·塑料降解物的生物高值转化·

王丹 重庆大学化学化工学院博士生导师，国家重点研发计划首席科学家，重庆市杰出青年基金获得者。中国科学院过程工程研究所博士，莱斯大学博士后，麻省理工学院访问学者，中国化工学会生物化工专业委员会常务委员，中国生物工程学会青年工作者委员会委员，第 8 届中国国际大学生创新创业大赛国家级银奖项目第一指导教师。长期从事生物合成化学品及新材料的化工过程研究，共发表 SCI 论文 90 多篇，Web of Science 总引 1 500 余次，H 指数为 24；参与编写中英文书籍 5 部；授权发明专利 22 项。

生物基塑料单体 5-氨基戊酸的生物合成新途径

康雅琦[1]，罗若诗[1]，林凡祯[1]，程杰[1]，周桢[3]，王丹[1,2*]

1 重庆大学化学化工学院，重庆 400044
2 煤矿灾害动力学与控制国家重点实验室，重庆 400044
3 重庆大学药学院，重庆 400044

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摘 要：5-氨基戊酸(5-aminovalanoic acid, 5AVA)可作为新型塑料尼龙 5 和尼龙 56 的前体，是合成聚酰亚胺的有前途的平台化合物。目前 5-氨基戊酸的生物合成法普遍产率较低且合成过程复杂，成本高。为实现 5AVA 的绿色生物合成，本研究通过组合表达来自日本白腹鲭(*Scomber japonicas*)的 L-赖氨酸 α-氧化酶、来自乳酸乳球菌(*Lactococcus lactis*)的 α-酮酸脱羧酶和来自大肠杆菌(*Escherichia coli*)的醛脱氢酶，在大肠杆菌中建立了一条以 L-赖氨酸为原料，以 2-酮-6-氨基己酸盐为中间产物生物合成 5AVA 的途径。在葡萄糖浓度为 55 g/L，赖氨酸盐酸盐 40 g/L 的初始条件下，最终消耗 158 g/L 的葡萄糖和 144 g/L 的赖氨酸盐酸盐，补料分批发酵产生了 57.52 g/L 的 5AVA，

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*Corresponding author. E-mail: dwang@cqu.edu.cn
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摩尔得率为 0.62 mol/mol。与文献报道的以 2-酮-6-氨基己酸盐为中间产物的 5AVA 生物合成途径相比，本文报道的新途径无需使用乙醇和双氧水，且 5AVA 产量进一步提高。

关键词：5-氨基戊酸；L-赖氨酸盐酸盐；人工途径；代谢工程

A new biosynthesis route for production of 5-aminovalanoic acid, a biobased plastic monomer

KANG Yaqi[1], LUO Ruoshi[1], LIN Fanzhen[1], CHENG Jie[1], ZHOU Zhen[3], WANG Dan[1,2*]

1 School of Chemistry and Chemical Engineering, Chongqing University, Chongqing 400044, China
2 State Key Laboratory of Coal Mine Disaster Dynamics and Control, Chongqing 400044, China
3 School of Pharmacy, Chongqing University, Chongqing 400044, China

Abstract: 5-aminovalanoic acid (5AVA) can be used as the precursor of new plastics nylon 5 and nylon 56, and is a promising platform compound for the synthesis of polyimides. At present, the biosynthesis of 5-aminovalanoic acid generally is of low yield, complex synthesis process and high cost, which hampers large-scale industrial production. In order to achieve efficient biosynthesis of 5AVA, we developed a new pathway mediated by 2-keto-6-aminohexanoate. By combinatory expression of L-lysine α-oxidase from *Scomber japonicus*, α-ketoacid decarcarboxylase from *Lactococcus lactis* and aldehyde dehydrogenase from *Escherichia coli*, the synthesis of 5AVA from L-lysine in *Escherichia coli* was achieved. Under the initial conditions of glucose concentration of 55 g/L and lysine hydrochloride of 40 g/L, the final consumption of 158 g/L glucose and 144 g/L lysine hydrochloride, feeding batch fermentation to produce 57.52 g/L of 5AVA, and the molar yield is 0.62 mol/mol. The new 5AVA biosynthetic pathway does not require ethanol and H_2O_2, and achieved a higher production efficiency as compared to the previously reported Bio-Chem hybrid pathway mediated by 2-keto-6-aminohexanoate.

