种无机碳转运蛋白, 其中包括3种HCO_3^-转运蛋白和2种CO_2转运蛋白[40], 利用这些蛋白, 蓝藻细胞可以主动从外界摄取无机碳进行光合作用. 本研究组通过在大肠杆菌细胞膜上单独或组合表达*Synechocystis* PCC6803中的HCO_3^-转运蛋白BicA和SbtA, 使大肠杆菌实现了对HCO_3^-的主动运输, 转运通量从30.0提高到71.1 μmol (g cell)$^{-1}$, 提高了1.4倍[41], 但同时发现这两种转运蛋白的表达对大肠杆菌细胞生长及琥珀酸合成产生了一定抑制作用, 这可能是由于胞内过多HCO_3^-积累对胞内pH的影响, 从而使细胞代谢发生紊乱. 因此, 单纯提高HCO_3^-的供给对于琥珀酸的合成是不够的, 还需要进一步提高CO_2固定反应的速率, 才能真正对琥珀酸合成起到促进作用.

3 CO_2固定过程

微生物通过糖酵解途径生成的PEP是琥珀酸代谢网络的节点, 其下游将出现C4与C3两条代谢支路: (ⅰ) C4支路上 PEP与HCO_3^-经羧化生成OAA, 进而转化为琥珀酸, 实现CO_2的固定; (ⅱ) C3支路上PEP经过脱羧还原反应生成甲酸和乳酸等杂酸. 可见, PEP在琥珀酸代谢途径中占据重要位置, 对该节点进行代谢流调控, 使主动运输到胞内的HCO_3^-及时有效地实现固定合成琥珀酸, 避免其转化、扩散和流失, 将引导C4支路占据代谢网络的主导地位.

促进CO_2固定的思路主要有两种: (ⅰ) 过量表达同源或异源的PPC和PCK, 旨在加强PEP到OAA的转化; (ⅱ) 通过敲除副产物合成的相关基因, 以减少杂酸合成, 从而减少PEP的消耗, 尤其是在*E. coli*中, 有半数的PEP会被糖磷酸转移系统(sugar phosphotransferase system, PTS)用于跨膜转运葡萄糖[42], 并生成丙酮酸, 进而生成乳酸/甲酸等副产物; 同时PEP还会在丙酮酸激酶(pyruvate kinase)编码基因*pyk*的作用下转化为丙酮酸, 造成PEP的大量损失. 可见, 为了促进琥珀酸合成, 过表达CO_2固定酶基因通常与副产物合成基因的敲除组合使用, 从两方面来强化琥珀酸的合成.

3.1 CO_2固定酶

能够与琥珀酸合成相关的CO_2固定功能酶主要有4种(图2): PPC、PCK、丙酮酸羧化酶(pyruvate carboxylase, PYC)和苹果酸酶(malic enzyme, MAE).

(1) PPC. PEP与CO_2在PPC的催化作用下生成OAA, 实现CO_2的固定, 生成草酰乙酸, 进而代谢转化为琥珀酸. PPC广泛存在于细菌中[43,44], 是大肠杆菌中主要行使催化功能的CO_2固定酶, 其底物亲和力高, 对HCO_3^-的K_m为0.1 μmol/L [36]. *ppc*基因的过表达对琥珀酸合成有促进作用, Millard等人[45]发现, 将大肠杆菌自身的*ppc*基因过表达, 可以使琥珀酸产量由3.0 g/L提高至10.7 g/L, 提高了3.5倍, 琥珀酸对糖得率达到约0.5 mol (mol glucose)$^{-1}$, 提高了2.8倍. 在敲除了乳酸脱氢酶(lactate dehydrogenase, *ldhA*)和丙酮酸甲酸裂解酶编码基因(pyruvate formate lyase, *pfl*)的*E. coli* SB202中, 过表达*Sorghum vulgare*的*ppc*基因, 琥珀酸产量由12.0 mmol/L提高至19.0 mmol/L, 提高了59.0%, 而琥珀酸对糖得率提高了50.0%[46]. 但是, 由于葡萄糖转运系统对PEP的需求, *ppc*的过表达也可能会使PTS系统可用的PEP减少, 造成葡萄糖摄取的降低, 从而影响细胞生长[47,48], 并且当PPC酶活超过一定阈值(约0.5 U (mg protein)$^{-1}$)时, 还会导致大量ATP(adenosine triphosphate)的损失, 并继而引起琥珀酸产量和转化率的降低[49].

