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Research Paper 研究报告

D-乳酸生产菌菊糖芽孢乳杆菌来源的D-乳酸脱氢酶同工酶

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摘要：【目的】菊糖芽孢乳杆菌(*Sporolactobacillus inulinus*)作为典型的同型发酵产D-乳酸的优势菌株，能够高效生产高纯度的D-乳酸。该菌株发酵受到多方面环境因素影响。糖代谢的关键酶例如葡萄糖激酶、磷酸果糖激酶、丙酮酸激酶以及乳酸脱氢酶均为由葡萄糖代谢成为乳酸的关键酶，该菌中相关代谢酶的研究是发酵调控至关重要的基础。分析*S. inulinus*的基因组表明有3个推测为D-乳酸脱氢酶的基因，其中已有报道研究了1个双功能蛋白[bifunctional protein (BP)]。本研究分别克隆并解析了另2个D-乳酸脱氢酶同工酶的性质。【方法】本研究以*S. inulinus* Y2-8基因组DNA为模板,克隆得到2个D-*ldh*基因(*dldh*、*dhdh*)，经测序分别为D-乳酸脱氢酶[D-lactic acid dehydrogenase (DLDH)]和D-羟基酸脱氢酶[D-isomer specific 2-hydroxyacid dehydrogenase (DHDH)]的基因。构建的重组菌表达蛋白DLDH，DHDH均具有催化丙酮酸生成D-乳酸的功能。【结果】重组菌表达的蛋白经镍柱亲和层析达到电泳纯。SDS-PAGE分析表明DLDH的表观分子量为37 kDa，DHDH的表观分子量为39 kDa。此外，DLDH以丙酮酸为底物时K_m值为(0.58±0.04) mmol/L，对底物有较高的亲和力，最适反应温度为35 °C，最适pH为6.5；而DHDH以丙酮酸为底物时K_m值为(1.70±0.08) mmol/L最适反应温度为30 °C，最适pH为7.5。另有报道的BP以丙酮酸为底物时K_m值为(3.40±0.02) mmol/L，最适反应温度为30 °C，最适pH为5.5。【结论】根据对底物丙酮酸的亲和力，最适温度及最适pH，推测DLDH是乳酸发酵中产D-乳酸的主导催化剂。结合相关酶学性质的分析可为今后的发酵调控提供理论依据。

关键词：菊糖芽孢乳杆菌，D-乳酸脱氢酶，克隆，酶学性质

近年来聚乳酸材料技术的创新突破提高了聚乳酸的力学、耐热、耐久性能及生物相容性，促进了聚乳酸在食品、药学、纺织、化工材料等高性能、高附加值材料领域的应用拓展[1]。研究表

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明通常需要通过聚D-乳酸[Poly(D-lactic acid)，PDLA]和聚L-乳酸[Poly(L-lactic acid)，PLLA]共聚实现聚乳酸材料的改性[2–3]，这又进一步带动了D-乳酸的生产研究。

鉴于高光学纯度D-乳酸的市场需求，生产高光学纯度D-乳酸的菌株及高产发酵工艺已成为了研究热点，例如通过分子改造，发酵调控、代谢调控等手段，以期获得高产高光学纯度的D-乳酸菌株[4–6]。常见的乳酸菌基因组中同时存在D-乳酸脱氢酶(D-lactate dehydrogenase, DLDH)和L-乳酸脱氢酶(L-lactate dehydrogenase, LLDH)，这2种酶的相对催化活性决定了最终发酵产物的光学纯度[7]，因此解析菌株中不同乳酸脱氢酶的作用，并通过分子手段对菌株进行改造及实现发酵调控具有重要的意义。Ishida等通过敲除酿酒酵母的L-乳酸脱氢酶以及重组来自肠膜明串珠菌的2个D-乳酸脱氢酶，最终获得高达99.9%的D乳酸[8]。2008年，Okino等将谷氨酸棒杆菌株中原有的L-乳酸脱氢酶基因失活，并表达来源于德氏乳酸杆菌的D-乳酸脱氢酶，改造后的基因工程菌产D-乳酸光学纯度能达到99.9%[9]。Okano等通过敲除植物乳杆菌菌株中的L-乳酸脱氢酶基因，并异源表达牛链球菌中的α-淀粉酶，新构建的菌株采用玉米淀粉为原料，发酵48 h，产光学纯度99.6%的D-乳酸达到73.2 g/L[10]。然而基因工程菌在实际生产中有培养体系复杂、生产效率较低等诸多不利因素。菊糖芽孢乳杆菌是一株野生的D-乳酸生产优势菌株，D-型乳酸脱氢酶是D-乳酸生产的关键酶，本研究对其基因组中存在的3个D-乳酸脱氢酶进行系统分析，有利于进一步的发酵调控。

