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MECHANISM AND RESEARCH PROGRESS OF ETHANOL FERMENTATION RELATED GENES *pdc* AND *adhII* INVOLVED IN FUEL ETHANOL PRODUCTION

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ABSTRACT

Fuel ethanol is important as one of the perfect energy sources in the replacement of fossil energy which is being used up. The use of biomass to produce fuel ethanol by fermentation are of interest. The product yield from fuel ethanol fermentation depends on high-level expression of the genes (*pdc* and *adhII*) which affect the rate and efficiency, key factors in the catabolic pathway of ethanol fermentation. In this paper, we review catabolic pathway of ethanol fermentation, the mechanism of actions of genes *pdc* and *adhII* and their applications in ethanol production and the research progress in the metabolic engineering of the genes *pdc* and *adhII* .

KEYWORDS: *pdc, adhII*, ethanol fermentation, mechanism

1. INTRODUCTION

The natural energy resources such as fossil fuel, petroleum and coal are being utilized at a rapid rate and these resources have been estimated certainly more than a few years. Therefore, alternative energy sources such as ethanol, methane, and hydrogen are being considered. Some biological processes have rendered possible alternative for producing ethanol and methane in large volumes. Cellulose is one of the most abundant renewable resources on the planet, and terrestrial plants can provide fifty billion tons of cellulose every year. In addition, it is the main biomass resource accounting for 60-80% of the all biomass on the planet. China is very rich in cellulose raw materials，the output of crop stalks only, is in excess of 700 million tons every year. In addition, the quantity of by-products from forestry, municipal solid waste and industrial wastes are considerable. The use of waste products as an alternative source of energy not only reduces the environmental pollution but also provides new opportunities to solve the world energy crisis. Therefore, it has great market potential and research value. Worldwide interest in the utilization of bio-ethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production [1]. Intense research has been carried out for obtaining efficient fermentative organisms, low-cost fermentation substrates, and optimum environmental conditions for fermentation.

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The main steps of fermentation of cellulose raw materials to ethanol are described below:

1. After pretreatment and enzmatic saccharification, cellulose is converted to several kinds of monosaccharides such as glucose, xylose and pentose.

2. Cellulose hydrolysates are converted by some ethanol fermenting microbial strains to pyruvate though EMP, ED pathway or some other channels.

3. With the catalysis by pyruvate decarboxylase (PDC) and alcohol dehydrogenase II (ADHII) in the presence of NADH, pyruvate is decarboxylated to acetaldehyde and then further converted to ethanol.

During ethanol fermentation, PDC and ADHII are crucial in since not only the direction of pyruvate metabolism but also the ethanol yield and efficiency of the fermentation are affected by the enzymes directly. Therefore, the genes encoding the enzymes have become the focus of research interest especially the construction of genetically engineered strains that , can efficiently convert biomass material to ethanol.

In this paper we review the catabolic pathway of ethanol fermentation and, the mechanism of actions of the genes *pdc* and *adhII* and their applications in fuel ethanol production, and also the research progress in the metabolic and incurring of the genes *pdc* and *adhII* .

2. RESEARCH PROGRESS

2.1 Characteristics of the genes *pdc* and *adhII*

After pretreatment and enzmatic saccharification, cellulose is hydrolyzed to monosaccharides such as glucose, xylose and pentose which can be utilized by microorganisms. All of these monosaccharides are converted to the important metabolic intermediate pyruvate through EMP, ED pathway or some other channels. Conversion of pyruvate to acetaldehyde and carbon dioxide catalyzed by PDC is a crucial step in the ethanol fermentation process. Ultimately, acetaldehyde is converted to ethanol by the catalysis of ADHII in the presence of NADH. In this process, PDC and ADHII are very important since many microorganisms produce little or no ethanol by fermentation in absence of these two genes. At present, due to presence of PDC and ADHII enzymes, *Saccharomyces cerevisiae* and *Zymomonas mobilis* are widely used in the ethanol fermentation process. However the coding sequences of these two genes are different in the two microorganisms.

