特约评述

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巴斯德毕赤酵母底盘细胞的工程化改造及应用

刘启，钱芷兰，宋丽丽，要超颖，徐名强，任燕娜，蔡孟浩
（华东理工大学生物工程学院，生物反应器工程国家重点实验室，上海 200237）

摘要： 优质的微生物底盘宿主是实现绿色、可持续生物制造的重要平台。巴斯德毕赤酵母底盘宿主因其在蛋白表达和发酵生产中的诸多优势受到了广泛的关注和应用。而作为一种工业甲基营养酵母，其可以有效地利用来源广泛的甲醇作为唯一碳源，使其成为碳一化合物潜在的生物转化平台。近年来，随着合成生物技术和生物制药技术的快速发展，围绕毕赤酵母底盘的工程化改造研究逐渐增多，并取得了卓有成效的进展，促进了毕赤酵母底盘的发展和升级。本文简述了毕赤酵母底盘细胞的发展和应用现状，从基因操纵技术、基因表达调控、代谢工程改造等方面介绍了毕赤酵母的工程化改造策略及应用效果，总结了毕赤酵母中合成生物技术、调控元器件、新型表达平台和生物转化体系的建立与开发情况。在此基础上，进一步强调了毕赤酵母中CRISPR介导的基因编辑及调控、转录系统的重构及人工设计，介绍了其在蛋白表达和化合物合成方面的应用，并分析了其在实际应用中的优势和问题。最后，对毕赤酵母在后续研究中的底盘升级方向和应用场景进行了展望。

关键词： 毕赤酵母；底盘细胞；甲基营养型酵母；细胞工厂；合成生物系统

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Rewiring and application of *Pichia pastoris* chassis cell

LIU Qi，QIAN Zhilan，SONG Lili，YAO Chaoying，XU Mingqiang，REN Yanna，CAI Menghao
（*State Key Laboratory of Bioreactor Engineering*，*School of Biotechnology*，*East China University of Science and Technology*，*Shanghai 200237*，*China*）

Abstract: Microbial chassis hosts are important platforms for green and sustainable biomanufacturing. *Pichia pastoris* has served as a preferred chassis for heterologous protein expression and fermentation production, which is attributed to its numerous advantages in expression capacity, post-translational modification, high cell density culture, and extracellular product purification. Moreover, as an industrial methylotrophic yeast, *P. pastoris* effectively utilizes cheap and widely sourced methanol as the sole carbon source, making it a potential biotransformation platform for C1 compounds. Recently, scientists have endowed this nonconventional yeast as an efficient microbial cell factory for biosynthesis of small molecule products beyond its traditional role of a protein expression workhorse. The growing of synthetic biology and biopharmaceutical technology has promoted the rapid development on the genetic rewiring of *P. pastoris* chassis host. A series of engineering strategies have been developed to break the restrictions and bottlenecks of *P. pastoris* in both academic

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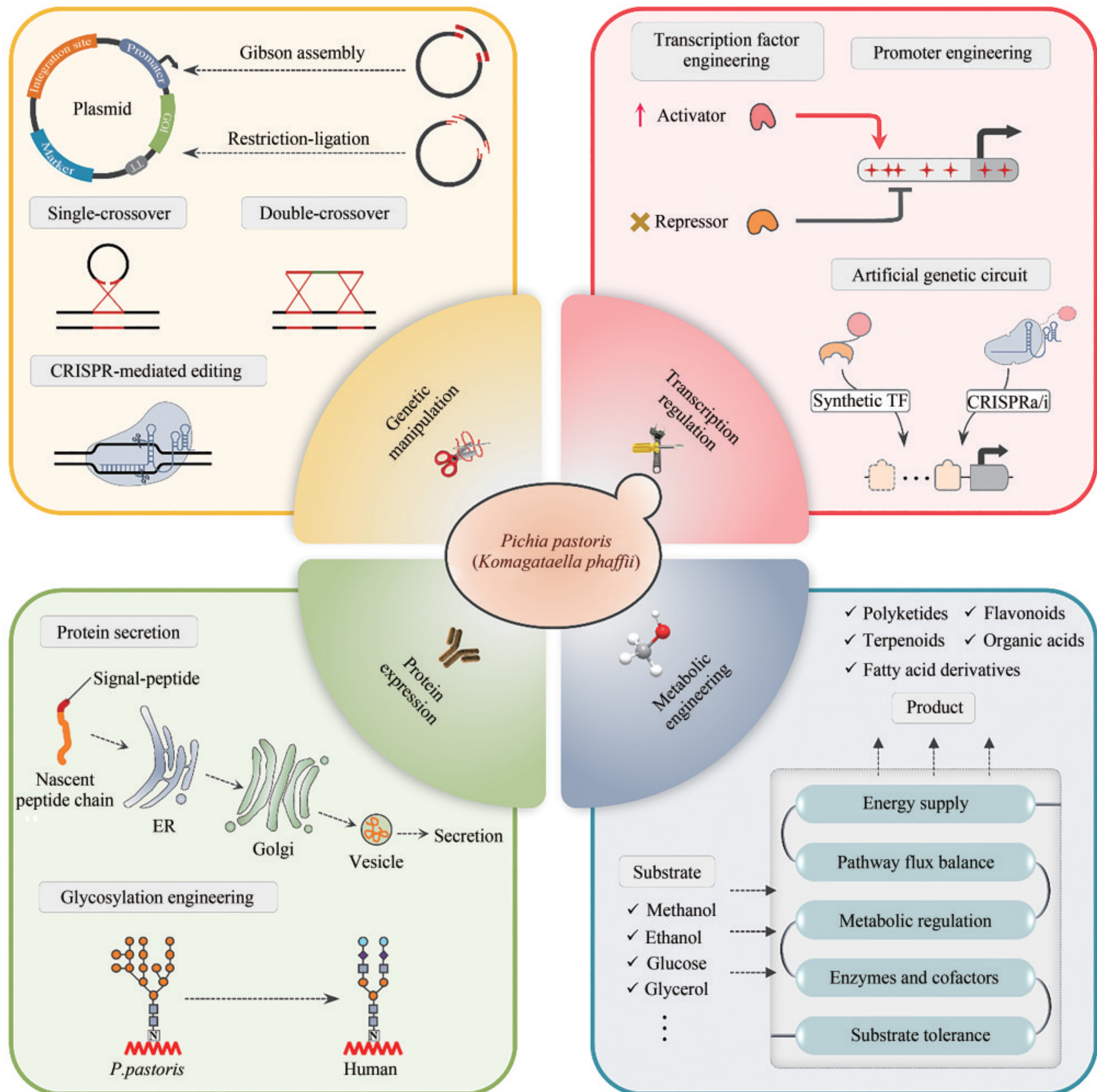
and industrial applications. This allowed the updated chassis versions adapting to diversified application scenarios. In this review, we briefly introduce the advances and current status of *P. pastoris*. We describe the development and application of this chassis from the genetic manipulation technology, regulation of gene expression, and metabolic engineering. We summarize the establishment and characterization of synthetic biological techniques, regulatory parts and devices, novel expression platform, and bioconversion system in *P. pastoris*. We emphasize the CRISPR-mediated gene editing, transcription regulation, rewiring of natural transcription system, and the design of artificial biosystems. Then the production of glycoprotein and the synthesis of natural products based on alcohols are concisely summarized. Also, the advantages and limitations of this host in practical application are analyzed and discussed. Finally, we propose the research directions for further updating versions of *P. pastoris* and provide a perspective on their future application scenarios.



Keywords: *Pichia pastoris*; chassis cell; methylotrophic yeast; cell factory; synthetic biological system

基于优质的微生物底盘宿主，开展高效、绿色、可持续的生物制造，已经成为合成生物学研究和生物经济发展的热点方向和重要目标[1-4]。作为一种甲基营养型微生物，巴斯德毕赤酵母（*Pichia pastoris*，又称*Komagataella phaffii*）在学术研究和工业生产中均受到了大量的研究和关注，是目前应用最为广泛的真核表达宿主之一[5-8]。目前广泛使用的毕赤酵母菌株是Phaff等于20世纪50年代在美国加利福尼亚州分离得到，并命名为*Pichia pastoris*[9]。1995年，Yamada等[10]将其重新归类于新属*Komagataella*。2005年，Kurtzman[11]进一步将*Komagataella*分为多个种，先前的*P. pastoris*被重新分类为*K. pastoris* 和*K. phaffii*。而现在应用于科研和工业生产的菌株大多数属于*K. phaffii*，例如CBS7435和GS115等。

巴斯德毕赤酵母（以下简称毕赤酵母）具有非常强的转录调控系统，且在遗传操作、翻译后修饰、分泌表达、高密度培养及产物纯化回收等方面均展现了诸多优势，适合用于大规模的工业化生产。而且，毕赤酵母对于廉价底物甲醇的强效同化作用，使其有望成为碳一化合物生物转化的优势底盘[5, 12-14]。此外，美国食品药品监督管理局（Food and Drug Administration，FDA）已将毕赤酵母认定为GRAS（generally recognized as safe，即公认安全的食品成分）菌株，进一步提升了其在药品、食品领域的应用潜力[15-16]。目前，毕赤酵母表达系统已有多种能够应对不同场景的商业化菌株及配套工具，在功能蛋白的生产和天然产物的合成中展现了出色的应用效果[6-7, 14, 16]。最近，使用毕赤酵母底盘系统生产的纳米抗体Caplacizumab（商品名Cablivi）和单克隆抗体Eptinezumab（商品名Vyepti）相继获得FDA批准上市，使其在生物医药领域的应用价值得到了更为广泛的关注。此外，FDA近期也批准了重组毕赤酵母生产的大豆血红蛋白用于新型食品（如ImpossibleTM植物牛肉），也进一步拓展了其在新食品领域的应用潜力。