(2) PCK. PEP与CO_2也可以在PCK的催化作用下生成OAA, 反应过程生成ATP. PCK对底物的亲和力低, 对HCO_3^-的K_m为13.0 μmol/L[37], 催化速度较慢. PCK

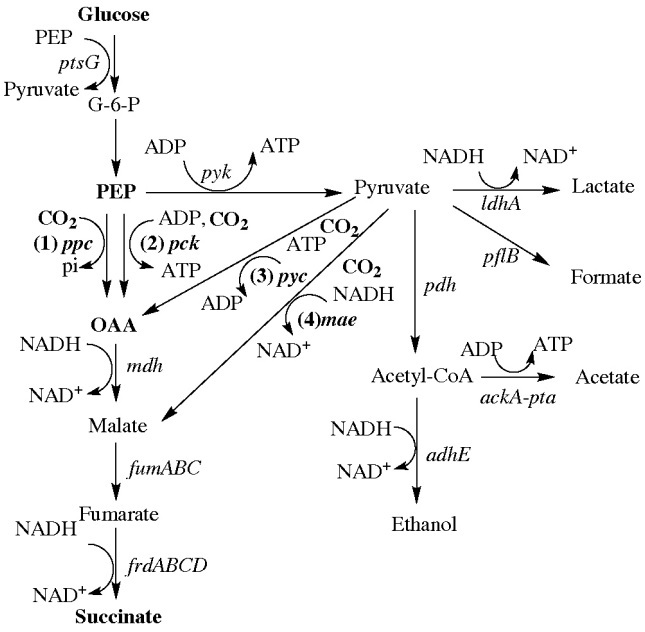

图 2　CO_2固定基因在琥珀酸代谢网络中催化的反应

在*A. succinogenes*中是主要的CO_2固定酶, 也是大肠杆菌自身具有的CO_2固定功能酶之一. 理论上, PCK的催化反应伴随着能量的产生, 更有利于细胞生长及琥珀酸的合成, 但*pck*基因的表达受葡萄糖抑制, 只有在糖异生、*ppc*缺陷及发酵环境中存在大量HCO_3^-的条件下才起正常催化作用[45,50,51]. Kim等人[52]通过在*E. coli*中表达*A. succinogenes*的*pck*基因, 使琥珀酸产量提高了6.5倍, 但是所获得的PCK酶活最高仅达到200.9 nmol (min mg protein)$^{-1}$, 远低于*A. succinogenes*中的酶活水平[53]. Zhang等人[54,55]研究发现, 通过转录水平激活可以显著提高大肠杆菌的PCK酶活, 从而产生更多的ATP用于细胞生长并促进琥珀酸合成.

(3) PYC. PYC广泛存在于真菌中[56,57], 丙酮酸与CO_2在PYC的催化作用下生成OAA, 在TCA中, 它是供给OAA的主要补充反应, 反应过程消耗ATP[57]. Sánchez等人[58]在敲除了乙醇脱氢酶(alcohol dehydrogenase)编码基因*adhE*和*ldhA*的*E. coli* SBS110MG中过表达*L. lactis*的*pyc*基因, 琥珀酸产量从0.6 g/L提高至15.6 g/L, 提高了25.0倍, 得率从0.2 mol (mol glucose)$^{-1}$提高到1.3 mol (mol glucose)$^{-1}$, 提高了5.5倍; 在此基础上, 他们还对*pyc*的启动子进行了系统优化[59], 发现同时使用trc启动子和*L. lactis*中*pyc*自身的启动子, 比单独使用lac, trc或自身启动子对PYC活力和琥珀酸得率的提高更有效. Liu等人[60]在敲除了*E. coli* NZN111(敲除了*ldhA*, *pflB*基因)自身*ppc*基因的基础上过表达*L. lactis*的*pyc*基因, 使CO_2固定速率从8.1 mg (L h)$^{-1}$上升至62.6 mg (L h)$^{-1}$, 琥珀酸产率从21.8 mg (L h)$^{-1}$提高至167.8 mg (L h)$^{-1}$, 均提高了6.7倍. 除了*L. lactis*的*pyc*, 在*E. coli*中表达来自于*Rhizobium etli*中的*pyc*基因也被证明可以显著促进琥珀酸合成[14,61]. Yang等人[62]利用计算机模拟对PPC, MAE和PYC这3个CO_2固定酶对琥珀酸产率的影响进行了分析, 结果显示PYC对琥珀酸产率影响最大, 在*E. coli* ZJG13中过表达*pyc*基因后, 琥珀酸的平均比生产速率(average specific productivity)可以从0.5 mmol (g CDW h)$^{-1}$进一步提升至0.8 mmol (g CDW h)$^{-1}$, 提高了60.0%.

