·综 述·

毕赤酵母细胞工厂工程化改造与应用

刘宇飞，曹颖，常立业，单聪慧，徐坤*

西北农林科技大学动物科技学院，陕西 杨凌 712100

刘宇飞, 曹颖, 常立业, 单聪慧, 徐坤. 毕赤酵母细胞工厂工程化改造与应用[J]. 生物工程学报, 2023, 39(11): 4376-4396.
LIU Yufei, CAO Ying, CHANG Liye, SHAN Conghui, XU Kun. Engineering and application of *Komagataella phaffii* as a cell factory[J]. Chinese Journal of Biotechnology, 2023, 39(11): 4376-4396.

摘 要：毕赤酵母作为细胞工厂在小分子代谢产物发酵和蛋白制品生物合成中扮演着重要角色，具有极其重要的工业应用价值。随着 CRISPR/Cas9 等新型编辑工具的开发和应用，对毕赤酵母细胞工厂进行多基因高效率的工程化改造已成为可能。本文首先对毕赤酵母工程化改造的遗传操作技术和目标方向进行了归纳总结，其次介绍了毕赤酵母作为细胞工厂的应用现状，同时探讨了毕赤酵母细胞工厂的优点及缺陷，并对其发展方向作出展望；以期为未来的毕赤酵母工程化改造研究提供参考和启示，推动毕赤酵母细胞工厂在生物产业中的创新应用。

关键词：毕赤酵母；细胞工厂；基因编辑；代谢工程；合成生物学

Engineering and application of *Komagataella phaffii* as a cell factory

LIU Yufei, CAO Ying, CHANG Liye, SHAN Conghui, XU Kun*

College of Animal Science and Technology, Northwest A&F University, Yangling 712100, Shaanxi, China

Abstract: Nowadays, engineered *Komagataella phaffii* plays an important role in the biosynthesis of small molecule metabolites and protein products, showing great potential and value in industrial productions. With the development and application of new editing tools such as CRISPR/Cas9, it has become possible to engineer *K. phaffii* into a cell factory with high polygenic efficiency. Here, the genetic manipulation techniques and objectives for engineering *K. phaffii* are first summarized. Secondly, the applications of engineered *K. phaffii* as a cell

资助项目：国家级大学生创新创业训练计划(202210712099)；陕西省大学生创新创业训练计划(S202310712277)；西北农林科技大学大学生创新创业训练计划(X202310712140)
This work was supported by the National College Students' Innovation and Entrepreneurship Training Program (202110712049), the Shaanxi Province College Students' Innovation and Entrepreneurship Training Program (S202210712476), and the Innovation and the Northwest A&F University Entrepreneurship Training Program for College Students (X202310712140).
*Corresponding author. E-mail: xukunas@nwafu.edu.cn
Received: 2023-02-10; Accepted: 2023-07-14; Published online: 2023-07-26

factory are introduced. Meanwhile, the advantages as well as disadvantages of using engineered *K. phaffii* as a cell factory are discussed and future engineering directions are prospected. This review aims to provide a reference for further engineering *K. phaffii* cell factory, which is supposed to facilitate its application in bioindustry.

Keywords: *Komagataella phaffii*; cell factory; gene editing; metabolic engineering; synthetic biology

毕赤酵母最早是由赫尔曼·法夫(Herman Phaff)于 1956 年从橡树上分离的一类甲醇营养型酵母，被命名为 *Pichia pastoris* 而成为一个种[1]。1995 年 *P. pastoris* 被重新归入 *Komagataella* 属，之后又被细分为 *Komagataella pastoris* 和 *Komagataella phaffii* 两个种[2-3]。目前，毕赤酵母模式菌株 CBS7435 及广泛应用的商业化底盘菌株 X33 和 GS115 均归属 *K. phaffii* 种，但大部分文献和数据库仍在使用旧名 *P. pastoris*，新名 *K. phaffii* 的使用尚有待学界进一步倡导和加强。

作为细胞工厂，毕赤酵母具有生长速度快、不易累积乙醇、适于大规模高密度发酵、可将甲醇作为唯一碳源和具有翻译后修饰能力等一系列优点。相对于其他真核表达系统，毕赤酵母在生产成本上存在显著优势。2006 年毕赤酵母和酿酒酵母(*Saccharomyces cerevisiae*)被美国食品及药物管理局(Food and Drug Administration, FDA)认定为公认安全(generally recognized as safe, GRAS)菌株，其安全性也得到了广泛认同[4]。

在众多酵母菌种中，毕赤酵母重组蛋白生产发酵工艺最为成熟，细胞干重可达 150 g/L，发酵规模可放大至 80 000 L[5-6]。据美国 Research Corporation Technologies 公司的统计(http://pichia.com/)，已有 5 000 余种蛋白成功在毕赤酵母中表达，1 000 多个研究机构在使用毕赤酵母表达系统，70 多个由毕赤酵母表达的商业化产品已经上市。毕赤酵母的生产性能已得到广泛认可[7]。

毕赤酵母作为底盘菌株也常用于生产各类代谢产物，包括但不限于乳酸、丙酮酸等[8-9]。在经过代谢工程改造后，还可生产其原本不易积累的乙醇以及聚酮类、萜类等高价值化合物[10-11]。毕赤酵母可利用甲醇、葡萄糖、甘油和羧甲基纤维素等多种碳源，且反应具有专一性，生产成本相比传统化学合成方法更加低廉[10]。

毕赤酵母遗传背景清晰，在 NCBI 中可以检索到 135 个菌株的全基因组测序数据。其中，模式菌株 CBS7435 及其商业化变体 GS115 基因组测序结果已经完成拼接并详细注释(GenBank 登录号：GCA_000223565.1、GCA_001746955.1)[12-13]，为毕赤酵母的代谢工程改造提供了详实可靠的参考。在此基础上，CRISPR/Cas9 等基因编辑工具的广泛应用进一步加快了毕赤酵母代谢工程改造的步伐[14]。不过，相比于大肠杆菌(*Escherichia coli*)和酿酒酵母等模式微生物，毕赤酵母各类调控元件仍有待进一步发掘[15]。

本文首先对毕赤酵母细胞工厂现有的改造技术和方向进行了综述，其次介绍了毕赤酵母细胞工厂的应用领域和优势，并探讨了毕赤酵母细胞工厂应用所面临的挑战和工程化改造的发展方向。

1 毕赤酵母细胞工厂工程化改造技术

1.1 基于线性化质粒载体的同源重组

在对毕赤酵母进行改造的过程中，最重要

的改造步骤是将外源基因的表达盒引入酵母。毕赤酵母因缺乏高效的质粒复制起点且质粒拷贝数低[16-17],在早期研究时往往通过线性化载体同源重组将表达盒稳定插入基因组,从而实现目标基因的插入或替换。目前,较为成熟的整合载体有 pPIC9K、pPIC3.5K 和 pAO815 等,可以实现单拷贝或多拷贝的基因整合[18-19]。该方法基于同源重组机制(homologous recombination, HR),因此线性载体转化效率、线性载体用量和同源重组效率都会影响阳性转化子数量。毕赤酵母的同源重组效率和线性载体转化效率均较低,因此需要选择更高效的转化方法和较大的线性载体数量(约 1−10 μg)。目前,电穿孔(electroporation)是所有转化方法中最为便捷且效率最高的方法(表 1)。电穿孔之前使用醋酸锂进行预处理可进一步提高转化效率[22-24]。受不同插入位点和同源臂长度影响,基因插入效率为<0.1%−80%不等,常在同源臂较短时观察到大量的假阳性转化子[24]。

1.2 基于 CRISPR/Cas9 的基因编辑

CRISPR/Cas9 是继归巢核酸酶、锌指核酸酶(zinc finger nucleases, ZFNs)、转录激活-效应器核酸酶(transcription activator-like effector nucleases, TALENs)后的新一代基因编辑技术,也是现有基因编辑技术中效率最高且操作最便捷的技术。CRISPR/Cas9 技术使基因编辑领域发生了革命性的变化。

CRISPR/Cas9 在酵母中的功能表达需要 2 个重要元件:Cas9 蛋白和 sgRNA[25]。目前最常见的 Cas9 蛋白是来源于酿脓链球菌(*Streptococcus pyogenes*)的 SpCas9,具有 2 个核酸酶结构域(HNH 和 RuvC)[26]。因其源于细菌,在进行酵母基因编辑时往往需要将核定位信号同 Cas9 蛋白融合表达,否则将无法进入核内发挥功能[27]。Cas9 需要 crRNA 和 tracrRNA 与之结合才能起到靶向切割的作用,在实际应用中,常将二者组合成一条 sgRNA[26]。Cas9 蛋白在与 sgRNA 结合后构象发生改变可以靶向识别原型间隔区相邻基序(protospacer adjacent motif, PAM)相邻的靶序列并产生 DNA 双链断裂(double-stranded break in DNA, DSB),激活同源重组或非同源末端连接(non-homologous end joining, NHEJ)两种 DNA 损伤修复途径从而实现基因编辑(图 1)。需要注意的是,被 CRISPR/Cas9 打靶后,毕赤酵母中 DSB 修复机制可能与酿酒酵母有所不同,具有较高的 NHEJ 修复效率[26]。目前,CRISPR/Cas9 系统已经成为一种极其有效的改造工具,已有多套毕赤酵母基因编辑系统可以实现单基因或多基因编辑[26-30]。

研究表明,密码子偏好性会影响 Cas9 在不同宿主细胞中的表达效果。比较人源优化 Cas9 (HsCas9)、毕赤酵母优化 Cas9 (PpCas9)和野生型 Cas9 (SpCas9)这 3 种 Cas9 在毕赤酵母中的编辑效率,发现使用 HsCas9 对 *GUT1* 基因的靶向效率高于 80%,而使用 PpCas9 的靶向效率低于 5%。野生型 SpCas9 在毕赤酵母中几乎不发挥作用[26]。目前,造成 HsCas9 和 PpCas9 效率

表 1 不同毕赤酵母转化方法比较

Table 1 Comparison of different methods for transforming *Komagataella phaffii*

Characteristics	PEG 1000	LiCl	Electroporation	Protoplast method
Transformation efficiency (colonies/μg DNA)	10^2–10^3	10^2–10^3	10^3–10^4	10^3–10^4
Enzyme treatment	None	None	None	Require
Special equipment	None	None	Require	None
Experimental complexity	Moderate	Moderate	Low	High
References	[20]	[21]	[22]	[20]

