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生物育种与工艺优化

脂肽-糖脂混合生物表面活性剂产生菌筛选和优化培养

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摘 要：结合脂肽和糖脂的性能优势，致力于产脂肽-鼠李糖脂混合型生物表面活性剂的新菌株选育和培养条件优化。采用血平板溶血圈法初筛菌株、改进排油圈法快速检测产量以及飞行时间质谱鉴定产物结构。对优选菌株的碳源、氮源和磷酸盐缓冲液、重要金属离子浓度等进行了单因子和正交试验，优化了培养基和培养条件。采用高压液相色谱和蒽酮比色法定量分析了产物组成。筛选获得了同时积累糖脂和脂肽的新菌株，鉴定命名为芽胞杆菌 *Bacillus subtilis* THY-7。摇瓶分批培养 48 h，细胞 OD_{600} 为 37.0，产物浓度 2.4 g/L，分别是优化前的 3.4 倍和 3.1 倍。发酵罐补料分批培养，泡沫中产物浓度达到 4.5 g/L，且 74% 为表面活性素，22% 为鼠李糖脂。*B. subtilis* THY-7 是具有脂肽-鼠李糖脂高产潜力的优选菌株。

关键词：脂肽和糖脂，混合型生物表面活性剂，枯草芽胞杆菌，质谱鉴定，正交优化，定量分析

Identification of *Bacillus subtilis* THY-7 and high titer optimization for the blend-biosurfactant of lipopeptide and glycolipid

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Abstract: Biosurfactants (BSs) are highlighted owing to their multiple advantages in diverse applications. To screen a

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superior strain that producing a blend-biosurfactant of lipopeptide and glycolipid, the hemolytic activity assay on blood agar plates, the modified oil-red spreading test and MALDI-TOF Mass Spectrometry identification of the purified products was carried out. *Bacillus subtilis* THY-7 was selected and its principal products were surfactin and dirhamnolipid. The medium component and culture conditions of THY-7 were optimized by both single factor and orthogonal experiments. After 48 h optimal batch culture in flask, the cell density (OD_{600}) was 37.0 and the product titer was 2.4 g/L, which was 3.4 folds and 3.1 folds of that under original condition, respectively. A fed-batch culture in a 5 L fermentor was further performed coupling with *in situ* recovery of foam, in which the titer of blend-BS increased to 4.5 g/L at 25 h. Quantification by HPLC and anthrone colorimetry revealed that surfactin and dirhamnolipid accounted for 74% and 22% of the blend-BS, respectively.

Keywords: lipopeptide and glycolipid, blend-biosurfactant, *Bacillus subtilis*, mass spectrometry identification, orthogonal optimization, quantification analysis

由于结构独特、性能优异、环境友好，微生物合成生物表面活性剂的研究方兴未艾。生物表面活性剂在油气开采、工农业生产、医药农药、化妆品等领域都具有良好的应用前景[1-3]。糖脂和脂肽是两种主要的生物表面活性剂。糖脂 (鼠李糖脂、槐糖脂等) 由亲水性糖基和疏水性脂肪酸侧链构成[2-4]。其中，鼠李糖脂的乳化和增溶性能突出[1]，洗油效率和热稳定性较好[1,5]，受到较多关注[6-7]。表面活性素、芬芥素和伊枯草菌素是由芽胞杆菌产生的 3 种主要脂肽产物[8-9]，分别由 5~10 个氨基酸残基形成的环肽和一个长链脂肪酸侧链构成。脂肽可降低水的表面张力至 26~27 mN/m，相对于辛烷的界面张力是 10^{-2} mN/m，且在 75 ℃下至少稳定 140 h[1]，可用于提高石油采收率[10]。然而，脂肽的分子结构复杂，生物合成效率低，提高脂肽产量的研究正在起步[11-13]。

结合糖脂和脂肽的性能优势，从菌株选育出发，获得了产生混合型生物表面活性剂的新菌株，并通过培养基和培养条件优化，提高了发酵产量，为脂肽-糖脂混合型生物表面活性剂开发和三次采油等重要应用提供了新思路和新方法。

1 材料与方法

1.1 培养基及溶血圈法筛选菌株

富集培养基、初始培养基和溶血圈法筛选菌株参见文献[14]；其中初始培养基改用蔗糖为碳源，17 g/L $NaNO_3$ 为无机氮源。

优化培养基 (/L)：红糖 70 g，酵母膏 1.0 g，$NaNO_3$ 25 g，$Na_2HPO_4{\cdot}12H_2O$ 1.0 g，KH_2PO_4 0.333 g，$MgSO_4{\cdot}7H_2O$ 0.15 g，$FeSO_4{\cdot}7H_2O$ 0.006 g，$MnSO_4{\cdot}H_2O$ 0.006 g，pH 7.0。

