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(54)发明名称

N-甲基吡咯啉的生物合成方法

(57)摘要

本发明提供了一种通过三酶联合催化制备N-甲基吡咯啉的方法,包括如下步骤:以L-鸟氨酸为底物,用鸟氨酸脱羧酶EcODC、腐胺-N-甲基转移酶AtPMT、胺氧化酶AaDAO3进行联合催化而得。上述方法也可以通过表达这三种酶的基因工程菌的发酵来实现。本发明的方法为环境友好型,反应条件温,具有工业化开发前景。

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1.一种制备N-甲基吡咯啉的方法,包括如下步骤:以L-鸟氨酸为底物,用氨基酸序列为SEQ ID NO:2的鸟氨酸脱羧酶EcODC、氨基酸序列为SEQ ID NO:6的腐胺-N-甲基转移酶AtPMT和三分三来源的胺氧化酶进行联合催化而得,其中

所述胺氧化酶是氨基酸序列为SEQ ID NO:14的AaDAO2或者氨基酸序列为SEQ ID NO:18的AaDAO3。

2.如权利要求1所述的方法,其特征在于,所述胺氧化酶是氨基酸序列为SEQ ID NO:18的AaDAO3。

3.一种微生物,其表达如权利要求1或2所述的鸟氨酸脱羧酶EcODC、腐胺-N-甲基转移酶AtPMT和胺氧化酶。

4.如权利要求3所述的微生物,其特征在于,所述微生物选自大肠杆菌和酿酒酵母。

5.如权利要求3所述的微生物,其特征在于,所述微生物是酿酒酵母,并且敲除了基因组中代谢N-甲氨基丁醛的基因ALD4、ALD5和HFD1。

6.如权利要求5所述的微生物,其特征在于,所述酿酒酵母过表达序列为SEQ ID NO:84的辅因子合成基因SAM2。

7.如权利要求3-6中任一项中所述的微生物在制备N-甲基吡咯啉中的应用。

8.如权利要求7所述的应用,其特征在于,通过如权利要求3-6中任一项中所述的微生物的发酵来生产N-甲基吡咯啉。

9.如权利要求8所述的应用,其特征在于,当所述微生物是大肠杆菌时,培养基是LB培养基;当所述微生物是酿酒酵母时,培养基是YPD培养基。

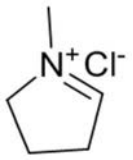
N-甲基吡咯啉的生物合成方法

技术领域

[0001] 本发明属于生物催化和代谢工程技术领域，具体地说，涉及一种N-甲基吡咯啉的生物合成方法。

背景技术

[0002] 式I所示化合物N-甲基吡咯啉(N-methylpyrrolinium)，主要以盐的形式存在，分子式为$C_5H_{10}N^+$，在一定的溶液环境下与N-甲氨基丁醛形成平衡)是植物来源的尼古丁及植物天然产物药物托品类生物碱生物合成途径中共有的关键中间体。从L-鸟氨酸开始的N-甲基吡咯啉生物合成路线报道((Chase et al.2003；Dräger 2004；Häkkinen et al.2005；Ruetsch et al.2001.)，参见附图1，包括三步：L-鸟氨酸由鸟氨酸脱羧酶催化生成腐胺，腐胺在N-甲基转移酶的催化作用下生成N-甲基化腐胺，N-甲基化腐胺进一步被胺氧化酶催化生成N-甲氨基丁醛，N-甲氨基丁醛通过希夫碱的自发反应自发成环形成N-甲基吡咯啉。

[0003] 

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[0004] 目前为止，以N-甲基吡咯啉为生物合成中间体的尼古丁及托品类生物碱主要从植物中提取得到。近年来发展的合成生物学技术，通过微生物菌株作为底盘细胞，将目标化合物生物合成途径相关基因导入，实现了植物来源的一些重要药用化合物的异源高效合成(Kotopka et al.2018;Li et al.2018.)，建立N-甲基吡咯啉的生物合成方法不仅能弥补化学合成方法的不足之处，同时也为以N-甲基吡咯啉为中间体的尼古丁及托品生物碱的途径解析及异源合成提供基础。

[0005] 在N-甲基吡咯啉的生物合成途径中，只有腐胺的代谢在微生物宿主中得到工程化改造(Nguyen et al.2015;Qian et al.2010)。目前许多物种中N-甲基吡咯啉生物合成途径中的相关酶也被鉴定(Docimo et al.2012;Heim etal.2007;Liu et al.2005)，由于烟草来源胺氧化酶在大肠中的可溶性低，发现并鉴定其它物种中胺氧化酶成为实现生物合成N-甲基吡咯啉的关键步骤。茄科植物三分三是我国特有的产托品类生物碱的资源植物，又名野烟，其干燥品总生物碱含量高达1.2％，托品生物碱总含量比常见的一些茄科植物如颠茄、莨菪、曼陀罗等都要高(Kai et al.2012.)，并且其转录组数据也被发表(Cui et al.2015.)，因此我们对植物三分三的胺氧化酶(AaDAOs)的功能进行了研究。

[0006] 众所周知，化学法合成N-甲基吡咯啉存在环境污染大的问题，导致开发环境友好型的绿色制备工艺很有必要。因此具有反应条件温和、催化效率高、副产物少等优点的生物制备法比如生物酶催化法成为有吸引力和有前途的方法。

发明内容

[0007] 为了实现N-甲基吡咯啉的生物合成，我们选择功能已知、活性高的古柯来源鸟氨酸脱羧酶(EcODC)、山崑宕来源腐胺-N-甲基转移酶(AtPMT)以及三分三来源胺氧化酶(AaDAOs)进行体外共催化底物L-鸟氨酸的反应，验证所选酶组合的可行性。由于大肠及酵母的遗传背景比较清晰，并且遗传操作容易，我们选其作为底盘细胞探究N-甲基吡咯啉的体内从头合成，希望为工业生产N-甲基吡咯啉及N-甲基吡咯啉来源重要生物碱的异源合成提供基础。

[0008] 具体而言，本发明提供了一种制备N-甲基吡咯啉的方法，包括如下步骤：以L-鸟氨酸为底物，用氨基酸序列为SEQ ID NO:2的鸟氨酸脱羧酶EcODC、氨基酸序列为SEQ IDNO:6的腐胺-N-甲基转移酶AtPMT和三分三来源的胺氧化酶进行联合催化而得。

[0009] 优选地，上述反应体系中还加入吡哆醛磷酸醛PLP、S-腺苷基甲硫氨酸SAM、$CuSO_4$。

[0010] 上述三分三来源的胺氧化酶优选是氨基酸序列为SEQ ID NO:10的AaDAO1、氨基酸序列为SEQ ID NO:14的AaDAO2或者氨基酸序列为SEQ ID NO:18的AaDAO3。更优选胺氧化酶是氨基酸序列为SEQ ID NO:18的AaDAO3。

[0011] 根据本发明的第二个方面，提供了一种微生物，其表达上述的鸟氨酸脱羧酶EcODC、腐胺-N-甲基转移酶AtPMT和胺氧化酶。

[0012] 优选地，上述微生物选自大肠杆菌和酿酒酵母。

[0013] 当上述微生物是大肠杆菌时，优选将EcODC基因和AtPMT基因构建在一个质粒上，将胺氧化酶基因构建在另一个质粒上，这两种质粒转化入大肠杆菌后，可形成用于生产N-甲基吡咯啉的基因工程菌。

[0014] 上述大肠杆菌优选是BL21(DE3)。

[0015] 优选地，用于克隆EcODC基因和AtPMT基因的质粒是pET24a或者pACYCDuet-1，用于克隆胺氧化酶AaDAO1、AaDAO2或者AaDAO3基因的质粒是pET30a。优选胺氧化酶是AaDAO3。

[0016] 当上述微生物是酿酒酵母时，优选将EcODC基因和AtPMT基因构建在一个质粒上，转化入酿酒酵母，得到包含EcODC和AtPMT基因的酿酒酵母；将胺氧化酶基因AaDAO1、AaDAO2或者AaDAO3基因整合入包含EcODC和AtPMT基因的酿酒酵母的基因组中，可形成用于生产N-甲基吡咯啉的基因工程菌。

[0017] 上述酿酒酵母优选是BY4742。

[0018] 优选地，用于克隆EcODC基因和AtPMT基因的质粒是序列为SEQ ID NO:35的质粒p2M(中国科学院上海生命科学研究院周志华实验室构建)。

[0019] 在一种优选的实施方式中，上述微生物是酿酒酵母，并且敲除了基因组中代谢N-甲氨基丁醛的基因ALD4、ALD5和HFD1。

[0020] 优选地，上述酿酒酵母过表达辅因子合成基因SAM2。

[0021] 上述辅因子合成基因SAM2的序列优选为SEQ ID NO:84。

[0022] 根据本发明的第二个方面，提供了上述微生物在制备N-甲基吡咯啉中的应用。

[0023] 优选地，通过上述微生物的发酵来生产N-甲基吡咯啉。

[0024] 当微生物是大肠杆菌时，优选培养基是LB培养基、或者任何适合于大肠杆菌发酵的培养基；当上述微生物是酿酒酵母时，优选培养基是YPD培养基、或者任何适合于酿酒酵母发酵的培养基。

[0025] 上述鸟氨酸脱羧酶EcODC、腐胺-N-甲基转移酶AtPMT和胺氧化酶AaDAO1、AaDAO2或者AaDAO3可以呈现酶的形式或者包涵酶的菌体形式。

[0026] 相应地，就催化体系而言，上述三种酶的组合可以是酶+酶的形式、酶+菌体的形式、或者菌体+菌体的形式。

[0027] 本发明的三酶联合催化体系不仅可以“一锅法”催化L-鸟氨酸反应得到N-甲基吡咯啉，而且表达上述鸟氨酸脱羧酶EcODC、腐胺-N-甲基转移酶AtPMT和胺氧化酶的大肠杆菌经过发酵可以产生4.33mg/L的N-甲基吡咯啉；表达上述三种酶的酿酒酵母经过发酵可以产生3.22mg/L的N-甲基吡咯啉；表达上述三种酶、基因组中敲除了基因ALD4、ALD5、HFD1的酿酒酵母经过发酵可以产生9.88mg/L的N-甲基吡咯啉；表达上述三种酶、基因组中敲除了基因ALD4、ALD5和HFD1、并且过表达SAM2基因的酿酒酵母经过发酵可以产生21.92mg/L的N-甲基吡咯啉，具有工业化开发和应用前景。

附图说明

[0028] 图1显示了从L-鸟氨酸开始反应直至产物N-甲基吡咯啉的生物合成途径。其中方框内显示了N-甲基吡咯啉生成N-甲基吡咯烷的检测反应。

[0029] 图2A显示了AaDAO2及AaDAO3催化N-甲基化腐胺转化成N-甲基吡咯啉的反应结果质谱图。其中(i)是氘代-N-甲基吡咯烷标准品；(ii)是AaDAO2煮沸对照；(iii)是AaDAO2反应；(iv)是AaDAO3煮沸对照；(v)是AaDAO3反应产物。

[0030] 图2B显示了EcODC、AtPMT和AaDAO3混合酶联合催化L-鸟氨酸转化成N-甲基吡咯啉的反应结果质谱图。其中(i)是氘代-N-甲基吡咯烷标准品；(ii)是AtPMT及AaDAO3空白对照；(iii)是AaDAO3空白对照；(iv)混合酶反应产物。

