

海洋破囊壶菌 Δ^5 -延长酶和 Δ^4 -脱饱和酶 在酿酒酵母中的共表达*

刘艳如** 江贤章 高媛媛 田宝玉 陈晓峰 陈金卿 黄建忠

(福建师范大学工业微生物教育部工程研究中心, 生命科学学院, 福建省现代发酵技术工程研究中心 福州 350108)

摘要 二十二碳六烯酸 (Docosahexaenoic acid, DHA C22:6n-3) 是具有各种重要生理功能的高度不饱和脂肪酸. 分别以质粒pYTFD5和pYFAD4为模板, 扩增获得860 bp的 Δ^5 -延长酶基因 (*elo5*) 和1 600 bp的 Δ^4 -脱饱和酶基因 (*fad4*). 利用重叠延伸PCR构建*elo5-fad4*融合基因, *Hind* III/*Sph* I双酶切后连接到经同样处理过的pYES2.0载体, 构建重组表达质粒pYELO5-FAD4. 转化酿酒酵母尿嘧啶缺陷型菌株INVSc1, 通过缺少尿嘧啶的选择性培养基筛选阳性克隆子. 添加外源脂肪酸C20:5底物, 半乳糖诱导表达. 气相色谱-质谱分析表明, 重组酵母总脂肪酸中出现了DHA (二十二碳六烯酸, C22:6n-3) 新产物, 融合基因*elo5-fad4*在酿酒酵母中得到了表达. 图7 表1 参18

关键词 破囊壶菌; 二十二碳六烯酸; 延长酶; 脱饱和酶; 共表达

CLC Q936

Co-expression of *Thraustochytrium* sp. FJN-10 Δ^5 -elongase and Δ^4 -desaturase in *Saccharomyces cerevisiae**

LIU Yanru**, JIANG Xianzhang, GAO Yuanyuan, TIAN Baoyu, CHEN Xiaofeng,

CHEN Jinqing & HUANG Jianzhong

(Engineering Research Center of Industrial Microbiology, Ministry of Education, College of Life Sciences, Fujian Normal University, Fuzhou 350108, China)

Abstract Docosahexaenoic acid (DHA C22:6n-3), a typical long chain polyunsaturated fatty acids (PUFAs) has many positive effects on diseases. Δ^5 -elongase gene (*elo5*, 860 bp) and Δ^4 -desaturase gene (*fad4*, 1 600 bp) were amplified by PCR using plasmid pYTFD5 and pYFAD4 as templates, respectively. *elo5-fad4* fusion gene amplified by overlap extension PCR was digested by *Hind* III and *Sph* I and subcloned into the yeast-*Escherichia coli* shuttle vector pYES2.0. The recombinant plasmid pYELO5-FAD4 containing target gene was transformed into *Saccharomyces cerevisiae* strain INVSc1 and the recombinant yeast cells were selected on agar synthetic medium lacking uracil. Expression of the fusion gene in transformant was induced by the addition of galactose to 2% (w/V). The yeast culture medium was supplemented with exogenous fatty acid substrate, eicosapentaenoic acid. Total fatty acids were extracted from the induced cells and subjected to methyl-esterification. The resultant fatty acid methyl esters were analyzed by GC detection. A novel peak corresponding to DHA (docosahexaenoic acid, C22:6n-3) methyl ester standards which was absent in the cell transformed with empty vector was detected with the same retention time and mass analysis. These results indicated that the protein encoded by *elo5-fad4* could specifically catalyze EPA to DHA. Fig 7, Tab 1, Ref 18

Keywords *Thraustochytrium*; docosahexaenoic acid; elongase; desaturase; co-expression

CLC Q936

二十二碳六烯酸 (Docosahexaenoic acid, DHA) 是人体必需的 ω -3系列长链高度不饱和脂肪酸, 具有重要的生理功能^[1-2]. 由于人体自身不能合成DHA且其来源有限, 因此探求新的DHA资源日益迫切^[3], 克隆DHA合成途径的相