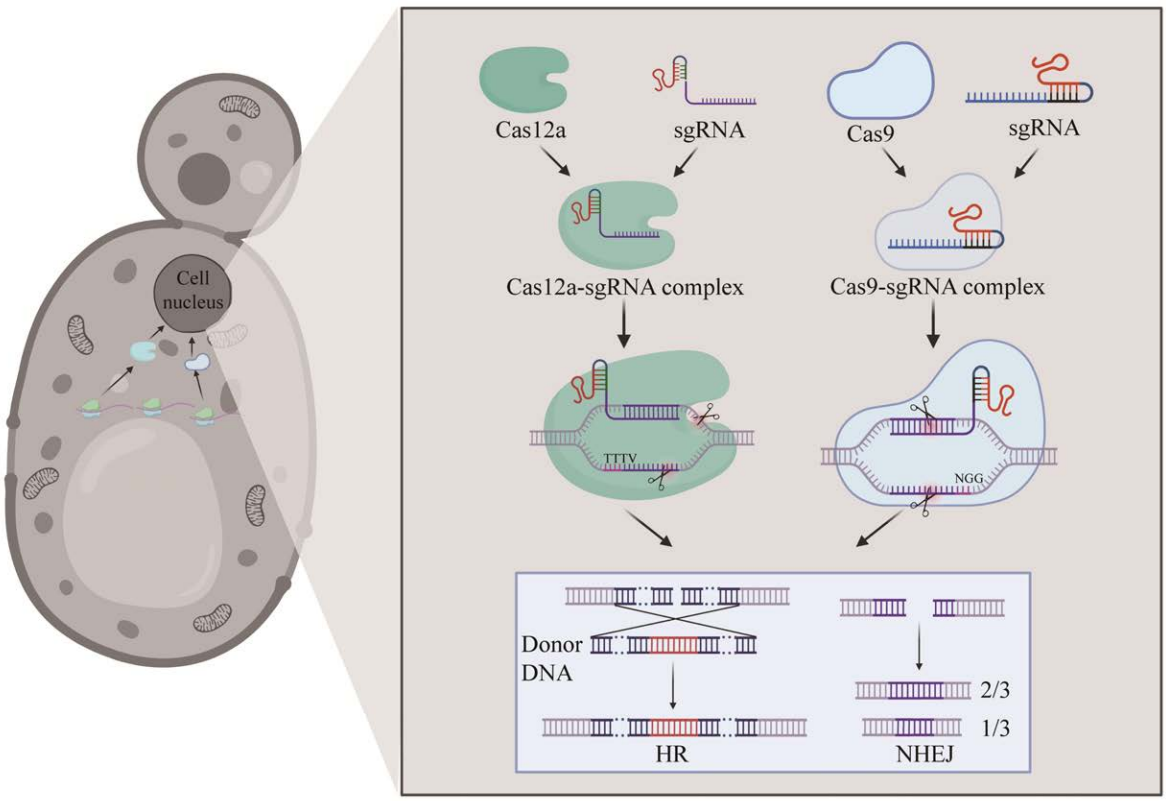

图 1　CRISPR/Cas9 与 CRISPR/Cas12a 介导的酵母基因组编辑　使用 BioRender (BioRender.com)绘制. 下同

Figure 1　Yeast genome editing mediated by CRISPR/Cas9 and CRISPR/Cas12a. Draw using BioRender (BioRender.com). The same below.

差异的原因尚不明确，推测其效率的差异可能来源于过度优化导致的蛋白折叠异常。

Cas9 的靶向识别功能依赖于 PAM 序列。SpCas9 的 PAM 序列 NGG 在每 8−16 bp 随机序列中仅存在一个，极大地限制了编辑位点的选择[31]。幸运的是，不同来源的 Cas9 蛋白可以识别不同的 PAM 序列，对 SpCas9 进行突变后得到的新变体亦可识别不同的 PAM 序列。目前，StCas9、NmCas9、GeoCas9、SaCas9、CjCas9 和 xCas9 等一系列可以识别不同 PAM 序列的 Cas9 蛋白已成功应用于基因编辑，有望在未来成为毕赤酵母基因编辑的有效工具[32-37]。

dCas9 是 Cas9 蛋白的突变体，突变位点为 D10A/H840A[38]。dCas9 结合 sgRNA 后仅有靶向识别活性，无核酸酶活性，因此无法引起 DSB。由此发展出了 CRISPR 抑制(CRISPR inhibition, CRISPRi)和 CRISPR 激活(CRISPR activation, CRISPRa)两种技术，目前已广泛地应用于基因的抑制与激活(图 2)。CRISPRi 可以通过两种方式实现对靶标基因的抑制。一是可以通过结合启动子来阻止 RNA 聚合酶的组装和延伸，效率较差。二是可以通过融合表达转录抑制结构域(transcription repression domain, TRD)增强抑制效果。CRISPRa 原理与 CRISPRi 相似，通过融合表达转录激活结构域(transcriptional activation domain, TAD)增强基因表达。目前，毕赤酵母的 CRISPRi 和 CRISPRa 主要基于对 P_{AOX1} 进行影响[39-41]。研究表明，dCas9 融合表

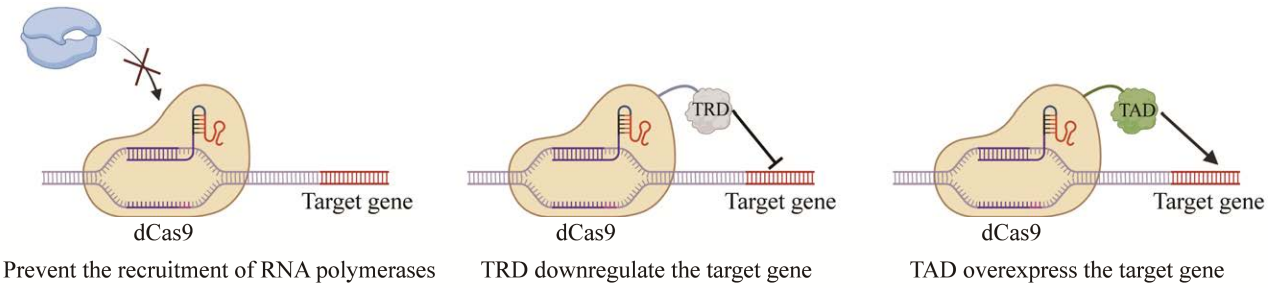

图 2　CRISPRi (CRISPRa)抑制(激活)靶基因表达

Figure 2　CRISPRi (CRISPRa) inhibits (activates) the expression of target genes.

达 Mxr1 和 Nrg1 可以对报告基因表达水平进行调控，Nrg1 组的报告基因表达量降低至对照组的 50%，Mxr1 组的报告基因表达量提升至对照组的 4−6 倍[40]。基于类似的原理，研究者开发出了高强度可编程的毕赤酵母平台 SynPic-X，实现了蛋白的高水平表达和表达量的精准调控，具有重要的应用价值[41]。

1.3　基于 CRISPR/Cas12a 的基因编辑

Cas12a (Cpf1)是一种V-A 型 Cas 蛋白，具有类似于 Cas9 的核酸酶活性。Cas12a 拥有类似于 Cas9 蛋白的 RuvC 结构域以及相对特殊的 NUC 结构域，产生 DSB 时形成的缺口具有 5′悬臂[42]。

目前，常见的 Cas12a 蛋白有 LbCas12a、AsCas12a 和 FnCas12a[43-45]。与 Cas9 靶向 CG 含量高的区域不同，Cas12a 已被证明可以有效地靶向 AT 含量高的区域。LbCas12a 和 AsCas12a 的 PAM 序列为 TTTN，FnCas12a 的 PAM 序列为 TTN。同时，Cas12a 往往可以对多种次优 PAM 序列起到识别作用，但切割活性会有所降低[46]。这些特点极大扩展了 CRISPR-Cas 系统的编辑位点。

CRISPR/Cas12a 已经成为了毕赤酵母改造的有效工具之一。在对相关元件进行优化后，可在毕赤酵母中实现基于 FnCas12a 的多基因快速导入和大片段 DNA 删除[47]。该系统的单基因编辑效率达 99%±0.8%，双基因编辑效率达 65%±2.5%至 80%±3%，三基因编辑效率达 30%±2.5%，可作为 CRISPR/Cas9 系统的有效补充。

综上，毕赤酵母改造技术近年来得到了长足的发展，在编辑效率和通量方面都实现了突破性的提升，为毕赤酵母的复杂多基因编辑改造提供了强有力的技术支撑。

2　毕赤酵母细胞工厂的应用

2.1　基于毕赤酵母的重组蛋白生产

重组蛋白是生物制品的核心产品之一。近年来，重组蛋白在疾病预防、疾病治疗、工业生产等领域中发挥的作用越来越大，推动了相关技术的快速发展。目前，常见的蛋白生产系统主要有 4 类，分别为大肠杆菌表达系统、酵母表达系统、昆虫细胞杆状病毒表达系统(baculovirus expression vector system, BEVS)和哺乳动物细胞表达系统(表 2)[48-52]。其中，酵母表达系统的典型代表是毕赤酵母系统。作为最简单的真核表达系统，相较于原核表达系统，毕赤酵母系统无内毒素且在生产蛋白时具有更加丰富的翻译后修饰机制[53]。相比于昆虫细胞和哺乳动物细胞表达系统，其表达速度更快，生产成本更低[54-55]。目前，研究者已在毕赤酵母中实现了多种抗原、工业酶等蛋白制品的高效生产(表 3，表 4)[56-87]。

表 2　不同重组蛋白生产系统比较

Table 2　Comparison of different systems for recombinant protein production

Characteristics	*E. coli*	*K. phaffii*	BEVS	CHO
Doubling time	20−30 min	60−120 min	18−24 h	24 h
Complexity of culture medium	Low	Low	High	High
Cost of culture medium	Low	Low	High	High
Secretory expression	Secreted into periplasmic space	Secreted into culture medium	Secreted into culture medium	Secreted into culture medium
Expression level	High	Low to high	Low to high	Low to moderate
N-linked glycosylation	×	Hypermannosylation	Simple	Complex
O-linked glycosylation	×	√	√	√
Phosphorylation	×	√	√	√
Acetylation	×	√	√	√
Endotoxin	√	×	×	×
References	[48]	[49]	[50]	[51]

CHO: Chinese hamster ovary, is the most common mammalian cell expression system.

表 3　由毕赤酵母生产的代表性重组蛋白疫苗

Table 3　Representative recombinant vaccines produced by *Komagataella phaffii*

Antigen	Pathogen	Secretory expression	Expression pattern	Yield (mg/L)	Immune mode	References
gp350	EBV	√	Methanol-induced expression	120.34	Injection	[56]
EBNA1	EBV	√	Methanol-induced expression	210.53	Injection	[57]
NA	HPAI (H5N1)	√	Methanol-induced expression	Not tested	Injection	[58]
VP1	EV71	√	Methanol-induced expression	~500	Injection	[59]
P1/3CD/3C	EV71/CVA16	×	Methanol-induced expression	Not tested	Injection	[60]
HBsAg	HBV	×	Methanol-induced expression	6 000−7 000	Injection	[61]
H5HA	HPAI	√	Methanol-induced expression	200	Intranasal instillation/ Injection	[62]

分泌表达重组蛋白可简化后续分离纯化工艺。毕赤酵母因其具有编码 Kex2p、Ste13p 等的蛋白酶的基因，可对 α 信号肽进行加工以实现分泌表达且分泌效果优于其他常见酵母[88]。多种哺乳动物的分泌信号在毕赤酵母中可以正常发挥功能，进一步丰富了分泌表达的选择[89-90]。细胞内源分泌的蛋白往往会影响目的蛋白的分离纯化。毕赤酵母内源分泌蛋白数量较少，大大降低了分泌型重组蛋白的纯化难度和成本[91]。

膜蛋白承担着重要的生物学功能，因此往往需要大量表达以用于研究。膜蛋白通常需要进行特定的共翻译和翻译后修饰才能具有正确的结构和功能。目前，毕赤酵母已成功表达 NRT1.1、PiPT 和 P 糖蛋白等数十种膜蛋白[92-95]。因其生产成本低、表达量高且具有多种翻译后修饰途径，有望在膜蛋白研究领域替代 CHO 系统和 BEVS 系统[93]。

研究者已利用毕赤酵母表达系统成功实现了多种重组蛋白的生产，但仍存在诸多问题。其首要问题是毕赤酵母的 N-连接糖基化(N-linked glycosylation)存在高甘露糖化且 O-连接糖基化(O-linked glycosylation)较为简单，这