Keywords: 5-aminovaleric acid; l-lysine; synthetic route; metabolic engineering

由于全球水资源的污染、气候变迁及石油供应不足等问题，人类社会的可持续发展受到严峻挑战，用生物基化学品代替传统石化衍生化学品受到了学术界和企业界的重点关注。近年来，研究者们已经利用微生物生产了许多重要的化学品，例如 6-氨基己酸[1]、果糖[2]、扁桃酸[3]、维生素 B12[4]、柚皮素[5]、4-羟基苯甲酸[6]、姜黄素[7]、羟基酪醇[8]等。生物基塑料单体是一种新型的化工原料，它带有适当官能团，能够聚合生成结构和性能可控的高分子材料。目前，运用生物技术合成了许多生物基材料单体，如己二酸[9]、戊二酸[10]、1,3-二氨基丙烷[11]、二氨基戊烷[12-13]、1,3-丙二醇[14]和 1,2-丙二醇[15]。值得一提的是，5-氨基戊酸(5-aminovalanoic acid, 5AVA)是合成聚酰亚胺的有前途的平台化合物，其聚合成的聚酰胺材料如尼龙 5[16]和尼龙 56[17]具有耐高温和耐有机溶剂的特性，可作为一次性用品、衣服、汽车、飞机和建筑材料等的原材料。

5AVA 可用于戊二酸[18-19]、δ-戊内酰胺[20]、

1,5-戊二醇[21]和5-羟基戊酸[22]等C5平台化学品的生产。目前，5AVA可由二氧化铈负载的纳米金催化哌啶氧化物进行合成[23]。然而，这种化学合成方法不仅需要很高的温度，产率较低，且污染较大[23]，因此寻找生产5AVA的替代方法是十分必要的。随着生物技术的快速发展，通过代谢工程和合成生物学合成5AVA引起了研究者广泛的关注[19]。

5AVA的合成与恶臭假单胞菌(*Pseudomonas fulticans*)中的L-赖氨酸分解代谢密切相关[24]。通过L-lys2-单加氧酶(DavB)和5-氨基戊酸酰胺水解酶(DavA)的过表达，产生了5AVA(图1A)[25]。Park等[26]利用大肠杆菌(*Escherichia coli*) WL3110/DavA-DavB生产了3.6 g/L的5AVA，滴度相对较低。因此，他们进一步在谷氨酸棒杆菌(*Corynebacterium glutamicum*)中采用人工H36启动子，产生了33.1 g/L的5AVA[27]。值得一提的是，Li等[28]采用L-赖氨酸特异性渗透酶(L-lysine permease, LysP)将5AVA滴度提高至63.2 g/L (表1)，Wang等[29]采用全细胞催化法，利用DavB和DavA将5AVA滴度提高至240.70 g/L (表1)。此外，Jorge等[30]以1,5-戊二胺和5-氨基戊醛为中间体，利用L-赖氨酸产生5AVA (图1B)。研究发现，通过日本鲭(*Scomber japonicas*) L-赖氨酸α-氧化酶(RaiP)的表达，可以L-赖氨酸盐酸盐(L-lys HCl)为底物，以2-酮基-6-氨基己酸(2-keto-6-aminohexanoic acid, 2K6AC)为中间体，生产5AVA，产量可达到29.12 g/L[31] (图1C)。但是由于此途径添加了乙醇和H_2O_2，是不安全和不经济的[31]。除此之外，研究发现通过固定在载体上的RaiP可以获得13.4 g/L 5AVA[32]。进一步地，大孔吸附树脂AK-1的使用可以实现从生物转化液中分离出5AVA[33]，纯度可达99.3%。