关基因并研究其功能已成为当前研究热点^[4-6]. 破囊壶菌 *Thraustochytrium* sp. FJN-10为本实验室采用松花粉垂钓法分离获得的DHA理想生产菌株^[7], 我们已通过RACE结合RT-PCR技术成功克隆了*Thraustochytrium* sp. FJN-10 DHA生物合成途径相关的 Δ^5 -延长酶基因^[8] (*elo5*) 和 Δ^4 -脱饱和酶基因^[9] (*fad4*) 的全长序列, 并进行了功能鉴定: Δ^5 -延长酶可延长EPA (20:5n-3 $\Delta^{5,8,11,14,17}$) 碳链骨架生成DPA (22:5n-3 $\Delta^{7,10,13,16,19}$), Δ^4 -脱饱和酶可催化DPA脱氢生成DHA (22:6n-3 $\Delta^{4,7,10,13,16,19}$). 但是, 仅克隆与表达碳链延长酶与脱饱和酶, 还不能实现转基因技术生产高度不饱和脂肪酸, 还需要对这两种酶进行共表达. 本研究利用重叠延伸PCR技术获得其融合基因*elo5-fad4*并转化酿酒酵母 (*Saccharomyces cerevisiae*) 尿嘧啶缺陷型菌株, 通过添加底物EPA进行诱导

收稿日期: 2008-11-07 接受日期: 2009-02-17

*国家自然科学基金 (Nos. 30370028, 30970047), 福建省自然科学基金重大项目 (No. 2003F005), 福建省自然科学基金 (No. 2008F3036), 福建省发改委科技产业化项目 (No. [2005]847) 和福建省科技平台 (No. 2005Q007) Supported by the National Natural Science Foundation of China (General Program Nos. 30370028, 30970047), the Natural Science Foundation of Fujian, China (General Program No. 2003F005, Major Program No. 2008F3036), the Project of Fujian Development and Reform Commission of China (No. [2005]847) and the Sci-tech Platform of Fujian, China (No. 2005Q007)

**通讯作者 Corresponding author (E-mail: yrlu@fjnu.edu.cn)

表达, 构建了能够合成DHA的酿酒酵母基因工程菌株。

1 材料与方法

1.1 材料

1.1.1 菌种与质粒 质粒pYTFD5、质粒pYFAD4、*Escherichia coli* TOP10 [F- *mcrA* Δ (*mrr*- *hsdRMS*- *mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*araleu*) 7697 *galU* *galK* *rpsL* (StrR) *end A1* *nupG*], 由福建师范大学工业微生物教育部工程研究中心提供。*S. cerevisiae*营养缺陷型菌株INVSc1、质粒pYES2.0购自Invitrogen公司。

1.1.2 培养基 (1) LB培养基 ($\rho/g L^{-1}$): 蛋白胨 10, 氯化钠 10, 酵母浸出物 5; (2) YPD培养基 ($\rho/g L^{-1}$): 酵母提取物 10, 葡萄糖 20, 蛋白胨 20, pH自然; (3) Sc-U合成培养基 (不含尿嘧啶, $\rho/g L^{-1}$): 无氨基酸氮源YNB 6.7, 碳源棉子糖 20, 氨基酸混合物I (Adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan) 0.1, 氨基酸混合物II (Aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine) 0.05; (4) Sc+U合成培养基 (含尿嘧啶): 在Sc-U培养基中添加0.1 g/L Uracil; (5) 诱导培养基: 在Sc-U液体培养基中添加2%半乳糖和1% NP-40进行诱导表达。

1.1.3 工具酶及主要试剂: T4 DNA连接酶、rTaq 酶、Ex Taq 酶、pMD-18T simple vector、X-gal、IPTG、Marker购自TaKaRa公司; 限制性内切酶 *Hind* III 和 *Sph* I、Pfu DNA polymerase, 购自Promega公司; RNaseA购自Fementer公司; Sc-U培养基所需的各种氨基酸、NP-40、各种脂肪酸甲酯标准品, 购自Sigma公司; 胶回收试剂盒购自上海申能博采公司。其余试剂购自上海生工生物工程公司。