本课题组前期利用低能氮离子注入诱变，筛选得到了1株稳定高产的菌株*S. inulinus* Y2-8，并对其产乳酸代谢的关键酶葡萄糖激酶、磷酸果糖激酶、丙酮酸激酶及其相关调控机理进行了研究[11–12]。本研究首先分析了*S. inulinus* Y2-8的基因组中推测的3个D-乳酸脱氢酶同工酶基因[13]，分别为DLDH (ZP_09713864)、DHDH (ZP_09713594)、BP (ZP_09713297)。在此基础上，本研究进一步克隆表达了该菌株中的D-型乳酸脱氢酶同工酶DLDH及DHDH，并分析其酶学性质。最后结合Zhu等对BP的相关报道[14]，本文推测了*S. inulinus* Y2-8中3个D-乳酸脱氢酶同工酶在产乳酸发酵中的作用，为后期发酵调控提供理论依据。

1 材料和方法

1.1 菌株和载体

所用菌株及质粒见表1。

1.2 酶和试剂

*Taq*mix酶，DNA限制性内切酶，DNA-T4连接酶，DL-2000 Marker，Protein Marker，基因组抽提试剂盒等购于TaKaRa公司；凝胶回收试剂盒

表1. 实验所用菌株和质粒

Table 1. The strains and plasmids used in the study

Strains and plasmids	Genotype	Source
S. inulinus Y2-8	High D-lactic-acid-producing strains breeding by the low-energy nitrogen ion beam	Stored in this lab
E. coli BL21(DE3)	F^-ompT hsdSB(rB^-, mB^-) gal dcm (DE3)	Stored in this lab
E-pET-28a/*dldh*	*E. coli* BL21 (pET-28a/*dldh*)	This study
E-pET-28a/*dhdh*	*E. coli* BL21 (pET-28a/*dhdh*)	This study
pET-28a(+)	$Kana^r$, T7 promoter, containing His-tag before multiple cloning site *Nde* I	Novagen
pET-28a/*dldh*	pET-28a containing a 1 kb *dldh* gene from *S. inulinus* Y2-8	This study
pET-28a/*dhdh*	pET-28a containing a 1 kb *dhdh* gene from *S. inulinus* Y2-8	This study

和质粒提取试剂盒以及PCR产物回收试剂盒均购于康宁生命科学(吴江)有限公司；丙酮酸钠、IPTG、NADH、NADPH等购于Sigma；卡那霉素购于南京生兴生物有限公司。

1.3 培养基和培养条件

LB培养基(g/L)：胰蛋白胨10、酵母粉5、NaCl 10，pH 7.0。

LB+Kana培养基：LB培养基灭菌后冷却至60 °C再加入过滤除菌的Kana使其终浓度为50 μg/mL。

S. inulinus Y2-8平板培养基(g/L)：葡萄糖20.0，酵母膏2.0，蛋白胨2.0，玉米浆 2.0 mL，KH_2PO_4 1.0，无水乙酸钠2.0，$MgSO_4$ 0.2，pH 7.0。

S. inulinus Y2-8种子培养基(g/L)：葡萄糖20.0，酵母膏2.0，蛋白胨2.0，玉米浆5.0 mL，$MgSO_4$ 0.2，麸皮2.0，$CaCO_3$14.0，pH 7.0。

相应的固体培养基，在以上成分基础上再加入20 g/L的琼脂。

首先将保存在–80 °C冰箱中的*S. inulinus* Y2-8接种到平板上活化，37 °C厌氧条件下培养48 h后再次转接到平板上生长48 h，接入种子培养液后在37 °C、150 r/min培养16 h，用于*S. inulinus* Y2-8的基因组的提取。