随着组学技术及合成生物学技术的快速发展，研究者们对毕赤酵母底盘的探索和认识也在不断深入，在基因组测序[17-19]、调控机制解析[20-22]、特色生物元件发掘[23-25]、新型表达平台设计[26-28]、基因组遗传编辑[29-31]及代谢工程改造[32-34]等方面均取得了显著成果，促进了毕赤酵母底盘的发展和升级，进一步拓宽了其应用场景。本文总结了近年来针对毕赤酵母底盘细胞的工程化改造策略及应用情况，并讨论了毕赤酵母在实际应用中的优势，以及面临的限制和解决方案。同时，也介绍了各类合成生物技术在毕赤酵母中的最新进展，包括基因编辑、转录调控、翻译后修饰、代谢途径调节等。最后，对毕赤酵母底盘系统的应用前景和发展方向进行了展望。

1 基因工程技术及工具开发

1.1 质粒的设计与构建

质粒的设计与构建是实现蛋白高效表达的重要前提。与大肠杆菌和酿酒酵母等传统模式底盘宿主不同，毕赤酵母中没有稳定的游离型质粒[5, 35]。近年来，尽管通过对自主复制序列的研究改造提高了游离型质粒在毕赤酵母中的稳定性[36-39]，但是整合型质粒仍旧是毕赤酵母中基因操作的主要载体。目前，毕赤酵母表达系统已有成熟且多样的商业化质粒载体[5, 16]，可通过无缝克隆及酶切连接等常规技术快速装载目的基因。然而，面对毕赤酵母日益丰富的应用场景和底盘升级需求，已有的商业化载体工具在构建带有多表达盒的复杂质粒时面临较大的挑战。Prielhofer等[40]基于Ⅱs型限制性内切酶，在毕赤酵母中建立了一套可用于多表达盒体外组装的模块化载体工具箱Gold*Pi*CS，为复杂质粒的设计和构建提供了更为便捷、快速的工具和方法。而Nishi等[41]则通过敲除毕赤酵母内源DNA连接酶Ⅳ（Dnl4），实现了多片段的体内一步组装，进一步优化了复杂质粒的构建及转化流程。

此外，筛选标记的选择对于质粒设计和菌株筛选也非常关键[42-43]。虽然已有一系列适用于毕赤酵母的营养缺陷型筛选标记（如*HIS4*、*ARG1*、*ADE2*等）和抗性基因筛选标记（如zeocin/*Sh ble*^{R}、G418/*kan*^{R}、hygromycin/*hph*^{R}、nourseothricin/*nat*^{R}等）被开发应用[40, 44-46]，但是对于复杂代谢途径的体内组装和底盘改造仍有较大限制。针对这一

问题，一些研究在质粒的设计中引入Cre/*loxP*重组酶系统，在筛选标记两侧添加*loxP*位点，通过Cre介导的*loxP*位点特异性重组实现筛选标记的删除回收[47-50]。同时，为了提高标记回收效率，一些反向筛选标记在毕赤酵母中被开发应用，例如*URA3*[51]、*amdS*[52]、*mazF*[53]等。携带反向筛选标记的菌株在特定条件下生长会严重受限甚至死亡，从而实现无标记工程菌株的快速有效筛选。位点特异性重组结合反向筛选的标记回收策略，为解决毕赤酵母中筛选标记的限制提供了有效方案。尽管如此，筛选标记的循环利用依然存在周期较长、操作烦琐等问题，仍有必要进一步优化并探索更为高效的菌株筛选方法，而近年来不断发展的CRISPR/Cas9基因编辑技术为此提供了新的策略和手段。

1.2 CRISPR基因编辑技术在毕赤酵母中的应用

作为目前应用最为广泛的基因编辑技术，CRISPR/Cas9系统在毕赤酵母中已有深入的研究和发展[54-55]。2016年，Weninger等[56]通过对Cas9的密码子和gRNA表达方式进行探索优化，首先在毕赤酵母中建立了可高效打靶的CRISPR/Cas9系统。此后，研究者们相继对Cas9和sgRNA的启动子、供体片段及质粒自主复制序列ARS等关键元件进行了改造优化[39-40，57-60]，进一步提升了CRISPR系统在毕赤酵母中的打靶能力。相比于传统基因操作技术，CRISPR系统主要通过Cas9切割产生的双链断裂（double strand break，DSB）来引发细胞内源修复机制，无需在整合位点处引入筛选标记，可以实现基因组的无标记改造，有效缓解毕赤酵母筛选标记不足的限制。Cas9/sgRNA通常以游离质粒的形式进行表达，质粒上携带的筛选标记可以通过细胞传代培养而丢失，操作更为简单快捷。而且，CRISPR系统具备在基因组多个位点同时产生DSB的能力，也为基因组的多位点协同编辑提供了可能[61-62]。

在天然生物体内，Cas9切割基因组DNA产生的DSB主要依靠非同源末端连接（non-homologous end joining，NHEJ）和同源定向修复（homology directed repair，HDR）两种机制进行修复[63-64]。NHEJ过程会随机引入碱基的插入、突变和缺失，其修复效果难以预测和控制。而HDR则是一种精确的修复机制，通过供体片段的不同设计可以分别实现基因的插入、删除及替换，是外源基因整合入基因组及基因组定向改造的主要手段（图1）。天然毕赤酵母主要偏向于NHEJ机制，导致HDR效率非常有限[56]。为了提升毕赤酵母基因组的精准编辑效率，首先需要对NHEJ过程进行抑制，使DSB的修复偏向于HDR机制。Weninger等[57]研究发现，敲除参与NHEJ的关键基因*KU70*可以有效阻断NHEJ的发生，从而显著提高基因组的精准编辑效率。本课题组Liu等[65]进一步在Δ*ku70*菌株中探究了基因组多位点的多基因共整合，实现双位点整合效率达到70%，而三位点共整合效率达到32%。Nishi等[41]在*DNL4*缺失株中，通过50 bp的同源臂实现了多个外源DNA片段的一步整合，证明缺失*DNL4*也是抑制NHEJ的有效策略。此外，多项研究还尝试增强HDR关键基因的表达，以提高HDR机制的修复效率（表1）。Cai等[29]过表达毕赤酵母HDR过程的关键基因*RAD52*，并敲除影响HDR的*MPH1*基因，使用50 bp同源臂实现基因敲除效率达到90%，而且三位点整合效率达到25%。Gao等[30]则通过异源表达酿酒酵母来源的*RAD52*、*RAD59*、*MRE11*等HDR关键基因，显著地提高了毕赤酵母同源重组的能力，仅需提供40 bp的同源臂就可使单位点、双位点、三位点基因整合效率分别达到100%、98%和81%。通过抑制NHEJ及增强HDR的工程化策略，大大提高了CRISPR系统在毕赤酵母中的精准编辑效率，为毕赤酵母中的基因操作提供了灵活且高效的技术工具。目前，CRISPR系统在HDR增强型的毕赤酵母底盘中已可以较好地实现三位点的同时编辑，但是在更多位点及较长片段的整合应用中还面临一定的挑战，值得进一步研究和优化。

除了Cas9以外，针对同属于Ⅱ类CRISPR系统的Cpf1近年来也有广泛的研究和应用[66]。与Cas9识别富含GC的PAM序列不同，Cpf1主要识别富含T的PAM序列，可以与Cas9的功能进行有效互补。最近，Zhang等[31]基于来源于*Francisella novicida*的*Fn*Cpf1，在毕赤酵母中建立了CRISPR/Cpf1系统介导的基因组编辑技术，可以实现大片段DNA的

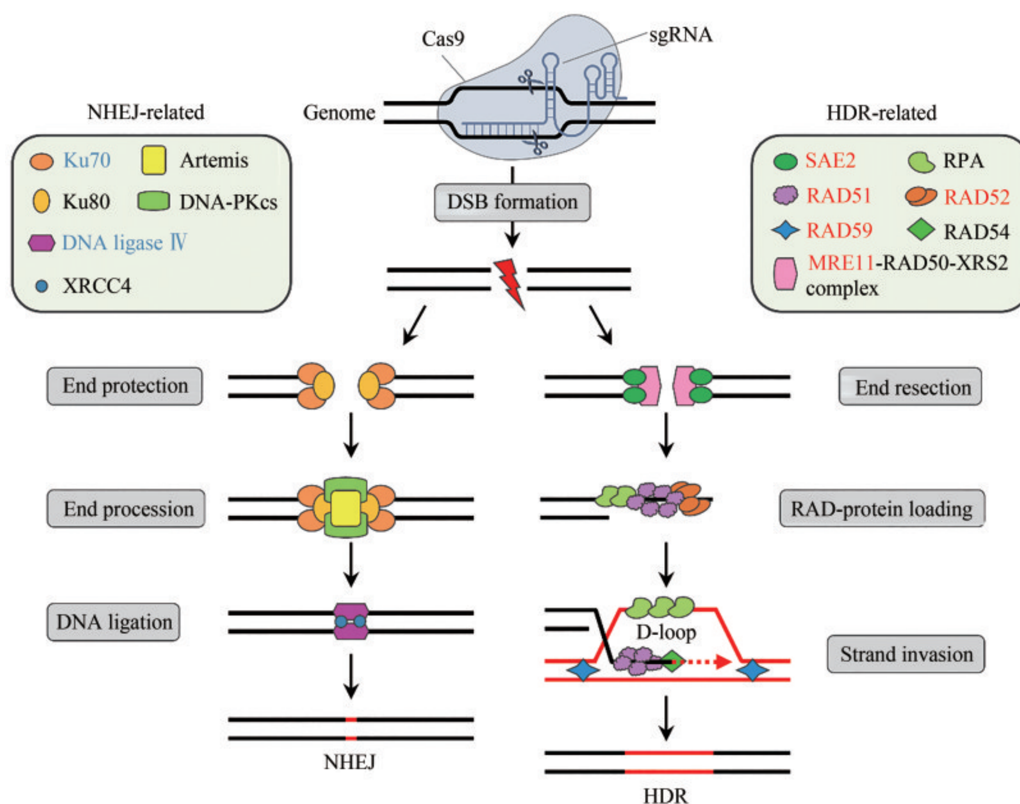


图1 毕赤酵母CRISPR介导的基因编辑过程及工程化改造策略[29-30，41，57]