The coding region of the structural gene encoding PDC [2] from *Z. mobilis* is 1707 nucleotides long and encodes a polypeptide of 568 amino acids with a calculated subunit mass of 60,790 Daltons. Three structural genes for PDC [3] from *S. cerevisiae*, *pdc1*, *pdc5*, *pdc6* have been characterized. The gene *pdc1* of *S. cerevisiae* has been sequenced [4]. The gene contains a cen reading frame of 1647 base pairs. The codon usage shows the same strong bias as fcurl for some other glycolytic enzymes. Transcription starts mainly at -30 and terminates 100 base pairs downstream of the termination codon. There are two structural genes for PDC from *Pichia stipitis* CBS 6054 [5]. PsPDC1 and PsPDC2 have diverged almost as far from one another as they have from the next most closely related known yeast gene. PsPDC1 contains an open reading frame of 1,791 nucleotides encoding 597 amino acids. PsPDC2 contains a reading frame of 1,710 nucleotides encoding 570 amino acids.

The DNA sequence for ADHII from *Z. mobilis* is 1.4kb long and contains an open reading frame encoding a polypeptide of 383 amino acids, with a molecular weight of 40,141 Daltons [6]. It is different from the corresponding sequence from *S. cerevisiae* [7, 8].

Due to defects such as ethanol tolerance, acid tolerance, the ability to degrade cellulose and the limited scope of monosaccharide metabolism, the use of *S. cerevisiae* and *Z. mobilis* in industrial production of ethanol is subject to certain constraints. Therefore, cloning of *pdc* and *adhII* genes from *S. cerevisiae* and *Z. mobilis* by constructing an expression plasmid and expressing the genes in other genetically engineered strains are the main research directions in the application of genetic engineering to improve cellulose degradation and

ethanol production.

2.2 *pdc* and *adh II* from *S. cerevisiae* and *Z. mobilis*

Owing to their important role in pyruvate metabolism, it is of great significance to research *pdc* and *adhII* genes for constructing genetically engineered strains which can be used to ferment cellulose hydrolysates to ethanol [9].

D-Xylose is a pentose sugar derived from agricultural or forest residues. Although enteric bacteria such as *Klebsiella* spp. ferment D-xylose to form mixed acids and butanediol in addition to ethanol, ethanol not high due to the absence of the key enzyme pyruvate decarboxylase. Tolan and Finn [10] constructed a multicopy plasmids containing the *pdc* gene from *Z. mobilis* and transferred it into *Klebsiella* spp. After screening, he got the genetically engineered strain, *Klebsiella planticola* ATCC 33531 in which the expression of the gene markedly increased the yield of ethanol to 1.3 mol/mol of xylose, or 25.1g/liter. Concurrently, there were significant decreases in the yields of formate, acetate, lactate, and butanediol. Alterthum and Ingram [11] cloned the *pdc* gene from *Z. mobilis* and constructed a recombinant *E. coli* that has been shown to ferment glucose, xylose and lactose to produce ethanol. Jiang *et al*. and Ren and Jiang [12-13] cloned the pyruvate decarboxylase genes (PDCsc/PDCzm) from *S. cerevisiae* and *Z. mobilis*, and then constructed high expressing plasmids pSC-22b and pZM-22b. The SDS-PAGE results showed that the over expression of PDCsc and PDCzm were achieved and the target proteins reached 18.6% and 37.9% among the total soluble proteins in their host strains . Li [14] joined the *pdc* gene from *Z. mobilis* with the vector pGM-T and transferred them into *E. coli* TOP10 under the control of promoter T7, to achieve high expression of the gene. The recombinant *E. coli* did not only ferment glucose but also xylose to ethanol. Research shows that the accumulation of PHB in microorganisms can improve the resistance ability of the host bacteria. *Z. mobilis* was transformed with polyhydroxybutyrate synthesis operon phbCAB equipped with a *pdc* promoter from *Z. mobilis*. For the first time, PHB was produced in recombinant *Z. mobilis* [15] and shake flask studies indicated that accumulation of PBH in *Z. mobilis* increased ethanol productivity by approximately 10% during the first 48h of anaerobic fermentation.