1.4 基因扩增及重组质粒的构建

参照操作手册，用TaKaRa的基因组抽提试剂盒提取*S. inulinus* Y2-8的基因组，以*S. inulinus* Y2-8的基因组为扩增模板，分别设计带有肠激酶酶切位点(GACGACGACGACAAG)及双酶切位点的扩增引物如表2所示，经PCR扩增后用试剂盒纯化回收PCR产物后与载体pET-28a连接，转入大肠杆菌BL21，经带有Kana抗性的平板筛选后送金维智测序。

1.5 诱导表达条件的优化，纯化及SDS-PAGE检测

对重组菌株的诱导表达条件(包括诱导时间，诱导温度，诱导剂浓度)进行优化，在含有50 μg/mL卡那霉素的LB培养基中按1%接种培养的种子液，37 °C摇瓶发酵至菌液OD_{600}值为0.6–0.8后加入0.01 mmol/L IPTG并在25 °C、150 r/min条件下诱导12 h，冷冻离心收集菌泥，用50 mmol/L Tris-HCl，pH 7.5缓冲液洗涤悬浮，采用高压匀浆破碎仪破碎后离心取上清。参照泰纯镍离子金属螯合亲和层析介质使用说明书的方法，利用重组蛋白中带有的His-Tag进行镍柱亲和层析，获得电泳纯的DLDH及DHDH酶液，随后将镍柱纯化的酶进行rEK酶切除去His-Tag。反应体系为：20 mmol/L Tris-HCl，100 mmol/L NaCl，酶液(0.2 mg)和1 μL rEK，pH 7.0，25 °C反应6 h，酶切产物使用镍柱亲和层析二次纯化获得纯化的蛋白，并采用SDS-PAGE分析。

表2. 本研究所用引物

Table 2. Primers used in this study

Primer	Sequence (5′→3′)
DHDH-F	GCGGCATATGGACGACGACGACAAGATGGCTTTTAAAATTATTGCG (*Nde* I)
DHDH-R	GCGCAAGCTTTCATTTTTTTGCTGGTTC (*Hin*d III)
DLDH-F	GCGGGCTAGCGACGACGACGACAAGATGAAGCTATTCATGTATGGTGTCC (*Nhe* I)
DLDH-R	GCGGAAGCTTTTATTGAGTGACAGCCGGCTTC (*Hin*d III)

1.6 D-羟基酸脱氢酶酶活检测及酶学性质研究

酶活力定义：每分钟催化1 μmol辅酶NADH/NADPH氧化形成NAD^+/$NADP^+$所需的酶量为1个酶活单位。

乳酸脱氢酶酶活检测反应在酶标板中进行，整个反应体系为250 μL (100 mmol/L pH 5.6的磷缓冲液，20 mmol/L丙酮酸钠，0.5 mmol/L NADH/NADPH，10 μL酶液)，通过最后加入丙酮酸钠溶液启动反应，测定340 nm处光吸收的变化来检测NADH/NADPH的变化。以灭活的酶为空白对照。并采用考马斯亮蓝法检测酶液的蛋白浓度。

最适反应温度分析：分别测定纯化的酶在不同温度下(25–45 °C)的酶活力。

最适反应pH分析：分别配制浓度为50 mmol/L的不同pH的反应缓冲液，其中pH 5.0–7.0为柠檬酸-柠檬酸钠($C_6H_8O_7$-$Na_3C_6H_5O_7$)缓冲液，pH 7.0–9.0为Tris-HCl缓冲液，pH 9.0–10.5为Glycine-NaOH缓冲液，测定纯酶在不同pH反应缓冲液中的酶活力。