1.2 发酵培养、产物提取和浓度测定

摇瓶培养在 37 ℃、200 r/min 摇床上进行。发酵罐培养采用 5 L Biostat B plus 发酵系统。发酵液于 4℃、8 000 r/min 离心 40 min，取上清液。采用酸沉碱溶、甲醇抽提和旋转蒸发制备金黄或亮黄色产物粉末[14]。

改进排油圈法粗测产物：在玻璃平板内加 40 mL 去离子水，取 200 μL 油红 O 染色的正十二烷平铺在水面上，稳定后加 3 μL 待测液，照相测排油圈直径 D，它和产物干重浓度 C 呈线性：C (mg/L)=34.434×D (mm)−349.92 (D 为 10~40 mm)。

脂肽 (表面活性素) 通过 HPLC-UV 法定量测定。采用 LC-20AT (Shimadzu, Kyoto, Japan) 系统，Intersil ODS-SP (GL Sciences, Kyoto, Japan) 色谱柱。流动相为乙腈：水：三氟乙酸=85：15：0.1，流速 0.8 mL/min，检测波长 205 nm。表面活性素标准品购自 Sigma 公司，含 4 种同系物。

鼠李糖脂定量分析采用蒽酮比色法。向 1 mL 样品中加入 4 mL 蒽酮试剂 (0.2 g 蒽酮溶于 100 mL 88% 硫酸)，混匀后沸水浴 10 min，再在冷水浴中冷却 20 min，测定 OD_{580}。脂肽对糖脂测定无干扰。

1.3 产物结构质谱分析和鉴定

脂肽和糖脂鉴定采用飞行时间质谱法 (Matrix-Assisted Laser Desorption /Ionization Time

of Flight Mass Spectrometry, MALDI-TOF MS) [14]。

2 结果与分析

2.1 菌株筛选和生物表面活性剂质谱鉴定

采集油田地下废水、清华校园餐厅油污地面和荷塘底泥、南海底泥等数十个土壤样品，富集培养后采用溶血圈法初筛表面活性剂高产菌株。根据脂肽和鼠李糖脂表面活性高、稳定性好的特性，将不同菌株产物放置 70 ℃烘箱 5 d，选取排油圈直径未减小的菌株在初始培养基中培养，提取发酵产物，用于 MALDI-TOF 质谱分析 (图 1)。其中，质荷比 (*m*/*z*) 656.07 为双鼠李糖脂，1 044.66 和 1 058.67 为表面活性素[15]，即产物为脂肽-糖脂混合型生物表面活性剂。对菌株进行 16S rRNA 基因序列测定和生理生化鉴定，结果为 *Bacillus subtilis* subsp. *inaquosorum*，命名为 THY-7。

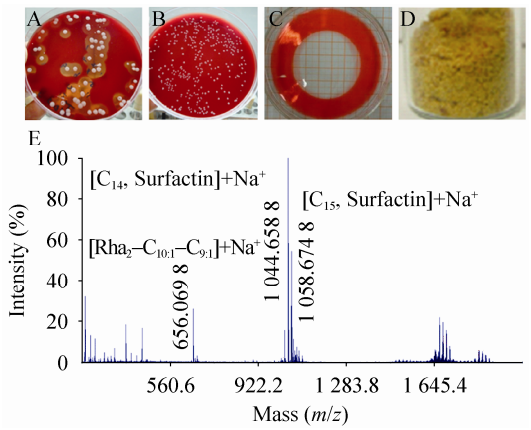

图 1 菌株筛选、产物制备和质谱鉴定

Fig. 1 Bacteria screening, product preparation and MALDI-TOF MS analysis. (A) Colonies with transparent circle on the blood plate. (B) Colonies without transparent circle. (C) Oil-red spreading test of THY-7 broth at 48 h. (D) Dried powder of products. (E) MALDI-TOF MS spectrum of THY-7 products. $C_{10:1}$/$C_{9:1}$, fatty acid chain C10 or C9 with an unsaturated bond at unclear position. Rha, the rhamnolipid group. C_{14}/C_{15}: fatty acid chain C14 or C15.