（标注蓝色字体代表该蛋白敲除后可以有效抑制NHEJ作用；标注红色字体代表该蛋白过表达可以提升HDR介导的修复效率）

Fig. 1 CRISPR mediated gene editing process and engineering strategy in *Pichia pastoris*[29-30, 41, 57]

(Blue font indicates that the knockout of target protein can inhibit NHEJ; red font indicates that the overexpression of target protein can enhance HDR efficiency)

删除和多位点的同时编辑，在单基因敲除（99%）、双基因编辑（65%～80%）和三基因编辑（30%）方面均展现了较高的效率，进一步丰富和充实了毕赤酵母的基因操作工具箱（表1）。

1.3 高拷贝菌株的筛选

构建高拷贝稳定整合菌株是提高蛋白表达水平的有效方法。对于一些特定抗生素而言，其浓度与抗性基因剂量之间存在一定的对应关系，因此，可以通过提升抗生素浓度以增强筛选压力，从而获取高拷贝菌株。在毕赤酵母中，基于遗传霉素（G418）、博来霉素（zeocin）、潮霉素（hygromycin）等抗生素均已建立了较为成熟的高拷贝菌株筛选体系[45，67-68]，并有广泛的研究和应用。此外，还可以在体外构建高拷贝质粒，或通过筛选标记回收实现质粒多次整合，进一步提高目的基因的拷贝数[48-49，69]。但是，高浓度的抗生素同时也会对细胞生长造成较大影响，使阳性菌落数量减少，给高拷贝转化子的筛选带来一定的困难。针对这一问题，Sunga等[70]提出了转化后载体扩增法（PTVA），将转化后得到的低拷贝菌株在抗生素浓度逐渐提高的平板上反复传代筛选，促使目标载体在基因组上多次重组，最终获得高拷贝转化子。Aw等[71]将PTVA法拓展至液体培养基的筛选中，有效提升了高拷贝转化子的筛选效率，并缩短了操作周期。在此基础上，Marx等[72]将基因组上高度重复的rDNA序列作为整合位点，在转化后通过PTVA法使目的基因在多个rDNA位点间发生重组，使高拷贝菌株的筛选效率进一步提高。目前，通过rDNA位点整合并结合PTVA的高拷贝菌株筛选方法，已经在毕赤酵母中成功用于蛋白表达及化合物合成，是提高目的基因拷贝数的有效策略[73-75]。在酿酒酵母、汉逊酵母等酵母宿主中，rDNA位点整合还被用于和CRISPR系统联合使

表1 毕赤酵母中CRISPR介导的HDR效率及工程化改造策略

Tab. 1 CRISPR mediated HDR efficiency and engineering strategy in *Pichia pastoris*

菌株改造策略	Cas9/gRNA表达方式	游离质粒ARS	HDR效率		参考文献
			敲除	敲入	
Wild type	P_{HTX1}-Cas9 P_{HTX1}-HH-sgRNA-HDV	PARS1	100%（单基因）	—	[56]
敲除*ku70*	P_{HTX1}-Cas9 P_{HTX1}-HH-sgRNA-HDV	PARS1	100%（单基因）	—	[57]
敲除*ku70*	P_{HTX1}-Cas9 P_{HTX1}-HH-sgRNA-HDV	PARS1	—	75%～97.9%（单位点） 57.7%～70.0%（双位点） 12.5%～32.1%（三位点）	[65]
Wild type	P_{GAP}-Cas9 P_{SER}-sgRNA	panARS	80%（单基因）	—	[39]
敲除*ku70*	P_{ENO1}-Cas9 P_{tRNA1}-tRNA1-sgRNA	PARS1	—	40%（三位点）	[58]
敲除*MPH1*和 过表达*Pp*RAD52	P_{HTX1}-Cas9 P_{HTX1}-HH-sgRNA-HDV	panARS	90%（单基因）	43%～70%（单位点） 25%（三位点）	[29]
Wild type	P_{GAP}-Cas9（整合） P_{SER}-sgRNA	panARS PARS1	—	98.6%（单位点） 33.3%～93.1%（双位点） 10%～75%（三位点）	[60]
敲除*ku70*	P_{GAP}-Cas9（整合） P_{SER}-sgRNA	PARS1	—	33.3%～57%（三位点）	[60]
过表达*Sc*RAD52 *Sc*RAD59 *Sc*MRE11	P_{GAP}-Cas9（整合） P_{SER}-sgRNA	PARS1	—	100%（单位点） 95.7%～97.9%（双位点） 64.5%～80.9%（三位点）	[30]
Wild type	P_{GAP}-*Fn*Cpf1（整合） P_{SER}-crRNA	panARS	99%（单基因） 65%～80%（双基因） 30%（三基因）	—	[31]

注：HH和HDV为具有自剪切活性的核酶。使用RNA聚合酶Ⅱ型启动子表达sgRNA时，在sgRNA两端添加核酶HH和HDV，通过核酶自剪切去除mRNA两端的冗余序列。

用[76-77]，通过设计sgRNA靶向重复的rDNA位点以同时产生多个DSB，然后提供携带目的基因的相应供体片段，从而实现基因的多位点高效整合。而毕赤酵母中尚未有类似研究报道，可以作为高拷贝菌株筛选的新型潜在策略。

2 基因的表达与调控策略

2.1 基因的转录调控

2.1.1 天然转录调控系统及启动子工程

高效、精准、可控的基因转录是实现生产效能最大化的关键步骤。而启动子作为转录调控系统的核心元件，决定着基因转录的强度与调控模式[5, 14]。因此，对于天然启动子的发掘与鉴定一直以来都是生物科学领域的重要研究方向之一。在毕赤酵母中，已有多种天然启动子被开发应用，包括组成型启动子和诱导型启动子[78-79]。其中，诱导型启动子可以有效解耦细胞生长和产物合成阶段，精准控制蛋白表达的时机和水平。醇氧化酶启动子P_{AOX1}是毕赤酵母中首先被鉴定开发的一种诱导型启动子[80]，同时也是目前应用最为广泛的天然启动子之一。P_{AOX1}受甲醇的单一调控和强力诱导，同时被葡萄糖、甘油、乙醇等多种常用碳源严格阻遏[78]。高效的启动能力及严谨的调控特性使P_{AOX1}受到了研究者们的普遍关注。基于P_{AOX1}的甲醇诱导培养体系已成为毕赤酵母最为成熟的生产工艺，大量应用于重组蛋白的表达及调控[14, 81]。除P_{AOX1}以外，在毕赤酵母的天然甲醇代谢途径中还存在一系列甲醇诱导型启动子[79, 82]，例如活性较

强的P_{DAS1}和P_{DAS2}、中等强度的P_{FBA2}和P_{FLD1}以及活性较弱的P_{FGH1}和P_{AOX2}等。这些强度各异的启动子也为甲醇诱导的蛋白表达和代谢调控提供了多样化的工具。而随着组学技术的发展以及对毕赤酵母底盘研究的不断深入，更多种类的诱导型启动子也相继被研究鉴定，进一步拓宽了毕赤酵母的应用范围[35]，例如响应鼠李糖诱导的P_{LRA3}和P_{LRA4}[83-84]、响应乙醇诱导的P_{ADH2}和P_{ICL1}[33，85-86]、响应葡萄糖浓度的P_{GTH1}[87-88]以及硫胺素饥饿诱导型启动子P_{THI11}[89]等。

与诱导型启动子相比，组成型启动子可以在不同条件下提供较为稳定的持续输出，可以用于底盘生理特性研究、元器件组装及大规模连续发酵生产等[78，90-91]。甘油醛三磷酸脱氢酶启动子P_{GAP}是毕赤酵母中应用最为广泛的组成型启动子[90，92]，虽然其强度明显弱于P_{AOX1}，不过由于工艺简单、操作方便及能够兼容多种培养条件，因此在研究和生产中具有特殊的应用价值。此外，毕赤酵母中其他组成型启动子，例如P_{TEF1}、P_{GCW14}、P_{GPM1}、P_{PGK1}等，也有相关的报道和应用[78，81，93]。