在天然途径中，α-酮酸脱羧酶(KivD)可催化多种α-酮酸生成醛[34-36]，在α-酮酸的脱羧中展现出高效活性[37-38]。与野生型KivD的底物相比，KivD突变体催化的底物链长相对较长，如2-丙酮-4-甲基己酸和2-酮-3-甲基戊酸[39]，而天然途径中的KivD催化的底物链长较短，如2-酮异戊酸和α-酮己二酸[36,39]。在大肠杆菌中，以2-酮丁酸盐为底物表达来自乳酸乳球菌(*Lactococcus lactis*)中的醇脱氢酶2 (alcohol dehydrogenase 2, ADH2)和KivD，可以生产2 g/L 1-丙醇[40]。L-赖氨酸α-氧化酶(RaiP)将L-赖氨酸的α-氨基氧化成羰基，同时产生NH_3和H_2O_2，生成中间体2K6AC。乙醛脱氢酶(PadA)可催化醛基转变为羧基。

与野生型相比，F381和M461中的2个KivD突变体表现出更高的底物识别和催化效率。此外，KatE和LysP的过表达有助于H_2O_2的去除和L-赖氨酸的转运，从而增加5AVA的

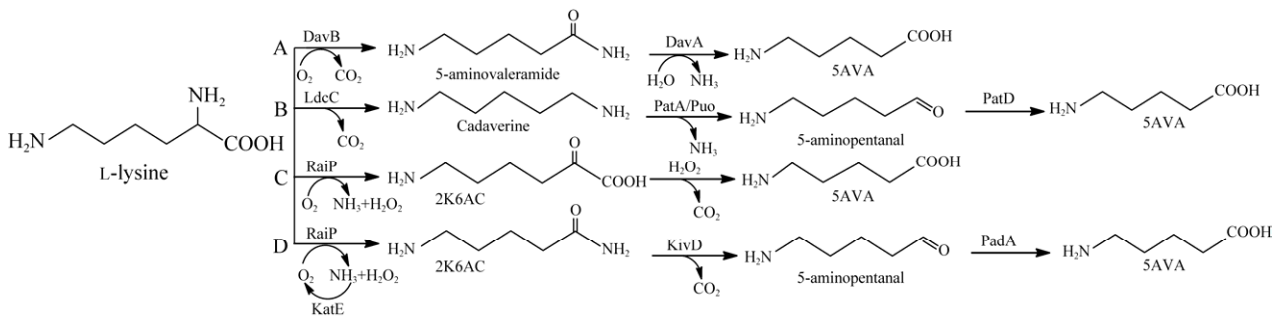

图1　L-赖氨酸合成5AVA的微生物途径

Figure 1　Microbial pathway for the synthesis of 5AVA from L-lysine.

表 1　5AVA 的不同合成途径

Table 1　Different biosynthetic pathways of 5AVA

Synthetic pathway	Host strain	Strategy	Description	5AVA titer (g/L)	Yield (g/g)	Substrate/Feedstock	References
A	*E. coli*	Whole-cell biotransformation	Expression of DavB and DavA in *E. coli*	240.70	0.70	L-lysine	[29]
A	*E. coli*	Enzymatic catalysis	Overexpression of DavB, DavA, PP2911 from *P. putida* and LysP from *E. coli*	63.20	0.62	L-lysine	[28]
A	*C. glutamicum*	Fed-batch fermentation	Expression of codon-optimized *dav*A and *dav*B, promoter engineering	33.10	0.10	Glucose	[27]
A	*C. glutamicum*	Fed-batch fermentation	Pretreatment, hydrolysis, purification and concentration of the *Miscanthus* hydrolyzate solution	12.51	0.10	*Miscanthus* hydrolyzate	[26]
B	*C. glutamicum*	Fermentation	N-acetylcadaverine and glutarate in a genome-streamlined L-lysine producing strain expressing *ldc*C, *pat*A, and *pat*D from *E. coli*	5.10	0.13	Glucose and alternative carbon sources	[30]
B	*C. glutamicum*	Fermentation	*C. glutamicum* GSLA21gabTDP with overexpression of LdcC, Puo, and PatD	3.70	0.09	Glucose	[46]
C	*E. coli*	Whole-cell biotransformation	Overexpression of RaiP from *S. japonicus* and addition of 4% ethanol and 10 mmol/L H_2O_2	29.12	0.44	L-lysine HCl	[31]
C	*E. coli*	Whole-cell biotransformation	Overexpression of RaiP from *S. japonicus* and whole-cell catalysts with ethanol pretreatment	50.62	0.51	L-lysine HCl	[43]
D	*E. coli*	Whole-cell biotransformation	Combination of native RaiP, KivD, PadA, KatE, and LysP, without addition of ethanol and H_2O_2	57.52	0.40	L-lysine HCl	This study