酶动力学参数分析[15]：在最适反应条件下，分别检测DLDH、DHDH在NAD(P)H浓度为0.40 mmol/L时不同丙酮酸钠浓度(0.05、1.00、2.00、4.00、8.00、10.00、20.00、40.00 mmol/L)浓度下各乳酸脱氢酶的酶活。采用Lineweaver-Burk双倒数作图法，以底物浓度的倒数(1/[*S*])为横坐标(X)，酶反应速度的倒数(1/*V*)为纵坐标(Y)作图，进行直线拟合，计算得米氏常数K_m和最大反应速率V_{max}。

2 结果和分析

2.1 菊糖芽孢乳杆菌D-乳酸脱氢酶及D-羟基酸脱氢酶基因的克隆与表达

在NCBI上搜索到菊糖芽孢乳杆菌[13]可能具有D-乳酸脱氢酶功能的基因有3个，分别为DLDH、DHDH及BP。本研究以提取的*S. inulinus* Y2-8基因组为模板，分别以方法1.4所示引物扩增得到带肠激酶酶切位点的*dldh*和*dhdh*基因，将纯化后的产物与PMD-18-T载体连接，经测序分析，与预测的一致，长度分别为1020 bp和1041 bp。将扩增片段双酶切插入表达载体pET-28a(+)中，得到pET-28a/*dldh*和pET-28a/*dhdh*，将重组质粒导入*E. coli* BL21 感受态细胞中，获得重组子*E*-pET-28a/*dldh*和*E*-pET-28a/*dhdh*。重组子提取质粒经双酶切鉴定，结果如图1所示，pET-28a/*dldh*和pET-28a/*dhdh*在1000 bp左右比空载体pET-28a多出一条带，与插入的外源基因大小一致，表明外源基因已成功插入到载体中。

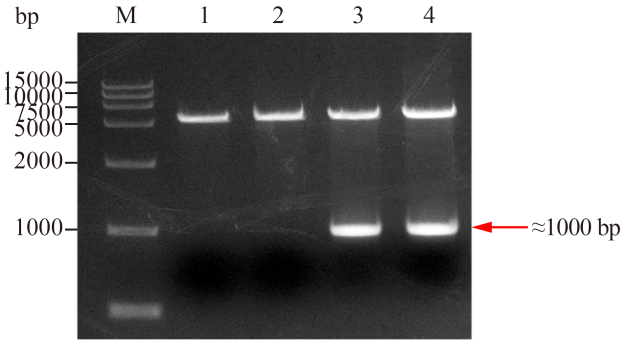

图 1. 重组质粒pET-*dldh*及pET-*dhdh*的双酶切验证电泳图谱

Figure 1. Agarose gel electrophoresis analysis for enzymatic digestion result of recombinant plasmids pET-*dldh* and pET-*dhdh*. M: DNA marker DL15000; lane 1: pET-28a/*Nhe* I+*Hin*d III; lane 2: pET-28a/*Nde* I+*Hin*d III; lane 3: pET-28a/*dldh*/*Nhe* I+*Hin*d III; lane 4: pET-28a/*dhdh*/*Nde* I+*Hin*d III.

重组子*E*-pET-28a/*dldh*和*E*-pET-28a/*dhdh*经诱导表达优化，初步优化后的表达条件为37 °C摇瓶发酵至菌液OD_{600}的值为0.6–0.8后加入IPTG，25 °C诱导12 h，收集菌液破碎离心后的上清液，通过镍柱纯化，纯化蛋白在SDS-PAGE中呈单一条带如图2所示。图2-A显示DLDH的大小约为37 kDa (DLDH含334个残基，理论分子量为36.8 kDa)，与理论分子量相近；图2-B显示DHDH的分子量约为39 kDa (DHDH含有341个氨基酸，理论分子量为37.3 kDa)，略高于理论分子量。本研究克隆的*S. inulinus* Y2-8来源的2个D-乳酸脱氢酶同工酶分子量与现报道的来源于鼠李糖乳杆菌、肠膜明串珠菌肠膜亚种中D-型乳酸脱氢酶的分子量相似[15–16]。