Mackenzie *et al.* [16] cloned the *adhII* gene from *Z. mobilis* and constructed an *adhII*-lacZ fusion with lacZ promoter. In the recombinant *Z. mobilis*, the *adhII* gene was overexpressed 7- to 14-fold. The 1059bp *adhII* encoding S-ADH was amplified by PCR using primers based on high conserved sequences, and the recombinant S-ADH was expressed in *E. coli* with his-tag [17]. The fusion protein was purified by affinity chromatography and characterized as a bifunctional aldehyde-alcohol dehydrogenase. The results suggested that a new pathway in ethanol formation in *T. ethanolicus* JW200.

The genes *pdc* and *adhII* from *Z. mobilis* were first transferred into *E. coli* by Ingram *et al.* [18]. The results demonstrated that it is possible to change the fermentation products of an organism, such as *E. coli*, by the addition of genes encoding appropriate enzymes which form an alternative system for the regeneration of NAD^+. This was the beginning of research on the genes *pdc* and *adhII* for constructing genetically engineered strains. Compared with *Z. mobilis*, *Erwinia* spp. are gram-negative facultative anaerobes within the family *Enterobacteriacae* which possess several desirable traits for the conversion of pentose sugars to ethanol, such as the ability to ferment a broad range of carbohydrates and the ease with which they can be genetically modified, but the yield of ethanol fermentation is low. Tolan and Finn [19] transferred *pdc* and *adhII* genes from *Z. mobilis* into *Erwinia spp*. Expression of the genes markedly increased the yields of ethanol (from 0.7 up to 1.45 mol/mol of xylose) and decreased the yields of formate, acetate, and lactate. However, the cells with pyruvate decarboxylase grew only one-fourth as fast as the wild type and tolerated only 2% ethanol. Alcohol tolerance was stimulated by the addition of yeast extract to the growth medium. In the 1990s, the research on the genes *pdc* and *adhII* reached an upsurge. The *pdc* and *adhII* genes were transferred into *Klebsiella oxytoca* M5A1 by Ohta. [20]. Final ethanol concentration of the fermented mash was in excess of 40 g/liter with an efficiency of 0.48 g of

ethanol (xylose) and 0.50g of ethanol (glucose) per g of sugar, as compared with a theoretical maximum of 0.51g of ethanol per g of sugar. The maximal volumetric productivity per hour for both sugars was 2.0g/liter. This volumetric productivity with xylose is almost twice that previously obtained with ethanologenic *E. coli*. In the same year, *Z. mobilis* genes for PDC and ADHII were integrated into the *E. coli* chromosome within or near the pyruvate formate-lyase gene (*pfl*) [21]. Integration improved the stability of the *Z. mobilis* genes in *E. coli*, but further selection was required to increase expression. Spontaneous mutants were selected for resistance to high level of chloramphenicol that also expressed high levels of the *Z. mobilis* genes. Analogous mutants were selected for increased expression of *adhII* on aldehyde indicator plates. These mutants were functionally equivalent to the previous plasmid-based strains for the fermentation of xylose and glucose to ethanol. Ethanol concentrations of 54.4 and 41.6g/liter were obtained from 10% glucose and 8% xylose, respectively. The efficiency of conversion exceeded theoretical limits (0.51g of ethanol/g of sugar) on the basis of added sugars because of the additional production of ethanol from the catabolism of complex nutrients. The *Z. mobilis* genes encoding *adhI*, *adhII*, and *pdc* were overexpressed in *E. coli* and *Z. mobilis* by using a broad-host-range vector containing the tac promoter and the lacIq repressor gene. Maximal IPTG (isopropyl-beta-D-thiogalactopyranoside) induction of these plasmid-borne genes in *Z. mobilis* resulted in a 35-fold increase in *adhI* activity, a 16.7-fold increase in *adhII* activity, and a 6.3-fold increase in *pdc* activity [22]. In these studies, the screening of the genetically engineered *E. coli* KO11 strain is of great significance [23]. *E. coli* KO11 was constructed to produce ethanol from acid hydrolysates of hemicellulose (pentoses and hexoses) by the chromosomal integration of *Z. mobilis* genes encoding PDC and ADHII. In this study, KO11 was further engineered for the fermentation of cellulose by adding the *K. oxytoca* casAB genes encoding enzyme II cellobiose and phospho-beta-glucosidase. KO11 harboring mutant plasmids (pLOI1908, pLOI1909, or pLOI1910) rapidly fermented cellobiose to ethanol, and the yield was more than 90% of the theoretical yield. Two of these strains were used with commercial cellulase to ferment mixed-waste office paper to ethanol.