[0031] 图3显示了与N-甲基吡咯啉合成途径相关的多种酶的纯化蛋白SDS-PAGE凝胶电泳照片。

[0032] 图4显示了产N-甲基吡咯啉的大肠杆菌工程菌的构建示意图。

[0033] 图5显示了大肠杆菌工程菌BL21-OP-A3的发酵历程及N-甲基吡咯啉产量图。

[0034] 图6显示了产N-甲基吡咯啉的酿酒酵母工程菌的构建示意图。

[0035] 图7A显示了酿酒酵母工程菌BY4742-OP-A3的发酵历程及N-甲基吡咯啉产量图。

[0036] 图7B显示了酿酒酵母工程菌ΔBY4742-OP-A3的发酵历程及N-甲基吡咯啉产量图。

[0037] 图7C显示了酿酒酵母工程菌ΔBY4742-OP-A3-SAM2的发酵历程及N-甲基吡咯啉产量图。

[0038] 图8A是N-甲基化腐胺的核磁共振氢谱图(^{1}H NMR)。

[0039] 图8B是N-甲基化腐胺的核磁共振碳谱图(^{13}C NMR)。

[0040] 图9A是N-甲基吡咯啉的核磁共振氢谱图(^{1}H NMR)。

[0041] 图9B是N-甲基吡咯啉的核磁共振碳谱图(^{13}C NMR)。

具体实施方式

[0042] 以下结合具体实施例对本发明做进一步详细说明。应理解，以下实施例仅用于说明本发明而非用于限定本发明的范围。

[0043] 本文中涉及到多种物质的添加量、含量及浓度，其中所述的百分含量，除特别说明

外，皆指质量百分含量。

[0044] 本发明通过三种酶组成的联合催化体系“一锅法”催化L-鸟氨酸进行数步反应，合成得到N-甲基吡咯啉。其中术语“联合催化体系”是指鸟氨酸脱羧酶EcODC、腐胺-N-甲基转移酶AtPMT和胺氧化酶(AaDAO1、AaDAO2或AaDAO3)的组合，包括但不限于酶表达菌株的组合。

[0045] 当作为生物催化剂用于生产化合物I时，本发明的酶EcODC、AtPMT、AaDAO1、AaDAO2和AaDAO3可以呈现酶的形式或者菌体的形式。所述酶的形式包括游离酶、固定化酶，包括纯化酶、粗酶、发酵液、载体固定的酶等。而且这些酶的分离纯化、包括固定化酶制备技术也是本领域技术人员所熟知的。所述菌体的形式包括存活菌体和死亡菌体，包括冻融的菌体、固定化的菌体。

[0046] 当采用微生物发酵来生产N-甲基吡咯啉时，就不需要另外添加L-鸟氨酸作为底物，因为大肠杆菌和酿酒酵母在生长过程中会在细胞内产生L-鸟氨酸，仅需要发酵就可以作为“微型生物工厂”将培养液中的碳源和氮源通过代谢转化为N-甲基吡咯啉。

[0047] 在本文中，有时为了描述简便，会将酶比如鸟氨酸脱羧酶EcODC蛋白质名称与其编码基因(DNA)名称混用，本领域技术人员应能理解它们在不同描述场合表示不同的物质。例如，对于EcODC(基因)，用于描述鸟氨酸脱羧酶功能或类别时，指的是蛋白质；在作为一种基因描述时，指的是编码该鸟氨酸脱羧酶的基因，以此类推，这是本领域技术人员容易理解的。

[0048] 本发明中使用的酶EcODC、AtPMT、AaDAO1、AaDAO2和AaDAO3序列明确，因此本领域技术人员能够容易地获得其编码基因、包含这些基因的表达盒和质粒、以及包含该质粒的转化体。这些基因、表达盒、质粒、转化体可以通过本领域技术人员所熟知的基因工程构建方式获得。

[0049] 本发明还建立了一种检测N-甲基吡咯啉含量的方法，通过使$NaBD_4$同位素(优选1M溶于0.1M硼酸钠缓冲溶液，pH10.0)还原N-甲基吡咯啉后生成氘代-N-甲基吡咯烷，结合液相-质谱检测，确定液体中的N-甲基吡咯啉含量。借由该检测方法，可以测定胺氧化酶(即AaDAO1、AaDAO2、AaDAO3)催化N-甲基化腐胺转化成N-甲基吡咯啉的活性；并且可以测定EcODC、AtPMT和胺氧化酶(即AaDAO1、AaDAO2或者AaDAO3)混合酶联合催化L-鸟氨酸转化成N-甲基吡咯啉的活性。图1中的方框内显示了N-甲基吡咯啉与$NaBD_4$的同位素还原反应，图2A和图2B显示了氘代-N-甲基吡咯烷的质谱检测结果。

[0050] 实施例

[0051] 材料和方法

[0052] 实施例中的全基因合成、引物合成及测序皆由苏州金唯智生物科技有限公司完成。

[0053] 实施例中的分子生物学实验包括质粒构建、酶切、连接、感受态细胞制备、转化、培养基配制等等，主要参照《分子克隆实验指南》(第三版)，J.萨姆布鲁克，D.W.拉塞尔(美)编著，黄培堂等译，科学出版社，北京，2002)进行。必要时可以通过简单试验确定具体实验条件。

[0054] PCR扩增实验根据质粒或DNA模板供应商提供的反应条件或试剂盒说明书进行。必要时可以通过简单试验予以调整。

[0055] LB培养基:10g/L胰蛋白胨、5g/L酵母提取物、10g/L氯化钠,pH7.0。

[0056] YPD培养基:1wt%酵母提取物,2wt%蛋白胨,2wt%葡萄糖。

[0057] YPAD培养基:10.0g/L酵母提取物、20.0g/L蛋白胨、20.0g/L D-葡萄糖、0.4g/L硫酸腺嘌呤。

[0058] HPLC测定条件:液相-质谱联用仪为Agilent 1290UHPLC-Agilent 6545Q-TOF ESI质谱;色谱柱为Agilent C18(4.6×150mm,3.5μm),流动相A为含0.1%甲酸水溶液,流动相B为0.1%乙腈,流速为0.35mL/min。分离程序为:0-1min,2%B;1-2min,2-3%B;2-10min,3-5%B;10-12min,5-95%B;12-15min,95%B;15-16min,95-2%B。质谱使用正模式,N-甲基吡咯啉被$NaBD_4$还原后生成N-甲基吡咯烷,目标化合物分子量为87.1027。

[0059] 实施例1N-甲基吡咯啉生物合成途径相关酶的制备

[0060] 1.制备鸟氨酸脱羧酶EcODC

[0061] 1.1以苏州金唯智生物科技有限公司合成的EcODC编码基因(SEQ ID NO:1)为模板,以引物对(SEQ ID NO:3、4)用KOD DNA聚合酶进行PCR扩增(94℃ 2min;98℃ 10s,55℃ 30s,68℃ 1min;30个循环;68℃ 7min),利用Axygen胶回收试剂盒(AxyPrep DNAGel ExtractionKit试剂盒)回收目的DNA片段。

[0062] 1.2用Thermofisher公司的NdeI及NotI双酶切胶回收片段及pET-24a(+)(Novagen),37℃酶切2h,用AXYGEN公司的AxyPrep PCR Cleanup Kit对酶切产物进行清洁回收,Axygen胶回收试剂盒(AxyPrep DNA Gel Extraction Kit试剂盒)胶回收目的片段。用NEB公司的T4DNA连接酶20℃连接2h,连接体系加入Top10感受态细胞,冰浴10min,42℃热激1min30s,置冰上5min,加入1mL LB,37℃摇床培养,200rpm,孵育45min。12000rpm离心1min,弃800微升上清,剩余200微升涂布含有50μg/mL卡那抗生素的固体LB平板,37℃培养箱培养过夜。

[0063] 1.3挑取单克隆转化子测序验证,测序正确的质粒pET24a-EcODC转化大肠杆菌E.coliBL21(DE3),涂布到含有卡那霉素的LB平板。挑单菌落于10mL含卡那抗生素(50μg/mL)LB中,37℃,200rpm培养过夜。将10mL菌液转接于1L摇瓶中,37℃,200rpm,培养至OD_{600}约为0.6左右,加入500μL 1M IPTG(终浓度为0.5mM),16℃诱导20h。

[0064] 1.4收集摇瓶中菌体,加入5倍重量体积裂解缓冲液(50mM磷酸钾,pH 8.0,150mMNaCl),压榨破碎,800bar,3次。于4℃,18000rpm下离心45min。上清液与Ni柱混合孵育40min,收集流出的上清流穿液(Flow-through,FT),用含25mM咪唑的裂解缓冲液洗脱杂蛋白20个柱体积,250mM洗脱目的蛋白,SDS-PAGE检测,得到纯度高的目的蛋白EcODC,其氨基酸序列为SEQ ID NO:2。

[0065] 2.制备腐胺N-甲基转移酶AtPMT、胺氧化酶AaDAO1、AaDAO2和AaDAO3

[0066] 按照与上述步骤相同的方法分别获得质粒pET24a-AtPMT、pET30a-AaDAO1、pET30a-AaDAO2及pET30a-AaDAO3,并且纯化目的蛋白。

[0067] 这些酶的纯化蛋白的SDS-PAGE凝胶电泳图如图3所示,得到了可溶、且纯度高的蛋白EcODC、AaDAO2及AaDAO3,可用于下一步酶活实验。其中

[0068] AtPMT基因序列为SEQ ID NO:5,用引物对(SEQ ID NO:7、8)扩增获得,编码的酶AtPMT的氨基酸序列为SEQ ID NO:6。

[0069] AaDAO1基因(SEQ ID NO:9)以植物三分三cDNA为模板,用引物对(SEQ ID NO:11、

12)扩增获得,编码的酶AaDAO1的氨基酸序列为SEQ ID NO:10。

[0070] AaDAO2基因(SEQ ID NO:13)以三分三cDNA为模板,用引物对(SEQ ID NO:15、16)扩增获得,编码的酶AaDAO2的氨基酸序列为SEQ ID NO:14。

[0071] AaDAO3基因(SEQ ID NO:17)以三分三cDNA为模板,用引物对(SEQ ID NO:19、20)扩增获得,编码的酶AaDAO3的氨基酸序列为SEQ ID NO:18。

[0072] 实施例2AaDAO2、AaDAO3活性测试及体外混合酶反应合成N-甲基吡咯啉

[0073] 2.1胺氧化酶AaDAO2及AaDAO3活性实验(100微升):

[0074] 2mM底物N-甲基化腐胺、20μM $CuSO_4$、50mM磷酸钾(pH 8.0)、酶蛋白10μM,用水补齐形成100微升体系,30℃反应30min。加入10微升$NaBD_4$(母液1M溶于0.1M硼酸钠缓冲液,pH10.0)还原。液相-质谱检测到,N-甲基吡咯啉可被还原生成N-甲基吡咯烷,目标化合物分子量为87.1027。如图2A所示,实验结果表明,AaDAO2及AaDAO3均可催化N-甲基化腐胺生成N-甲基吡咯啉。

[0075] 2.2EcODC、AtPMT及AaDAO3混合酶反应(100微升):

[0076] 5mM底物L-鸟氨酸、5mM PLP(吡哆醛磷酸醛)、5mM SAM(S-腺苷基甲硫氨酸)、20μM $CuSO_4$、10μM EcODC,10μM AtPMT,10μM AaDAO3。反应液30℃孵育2h,产物检测步骤与上述步骤1实验相同。如图2B所示,实验结果表明,EcODC、AtPMT、AaDAO3三酶联合可以体外催化从L-鸟氨酸到N-甲基吡咯啉的转化。

[0077] 实施例3产N-甲基吡咯啉的大肠杆菌工程菌的构建和发酵

[0078] 3.1以EcODC的编码基因(SEQ ID NO:1)为模板,以引物对(SEQ ID NO:21、22)用KOD DNA聚合酶进行PCR扩增(94℃ 2min;98℃ 10s,55℃ 30s,68℃ 1min;30个循环;68℃ 7min)。琼脂糖凝胶电泳,回收目的片段。