1.2 引物设计

根据本实验室已克隆的*elo5*序列 (GenBank accession No. ABC18313) 和*fad4*序列 (GenBank accession No. DQ133575) 及Kozak提出的真核生物偏好转译起始密码子周边序列^[10-11], 设计特异性引物 (表1)。其中, 引物P1与*elo5*开放阅读框起始密码子附近的序列相对应, 并在5'末端添加 *Hind* III 酶切位点和3个保护碱基; 引物P2与*elo5*终止密码子附近的序列及*fad4*5'末端起始密码子附近的序列互补, 并去除*elo5*的终止密码子TAA; 引物P3与*elo5*终止密码子附近的序列及*fad4*5'末端起始密码子附近的序列相对应, 同样去除*elo5*终止密码子TAA; 引物P4与*fad4*的开放阅读框终止密码子序列互补, 并在5'末端添加*Sph* I酶切位点和3个保护碱基。*elo5* 和*fad4*间以编码Gly的两个三联密码子GGAAGC相连^[12]。同时, 为提高第

表1 引物核苷酸序列

Table 1 Nucleotides sequence of primers

Primer	Sequence (5'→3')	Length (nt)
P1	<u>CCCAAGCTT</u> ACCATGGATGTCGTCGAGCAGC	31
P2	GTAGCCGACCGCCATAATGGAAGCGATGGTC-TTCTGCTTCTGGG	45
P3	CCCAAGAAGCAGAAGACCATCGCTTCCATT-ATGGCGGTCCGGCTAC	45
P4	CATGCATGCTCAGGCAGCGGCTGC	25
P5	TCGATTCCCAAGCTTACCATGGATGTC	27

下划线分别表示 *Hind* III 和 *Sph* I 的酶切位点。The underlined indicate *Hind* III and *Sph* I sites

二轮PCR扩增的特异性, 在引物P1的基础上设计引物P5与P4配对, 进行重叠延伸PCR。

1.3 *elo5*和*fad4*基因的扩增

以质粒pYTFD5为模板, 引物P1、P2扩增*elo5*基因; 以质粒pYFAD4为模板, 引物P3、P4扩增*fad4*基因。PCR反应体系: 10×Pfu buffer 5 μ L, dNTPs (10mmol/L) 4 μ L, DNA模板 1 μ L, 引物P1 (P3) (10 μ mol/L) 1 μ L, 引物P2 (P4) (10 μ mol/L) 1 μ L, Pfu polymerase (5 U/ μ L) 0.25 μ L, 去离子水补足至50 μ L; 扩增条件: 94 $^{\circ}$ C 4 min; 94 $^{\circ}$ C 1 min, 55 $^{\circ}$ C 1 min, 72 $^{\circ}$ C 2 min, 5个循环; 94 $^{\circ}$ C 1 min, 65 $^{\circ}$ C 1 min, 72 $^{\circ}$ C 2 min, 28个循环; 72 $^{\circ}$ C 10 min。1.0%琼脂糖凝胶电泳鉴定PCR产物, 胶回收。

1.4 *elo5-fad4*融合基因的PCR扩增

根据重叠延伸PCR的原理, 以回收纯化的*elo5*和*fad4*为模板, 利用外侧引物P4、P5进行第二轮PCR扩增, 获得*elo5*和*fad4*的融合基因*elo5-fad4*。重叠延伸PCR反应体系: 10×Pfu buffer 5 μ L, dNTPs (10mmol/L) 4 μ L, *elo5*和*fad4*胶回收产物各1 μ L, 引物P4 (10 μ mol/L) 1 μ L, 引物P5 (10 μ mol/L) 1 μ L, Pfu polymerase (5 U/ μ L) 0.25 μ L, 去离子水补足至50 μ L; 扩增条件: 94 $^{\circ}$ C 4 min; 94 $^{\circ}$ C 1 min, 65 $^{\circ}$ C 1 min, 72 $^{\circ}$ C 3 min, 30个循环; 72 $^{\circ}$ C 10 min。1.0%琼脂糖凝胶电泳鉴定PCR产物。