(4) MAE. 丙酮酸与CO_2在MAE的催化作用下生成*L*-苹果酸, 进而代谢转化为琥珀酸. 当催化反应以NAD^+(nicotinamide adenine dinucleotide)为氢受体时, 编码基因为*maeA*; 以$NADP^+$(nicotinamide adenine dinucleotide phosphate)为氢受体时, 编码基因为*maeB*[63]. 由于固定反应是由丙酮酸到苹果酸, 因而从PEP到丙酮酸这一步产生的ATP得以积累. 丙酮酸到苹果酸的催化反应是可逆的, MAE催化丙酮酸到苹果酸的K_m约为0.3 mmol/L, 逆反应的K_m为16.0 mmol/L[64]. 在*E. coli*中表达苹果酸酶编码基因, 可以有效提高琥珀酸产量[65,66]. Stols和Donnelly[64]在*E. coli* NZN111中过表达*maeA*, 琥珀酸产量提高4.5倍.

3.2　多个CO_2固定酶的组合调控

由于不同CO_2固定酶催化的底物不同, 或者每种酶的催化特性存在差异, 将不同的酶进行组合表达也是常用的策略. Lin等人[46]通过在*E. coli* SB202(敲除了*ldhA*和*pfl*)中同时引入*S. vulgare*中的*ppc*基因以及*L. lactis*的*pyc*基因, 琥珀酸的产量比对照组提高了65.0%, 虽然与单独表达*ppc*时获得的琥珀酸产量相比没有显著提高, 但丙酮酸浓度从单独表达*ppc*时的19.0 mmol/L降至无法检测.

Zhang研究组[49]在中等PCK酶活(约0.5 U (mg protein)$^{-1}$)条件下协同表达PPC, 所获得的琥珀酸产量比单表达PPC或PCK的菌株所获得的琥珀酸产量分别提高66.0%和184.0%; 在高PCK酶活(1.6 U/mg)条件下协同表达PPC, 所获得的琥珀酸产量比单表达PPC或PCK的菌株所获得的琥珀酸产量分别提高200.0%和24.0%.

4 协同调控策略

4.1 与CO_2供给的协同调控

当菌种改造策略使大肠杆菌具备良好的CO_2固定能力后, CO_2能否及时供给必将成为琥珀酸合成的制约因素. 因此, 有必要对供给与固定这两个过程进行协同代谢调控, 一方面提高供给效率, 另一方面保证CO_2固定代谢活跃, 从而使更多的CO_2参与琥珀酸的生物合成.

本研究组在前期实现HCO_3^-转运通量提高1.4倍的基础上, 将转运和固定过程进行协同优化, 发现当HCO_3^-转运基因 *sbtA*和固定基因 *pck* 组合表达时, 琥珀酸产量比出发菌株提高了15.0%, 解除了单表达转运基因时对细胞代谢的抑制作用[41]; 并进一步构建不同强度的启动子, 与 HCO_3^-转运基因 *bicA*, *sbtA*和固定基因 *ppc*, *pck* 分别进行组装, 成功实现了转运和固定基因表达水平的精细匹配和 CO_2代谢途径的协同调控, 当 *sbtA*, *bicA*和 *ppc* 使用弱启动子, *pck* 使用强启动子时, 琥珀酸产量达到 89.4 g/L, 比出发菌株提高了37.5%[67].