[0079] 3.2用BamHI及HindIII双酶切回收片段及pACYCDuet-1质粒,37℃酶切2h,酶切产物进行清洁回收,胶回收酶切后的pACYCDuet-1质粒,用T4DNA连接酶20℃连接2h。连接体系加入E.coli TOP10感受态,冰浴10min,42℃热激1min30s,置冰上5min,加入1mL LB,37℃,200rpm摇床孵育45min。12000rpm离心1min,弃800微升上清,剩余200微升涂布含有34μg/mL氯霉素的固体LB平板,37℃培养箱培养过夜。挑取单克隆转化子测序,得到正确的质粒pACYCDuet-EcODC。

[0080] 3.3以AtPMT的编码基因(SEQ ID NO:5)为模板,以引物对(SEQ ID NO:23、24)用KOD DNA聚合酶进行PCR扩增(94℃ 2min;98℃ 10s,55℃ 30s,68℃ 1min;30个循环;68℃ 7min),琼脂糖凝胶电泳,回收目的片段。

[0081] 3.4用NdeI及KpnI双酶切胶回收片段及pACYCDuet-EcODC质粒,37℃酶切2h,胶回收目的片段,用T4DNA连接酶20℃连接2h。连接体系加入E.coli TOP10感受态,冰浴10min,42℃热激1min30s,置冰上5min,加入1mL LB,37℃,200rpm摇床孵育45min。12000rpm离心1min,弃800微升上清,剩余200微升涂布含有34μg/mL氯霉素的固体LB平板,37℃培养箱培养过夜。挑取单克隆转化子测序,得到正确的质粒pACYCDuet-EcODC-AtPMT。该质粒包含了EcODC和AtPMT两种基因。

[0082] 3.5将1微升pACYCDuet-EcODC-AtPMT质粒转化入大肠杆菌BL21(DE3),得到菌株BL21-OP。将BL21-OP菌株制备感受态,将实施例1中制备的质粒pET30a-AaDAO3转化感受态细胞,得到菌株BL21-OP-A3,菌株构建示意图如图4所示。

[0083] 3.6从平板挑取菌株单克隆，在含有LB液体培养基(含有34μg/mL氯霉素及50μg/mL卡那霉素)的试管中摇床培养过夜。转接含50毫升LB的锥形瓶，待OD_{600}至0.6-0.8，加入0.15mM IPTG，及50μM $CuSO_4$，菌液置于25℃摇床，120rpm进行发酵，不同时间点取样检测OD_{600}及N-甲基吡咯啉产量。如图5所示，实验结果表明，菌株可以合成N-甲基吡咯啉，在48h产量达到4.33mg/L。

[0084] 实施例4产N-甲基吡咯啉的酿酒酵母工程菌的构建

[0085] 4.1构建BY4742-OP酵母菌株：

[0086] 4.1.1利用表1中所述引物和模板，通过PCR的方法扩增EcODC、AtPMT基因和酵母内源启动子PGK1(SEQ ID NO:33)、终止子FBA1(SEQ ID NO:34)，相邻片段之间通过引物引入15～21bp同源序列。DNA聚合酶选用TSINGKE公司的高保真DNA聚合酶I-5™ 2X HighFidelity Master Mix，参考其说明书设定PCR程序：98℃ 2min；98℃ 15s，58℃ 10s，72℃ 1min，共35个循环；72℃ 5min；10℃保温。

[0087] 4.1.2对PCR产物进行琼脂糖凝胶电泳检测，在紫外光下，切下与目标DNA大小一致的条带。然后采用AXYGEN公司的AxyPrep DNAGel ExtractionKit从琼脂糖凝胶中回收DNA片段。用Takara公司的QuickCut限制性内切酶BamHI和EcoRI双酶切酵母高拷贝质粒p2M(中国科学院上海生命科学研究院周志华实验室提供，全长序列为SEQ ID NO:35)30min，用AXYGEN公司的AxyPrep PCR Cleanup Kit对酶切产物进行清洁回收。利用Yeasen公司的Hieff Clone™ Plus Mlti One Step Cloning Kit重组酶将胶回收产物与p2M双酶切质粒进行重组连接(反应条件参照试剂盒说明书)。

[0088] 4.1.3连接产物转化E.coli TOP10感受态细胞，并涂布于添加100μg/mL氨苄青霉素的LB平板上。通过菌落PCR验证阳性转化子，并测序进一步验证，结果表明酵母表达质粒p2M-EcODC-AtPMT构建成功。该质粒包含了EcODC和AtPMT两种基因。

[0089] 4.1.4将酵母表达质粒p2M-EcODC-AtPMT转入酿酒酵母BY4742感受态中(感受态制作与转化方法参照(Gietz and Schiestl 2007.)，用无菌接种环挑取BY4742单克隆至5mLYPAD液体培养基，200rpm，30℃培养；12h-16h后转接至50mL YPAD培养基中，200rpm，30℃，培养至OD_{600}为2时，3000g离心5min，收集菌体，重悬于25mL无菌水；3000g，离心5min收集菌体，菌体重悬于1mL无菌水，吸取100微升菌液至1.5mL离心管中，13000g，离心30s，弃上清。配制转化体系：240μL PEG 3350(50％w/v)，36μL 1.0M LiAc，50μL Single-stranded carrier DNA(Invitrogen)(2.0mg/mL)，34μL质粒p2M-EcODC-AtPMT，总体积为360μL。将此转化体系加入BY4742感受态中，重悬菌体，42℃热激40min，13000g离心30s，弃上清，加入1mL YPD培养基，30℃，200rpm，孵育2h。取200微升涂布于添加300μg/mL潮霉素B(Hygromycin B)的YPAD平板上，30℃培养48h。通过酵母菌落PCR验证得到阳性转化子，即得BY4742-OP菌株。

[0090] 表1

[0091]

片段名称	模板	正向引物	反向引物
EcODC基因	pACYCDuet-EcODC-AtPMT质粒	SEQ ID NO:25	SEQ ID NO:26
FBA1终止子	酿酒酵母BY4742基因组	SEQ ID NO:27	SEQ ID NO:28
PGK1启动子	酿酒酵母BY4742基因组	SEQ ID NO:29	SEQ ID NO:30
AtPMT基因	pACYCDuet-EcODC-AtPMT质粒	SEQ ID NO:31	SEQ ID NO:32

[0092] 4.2构建BY4742-OP-A3酵母菌株：

[0093] 4.2.1利用表2中引物和模板，通过PCR的方法扩增转化片段：X-4位点上游同源序列(SEQ ID NO:48)、TEF1启动子(SEQ ID NO:49)、AaDAO3基因、PRM9终止子(SEQ ID NO:50)、KanMX表达盒(SEQ ID NO:51)、X-4位点下游同源序列(SEQ ID NO:52)，相邻片段之间通过引物引入60～80bp同源序列。DNA聚合酶选用TSINGKE公司的高保真DNA聚合酶I-5™ 2X High Fidelity Master Mix，参考其说明书设定PCR程序：98℃ 2min；98℃ 15s，58℃ 10s，72℃ 1min，共35个循环；72℃ 5min；10℃保温。

[0094] 4.2.2对PCR产物进行琼脂糖凝胶电泳检测，在紫外光下，切下与目标DNA大小一致的条带。然后采用AXYGEN公司的AxyPrep DNA Gel Extraction Kit从琼脂糖凝胶中回收DNA片段。

[0095] 4.2.3将以上6个片段转化BY4742-OP感受态中(感受态制作与转化方法参照(Gietz and Schiestl 2007.)，并涂布于添加300μg/mL潮霉素B(Hygromycin B)和200mg/L G418的YPAD平板上，30℃培养48h。通过酵母菌落PCR验证得到阳性转化子，即为BY4742-OP-A3菌株。该酿酒酵母菌中导入了EcODC、AtPMT和AaDAO3三种酶的基因。

[0096] 表2

[0097]

片段名称	模板	正向引物	反向引物
X-4位点上游同源序列	酿酒酵母BY4742基因组	SEQ ID NO:36	SEQ ID NO:37
TEF1启动子	酿酒酵母BY4742基因组	SEQ ID NO:38	SEQ ID NO:39
AaDAO3基因	pET30a-AaDAO3质粒	SEQ ID NO:40	SEQ ID NO:41
PRM9终止子	酿酒酵母BY4742基因组	SEQ ID NO:42	SEQ ID NO:43
KanMX表达盒	pMEL13质粒*	SEQ ID NO:44	SEQ ID NO:45
X-4位点下游同源序列	酿酒酵母BY4742基因组	SEQ ID NO:46	SEQ ID NO:47

[0098] *pMEL13质粒是商业化质粒，可用来转录sgRNA，从euroscarf购买得到。

[0099] 4.3构建脱氢酶敲除菌株△BY4742：

[0100] 4.3.1在Yeastriction网站(http://yeastriction.tnw.tudelft.nl/)上设计敲除酿酒酵母内源基因ALD4(SEQ ID NO:53)、ALD5(SEQ ID NO:54)、HFD1(SEQ ID NO:55)的sgRNA序列(粗体为PAM序列)：ALD4：5’-CTTGTCCTGTTCAATTAATTCGG-3’；ALD5：5’-TGGCAAATGATTCTCAATATGGG-3’；HFD1：5’-AGGGTAAAATCATTCCAATATGG-3’。

[0101] 利用表3中引物和模板，通过PCR的方法扩增得到CAS9基因(SEQ ID NO:56)片段，两端通过引物引入21bp同源序列。DNA聚合酶选用TSINGKE公司的高保真DNA聚合酶I-5™ 2X High Fidelity Master Mix，参考其说明书设定PCR程序：98℃ 2min；98℃15s，58℃ 10s，72℃ 1.5min，共35个循环；72℃ 5min；10℃保温。

[0102] 4.3.2对PCR产物进行琼脂糖凝胶电泳检测，在紫外光下，切下与目标DNA大小一致的条带。然后采用AXYGEN公司的AxyPrep DNA Gel Extraction Kit从琼脂糖凝胶中回收DNA片段。用Takara公司的QuickCut限制性内切酶BamHI和XhoI双酶切酵母高拷贝质粒p2M(中国科学院上海生命科学研究院周志华实验室提供，全长序列见SEQ ID NO:43)30min，用AXYGEN公司的AxyPrep PCR Cleanup Kit对酶切产物进行清洁回收。利用Yeasen公司的Hieff Clone™ Plus Mlti One Step Cloning Kit重组酶将胶回收产物与p2M双酶切质粒进行重组连接。

[0103] 4.3.3将重组产物转化E.coli TOP10感受态细胞,并涂布于添加100μg/mL氨苄青霉素的LB平板上。通过菌落PCR验证阳性转化子,并测序进一步验证,结果表明CAS9表达质粒p2M-CAS9的构建成功。

[0104] 表3

[0105]

片段名称	模板	正向引物	反向引物
CAS9片段	IMX673菌株*基因组	SEQ ID NO:63	SEQ ID NO:64
线性化pMEL13-ALD4	pMEL13质粒	SEQ ID NO:65	SEQ ID NO:66
ALD4-repair-UP	酿酒酵母BY4742基因组	SEQ ID NO:67	SEQ ID NO:68
ALD4-repair-DOWN	酿酒酵母BY4742基因组	SEQ ID NO:69	SEQ ID NO:70
线性化pMEL13-ALD5	pMEL13质粒	SEQ ID NO:71	SEQ ID NO:72
ALD5-repair-UP	酿酒酵母BY4742基因组	SEQ ID NO:73	SEQ ID NO:74
ALD5-repair-DOWN	酿酒酵母BY4742基因组	SEQ ID NO:75	SEQ ID NO:76
线性化pMEL13-HFD1	pMEL13质粒	SEQ ID NO:77	SEQ ID NO:78
HFD1-repair-UP	酿酒酵母BY4742基因组	SEQ ID NO:79	SEQ ID NO:80
HFD1-repair-DOWN	酿酒酵母BY4742基因组	SEQ ID NO:81	SEQ ID NO:82