Cyanobacteria are autotrophic prokaryotes which carry out oxygenic photosynthesis and accumulate glycogen as the major form of stored carbon. Deng and Coleman [24] introduced new genes into a C*yanobacterium* in order to create a novel pathway for fixed carbon utilization which results in the synthesis of ethanol. The coding sequences of *pdc* and *adhII* from *Z. mobilis* were cloned into the shuttle vector pCB4 and then used to transform the *Cyanobacterium Synechococcus sp.* strain PCC 7942. Under control of the promoter from the *rbcLS* operon encoding the cyanobacterial ribulose-1, 5-bisphosphate carboxylase/ oxygenase, the *pdc* and *adhII* genes were expressed at high levels, as demonstrated by Western blotting and enzyme activity analyses. As *cyanobacteria* have simple growth requirements and use light, CO_2, and inorganic elements efficiently, production of ethanol by *cyanobacteria* is a potential system for bioconversion of solar energy and CO_2 into a valuable resource. *Cellulolytic clostridia* have evolved to catabolize lignocellulosic materials at a seasonal biorhythm, so their biotechnological exploitation requires genetic improvements. As high carbon flux leads to pyruvate accumulation, which is responsible for the cessation of growth of *Clostridium cellulolyticum*, this accumulation is decreased by heterologous expression of PDC and ADH from *Z. mobilis*. Guedon *et al.* [25] transferred the *pdc* and *adhII* genes into *C. cellulolyticum*. In comparison with that of the wild strain, growth of the recombinant strain at the same specific rate but for 145 h instead of 80 h led to a 150% increase in cellulose consumption and a 180% increase in cell dry weight. The fermentation pattern was shifted significantly, lactate production decreased by 48%, whereas the concentrations of acetate and ethanol increased by 93 and 53%, respectively. This study demonstrates that the fermentation of cellulose, the most abundant and renewable polymer on earth, can be greatly improved by using genetically engineered *C. cellulolyticum*. Wild-type *Bacillus subtilis* ferments 20g/liter glucose in 48 h, producing lactate and butanediol, but not ethanol or acetate. To construct an ethanologenic *B. subtilis* strain, homologous recombination was used by Susana Romero's group [26] to disrupt the native lactate dehydrogenase (LDH)

gene (*ldh*) by chromosomal insertion of the *Z. mobilis* genes *pdc* and *adhII* under the control of the *ldh* native promoter. The values of the intracellular PDC and ADHII enzymatic activities of the engineered *B. subtilis* BS35 strain were similar to those found in an ethanologenic *E. coli* strain. BS35 produced ethanol and butanediol. Biodiesel is an alternative energy source and a substitute for petroleum-based diesel fuel. Despite numerous environmental benefits, a broader use of biodiesel is hampered by the extensive acreage required for sufficient production of oilseed crops. Therefore, processes are urgently needed to enable biodiesel production from more readily available bulk plant materials like sugars or cellulose. Toward this goal, Kalscheuer *et al.* [27] established biosynthesis of biodiesel-adequate FAEEs, referred to as microdiesel, in metabolically engineered *E. coli*. This was achieved by heterologous expression in *E. coli* of the *Z. mobilis* PDC and ADHII and the unspecific acyltransferase from *Acinetobacter baylyi* strain ADP1. By this approach, ethanol formation was combined with subsequent esterification of the ethanol with the acyl moieties of coenzyme A thioesters of fatty acids if the cells were cultivated under aerobic conditions in the presence of glucose and oleic acid. Ethyl oleate was the major constituent of these FAEEs, with minor amounts of ethyl palmitate and ethyl palmitoleate. FAEE concentrations of 1.28 g/liter and a FAEE content of the cells of 26% of the cellular dry mass were achieved by fed-batch fermentation using renewable carbon sources. This novel approach might pave the way for industrial production of biodiesel equivalents from renewable resources by employing engineered micro-organisms, enabling a broader use of biodiesel-like fuels in the future.

2.3 *pdc* and *adh II* from other microorganisms

PDC is the key enzyme in ethanol fermentations. Although widely distributed among plants, yeasts, and fungi, PDC is absent in animals and rare in bacteria, the related research for *pdc* gene in other bacteria is few.