在琥珀酸大规模发酵过程中, CO_2的大量供给势必会提高生产成本. 因此, 廉价的CO_2来源对琥珀酸生产非常重要. 在乙醇发酵时, 每消耗1 mol葡萄糖会产生2 mol乙醇和2 mol CO_2. 如果将乙醇和琥珀酸发酵过程进行组合, 既可以解决乙醇发酵时CO_2排放问题[68], 也可以为琥珀酸合成提供大量的CO_2作为底物. Nghiem等人[69]将乙醇发酵产生的 CO_2直接通入琥珀酸发酵液中, 同时与碱性物质联合使用控制 pH, 当与 NH_4OH联用时, 琥珀酸产量和得率均比不通气时有明显提高, 琥珀酸产量从 18.6 g/L提高到 51.4 g/L, 得率从0.5 mol (mol glucose)$^{-1}$ 提高至1.1 mol (mol glucose)$^{-1}$.

4.2 与还原力供给的协同调控

还原力是琥珀酸合成过程中的另一个关键因素, 每合成1 mol琥珀酸需要2 mol的NADH, 但每消耗1 mol葡萄糖只能产生2 mol的NADH, 因而NADH的不足会限制琥珀酸的合成, 同时由于CO_2固定反应的激活, 使PEP到琥珀酸的代谢通量升高, 对还原力的需求也会增强. 因而, 在CO_2固定反应增强的同时补充还原剂, 也是促进琥珀酸合成的有效方法. Balzer等人[70]尝试将一种来自于*Candida boidinii*中的依赖于NAD^+的甲酸脱氢酶基因(formate dehydrogenase, *fdh*)与*L. lactis*的*pyc*在*E. coli*中进行共表达, 这种甲酸脱氢酶能够将1 mol甲酸转化为1 mol CO_2, 同时生成1 mol NADH. 结果发现与单独表达*pyc*相比, *fdh*与*pyc*共表达所获得的平均糖消耗速率从1.4 g (L h)$^{-1}$提高到2.0 g (L h)$^{-1}$, 琥珀酸产率从1.0 g (L h)$^{-1}$提高到2.0 g (L h)$^{-1}$, 副产物甲酸产量从17.0 mmol/L下降至0~3.0 mmol/L. Ma等人[71]在*E. coli* BA002(敲除了*ldhA*和*pflB*)中过表达了烟碱酸磷酸核糖基转移酶(NAPRTase, *pncB*)和*L. lactis*的*pyc*基因, 使NAD(H)的浓度提高了9.8倍, 琥珀酸产量提高了8.0倍, 达到14.1 g/L, 并在此基础上利用铁氰化钾和二硫苏糖醇来调控氧化还原电位(oxido-reduction potential, ORP), 检测不同ORP条件下NADH和NAD^+的浓度, 以及对应的琥珀酸合成情况, 发现ORP越低(−400 mV), NADH/NAD^+的比例越高, 对应地PEP到OAA和丙酮酸到OAA的代谢通量越高, 琥珀酸产量达到28.6 g/L, 比ORP为−200 mV时提高了73.0% [72].

除了NADH, H_2作为一种电子供体也对琥珀酸的合成有显著促进作用. McKinlay和Vieille[73]通过^{13}C标记的方法监测不同$NaCO_3$和H_2条件下的代谢通量变化, 发现在100 mmol/L $NaCO_3$条件下, H_2通入前后富马酸到琥珀酸的反应通量从74.6上升到83.5. 丙酮-丁醇-乙醇(acetone-butanol-ethanol, ABE)发酵过程产生的尾气中有约60%的CO_2, 同时还有超过30%的H_2[74,75], 因而比乙醇发酵的尾气更适合与琥珀酸发酵过程进行组合. He等人[76]将ABE发酵尾气通入琥珀酸发酵液中, 琥珀酸产量达到65.7 g/L, 比只通纯CO_2时获得的产量提高了13.5%; 琥珀酸产率达到0.8 g (L h)$^{-1}$, 提高了13.4%.