[0106] *IMX673菌株是商业化酿酒酵母菌株,从euroscarf购买得到。

[0107] 4.3.4敲除ALD4基因:

[0108] 利用表3中引物和模板,通过PCR的方法扩增得到线性化pMEL13-ALD4质粒,两端通过引物引入20bp的sgRNA同源序列。DNA聚合酶选用TSINGKE公司的高保真DNA聚合酶I-5™ 2X High Fidelity Master Mix,参考其说明书设定PCR程序:98℃ 2min;98℃15s,58℃ 10s,72℃ 1.5min,共35个循环;72℃ 5min;10℃保温。

[0109] 对PCR产物进行琼脂糖凝胶电泳检测,在紫外光下,切下与目标DNA大小一致的条带。然后采用AXYGEN公司的AxyPrep DNA Gel Extraction Kit从琼脂糖凝胶中回收DNA片段。利用Yeasen公司的Hieff Clone™ Plus Mlti One Step Cloning Kit重组酶将胶回收产物进行重组环化(反应条件参照试剂盒说明书)。

[0110] 将环化产物转化E.coli TOP10感受态细胞,并涂布于添加100μg/mL氨苄青霉素的LB平板上。通过菌落PCR验证阳性转化子,并测序进一步验证,结果表明sgRNA表达质粒pMEL13-ALD4的构建成功。

[0111] 利用表3中引物和模板,通过PCR的方法扩增得到ALD4-repair-UP(SEQ ID NO:57)和ALD4-repair-DOWN(SEQ ID NO:58),连接处通过引物引入53bp同源序列。DNA聚合酶选用TSINGKE公司的高保真DNA聚合酶I-5™ 2X High Fidelity Master Mix,参考其说明书设定PCR程序:98℃ 2min;98℃ 15s,58℃ 10s,72℃ 0.5min,共35个循环;72℃5min;10℃保温。

[0112] 对PCR产物进行琼脂糖凝胶电泳检测,在紫外光下,切下与目标DNA大小一致的条带。然后采用AXYGEN公司的AxyPrep DNA Gel Extraction Kit从琼脂糖凝胶中回收DNA片段。

[0113] 将得到的两个片段与构建好的两个质粒(p2M-CAS9和pMEL13-ALD4)一同转化到酿酒酵母BY4742感受态中,并涂布于添加300μg/mL潮霉素B(Hygromycin B)和200mg/LG418的YPAD平板上,30℃培养48h。通过酵母菌落PCR验证得到ALD4敲除转化子。通过传代培养丢失p2M-CAS9和pMEL13-ALD4质粒,得到△BY4742-1菌株。

[0114] 4.3.5敲除ALD5和HFD1基因：

[0115] 参照上述步骤4.3.4中敲除ALD4基因的方法，构建pMEL13-ALD5质粒，扩增ALD5-repair-UP(SEQ ID NO:59)和ALD5-repair-DOWN(SEQ ID NO:60)片段，在△BY4742-1的基础上继而敲除ALD5，通过质粒丢失得到△BY4742-2菌株。

[0116] 参照上述步骤4.3.4中敲除ALD4基因的方法，构建pMEL13-HFD1质粒，扩增HFD1-repair-UP(SEQ ID NO:61)和HFD1-repair-DOWN(SEQ ID NO:62)片段，在△BY4742-2的基础上继续敲除基因HFD1，通过质粒丢失最终得到ALD4-ALD5-HFD1敲除菌株，即△BY4742菌株。

[0117] 4.3.6构建ΔBY4742-OP-A3菌株：

[0118] 参照上述步骤4.2中构建BY4742-OP-A3菌株的方法，将EcODC、AtPMT和AaDAO3基因转入△BY4742菌株中，即得到ΔBY4742-OP-A3菌株。该酿酒酵母菌中导入了EcODC、AtPMT和AaDAO3三种酶的基因，并且敲除了ALD4、ALD5和HFD1基因。

[0119] 4.4构建过表达基因SAM2的ΔBY4742-OP-A3-SAM2酵母菌株：

[0120] 4.4.1利用表4中引物和模板，通过PCR的方法扩增转化片段：X-4位点上游同源序列、TEF1启动子、AaDAO3基因、PRM9终止子、PGI终止子(SEQ ID NO:83)、SAM2基因(SEQ ID NO:84)、TCCTDH启动子(经强化后的TDH3启动子，序列SEQ ID NO:85)、KanMX表达盒、X-4位点下游同源序列，相邻片段之间通过引物引入60～80bp同源序列。DNA聚合酶选用TSINGKE公司的高保真DNA聚合酶I-5^{TM} 2X High Fidelity Master Mix，参考其说明书设定PCR程序：98℃ 2min；98℃ 15s，58℃ 10s，72℃ 1min，共35个循环；72℃ 5min；10℃保温。

[0121] 4.4.2对PCR产物进行琼脂糖凝胶电泳检测，在紫外光下，切下与目标DNA大小一致的条带。然后采用AXYGEN公司的AxyPrep DNA Gel ExtractionKit从琼脂糖凝胶中回收DNA片段。

[0122] 4.4.3将以上9个片段和p2M-EcODC-AtPMT质粒一起转化△BY4742感受态中(感受态制作与转化方法参照(Gietz and Schiestl 2007)，并涂布于添加300μg/mL潮霉素B(Hygromycin B)和200mg/L G418的YPAD平板上，30℃培养48h。通过酵母菌落PCR验证得到阳性转化子，即△BY4742-OP-A3-SAM2菌株。该酿酒酵母菌中导入了EcODC、AtPMT和AaDAO3三种酶的基因，并且敲除了ALD4、ALD5和HFD1基因，同时又导入了辅因子合成基因SAM2。酵母菌株的构建示意图如图6所示。

[0123] 表4

[0124]

片段名称	模板	正向引物	反向引物
X-4 位点上游同源序列	酿酒酵母 BY4742 基因组	SEQ ID NO: 36	SEQ ID NO: 37
TEF1 启动子	酿酒酵母 BY4742 基因组	SEQ ID NO: 38	SEQ ID NO: 39
*Aa*DAO3 基因	pET30a-*Aa*DAO3 质粒	SEQ ID NO: 40	SEQ ID NO: 41
PRM9 终止子	酿酒酵母 BY4742 基因组	SEQ ID NO: 42	SEQ ID NO: 86
PGI 终止子	酿酒酵母 BY4742 基因组	SEQ ID NO: 87	SEQ ID NO: 88
SAM2 基因	酿酒酵母 BY4742 基因组	SEQ ID NO: 89	SEQ ID NO: 90
TCCTDH 启动子	TCCTDH 质粒*	SEQ ID NO: 91	SEQ ID NO: 92

[0125]

KanMX 表达盒	pMEL13 质粒	SEQ ID NO: 93	SEQ ID NO: 45
X-4 位点下游同源序列	酿酒酵母 BY4742 基因组	SEQ ID NO: 46	SEQ ID NO: 47

[0126] *TCCTDH序列连接到pUC57质粒中。

[0127] 实施例5产N-甲基吡咯啉的酿酒酵母工程菌的发酵

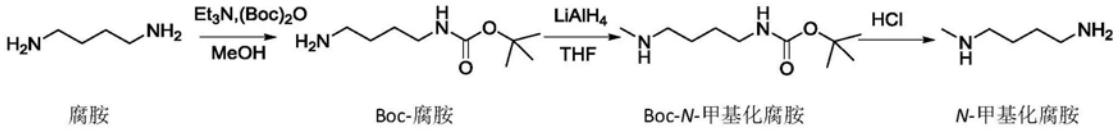
[0128] 从平板挑取野生型酿酒酵母BY4742、上述实施例3和4中构建的酿酒酵母工程菌BY4742-OP、BY4742-OP-A3、ΔBY4742-OP-A3、△BY4742-OP-A3-SAM2，在含有5mL YPD培养基的试管中摇床培养过夜。转接含50毫升YPD的锥形瓶中，加入50μM $CuSO_4$，菌液置于30℃摇床，200rpm进行发酵培养，不同时间点取样检测OD_{600}及N-甲基吡咯啉产量。如图7A所示，含有EcODC、AtPMT及AaDAO3基因的酵母菌株BY4742-OP-A3积累了N-甲基吡咯啉，发酵时间达到120h时，N-甲基吡咯啉产量为3.22mg/L。为了进一步提高目标产物产量，我们敲除了用于将N-甲氨基丁醛转化为N-甲氨基丁酸的脱氢酶基因ALD4、ALD5和HFD1，发酵结果表明酵母菌株ΔBY4742-OP-A3的N-甲基吡咯啉产量提高至9.88mg/L(图7B)。为了进一步提高N-甲基吡咯啉产量，我们过表达了SAM2来提高AtPMT催化所需辅因子SAM的供应量，优化的菌株△BY4742-OP-A3-SAM2最终生成21.92mg/L的N-甲基吡咯啉(图7C)，相比酵母菌株BY4742-OP-A3，产量提高了7倍左右。

[0129] 实施例7化学合成N-甲基化腐胺及N-甲基吡咯啉

[0130] 由于N-甲基化腐胺及N-甲基吡咯啉难以通过商业采购得到，我们通过化学方法对其进行合成作为反应底物及产物标准品。具体方法如下：

[0131] 7.1合成N-甲基化腐胺

[0132]



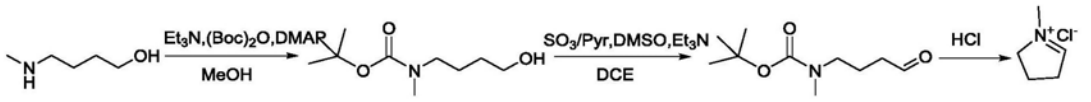
[0133] 将Et_3N(1mmol)及$(Boc)_2O$(262mg，1.2mmol)加入10mL含有1.0mol腐胺的甲醇中，室温搅拌2h，加入10mL 0.1N NaOH水溶液，并用15mL乙酸乙酯萃取。然后用20mL 0.1N HCl反萃有机相，得到的水相调节至pH 10，用20mL乙酸乙酯萃取三次，合并有机相，用饱和食盐水清洗一次，无水硫酸钠干燥，过滤，旋转干燥，得到Boc-腐胺。将$LiAlH_4$(6.33mmol)加入10mL无水THF中，冰浴，反应瓶充氮气保持无氧环境，将Boc-腐胺(1mmol)溶解于5mL无水THF，逐滴加入$LiAlH_4$/THF溶液中，反应液温度逐渐升至85℃，回流18h，冷却至室温后置于冰浴，缓慢加入硫酸钠终止反应，过滤，滤液中加入20mL 0.1N NaOH水溶液，加入25mL乙酸乙酯萃取，然后加入25mL 0.1N HCl反萃有机相，将得到的水溶液旋转干燥，得到N-甲基化腐胺纯品。

[0134] 图8A和图8B分别显示了N-甲基化腐胺的核磁共振氢谱(^1H NMR)、以及核磁共振碳谱(^{13}C NMR)。

[0135] ^1H NMR(400MHz，D_2O) δ3.09(dd，J＝15.2，7.4Hz，4H)，2.74(s，3H)，1.94-1.64(m，4H)。

[0136] ^{13}C NMR(101MHz，D_2O) δ48.26，38.79，32.68，23.83，22.56。

[0137] 7.2合成N-甲基吡咯啉

[0138] 

