重组嗜水气单胞菌 *Aeromonas hydrophila* 4AK4 中 3-羟基丁酸 3-羟基己酸共聚酯 PHBHHx 的发酵生产

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摘 要 3-羟基丁酸和 3-羟基己酸共聚酯(PHBHHx)是一种性能优良的新型生物可降解材料，其机械和加工性能与 3-羟基己酸(3HHx)在共聚物中的含量密切相关。在嗜水气单孢菌 *Aeromonas hydrophila* 4AK4 中引入了编码 β-酮基硫解酶 (β-ketothiolase)的 *phbA* 基因和编码乙酰乙酰辅酶 A 还原酶(Acetoacetyl-CoA reductase)的 *phbB* 基因，使重组菌增加了一条利用乙酰辅酶 A 合成 3-羟基丁酸-CoA 的代谢途径，这使得利用非相关性碳源调控 PHBHHx 的单体组成比例成为可能。利用葡萄糖酸钠和月桂酸作为碳源，对重组 *Aeromonas hydrophila* 4AK4 进行了摇瓶培养及 5 L 发酵罐培养的研究。在摇瓶实验中，通过改变碳源中两种组分的比例，可以使 *A. hydrophila* 4AK4 合成的 PHBHHx 中的 3HHx 摩尔含量由原来的 15%左右降低到 3% ~ 12 %，成功地实现了对 PHBHHx 单体组成的调控；当以月桂酸为唯一碳源时，在 5 L 发酵罐中，经过 56 h 的培养，获得了 51.5 g/L 的细胞干重(CDW)，其中 62 %为 PHBHHx，3HHx 在 PHBHHx 中的摩尔含量为 9.7 %；当以 1∶1 的葡萄糖酸钠和月桂酸为碳源时，48 h 的 5 L 发酵罐培养获得了 32.8 g/L 的 CDW 和 52 %的 PHBHHx 含量，其中 3HHx 在 PHBHHx 中的摩尔含量为 6.7 %。结果证明了该重组菌在大规模生产单体组成可控 PHBHHx 方面具有很大的应用潜力。

关键词 3-羟基丁酸 3-羟基己酸共聚酯，嗜水气单孢菌，3-羟基己酸

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聚 3-羟基脂肪酸酯(PHA)家族中的 3-羟基丁酸 3-羟基己酸共聚物(PHBHHx)是一种新型的、性能优良的生物可降解、生物可相容的材料[1-3]，研究表明，PHBHHx 的材料性能与其单体之一的 3-羟基己酸(3HHx)的含量有着非常密切的关系[4]。随着 3HHx 含量的提高，PHBHHx 的结晶度降低[4]、熔点降低、柔软性和断裂伸长率增加[5]。据报道[1]，3HHx 摩尔含量为 10 %的 PHBHHx 熔点只有 127℃，而断裂伸长率高达 400%，与前两代的 PHA 材料聚 3-羟基丁酸酯(PHB，熔点 177℃，断裂伸长率 5%)和 3-羟基丁酸 3-羟基戊酸共聚酯(PHBV，3-羟基戊酸摩尔含量为 10 %的 PHBV 熔点 150℃，断裂伸长率为 20 %)相比，具有优越的材料性能及加工性能。此外，不同单体组成的 PHBHHx 还可以满足生产、科研的各种不同的要求。

嗜水气单胞菌(*Aeromonas hydrophila*)因能合成性状优良的 PHBHHx，已成为研究的热点[4-9]。*Aeromonas hydrophila* 中合成 PHBHHx 所需要的前体仅依赖于脂肪酸的 β-氧化过程提供[8]，因此不能以非相关性碳源(如糖类)为底物合成 PHBHHx，而只能以脂肪酸、油脂等相关性碳源为底物积累 PHBHHx。近来的研究表明，改变培养基、改变发酵条件等方法对其合成的 PHBHHx 中单体组成的影响很小[8,9]。研究发现，在 *Aeromonas hydrophila* 合成的 PHBHHx 中，3-羟基丁酸与 3-羟基己酸的组成通常有其固有的比例。*Aeromonas hydrophila* WQ 合成的 PHBHHx 中，3HHx 摩尔含量为 3% ~ 5%[9]，而 *Aeromonas hydrophila* 4AK4 中的这一摩尔含量为 12% ~ 15 %[7-9]，难以满足生产、研究的多种需要。

本研究在 *Aeromonas hydrophila* 4AK4 中引入了 *phaA*、*phaB* 基因，*phaA* 基因编码 β-酮基硫解酶 (β-ketothiolase)，可以催化两个乙酰辅酶 A 生成乙酰乙酰辅酶 A；*pha*B 基因编码乙酰乙酰辅酶 A 还原酶(Acetoacetyl-CoA reductase)，可以把乙酰乙酰辅酶 A

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转化为 3HB-CoA[10-11]。这条新的提供 PHBHHx 合成前体的代谢途径使重组菌可以利用 β-氧化途径或糖类代谢途径产生的乙酰辅酶 A 合成 3HB 前体，这使得调控 PHBHHx 的单体组成成为可能。本研究在重组菌摇瓶培养的基础上进行了发酵罐发酵的实验，为工业化规模生产单体组成可控的优良生物可降解材料奠定了基础。

1 材料和方法

1.1 菌株

嗜水气单孢菌（*Aeromonas hydrophila*）4AK4 为清华大学微生物实验室收藏。

1.2 重组菌株的构建

编码有 *phbA* 和 *phbB* 基因的质粒 pTG01（构建过程见下面结果与讨论）通过电转化的方法转入到 *A. hydrophila* 4AK4 中。

1.3 摇瓶培养基及培养条件

上述微生物的培养基组成（g/L）：月桂酸 4，$(NH_4)_2SO_4$ 2，$MgSO_4 \cdot 7H_2O$ 0.2，$Na_2HPO_4 \cdot 12H_2O$ 9.65，KH_2PO_4 1.5，酵母粉 1，微量元素溶液 1 mL/L 和卡那霉素 50 μg/mL。微量元素溶液含有（g/L）：$FeCl_3 \cdot 6H_2O$ 20，$CaCl_2 \cdot H_2O$ 10，$CuSO_4 \cdot 5H_2O$ 0.03，$MnCl_2 \cdot 4H_2O$ 0.05，$ZnSO_4 \cdot 7H_2O$ 0.1 和 HCl 0.5 mol/L。