表 4 由毕赤酵母生产的重要工业酶

Table 4 Important industrial enzymes produced by *Komagataella phaffii*

EC No.	Name	Promoter	Expression pattern	Yield (U/L)	Codon optimization	References
EC 1.1.3.4	Glucose oxidase	P_{AOX1}	Methanol-induced	98 400	×	[63]
EC 1.1.3.5	Hexose oxidase	P_{AOX1}	Methanol-induced	42	×	[64]
EC 1.1.99.18	Cellobiose dehydrogenase	P_{AOX1}	Methanol-induced	7 800	×	[65]
EC 1.10.3.2	Laccase	P_{AOX1}	Methanol-induced	51 000	×	[66]
EC 1.11.1.6	Catalase	P_{AOX1}	Methanol-induced	1 380 000	×	[67]
EC 1.11.1.7	Peroxidase	P_{AOX1}	Methanol-induced	31 000	×	[68]
EC 2.3.2.13	Protein-glutamine glutamyltransferase	P_{AOX1}	Methanol-induced	3.91	×	[69]
EC 3.1.1.1	Carboxylesteras	P_{AOX1}	Methanol-induced	Not tested	×	[70]
		P_{GAP}	Constitutive	1 747.5 329 (secretion)		
EC 3.1.1.3	Triacylglycerol lipase	P_{AOX1}	Methanol-induced	20 000	×	[71]
EC 3.1.1.20	Tannase	P_{AOX1}	Methanol-induced	7 000	×	[72]
EC 3.1.1.32	Phospholipase A1	P_{AOX1}	Methanol-induced	Not tested	√	[73]
EC 3.1.1.73	Feruloyl esterase	P_{AOX1}	Methanol-induced	830	×	[74]
EC 3.1.3.8	Phytase	P_{AOX1}	Methanol-induced	865 000	√	[75]
EC 3.1.4.1	Phosphodiesterase	P_{AOX1}	Methanol-induced	369.81	×	[76]
EC 3.2.1.1	Amylase	P_{GAP}	Constitutive	68 250	×	[77]
EC 3.2.1.4	Cellulase	P_{AOX1}	Methanol-induced	Not tested	√	[78]
EC 3.2.1.6	Endo glucanase	P_{AOX1}	Methanol-induced	10 017 000	√	[79]
EC 3.2.1.20	α-glucosidases	P_{AOX1}	Methanol-induced	Not tested 1 747.5	×	[80]
EC 3.4.21.4	Trypsin	P_{AOX1}	Methanol-induced	689 470	×	[81]
EC 3.4.21.26	Prolyloligopeptidase	P_{AOX1}	Methanol-induced	500	×	[82]
EC 3.4.21.62	Subtilisin	P_{AOX1}	Methanol-induced	30 000	×	[83]
EC 3.4.21.63	Oryzin	P_{AOX1}	Methanol-induced	Not tested	×	[84]
EC 3.5.1.1	Asparaginase	P_{AOX1}	Methanol-induced	85 600	×	[85]
EC 4.2.2.2	Pectate lyase	P_{AOX1}	Methanol-induced	166 740	√	[86]
EC 4.2.2.10	Pectin lyase	P_{AOX1}	Methanol-induced	Not tested	×	[87]

可能改变重组蛋白的构象并影响其生物活性[96-97]。有研究表明，毕赤酵母表达的抗凝血酶III的活性受高甘露糖化影响被抑制 50%[98]。其次，毕赤酵母液泡承担着类似于溶酶体的功能，含有大量不同种类的蛋白酶，易导致蛋白降解从而降低蛋白产量[49]。另外，在使用甲醇诱导时，毕赤酵母对甲醇的浓度和甘油、葡萄糖等碳源杂质较为敏感，需要进行诱导条件的优化[99]。毕赤酵母还存在转化效率低、可用筛选标记较少等和其他真核系统相似的问题[100]。

2.2 基于毕赤酵母的代谢产物生产

相较大肠杆菌和酿酒酵母等模式生物，研究者对毕赤酵母的代谢途径的了解较为粗浅。毕赤酵母作为甲醇营养型酵母，能够氧化甲醇并以其作为合成其他产物的唯一碳源。此外，葡萄糖和甘油亦可用作发酵碳源。作为典型的

Crabtree-negative 酵母，毕赤酵母摄取葡萄糖的速率较慢(约为酿酒酵母的 1/8−1/9)[101]。在完全好氧的条件下，毕赤酵母不会产生和积累乙醇。加之毕赤酵母 pH 值适应范围广(约 3.0−7.0)，较为适合大规模高密度发酵生产各类代谢产物[102]。

2009 年毕赤酵母完成全基因组测序，随后研究者对多个标准株进行了详细的注释，开启了毕赤酵母基因组学和合成生物学研究的大门。毕赤酵母进行代谢工程改造或发酵条件优化后，可实现多种代谢产物的生产，包括但不限于乳酸、丙酮酸、乙醇、聚酮类和萜类等(表 5)[103-114]。目前，受限于落后的改造手段，大部分针对毕赤酵母的代谢工程改造较为简单。

综上，毕赤酵母细胞工厂已经成为一种高效的生物合成平台并广泛应用于各个领域。随着合成生物学研究的深入，毕赤酵母细胞工厂应用范围有望进一步拓展，一些现有的不足也有望通过定向改造得以克服。

3 毕赤酵母细胞工厂工程化改造方向

为了克服毕赤酵母作为细胞工厂的诸多缺陷，研究者对其进行了大量的工程化遗传改造，以期得到性能优良的毕赤酵母底盘菌株(图 3)。

3.1 提升同源重组效率

在 DNA 双链断裂修复机制上，毕赤酵母和酿酒酵母存在一定差异，主要为 NHEJ 而非 HR。NHEJ 修复速度快但随机性强，HR 修复过程较为复杂但更加精确(图 4)。NHEJ 修复占优势时会影响 HR 修复并加大筛选工作量[115]。如需进行精确高效的基因编辑工作，有必要将毕赤酵母改造为高 HR 效率的底盘菌株。目前，研究者已经探索出多种改造方案，例如敲除 NHEJ 关键基因 *Ku70*、过表达促进 HR 的 *Rad52* 等基因、敲除 HR 抑制基因 *Mph1* 等[115-117]。

表 5 由毕赤酵母生产的代谢产物

Table 5 Metabolites produced by *Komagataella phaffii*

Product	Classification	Knock in/Upregulated	Chassis strain	Yield (mg/L)	References
Pyruvic acid	Organic acid	(S)-2-hydroxyacid oxidase	GS115	57 000	[103]
Glyoxylate	Organic acid	catT	MSP10	Not tested	[104]
Nitrile hydratase activity	Amide	NHα, NHβ, P14K	GS115	Not tested	[105]
Riboflavin	Vitamin	RIB1-7	X33, GS115	175	[106]
Long-chain fatty acids	Fatty acid	PhtELO5, IsFAD4	GS115	Not tested	[107]
Citrinin	Polyketide	PksCT, npgA, mpl1-7	GS115	2 200	[108]
Bioethanol	Alcohol	BGL, EG1, CHB1, EX1, BXYL	GS115	32 600	[109]
Dammarenediol-II	Terpenoid	DDS, ERG1	GS115	500	[110]
Δ9-tetrahydrocannabinolic acid	Cannabinoid precursor	THCAS	PichiaPink	Not tested	[111]
1′-hydroxybufuralol	Beta blocker	CYP2D6, CPR	X-33, CBS7435	171.1	[112]
Catharanthine	Vinblastine precursor	33 genes, including SGD, GS, GO, etc.	CAN1	2.57	[113]
Chondroitin sulfate A	Polysaccharide	*tua*D, *kfo*C, *kfo*A	GS115	2 600	[114]

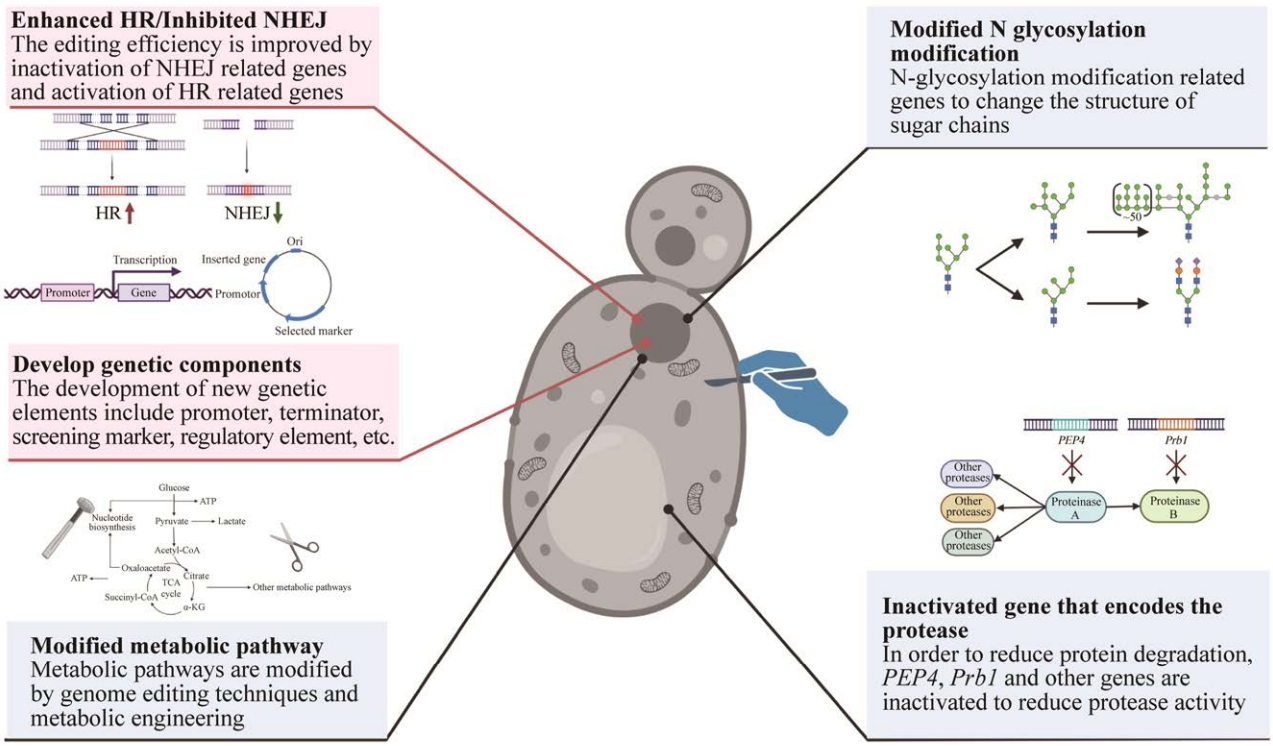

图 3　毕赤酵母细胞工厂工程化遗传改造方向

Figure 3　Proposed direction for genetic modification of *Komagataella phaffii* cell factory.