[0139] 将1mL Et_3N和0.1mmol DMAP逐滴加入含有1mmol N-甲氨基丁醇的5mL THF溶液中，缓慢加入1.1mmol$(Boc)_2O$，溶液室温搅拌1h，然后加入10mL 0.1N NaOH水溶液，并用15mL乙酸乙酯萃取。有机相中加入15mL 0.1N HCl，获得的水溶液调至pH 10，然后用15mL乙酸乙酯萃取3次，合并有机相，饱和食盐水干燥，过滤，旋转干燥，得到Boc-N-甲氨基丁醇。将0.5mmol Boc-N-甲氨基丁醇、1mL DMSO及0.5mL Et_3N溶解于4mL无水二氯甲烷中，加入1.5mmol SO_3/Pyr复合物，保持0℃，搅拌30min，硅胶柱纯化得到Boc-N-甲氨基丁醛，将0.4mmol Boc-N-甲氨基丁醛溶于10mL水中，加入2N HCl，室温搅拌1h，旋转干燥，得到N-甲基吡咯啉纯品。

[0140] 图9A和图9B分别显示了N-甲基吡咯啉的核磁共振氢谱(^1H NMR)、以及核磁共振碳谱(^{13}C NMR)。

[0141] ^1H NMR(500MHz,D_2O)δ8.63(s,1H),4.16(t,J＝7.4Hz,2H),3.63(s,3H),3.16(d,J＝29.4Hz,2H),2.44-2.27(m,2H)。

[0142] ^{13}C NMR(101MHz,D_2O)δ181.56,60.59,40.39,35.74,19.48。

[0143] 总之，本发明的EcODC、AtPMT和AaDAO2-3三酶联合催化体系可以“一锅法”催化L-鸟氨酸反应得到N-甲基吡咯啉，而且构建的大肠杆菌工程菌和酿酒酵母工程菌可以经过发酵直接产生N-甲基吡咯啉。本领域技术人员显然容易理解，与化学合成法相比，本发明的生物合成法具有绿色环保的天然优势，因而具有工业化开发和应用前景。

[0144] 还需说明的是，本说明书中对先前公开的文献的列举和论述不应视为承认该文献是现有技术或者是公知常识。

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[0001] 序列表

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[1267] tatatataaa atgcatgtaa aaatctttca tcatgaacta ctcggaattt ttggctggaa 420

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[1278] <213> 人工序列()

[1279] <400> 64

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[1283] <212> DNA

[1284] <213> 人工序列()

[1285] <400> 65

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[1300] <211> 48

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[1305] <210> 69

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[1434] ctcgtttctt tttcttcgtc gaaaaaggct taattaatag agattactac atattccaac 240
[1435] aagaccttcg caggaaagta tacctaaact aattaaagaa atctccgaag ttcgcatttc 300
[1436] attgaacggc tcaattaatc tttgtaaata tgagcgtttt tacgttcaca ttgccttttt 360
[1437] ttttatgtat ttaccttgca tttttgtgct aaaaggcgtc acgttttttt ccgccgcagc 420
[1438] cgcccggaaa tgaaaagtat gacccccgct agaccaaaaa tacttttgtg ttattggagg 480
[1439] atcgcaatcc ctaagcttag tggaattatt agaatgacca ctactccttc taatcaaaca 540
[1440] cgcggaaata gccgccaaaa gacagatttt attccaaatg cgggtaacta tttgtataat 600
[1441] atgtttacat attgagcccg tttaggaaag tgcaagttca aggcactaat caaaaaagga 660
[1442] gatttgtaaa tatagcgacc gaatcaggaa aaggtcaaca acgaagttcg cgatatggat 720
[1443] gaacttcggt gcctgtccgt ttaaacatcg gatcccatac tagcgttgaa tgttagcgtc 780
[1444] aacaacaaga agtttaatga cgcggaggcc aaggcaaaaa gattccttga ttacgtaagg 840
[1445] gagttagaat cattttgaat aaaaaacacg ctttttcagt tcgagtttat cattatcaat 900
[1446] actgccattt caaagaatac gtaaataatt aatagtagtg attttcctaa ctttatttag 960
[1447] tcaaaaaatt agccttttaa ttctgctgta acccgtacat gcccaaaata gggggcgggt 1020
[1448] tacacagaat atataacatc gtaggtgtct gggtgaacag tttattcctg gcatccacta 1080
[1449] aatataatgg agcccgcttt ttaagctggc atccagaaaa aaaaagaatc ccagcaccaa 1140
[1450] aatattgttt tcttcaccaa ccatcagttc ataggtccat tctcttagcg caactacaga 1200
[1451] gaacaggggc acaaacaggc aaaaaacggg cacaacctca atggagtgat gcaacctgcc 1260
[1452] tggagtaaat gatgacacaa ggcaattgac ccacgcatgt atctatctca ttttcttaca 1320
[1453] ccttctatta ccttctgctc tctctgattt ggaaaaagct gaaaaaaaag gttgaaacca 1380
[1454] gttccctgaa attattcccc tacttgacta ataagtatat aaagacggta ggtattgatt 1440
[1455] gtaattctgt aaatctattt cttaaacttc ttaaattcta cttttatagt tagtcttttt 1500
[1456] tttagtttta aaacaccaag aacttagttt cgaataaaca cacataaaca aacaaa 1556
[1457] <210> 86
[1458] <211> 58
[1459] <212> DNA
[1460] <213> 人工序列()
[1461] <400> 86
[1462] gcgcgaagga cataactcat gaagcctcca gtataccatt ttcaacatcg tattttcc 58
[1463] <210> 87
[1464] <211> 53
[1465] <212> DNA
[1466] <213> 人工序列()
[1467] <400> 87
[1468] atgcaacgct tcggaaaata cgatgttgaa aatggtatac tggaggcttc atg 53
[1469] <210> 88

[1470] <211> 57
[1471] <212> DNA
[1472] <213> 人工序列()
[1473] <400> 88
[1474] agtactcatg ggaaaaacca aagaaattgg aatttacaaa tcgctcttaa atatata 57
[1475] <210> 89
[1476] <211> 56
[1477] <212> DNA
[1478] <213> 人工序列()
[1479] <400> 89
[1480] aatgttcttt aggtatatat ttaagagcga tttgtaaatt ccaatttctt tggttt 56
[1481] <210> 90
[1482] <211> 56
[1483] <212> DNA
[1484] <213> 人工序列()
[1485] <400> 90
[1486] aacttagttt cgaataaaca cacataaaca aacaaaatgt ccaagagcaa aacttt 56
[1487] <210> 91
[1488] <211> 55
[1489] <212> DNA
[1490] <213> 人工序列()
[1491] <400> 91
[1492] tcagaggtaa ataagaaagt tttgctcttg gacattttgt ttgtttatgt gtgtt 55
[1493] <210> 92
[1494] <211> 53
[1495] <212> DNA
[1496] <213> 人工序列()
[1497] <400> 92
[1498] gtcgattcga tactaacgcc gccatccagt gtcgacgcgc catagcttca aaa 53
[1499] <210> 93
[1500] <211> 53
[1501] <212> DNA
[1502] <213> 人工序列()
[1503] <400> 93
[1504] aaaaaggagt agaaacattt tgaagctatg gcgcgtcgac actggatggc ggc 53

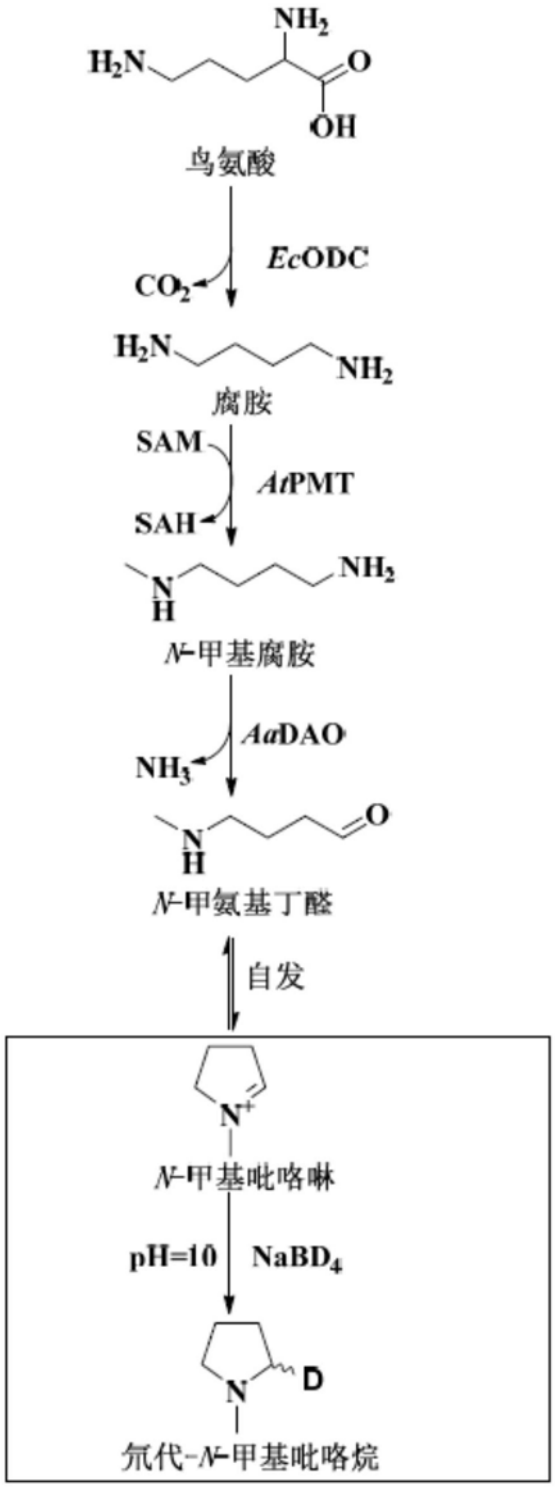
NH2
H2N
O
OH
鸟氨酸
EcODC
CO2
H2N
NH2
腐胺
SAM
AtPMT
SAH
NH2
N
H
N-甲基腐胺
AaDAO
NH3
O
N
H
N-甲氨基丁醛
自发
N+
N-甲基吡咯啉
pH=10
NaBD4
N
D
氘代-N-甲基吡咯烷

图1

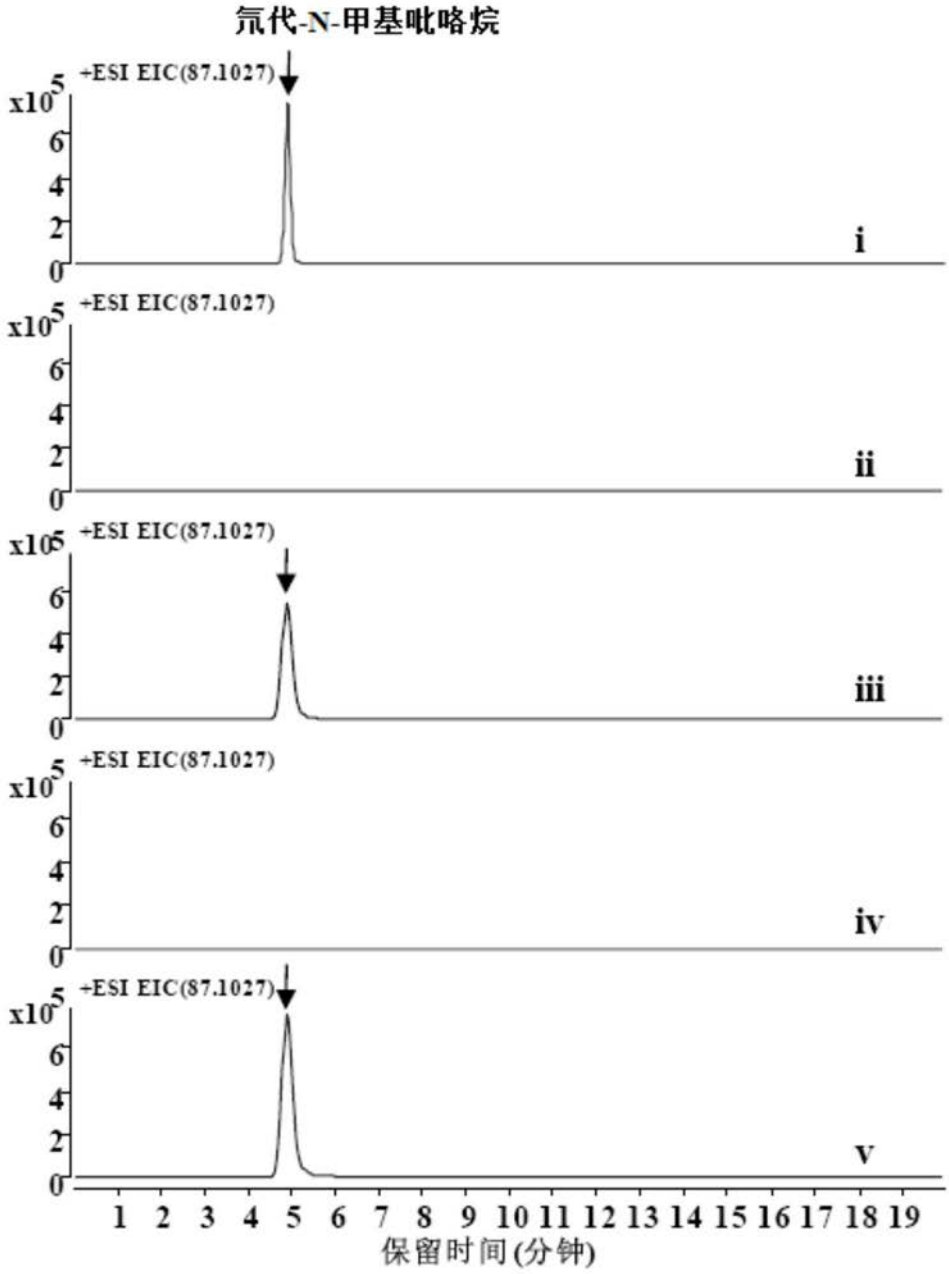
氘代-N-甲基吡咯烷
+ESI EIC(87.1027)
x10^5
i
ii
iii
iv
v
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
保留时间(分钟)