☎: 010-64807509
✉: cjb@im.ac.cn

产量。在本研究中，将来自 *S. japonicus* 的 L-赖氨酸 α-氧化酶、乳球菌的 α-酮酸脱羧酶和大肠杆菌的醛脱氢酶，在大肠杆菌 BL21(DE3)中过表达，以 2-酮基-6-氨基己酸酯为中间体，构建了生物合成 5AVA 的新途径(图 1D)。这种新途径在工业应用中具有广阔的前景，它不仅提高了 L-赖氨酸的价值，而且实现了 5AVA 在大肠杆菌中高效地合成，将为尼龙 5 和尼龙 56 发展成为一种新型的生物基塑料奠定基础。

1 材料与方法

1.1 菌株和质粒

本研究涉及的菌株和质粒如表 2 所示。将质粒 pCJ01、pETaRPK、pETaRPK$^{\#}$、pEAkatE 或 pEAKL 转入敲除了 *cadA* 的大肠杆菌 BL21(DE3)，分别获得菌株 CJ02、CJ06、CJ07、CJ08 或 CJ09。

1.2 培养条件

将携带相应质粒的大肠杆菌在 100 mg/L 氨苄青霉素的 LB 琼脂平板上培养，并在 37 °C 下生长 12 h。单个菌落接种到补充有 100 mg/L 氨苄青霉素的 2 mL LB 肉汤中，在 37 °C、250 r/min 条件下培养 12 h。

培养基为基本培养基：5 g/L 酵母提取物，10 g/L 胰蛋白酶，15 g/L 葡萄糖，0.1 g/L $FeCl_3$，2.1 g/L 柠檬酸，2.5 g/L $(NH_4)_2SO_4$，0.5 g/L

表 2 本研究使用的菌株和质粒

Table 2 Strains and plasmids used in the study

Strains and plasmids	Description	Sources
Strains		
DH5α	Wild type	Novagen
BL21(DE3)	Wild type	Novagen
ML03	*E. coli* BL21(DE3) Δ*cadA*	[41]
CJ00	*E. coli* BL21(DE3) harboring plasmid pET21a	[31]
CJ01	*E. coli* BL21(DE3) harboring plasmid pCJ01	[31]
CJ02	*E. coli* ML03 harboring plasmid pCJ01	[31]
CJ05	*E. coli* BL21(DE3) harboring plasmid pETaRPK	This study
CJ06	*E. coli* ML03 harboring plasmid pETaRPK	This study
CJ07	*E. coli* ML03 harboring plasmid pETaRPK$^{\#}$	This study
CJ08	*E. coli* ML03 harboring plasmid pETaRPK$^{\#}$ and pZAkatE	This study
CJ09	*E. coli* ML03 harboring plasmid pETaRPK$^{\#}$ and pZAKL	This study
Plasmids		
pZA22	Empty plasmid used as control, Kan^{R}	[1]
pCJ01	pET21a-*raiP*, pET21a carries a L-lysine α-oxidase gene (*raiP*) from *S. japonicus* with *Nde* I and *Bam*H I restrictions, Amp^{R}	[31]
pETaRPK	pET21a-*raiP-kivD-padA*, pET21a carries a L-lysine α-oxidase gene (*raiP*) from *S. japonicus*, a α-ketoacid decarboxylase gene (*kivD*) from *L. lactis* and a aldehyde dehydrogenase gene (*padA*) from *E. coli*, Amp^{R}	This study
pETaRPK$^{\#}$	pET21a-*raiP-kivD*$^{\#}$*-padA*, pET21a carries a L-lysine α-oxidase gene (*raiP*) from *S. japonicus*, a α-ketoacid decarboxylase mutant (F381A/V461A) gene from *L. lactis* and an aldehyde dehydrogenase gene (*padA*) from *E. coli*, Amp^{R}	This study
pZAkatE	pZA22-*katE*, pZA22 carries a catalase gene (*katE*) from *E. coli*, Kan^{R}	This study
pZAKL	pZA22-*katE-lysP*, pZA22 carries a catalase gene (*katE*) from *E. coli* and a lysine permease gene (*lysP*) from *E. coli*, Kan^{R}	This study