所有微生物的培养条件均为：30 ℃，48 h，摇床转速 200 r/min（NBS Series 25D，New Brunswick，NJ，美国）。

1.4 发酵罐培养基及培养条件

发酵罐培养基 1（g/L）：月桂酸 10，葡萄糖酸钠 10，$(NH4)_2SO_4$ 4，$MgSO_4 \cdot 7H_2O$ 0.5，$Na_2HPO_4 \cdot 12H_2O$ 2.0，KH_2PO_4 1.85，酵母粉 1，微量元素溶液 1 mL/L。补料中含 30 g 月桂酸、30 g 葡萄糖酸钠，9 g $(NH_4)_2SO_4$，于发酵 12 h 之后每隔 6 h 补料 1 次。

发酵罐培养基 2（g/L）：月桂酸 10，$(NH_4)_2SO_4$ 4，$MgSO_4 \cdot 7H_2O$ 0.5，$Na_2HPO_4 \cdot 12H_2O$ 2.0，KH_2PO_4 1.85，酵母粉 1，微量元素溶液 1 mL/L。补料中含 30 g 月桂酸、6 g $(NH_4)_2SO_4$，于发酵 12 h 之后每隔 4 h 补料 1 次。

发酵罐培养条件：发酵有效体积 3 L，30 ℃，pH 6.5，通气量 5 L/min 过滤空气；溶氧 DO 通过搅拌自动控制在 15 % 之上，搅拌转速控制范围为 200 ~ 800 r/min；分批补料培养 48 h；接种量：5 %（*V*/*V*）。种子用 LB 培养基（5 g/L 酵母粉，10 g/L 蛋白胨，10 g/L NaCl）（加入卡那霉素 50 μg/mL）在 30 ℃、转速 200 r/min 摇床（NBS Series 25D，New Brunswick，美国）中培养 12 h。所用发酵罐为自动控制的 NBS 3000（NBS，New Brunswick，美国）。

1.5 分析方法

细胞干重（cell Dry Weight，CDW）、NH_4^+、PHBHHx 含量测量方法见文献[13]；*P* 的测量方法见文献[14]。

2 结果及讨论

2.1 构建编码 *phbA*、*phbB* 基因的 *Aeromonas hydrophila* 4AK4 重组菌 *A. hydrophila* 4AK4（pTG01）

野生型 *A. hydrophila* 4AK4 中没有 *phbA*、*phbB* 基因，它主要依赖 β-氧化过程产生 3HB 及 3HHx 的前体，因此不能利用糖类等非相关性碳源积累 PHBHHx，仅能利用相关性碳源（各种脂肪酸，油脂等）合成 PHBHHx。其积累的 PHBHHx 中 3HHx 的摩尔百分含量通常为 12% ~ 15%。在 *A. hydrophila* 4AK4 中引入 *phbA*、*phbB* 基因，使得重组菌 4AK4（pTG01）可以利用 β-氧化过程以及糖类代谢过程中产生的乙酰辅酶 A 合成 3HB 前体，额外增加了一条合成 3HB 前体的途径。重组菌 4Ak4（pTG01）可以以糖类作为唯一碳源合成聚羟基丁酸酯 PHB。这样，便可以通过改变底物中糖类碳源和脂肪酸碳源的比例，调节细菌积累的 PHBHHx 的结构组成。

用 *Hin*dⅢ 和 *Kpn* Ⅰ 对质粒 pUC-AB（质粒 pUC-AB 构建见 Zhao *et al*（2003）[15]）进行酶切，得到含有 *phbA*、*phbB* 基因的片段，并插入到质粒 pBBR1MCS-2（pBBR1MCS-2 由 Kovach *et al*（1995）构建[16]）中，得到了带有卡那霉素抗性基因的新质粒 pTG01（如图 1 所示）。

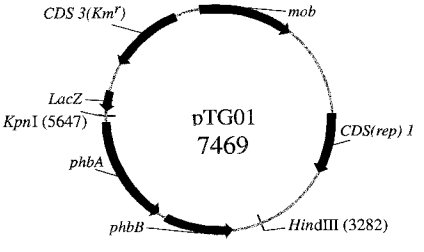

图 1 质粒 pTG01

Fig. 1 Plasmid pTG01

2.2 表达 *phbA*、*phbB* 基因的重组菌 *A. hydrophila* 4AK4（pTG01）降低了 3HHx 在 PHBHHx 中的含量

摇瓶实验的结果（如表 1 所示）表明，重组菌 4AK4（pTG01）中引入的合成 3HB 代谢途径可以有效降低 PHBHHx 中 3HHx 的含量。使用纯月桂酸作

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为唯一碳源时,重组菌 4AK4(pTG01)合成的 PHBHHx 中,3HHx 摩尔含量为 12.29 %,野生菌 4AK4 中这一含量为 16.85 %,降低了 4.56 %。在 β-氧化过程中产生的乙酰辅酶 A 主要用于 TCA 循环等提供能量的代谢途径,流入 3HB 合成途径的乙酰辅酶 A 较少,因此 3HHx 降低的幅度较小。如果在底物中加入糖类,其代谢之后产生大量的乙酰辅酶 A,将会有更多的乙酰辅酶 A 流入 3HB 合成途径,这样就可以根据需要调节 PHBHHx 中 3HHx 的比例。

表 1　PHBHHx 在 *A. hydrophila* 4AK4 及 *A. hydrophila* 4AK4(pTG01)中的积累

Table 1　Production of PHBHHx by *A. hydrophila* 4AK4 and *A. hydrophila* 4AK4(pTG01) in flask culture