随着毕赤酵母应用范围的不断拓展，天然启动子逐渐难以满足学术研究和工业应用的多样化需求。启动子工程是一项有效的应对策略，通过对天然启动子序列的随机或定向改造，构建启动子突变体或文库，可以在一定程度上改变启动子的转录强度和调控模式。毕赤酵母中，这类工作主要集中于P_{AOX1}的改造上，包括序列分段缺失和重组、潜在转录因子结合位点改造以及对特定区域的突变等[94-98]。基于上述策略，一系列P_{AOX1}突变文库相继被报道，为毕赤酵母甲醇调控提供了更加丰富的可选工具。此外，针对P_{GAP}、P_{CAT1}和P_{GTH1}的序列改造和文库构建也有相关报道[88，99-101]。还有一些研究则基于核心启动子区域的序列参数分析，直接人工设计并构建合成型启动子，为启动子的设计提供了新的方法和思路[25，102]。这类启动子工程策略进一步丰富了毕赤酵母中的转录调控元件库。不过，受限于细胞遗传背景，仅依靠序列改造的启动子突变体在转录强度和调控模式上往往很难取得颠覆性突破。

终止子也是决定转录活性的关键元件，但在毕赤酵母中，对于终止子的研究相对较少。除了一些内源性终止子被开发报道以外，一些来源于其他酵母宿主的异源终止子也被证实可在毕赤酵母中使用[40，82]。最近，Ito等[24]基于P_{GAP}系统比较了72个终止子的活性，包括28个内源性终止子和来源于酿酒酵母的41个终止子，以及3个合成型终止子。不同的终止子之间可以产生高达17倍的表达强度差异。此外，Ramakrishnan等[103]研究发现，启动子的选择也会对终止子的作用效果产生影响，多个终止子在分别与P_{AOX1}和P_{GAP}组合时，会呈现较大的活性差异。因此，选择合适的启动子/终止子组合在实际应用中非常重要，而启动子与终止子之间的适配关系和作用机理也值得进一步探究。

2.1.2 甲醇诱导转录调控系统的改造

基于P_{AOX1}的甲醇诱导转录调控系统是毕赤酵母中最常用的基因表达工具，也是毕赤酵母的主要应用优势之一[14，78，81]。但是，一些常规碳源和底物前体对P_{AOX1}的严格阻遏为其实际应用带来了一定限制。如何在保留P_{AOX1}高效活性的同时拓展其应用范围，是研究者们一直希望解决的关键问题。很多研究致力于解析P_{AOX1}的甲醇调控机制，以期通过转录体系的改造而开发更加灵活的调控工具[35，104]。目前，关于P_{AOX1}的转录调控体系已有初步的研究和阐述，其转录激活因子Mxr1、Prm1、Mit1及转录阻遏因子Mig1、Mig2、Nrg1等相继被鉴定[20，105-109]。本课题组Wang等[108]对这些转录因子与P_{AOX1}之间的相互作用进行了研究分析，建立了甲醇调控P_{AOX1}的信号传递模型。在此基础上，通过敲除三个阻遏因子，并过表达关键激活因子Mit1，实现了甘油条件下的脱阻遏表达，达到野生型P_{AOX1}甲醇诱导活性的77%[109]。随后，Vogl等[110]使用P_{GAP}和P_{CAT1}分别对三个转录激活因子以及其他多种调控蛋白进行过表达，测试了不同因子的脱阻遏效果，并且发现Mit1的过表达可使野生型在甲醇条件下的活性提升82%。而Chang等[111]则基于Mxr1设计了一套合成型正反馈基因线路，在甘油饥饿条件下实现了一定的脱阻遏活性。Yang等[112]使用甲醇诱导型启动子P_{SUT2}过表达Mxr1，使P_{AOX1}的活性显著提高。Takagi等[113]过表达Prm1并改造甲醇诱导型启动子P_{DAS1}，使P_{DAS1}活性大幅提升并实现了葡萄糖条件下的脱阻遏活性。基于缺失阻

遏因子和过表达激活因子的转录因子工程，可以在一定程度上改造细胞遗传背景，是提升天然启动子强度及改善调控模式的有效策略（图2）。此外，一些蛋白激酶、碳源转运体和传感器的缺失也可以有效改造P_{AOX1}的调控模式[116-119]，实现特定条件的脱阻遏。不过，天然转录系统的简单改造仍旧无法完全摆脱内源调控遗传背景的限制，而且还可能会对细胞自身代谢网络产生干扰，因此存在一定的局限性。

2.1.3 合成型转录调控系统的设计

随着合成生物学的发展，通过生物元器件的工程化理性设计来构建独立于宿主内源调控网络的人工调控系统，逐渐成为新的研究热点和方向[104, 120-121]。真核生物中，将不同来源的转录因子、启动子及其他类型的调控元件进行组合和重构，可以构建不依赖细胞遗传背景的新型转录调控系统[120, 122-123]（图2）。Perez-Pinera等[27]在毕赤酵母中基于雌二醇诱导元件和VP64激活因子，构建了一套可以响应雌二醇信号的人工转录级联调控系统，能够以雌二醇作为诱导剂实现精准调控。Rantasalo等[26]通过一些通用功能元件的组合优化，设计了一套合成型表达系统SES，在包括毕赤酵母在内的6种真核宿主中均展现了不错的效果。本课题组Liu等[28]则利用毕赤酵母内源转录因子激活域和核心启动子，结合原核生物操纵子调控元件，构建了一套转录信号增益器件iTSAD，在多种碳源条件下均可实现蛋白的强力表达，最高可达甲醇诱导条件下P_{AOX1}的5.2倍。可以发现，相较于天然转录调控系统，人工设计的合成型系统具备更好的灵活性，能够摆脱内源调控网络的限制，在强度和调控模式上实现显著突破。

近年来，基于无核酸酶活性的dCas9蛋白的CRISPR调控技术，受到了广泛关注并有非常快速的发展[62, 124-125]。在毕赤酵母中，CRISPR系统介导的转录激活（CRISPRa）和转录干扰（CRISPRi）已有相关报道（图2）。Yang等[59]首先在毕赤酵母中测

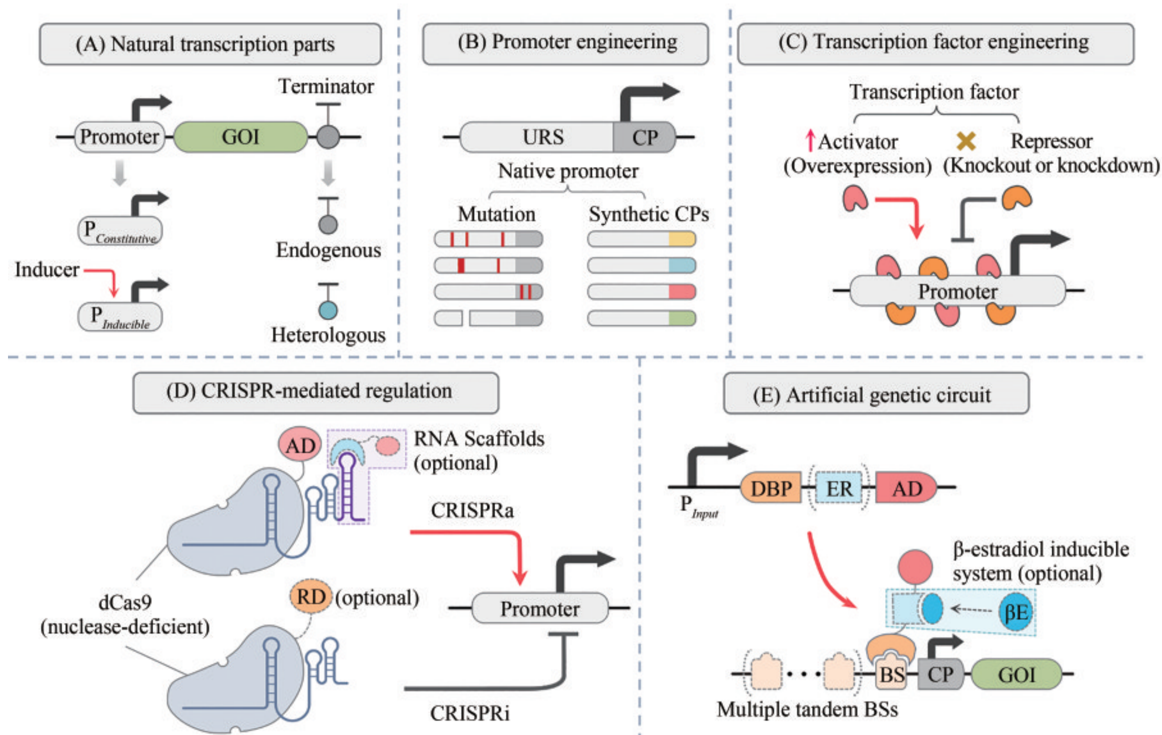


图2 毕赤酵母中转录调控系统的改造及工程化设计[24-28, 40, 59, 78, 82, 88, 94-103, 108-115]

URS—上游调控序列；CP—核心启动子；AD—激活结构域；RD—阻遏结构域；
DBP—DNA结合蛋白；BS—结合位点；ER—雌二醇受体；βE—β-雌二醇

Fig. 2 Rewiring and engineering design of transcription regulation system in *Pichia pastoris*[24-28, 40, 59, 78, 82, 88, 94-103, 108-115]

URS—upstream regulatory sequence; CP—core promoter; AD—activation domain; RD—repression domain;
DBP—DNA binding protein; BS—binding site; ER—estradiol receptor; βE—β-estradiol