图2A

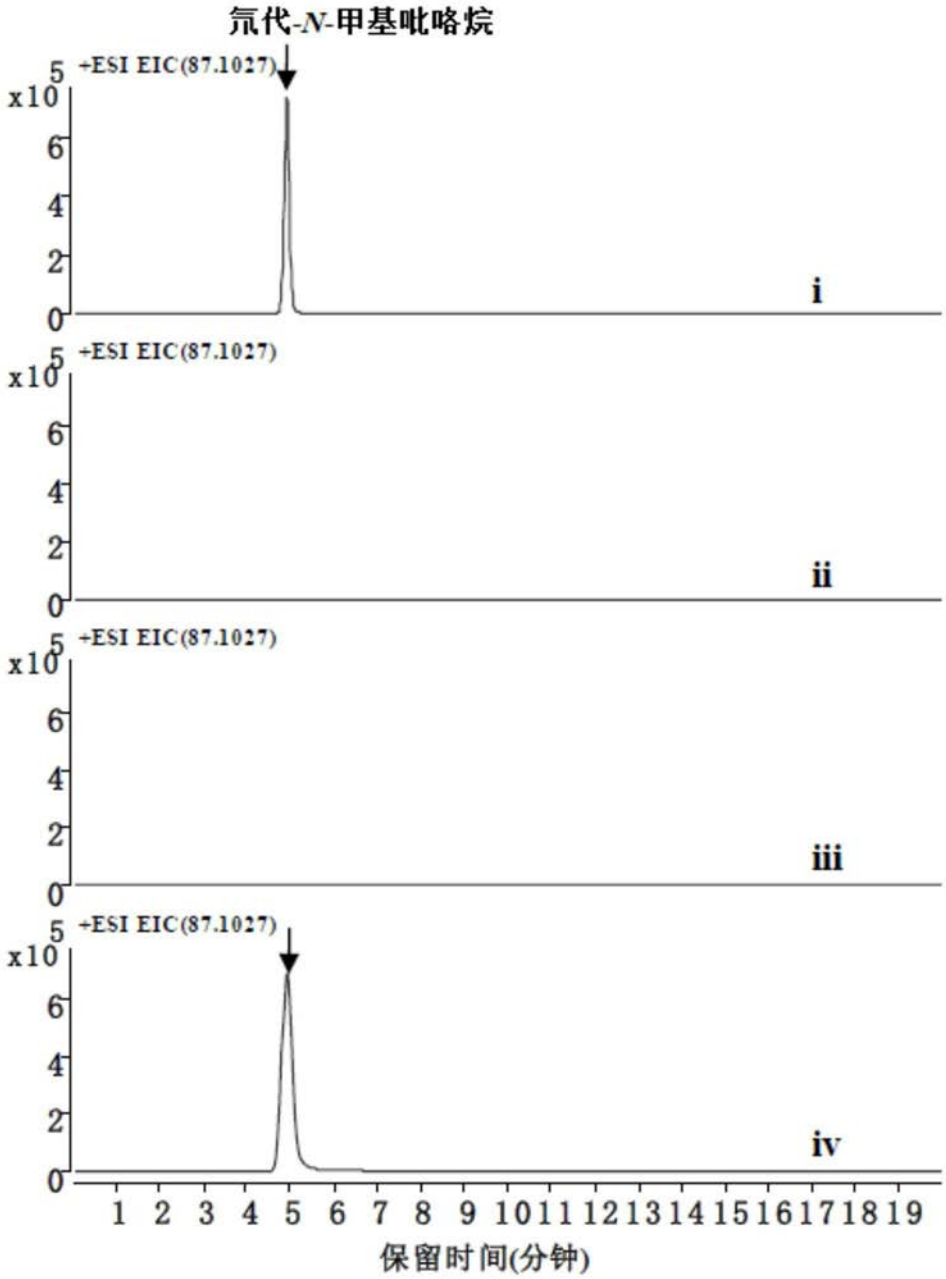
氘代-N-甲基吡咯烷
+ESI EIC(87.1027)
x10 5
6
4
2
0
i
ii
iii
iv
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
保留时间(分钟)

图2B

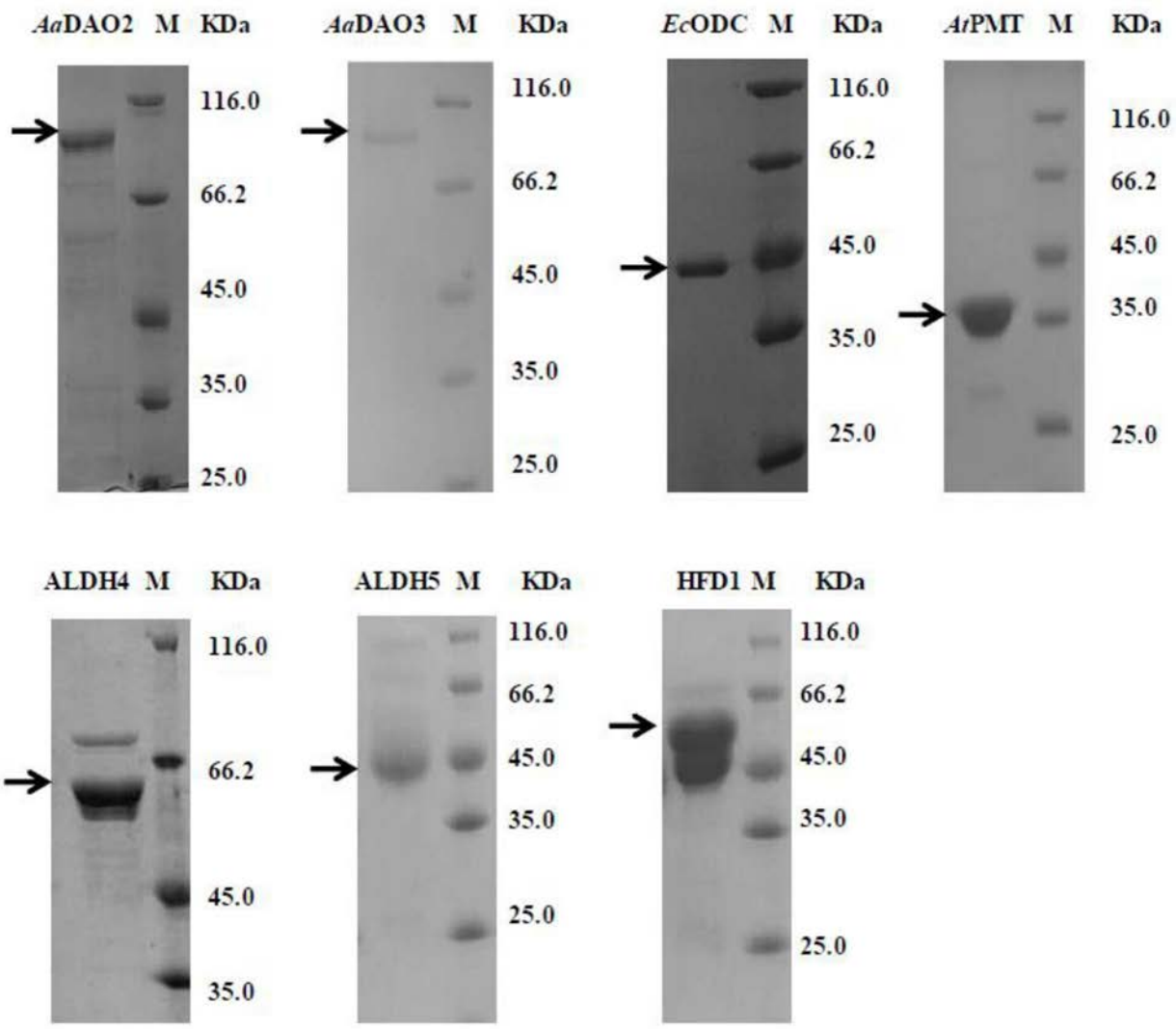
AaDAO2 M KDa
116.0
66.2
45.0
35.0
25.0
AaDAO3 M KDa
116.0
66.2
45.0
35.0
25.0
EcODC M KDa
116.0
66.2
45.0
35.0
25.0
AtPMT M KDa
116.0
66.2
45.0
35.0
25.0
ALDH4 M KDa
116.0
66.2
45.0
35.0
ALDH5 M KDa
116.0
66.2
45.0
35.0
25.0
HFD1 M KDa
116.0
66.2
45.0
35.0
25.0

图3

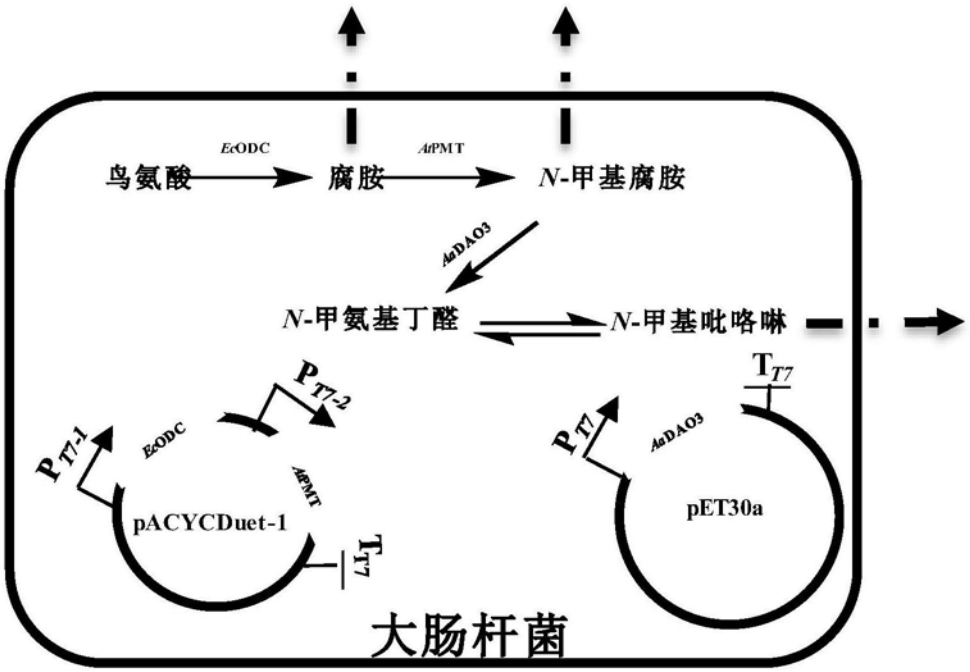
EcODC
AtPMT
鸟氨酸
腐胺
N-甲基腐胺
AaDAO3
N-甲氨基丁醛
N-甲基吡咯啉
PT7-1
EcODC
PT7-2
AtPMT
pACYCDuet-1
TT7
PT7
AaDAO3
TT7
pET30a
大肠杆菌