2.2 D-型乳酸脱氢酶DLDH、DHDH酶学性质分析

D-型乳酸脱氢酶DLDH和DHDH的最适催化温度和pH及两酶对底物丙酮酸的亲和力K_m等相关酶动力学参数见表3，并与该菌中已报道的另一种推测为D-型乳酸脱氢酶(同时标注为BP)一同分析。其中DLDH对丙酮酸显示了最高的亲和力，K_m

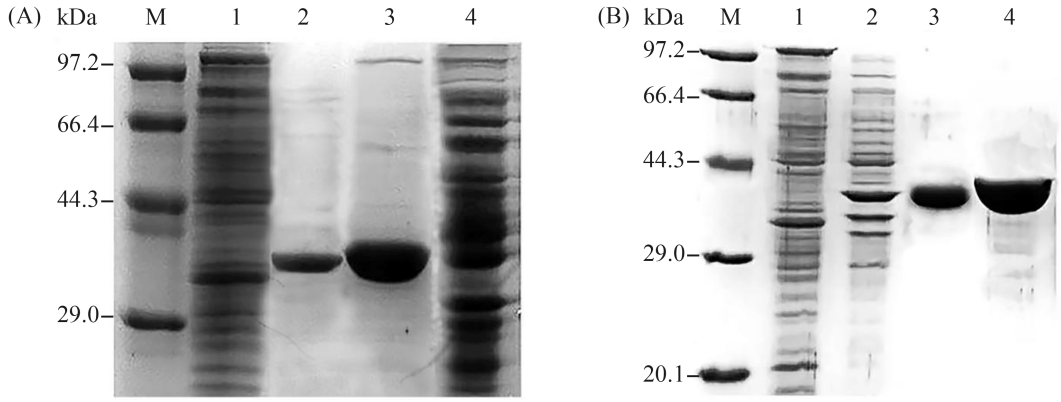

图 2. 纯化的乳酸脱氢酶的SDS-PAGE图谱

Figure 2. SDS-PAGE analysis of purified DLDH (A) from recombinant *E*-pET-28a/*dldh* and DHDH (B) from recombinant *E*-pET-28a/*dhdh*. A: lane M, protein marker; lane 1, crude extracts of *E*-pET28a; lane 2, purified DLDH without His tag; lane 3, DLDH purified by Ni^{2+} affinity chromatography; lane 4, crude extracts of *E*-pET-28a/*dldh*. B: lane M, protein marker; lane 1, crude extracts of *E*-pET28a; lane 2, crude extracts of *E*-pET-28a/*dhdh*; lane 3, purified DHDH without His tag; lane 4, DHDH purified by Ni^{2+} affinity chromatography.

表3. 菊糖芽孢乳杆菌CASD中几种D-乳酸脱氢酶同工酶的对比

Table 3. Comparison of d-(-)-lactate-dehydrogenase isozyme from *S. inulinus* CASD

Parameters	D-lactic acid Dehydrogenase (DLDH)	D-isomer specific 2-hydroxyacid dehydrogenase (DHDH)	Bifunctional protein (BP)
Molecular mass/kDa	37	39	37
Activity/(U/mg)	56.20±0.43	43.30± 0.21	7.50 ± 0.17
Optimum T[a]/°C	35[b]	30[b]	30[b]
Optimum pH	6.5[b]	7.5[b]	5.5[b]
K_m/(mmol/L)	0.58±0.04	1.70±0.08	3.40±0.02
Reference	This study	This study	[14, 17]

[a]: T means temperature. [b]: optimal temperature and pH values are averages of results from three replicates.