研究表明，使用 *Ku70* 缺陷菌株在同源臂长仅 250 bp 的条件下可对 *His4* 和 *ADE1* 位点实现 90%以上的编辑效率[115]。与之伴随的是，敲除 *Ku70* 基因后菌株抗紫外线的能力低于野生型菌株。在使用 *Ku70* 缺陷菌株敲除 *GUT1* 基因时未检测到 NHEJ 且相较野生型菌株转化率大大降低，因此认为敲除 *Ku70* 基因可以完全失活 NHEJ 相关途径，进一步解释了失活 *Ku70* 基因提升编辑效率的原理[27]。目前，使用 *Ku70* 缺陷株进行毕赤酵母基因编辑已经成为了毕赤酵母改造的常用方法[116,118-120]。

HR 相关基因的表达水平与同源重组效率息息相关，过表达 *Rad52* 等 HR 相关基因可显著增强重组的稳定性。在对多种 HR 相关基因进行研究时发现，高表达 *Rad52* 可将单基因编辑效率提升至 90%，敲除 *Mph1* 基因可使多片段重组效率提升 13.5 倍[116]。在 Cas9 蛋白 C 端融合表达 *Mre11* 且过表达 *Rad52* 的情况下，双基因和三基因编辑效率均得到显著提升，达到了 86.7%和 16.7%[121]。基于上述研究结果，研究者通过过表达 *RAD52*、*RAD59*、*MRE11* 和 *SAE2* 基因进一步提高 HR 效率，使用含 40 bp 短同源臂的供体即可实现 100%的单基因编辑效率和 81%的三基因编辑效率，得到了可进行高效改造的底盘菌株[117]。

3.2　挖掘和开发遗传元件

毕赤酵母作为非模式酵母，其遗传元件(genetic element)的挖掘和开发相对滞后。与大肠杆菌、酿酒酵母等模式生物相比缺乏多样的启动子和筛选标记[122-123]。虽然已经开发出诸如 GoldenPiCS 等毕赤酵母表达和改造工具包，极大地方便了对毕赤酵母的改造[124-125]，但可用启

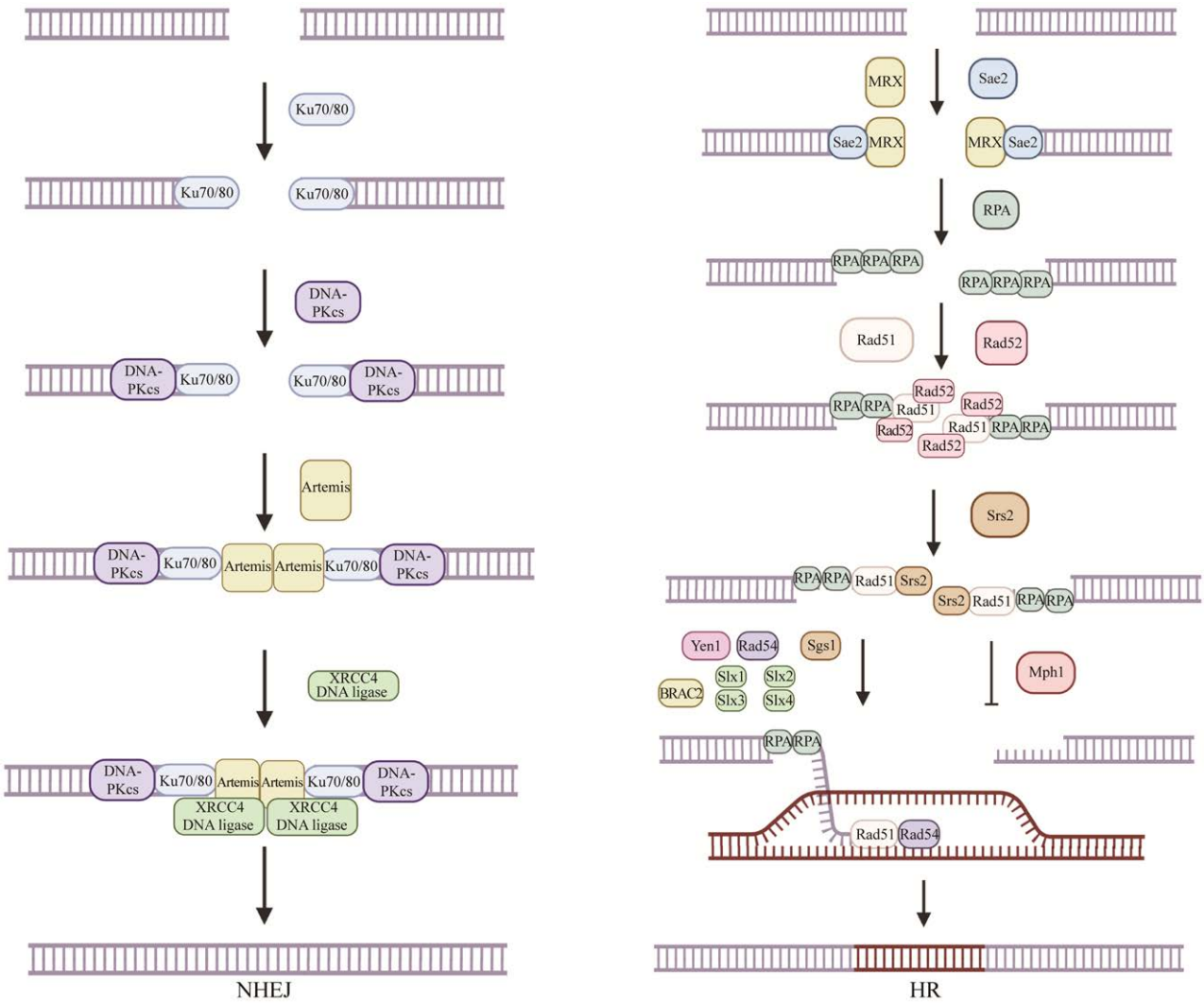

图 4　DNA 双链断裂修复机制

Figure 4　The mechanisms for repairing DNA double-stranded break.

动子仍局限于 P_{GAP}、P_{ADH2}、P_{AOX1} 和 P_{TEF1} 等约 20 种启动子，筛选标记仅局限于 *ADE2*、*HIS4*、*Trp2* 等为数不多的营养缺陷型基因和 *BleoR*、*KanMX*、*hphMX* 和 *nrsR* 等抗性基因，诱导方式也只有甲醇诱导、甘油诱导和葡萄糖诱导[122,124-125]。这些遗传元件在进行复杂的多基因编辑改造代谢途径时显得捉襟见肘，需进一步发掘和开发新的遗传元件和工具包。

3.3　改造蛋白糖基化修饰

糖蛋白的糖基分为 N-连接糖基和 O-连接糖基两种类型。N-连接糖基连接在 Asn-X-Thr/Ser 保守序列中的天冬酰胺(Asn)上(其中的 X 为除脯氨酸以外的任意氨基酸残基)[126]。O-连接糖基的结构比 N-连接糖基更加简单且连接位点更多，常出现在丝氨酸(Ser)、苏氨酸(Thr)上[126]。抗体、酶等大部分有生物活性的重组蛋白产品需要进行适当的糖基化修饰。研究表明，糖基化(特别是 N-连接糖基化)对于蛋白的构象、半衰期和免疫原性等多个方面具有重要影响[127]。毕赤酵母作为最简单的真核表达系统，能够进

行高甘露糖化修饰，但会影响蛋白结构并提高其免疫原性，可能降低毕赤酵母生产的重组蛋白的活性和半衰期[128]。因此，对毕赤酵母糖基化修饰进行改造(特别是人源化改造)对提高毕赤酵母细胞工厂生产能力具有重大意义。

针对毕赤酵母的糖基化修饰改造最早通过失活 *Och1* 基因并导入 UDP-GlcNAc 转运体和 α-1,2-甘露糖苷酶基因得到了具有 GlcNAc-Man_5-$GlcNAc_2$ 糖基化修饰的菌株[129]。在此基础上，进一步导入 N-乙酰氨基葡萄糖转移酶 I 和 β-1,4-半乳糖转移酶等基因可更加稳定地解决高甘露糖化问题[130]。失活 *Och1* 和 *Alg3* 基因并导入 *Mns I*、*GnTI*、*GnTII*和 *GalTI* 基因可以将毕赤酵母糖基化修饰改造为 $GlcNAc_2$-Man_3-$GlcNAc_2$，这向糖基人源化迈出了重要的一步。Hamilton 等通过复杂的改造，实现了毕赤酵母糖基的人源化并使用毕赤酵母生产了具有人源化糖基修饰的免疫球蛋白 G (IgG)[131-132]。

3.4 降低蛋白酶活性

毕赤酵母在生产蛋白的过程中会因受到胁迫产生蛋白酶，导致目的蛋白产率下降、分离纯化困难和蛋白活性降低[133]。在毕赤酵母中，液泡是外源蛋白降解最主要的场所，约 80%的蛋白质会在酵母液泡中被降解，还有一部分在细胞基质被降解[134-135]。目前，工业生产中常用的解决办法有 3 种[136]：(1) 在培养基中添加过量的蛋白酶水解底物；(2) 添加对应的蛋白酶抑制剂；(3) 优化发酵条件，特别是优化温度和 pH 值。但是这些方法都不能从根本上解决蛋白酶水解的问题，而且还会给下游分离纯化增加困难。根本解决蛋白降解问题需要对毕赤酵母进行基因工程改造。

目前可以确定 *PEP4* 和 *Prb1* 两个基因在水解外源蛋白中发挥重要作用。*PEP4* 基因编码蛋白酶 A (proteinase A)前体。蛋白酶 A 前体在内质网进行糖基化并在高尔基体中进行切割后转运至液泡[137]。蛋白酶 A 可以催化蛋白质水解并与蛋白酶 B、羧肽酶 Y、氨肽酶I等多种酶的激活相关，因此，*PEP4* 基因是液泡蛋白酶系统的关键基因，敲除 *PEP4* 基因可解决多数蛋白降解问题。*Prb1* 编码蛋白酶 B，在被蛋白酶 A 激活后具有蛋白酶活性[99]。蛋白酶 B 的酶原在一定的条件下也具有蛋白酶活性，因此在解决蛋白降解问题的过程中敲除 *Prb1* 基因具有较大意义。目前已有多种成功的商业化蛋白酶缺陷菌株，例如 SMD1163 (*PEP4-Prb1-His4-*)、SMD1168 (*PEP4-His4-*)、PichiaPink 2 (*ADE2-PEP4-*)、PichiaPink3 (*ADE2-Prb1-*)和 PichiaPink4 (*ADE2-Prb1-PEP4-*)等。此外，对 *YPS1-3*、*YPS7* 和 *MKC7* 等编码蛋白酶的基因进行改造也具有减少蛋白降解的效果[138]。

3.5 改造代谢通路

生产小分子代谢产物是毕赤酵母作为细胞工厂的重要职责之一。随着作为代谢工厂优势的逐步显现和多基因一步编辑技术的发展，通过合成生物学和代谢工程的方法挖掘毕赤酵母生产潜力已成为热点领域。目前，对毕赤酵母代谢通路的改造有两大思路：引入异源酶优化天然代谢产物的合成和整体敲入多个异源基因导入新的代谢通路。

对 S-腺苷-L-甲硫氨酸(S-adenosyl-L-methionine, SAM)合成途径的代谢工程改造是引入异源酶优化天然代谢产物的经典案例。该研究通过过表达 SAM2 基因使 SAM 产量提高 45 倍[139]。与之类似，通过表达异源 D-阿拉伯糖醇和木糖醇脱氢酶能够使木糖醇的产量提升至 0.29 g/(L·h)[140]。研究者通过 pGAPZB 质粒导入 3 个外源基因，使自身无法生产番茄红素的 X33 菌株成功表达了番茄红素，产量可达 73.9 mg/L[126]。但该种方法存在着生产性能不稳定和质粒可能丢失的

缺陷。随着多基因一步编辑技术的发展和众多中性位点被鉴定，多个异源基因稳定整合建立新的代谢通路已经成为可能[126]。使用多基因一步编辑技术可以实现番茄红素关键基因的一步快速导入，使酵母菌株可更加稳定地生产番茄红素[127]。随着多基因一步编辑技术的突破，研究者通过导入数十个外源基因实现了在毕赤酵母中长春质碱的高效合成，相比于同类型的酿酒酵母细胞工厂生产能力提高了 25 倍，开启了毕赤酵母代谢通路改造的新纪元[113,141]。

综上所述，在对毕赤酵母细胞工厂进行定向改造后可以解决其存在的诸多缺陷并赋予其新的生产功能，从而提高生产效率并拓展应用领域。

4 总结与展望

经过近 50 年的推广应用，毕赤酵母的生物合成能力、发酵生产成本和生物安全性已经得到了业界广泛认可，但作为新时代合成生物学细胞工厂，其工程化改造和利用仍有待进一步挖掘和探索。

首先，相比于酿酒酵母，毕赤酵母缺乏高效率的编辑手段和充足的合成生物学元件。在基因编辑手段方面，毕赤酵母的相关研究远远落后于酿酒酵母。目前，在酿酒酵母中已成功应用的 StCas9、XCas9、Cas13a、Base editing 和 Prime editing 等新型编辑工具以及供体适配等新型基因编辑策略还未拓展至毕赤酵母，有待进一步研究[31,142-146]。与此同时，毕赤酵母改造中可使用的遗传元件较少，仍需进一步发掘。相比于酿酒酵母，毕赤酵母同源重组效率低也是限制其改造和发展的重要因素。目前已有的解决方案在提升同源重组效率的同时仍存在菌株致死率高和适应性差等问题。