2.2 THY-7 培养基和培养条件的优化

温度和初始 pH 值优化表明，THY-7 的最优培养温度和初始 pH 分别为 37 ℃和 pH 7.0。考察了 6 种碳源、各 5 种有机氮源和无机氮源对细胞生长和产物积累的影响 (图 2)。红糖优于蔗糖、甘油和葡萄糖，是优选的较廉价碳源。酵母膏和玉米浆的效果虽略差于蛋白胨和牛肉膏，但更有工业价值。因玉米浆中杂质较多，本文选用 1 g/L 酵母膏为有机氮进行后续研究。无机氮则优选硝酸钠。

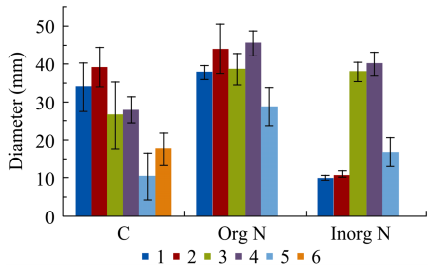

图 2 THY-7 培养基的碳源和氮源

Fig. 2 Carbon and nitrogen sources of THY-7. C: 6 carbon sources; 1−6: sucrose, brown sugar, glucose, glycerol, starch and molasses at 30, 30, 31.5, 32.2, 28.4 and 40 g/L, respectively. Org N: 5 organic nitrogen; 1−5: yeast extract, tryptone, corn steep liquor, beef extract and soybean powder at 1, 1, 3, 1 and 1 g/L, respectively. Inorg N: 5 in-organic nitrogen; 1−5: NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and urea at 8.7, 10.87, 6.59, 17 and 4.94 g/L (equal N-element), respectively. Experiments were performed 3 times.

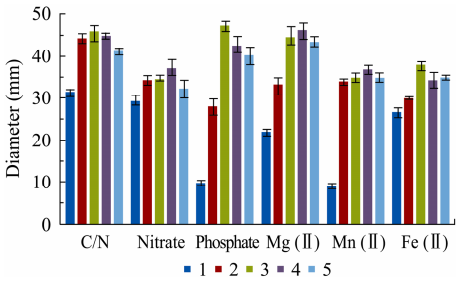

图 3 THY-7 培养基的单因子优化

Fig. 3 Medium optimization of THY-7. C/N: sucrose/yeast extract; Nitrate: $NaNO_3$; Phosphate: KH_2PO_4/$Na_2HPO_4{\cdot}12H_2O$; Mg (II): $MgSO_4{\cdot}7H_2O$; Mn (II): $MnSO_4{\cdot}H_2O$; Fe (II): $FeSO_4{\cdot}7H_2O$; 1−5: for C/N: 30:1, 40:1, 50:1, 60:1 and 70:1; for nitrate: 4.25, 8.5, 17.0, 34.0 and 51.0 g/L; for phosphate: 0.1/0.3, 0.333/1.0, 1.0/3.0, 2.0/6.0, 3.0/9.0 g/L; for Mg (II): 0, 0.03, 0.15, 0.6, 1.0 g/L; for Mn (II): 0, 0. 67, 6, 20, 60 mg/L; for Fe (II): 0, 0.67, 2, 6, 20 mg/L, respectively. Experiments reproduced 3 times.

选择碳氮比 (红糖/酵母膏的质量浓度)、硝酸盐、磷酸盐、镁、锰和铁离子浓度 6 种因素进行

单因子优化实验 (图 3)，以排油圈直径最大为指标，分别获得了各因素的优选单因子浓度。

进一步设计了如表 1 所示的六因素五水平正交实验。以排油圈直径为指标，完成了 25 组不同实验条件下的产物产量评估。结果表明，A-V、B-II、C-I、D-II、E-IV 和 F-II 分别是 6 个因素的优选水平，其中，C/N 和硝酸钠的极差分别是 61 和 54，其他成分的极差则均小于 20，即 C/N 和硝酸钠是产物积累的主要影响因素。最终获得了如“材料与方法 1.1”所示优化培养基。

表 1　THY-7 的 L25(5^6)正交试验设计

Table 1　L25(5^6) orthogonal test design for THY-7

Ls	A	B	C	D	E	F
I	30	17	0.3:1.0	0.05	0. 5	2.0
II	40	25	0.7:2.0	0.15	1.0	6.0
III	50	34	1.0:3.0	0.60	2.0	20.0
IV	60	42	1.5:4.5	1.00	6.0	30.0
V	70	51	2.0:6.0	1.50	20.0	60.0

Ls: levels; A: C/N ratio; B: $NaNO_3$, g/L; C: KH_2PO_4 (g/L): $Na_2HPO_4{\cdot}12H_2O$ (g/L); D: $MgSO_4{\cdot}7H_2O$, (g/L); E, $FeSO_4{\cdot}7H_2O$, (mg/L); F: $MnSO_4{\cdot}H_2O$, (mg/L). Experiments were performed two times.