1.5 重组质粒pMD-ELO5FAD4的构建

回收纯化重叠延伸PCR产物, 按常规程序进行加A反应并取1 μ L和pMD-18T simple vector进行连接反应。转化*E. coli* TOP10, 经蓝白斑筛选, 菌落PCR和*Hind* III / *Sph* I双酶切鉴定阳性克隆, 命名为pMD-ELO5FAD4。用M13通用引物对重组质粒进行测序验证。

1.6 *elo5-fad4*融合基因共表达载体的构建和转化

用限制性内切酶*Hind* III 和*Sph* I对pMD-ELO5FAD4和表达载体pYES2.0分别进行双酶切、回收, 16 $^{\circ}$ C过夜连接。反应结束, 加1/10体积的3 mol/L NaAc溶液和2倍体积的无水乙醇, -20 $^{\circ}$ C放置30 min, 离心 (12 000 r/min, 4 $^{\circ}$ C, 10 min); 用预冷的75%乙醇洗涤2次, 自然晾干后溶于适量去离子水中备用。

连接产物转化*E. coli* TOP10感受态细胞。涂布在含氨苄青霉素 (100 μ g/mL) 的LB平板上, 37 $^{\circ}$ C过夜培养, 碱裂解法提取重组质粒。重组质粒和载体pYES2.0分别用限制性内切酶*Hind* III和*Sph* I进行双酶切鉴定, 最终获得重组表达质粒pYELO5-FAD4。表达载体pYES2.0与重组表达质粒pYELO5-FAD4分别转化*S. cerevisiae*尿嘧啶缺陷型菌株INVSc1, 在缺少尿嘧啶的合成培养基上筛选阳性克隆子。*S. cerevisiae*感受态细胞的制备和转化参照*S.c.EasyComp*TM Transformation Kit (Invitrogen) 试剂盒的说明。

1.7 融合基因*elo5-fad4*在酿酒酵母中的诱导表达

挑取Sc-U选择性固体培养基上阳性克隆子YELO5-FAD4和YES2.0, 分别接种于5 mL Sc-U选择性培养基, 过夜振荡培养 (30 $^{\circ}$ C, 250 r/min)。以5%接种量加入到含1% NP-40的100 mL Sc-U液体培养基中, 添加外源脂肪酸底物EPA至终浓度为0.3 mmol/L, 30 $^{\circ}$ C继续培养至 $D_{600\text{nm}}$ 达到0.2时, 加入2%半乳糖诱导, 20 $^{\circ}$ C继续培养72 h, 收集菌体, 提

取油脂并甲酯化. 利用安捷伦6890N/5975MS气相色谱-质谱分析分析脂肪酸组成^[15].

2 结果与分析

2.1 *elo5-fad4*融合基因的PCR扩增

分别以质粒pYTFD5为模板, 引物P1、P2 PCR扩增*elo5*基因; 以质粒pYFAD4为模板, 引物P3、P4扩增*fad4*基因, 琼脂糖凝胶电泳验证条带大小后, 回收纯化*elo5*和*fad4*条件. 以引物P4、P5进行重叠延伸PCR第二轮扩增, PCR扩增产物大小约2500 bp (图1), 与目的条带大小相符, 获得融合基因*elo5-fad4*.

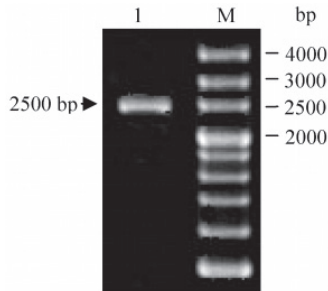


图1 融合基因*elo5-fad4*的扩增

Fig. 1 Amplification of fusion *elo5-fad4* gene by overlap extension PCR
M: 200 bp DNA marker. I: 融合基因
M: 200 bp DNA marker. I: Recombinant fusion gene

2.2 重组质粒pMD-ELO5FAD4的构建

融合基因*elo5-fad4* PCR加A产物与pMD-18T simple vector进行连接, 转化*E. coli* TOP10. 随机挑选4个白斑进行菌落PCR. 所挑取的菌落均出现2500 bp的目的条带. 利用*Hind* III和*Sph* I双酶切进一步鉴定表明重组质粒含有融合基因*elo5-fad4* (图2). 同时, 测序结果也表明重组质粒含有融合基因且正确融合. 将鉴定正确的重组质粒命名为pMD-ELO5FAD4.