Strains	IPTG[a]	Sodium gluconate[b]/(g/L)	CDW/(g/L)	PHBHHx/CDW/%	3HHx mol faction in PHBHHx/%
4AK4	–	0	1.94 ± 0.02	35.64 ± 0.36	16.85 ± 0.06
4AK4	–	8	2.25 ± 0.96	20.61 ± 1.47	13.84 ± 0.69
4AK4(pTG01)	–	0	1.92 ± 0.15	46.75 ± 5.24	12.29 ± 1.28
4AK4(pTG01)	–	8	2.55 ± 0.53	50.65 ± 2.36	5.94 ± 0.20
4AK4(pTG01)	+	0	2.20 ± 0.20	41.45 ± 2.84	12.25 ± 0.63
4AK4(pTG01)	+	4	2.79 ± 0.54	40.09 ± 1.29	7.69 ± 0.06
4AK4(pTG01)	+	8	2.66 ± 0.32	39.71 ± 1.24	5.72 ± 0.11
4AK4(pTG01)	+	12	3.09 ± 0.29	37.52 ± 1.84	2.80 ± 0.39

"–" No IPTG added; "+" IPTG added

a: 0.2 g/L IPTG added in 12 h after inoculation

b: Sodium gluconate was divided into two equal amounts and was added 12 h and 24 h after inoculation, respectively

在重组菌的摇瓶培养中,当加入的葡糖酸钠与月桂酸的比例为 1∶1、2∶1、3∶1 时,3HHx 的摩尔百分含量分别降低到 7.69%、5.72%、2.80%。3HHx 的含量在一定范围内具有可控性。

添加异丙基硫代半乳糖苷(Isopropyl thiogalactoide,IPTG)的重组菌,合成的 PHBHHx 中,3HHx 含量与未添加的对照组没有显著差别,说明质粒本底表达的水平较高,并不需要额外添加 IPTG 来增强质粒的表达。

野生型 4AK4 也可以通过添加葡糖酸钠降低 3HHx 的摩尔含量,不过降低的幅度很小(16.85 % 降低至 13.84 %)。但是与此同时 PHBHHx 的含量也从 35.64 %降低至 20.61 %。这种调控 3HHx 含量的方法对于 PHBHHx 的积累是不利的。

2.3　重组菌 *A. hydrophila* 4AK4 (pTG01)在发酵罐中以葡萄糖酸钠和月桂酸(1∶1)为碳源的生长和 PHBHHx 合成

使用比例为 1∶1 的混合碳源,在 48 h 的发酵罐发酵培养中,获得了 32.8 g/L 的细胞干重(CDW),52%的 PHBHHx 含量,其中 3HHx 在 PHBHHx 中的摩尔含量为 6.7 %(如图 2 所示)。整个培养过程使用了 60 g/L 月桂酸和 60 g/L 葡萄糖酸钠,细菌共消耗了 15.3 g/L $(NH_4)_2SO_4$ 和 0.40 g/L 的磷元素。PHBHHx 的产率为 0.355 $g \cdot L^{-1} \cdot h^{-1}$。

在整个培养过程中,$(NH_4)_2SO_4$ 的浓度在 4 ~ 10 g/L 之间,氮源的供给充足,并没有起到限制作用。磷元素的浓度在整个过程中逐渐降低,当发酵结束时,还有 0.2 g/L。PHBHHx 的含量在发酵过程中比较稳定,一直在 50 % 左右。许多文献报导 N、P 元素的限制可以有效地提高 PHA 的含量。N、P 元素的限制对于提高重组菌 *A. hydrophila* 4AK4 (pTG01)生产的 PHBHHx 含量的作用有待于进一步的实验验证。

在发酵初期 3HHx 摩尔含量较高,约为 9 %,很快就逐渐降低并长时期稳定在 6.8 %左右。由于重组菌是通过把乙酰辅酶 A 转化为 3HB-CoA 的新的代谢途径提供额外的 3HB 前体,乙酰辅酶 A 在代谢中的流向会影响到 PHBHHx 中单体的组成。在发酵前期,可能是因为 *phbA*、*phbB* 所编码的酶的表达量还不够高,乙酰辅酶 A 更多流向 TCA 循环等提供能量的代谢途径,因此 3HHx 的含量在发酵前期较高。

发酵培养的结果验证了摇瓶实验的结果,使用糖类-脂肪酸作为混合碳源,可以通过改变两种碳源的比例来调节重组 *A. hydrophila* 4AK4 (pTG01)生产的 PHBHHx 的单体组成。

2.4　重组菌 *A. hydrophila* 4AK4 (pTG01)在发酵罐中以月桂酸为单一碳源的生长和 PHBHHx 合成

使用月桂酸为单一碳源,在 56 h 的发酵罐发酵

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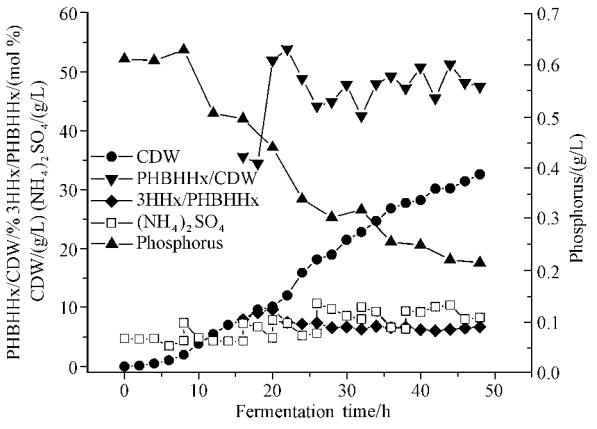

图 2 重组菌 *A. hydrophila* 4AK4 (pTG01)在发酵罐中以葡萄糖酸钠和月桂酸(1∶1)为碳源的菌体生长、PHBHHx 合成和底物消耗曲线图

Fig. 2 Time profile of cell growth, PHBHHx production, $(NH_4)_2SO_4$ and phosphorus uptake during the fermentation of *A. hydrophila* 4AK4 (pTG01) in a 5 L fermentor using sodium gluconate and lauric acid (1∶1) as carbon sources

培养中,获得了 51.5 g/L 的细胞干重(CDW),62 % 的 PHBHHx 含量,其中 3HHx 在 PHBHHx 中的摩尔含量为 9.7 %(如图 3 所示)。整个培养过程使用了 100 g/L 月桂酸,细菌共消耗了 19.6 g/L $(NH_4)_2SO_4$ 和 0.50 g/L 的磷元素。PHBHHx 的产率为 0.570 g·L^{-1}·h^{-1}。