5 展望

微生物发酵法生产琥珀酸之所以倍受青睐, 除了清洁高效的生产方式, 还因为该生物转化过程具备固定温室气体CO_2的能力, 具备了“温室气体资源化”这一显著优势. CO_2固定做为琥珀酸生物合成的关键环节, 经过对CO_2供给、溶解、转运、转化和固定等多个环节的系统解析和优化, 其固定效率已有了很大提高, 但对于CO_2相关代谢调控的研究主要集中于功能酶的同源或异源表达及对应的简单调控, 对调控机制的理解并不深入, 所获得的酶活还有很大上升空间. 因此, 拟解决此问题可以从以下3点着手:

(1) 继续深入对调控机制进行研究, 例如, 可以根

据关键酶的结构信息, 针对其核心功能区域进行突变, 识别关键催化位点, 优化其催化功能.

(2) 由于CO_2从供给到固定需要多个串联环节, 如何实现多环节之间的协调配合还需要进行更深层次的研究.

(3) 细胞的表型通常由多个基因共同调控, 同时细胞的代谢网络体系具有鲁棒性(Robust)特征, 会对发生变化的代谢途径进行调整, 致使整个网络的代谢流重新分配, 因而仅靠基因工程手段对个别基因的操作有时难以达到预期效果, 只有对控制琥珀酸合成的多个关键途径(如CO_2固定和乙醛酸支路等)相关基因进行全局性调控, 才能有效强化琥珀酸生物合成.

参考文献

1 Cukalovic A, Stevens C V. Feasibility of production methods for succinic acid derivatives: a marriage of renewable resources and chemical technology. Biofuels Bioprod Bioref, 2008, 2: 505–529

2 Zeikus J G, Jain M K, Elankovan P. Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotech, 1999, 51: 545–552

3 Bechthold I, Bretz K, Kabasci S, et al. Succinic acid: a new platform chemical for biobased polymers from renewable resources. Chem Eng Technol, 2008, 31: 647–654

4 Werpy T, Petersen G. Top Value Added Chemicals from Biomass. Washington: Department of Energy, 2004, 1: 1–76

5 Bozell J J, Petersen G R. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chem, 2010, 12: 539–554

6 Jansen M L A, van Gulik W M. Towards large scale fermentative production of succinic acid. Curr Opin Biotech, 2014, 30: 190–197

7 Choi S, Song C W, Shin J H, et al. Biorefineries for the production of top building block chemicals and their derivatives. Metabolic Eng, 2015, 28: 223–239

8 Guettler M V, Rumler D, Jain M K. *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int J Syst Bacteriol, 1999, 49: 207–216

9 Du C, Lin S K C, Koutinas A, et al. Succinic acid production from wheat using a biorefining strategy. Appl Microbiol Biotechnol, 2007, 76: 1263–1270

10 Lee P C, Lee S Y, Hong S H, et al. Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl Microbiol Biotech, 2002, 58: 663–668

11 Lee S J, Song H, Lee S Y. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl Environ Microbiol, 2006, 72: 1939–1948

12 Meynial-Salles I, Dorotyn S, Soucaille P. A new process for the continuous production of succinic acid from glucose at high yield, titer, and productivity. Biotechnol Bioeng, 2008, 99: 129–135

13 Nghiem N P, Davison B H, Suttle B E, et al. Production of succinic acid by *Anaerobiospirillum succiniciproducens*. Appl Biochem Biotechnol, 1997, 63–65: 565–576

14 Vemuri G N, Eiteman M A, Altman E. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J Ind Microbiol Biotech, 2002, 28: 325–332

15 Sánchez A M, Bennett G N, San K Y. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. Metab Eng, 2005, 7: 229–239

16 Yan D, Wang C, Zhou J, et al. Construction of reductive pathway in *Saccharomyces cerevisiae* for effective succinic acid fermentation at low pH value. Bioresour Technol, 2014, 156: 232–239

17 Raab A M, Gebhardt G, Bolotina N, et al. Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. Metab Eng, 2010, 12: 518–525

18 Okino S, Noburyu R, Suda M, et al. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl Microbiol Biotechnol, 2008, 81: 459–464

19 Okino S, Inui M, Yukawa H. Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. Appl Microbiol Biotechnol, 2005, 68: 475–480