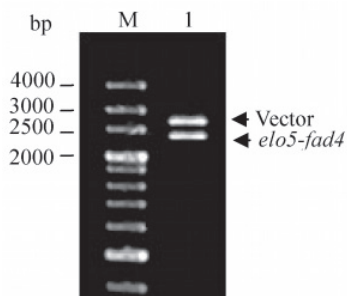


图2 重组质粒pMD-ELO5FAD4双酶切鉴定

Fig. 2 Identification of recombinant plasmid pMD-ELO5FAD4 digested by *Hind* III and *Sph* I

M: 200 bp DNA marker. I: *Hind* III和*Sph* I双酶切后的重组质粒
M: 200 bp DNA marker. I: Recombinant plasmid digested by *Hind* III and *Sph* I

2.3 *elo5-fad4*融合基因共表达载体的构建与鉴定

用限制性内切酶*Hind* III和*Sph* I对pMD-ELO5FAD4进行双酶切, 切胶回收融合基因*elo5-fad4*, 与同样双酶切处理的表达载体pYES2.0进行连接, 构建共表达载体pYELO5-FAD4. 将重组质粒转化*E. coli* TOP10, 挑取转化子, *Hind* III/*Sph* I双酶切验证 (图3) 表明阳性转化子含有2500 bp的目的基因. 上述结果充分表明重组表达质粒 (命名为pYELO5-

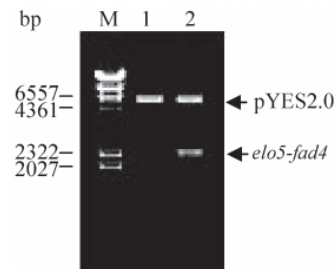


图3 重组表达质粒pYELO5-FAD4双酶切鉴定

Fig. 3 Identification of recombinant plasmid pYELO5-FAD4 digested with *Hind* III and *Sph* I

M: λ -*Hind* III marker. 1: *Sph* I与*Hind* III双酶切的pYES2.0; 2: *Sph* I与*Hind* III双酶切的重组质粒

M: λ -*Hind* III marker; 1: pYES2.0 digested with *Sph* I and *Hind* III; 2: Recombinant plasmid digested with *Hind* III and *Sph* I

FAD4) 构建成功, *elo5-fad4*融合基因片段插入正确.

2.4 融合基因*elo5-fad4*在酿酒酵母中的诱导表达

重组表达质粒pYELO5-FAD4及空载体pYES2.0分别转化酵母细胞, 从Sc-U平板筛选获得阳性转化子YELO5-FAD4及对照菌株INVSc1. 挑取少量菌落, 用引物P4、P5进行PCR验证重组成功. 添加底物EPA, 半乳糖诱导后, 分别提取转化子与对照菌细胞总脂肪酸并制备其甲酯. 对照菌株INVSc1 (图4-A) 中出现与脂肪酸底物EPA相对应的峰, 转化子YELO5-FAD4 (图4-B) 中不仅出现底物EPA峰, 而且在28.039 min出现了一个新的峰, 质谱鉴定表明该新峰是DHA (图5). 以上结果表明, *elo5-fad4*编码产物能特异地利用C20:5底物, 并将其转换成C22:6, 说明产DHA的酿酒酵母工程菌构建成功.