$K_3PO_4·3H_2O$，1.0 mmol/L $MgSO_4$，3 g/L KH_2PO_4，0.5 mmol/L 硫胺素二磷酸(thiamine diphosphate, ThDP)，抗生素。OD_{600} 达到 0.5 后，添加 0.5 mmol/L 异丙基-β-D-硫代半乳糖苷(isopropyl-β-D-thiogalactoside, IPTG)和 6.5 g/L L-赖氨酸盐酸，并继续培养。

工程菌株的补料分批生物转化在 5.0 L 发酵罐中进行。发酵培养基组成为：55 g/L 葡萄糖、1.6 g/L $MgSO_4·7H_2O$、0.007 56 g/L $FeSO_4·7H_2O$、1.6 g/L $(NH_4)_2SO_4$、2 g/L 柠檬酸、7.5 g/L $K_2HPO_4·3H_2O$、0.02 g/L Na_2SO_4、0.006 4 g/L $ZnSO_4$、0.000 6 g/L $Cu_2SO_4·5H_2O$、0.004 g/L $CoCl_2·6H_2O$[41]。通过添加氨水将 pH 控制在 6.7–6.9，将温度设定为 30 °C。发酵期间逐渐添加消泡剂 289，防止在生物转化过程中形成泡沫。在整个发酵过程中，葡萄糖和 L-赖氨酸的浓度分别保持在 15 g/L 和 20 g/L 左右。

1.3 蛋白质的表达和纯化

37 °C 时，向用于蛋白质表达的培养基的 LB 琼脂中补充 0.5 mmol/L ThDP。OD_{600} 达到 0.5 后，加入 0.5 mmol/L IPTG。20 °C 时，用磷酸钾缓冲液(KPB, 50 mmol/L, pH 8.0)洗涤细胞。在 50 mmol/L KPB 的冰浴中利用超声处理破坏细胞，并使用 Ni-NTA 柱用 AKTA 纯化器 10 纯化酶[1]。使用 SpectraMax $M2_e$ 在 280 nm 处测量蛋白质的浓度[31]。

1.4 酶测定

RaiP 的氧化活性是通过测量 H_2O_2 的生成速率来确定的[31]。利用耦合酶测定法，在 30 °C 下测定 KivD 和 KivD 突变(KivD*)的脱羧活性[36]。反应混合物包含 1.0 mmol/L NAD^+、1.1 μmol/L PadA、1.1 μmol/L RaiP、0.85 μmol/L KivD 或 KivD*以及不同浓度的 L-赖氨酸缓冲液(50 mmol/L KPB, pH 8.0, 1 mmol/L $MgSO_4$, 1.0 mmol/L TCEP, 0.5 mmol/L ThDP)。在刚开始反应时添加底物 L-赖氨酸，并在 340 nm 处监测 NADH 的形成，消光系数为 6.22 mmol/(L·cm)。

2 结果与讨论

2.1 大肠杆菌合成 5AVA 的人工合成路线的构建

5AVA 的生物合成途径包括 3 个步骤：(1) 通过 RaiP 将 L-赖氨酸脱氨转化为中间体 2K6AC；(2) 通过 KivD 使 2K6AC 脱羧产生 5-氨基戊醛；(3) 通过 PadA 将 5-氨基戊醛氧化为 5AVA (图 1D)。首先，构建质粒 pETaRPK，并将其导入大肠杆菌 BL21(DE3)中以获得菌株 CJ05，在 T7 启动子下共表达 RaiP、KivD 和 PadA。为了减少 L-赖氨酸降解为 5-戊二胺，敲除赖氨酸脱羧酶基因 *cadA* 以获得菌株 CJ06。值得注意的是，菌株 CJ01、CJ02、CJ05 和 CJ06 也可以生产 5AVA。菌株 CJ00 从 6.5 g/L L-lys HCl 生产了 0.06 g/L 5AVA，消耗量为 0.01 g/g L-lys (表 3)。对于工程菌株 CJ01，可生 0.23 g/L 5AVA。此外，菌株 CJ05 通过图 1D 所示的途径生产 1.66 g/L 的 5AVA，与单基因途径(图 1C)相比，产量增加了 774%。