其次，在进行工业生产的过程中，毕赤酵母的抗逆性会在很大程度上影响成本和产量。毕赤酵母具有较强的 pH 适应能力，但耐热性等抗逆性状与同属甲醇营养型酵母的多形汉逊酵母(*Ogataea polymorpha*)仍有较大差距[147]。因此，对毕赤酵母的抗逆性相关基因进行改造可能有助于进一步降低其发酵生产成本。

再次，毕赤酵母作为口服递送平台的研究仍然较少。现如今，对口服药物或口服疫苗进行递送时主要选择人造纳米颗粒、大肠杆菌和酿酒酵母等成熟的递送平台。特别是酿酒酵母，因其较强的安全性、成熟的表面展示机制以及在细胞壁中具有促进免疫的 β-葡聚糖，经简单改造即可对 DNA 疫苗或其他功能性分子进行口服递送[148-149]。毕赤酵母与酿酒酵母同属 GRAS 菌株，在具备上述优点的同时还具有更好的耐酸碱能力和蛋白表达能力，可以更好地在胃肠液中生存并发挥功能[150-151]。因此，毕赤酵母有望成为存在于人类或动物胃肠道中的细胞工厂。

另外，毕赤酵母作为一种甲醇营养型酵母存在甲醇利用率低和对甲醇浓度敏感的问题[152]。在生产部分低附加值的化学产品时，甲醇利用率将会极大地影响生产的经济性。因此，有必要对毕赤酵母的甲醇利用相关途径进行进一步的代谢工程优化以取得更加优良的底盘菌株。

总之，毕赤酵母在合成生物学领域具有独特的优势，应用前景广阔但仍具挑战性。相信随着高通量基因编辑平台的开发和代谢工程研究的深入，毕赤酵母细胞工厂将成为合成生物学领域不可或缺的角色。

REFERENCES

[1] PHAFF HJ, MILLER MW, SHIFRINE M. The taxonomy of yeasts isolated from *Drosophila* in the

Yosemite region of California[J]. Antonie Van Leeuwenhoek, 1956, 22(1): 145-161.

[2] YAMADA Y, MATSUDA M, MAEDA K, MIKATA K. The phylogenetic relationships of methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: the proposal of *Komagataella* gen. nov. (Saccharomycetaceae)[J]. Bioscience, Biotechnology, and Biochemistry, 1995, 59(3): 439-444.

[3] NAUMOV GI, NAUMOVA ES, TYURIN OV, KOZLOV DG. *Komagataella kurtzmanii* sp. nov., a new sibling species of *Komagataella* (*Pichia*) *pastoris* based on multigene sequence analysis[J]. Antonie Van Leeuwenhoek, 2013, 104(3): 339-347.

[4] ZHANG QQ, WANG XL, LUO HY, WANG YR, WANG Y, TU T, QIN X, SU XY, HUANG HQ, YAO B, BAI YG, ZHANG J. Metabolic engineering of *Pichia pastoris* for myo-inositol production by dynamic regulation of central metabolism[J]. Microbial Cell Factories, 2022, 21(1): 112.

[5] SONG KM, OKUYAMA M, KOBAYASHI K, MORI H, KIMURA A. Characterization of a glycoside hydrolase family 31 α-glucosidase involved in starch utilization in *Podospora anserina*[J]. Bioscience, Biotechnology, and Biochemistry, 2013, 77(10): 2117-2124.

[6] MATTANOVICH D, BRANDUARDI P, DATO L, GASSER B, SAUER M, PORRO D. Recombinant protein production in yeasts[J]. Methods in Molecular Biology (Clifton, NJ), 2012, 824: 329-358.

[7] 朱泰承, 李寅. 毕赤酵母表达系统发展概况及趋势[J]. 生物工程学报, 2015, 31(6): 929-938.
ZHU TC, LI Y. Recent development of Pichia pastoris system: current status and future perspective[J]. Chinese Journal of Biotechnology, 2015, 31(6): 929-938 (in Chinese).

[8] YAMADA R, OGURA K, KIMOTO Y, OGINO H. Toward the construction of a technology platform for chemicals production from methanol: D-lactic acid production from methanol by an engineered yeast *Pichia pastoris*[J]. World Journal of Microbiology & Biotechnology, 2019, 35(2): 37.

[9] GOUGH S, DOSTAL L, HOWE A, DESHPANDE M, SCHER M, ROSAZZA JNP. Production of pyruvate from lactate using recombinant *Pichia pastoris* cells as catalyst[J]. Process Biochemistry, 2005, 40(8): 2597-2601.

[10] DONG C, QIAO J, WANG XP, SUN WL, CHEN LX, LI ST, WU K, MA LX, LIU Y. Engineering *Pichia pastoris* with surface-display minicellulosomes for carboxymethyl cellulose hydrolysis and ethanol production[J]. Biotechnology for Biofuels, 2020, 13: 108.

[11] ZHU QY, LIU Q, YAO CY, ZHANG YX, CAI MH. Yeast transcriptional device libraries enable precise synthesis of value-added chemicals from methanol[J]. Nucleic Acids Research, 2022, 50(17): 10187-10199.

[12] KÜBERL A, SCHNEIDER J, THALLINGER GG, ANDERL I, WIBBERG D, HAJEK T, JAENICKE S, BRINKROLF K, GOESMANN A, SZCZEPANOWSKI R, PÜHLER A, SCHWAB H, GLIEDER A, PICHLER H. High-quality genome sequence of *Pichia pastoris* CBS7435[J]. Journal of Biotechnology, 2011, 154(4): 312-320.

[13] STURMBERGER L, CHAPPELL T, GEIER M, KRAINER F, DAY KJ, VIDE U, TRSTENJAK S, SCHIEFER A, RICHARDSON T, SORIAGA L, DARNHOFER B, BIRNER-GRUENBERGER R, GLICK BS, TOLSTORUKOV I, CREGG J, MADDEN K, GLIEDER A. Refined *Pichia pastoris* reference genome sequence[J]. Journal of Biotechnology, 2016, 235: 121-131.

[14] FISCHER JE, GLIEDER A. Current advances in engineering tools for *Pichia pastoris*[J]. Current Opinion in Biotechnology, 2019, 59: 175-181.

[15] DOU WW, ZHU QC, ZHANG MH, JIA ZY, GUAN WJ. Screening and evaluation of the strong endogenous promoters in *Pichia pastoris*[J]. Microbial Cell Factories, 2021, 20(1): 156.

[16] ABAD S, KITZ K, HÖRMANN A, SCHREINER U, HARTNER FS, GLIEDER A. Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*[J]. Biotechnology Journal, 2010, 5(4): 413-420.

[17] CAMATTARI A, GOH A, YIP LY, TAN AHM, NG SW, TRAN A, LIU GW, LIACHKO I, DUNHAM MJ, RANCATI G. Characterization of a panARS-based episomal vector in the methylotrophic yeast *Pichia pastoris* for recombinant protein production and synthetic biology applications[J]. Microbial Cell Factories, 2016, 15(1): 139.

[18] ZHANG YH, ZHU ZM, ZHAI WB, BI YH, YIN Y, ZHANG WZ. Expression and purification of asprosin in *Pichia pastoris* and investigation of its increase glucose uptake activity in skeletal muscle through activation of AMPK[J]. Enzyme and Microbial Technology, 2021, 144: 109737.

[19] SHAO YR, XUE CL, LIU WQ, ZUO SQ, WEI PL, HUANG L, LIAN JZ, XU ZN. High-level secretory production of leghemoglobin in *Pichia pastoris* through enhanced globin expression and heme biosynthesis[J]. Bioresource Technology, 2022, 363: 127884.

[20] CREGG JM, BARRINGER KJ, HESSLER AY, MADDEN KR. *Pichia pastoris* as a host system for transformations[J]. Molecular and Cellular Biology, 1985, 5(12): 3376-3385.

[21] KUMAR S, MANNIL A, MUTTURI S. Modified chemical method for efficient transformation and diagnosis in *Pichia pastoris*[J]. Protein Expression and Purification, 2020, 174: 105685.

[22] YANG X, WANG YM, ZHAO P, YU XY. Study on strategy improvement of yeast transformation experiment based on ANP method[J]. Applied Mechanics and Materials, 2012, 217/218/219: 931-936.

[23] WU SX, LETCHWORTH GJ. High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol[J]. BioTechniques, 2004, 36(1): 152-154.

[24] MADDEN K, TOLSTORUKOV I, CREGG J. Electroporation of *Pichia pastoris*[M]//Fungal Biology. Cham: Springer International Publishing, 2014: 87-91.

[25] JINEK M, CHYLINSKI K, FONFARA I, HAUER M, DOUDNA JA, CHARPENTIER E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity[J]. Science, 2012, 337(6096): 816-821.

[26] WENINGER A, HATZL AM, SCHMID C, VOGL T, GLIEDER A. Combinatorial optimization of CRISPR/Cas9 expression enables precision genome engineering in the methylotrophic yeast *Pichia pastoris*[J]. Journal of Biotechnology, 2016, 235: 139-149.

[27] WENINGER A, FISCHER JE, RASCHMANOVÁ H, KNIELY C, VOGL T, GLIEDER A. Expanding the CRISPR/Cas9 toolkit for *Pichia pastoris* with efficient donor integration and alternative resistance markers[J]. Journal of Cellular Biochemistry, 2018, 119(4): 3183-3198.

[28] HOU CL, YANG YK, XING Y, ZHAN CJ, LIU GQ, LIU XX, LIU CL, ZHAN JL, XU DH, BAI ZH. Targeted editing of transcriptional activator MXR1 on the *Pichia pastoris* genome using CRISPR/Cas9 technology[J]. Yeast (Chichester, England), 2020, 37(4): 305-312.

[29] GU Y, GAO JC, CAO MF, DONG C, LIAN JZ, HUANG L, CAI J, XU ZN. Construction of a series of episomal plasmids and their application in the development of an efficient CRISPR/Cas9 system in *Pichia pastoris*[J]. World Journal of Microbiology & Biotechnology, 2019, 35(6): 79.

[30] LIU Q, SHI XN, SONG LL, LIU HF, ZHOU XS, WANG QY, ZHANG YX, CAI MH. CRISPR-Cas9-mediated genomic multiloci integration in *Pichia pastoris*[J]. Microbial Cell Factories, 2019, 18(1): 144.

[31] GAUDELLI NM, KOMOR AC, REES HA, PACKER MS, BADRAN AH, BRYSON DI, LIU DR. Publisher correction: programmable base editing of A•T to G•C in genomic DNA without DNA cleavage[J]. Nature, 2018, 559(7714): E8.

[32] SUN YS, YAN NN, MU L, SUN B, DENG JR, FANG YY, SHAO SM, YAN Q, HAN FR, ZHANG ZY, XU K. sgRNA-shRNA structure mediated SNP site editing on porcine IGF_2 gene by CRISPR/StCas9[J]. Frontiers in Genetics, 2019, 10: 347.

[33] XU K, REN CH, LIU ZT, ZHANG T, ZHANG TT, LI D, WANG L, YAN Q, GUO LJ, SHEN JC, ZHANG ZY. Efficient genome engineering in eukaryotes using Cas9 from *Streptococcus thermophilus*[J]. Cellular and Molecular Life Sciences, 2015, 72(2): 383-399.

[34] HSU PD, SCOTT DA, WEINSTEIN JA, RAN FA, KONERMANN S, AGARWALA V, LI YQ, FINE EJ, WU XB, SHALEM O, CRADICK TJ, MARRAFFINI LA, BAO G, ZHANG F. DNA targeting specificity of RNA-guided Cas9 nucleases[J]. Nature Biotechnology, 2013, 31(9): 827-832.

[35] HOU ZG, ZHANG Y, PROPSON NE, HOWDEN SE, CHU LF, SONTHEIMER EJ, THOMSON JA. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*[J]. Proceedings of the National Academy of Sciences of the United States of America, 2013, 110(39): 15644-15649.