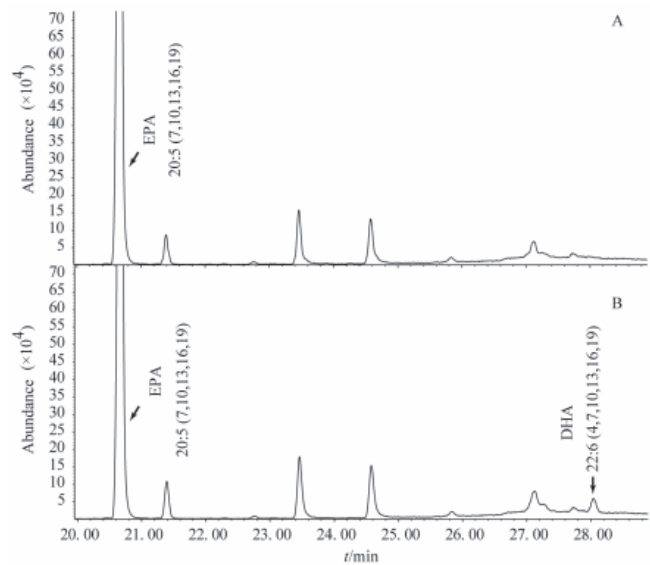


图4 转化子脂肪酸甲酯的气相色谱分析

Fig. 4 Identification of DHA in transformants by GC analysis

A: 带pYES2.0质粒的酵母; B: 带pYELO5-FAD4质粒的酵母

A: *S. cerevisiae* transformed with the control vector pYES2.0; B: *S. cerevisiae* transformed with pYELO5-FAD4

2.5 转化子遗传稳定性分析

将重组转化子涂布到Sc+U平板上, 长出菌落后影印到Sc-U平板上. Sc+U平板上长出的是所有转化子 (包括尿嘧啶缺陷挽救的酵母及尿嘧啶缺陷型酵母, 图6-A), 而Sc-U平板

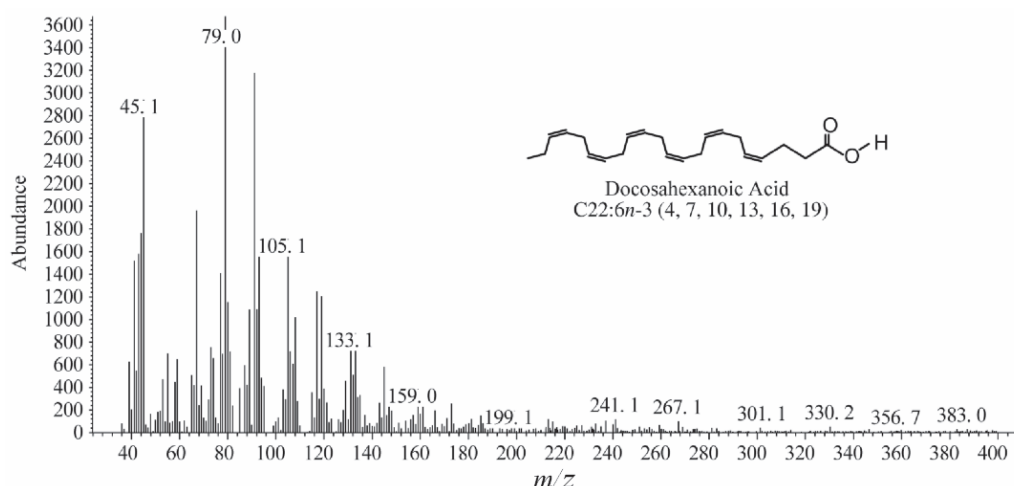
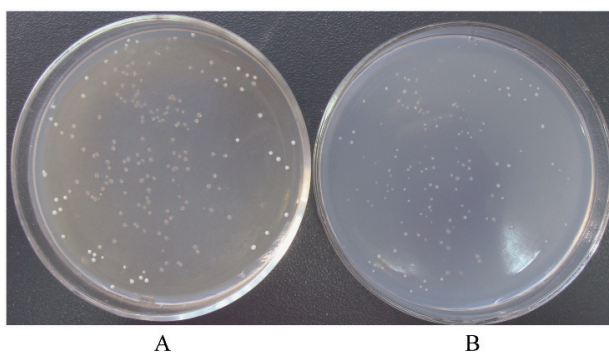