由此可见，利用 RaiP、KivD、PadA 这 3 种关键酶，2K6AC 作为中间产物生产 5AVA 途径具有可行性。

表 3 5AVA 生产途径对比表

Table 3 Comparison of 5AVA production approaches

Strains	Plasmids	L-lysine HCl (g/L)	5AVA titer (g/L)
CJ00	BL21(DE3)/pET21a	6.5	0.06
CJ01	BL21(DE3)/pET21a-*raiP*	6.5	0.23
CJ02	ML03/pET21a-*raiP*	6.5	0.32
CJ05	BL21(DE3)/pET21aRPK	6.5	1.66
CJ06	ML03/pET21aRPK	6.5	1.95

2.2 过氧化氢酶 KcatE 和赖氨酸渗透酶 LysP 的过表达有利于 5AVA 产量的增加

本研究采用了 4 种策略来提高 5AVA 的产量。首先，敲除赖氨酸脱羧酶基因 *cadA*。其次，选择 L-lys HCl 作为底物，以提高 L-lys 的利用率[31,41,42]。然后，H_2O_2 可以抑制细胞生长[43]，从而影响目标产物的产生。Liu 等[44]发现过氧化氢酶的表达使 H_2O_2 的含量显著降低，α-酮戊二酸的产量显著增加。在本研究中，菌株 CJ08 中 *katE*、*raiP*、*kivD*#和 *padA* 的共表达下产生 1.88 g/L 的 5AVA，与菌株 CJ07 相比没有显著差异(表 4)。事实上，KatE 的过表达会消除 RaiP 产生的 H_2O_2。如表 4 所示，在发酵期间，KatE 的引入并没有显著增加 OD_{600} 和 5AVA 的产量。相反，它降低了 OD_{600}，可能是由于过多的基因表达增加了细胞负担，导致细胞量减少[45]。然而，在发酵罐中，H_2O_2 可以显著抑制细胞生长，导致 5AVA 的产量有限[31,42]。

表 4 工程菌株在 250 mL 烧瓶中合成 5AVA

Table 4 The engineered strain synthesized 5AVA in a 250 mL flask

Strains	Time (h)	Cell density (OD_{600})	Glucose consumed (g/L)	5AVA production (g/L)	5AVA yield (g/g)[a]
CJ06	12	5.24±0.38	7.22±0.33	0.85±0.04	0.19±0.03
	24	8.15±0.52	11.36±0.46	1.69±0.03	0.35±0.03
CJ07	12	5.19±0.41	7.09±0.25	0.96±0.02	0.25±0.01
	24	8.08±0.55	11.25±0.48	1.85±0.02	0.39±0.03
CJ08	12	5.14±0.36	7.02±0.28	0.94±0.01	0.25±0.02
	24	7.91±0.46	11.17±0.41	1.88±0.02	0.40±0.03
CJ09	12	5.08±0.33	6.88±0.18	1.01±0.03	0.23±0.01
	24	7.85±0.42	11.11±0.39	1.93±0.01	0.41±0.02

Data are presented as $\bar{x} \pm s$ calculated from three replicate biotransformation experiments. Statistics were performed by the two-tailed student *t*-test. [a]: The yield of 5AVA was calculated based on L-lys consumption. 6.5 g/L L-lys HCl, 15 g/L glucose, 0.5 mmol/L IPTG, 1.0 mmol/L $MgSO_4$ and 0.5 mmol/L ThDP were added.