在 56 h 的发酵培养中,$(NH_4)_2SO_4$ 的浓度介于 2~6 g/L 之间,磷元素逐渐降低至 0.07 g/L。PHBHHx 的含量缓慢升高,由前期的 45 % 左右提高至 60% 左右。以月桂酸为单一碳源,无论是 CDW 还是 PHBHHx 含量,都要高于使用混合碳源的结果。这说明对于 *A. hydrophila* 4AK4 (pTG01),脂肪酸还是比糖类更有利于菌体的生长和 PHBHHx 的积累。但是,由于以脂肪酸为底物带来了高成本等问题,这也是短链-中长链 PHA 共聚物生产中的一个普遍问题,需要进一步的研究加以解决。

3HHx 含量在整个发酵培养过程中的变化非常有趣,开始 3HHx 摩尔含量高达 11 %,之后缓慢降低至 7.5 %,后期又缓慢升至 9.7 %。这可能与乙酰 CoA 在细菌生长不同时间的流向有关。在对数期前期,乙酰 CoA 主要流向 TCA 循环以提供细菌分裂、生长所需的大量能量,很少流入合成 3HB 前体途径。因此,重组菌在发酵前期 3HHx 含量较高。进入细菌生长的对数期后期及稳定期后,流入合成 3HB 前体途径的乙酰 CoA 增加,3HHx 含量下降。但由于长期培养,质粒有一定程度的丢失,因此后期 3HHx 含量上升。但质粒丢失的程度从 3HHx 上升的程度看来,不是很严重。另一种 3HHx 含量变化的可能是与 *phbA*、*phbB* 基因表达的时期有关。培养的前期,如果这两个基因表达还未启动,或者表达的量很少,重组菌的表现则与野生型无异。

以月桂酸为单一碳源,重组菌只能利用 β 氧化本身提供的乙酰辅酶 A 来供给额外的 3HB 前体合成途径,因此,其对 PHBHHx 单体组成的调节能力非常有限,只能把 3HHx 的摩尔含量降低到 9.7 % 左右。但由于其 PHBHHx 的产率(0.570 g·L^{-1}·h^{-1})要远远高于以糖类-脂肪酸为碳源的结果(0.355 g·L^{-1}·h^{-1}),目前仍是最有效的生产 PHBHHx 的方法。

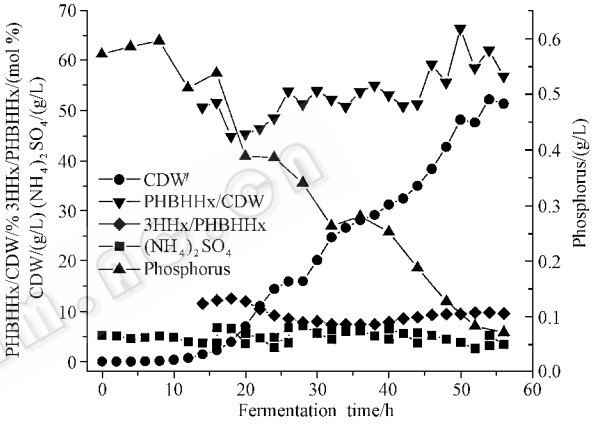

图 3 重组菌 *A. hydrophila* 4AK4 (pTG01)在发酵罐中以月桂酸为碳源的菌体生长、PHBHHx 合成和底物消耗曲线图

Fig. 3 Time profile of Cell growth, PHBHHx production, $(NH_4)_2SO_4$ and Phosphorus uptake during the fermentation of *A. hydrophila* 4AK4 (pTG01) in 5 L fermentor using lauric acid as sole carbon source

3 结 论

本研究在嗜水气单孢菌 *Aeromonas hydrophila* 4AK4 中引入了编码 β-酮基硫解酶(β-ketothiolase)的 *phbA* 基因和编码乙酰乙酰辅酶 A 还原酶(Acetoacetyl-CoA reductase)的 *phbB* 基因,以此构建了一条利用乙酰辅酶 A 合成 3-羟基丁酸-CoA 的代谢途径。利用葡萄糖酸钠和月桂酸作为碳源,对重组菌 *A. hydrophila* 4AK4 (pTG01)进行的摇瓶培养及 5 L 发酵罐培养的研究表明:可以通过改变碳源中葡萄糖酸钠和月桂酸两种组分的比例,使 *A. hydrophila* 4AK4 (pTG01)合成的 PHBHHx 中的 3HHx 摩尔含量则由原来的 15 % 左右降低到 3% ~ 12%,成功地实现了对 PHBHHx 单体组成的调控。在 5 L 发酵罐中,

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当以月桂酸为唯一碳源时,可以达到 0.570 g·L^{-1}·h^{-1} 的 PHBHHx 产率,获得 51.5 g/L 的细胞干重(CDW)、62% 的 PHBHHx 含量,其中 3HHx 在 PHBHHx 中的摩尔含量为 9.7 %.在 5 L 发酵罐中,当以 1∶1 的葡萄糖酸钠和月桂酸为碳源时,PHBHHx 的产率为 0.355 g·L^{-1}·h^{-1},获得了 32.8 g/L 的 CDW、52% 的 PHBHHx 含量,其中 3HHx 在 PHBHHx 中的摩尔含量为 6.7%。高密度生产高含量的、单体结构可控的 PHBHHx 成为可能。

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Fermentative Production of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) by Recombinant *Aeromonas hydrophila* 4AK4 (pTG01)

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Abstract　Copolyesters consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) (PHBHHx), a new type of biodegradable material, are receiving considerable attentions recently. The material properties are strongly related to the 3HHx fraction of PHBHHx. As the 3HHx fraction increase, crystallinity and melting point of PHBHHx decrease, flexibility and tractili-

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ty increase. PHBHHx of different 3HHx fraction can meet different demands of commercial application and research.

Aeromonas are the best studied PHBHHx-producing strains. Recent studies have been focused on optimizations of fermentative culture media and culture conditions for low-cost and efficient fermentative production. Aliphatic substrates such as long-chain fatty acid and soybean oil were used in the PHBHHx fermentation as the sole carbon source and energy source. Two-stage fermentation method was also developed for more efficient PHBHHx production. While studies on *Aeromonas hydrophila* revealed that the monomer composition of PHBHHx could not easily be regulated by fermentative process engineering methods such as changing substrates and fermentative conditions because precursors involved in the PHBHHx synthesis were all from the β-oxidation pathway.