图5 新产物峰脂肪酸甲酯质谱分析

Fig. 5 GC/MS analysis of fatty acid methyl esters of new peak



A B

图6 重组酵母营养缺陷分析

Fig. 6 Auxotrophic analysis of recombinant yeasts

A: Sc+U平板上的酵母; B: Sc-U平板上的酵母

A: Yeasts grown on Sc+U plate; B: Yeasts grown on Sc-U plate

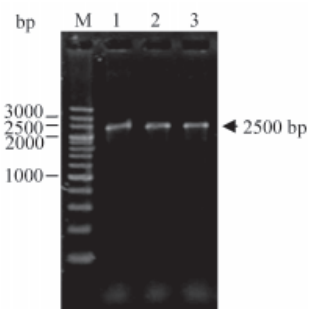


图7 酵母重组子传代稳定性分析

Fig. 7 Genetic stability of recombinant yeasts

M: 200 bp marker. 1: 第1代重组酵母; 2: 第5代重组酵母; 3: 第10代重组酵母

M: 200 bp marker. 1: The 1st generation; 2: The 5th generation; 3: The 10th generation

上长出的仅是尿嘧啶缺陷挽救的酵母(图6-B)。结果表明,重组转化子可在Sc+U和Sc-U的平板上长出一致的菌落,说明尿嘧啶互补基因已随着*elo5-fad4*基因整合到染色体。将重组转化子YELO5-FAD4连续传代10次,随机挑取单菌落,用引物P1和P4进行PCR验证,均获得2 500 bp的条带(图7)。试验表明所构建的菌重组菌YELO5-FAD4具有很高的遗传稳定性。

3 讨论

破囊壶菌与裂殖壶菌同属于破囊壶菌科,但是裂殖壶菌只能合成DHA与DPA(Docosapentaenoic acid,二十二碳五烯酸)^[14],而破囊壶菌却可以合成包括DHA、AA(Arachidonic acid,花生四烯酸)、GLA(γ -linolenic acid, γ -亚麻酸)在内的一系列高度不饱和脂肪酸^[7],这表明,破囊壶菌与裂殖壶菌的DHA合成途径不同,它是通过碳链延长与脱饱和的生化反应合成高度不饱和脂肪酸,因此,破囊壶菌是研究DHA生物合成途径的理想模式生物。常见的高度不饱和脂肪酸生物合成途径依赖于膜结合的碳链延长酶与脱饱和酶。以DHA为例,从乙酰辅酶A开始,合成DHA大约需要30多个酶、70多个生化反应,其中包括4个重复的饱和脂肪酸合成循环以及随后耗能、耗氧的脱饱和与延长反应。如何共表达这些复杂的基因,使转基因微生物能够从头或从中间合成长链高度不饱和脂肪酸是当前脂肪酸研究的热点之一。我们已经成功克隆并表达了 $\Delta 5$ -延长酶^[8]和 $\Delta 4$ -脱饱和酶^[9],为了让 $\Delta 5$ -延长酶和 $\Delta 4$ -脱饱和酶在时间与空间上共同表达,我们将 $\Delta 5$ -延长酶与 $\Delta 4$ -脱饱和酶进行融合表达。由于延长酶有7个跨膜区^[12],脱饱和酶有4个跨膜区^[13],这两个酶共表达后,成功结合于内质网膜上,由于内质网膜的锚定作用,其空间结构不会互相影响,添加外源底物EPA后,酵母可以合成DHA。这表明利用共表达合成脂肪酸的技术路线是可行的。目前,关于延长酶和脱饱和酶在酿酒酵母中共表达的研究国外只有为数不多的报道^[15-18],国内则尚未见相关研究报道。本研究首次利用重叠延伸PCR技术,构建融合基因*elo5-fad4*并转化酿酒酵母,通过添加来源较为丰富的底物EPA,在半乳糖的诱导下进行表达,成功构建产DHA的酵母工程菌,表明由EPA合成DHA的可行性,为寻找可替换资源合成DHA提供了科学依据。

References

- Rinaldi B, Di Piero P, Vitelli MR, D'Amico M, Berrino L, Rossi F, Filippelli A. Effects of docosahexaenoic acid on calcium pathway in adult rat cardiomyocytes. *Life Sci*, 2002, **71** (9): 993-1004
- Sijtsma L, de Swaaf ME. Biotechnological production and applications of