图4

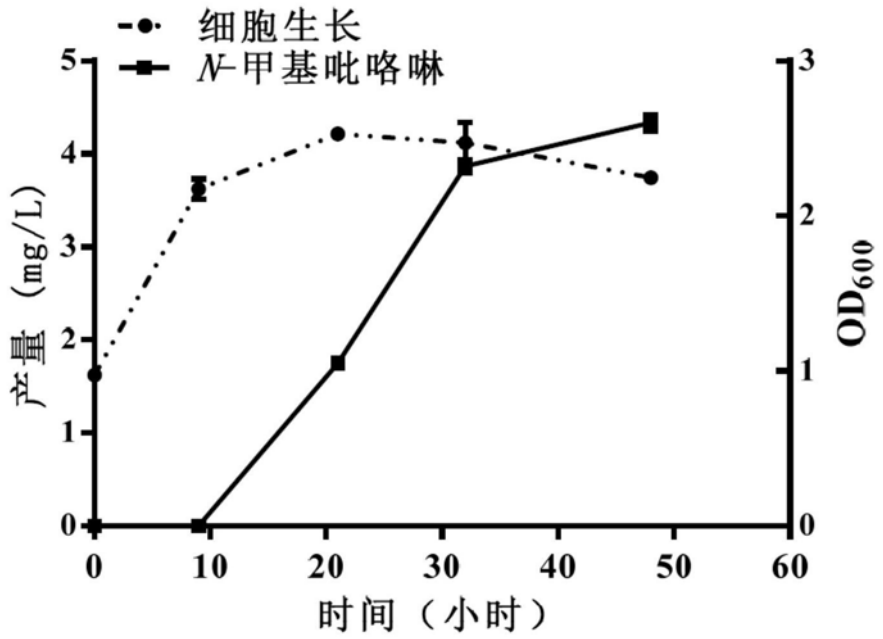
细胞生长
N-甲基吡咯啉
产量（mg/L）
OD600
时间（小时）
0
10
20
30
40
50
60
0
1
2
3
4
5

图5

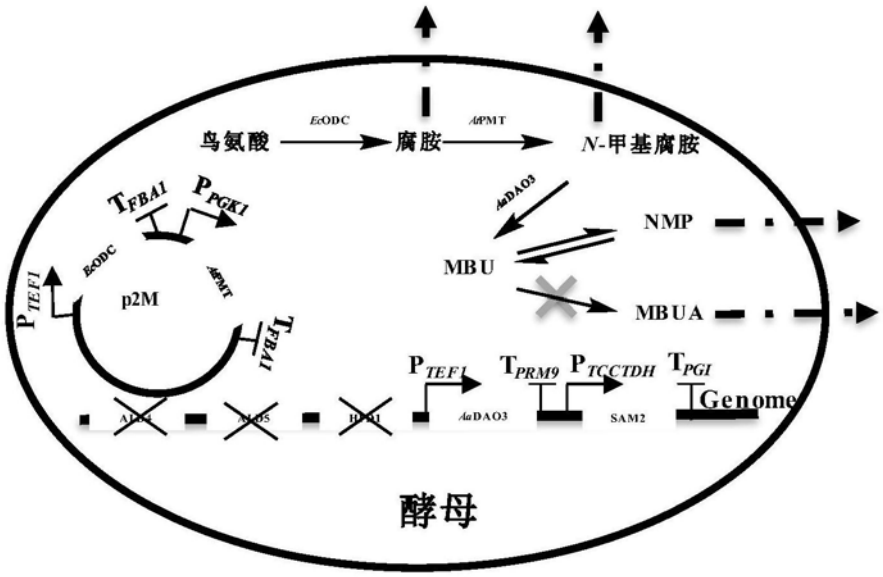
鸟氨酸
EcODC
腐胺
AtPMT
N-甲基腐胺
NMP
MBU
MBUA
AaDAO3
p2M
Genome
SAM2
酵母

图6

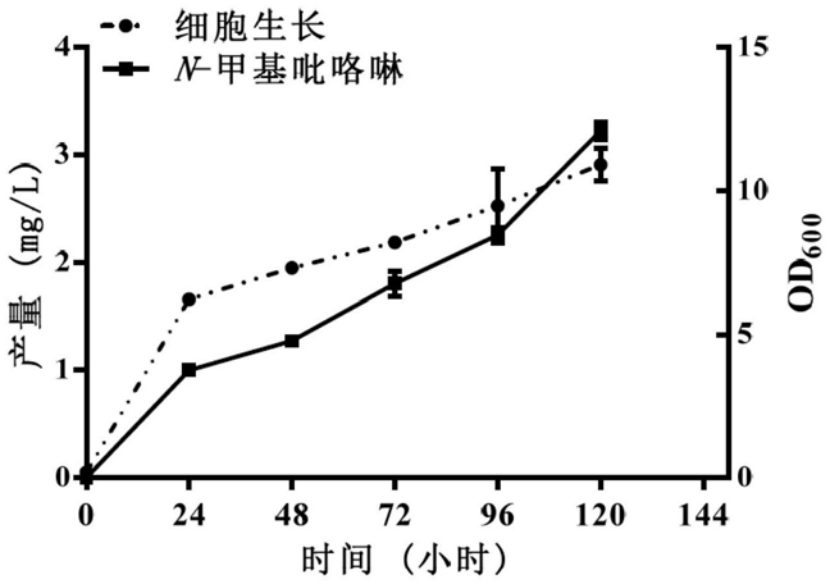
细胞生长
N-甲基吡咯啉
产量（mg/L）
OD600
时间（小时）
0
24
48
72
96
120
144
1
2
3
4
5
10
15

图7A

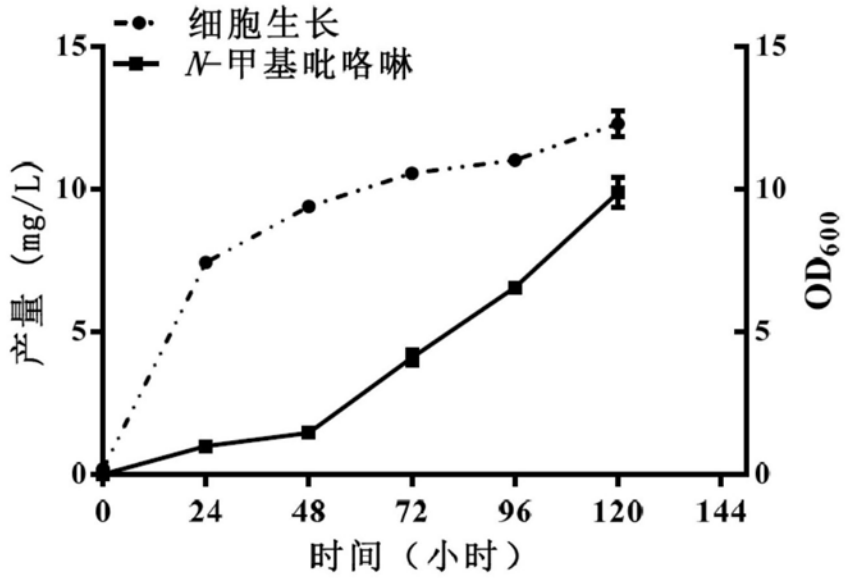
细胞生长
N-甲基吡咯啉
产量（mg/L）
OD600
时间（小时）
0
5
10
15
0
24
48
72
96
120
144

图7B

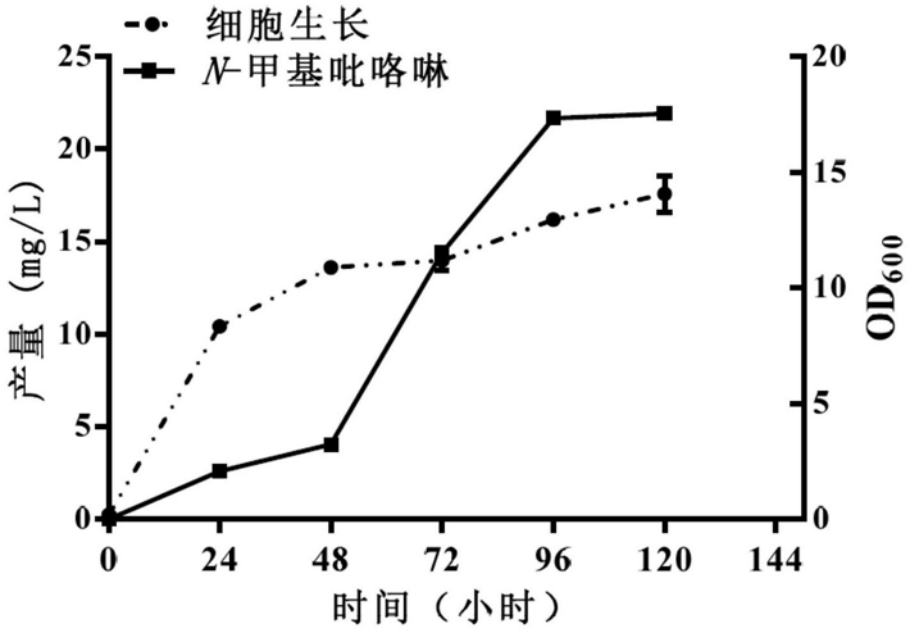
细胞生长
N-甲基吡咯啉
产量（mg/L）
OD600
时间（小时）
0
5
10
15
20
25
0
24
48
72
96
120
144