值为(0.58±0.04) mmol/L，进一步研究表明DLDH基本不利用NADPH辅酶，说明DLDH是NADH依赖性脱氢酶家族。而DHDH对丙酮酸的亲和力居中，以NADH为辅酶时的K_m值为(1.70±0.08) mmol/L，DHDH同时能利用NADPH，其K_m值为(1.76±0.05) mmol/L。Zhu等报道的该菌株中BP[11−12]对丙酮酸的亲和力较低，K_m值为(3.40±0.02) mmol/L[14]。

温度对酶活性的影响见图3-A。DLDH的最适反应温度为35 °C。在反应温度为20–35 °C阶段，随着温度的升高，酶活显著提高，当温度为40 °C时，酶活力约为最适活力的84%；DHDH的最适反应温度为30 °C，当反应温度大于30 °C时酶活开始缓慢下降，当温度高于35 °C时，酶活力快速下降。报道的BP的最适反应温度也为30 °C[17]，与DHDH的最适温度相近。pH对酶活性的影响见图3-B，DLDH的最适pH为6.5；DHDH的最适pH呈弱碱性为7.5，然而在pH 6.5时能呈现最适活力的85%；而BP的最适pH为5.5[17]。3个D-乳酸脱氢酶呈现了不同的最适催化pH。

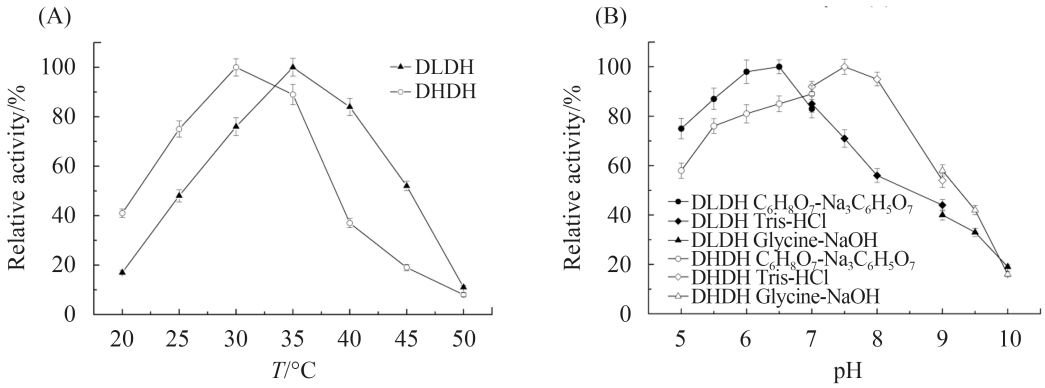

图 3. 温度(A)及pH (B)对DLDH、DHDH酶活的影响

Figure 3. Effect of temperatures (A) or pH (B) on the activity of DLDH (solid) and DHDH (open).

3 讨论

菊糖芽孢乳杆菌(*Sporolactobacillus inulinus*)是重要的高纯度的D-乳酸的生产菌，本课题组在前期研究中通过诱变筛选，发酵调控等手段在提高新获取的菌株*S. inulinus* Y2-8产量上取得了可喜的成效，并对影响其产乳酸的几个关键酶进行了相关研究[11−12]，另有Yu等解析了*S. inulinus* CASD的全基因[13]，Zhu等系统地研究了另1个D-型乳酸脱氢酶同工酶，该酶同时具有谷氨酸脱氢酶酶活，同时被注释为BP[14]，该酶以丙酮酸钠为底物时，K_m值为(3.40±0.02) mmol/L，酶催化的最适温度为30 °C，最适催化pH为5.5。本研究又进一步克隆了*S. inulinus* Y2-8中的*dldh*及*dhdh*基因并将其在大肠杆菌BL21(DE3)中进行了表达。对这2种乳酸脱氢酶同工酶的酶学性质展开了分析，该菌中3个D-乳酸脱氢酶的性质汇总见表3。本课题组前期研究结果表明该菌株在发酵过程中最适生长及产酸温度为36–38 °C，以$CaCO_3$为中和剂时发酵的自然pH为5.0，并通过荧光探针检测发现随着发酵的进行，该菌株的胞内pH始终维持在6.3–6.5[11]。由汇总表3可见，该菌的DLDH对丙酮酸显示了最高的亲和力，且该酶的最适催化温度为(35 °C)及pH (6.5)最接近优化后的实际发酵条件，推测在*S. inulinus* Y2-8发酵催化丙酮酸生成D-乳酸中起到最主要的作用。该菌的DLDH的最适催化pH为6.5，与菌株在发酵过程中的胞内pH非常接近，此外，DHDH在pH 6.5时仍呈现最适酶活力的85%，实际发酵条件下3个D-乳酸脱氢酶的贡献度应在对应发酵条件下进一步分析相关乳酸脱氢酶的转录来进一步阐述。