20 Guettler M V, Jain M K, Rumler D. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US Patent, US5573931, 1996-11-12

21 Guettler M V, Jain M K, Soni B K. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent, US5723322, 1998-03-03

22 Wendisch V F, Bott M, Eikmanns B J. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. Curr Opin Microbiol, 2006, 9: 268–274

23 Wang D, Li Q, Song Z, et al. High cell density fermentation via a metabolically engineered *Escherichia coli* for the enhanced production of succinic acid. J Chem Technol Biotechnol, 2011, 86: 512–518

24 Lin H, Bennett G N, San K Y. Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. Metab Eng, 2005, 7: 116–127

25 Lin H, Bennett G N, San K Y. Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. Biotechnol Bioeng, 2005, 90: 775–779

26 Rischbieter E, Schumpe A, Wunder V. Gas solubilities in aqueous solutions of organic substances. J Chem Eng Data, 1996, 41: 809–812

27 Weisenberger S, Schumpe A. Estimation of gas solubilities in salt solutions at temperatures from 273 K to 363 K. Aiche J, 1996, 42: 298–300

28 Song H, Lee J W, Choi S, et al. Effects of dissolved CO_2 levels on the growth of *Mannheimia succiniciproducens* and succinic acid production. Biotechnol Bioeng, 2007, 98: 1296–1304

29 Zou W, Zhu L W, Li H M, et al. Significance of CO_2 donor on the production of succinic acid by *Actinobacillus succinogenes* ATCC 55618. Microb Cell Fact, 2011, 10: 87

30 Wang C C, Zhu L W, Li H M, et al. Performance analyses of a neutralizing agent combination strategy for the production of succinic acid by *Actinobacillus succinogenes* ATCC 55618. Bioprocess Biosyst Eng, 2012, 35: 659–664

31 Liu Y P, Zheng P, Sun Z H, et al. Strategies of pH control and glucose-fed batch fermentation for production of succinic acid by *Actinobacillus succinogenes* CGMCC1593. J Chem Technol Biotechnol, 2008, 83: 722–729

32 Bazaes S, Toncio M, Laivenieks M, et al. Comparative kinetic effects of Mn^{2+}, Mg^{2+} and the ATP/ADP ratio on phosphoenolpyruvate carboxykinases from *Anaerobiospirillum succiniciproducens* and *Saccharomyces cerevisiae*. Protein J, 2007, 26: 265–269

33 Wu H, Li Q, Li Z, et al. Succinic acid production and CO_2 fixation using a metabolically engineered *Escherichia coli* in a bioreactor equipped with a self-inducing agitator. Bioresour Technol, 2012, 107: 376–384

34 Zeebe R E. On the molecular diffusion coefficients of dissolved CO_2, HCO_3^-, and CO_3^{2-} and their dependence on isotopic mass. Geochim Cosmochim Acta, 2011, 75: 2483–2498

35 Lu S, Eiteman M A, Altman E. Effect of CO_2 on succinate production in dual-phase *Escherichia coli* fermentations. J Biotech, 2009, 143: 213–223

36 Kai Y, Matsumura H, Inoue T, et al. Three-dimensional structure of phosphoenolpyruvate carboxylase: a proposed mechanism for allosteric inhibition. Proc Natl Acad Sci USA, 1999, 96: 823–828

37 Krebs A, Bridger W A. The kinetic properties of phosphoenolpyruvate carboxykinase of *Escherichia coli*. Can J Biochem, 1980, 58: 309–318

38 Wang D, Li Q, Li W, et al. Improvement of succinate production by overexpression of a cyanobacterial carbonic anhydrase in *Escherichia coli*. Enzyme Microbial Technol, 2009, 45: 491–497

39 Gutknecht J, Bisson M A, Tosteson F C. Diffusion of carbon dioxide through lipid bilayer membranes. Effects of carbonic anhydrase, bicarbonate, and unstirred layers. J General Physiol, 1988, 69: 779–794

40 Price G D. Inorganic carbon transporters of the cyanobacterial CO_2 concentrating mechanism. Photosynth Res, 2011, 109: 47–57