因此进一步地，本研究将过表达赖氨酸转运蛋白基因 *lysP* 插入质粒 pZAkatE 中以形成新的质粒 pZAKL。如表 4 所示，菌株 CJ09 可产生 1.93 g/L 的 5AVA。

2.3 补料分批生物转化生产 5AVA

工程菌株 CJ09 的补料分批生物转化结果如图 2 所示。CJ09 生长速度很快，在 18 h 内的最高细胞浓度(OD_{600})可达到 142。添加 L-lys HCl 后的 18–36 h 之间，5AVA 累积至 48.3 g/L。随着发酵时间增加至 48 h，5AVA 累积至 57.52 g/L，5AVA 的产率和产量分别为 1.09 g/(L·h)和 0.65 g/g L-赖氨酸。而菌株 CJ02 生产了 9.16 g/L 5AVA，产率为 0.11 g/g L-lys。菌株 CJ08 中 KatE 的表达不会影响 5AVA 的产量，而且其 5AVA 的产量却比 CJ07 明显增加达到 45.92 g/L，CJ07 的滴度仅为 16.48 g/L (表 4)。这是因为 H_2O_2 对 CJ07 的生长有明显的抑制作用，使其 OD_{600} 只有 40。上述结果表明，本文中的合成路线可以高效地生产 5AVA。

在反应机理方面，本文提出的 5AVA 合成策略主要包括 3 个步骤：(1) RaiP 催化生产中间体 6A2KCA；(2) KivD 将 2K6AC 脱羧为 5-氨基戊醛；(3) PadA 将 5-氨基戊醛氧化生成 5AVA。与先前的全细胞转化(表 1)相比，5AVA 的滴度从 29.12 g/L 增加至 57.52 g/L，增加了约 97.5%；结果证明，H_2O_2 会抑制细胞生长和酶活性，导致 5AVA 的产率较低[31]。本文中的途径与其他发酵生产 5AVA 的合成途径[30](表 1)相比，5AVA 的滴度从 5.1 g/L 提高至 57.52 g/L。与另一种全细胞催化工作[42]相比，5AVA 的滴度从 50.62 g/L 提高至 57.52 g/L，提高了约 13.60%。而且在不添加乙醇和 H_2O_2 的条件下，5AVA 的工业化生产具有更高的安全性和经济性。

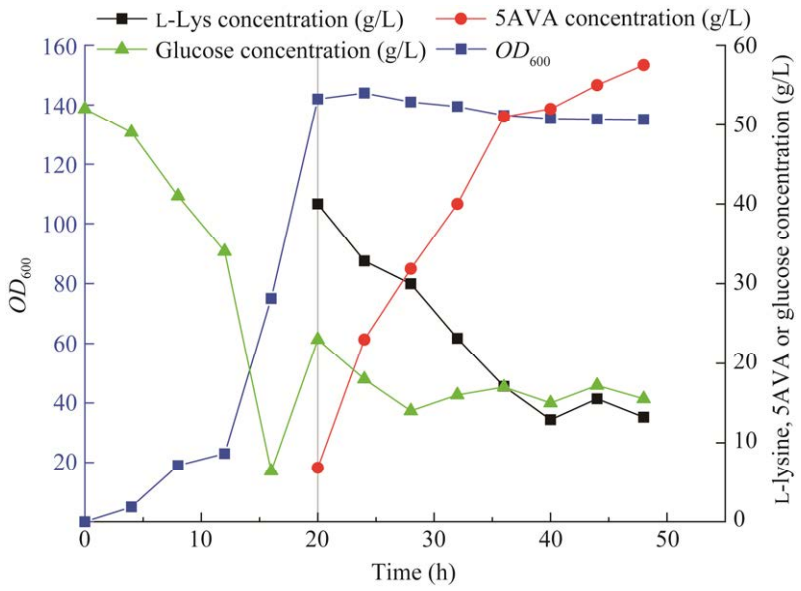

图 2　工程菌株 CJ09 在 5 L 发酵罐中合成 5AVA

Figure 2　Synthesis of 5AVA by engineered strain CJ09 in 5 L fermentor.

3　结论

本研究提出并优化了大肠杆菌生产 5AVA 的生物合成途径。通过引入过氧化氢酶 KatE 降解 H_2O_2，减少 H_2O_2 对酶活性和细胞生长的抑制，从而使 5AVA 有较高的产率。本实验采用可再生基质和简单的培养条件，具有较高的产率，对环境污染小。提高底物利用率和 H_2O_2 分解效率均有助于提高 5AVA 的产量，这有可能成为其他化学品可持续生物合成的普适性策略。

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