图7C

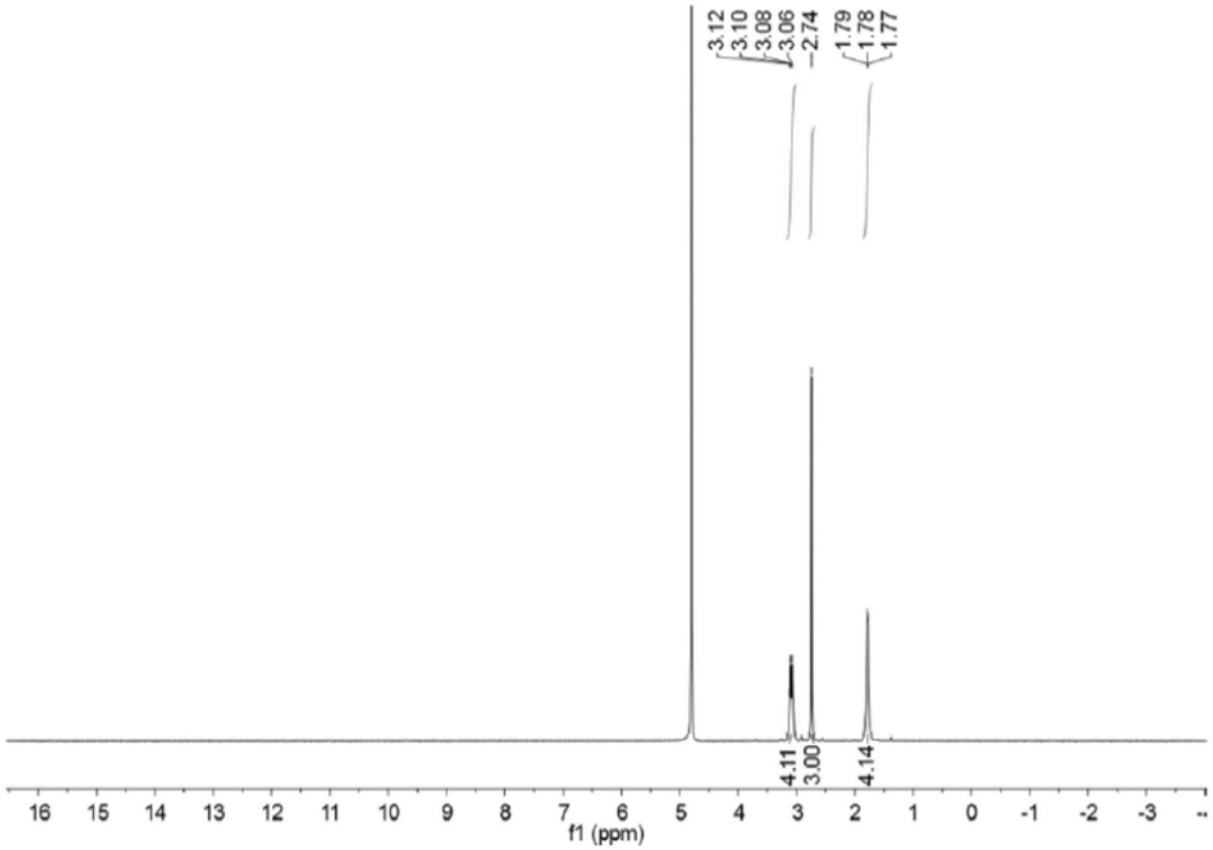
3.12
3.10
3.08
3.06
2.74
1.79
1.78
1.77
4.11
3.00
4.14
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3
f1 (ppm)

图8A

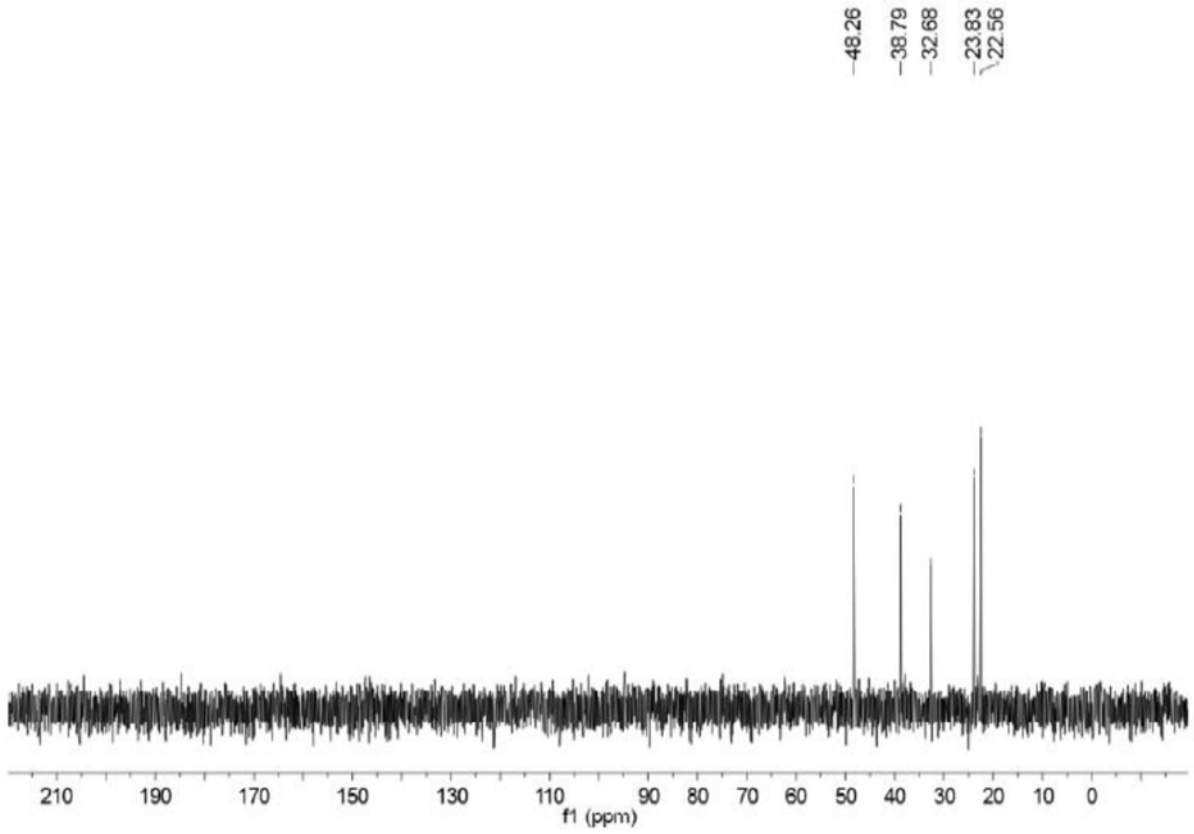
48.26
38.79
32.68
23.83
22.56
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

图8B

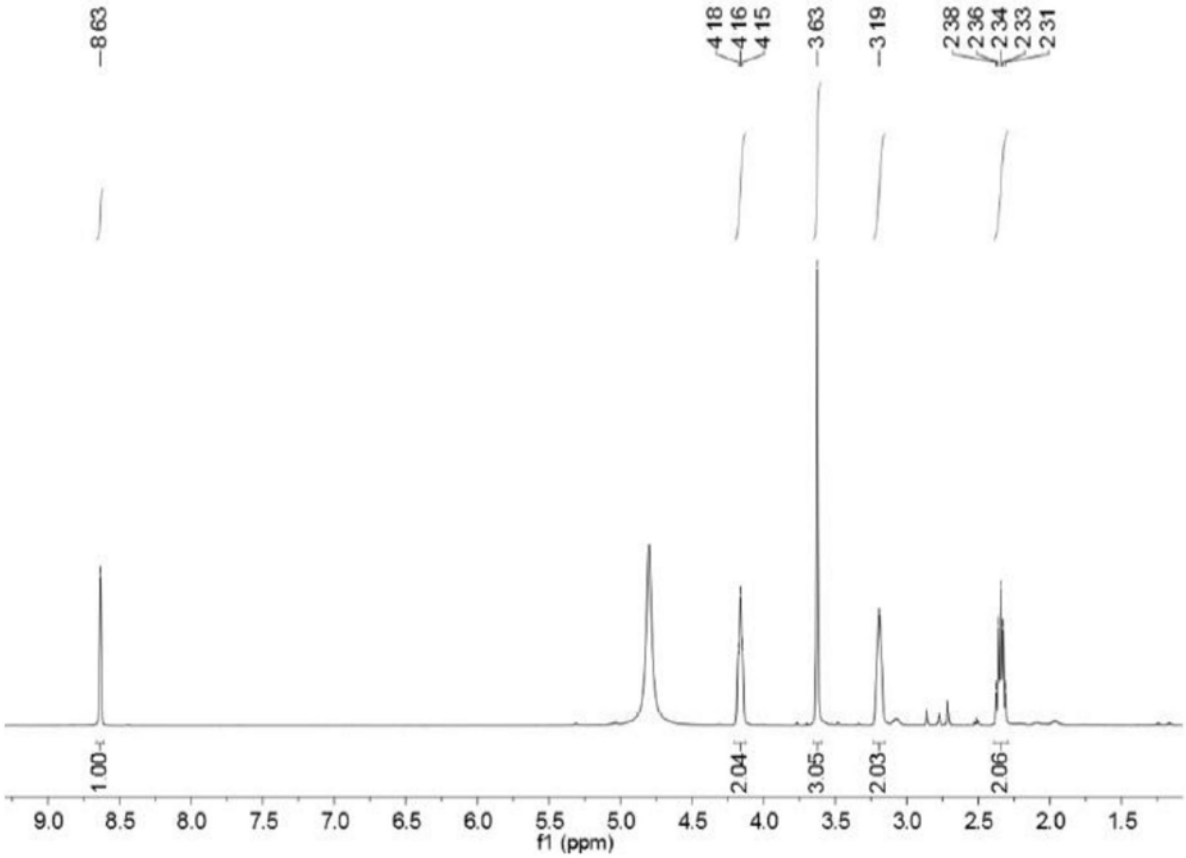
8.63
4.18
4.16
4.15
3.63
3.19
2.38
2.36
2.34
2.33
2.31
1.00
2.04
3.05
2.03
2.06
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
f1 (ppm)

图9A

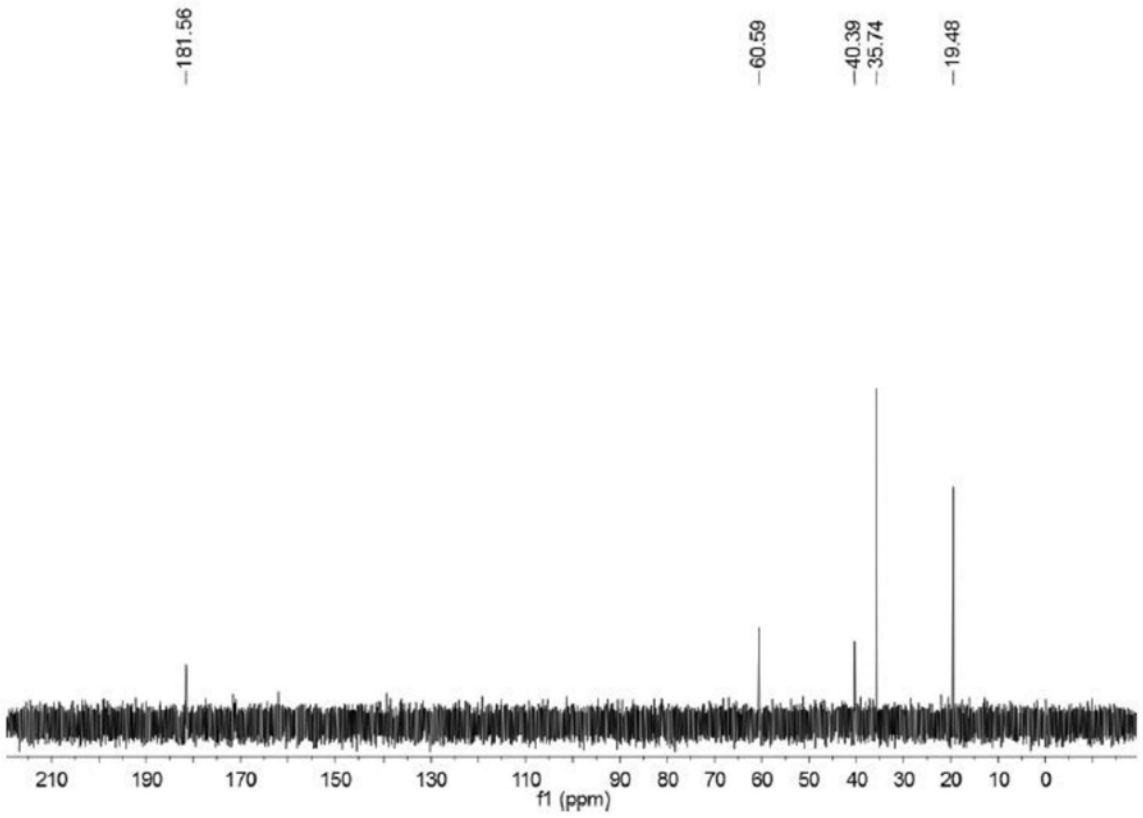
181.56
60.59
40.39
35.74
19.48
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

图9B