In this study, *phbA* gene encoding β-ketothiolase and *phbB* gene encoding acetoacetyl-CoA reductase were introduced into a PHBHHx-producing strain *Aeromonas hydrophila* 4AK4 so as to provide a new 3HB precursors synthesis way. *phbA* gene encodes β-ketothiolase which can catalyze two acetyl-CoA to form acetoacetyl-CoA; *phbB* gene encodes acetoacetyl-CoA reductase catalyzing acetoacetly-CoA into 3HB-CoA which is the precursor of 3HB. The introduced novel 3-hydroxybutyrate precursor synthesis pathway allowed the recombinant strain to use unrelated carbon source such as gluconate to provide 3HB precursors for PHBHHx synthesis. Shake-flask experiments were carried out to produce PHBHHx of controllable monomer composition and fermentations in 5 L fermentor were also proceeded for confirmation of these result in large-scale culture.

In flask culture, it was possible to reduce the 3HHx mol fraction in PHBHHx from 15 % in the wild type to 3% ~ 12% in the recombinant by simply changing the ratio of gluconate to lauric acid in the culture media. When lauric acid was used as the sole carbon source, 51.5 g/L Cell Dry Weight (CDW) containing 62 % PHBHHx with 9.7 % 3HHx mol fraction was obtained in 56 hours of fermentation in a 5 liter fermentor. When co-substrates of sodium gluconate and lauric acid (1∶1) were used as carbon sources, 32.8 g/L CDW containing 52 % PHBHHx with 6.7 % 3HHx mol fraction was obtained in 48 hours of fermentation. These results showed the possibility for fermentative production of PHBHHx with controllable monomer composition.

Key words PHBHHx, *Aeromonas hydrophila*, 3HHx

干细胞用于治疗心脏病的研究

美国研究人员曾利用取自老鼠体内的干细胞,对一名心肌梗塞患者进行了移植治疗;法国研究人员也进行过取自骨骼肌的干细胞注入梗塞心肌治疗试验;德国罗斯托克大学医学院科研人员也对一名心肌梗塞患者进行自体干细胞移植治疗,但均未取得最令人满意的结果,工作还在继续着。而国内外研究人员从自体骨髓中提取干细胞用于治疗经动物试验取得了成功,目前用此方法对心脏病患者进行治疗亦取得了重要进展。德国杜塞尔多夫大学医学院研究人员成功地用患者自体干细胞为一名心肌梗塞患者(46 岁男子)进行治疗,该患者发生大面积心肌梗塞,大约 1/4 的心脏肌肉组织坏死,住院第 4 天,对他进行自体干细胞移植手术,即从患者身上取出健康干细胞,并将其注射到患者心肌梗塞坏死部分,干细胞自行渗入其坏死组织,并重新生长出心肌细胞,术后 10 个星期患者心肌梗塞缩小近 1/3,心脏功能也得到明显改善。在美国波士顿一家医院研究人员做了一项类似的动物试验,曾用骨髓间质干细胞(mesenchymal stem cell)修复软骨等损伤取得成功,但用于修复心脏组织再生却效果不佳,为此,对这种干细胞进行改造,在其中加入一种叫 Akt 蛋白质的基因,这种基因编码的蛋白质能够预防细胞死亡,尔后将这种改造的干细胞移植到心脏病发作的实验鼠心脏后,这些干细胞与损伤的心脏组织结合,此实验鼠丧失的心肌能力从此可恢复 80% ~ 90%,其心肌功能恢复到接近正常水平,这项结果有望用于人的心脏病。我国第四军医大学研究人员应用自体骨髓干细胞移植技术治疗急性心肌梗塞患者取得成功;上海复旦大学中山医院研究人员同样应用自体骨髓干细胞移植促进了心肌梗塞患者的心功能的恢复;北京大学干细胞研究中心与北京阜外心血管病医院合作开展干细胞用于治疗心脏病的研究,为治疗心脏病开发自体骨髓干细胞资源。

总之,干细胞用于治疗心脏病的实验研究取得成功,并展现其美好的开发前景。(1)自体干细胞移植治疗的心脏病如心肌梗塞患者不会产生排斥反应,也不涉及伦理问题;(2)将干细胞用于治疗心脏病是这项研究成果的一次重要突破,将鼓舞研究者不断实践与创新,为挽救更多的生命开辟一条新的治疗途径。

(柯 为 供稿)

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Enhancements in Ethanol Tolerance of a Self-flocculating Yeast by Calcium Ion Through Decrease in Plasmalemma Permeability

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Abstract Ca^{2+} at 1.64 mmol/L markedly increased ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. After 9 h of exposure to 20% (*V*/*V*) ethanol at 30℃, no viability remained for the control whereas 50.0% remained for the cells both grown and incubated with ethanol in Ca^{2+}-added medium. Furthermore, when subjected to 15% (*V*/*V*) ethanol at 30℃, the equilibrium nucleotide concentration and plasma membrane permeability coefficient (*P*′) of the cells both grown and incubated with ethanol in Ca^{2+}-added medium accounted for only 50.0% and 29.3% those of the control respectively, indicating that adding Ca^{2+} can markedly reduce plasma membrane permeability of yeast cells under ethanol stress as compared with the control. Meanwhile, high viability levels acquired by the addition of Ca^{2+} exactly corresponded to the striking decreases in extracellular nucleotide concentration and *P*′ achieved with identical approach. Therefore, the enhancing effect of Ca^{2+} on ethanol tolerance of this strain is closely related to its ability to decrease plasma membrane permeability of yeast cells subjected to ethanol stress.

Key words calcium ion, ethanol tolerance, plasma membrane permeability, self-flocculating yeast, viability

The accumulation of ethanol in yeast cultures leads to declines in growth, viability and ethanol formation[1]. The maximal concentration of ethanol obtained in broths at the end of fermentation is influenced by several factors. Therefore, many attempts have been made to enhance alcoholic fermentation, such as induction of alcohol-tolerant cells[2,3], screening of alcohol-tolerant mutants[4] and alteration of nutritional conditions[5]. It has been shown that a number of compounds, including unsaturated fatty acids and sterols, proteins, amino acids, vitamins and metal ions, result in improvements in alcoholic fermentations[6]. Complex medium such as Jerusalem artichoke juice has been found to lead to enhancements in alcoholic fermentation by increasing yeast ethanol tolerance[7,8]. It is possible that supplements such as oryzenin, albumin and koji mold mycelia may act in a similar way[2,6].