试了CRISPRi技术，通过设计相应sgRNA靶向P_{AOX1}使其活性降至28%。Liao等[114]将dCas9分别与阻遏因子Mix1/RD1152和激活因子VPR融合表达，在毕赤酵母中实现了CRISPR介导的转录干扰和激活。此外，Baumschabl等[115]通过RNA支架（scgRNA）的设计，同样实现了对基因转录的激活和阻遏效果。本课题组Liu等[28]在毕赤酵母中分别设计并构建了CRISPRa激活器件和CRISPRi阻遏器件，进一步使其协同调控转录信号增益器件iTSAD，构建了新型毕赤酵母表达平台SynPic-X，实现了兼具高强度、低渗漏和可编程特性的基因表达调控，且通过简单切换低强度输入启动子即可实现对人工自定义诱导信号的灵活响应和高强度表达。总体而言，CRISPRa/CRISPRi技术的探索和发展，为毕赤酵母中人工遗传线路的设计提供了更为灵活的策略。

2.2 糖基化蛋白的表达策略

毕赤酵母底盘系统在重组蛋白的表达中具有诸多优势，不过在糖蛋白的生产方面则具有一定限制。糖蛋白是重组蛋白药物的重要组成部分，而糖基化修饰则对糖蛋白的功能非常关键[126-127]。目前，重组糖蛋白药物主要通过哺乳动物细胞表达系统进行生产。由于毕赤酵母糖基化结构为高甘露糖型，与人源的杂合型和复杂型有较大差异，导致其表达的糖蛋白对人具有免疫原性，功能和活性均会受到影响[7, 128]。因此，很多研究致力于探索并改造毕赤酵母的糖基化途径，开发能够产生人源糖基化的新型毕赤酵母底盘系统，为糖蛋白药物的生产提供新型工具和平台[7, 129-131]。

目前，关于毕赤酵母糖基化途径的研究和改造主要聚焦于*N*-糖基化修饰上[7, 129, 132]（图3）。*N*-糖基化是将寡糖链与蛋白质肽链Asn-X-Ser/Thr（X是除脯氨酸外的所有氨基酸）结构中的Asn酰胺氮通过β-1,4糖苷键特异性连接而成[131, 133]。毕赤酵母的*N*-糖基化起始于内质网膜，包括寡糖在脂质载体上的组装以及将寡糖转移至内质网腔内与肽链连接[129, 133]。首先，多萜醇脂及与其相连的一个焦磷酸基团组成多萜醇磷酸（dolichol-phosphate，Dol-P），将糖链的生物合成固定于内质网膜上。然后，通过一系列Alg家族蛋白的催化形成$Glc_3Man_9GlcNAc_2$-Dol-PP糖链结构[134-136]。接着，通过糖基转移酶OST将寡糖链转移至新生多肽的天冬氨酸残基上，并在葡萄糖苷酶Ⅰ和Ⅱ以及内质网甘露糖酶Ⅰ的作用下切除3个葡萄糖残基和1个甘露糖残基，最终形成$Man_8GlcNAc_2$糖型。这一糖链结构及形成过程在酵母和哺乳动物中是高度保守的[128-129, 131]。当糖蛋白被转运至高尔基体后，先在α-1,6-甘露糖转移酶Och1的作用下添加一个α-1,6-甘露糖，接着在甘露糖基转移酶和磷酸甘露糖基转移酶作用下添加大量甘露糖和磷酸甘露糖，从而形成高甘露糖型糖链结构[129, 133]。相比于常规酵母和丝状真菌，毕赤酵母的高甘露糖化程度较低，平均甘露糖残基数仅有9～14[6, 35, 133]，与人源化的糖基构型更为接近，这也为毕赤酵母的糖基化工程改造提供了便利条件。

酵母与人源的糖基化构型主要区别在于高尔基体中的修饰过程（图3）。敲除毕赤酵母内源Och1可以阻断高尔基体中的高甘露糖化，为进一步的糖工程改造提供基础[7, 131, 133]。Choi等[137]在Och1敲除菌株中分别构建了不同来源的甘露糖苷酶MnsⅠ文库和乙酰葡萄糖转移酶GnTⅠ文库，通过高通量筛选首次得到了能够表达杂合型糖链结构$GlcNAcMan_5GlcNAc_2$的毕赤酵母菌株。在此基础上，Hamilton等[138]进一步引入MnsⅡ和GnTⅡ，得到了更接近人源糖基化构型的糖链结构$GlcNAc_2Man_3GlcNAc_2$，使毕赤酵母的糖工程改造取得了显著进步。随后，Hamilton等[139]通过敲除4个毕赤酵母内源特异性糖基化基因，同时引入14个外源糖基化功能基因，成功得到人源复杂型糖链结构$Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$，使糖蛋白末端唾液酸化率超过90%。Jacobs等[132]和Thak等[133]则分别对毕赤酵母的人源糖基化改造策略进行了总结，并提出了相关操作流程。目前，已有多种重组糖蛋白使用毕赤酵母糖基化工程菌株进行表达[7]，例如单克隆抗体[140]、重组促红细胞生成素rEPO[139]、人干扰素α-2b[141]等。此外，通过单缺失*ALG3*或双缺失*ALG3*/*ALG11*可以直接得到$Man_5GlcNAc_2$或$Man_3GlcNAc_2$糖链结构，来作为GnTⅠ的底物进行人源糖基化修饰[142-143]。该策略在酿酒酵母和汉逊酵母中已有报道，也为毕赤酵母中的糖基化工程提供了新的思路和参考。

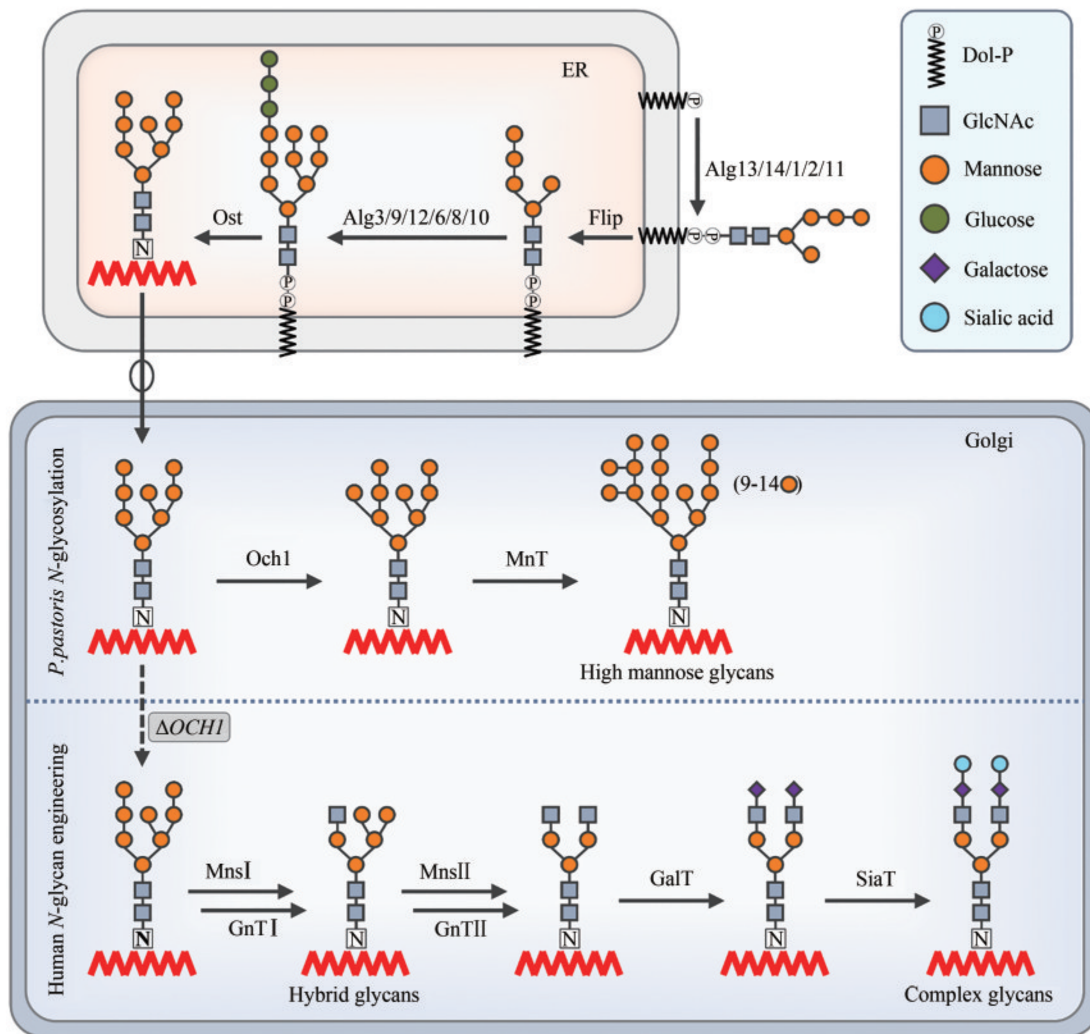


图3 毕赤酵母*N*糖基化过程及工程化改造[128-141]

Fig. 3 The *N*-glycosylation and *N*-glycan engineering in *Pichia pastoris*[128-141]