41 Zhu L W, Zhang L, Wei L N, et al. Collaborative regulation of CO_2 transport and fixation during succinate production in *Escherichia coli*. Sci Rep, 2015, 5: 17321

42 Ma X, Zhang X, Wang B, et al. Engineering microorganisms based on molecular evolutionary analysis: a succinate production case study. Evol Appl, 2014, 7: 913–920

43 Fraenkel D G. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. 2nd ed. Washington: American Society for Microbiology Press, 1996. 189–198

44 Keseler I M, Collado-Vides J, Gama-Castro S, et al. EcoCyc: a comprehensive database resource for *Escherichia coli*. Nucleic Acids Res, 2004, 33: D334–D337

45 Millard C S, Chao Y P, Liao J C, et al. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*. Appl Environ Microbiol, 1996, 62: 1808–1810

46 Lin H, San K Y, Bennett G N. Effect of *Sorghum vulgare* phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. Appl Microbiol Biotechnol, 2005, 67: 515–523

47 Chao Y P, Liao J C. Alteration of growth yield by overexpression of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in *Escherichia coli*. Appl Environ Microbiol, 1993, 59: 4261–4265

48 Gokarn R R, Eiteman M A, Altman E. Metabolic analysis of *Escherichia coli* in the presence and absence of the carboxylating enzymes phosphoenolpyruvate carboxylase and pyruvate carboxylase. Appl Environ Microbiol, 2000, 66: 1844–1850

49 Tan Z, Zhu X, Chen J, et al. Activating phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in combination for improvement of succinate production. Appl Environ Microbiol, 2013, 79: 4838–4844

50 Kao K C, Tran L M, Liao J C. A global regulatory role of gluconeogenic genes in *Escherichia coli* revealed by transcriptome network analysis. J Biol Chem, 2005, 280: 36079–36087

51 Kwon D Y, Lee S Y, Kim P. Influence of gluconeogenic phosphoenolpyruvate carboxykinase (PCK) expression on succinic acid fermentation in *Escherichia coli* under high bicarbonate condition. J Microbiol Biotechnol, 2006, 16: 1448–1452

52 Kim P, Laivenieks M, Vieille C, et al. Effect of overexpression of *Anaerobiospirillum succiniciproducens* phosphoenolpyruvate carboxykinase on succinate production in *Escherichia coli*. Appl Environ Microbiol, 2004, 70: 1238–1241

53 der Werf M J V, Guettler M V, Jain M K, et al. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z. Arch Microbiol, 1997, 167: 332–342

54 Zhang X, Jantama K, Moore J C, et al. Metabolic evolution of energy-conserving pathways for succinate production in *Escherichia coli*. Proc Natl Acad Sci USA, 2009, 106: 20180–20185

55 Zhang X, Jantama K, Shanmugam K T, et al. Reengineering *Escherichia coli* for succinate production in mineral salts medium. Appl Environ Microbiol, 2009, 75: 7807–7813

56 Scrutton M C. Fine control of the conversion of pyruvate (phosphoenolpyruvate) to oxaloacetate in various species. FEBS Lett, 1978, 89: 1–9

57 Attwood P V. The structure and the mechanism of action of pyruvate carboxylase. Int J Biochem Cell Biol, 1995, 27: 231–249

58 Sánchez A M, Bennett G N, San K Y. Efficient succinic acid production from glucose through overexpression of pyruvate carboxylase in an *Escherichia coli* alcohol dehydrogenase and lactate dehydrogenase mutant. Biotechnol Prog, 2008, 21: 358–365

59 Thakker C, Zhu J, San K Y, et al. Heterologous *pyc* gene expression under various natural and engineered promoters in *Escherichia coli* for improved succinate production. J Biotech, 2011, 155: 236–243

60 Liu R, Liang L, Wu M, et al. CO_2 fixation for succinic acid production by engineered *Escherichia coli* co-expressing pyruvate carboxylase and nicotinic acid phosphoribosyltransferase. Biochem Eng J, 2013, 79: 77–83

61 Gokarn R R, Evans J D, Walker J R, et al. The physiological effects and metabolic alterations caused by the expression of *Rhizobium etli* pyruvate carboxylase in *Escherichia coli*. Appl Microbiol Biotech, 2001, 56: 188–195