To support optimal growth and fermentation, yeasts require micro and millimolar concentrations of various inorganic cations[9]. These ionic species play either enzymatic role or structural role or both. Supplementing yeast fermentations with 0.5 mmol/L Mg^{2+} proved to prolong exponential growth and to reduce the decline in fermentation activity[10]. It has been suggested that some of the reported beneficial effects of complex nutrients on ethanol production could partially originate from the correction of a simple inorganic ion (such as magnesium) deficiency[10]. In recent years, a study has highlighted the role of magnesium in the amelioration of the detrimental effects of ethanol toxicity and temperature shock in a wine-making strain of *Saccharomyces cerevisiae*[11].

The addition of Ca^{2+} to a final concentration ranging from 2.5 to 10 mmol/L was found to enhance the thermostability of *Bacillus stearothermophilus*[12,13] by stimulating growth at supraoptimal temperatures and raising the maximum growth temperature. Ethanol and high temperatures interfere with membrane organization, increasing its fluidity and permeability to ions and small metabolites[1], and inhibiting the transport of nutrients[14]. Thus, the increase in the thermostability by supplementing calcium salts could possibly be extensible to an increase in ethanol tolerance of cells. In this work, a novel relationship between the enhancing effect of Ca^{2+} on yeast ethanol tolerance and its ability to decrease plasma membrane permeability of yeast cells subjected to ethanol stress was experimentally established. On the other hand, a self-flocculating yeast (a specific kind of immobilized cells containing no carrier)[15,16] was employed in this study to provide an opportunity for understanding ethanol tolerance displayed by non-free cells, with which few studies on yeast ethanol tolerance have ever been conducted up to now.

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1 MATERIALS AND METHODS

1.1 Strain, media and culture conditions

The strain SPSC used in this work was a self-flocculating yeast, a fusant of *Schizosaccharomyces pombe*[17] and *Saccharomyces cerevisiae*, which was constructed using protoplast fusion technology and preserved by our laboratory[15].

Agar slope medium for culture maintenance contained (g/L): glucose 10, yeast extract 3.85, peptone 3, agar 20. Growth medium for yeast aggregates formation consisted of (g/L): glucose 30, yeast extract 3.85, peptone 3. Fermentation medium for batch ethanol production was composed of (g/L): glucose 200, yeast extract 5, peptone 3. To form yeast aggregates, a loopful of cells from agar slope were used to inoculate 100-mL volumes of growth media and the cultures were incubated orbitally at 30 ℃ for 18 h. These prepared cell aggregates were used in the following experiments.

1.2 Evaluation of growth activity tolerant to ethanol

In this work, the above growth medium was used as basal medium. To examine the effect of Ca^{2+} (used as $CaCl_2$; all Ca^{2+} appearing in this paper represents $CaCl_2$) on strain SPSC growth activity resistant to ethanol, a series of initial concentrations of ethanol (6%, 8%, 9%, 10%, *V*/*V*) were formed by adding various amounts of ethanol to growth media which were supplemented or unsupplemented with 1.64 mmol/L Ca^{2+}. Then the growth media were inoculated with the prepared cell aggregates (with 18 h of culture time) and the cultures were incubated orbitally at 30 ℃ for 33 h. Evaluation of the effect of Ca^{2+} on growth activity tolerant to ethanol was based on comparison of the biomass formation of Ca^{2+}-supplemented cultures with that of unsupplemented cultures. Biomass was measured by determination of dry weight of cells in broth and expressed as gram (dry weight) of cells per liter of broth[15].

1.3 Evaluation of fermentation activity tolerant to ethanol

To test the influence of Ca^{2+} on strain SPSC fermentation activity tolerant to ethanol, different amounts of ethanol were respectively added to fermentation media supplemented with or without 1.64 mmol/L Ca^{2+} to produce a series of initial concentrations of ethanol (9%, 11%, 12%, 13%, *V*/*V*) and then the prepared cell aggregates were inoculated into the media and the cultures were incubated orbitally at 30 ℃ for 50 h. The effect of Ca^{2+} on fermentation activity tolerant to ethanol was evaluated by comparing the net ethanol production (not including initial added ethanol) of Ca^{2+}-supplemented fermentations with that of unsupplemented fermentations. The concentration of ethanol was determined by gas chromatography (Agilent 6890A GC, USA, equipped with flame ionization detector). The temperature of the inlet and of the detector was 160 ℃ and 230 ℃ respectively, and oven was operated isothermically at 90 ℃. The carrier gas was N_2 and n-butanol was used as the internal standard.

1.4 Measurement of viability

The above growth medium was also used in this research to prepare cell aggregates which would be incubated with 20% (*V*/*V*) ethanol to trigger ethanol stress. After centrifugation and two washes with distilled water, cells grown in Ca^{2+} (1.64 mmol/L)-supplemented or unsupplemented medium were incubated with 20% (*V*/*V*) ethanol buffer solution added with or without 1.64 mmol/L Ca^{2+} at 30 ℃. At suitable intervals, samples were taken in order to follow the concentrations of viable cells. Viable cells were counted by plating appropriate dilutions of cells on agar slope media and incubating at 30℃ for 2 or 3 days. Viability (%) = $(C_t/C_0) \times 100\%$, where C_0 and C_t represent number of viable cells per mL of cell suspension at the onset and a given time of incubation with ethanol respectively.