2.3　优化前后细胞生长和产物积累比较

对 THY-7 进行优化前后的摇瓶平行培养，测定细胞密度和生物表面活性剂产量，结果如图 4A 和 4B。培养 48 h 时，细胞 OD_{600} 为 37.0，产物浓度为 2.4 g/L，分别是优化前的 3.4 和 3.1 倍。显微观察细胞形态，发现 32 h 时大量营养细胞已转化为休眠芽胞 (图 4C)，因此产物合成基本终止。为此，在 5 L 发酵罐中进行补料分批培养，初始装液量 2 L，10 h 和 12 h 分别补充优化培养基 60 mL，发酵过程中提取泡沫收集产物。培养 25 h 后，泡沫产物浓度达到 4.5 g/L，是目前报道的国内外较好水平。

2.4　THY-7 产物中脂肽与糖脂含量的定量分析

表面活性素标准品以及 THY-7 产物的 HPLC 色谱图如图 5A 和 5B 所示。在 200 mg/L THY-7 产物中，与标准品出峰位置相同的 4 种表面活性素的总浓度为 147.8 mg/L，占 74%。蒽酮比色法检测鼠李糖脂标准品及 THY-7 产物，结果如图 5C 和 5D 所示。在 100 mg/L THY-7 产物中，测得鼠李糖脂为 22.0 mg/L，占 22%。

产物性能评价还表明，THY-7 脂肽-糖脂混合生物表面活性剂具有良好的界面/表面活性、乳化性能和热稳定性，对油砂原油具有高驱油效率，优于实验室前期筛选获得的 *B. subtilis* TU2[15]，有望在次采油等领域获得实际应用。

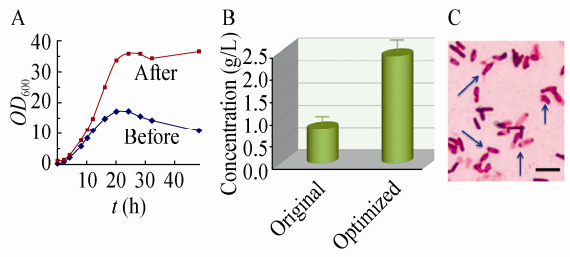

图 4　摇瓶优化前后比较及显微观察

Fig. 4　Flask comparison before and after optimization and microscope observation. (A) Cells' optical density at 600 nm (OD_{600}). (B) Biosurfactant concentration (dry weight) at 48 h. (C) Microscopic cell morphology of THY-7 at 32 h after optimization. Bar represents 4 μm.

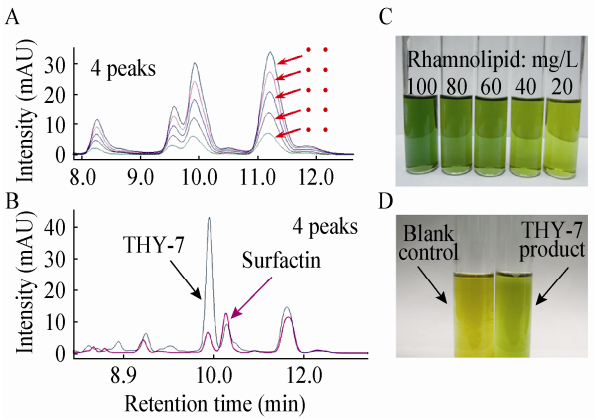

图 5　表面活性素和鼠李糖脂定量分析

Fig. 5　Quantification of surfactin and rhamnolipid. (A) HPLC-UV spectra of surfactin standards (Sigma) in 40, 80, 120, 160 and 200 mg/L, corresponding to ①－⑤, respectively. (B) HPLC of THY-7 product with respect to surfactin standard. (C) Anthrone colorimetry of rhamnolipid standards in 100, 80, 60, 40 and 20 mg/L, respectively. (D) Anthrone reaction of blank control (left) and 100 mg/L THY-7 product (right).

3 结论

通过血平板溶血圈快速筛选、改进排油圈法快速检测、高温筛选、产物分子质谱鉴定和菌株种属鉴定，获得了一株产脂肽-糖脂混合型生物表面活性剂的新菌株，命名为 *B. subtilis* THY-7。对其培养条件和培养基进行了优化，利用红糖为廉价碳源，产物主要为表面活性素和鼠李糖脂。摇瓶分批培养时，产量为 2.4 g/L。5 L 发酵罐补料分批培养并提取泡沫，产物浓度为 4.5 g/L。其中表面活性素约占 74%，鼠李糖脂约占 22%。实验表明，THY-7 合成的混合型生物表面活性剂的表面活性、乳化和驱油性能突出。可以预期，继续进行菌株改造以及补料培养耦合产物分离等工艺优化，产物产量将进一步提高，应用前景广阔。

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