- the ω -3 polyunsaturated fatty acid docosahexaenoic acid. *Appl Microbiol Biotechnol*, 2004, **64** (2): 146~153
- 3 Certik M, Shimizu S. Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *J Biosci Bioeng*, 1999, **87** (1): 1~14
- 4 Passorn S, Laoteng K, Rachadawong S, Tanticharoen M, Cheevadhanarak S. Heterologous expression of *Mucor rouxii* delta (12)-desaturase gene in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun*, 1999, **263** (1): 47~51
- 5 Sakuradani E, Shimizu S. Gene cloning and functional analysis of a second delta 6-fatty acid desaturase from an arachidonic acid-producing *Mortierella* fungus. *Biosci Biotechnol Biochem*, 2003, **67** (4): 704~711
- 6 Schneiter R, Tatzer V, Gogg G, Leitner E, Kohlwein SD. Elo1p-dependent carboxy-terminal elongation of C14:1 Delta (9) to C16:1 Delta (11) fatty acids in *Saccharomyces cerevisiae*. *J Bacteriol*, 2000, **182** (13): 3655~3660
- 7 Huang JZ (黄建忠), Liu LX (刘丽霞), Wu SG (吴松刚). Profile of fatty acids and analysis of 18S rDNA sequence in *Thraustochytrium* sp. FJN-10 highly producing DHA. *Pharm Biotechnol* (药物生物技术), 2005, **12** (1): 1~5
- 8 Jiang XZ (江贤章), Qin LN (秦丽娜), Tian BY (田宝玉), Shu ZY (舒正玉), Huang JZ (黄建忠). Cloning and expression of two elongase genes involved in the biosynthesis of docosahexaenoic acid in *Thraustochytrium* sp. FJN-10. *Acta Microbiol Sin* (微生物学报), 2008, **48** (2): 176~183
- 9 Liu LX (刘丽霞), Jiang XZ (江贤章), Qin WX (覃文新), Yao GF (姚根富), Huang JZ (黄建忠). Identification of the Δ^4 fatty acid desaturase gene from *Thraustochytrium* sp. FJN-10. *Ind Microbiol* (工业微生物), 2006, **36** (1): 1~6
- 10 Kozak M. Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. *Embo J*, 1997, **16** (9): 2482~2492
- 11 Kozak M. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res*, 1984, **12** (2): 857~872
- 12 Zheng Y (郑耘), Li HP (李华平), Xiao HG (肖火根), Fan HZ (范怀忠). Construction of plant expression vector of fusion genes with *Banana bunchy top virus* replicase and *Cucumber mosaic virus* coat protein. *J South China Agric Univ* (华南农业大学学报), 2005, **26** (3): 18~21
- 13 Qiu X, Hong H, Mackenzie SI. Identification of a Delta 4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexaenoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. *J Biol Chem*, 2001, **276** (34): 31561~31566
- 14 Huang JZ (黄建忠), Jiang XZ (江贤章). Isolation of *Schizochytrium* sp. FJU-512 with high yield of DHA and comparative analysis on its 18S rRNA gene sequence. *Chin J Appl Environ Biol* (应用与环境生物学报), 2005, **11** (2): 202~207
- 15 Sayanova O, Haslam R, Qi B, Lazarus CM, Napier JA. The alternative pathway C20 Δ 8-desaturase from the non-photosynthetic organism *Acanthamoeba castellanii* is an atypical cytochrome b5-fusion desaturase. *FEBS Lett*, 2006, **580** (8): 1946~1952
- 16 Beaudoin F, Michaelson LV, Hey SJ, Lewis MJ, Shewry PR, Sayanova O, Napier JA. Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway. *Proc Natl Acad Sci U S A*, 2000, **97** (12): 6421~6426
- 17 Pereira SL, Leonard AE, Huang YS, Chuang LT, Mukerji P. Identification of two novel microalgal enzymes involved in the conversion of the ω 3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid. *Biochem J*, 2004, **384** (2): 357~366
- 18 Domergue F, Abbadı A, Zähringer U, Moreau H, Heinz E. *In vivo* characterization of the first acyl-CoA Δ 6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. *Biochem J*, 2005, **389** (2): 483~490