1.5 Measurements of extracellular nucleotide concentration and plasma membrane permeability coefficient

In this work, the above growth medium was used to prepare cell aggregates for being incubated with 15% (*V*/*V*) ethanol for the purpose of measurements of extracellular nucleotide concentration and plasma membrane permeability coefficient (P'). After harvested and washed with distilled water repeatedly until the absorbance of the supernatant at 260 nm was negligible, cells grown in Ca^{2+} (1.64mmol/L)-supplemented or unsupplemented medium were suspended in an aqueous solution of 15% (*V*/*V*) ethanol added with or without 1.64 mmol/L Ca^{2+} and then mildly shaken on a rotary shaker at 30 ℃. The absorbances of the supernatant at 260 nm and 280 nm were measured periodically until they reached the equilibrium. The nucleotide concentration (expressed as microgram of phosphorus per mL of supernatant), was calculated from the following equation[18]:

$$\text{Nucleotide} = 11.87\,A_{260} - 10.40\,A_{280}$$

The membrane permeability coefficient of nucleotide, P' (cm/h), was evaluated by using the following equation (representative of a simple diffusion model)[18]:

$$\ln(C_e^{\infty} - C_e) = \ln(C_e^{\infty} - C_e^{0}) - (1 + V_i/V_e)(A/V_i)P't$$

where t is time (h), C_e is the extracellular solute concentration (mol/cm^3), A is the total surface area of the cell membrane (cm^2), V_i is the total intracellular liquid volume (cm^3), and V_e is the extracellular liquid volume (cm^3). C_e^0 and C_e^{∞} represent C_e when $t = 0$ and ∞ respectively.

1.6 Statistical methods

In this work, all experiments were performed in triplicate and all determinations were done in duplicate with mean values given.

2 RESULTS AND DISCUSSION

The optimal concentration of Ca^{2+} to be used as an additive in this work was determined to be 1.64 mmol/L based on batch ethanol fermentation employing strain SPSC (data not shown). A Ca^{2+} concentration below or above 1.64 mmol/L (but within the tested range of 0.20 to 2.40 mmol/L) led to decrease in the maximal concentration of

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ethanol finally obtained in broths, but its level was still higher than that of the control (unsupplemented with Ca^{2+}) (data not shown). As a result, a Ca^{2+} concentration of 1.64 mmol/L was chosen for subsequent studies.

2.1 Effect of Ca^{2+} addition on strain SPSC growth and fermentation activity tolerant to ethanol

Measurement of growth in the presence of ethanol is the frequently used method for determining ethanol tolerance[19]. Fig. 1 shows the effect of Ca^{2+} on growth activity tolerant to ethanol of strain SPSC. At each initial concentration of ethanol (6%, 8%, 9%, 10%, *V*/*V*) present in growth medium, the maximal growth of Ca^{2+}-supplemented cultures always remained higher than that of unsupplemented cultures. On the other hand, the increases in the maximal growth of Ca^{2+}-supplemented cultures over unsupplemented cultures were around 8.2%, 11.1%, 17.2% and 50.0% for 6%, 8%, 9% and 10% initial ethanol present in growth media respectively. These indicate that an optimal concentration of Ca^{2+} effectively promotes strain SPSC growth activity resistant to ethanol.

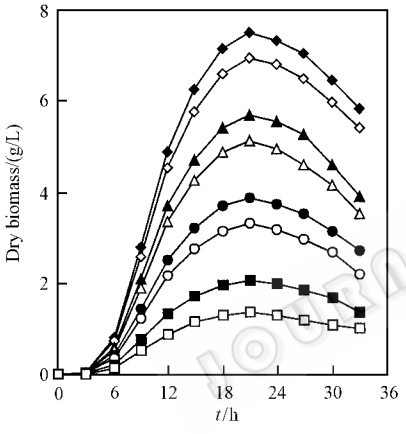

Fig. 1 Effect of Ca^{2+} addition on biomass formation of strain SPSC in the presence of initial ethanol

Closed and open symbols represent cultures supplemented and unsupplemented with 1.64 mmol/L Ca^{2+} respectively. Initial concentration of ethanol added to medium (*V*/*V*): ◆,◇ 6%; ▲,△ 8%; ●,○ 9%; ■,□ 10%

The inhibition of fermentation capacity (the maximal amount of ethanol produced during fermentation) by exogenously supplemented ethanol is another parameter usually employed to evaluate ethanol tolerance of yeast[6]. Fig. 2 illustrates the impact of Ca^{2+} on net ethanol production (not including initial added ethanol) of this strain. At each initial concentration of ethanol added to fermentation medium, the maximal net ethanol production of Ca^{2+}-supplemented cultures always remained higher than that of unsupplemented cultures. This shows that an optimal concentration of Ca^{2+} somehow protects this strain from toxic effects of ethanol, thus increasing its fermentation activity resistant to ethanol.

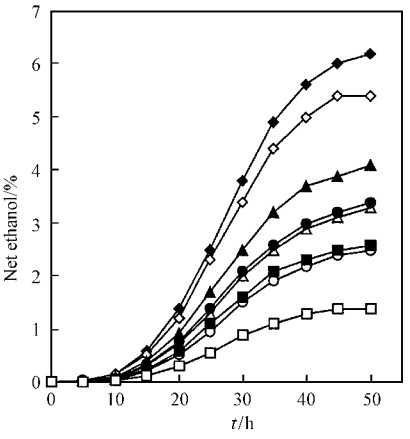

Fig. 2 Effect of Ca^{2+} addition on net ethanol production of strain SPSC in the presence of initial ethanol

Closed and open symbols represent fermentations supplemented and unsupplemented with 1.64 mmol/L Ca^{2+} respectively. Initial concentration of ethanol added to medium (*V*/*V*): ◆,◇ 9%; ▲,△11%; ●,○ 12%; ■,□ 13%

2.2 Effect of Ca^{2+} addition on viability of strain SPSC subjected to 20% (*V*/*V*) ethanol

The role of Ca^{2+} in ethanol tolerance of this strain was further tested by exposing cells to 20% (*V*/*V*) ethanol at 30 ℃. Meanwhile, the influence of different modes of Ca^{2+} addition on viability was also examined (Fig. 3). As shown in Fig. 3, whichever mode of Ca^{2+} supplementation was employed, cells in media added with Ca^{2+} always had higher viability levels as compared with the control. After 9 h of exposure to ethanol, no viability was observed for the control, whereas 50.0% remained for the cells both grown and incubated in