[36] HARRINGTON LB, PAEZ-ESPINO D, STAAHL BT, CHEN JS, MA EB, KYRPIDES NC, DOUDNA JA. A thermostable Cas9 with increased lifetime in human plasma[J]. Nature Communications, 2017, 8: 1424.

[37] KIM E, KOO T, PARK SW, KIM D, KIM K, CHO HY, SONG DW, LEE KJ, JUNG MH, KIM S, KIM JH, KIM JH, KIM JS. *In vivo* genome editing with a small Cas9 orthologue derived from *Campylobacter jejuni*[J]. Nature Communications, 2017, 8: 14500.

[38] BROCKEN DJW, TARK-DAME M, DAME RT. dCas9: a versatile tool for epigenome editing[J]. Current Issues in Molecular Biology, 2018, 26: 15-32.

[39] YANG YK, LIU GQ, CHEN X, LIU M, ZHAN CJ, LIU XX, BAI ZH. High efficiency CRISPR/Cas9 genome editing system with an eliminable episomal sgRNA plasmid in *Pichia pastoris*[J]. Enzyme and Microbial Technology, 2020, 138: 109556.

[40] 王小龙. 毕赤酵母AOX1启动子转录调控机制研究[D]. 上海: 华东理工大学博士学位论文, 2016.
WANG XL. Study on the transcriptional regulation mechanism of Pichia pastoris AOX1 promoter[D]. Shanghai: Doctoral Dissertation of East China University of Science and Technology, 2016 (in Chinese).

[41] LIU Q, SONG LL, PENG QQ, ZHU QY, SHI XN, XU MQ, WANG QY, ZHANG YX, CAI MH. A programmable high-expression yeast platform responsive to user-defined signals[J]. Science advances, 2022, 8(6): eabl5166.

[42] SWARTS DC, JINEK M. Cas9 versus Cas12a/Cpf1: structure-function comparisons and implications for genome editing[J]. Wiley Interdisciplinary Reviews RNA, 2018, 9(5): e1481.

[43] ZETSCHE B, GOOTENBERG JS, ABUDAYYEH OO, SLAYMAKER IM, MAKAROVA KS, ESSLETZBICHLER P, VOLZ SE, JOUNG J, van der OOST J, REGEV A, KOONIN EV, ZHANG F. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system[J]. Cell, 2015, 163(3): 759-771.

[44] ZHANG LY, ZURIS JA, VISWANATHAN R, EDELSTEIN JN, TURK R, THOMMANDRU B, RUBE HT, GLENN SE, COLLINGWOOD MA, BODE NM, BEAUDOIN SF, LELE S, SCOTT SN, WASKO KM, SEXTON S, BORGES CM, SCHUBERT MS, KURGAN GL, MCNEILL MS, FERNANDEZ CA, et al. AsCas12a ultra nuclease facilitates the rapid generation of therapeutic cell medicines[J]. Nature Communications, 2021, 12: 3908.

[45] CHENG PP, ZHANG ZH, YANG FY, CAI S, WANG LN, WANG CM, WANG M, LIU YC, FEI CZ, ZHANG LF, XUE FQ, GU F. FnCas12a/crRNA-mediated genome editing in *Eimeria tenella*[J]. Frontiers in Genetics, 2021, 12: 738746.

[46] ZETSCHE B, ABUDAYYEH OO, GOOTENBERG JS, SCOTT DA, ZHANG F. A survey of genome editing activity for 16 Cas12a orthologs[J]. The Keio Journal of Medicine, 2020, 69(3): 59-65.

[47] ZHANG XY, GU SJ, ZHENG XY, PENG SQ, LI YR, LIN Y, LIANG SL. A novel and efficient genome editing tool assisted by CRISPR-Cas12a/Cpf1 for *Pichia pastoris*[J]. ACS Synthetic Biology, 2021, 10(11): 2927-2937.

[48] BAESHEN MN, AL-HEJIN AM, BORA RS, AHMED MMM, RAMADAN HAI, SAINI KS, BAESHEN NA, REDWAN EM. Production of biopharmaceuticals in *E. coli*: current scenario and future perspectives[J]. Journal of Microbiology and Biotechnology, 2015, 25(7): 953-962.

[49] YANG ZL, ZHANG ZS. Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris*: a review[J]. Biotechnology Advances, 2018, 36(1): 182-195.

[50] JOSHI PRH, VENEREO-SANCHEZ A, CHAHAL PS, KAMEN AA. Advancements in molecular design and bioprocessing of recombinant adeno-associated virus gene delivery vectors using the insect-cell baculovirus expression platform[J]. Biotechnology Journal, 2021, 16(4): e2000021.

[51] GUTIÉRREZ-GONZÁLEZ M, LATORRE Y, ZÚÑIGA R, AGUILLÓN JC, MOLINA MC, ALTAMIRANO C. Transcription factor engineering in CHO cells for recombinant protein production[J]. Critical Reviews in Biotechnology, 2019, 39(5): 665-679.

[52] MALM M, SAGHALEYNI R, LUNDQVIST M, GIUDICI M, CHOTTEAU V, FIELD R, VARLEY PG, HATTON D, GRASSI L, SVENSSON T, NIELSEN J, ROCKBERG J. Evolution from adherent to suspension: systems biology of HEK293 cell line development[J]. Scientific Reports, 2020, 10: 18996.

[53] EJIKE UC, CHAN CJ, LIM CSY, LAY HONG LIM R. Functional evaluation of a recombinant fungal immunomodulatory protein from *L. rhinocerus* produced in *P. pastoris* and *E. coli* host expression systems[J]. Applied Microbiology and Biotechnology, 2021, 105(7): 2799-2813.

[54] ERGÜN BG, LAÇıN K, ÇALOĞLU B, BINAY B. Second generation *Pichia pastoris* strain and bioprocess designs[J]. Biotechnology for Biofuels and Bioproducts, 2022, 15(1): 150.

[55] de SÁ MAGALHÃES S, KESHAVARZ-MOORE E. *Pichia pastoris* (*Komagataella phaffii*) as a cost-effective tool for vaccine production for low- and middle-income countries (LMICs)[J]. Bioengineering (Basel, Switzerland), 2021, 8(9): 119.

[56] WANG M, JIANG S, HAN ZW, ZHAO B, WANG LA,

ZHOU ZX, WANG YF. Expression and immunogenic characterization of recombinant gp350 for developing a subunit vaccine against Epstein-Barr virus[J]. Applied Microbiology and Biotechnology, 2016, 100(3): 1221-1230.

[57] WANG M, JIANG S, LIU XY, WANG YF. Expression, purification, and immunogenic characterization of Epstein-Barr virus recombinant EBNA1 protein in *Pichia pastoris*[J]. Applied Microbiology and Biotechnology, 2013, 97(14): 6251-6262.

[58] SUBATHRA M, SANTHAKUMAR P, NARASU ML, BEEVI SS, LAL SK. Evaluation of antibody response in mice against avian influenza A (H5N1) strain neuraminidase expressed in yeast *Pichia pastoris*[J]. Journal of Biosciences, 2014, 39(3): 443-451.

[59] WANG M, JIANG S, WANG YF. Recombinant VP1 protein expressed in *Pichia pastoris* induces protective immune responses against EV71 in mice[J]. Biochemical and Biophysical Research Communications, 2013, 430(1): 387-393.

[60] ZHANG C, LIU QW, KU ZQ, HU Y, YE XH, ZHANG YY, HUANG Z. Coxsackievirus A16-like particles produced in *Pichia pastoris* elicit high-titer neutralizing antibodies and confer protection against lethal viral challenge in mice[J]. Antiviral Research, 2016, 129: 47-51.

[61] GURRAMKONDA C, ZAHID M, NEMANI SK, ADNAN A, GUDI SK, KHANNA N, EBENSEN T, LÜNSDORF H, GUZMÁN CA, RINAS U. Purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris* and *in vivo* analysis of their immunogenic properties[J]. Journal of Chromatography B, 2013, 940: 104-111.

[62] 范文辉, 王萌, 刘丽蓉, 张鹤, 张爽, 凌红丽, 刘文军, 李晶. H5亚型流感病毒HA头部球状结构域在毕赤酵母中的高效表达及其免疫原性分析[J]. 生物工程学报, 2019, 35(1): 49-58.
FAN WH, WANG M, LIU LR, ZHANG H, ZHANG S, LING HL, LIU WJ, LI J. High expression and immunogenicity analysis of HA globular head domain of H5 subtype avian influenza virus produced in Pichia pastoris[J]. Chinese Journal of Biotechnology, 2019, 35(1): 49-58 (in Chinese).

[63] MENG Y, ZHAO M, YANG M, ZHANG Q, HAO J, MENG Y. Production and characterization of recombinant glucose oxidase from *Aspergillus niger* expressed in *Pichia pastoris*[J]. Letters in Applied Microbiology, 2014, 58(4): 393-400.

[64] HANSEN OC, STOUGAARD P. Hexose oxidase from the red *Alga Chondrus crispus*[J]. Journal of Biological Chemistry, 1997, 272(17): 11581-11587.

[65] BEY M, BERRIN JG, POIDEVIN L, SIGOILLOT JC. Heterologous expression of *Pycnoporus cinnabarinus* cellobiose dehydrogenase in *Pichia pastoris* and involvement in saccharification processes[J]. Microbial Cell Factories, 2011, 10: 113.

[66] KITTL R, MUEANGTOOM K, GONAUS C, KHAZANEH ST, SYGMUND C, HALTRICH D, LUDWIG R. A chloride tolerant laccase from the plant pathogen ascomycete *Botrytis aclada* expressed at high levels in *Pichia pastoris*[J]. Journal of Biotechnology, 2012, 157(2): 304-314.

[67] SHI XL, FENG MQ, SHI J, SHI ZH, ZHONG J, ZHOU P. High-level expression and purification of recombinant human catalase in *Pichia pastoris*[J]. Protein Expression and Purification, 2007, 54(1): 24-29.

[68] SPADIUT O, ROSSETTI L, DIETZSCH C, HERWIG C. Purification of a recombinant plant peroxidase produced in *Pichia pastoris* by a simple 2-step strategy[J]. Protein Expression and Purification, 2012, 86(2): 89-97.

[69] LI HB, ZHANG LW, CUI YH, LUO X, XUE CH, WANG SM. Expression of soluble recombinant transglutaminase from *Zea mays* in *Pichia pastoris*[J]. World Journal of Microbiology Biotechnology, 2013, 29(5): 939-947.

[70] DELROISSE JM, DANNAU M, GILSOUL JJ, EL MEJDOUB T, DESTAIN J, PORTETELLE D, THONART P, HAUBRUGE E, VANDENBOL M. Expression of a synthetic gene encoding a *Tribolium castaneum* carboxylesterase in *Pichia pastoris*[J]. Protein Expression and Purification, 2005, 42(2): 286-294.

[71] SHI BH, ZENG LQ, SONG HL, SHI QQ, WU SG. Cloning and expression of *Aspergillus tamarii* FS132 lipase gene in *Pichia pastoris*[J]. International Journal of Molecular Sciences, 2010, 11(6): 2373-2382.

[72] ZHONG XF, PENG LS, ZHENG SL, SUN ZZ, REN YF, DONG ML, XU AL. Secretion, purification, and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*[J]. Protein Expression and Purification, 2004, 36(2): 165-169.

[73] BORODINA I, JENSEN BM, WAGNER T, HACHEM MA, SØNDERGAARD I, POULSEN LK. Expression of enzymatically inactive wasp venom phospholipase

A1 in *Pichia pastoris*[J]. PLoS One, 2011, 6(6): e21267.

[74] MOUKOULI M, TOPAKAS E, CHRISTAKOPOULOS P. Cloning, characterization and functional expression of an alkalitolerant type C feruloyl esterase from *Fusarium oxysporum*[J]. Applied Microbiology and Biotechnology, 2008, 79(2): 245-254.