2.3 蛋白的分泌表达

毕赤酵母自身分泌的胞外蛋白数量较少，因此胞外分泌表达是毕赤酵母中外源蛋白的主要生产方式[6, 14]。而信号肽则是引导分泌过程并决定分泌效率的关键因素[144]。目前，毕赤酵母中应用最广泛的信号肽主要为来自酿酒酵母的α交配因子信号肽（α信号肽）[6]。通过密码子优化、疏水区域改造等策略，可以有效提高α信号肽在毕赤酵母中介导的分泌效率[145-146]。然而，α信号肽虽然在毕赤酵母中可以发挥良好的分泌功能，但其“翻译后转运”的易位方式可能导致新生肽链在胞质中折叠，从而影响转运效率。Barrero等[147]将酿酒酵母Ost1信号肽与α信号肽的pro区进行融合，有效减少了新生肽链的聚集，将脂肪酶BTL2和红色荧光蛋白的分泌表达量分别提升了10倍和20倍。此外，开发更多种类的高效信号肽也是研究者们的一项重要目标。一些毕赤酵母内源信号肽相继被开发报道，并展现了出色的分泌表达能力[6]，例如来源于*PHO1*[148]、*EXG1*[149]、*MSB2*[150]和PAS_chr3_0030[151]等基因的自身信号肽。Massahi等[152-153]则利用SignalP 4.1等软件对56个潜在的内源和外源信号肽进行评分，并使用重组人生长激素对分泌效率进行评估。总体而言，信号肽对于胞外蛋白的表达非常重要，在实际应用中应当灵活选择，以取得更佳的分泌效果。

在信号肽引导的分泌过程中，蛋白质在内质

网中的正确折叠非常关键。新生肽链以未折叠状态进入内质网，并在分子伴侣的辅助下进行折叠和组装[6, 8, 14, 154]。然而，外源蛋白的高效表达可能会引发分子伴侣的短缺，从而导致大量新生肽链不能及时正确折叠而聚集并滞留在内质网腔内，造成内质网压力，进而激活未折叠蛋白响应（unfolded protein response，UPR）[8, 154]。共表达分子伴侣是缓解内质网压力的重要策略。作为UPR转录激活因子，Hac1可以响应UPR并调节蛋白折叠和转运。过表达Hac1可以有效提升毕赤酵母中分泌蛋白的表达水平[68, 155-158]（表2）。此外，其他多种分子伴侣的过表达也在毕赤酵母中取得了显著效果，例如二硫键异构酶Pdi1、氧化还原蛋白Ero1、内质网伴侣蛋白Kar2等[158-161]，而且分子伴侣的过表达对于高拷贝菌株的产量提升效果尤为明显[162-163]。不过，毕赤酵母中许多蛋白分泌途径的关键作用因子目前仍未发现与阐明，分泌机制的探究和关键因子的鉴定仍是毕赤酵母底盘升级的重要方向。

表2 分子伴侣共表达提高毕赤酵母蛋白表达量

Tab. 2 Co-expression of chaperone to enhance protein expression in *Pichia pastoris*

外源蛋白	分子伴侣	效价提升倍数	参考文献
狂犬病病毒糖蛋白	PDI1	9.6倍	[159]
	ERO1	3倍	
	GPX1	8.2倍	
白介素-2-人血清白蛋白融合蛋白	PDI1	2.2倍	[161]
	KAR2	1.9倍	
	ERO1	2.3倍	
猪肽聚糖识别蛋白	PDI1	5倍（高拷贝）	[162]
疏水蛋白HFBI	KAR2	14倍（单拷贝） 9.8倍（双拷贝） 22倍（三拷贝）	[163]
	PDI	7.8倍（三拷贝）	
	ERO1	30倍（三拷贝）	
人溶菌酶	HAC1	1.2倍	[156]
铜绿假单胞菌弹性蛋白酶	HAC1	1.8～3.9倍	[155]
大肠杆菌植酸酶	HAC1	1.36倍	[158]
	PDI1	1.40倍	
家蚕乙酰胆碱酯酶	PDI1	5倍	[160]
几丁质酶	HAC1	1.3倍	[157]

注：表中所列拷贝数（高拷贝、单拷贝、双拷贝等）是指外源蛋白编码基因在基因组上的拷贝数。

此外，蛋白质在细胞器间的转运也是分泌表达的关键环节。Baumann等[164]在毕赤酵母中过表达了涉及内质网转运的关键因子WSC4，使重组Fab抗体片段产量提升1.2倍。而蛋白从内质网到高尔基体和细胞膜的运输也是限制分泌效率的主要瓶颈之一。在内质网中经过加工后，正确折叠的蛋白质被包装在COP-Ⅱ囊泡中，并转运至高尔基体。通过增强囊泡运输相关蛋白的表达，可以有效提升分泌蛋白的产量。Gasser等[165]基于转录组分析筛选到了多个蛋白分泌的辅助因子，包括参与蛋白运输的BFR2和Bmh2，以及液泡ATP酶亚基Cup5和胞外分泌相关的蛋白激酶Kin2，这些关键因子的过表达对蛋白的分泌表达均展现了积极影响。Guan等[161]则过表达了参与囊泡运输Sec1和Sly1，分别使IL2-HAS的产量提升2.5倍和1.9倍。可以发现，强化蛋白转运过程也是提升分泌蛋白表达水平的有效策略。

3 代谢工程改造及应用

3.1 以甲醇为底物的代谢工程研究

甲醇作为一种来源广泛、成本低廉的碳一底物，具有成为新一代大宗生物制造原料的应用潜力[166-167]。作为一种甲基营养型酵母，毕赤酵母独特的甲醇同化能力和诱导调控体系，使其成为甲醇生物转化的优质底盘宿主之一[12, 168]。在毕赤酵母中，甲醇的代谢主要发生在过氧化物酶体当中，细胞器的间隔有利于缓解甲醇及其代谢物对细胞的毒性。甲醇进入过氧化物酶体后，首先被醇氧化酶（Aox）氧化为甲醛及过氧化氢，然后由过氧化氢酶（Cat）将后者分解为水与氧气。甲醛的代谢则分为异化作用和同化作用两部分进行。异化作用是在甲醛脱氢酶（Fld）、*S*-甲酰基谷胱甘肽水解酶（Fgh）、甲酸脱氢酶（Fdh）等的介导下，依次脱氢代谢生成甲酸和二氧化碳，并产生NADH为细胞提供能量。同化作用则是甲醛进入单磷酸木酮糖循环途径，最终生成3-磷酸甘油醛，随后在胞质中代谢为丙酮酸，进入中心碳代谢，为细胞生长提供所需的生物质组分[35, 168-169]。相比于葡萄糖等传统碳源，甲醇代谢过程能够产生较高的还原

力，对于天然产物的生物合成更具优势[166，170]。近年来，以甲醇为底物的毕赤酵母细胞工厂已有较多研究和成果[166，168]。Zhang等[171]通过增强毕赤酵母内源TCA循环，实现了由甲醇从头生产多种四碳二羧酸。Guo等[172]对毕赤酵母甲醇同化途径进行改造，并阻断副产物合成途径，以甲醇为原料实现了苹果酸的高效合成。最近，Cai等[173]对毕赤酵母中心代谢网络及甲醇利用途径进行优化，实现了甲醇向脂肪酸和脂肪醇的高效生物转化。同时，毕赤酵母甲醇表达系统还被用于多种脂肪酸衍生物（长链α-烯烃、蓖麻油酸、花生四烯酸等）[174-176]及萜类化合物（番茄红素、β-胡萝卜素、虾青素、诺卡酮、达玛烯二醇等）[60，82，177-180]的生产。本课题组则以甲醇为碳源底物，在毕赤酵母中实现了一系列聚酮化合物的合成，包括6-甲基水杨酸、土曲霉酸、橘霉素、洛伐他汀及莫纳可林J等[33，181-184]。这些小分子化合物的成功合成，进一步证实了毕赤酵母具备成为高效微生物细胞工厂和优质甲醇生物转化底盘的应用潜力和开发价值。

在实际应用中，毕赤酵母的甲醇代谢存在耗氧量大、产热高且碳利用效率有限等问题。而且，甲醇毒性对细胞生长的影响也是限制生产效率的关键因素。一些研究使用混合补料策略[185-189]，将甲醇与甘油或山梨醇等其他碳源混合添加，可以在缓解甲醇毒性的同时降低耗氧和产热。乙酸盐、乳酸、葡萄糖酸盐等也有与甲醇混合应用的报道[190-191]。此外，本课题组Wang等[192]基于P_{AOX1}的转录调控体系改造，实现了低剂量甲醇补料条件下的蛋白高效生产，有效降低了甲醇代谢过程的耗氧和产热量，为以甲醇为底物的工业生产提供了新的方法和策略。不过，在天然产物的合成以及大规模的生产应用中，甲醇生物转化所带来的耗氧和产热仍是毕赤酵母面临的关键挑战之一。如何调节或改造甲醇的代谢网络，进一步提升甲醇的生物利用效率，是毕赤酵母底盘改造升级的一个重要方向。

近年来，随着“碳中和”国家战略的提出，以CO_2为底物的生物转化成为绿色生物制造的研究热门和重要目标之一。Gassler等[32]基于卡尔文（CBB）循环及单磷酸木酮糖（XuMP）循环的相似性，对毕赤酵母的甲醇代谢途径进行了改造，通过引入8个异源功能酶、删除3个内源甲醇代谢关键酶，在毕赤酵母中实现了以CO_2为碳源的自养模式。以此为基础，通过适应性进化进一步增强了CO_2的利用效率[193]，使新型毕赤酵母底盘菌株能够以CO_2为唯一碳源进行生长。同时，该研究保留了毕赤酵母的甲醇异化作用途径，以甲醇作为能量和还原力的供给底物。CO_2自养模式的实现进一步丰富了毕赤酵母的应用范围，提升了毕赤酵母在碳中和战略体系下的开发价值。

3.2 乙醇驱动的生物合成体系

对于很多天然产物分子的生物合成而言，胞质乙酰辅酶A的供应是限制合成效率的关键因素[194]。在天然酵母中，乙醇可以在细胞质内通过三步代谢直接产生乙酰辅酶A，因而非常适合作为胞质乙酰辅酶A的供给前体[85，195]。相比于酿酒酵母，毕赤酵母具有更加高效的乙醇同化能力[33]，在乙醇为碳源时生长情况明显优于甲醇，与葡萄糖条件接近，展现了毕赤酵母中乙醇驱动型生物合成体系的潜在开发价值。