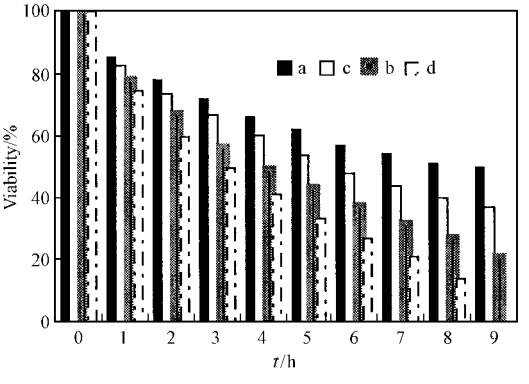

Fig. 3 Effect of Ca^{2+} addition on viability of strain SPSC subjected to 20% (*V*/*V*) ethanol at 30℃

Letters (a, b, c, d) denote: a. Cells grown and incubated with ethanol in Ca^{2+} (1.64 mmol/L)-supplemented medium; b. Cells grown in Ca^{2+} (1.64 mmol/L)-supplemented medium but incubated with ethanol in unsupplemented medium; c. Cells grown in unsupplemented medium but incubated with ethanol in Ca^{2+} (1.64 mmol/L)-supplemented medium; d. Cells grown and incubated with ethanol in unsupplemented medium (control)

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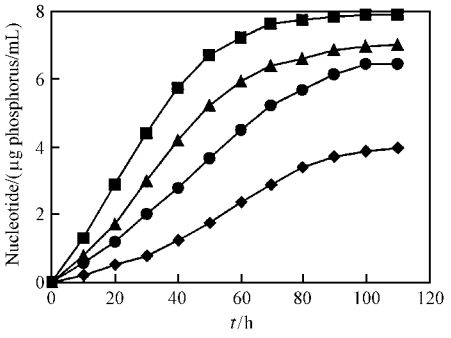

Fig.4 Effect of Ca^{2+} addition on extracellular nucleotide concentration in cell suspension of strain SPSC subjected to 15% (*V*/*V*) ethanol at 30℃

The denotation of each letter (a, b, c, d) is identical to that described in Fig. 3 except that the concentration of ethanol with which cells were incubated was 15% (*V*/*V*). —◆— a; —▲— b; —●— c; —■— d

Ca^{2+}-supplemented medium. Viability was highest in the order of both growth and incubation supplementation of Ca^{2+}, only incubation supplementation, only growth supplementation and no supplementation.

2.3 Effect of Ca^{2+} addition on extracellular nucleotide concentration and plasma membrane permeability coefficient of strain SPSC subjected to 15% (*V*/*V*) ethanol

The above experimental data show that an optimal concentration of Ca^{2+} is able to markedly enhance ethanol tolerance of strain SPSC. But in what way does Ca^{2+} play such a role? In order to gain insight into the way employed by Ca^{2+} to protect yeast cells from toxic effects of ethanol and thus to increase alcohol tolerance, experiment was carried out to test the influence of Ca^{2+} on plasma membrane permeability of strain SPSC subjected to 15% (*V*/*V*) ethanol at 30 ℃. Also, different modes of Ca^{2+} addition (Fig.4) were performed in the same way as the viability experiment (Fig.3). Fig.4 shows the effect of Ca^{2+} on diffusion of intracellular nucleotide across plasma membrane. Whichever mode of Ca^{2+} addition was adopted, cells in Ca^{2+}-supplemented media always retained lower extracellular nucleotide concentrations as compared with the control. The equilibrium nucleotide concentration of the cells both grown and incubated in Ca^{2+}-supplemented medium accounted for only 50.0% of the control level. The extracellular nucleotide concentration was lowest in the order of both growth and incubation supplementation of Ca^{2+}, only incubation supplementation, only growth supplementation and no supplementation, which exactly corresponded to the ranked order of viability (from the highest to the lowest, representing ethanol tolerance from the strongest to the weakest) (Fig.3). These indicate that strong ethanol tolerance acquired by adding Ca^{2+} is closely associated with striking decrease in extracellular nucleotide concentration achieved with identical approach. Based on these results, it appears that Ca^{2+} somehow strengthens plasma membrane permeability barrier of strain SPSC under ethanol stress. This hypothesis prompted an investigation into the effect of Ca^{2+} on plasma membrane permeability coefficient of yeast cells subjected to 15% (*V*/*V*) ethanol at 30 ℃, with the results listed in Table 1.

Table 1 Effect of Ca^{2+} addition on plasma membrane permeability coefficient (*P*′) of strain SPSC subjected to 15% (*V*/*V*) ethanol at 30 ℃

Culture condition*	*P*′/(cm/h)
Ⅰ	6.33×10^{-7}
Ⅱ	9.65×10^{-7}
Ⅲ	1.32×10^{-6}
Ⅳ	2.16×10^{-6}

* Ⅰ: cells grown and incubated with ethanol in Ca^{2+} (1.64 mmol/L)-supplemented medium;

Ⅱ: cells grown in unsupplemented medium but incubated with ethanol in Ca^{2+} (1.64 mmol/L)-supplemented medium;

Ⅲ: cells grown in Ca^{2+} (1.64 mmol/L)-supplemented medium but incubated with ethanol in unsupplemented medium;

Ⅳ: cells grown and incubated with ethanol in unsupplemented medium (control)

As shown in Table 1, whichever mode of Ca^{2+} addition was adopted, cells in media supplemented with Ca^{2+} always had lower membrane coefficients than the control level. For instance, *P*′ of the cells both grown and incubated in Ca^{2+}-supplemented medium accounted for only 29.3% that of the control. This just confirms that Ca^{2+} is indeed able to markedly reduce plasma membrane permeability of this strain under ethanol stress as compared with the control, indicating its potential to strengthen plasma membrane permeability barrier. Meanwhile, mode response of *P*′ to added Ca^{2+} also occurred: *P*′ became smallest in the order of Ⅰ, Ⅱ, Ⅲ and Ⅳ (Table 1), precisely corresponding to the ranked order of extracellular nucleotide concentration (from the lowest to the highest) (Fig.4), and of viability (from the highest to the lowest) (Fig.3). Thus, the enhancement by Ca^{2+} on ethanol tolerance of strain SPSC is closely relevant to its ability to decrease plasma membrane permeability of yeast cells subjected to ethanol stress.

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