[75] XIONG AS, YAO QH, PENG RH, HAN PL, CHENG ZM, LI Y. High level expression of a recombinant acid phytase gene in *Pichia pastoris*[J]. Journal of Applied Microbiology, 2005, 98(2): 418-428.

[76] JOYE IJ, BELIËN T, BRIJS K, PROOST P, SOETAERT W, DELCOUR JA. Characterisation of the first wheat (*Triticum aestivum* L.) nucleotide pyrophosphatase/phosphodiesterase resembling mammalian counterparts[J]. Journal of Cereal Science, 2010, 51(3): 326-336.

[77] LIU ZW, YIN HX, YI XP, ZHANG AL, LUO JX, ZHANG TY, FU CY, ZHANG ZH, SHEN JC, CHEN LP. Constitutive expression of barley α-amylase in *Pichia pastoris* by high-density cell culture[J]. Molecular Biology Reports, 2012, 39(5): 5805-5810.

[78] MELLITZER A, WEIS R, GLIEDER A, FLICKER K. Expression of lignocellulolytic enzymes in *Pichia pastoris*[J]. Microbial Cell Factories, 2012, 11(1): 1-11.

[79] CHEN XY, MENG K, SHI PJ, BAI YG, LUO HY, HUANG HQ, YUAN TZ, YANG PL, YAO B. High-level expression of a novel *Penicillium* endo-1,3(4)-β-D-glucanase with high specific activity in *Pichia pastoris*[J]. Journal of Industrial Microbiology and Biotechnology, 2012, 39(6): 869-876.

[80] WU KY, HUANG SH, DING S, ZHANG YK, CHEN GG, LIANG ZQ. Expression, purification and characterization of recombinant α-glucosidase in *Pichia pastoris*[J]. Folia Microbiologica, 2010, 55(6): 582-587.

[81] LING ZM, MA TB, LI JH, DU GC, KANG Z, CHEN J. Functional expression of trypsin from *Streptomyces griseus* by *Pichia pastoris*[J]. Journal of Industrial Microbiology & Biotechnology, 2012, 39(11): 1651-1662.

[82] KANG C, YU XW, XU Y. Gene cloning and enzymatic characterization of an endoprotease endo-pro-*Aspergillus niger*[J]. Journal of Industrial Microbiology & Biotechnology, 2013, 40(8): 855-864.

[83] SALAMIN K, SRIRANGANADANE D, LÉCHENNE B, JOUSSON O, MONOD M. Secretion of an endogenous subtilisin by *Pichia pastoris* strains GS115 and KM71[J]. Applied and Environmental Microbiology, 2010, 76(13): 4269-4276.

[84] GUO JP, MA Y. High-level expression, purification and characterization of recombinant *Aspergillus oryzae* alkaline protease in *Pichia pastoris*[J]. Protein Expression and Purification, 2008, 58(2): 301-308.

[85] FERRARA MA, SEVERINO NMB, MANSURE JJ, MARTINS AS, OLIVEIRA EMM, SIANI AC, PEREIRA N JR, TORRES FAG, BON EPS. Asparaginase production by a recombinant *Pichia pastoris* strain harbouring *Saccharomyces cerevisiae* ASP3 gene[J]. Enzyme and Microbial Technology, 2006, 39(7): 1457-1463.

[86] ZHANG CJ, YAO J, ZHOU C, MAO LW, ZHANG GM, MA YH. The alkaline pectate lyase PEL168 of *Bacillus subtilis* heterologously expressed in *Pichia pastoris*is more stable and efficient for degumming ramie fiber[J]. BMC Biotechnology, 2013, 13(1): 1-9.

[87] PÉREZ-FUENTES C, CRISTINA RAVANAL M, EYZAGUIRRE J. Heterologous expression of a *Penicillium purpurogenum* pectin lyase in *Pichia pastoris* and its characterization[J]. Fungal Biology, 2014, 118(5/6): 507-515.

[88] DALY R, HEARN MTW. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production[J]. Journal of Molecular Recognition: JMR, 2005, 18(2): 119-138.

[89] HE ZY. Comparison of alpha-factor preprosequence and a classical mammalian signal peptide for secretion of recombinant xylanase xynB from yeast *Pichia pastoris*[J]. Journal of Microbiology and Biotechnology, 2012, 22(4): 479-483.

[90] NEIERS F, BELLOIR C, POIRIER N, NAUMER C, KROHN M, BRIAND L. Comparison of different signal peptides for the efficient secretion of the sweet-tasting plant protein brazzein in *Pichia pastoris*[J]. Life, 2021, 11(1): 46.

[91] KARBALAEI M, REZAEE SA, FARSIANI H. *Pichia pastoris*: a highly successful expression system for optimal synthesis of heterologous proteins[J]. Journal of Cellular Physiology, 2020, 235(9): 5867-5881.

[92] BERTHELEME N, CHAE PS, SINGH S, MOSSAKOWSKA D, HANN MM, SMITH KJ, HUBBARD JA, DOWELL SJ, BYRNE B. Unlocking

the secrets of the gatekeeper: methods for stabilizing and crystallizing GPCRs[J]. Biochimica et Biophysica Acta (BBA)-Biomembranes, 2013, 1828(11): 2583-2591.

[93] BYRNE B. *Pichia pastoris* as an expression host for membrane protein structural biology[J]. Current Opinion in Structural Biology, 2015, 32: 9-17.

[94] PARKER JL, NEWSTEAD S. Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1[J]. Nature, 2014, 507(7490): 68-72.

[95] ASADA H, UEMURA T, YURUGI-KOBAYASHI T, SHIROISHI M, SHIMAMURA T, TSUJIMOTO H, ITO K, SUGAWARA T, NAKANE T, NOMURA N, MURATA T, HAGA T, IWATA S, KOBAYASHI T. Evaluation of the *Pichia pastoris* expression system for the production of GPCRs for structural analysis[J]. Microbial Cell Factories, 2011, 10: 24.

[96] KATLA S, YOGANAND KNR, HINGANE S, RANJITH KUMAR CT, ANAND B, SIVAPRAKASAM S. Novel glycosylated human interferon alpha 2b expressed in glycoengineered *Pichia pastoris* and its biological activity: N-linked glycoengineering approach[J]. Enzyme and Microbial Technology, 2019, 128: 49-58.

[97] JOSHI HJ, NARIMATSU Y, SCHJOLDAGER KT, TYTGAT HLP, AEBI M, CLAUSEN H, HALIM A. SnapShot: O-glycosylation pathways across kingdoms[J]. Cell, 2018, 172(3): 632-632.e2.

[98] MOCHIZUKI S, HAMATO N, HIROSE M, MIYANO K, OHTANI W, KAMEYAMA S, KUWAE S, TOKUYAMA T, OHI H. Expression and characterization of recombinant human antithrombin III in *Pichia pastoris*[J]. Protein Expression and Purification, 2001, 23(1): 55-65.

[99] MASTROPIETRO G, AW R, POLIZZI KM. Expression of proteins in *Pichia pastoris*[M]//Methods in Enzymology. Amsterdam: Elsevier, 2021: 53-80.

[100] JUTURU V, WU JC. Heterologous protein expression in *Pichia pastoris*: latest research progress and applications[J]. Chembiochem: a European Journal of Chemical Biology, 2018, 19(1): 7-21.

[101] BAUMANN K, DATO L, GRAF AB, FRASCOTTI G, DRAGOSITS M, PORRO D, MATTANOVICH D, FERRER P, BRANDUARDI P. The impact of oxygen on the transcriptome of recombinant *S. cerevisiae* and *P. pastoris*-a comparative analysis[J]. BMC Genomics, 2011, 12: 218.

[102] CREGG JM, VEDVICK TS, RASCHKE WC. Recent advances in the expression of foreign genes in *Pichia pastoris*[J]. Bio/Technology, 1993, 11(8): 905-910.

[103] EISENBERG A, SEIP JE, GAVAGAN JE, PAYNE MS, ANTON DL, DICOSIMO R. Pyruvic acid production using methylotrophic yeast transformants as catalyst[J]. Journal of Molecular Catalysis B: Enzymatic, 1997, 2(4/5): 223-232.

[104] PAYNE MS, PETRILLO KL, GAVAGAN JE, DICOSIMO R, WAGNER LW, ANTON DL. Engineering *Pichia pastoris* for biocatalysis: co-production of two active enzymes[J]. Gene, 1997, 194(2): 179-182.

[105] WU S, FALLON RD, PAYNE MS. Engineering *Pichia pastoris* for stereoselective nitrile hydrolysis by co-producing three heterologous proteins[J]. Applied Microbiology and Biotechnology, 1999, 52(2): 186-190.

[106] MARX H, MATTANOVICH D, SAUER M. Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris*[J]. Microbial Cell Factories, 2008, 7: 23.

[107] JIANG ML, GUO B, WAN X, GONG YM, ZHANG YB, HU CJ. Isolation and characterization of the diatom *Phaeodactylum* Δ5-elongase gene for transgenic LC-PUFA production in *Pichia pastoris*[J]. Marine Drugs, 2014, 12(3): 1317-1334.

[108] XUE Y, KONG CX, SHEN W, BAI CX, REN YN, ZHOU XS, ZHANG YX, CAI MH. Methylotrophic yeast *Pichia pastoris* as a chassis organism for polyketide synthesis *via* the full citrinin biosynthetic pathway[J]. Journal of Biotechnology, 2017, 242: 64-72.

[109] ZHANG YY, WANG CY, WANG LL, YANG RX, HOU PL, LIU JH. Direct bioethanol production from wheat straw using xylose/glucose co-fermentation by co-culture of two recombinant yeasts[J]. Journal of Industrial Microbiology & Biotechnology, 2017, 44(3): 453-464.

[110] LIU XB, LIU M, TAO XY, ZHANG ZX, WANG FQ, WEI DZ. Metabolic engineering of *Pichia pastoris* for the production of dammarenediol-II[J]. Journal of Biotechnology, 2015, 216: 47-55.

[111] ZIRPEL B, STEHLE F, KAYSER O. Production of Δ9-tetrahydrocannabinolic acid from cannabigerolic acid by whole cells of *Pichia* (*Komagataella*) *pastoris* expressing Δ9-tetrahydrocannabinolic acid synthase

from *Cannabis sativa*[J]. Biotechnology Letters, 2015, 37(9): 1869-1875.

[112] GEIER M, BRAUN A, EMMERSTORFER A, PICHLER H, GLIEDER A. Production of human cytochrome P450 2D6 drug metabolites with recombinant microbes-a comparative study[J]. Biotechnology Journal, 2012, 7(11): 1346-1358.

[113] GAO JC, ZUO YM, XIAO F, WANG YL, LI DF, XU JH, YE CF, FENG LJ, JIANG LJ, LIU TF, GAO D, MA B, HUANG L, XU ZN, LIAN JZ. Biosynthesis of catharanthine in engineered *Pichia pastoris*[J]. Nature Synthesis, 2023, 2(3): 231-242.

[114] 盛靖雨, 金学荣, 胥睿睿, 王阳, 康振. 基于工程化毕赤酵母一锅法合成硫酸软骨素 A[J]. 生物工程学报, 2022, 38(7): 2594-2605.
SHENG JY, JIN XR, XU RR, WANG Y, KANG Z. One-pot synthesis of chondroitin sulfate A by engineered *Pichia pastoris*[J]. Chinese Journal of Biotechnology, 2022, 38(7): 2594-2605 (in Chinese).

[115] NÄÄTSAARI L, MISTLBERGER B, RUTH C, HAJEK T, HARTNER FS, GLIEDER A. Deletion of the *Pichia pastoris* KU70 homologue facilitates platform strain generation for gene expression and synthetic biology[J]. PLoS One, 2012, 7(6): e39720.