目前，毕赤酵母中乙醇的代谢途径尚未表征完全，许多相关酶都是基于酿酒酵母中的同类酶开展功能研究[85，195]。乙醇先在细胞质中代谢为乙醛，该过程由乙醇脱氢酶（Adh2，也有研究称作Adh3）催化完成[85-86，196]，而Adh6、Adh7、Adh900等酶则参与乙醛向乙醇的合成[85，195]。乙醛接着在乙醛脱氢酶（Ald1）的作用下生成乙酸，再经乙酰辅酶A合酶（Acs1）催化产生乙酰辅酶A[85]。乙醇的代谢途径中存在多个乙醇诱导型启动子，包括P_{ADH2}、P_{ACS1}、P_{ALD1}及P_{ICL1}等。其中，P_{ADH2}强度较高，最高表达水平接近P_{AOX1}，且其活性受到乙醇浓度的剂量调控[196-197]。不同于P_{AOX1}受甲醇的严格诱导及其他碳源的严格阻遏，P_{ADH2}则受乙醇诱导的同时也可以在葡萄糖条件下表达，且两者同时存在时表达强度并不会受到葡萄糖影响[196]。这些乙醇调控型启动子为乙醇生物合成体系提供了有效的工具。同时，对于乙醇调控体系的研究和改造也已有一些报道和进展。研究发现，P_{AOX1}的转录因子Mxr1同时也涉及P_{ADH2}的激活[21，196]，而激活因子Cat8-1及Cat8-

2则参与P_{ADH2}、P_{ALD1}及P_{ACS1}等的调控[198]。基于P_{ADH2}转录因子结合序列改造的合成启动子P_{SNT5}和$P_{ADH2-Cat8-L2}$，在乙醇诱导下的表达强度分别达到P_{ADH2}的2.2倍和4.8倍[196-197]。本课题组Liu等[33]结合P_{ICL1}和信号增益器件，在毕赤酵母中开发了乙醇诱导型调控系统ESAD，建立了乙醇同时作为碳源、前体和诱导物的新型生物合成系统，并实现了由乙醇从头合成聚酮类化合物莫纳可林，产量达到3.2 g/L。本课题组Qian等[34]进一步开发了基于ESAD的乙醇诱导型器件组合，并应用于植物黄酮类化合物的生物合成调控，通过途径装配与通量平衡，最终实现由乙醇从头合成黄芩素（401.9 mg/L），并实现千层纸素的首次异源生物合成（339.5 mg/L）（图4，表3）。

在乙醇代谢过程中，中间代谢物乙酸的积累会对细胞生长产生明显的影响。当乙酸浓度超过40 mmol/L时，毕赤酵母的生长就会受到严重抑制[199]。因此，降低乙酸积累及增强细胞耐受性，是提升乙醇利用效率的关键。本课题组Xu等[199]基于毕赤酵母激酶缺失文库筛选到了多个与乙酸耐受相关的基因，其中激酶Hrk1的过表达能够明显提高毕赤酵母对于乙酸的耐受浓度。同时，增

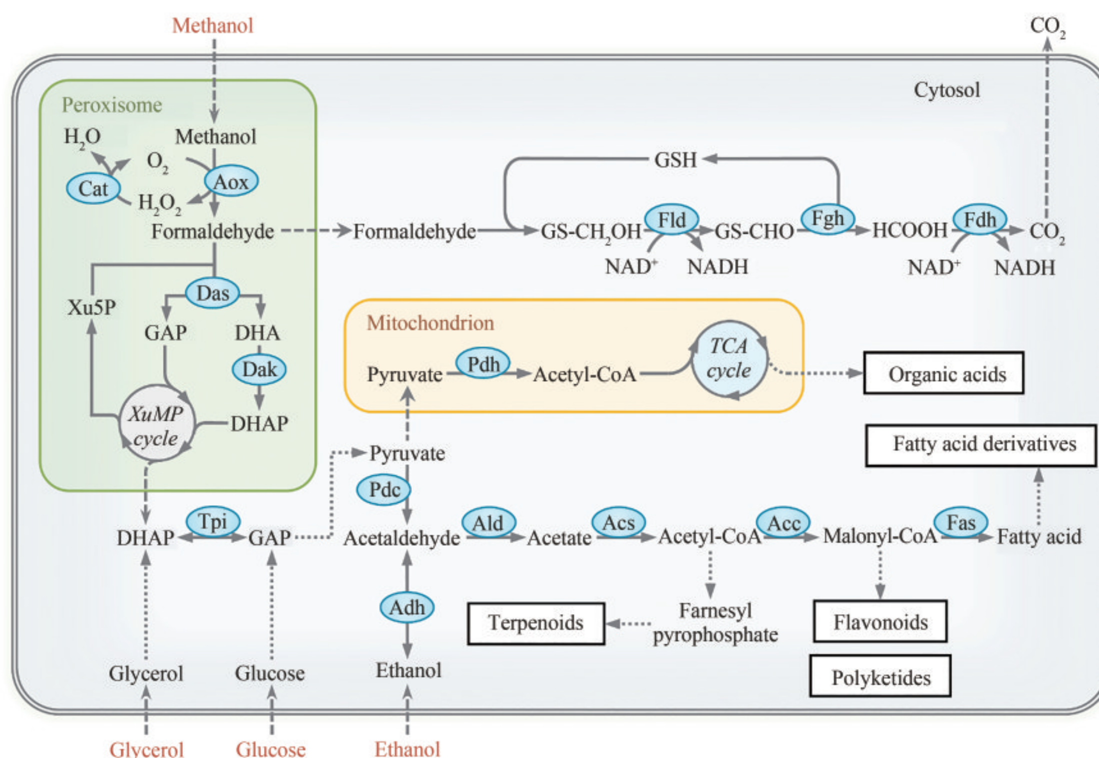


图4 毕赤酵母中不同碳源底物的代谢途径及各类天然产物的合成[33-34, 60, 82, 171-184, 199-203]

DHA—二羟丙酮；GAP—3-磷酸甘油醛；Xu5P—5-磷酸木酮糖；DHAP—磷酸二羟丙酮；XuMP cycle—单磷酸木酮糖循环；Aox—醇氧化酶；Cat—过氧化氢酶；Das—二羟丙酮合成酶；Dak—二羟丙酮激酶；Tpi—磷酸丙糖异构酶；Fld—甲醛脱氢酶；Fgh—*S*-甲酰基谷胱甘肽水解酶；Fdh—甲酸脱氢酶；Pdh—丙酮酸脱氢酶；Pdc—丙酮酸脱羧酶；Adh—乙醇脱氢酶；Ald—乙醛脱氢酶；Acs—乙酰辅酶A合成酶；Acc—乙酰辅酶A羧化酶；Fas—脂肪酸合成酶；→表示酶催化反应；-->表示代谢物在细胞内外或不同细胞器间的穿梭；···>表示多步代谢途径

Fig. 4 Metabolic pathways of different carbon sources and synthesis of various natural products based on in *Pichia pastoris*[33-34, 60, 82, 171-184, 199-203]

DHA—dihydroxyacetone; GAP—glyceraldehyde-3-phosphate; Xu5P—xylulose 5-phosphate; DHAP—dihydroxyacetone phosphate; XuMP cycle—xylulose monophosphate cycle; Aox—alcohol oxidase; Cat—catalase; Das—dihydroxyacetone synthase; Dak—dihydroxyacetone kinase; Tpi—triosephosphate isomerase; Fld—formaldehyde dehydrogenase; Fgh—*S*-formylglutathione hydrolase; Fdh—formate dehydrogenase; Pdh—Pyruvate dehydrogenase; Pdc—pyruvate decarboxylase; Adh—alcohol dehydrogenase; Ald—acetaldehyde dehydrogenase; Acs—acetyl-CoA synthetase; Acc—acetyl-CoA carboxylase; Fas—fatty acid synthetase; → indicate enzyme catalyzed reaction; --> indicate the shuttle of metabolites between extracellular and intracellular or between different organelles; ···> indicate a multistep metabolic pathway.