62 Yang J, Wang Z, Zhu N, et al. Metabolic engineering of *Escherichia coli* and *in silico* comparing of carboxylation pathways for high succinate productivity under aerobic conditions. Microbiol Res, 2014, 169: 432–440

63 Wedding R T. Malic enzymes of higher plants: characteristics, regulation, and physiological function. Plant Physiol, 1989, 90: 367–371

64 Stols L, Donnelly M I. Production of succinic acid through overexpression of NAD^+-dependent malic enzyme in an *Escherichia coli* mutant. Appl Environ Microbiol, 1997, 63: 2695—2701

65 Hong S H, Lee S Y. Metabolic flux analysis for succinic acid production by recombinant *Escherichia coli* with amplified malic enzyme activity. Biotechnol Bioeng, 2001, 74: 89–95

66 Hong S H, Lee S Y. Enhanced production of succinic acid by metabolically engineered *Escherichia coli* with amplified activities of malic enzyme and fumarase. Biotechnol Bioprocess Eng, 2004, 9: 252–255

67 Yu J H, Zhu L W, Xia S T, et al. Combinatorial optimization of CO_2 transport and fixation to improve succinate production by promoter engineering. Biotechnol Bioeng, 2016, 113: 1531–1541

68 Xu Y, Isom L, Hanna M A. Adding value to carbon dioxide from ethanol fermentations. Bioresour Technol, 2010, 101: 3311–3319

69 Nghiem N P, Hicks K B, Johnston D B. Integration of succinic acid and ethanol production with potential application in a corn or barley biorefinery. Appl Biochem Biotechnol, 2010, 162: 1915–1928

70 Balzer G J, Thakker C, Bennett G N, et al. Metabolic engineering of *Escherichia coli* to minimize byproduct formate and improving succinate productivity through increasing NADH availability by heterologous expression of NAD^+-dependent formate dehydrogenase. Metab Eng, 2013, 20: 1–8

71 Ma J, Gou D, Liang L, et al. Enhancement of succinate production by metabolically engineered *Escherichia coli* with co-expression of nicotinic acid phosphoribosyltransferase and pyruvate carboxylase. Appl Microbiol Biotechnol, 2013, 97: 6739–6747

72 Liu R, Liang L, Jiang M, et al. Effects of redox potential control on succinic acid production by engineered *Escherichia coli* under anaerobic conditions. Process Biochem, 2014, 49: 740–744

73 McKinlay J B, Vieille C. 13C-metabolic flux analysis of *Actinobacillus succinogenes* fermentative metabolism at different $NaHCO_3$ and H_2 concentrations. Metab Eng, 2008, 10: 55–68

74 Lin P Y, Whang L M, Wu Y R, et al. Biological hydrogen production of the genus *Clostridium*: metabolic study and mathematical model simulation. Int J Hydrogen Energ, 2007, 32: 1728–1735
75 Kong X, He A, Zhao J, et al. Efficient acetone-butanol-ethanol production (ABE) by *Clostridium acetobutylicum* XY16 immobilized on chemically modified sugarcane bagasse. Bioprocess Biosyst Eng, 2015, 38: 1365–1372
76 He A, Kong X, Wang C, et al. Efficient carbon dioxide utilization and simultaneous hydrogen enrichment from off-gas of acetone-butanol-ethanol fermentation by succinic acid producing *Escherichia coli*. Bioresour Tech, 2016, 214: 861–865

Research progress in metabolic regulation of CO_2 associated with succinate biosynthesis

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As an important C4 platform compound, succinic acid has been classified as the most promising chemical among the 12 bio-based chemicals by the US Department of Energy. As the essential substrate for the biosynthesis of succinic acid, CO_2 is regarded as the key factor to determine the efficiency of succinic acid production. In this review, we comment the current status of metabolic regulation of CO_2 associated with succinate biosynthesis from the following aspects: CO_2 dissolution, transport and uptake, fixation, and combined regulation with strain development and bioprocess engineering. Finally, the trends of CO_2 regulation strategy are prospected.

succinic acid, CO_2, biosynthesis, metabolic regulation

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