[116] CAI P, DUAN XP, WU XY, GAO LH, YE M, ZHOU YJ. Recombination machinery engineering facilitates metabolic engineering of the industrial yeast *Pichia pastoris*[J]. Nucleic Acids Research, 2021, 49(13): 7791-7805.

[117] GAO JC, YE CF, CHENG JT, JIANG LH, YUAN XH, LIAN JZ. Enhancing homologous recombination efficiency in *Pichia pastoris* for multiplex genome integration using short homology arms[J]. ACS Synthetic Biology, 2022, 11(2): 547-553.

[118] MELO N, MULDER K, NICOLA A, CARVALHO L, MENINO G, MULINARI E, PARACHIN N. Effect of pyruvate decarboxylase knockout on product distribution using *Pichia pastoris* (*Komagataella phaffii*) engineered for lactic acid production[J]. Bioengineering, 2018, 5(1): 17.

[119] GUO F, DAI ZX, PENG WF, ZHANG SJ, ZHOU J, MA JF, DONG WL, XIN FX, ZHANG WM, JIANG M. Metabolic engineering of *Pichia pastoris* for malic acid production from methanol[J]. Biotechnology and Bioengineering, 2021, 118(1): 357-371.

[120] LIAO XH, LI L, JAMEEL A, XING XH, ZHANG C. A versatile toolbox for CRISPR-based genome engineering in *Pichia pastoris*[J]. Applied Microbiology and Biotechnology, 2021, 105(24): 9211-9218.

[121] ZHANG K, DUAN XP, CAI P, GAO LH, WU XY, YAO L, ZHOU YJ. Fusing an exonuclease with Cas9 enhances homologous recombination in *Pichia pastoris*[J]. Microbial Cell Factories, 2022, 21(1): 182.

[122] VOGL T, GLIEDER A. Regulation of *Pichia pastoris* promoters and its consequences for protein production[J]. New Biotechnology, 2013, 30(4): 385-404.

[123] GASSER B, PRIELHOFER R, MARX H, MAURER M, NOCON J, STEIGER M, PUXBAUM V, SAUER M, MATTANOVICH D. *Pichia pastoris*: protein production host and model organism for biomedical research[J]. Future Microbiology, 2013, 8(2): 191-208.

[124] PRIELHOFER R, BARRERO JJ, STEUER S, GASSLER T, ZAHRL R, BAUMANN K, SAUER M, MATTANOVICH D, GASSER B, MARX H. GoldenPiCS: a Golden Gate-derived modular cloning system for applied synthetic biology in the yeast *Pichia pastoris*[J]. BMC Systems Biology, 2017, 11(1): 123.

[125] GAO JC, XU JH, ZUO YM, YE CF, JIANG LJ, FENG LJ, HUANG L, XU ZN, LIAN JZ. Synthetic biology toolkit for marker-less integration of multigene pathways into *Pichia pastoris via* CRISPR/Cas9[J]. ACS Synthetic Biology, 2022, 11(2): 623-633.

[126] GEMMILL TR, TRIMBLE RB. Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species[J]. Biochimica et Biophysica Acta (BBA)-General Subjects, 1999, 1426(2): 227-237.

[127] SINCLAIR AM, ELLIOTT S. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins[J]. Journal of Pharmaceutical Sciences, 2005, 94(8): 1626-1635.

[128] WILDT S, GERNGROSS TU. The humanization of N-glycosylation pathways in yeast[J]. Nature Reviews Microbiology, 2005, 3(2): 119-128.

[129] CHOI BK, BOBROWICZ P, DAVIDSON RC, HAMILTON SR, KUNG DH, LI HJ, MIELE RG, NETT JH, WILDT S, GERNGROSS TU. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*[J]. Proceedings of the National Academy of Sciences of the United States of America, 2003, 100(9): 5022-5027.

[130] VERVECKEN W, KAIGORODOV V, CALLEWAERT N, GEYSENS S, de VUSSER K, CONTRERAS R. *In*

vivo synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*[J]. Applied and Environmental Microbiology, 2004, 70(5): 2639-2646.

[131] HAMILTON SR, DAVIDSON RC, SETHURAMAN N, NETT JH, JIANG YW, RIOS S, BOBROWICZ P, STADHEIM TA, LI HJ, CHOI BK, HOPKINS D, WISCHNEWSKI H, ROSER J, MITCHELL T, STRAWBRIDGE RR, HOOPES J, WILDT S, GERNGROSS TU. Humanization of yeast to produce complex terminally sialylated glycoproteins[J]. Science, 2006, 313(5792): 1441-1443.

[132] LI HJ, SETHURAMAN N, STADHEIM TA, ZHA DX, PRINZ B, BALLEW N, BOBROWICZ P, CHOI BK, COOK WJ, CUKAN M, HOUSTON-CUMMINGS NR, DAVIDSON R, GONG B, HAMILTON SR, HOOPES JP, JIANG YW, KIM N, MANSFIELD R, NETT JH, RIOS S, et al. Optimization of humanized IgGs in glycoengineered *Pichia pastoris*[J]. Nature Biotechnology, 2006, 24(2): 210-215.

[133] AHMAD M, HIRZ M, PICHLER H, SCHWAB H. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production[J]. Applied Microbiology and Biotechnology, 2014, 98(12): 5301-5317.

[134] AHMAD M, WINKLER CM, KOLMBAUER M, PICHLER H, SCHWAB H, EMMERSTORFER-AUGUSTIN A. *Pichia pastoris* protease-deficient and auxotrophic strains generated by a novel, user-friendly vector toolbox for gene deletion[J]. Yeast (Chichester, England), 2019, 36(9): 557-570.

[135] GLEESON MAG, WHITE CE, MEININGER DP, KOMIVES EA. Generation of protease-deficient strains and their use in heterologous protein expression[M]//Methods in Molecular Biology. Totowa, NJ: Humana Press, 1998: 81-94.

[136] STRATTON J, CHIRUVOLU V, MEAGHER M. High cell-density fermentation[M]//Methods in Molecular Biology. Totowa, NJ: Humana Press, 1998: 107-120.

[137] SENEROVIC L, STANKOVIC N, SPIZZO P, BASSO A, GARDOSSI L, VASILJEVIC B, LJUBIJANKIC G, TISMINETZKY S, DEGRASSI G. High-level production and covalent immobilization of *Providencia rettgeri* penicillin G acylase (PAC) from recombinant *Pichia pastoris* for the development of a novel and stable biocatalyst of industrial applicability[J]. Biotechnology and Bioengineering, 2006, 93(2): 344-354.

[138] WU M, SHEN Q, YANG Y, ZHANG S, QU W, CHEN J, SUN HY, CHEN SQ. Disruption of *YPS1* and *PEP4* genes reduces proteolytic degradation of secreted HSA/PTH in *Pichia pastoris* GS115[J]. Journal of Industrial Microbiology & Biotechnology, 2013, 40(6): 589-599.

[139] HE JY, DENG JJ, ZHENG YH, GU J. A synergistic effect on the production of S-adenosyl-L-methionine in *Pichia pastoris* by knocking in of S-adenosyl-L-methionine synthase and knocking out of cystathionine-β synthase[J]. Journal of Biotechnology, 2006, 126(4): 519-527.

[140] BHATAYA A, SCHMIDT-DANNERT C, LEE PC. Metabolic engineering of *Pichia pastoris* X-33 for lycopene production[J]. Process Biochemistry, 2009, 44(10): 1095-1102.

[141] ZHANG J, HANSEN LG, GUDICH O, VIEHRIG K, LASSEN LMM, SCHRÜBBERS L, ADHIKARI KB, RUBASZKA P, CARRASQUER-ALVAREZ E, CHEN L, D'AMBROSIO V, LEHKA B, HAIDAR AK, NALLAPAREDDY S, GIANNAKOU K, LALOUX M, ARSOVSKA D, JØRGENSEN MAK, CHAN LJG, KRISTENSEN M, et al. A microbial supply chain for production of the anti-cancer drug vinblastine[J]. Nature, 2022, 609(7926): 341-347.

[142] GONG GP, ZHANG YP, WANG ZB, LIU L, SHI SB, SIEWERS V, YUAN QP, NIELSEN J, ZHANG X, LIU ZH. GTR 2.0: gRNA-tRNA array and Cas9-NG based genome disruption and single-nucleotide conversion in *Saccharomyces cerevisiae*[J]. ACS Synthetic Biology, 2021, 10(6): 1328-1337.

[143] JING XY, XIE BR, CHEN LX, ZHANG NB, JIANG YY, QIN H, WANG HB, HAO P, YANG S, LI X. Implementation of the CRISPR-Cas13a system in fission yeast and its repurposing for precise RNA editing[J]. Nucleic Acids Research, 2018, 46(15): e90.

[144] PAN YJ, XIA SY, DONG C, PAN HJ, CAI J, HUANG L, XU ZN, LIAN JZ. Random base editing for genome evolution in *Saccharomyces cerevisiae*[J]. ACS Synthetic Biology, 2021, 10(10): 2440-2446.

[145] DONG C, GOU YW, LIAN JZ. SgRNA engineering for improved genome editing and expanded functional assays[J]. Current Opinion in Biotechnology, 2022, 75: 102697.

[146] 张潇筠, 徐坤, 沈俊岑, 穆璐, 钱泓润, 崔婕妤, 马宝霞, 陈知龙, 张智英, 魏泽辉. 一种新型提高 HDR 效率的 CRISPR/Cas9-Gal4BD 供体适配基因编辑系统[J]. 遗传, 2022, 44(8): 708-719.

ZHANG XJ, XU K, SHEN JC, MU L, QIAN HR, CUI JY, MA BX, CHEN ZL, ZHANG ZY, WEI ZH. A CRISPR/Cas9-Gal4BD donor adapting system for enhancinghomology-directed repair[J]. Hereditas (Beijing), 2022, 44(8): 708-719 (in Chinese).

[147] 高琳惠, 蔡鹏, 周雍进. 甲醇酵母代谢工程研究进展[J]. 生物工程学报, 2021, 37(3): 966-979.
GAO LH, CAI P, ZHOU YJ. Advances in metabolic engineering of methylotrophic yeasts[J]. Chinese Journal of Biotechnology, 2021, 37(3): 966-979 (in Chinese).

[148] ZAKRIA HM, HAN BQ, YUE F, MU L, FANG YY, LI XY, XU K, ZHANG ZY. Significant body mass increase by oral administration of a cascade of shIL21-MSTN yeast-based DNA vaccine in mice[J]. Biomedicine & Pharmacotherapy, 2019, 118: 109147.

[149] HAN BQ, XU K, LIU ZT, GE W, SHAO SM, LI PC, YAN NN, LI XY, ZHANG ZY. Oral yeast-based DNA vaccine confers effective protection from *Aeromonas hydrophila* infection on *Carassius auratus*[J]. Fish & Shellfish Immunology, 2019, 84: 948-954.

[150] FRANÇA RC, CONCEIÇÃO FR, MENDONÇA M, HAUBERT L, SABADIN G, DIAZ de OLIVEIRA P, AMARAL MG, DA SILVA WP, MOREIRA ÂN. *Pichia pastoris* X-33 has probiotic properties with remarkable antibacterial activity against *Salmonella typhimurium*[J]. Applied Microbiology and Biotechnology, 2015, 99(19): 7953-7961.

[151] Zhang XF, AI YH, XU Y, YU XW. High-level expression of *Aspergillus niger* lipase in *Pichia pastoris*: characterization and gastric digestion *in vitro*[J]. Food Chemistry, 2019, 274: 305-313.

[152] GUO YK, LIAO Y, WANG J, MA C, QIN JL, FENG J, LI Y, WANG X, CHEN KQ. Methylotrophy of *Pichia pastoris*: current advances, applications, and future perspectives for methanol-based biomanufacturing[J]. ACS Sustainable Chemistry & Engineering, 2022, 10(5): 1741-1752.

(本文责编 陈宏宇)