表3 基于毕赤酵母底盘细胞的天然产物合成

Tab. 3 Production of natural products in *Pichia pastoris* chassis cell

种类	化合物	底物及培养方式	产量	参考文献
四碳有机酸	苹果酸	甲醇（摇瓶）	2.79 g/L	[172]
		葡萄糖（摇瓶）	8.55 g/L	
		甲醇（反应器）	42.28 g/L	[171]
	富马酸	甲醇（反应器）	0.76 g/L	
	琥珀酸	甲醇（反应器）	9.42 g/L	
脂肪酸衍生物	蓖麻油酸	甲醇（摇瓶）	171.44 mg/L	[175]
	长链α-烯烃	甲醇（摇瓶）	1.6 mg/L	[174]
	脂肪酸	甲醇（反应器）	23.4 g/L	[173]
	脂肪醇	甲醇（反应器）	2.0 g/L	
萜类化合物	番茄红素	葡萄糖（摇瓶）	1.141 μg/g DCW	[202]
		甲醇（反应器）	714 mg/L	[180]
	β-胡萝卜素	葡萄糖（摇瓶）	339 μg/g DCW	[202]
	虾青素	葡萄糖（摇瓶）	3.7 μg/g DCW	[203]
	诺卡酮	甲醇（反应器）	208 mg/L	[178]
	达玛烯二醇	甲醇（摇瓶）	1.073 mg/g DCW	[179]
聚酮类化合物	6-甲基水杨酸	甲醇（反应器）	2.2 g/L	[181]
	橘霉素	甲醇（摇瓶）	0.6 mg/L	[182]
	洛伐他汀	甲醇（反应器）	250.8 mg/L	[184]
		甲醇（反应器）	419.0 mg/L	[201]
	莫纳可林J	甲醇（反应器）	593.9 mg/L	[184]
		乙醇（反应器）	3.2 g/L	[33]
黄酮类化合物	黄芩素	乙醇（摇瓶）	401.9 mg/L	[34]
	千层纸素	乙醇（摇瓶）	339.5 mg/L	

强乙酰辅酶A合成酶（Acs1）的表达强度，促进乙酸的代谢转化，也是缓解乙酸抑制作用的有效策略。此外，Paes等[200]通过转录组分析发现乙酸代谢相关基因涉及RNA加工、中心碳代谢及氧化还原酶活性等，为进一步优化乙酸代谢提供了方向和参考。

3.3 代谢途径的调控与平衡

作为一种新型微生物细胞工厂，毕赤酵母在代谢工程领域已有较多报道和应用[6，8，14，204]。在天然产物的生物转化过程中，代谢途径的调控和平衡对于提升底物利用效率及减少中间产物积累非常关键。为了提升毕赤酵母底盘的生产效率，一些经典的代谢工程调控策略被应用于毕赤酵母中并取得了明显效果，包括增强前体供应[205]、降低副产物分支途径通量[34]及促进毒性产物外排[201]等。此外，部分研究则在转录层面对途径关键酶的转录水平进行精细调控，以平衡各途径节点的代谢通量。通常而言，构建强度跨度大、分布均匀的启动子文库，是对转录水平进行精细调控的有效策略之一[81]。Vogl等[23]在毕赤酵母中构建了合成型双向启动子文库，并将其应用于紫杉烯和β-胡萝卜素的代谢途径调控与平衡。本课题组也构建了P_{GAP}突变文库和人工转录调控器件组合，并分别应用于聚酮类和黄酮类化合物的代谢工程优化[34，206]。而基于关键代谢产物浓度的动态调控，也是控制代谢节点流量、提升产物合成效率的有效手段。本课题组Wen等[207]基于丙二酰辅酶A的感应蛋白FapR设计了一套响应胞内丙二酰辅酶A浓度的动态调控系统，为丙二酰辅酶A衍生物的高效合成提供了新的策略和工具。

能量的供应和调控也是决定代谢工程效率的关键因素[8，208]。添加能源底物、调控碳源补给策略以及对底盘宿主进行能量代谢体系改造，均为提升细胞内ATP水平的有效策略[208-209]。柠檬酸钠是增加ATP供应的常用能源底物。在毕赤酵母的培养过程中，添加柠檬酸钠可以有效提升耗能产物的合成效率[205，210]。而关于毕赤酵母能量代谢体系的改造已有一定的成果和报道。例如，Chen等[211]将透明颤菌血红蛋白（VHb）在毕赤酵母中异源表达，可以有效增强胞内ATP的合成效率，使细胞生长和产物合成能力均大幅提升。本课题组Ren等[205]在毕赤酵母中表达拟南芥来源的ATP合成酶，也可将胞磷胆碱的产量提升22%。另外，有研究者对毕赤酵母内源腺苷酸激酶进行过表达，也可以使谷胱甘肽、*S*-腺苷甲硫氨酸、CALB的产量分别提升25.6%、33.8%和12.8%[210，212-213]。此外，辅因子的供应与调控以及维持细胞内氧化还原稳态也是影响产物合成的重要因素。一些外源蛋白或代谢途径的引入和改造往往会涉及辅因子（NADH、NADPH、$FADH_2$等）的消耗、生成和转化，进而引起细胞内氧化还原状态的波动，影响产物合成效率[13，214]。因此，通过辅因子工程技术调节细胞内的还原力平衡是提升生物合成效率的有效策略。Schroer等[215]通过增强毕赤酵母的甲醇异化代谢途径，实现了NADH的高效再生，有

效提高了丁二醇的合成效率。Fina等[216]通过过表达酿酒酵母来源的NADH激酶cPos5，增强了细胞内NADPH的供应，实现了毕赤酵母中3-羟基丙酸的高效合成。Cai等[173]则在毕赤酵母中引入酿酒酵母来源的异柠檬酸脱氢酶（Idp2），同样实现了NADPH的高效供应，从而增强了毕赤酵母中脂肪酸的合成。综上，ATP及辅因子的代谢调控是增强细胞生长和产物合成效率的有效策略，对于毕赤酵母底盘的开发和代谢工程优化具有重要意义。

4　总结与展望

毕赤酵母在蛋白表达和天然产物合成的应用中均展现了独特的优势。然而，与传统模式微生物（如大肠杆菌、酿酒酵母等）相比，毕赤酵母中的遗传操作仍然存在工具少、效率低、操作烦琐等问题，在遗传线路构建、底盘系统化改造和复杂途径组装等方面存在较大的挑战[5-6，63]。近年来，毕赤酵母中CRISPR/Cas9基因编辑技术的应用及NHEJ/HDR机制的工程化改造[29-31，54，60，63]，大大提升了遗传操作效率并丰富了基因工程工具箱，为毕赤酵母底盘的进一步改造升级奠定了基础。不过，目前关于毕赤酵母的大多数研究和应用主要还是基于已有的商业化菌株和质粒进行。因此，后续研究中有必要建立更多的通用型底盘菌株、工具质粒和基因操作流程，为毕赤酵母的应用提供更加多样化的选择。此外，整合位点的选择也是影响基因表达的关键因素。在基因组上启动子和终止子的间隔区选取整合位点，可以最大限度保证表达盒区域的完整性，是目前毕赤酵母中CRISPR介导基因整合的主要方法[29-30，60，65]。但是，目前对于整合位点的效果评估主要集中于基因整合效率和菌株生长情况，而对于细胞内源调控网络的影响和工程菌株的遗传稳定性的关注较少。接下来，综合已有成果开展进一步探索评估以筛选更加高效且具备遗传稳定性的整合位点，是毕赤酵母底盘研究的一项重要任务。而且，在此基础上，CRISPR系统介导的多位点共编辑的应用潜力也值得深入探索和发掘，为毕赤酵母中的遗传操作提供更加高效、便捷的解决方案。

毕赤酵母具有非常强力的天然转录调控体系，可以高效地启动转录。而且，近年来对于毕赤酵母天然转录系统的改造以及人工表达平台的设计，进一步增强了毕赤酵母底盘系统的转录能力，使毕赤酵母可以适配更加多样化的应用场景[25-26，28，110]。不过，强力的转录水平不一定能够带来更高的表达量[217]，而且，新生肽链的快速产生也容易导致未折叠蛋白反应（UPR）[154]，反而影响蛋白表达水平。因此，在具备更强转录能力的新型毕赤酵母底盘平台中，蛋白的折叠和分泌则成为主要的限制因素。信号肽改造和分子伴侣共表达是提高蛋白分泌水平的有效策略[6]，可以进一步研究优化以适配新型的强效转录调控系统。此外，糖基化蛋白的生产也逐渐成为毕赤酵母的重要发展方向[131]。目前针对毕赤酵母底盘的糖工程改造可以实现人源糖基化蛋白的表达，并已有商业化的表达菌株（GlycoSwitch®，BioGrammatics）[218]。尽管如此，在高均一性、高产量和高活性的重组糖蛋白生产需求方面，毕赤酵母底盘还面临较大的挑战，有待进一步的深入研究。

毕赤酵母在重组蛋白的生产中有广泛的应用和报道。相比之下，毕赤酵母在小分子化合物合成中的应用案例仍旧比较有限[5，13]。其中，底物转化效率是亟待优化的关键因素之一。尽管毕赤酵母能够利用廉价且来源广泛的甲醇作为碳源底物，但是部分甲醇会通过甲醛的异化代谢途径分解为CO_2，造成碳浪费并影响甲醇向产物的转化效率[6]。因此，如果能够对甲醇代谢途径进行重设计和理性改造，提高碳利用效率，则可以进一步提升毕赤酵母底盘系统的应用价值。此外，作为能够快速生成胞质乙酰辅酶A的前体碳源，乙醇在毕赤酵母中的应用也展现了出色的效果和潜力[33-34]。因此，开发以乙醇为底物的新型生物合成体系，也可以为毕赤酵母中的生物转化提供有效策略。

综上所述，近年来针对毕赤酵母底盘的工程化改造，在基因操作、转录调控、蛋白表达、代谢工程等方面均取得了出色的效果，使毕赤酵母成为蛋白生产和天然产物合成的优质底盘宿主之一。而面临学术研究和工业生产日益多样化的应用场景需求，毕赤酵母底盘系统仍有进一步的改造和升级空间，未来仍值得更为深入的探索和研究。

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通讯作者：蔡孟浩（1984—），男，博士，副教授，博士生导师。研究方向为合成生物学与发酵工程，通过遗传元件挖掘与工具开发、真核微生物底盘设计与改造、细胞代谢重构与发酵优化，实现药/食源功能分子的规模化发酵制备。

E-mail：cmh022199@ecust.edu.cn

第一作者：刘启（1991—），男，博士后。研究方向为酵母底盘的改造优化及合成遗传线路设计。

E-mail：liuqi0496@ecust.edu.cn