S. inulinus Y2-8来源的3个D-型乳酸脱氢酶的最适催化温度都相对较低，DLDH的最适温度仅为35 °C，在40 °C时的活力约为最适活力的84%，DHDH与BP的最适温度均为30 °C。当温度高于40 °C时，除DLDH外，其它2个酶(DHDH与BP)的酶活力呈现较快的下降趋势，鉴于3个D-乳酸脱氢酶对高温相对敏感，说明该菌株可能并不适应传统的高温驯化[18−19]，过高的发酵温度会降低D-型乳酸脱氢酶的活力，进而影响菌株产D-乳酸的能力[20−21]，但实际上也不排除酶在细胞内稳定性的增强。综上表明，该研究对*S. inulinus* Y2-8的产D-乳酸关键代谢的3个D-乳酸脱氢酶的研究可为实际的菌种选育和产乳酸发酵调控奠定理论基础。

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Characterization of D-lactate dehydrogenase isozymes from a D-lactic acid producing bacterium *Sporolactobacillus inulinus*

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Abstract: [**Objective**] *Sporolactobacillus inulinus*, a typical homofermentative lactic acid bacterium, is an efficient D-lactic acid producer. Various environment factors affect the productivity of *S. inulinus*. Glucokinase, phosphofructokinase, pyruvate kinase and lactic dehydrogenase are the key enzymes of D-lactic acid production from glucose by *S. inulinus*. The characteristics of these enzymes are important in controlling and regulating the fermentation process. According to the genome bioinformatics analysis of *S. inulinus* CASD, three putative D-lactate dehydrogenases were identified, among which the bifunctional protein had been reported. In this study, we provided insights into the characteristics of the other two D-lactate dehydrogenase isozymes. [**Methods**] *S. inulinus* Y2-8 genome was used as the template to amplify D-lactate dehydrogenase gene (*dldh*) and D-isomer specific 2-hydroxyacid dehydrogenase gene (*dhdh*). The two recombinant strains *E*-pET-28a/*dldh* and *E*-pET-28a/*dhdh* were constructed for enzyme expression. Both recombinants DLDH and DHDH could convert pyruvic acid into D-lactic acid. [**Results**] Enzymes expressed by recombinant strains were purified by Ni-NTA chromatography. The apparent molecular mass of DLDH was approximately 37 kDa by SDS-PAGE analysis, and DLDH showed a high affinity to pyruvate with the K_m value of (0.58±0.04) mmol/L. The optimal reaction temperature and pH for DLDH was 35 °C and 6.5, respectively. The apparent molecular mass of DHDH was approximately 39 kDa, and the K_m of DHDH toward pyruvate was (1.70±0.08) mmol/L. The optimum catalysis temperature and pH of DHDH were 30 °C and 7.5, respectively. [**Conclusion**] According to the K_m and optimal reaction pH, DLDH was suggested as the main catalyst in formation D-lactic acid from pyruvate during the fermentation. The enzymatic properties would contribute to the regulation of the fermentation of *S. inulinus*.

Keywords: *Sporolactobacillus inulinus*, D-lactate dehydrogenase, cloning, characteristics

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