The *pdc* gene from *Sarcina. ventriculi* is the first to be cloned and characterized from a Gram-positive bacterium [28]. In *E. coli*, the recombinant *pdc* gene from *S. ventriculi* was poorly expressed due to differences in codon usage. Expression was improved by the addition of supplemental codon genes and this facilitated the 136-fold purification of the recombinant enzyme as a homo-tetramer of 58 kDa subunits. Unlike *Z. mobilis* PDC, which exhibits Michaelis–Menten kinetics, *S. ventriculi* PDC is activated by pyruvate and exhibits sigmoidal kinetics similar to fungal and higher plant PDCs. Amino acid residues involved in the allosteric site for pyruvate in fungal PDCs were conserved in *S. ventriculi* PDC, consistent with a conservation of mechanism. Cluster analysis of deduced amino acid sequences confirmed that *S. ventriculi* PDC is quite distant from *Z. mobilis* PDC and plant PDCs. *S. ventriculi* PDC appears to have diverged very early from a common ancestor which included most fungal PDCs and eubacterial indole-3-pyruvate decarboxylases. These results suggest that the *S. ventriculi pdc* gene is quite ancient in origin, in contrast to the *Z. mobilis pdc*, which may have originated by horizontal transfer from higher plants. Three known bacterial *pdc* genes have been previously described and expressed as active recombinant proteins. Raj *et al* [29] they described a new bacterial *pdc* gene from *Zymobacter palmae*. The pattern of codon usage for this gene appears quite similar to that for *E. coli* genes. In *E. coli* recombinants, the *Z. palmae* PDC represented approximately 1/3 of the soluble protein. Biochemical and kinetic properties of the *Z. palmae* enzyme were compared to purified PDCs from three other bacteria. Of the four bacterial PDCs, the *Z. palmae* enzyme exhibited the highest specific activity (130U mg of protein-1) and the lowest K_m for pyruvate (0.24mM). Differences in biochemical properties, thermal stability, and codon usage may offer unique advantages for the development of new biocatalysts for fuel ethanol. To further examine this, *pdc* genes from bacteria to yeast were expressed in the Gram-positive host *Bacillus megaterium*. The PDC activity and protein levels were determined for each strain. In addition, the levels of *pdc*-specific mRNA transcripts and stability of recombinant proteins were assessed. From analysis by Talarico *et al* [30], the *pdc* gene of Gram-positive *Sarcina ventriculi* was found to be the most advantageous for engineering high-level synthesis of PDC

in a Gram-positive host. This gene was thus selected for transcriptional coupling to the *adhII* of *Geobacillus stearothermophilus*. The resulting Gram-positive ethanol production operon was expressed at high levels in *B. megaterium*. Extracts from this recombinant were shown to catalyse the production of ethanol from pyruvate.

At the same time, the research for *adhII* gene is under way. In the study of *Alcaligenes eutrophus* [31], the nucleotide sequence of the gene that encodes the fermentative, multifunctional ADHII in *Alcaligenes eutrophus*, and of adjacent regions on a 1.8-kilobase-pair PstI fragment was determined. From the deduced amino acid sequence, a molecular weight of 38,549 was calculated for the ADHII subunit. The amino acid sequence reveals homologies from 22.3 to 26.3% with zinc-containing alcohol dehydrogenases from eucaryotic organisms (*Schizosaccharomyces pombe*, *Zea mays*, mouse, horse liver, and human liver). Most of the 22 amino acid residues, which are strictly conserved in this group of ADHs, either were present in the *A. eutrophus* enzyme or had been substituted by related amino acids. The *A. eutrophus adhII* gene was transcribed in *E. coli* only under the control of the lac promoter, but was not expressed by its own promoter. An *adhII* gene from *Clostridium acetobutylicum* was cloned on a recombinant plasmid, pCADH100 [32]. *E. coli* HB101, and an allyl alcohol-resistant mutant, HB101-adh1, containing this plasmid were unable to grow aerobically or anaerobically on agar media containing sublethal concentrations of allyl alcohol. *E. coli* HB101 and HB101-adh1 transformed with the plasmid pCADH100 produced increased levels of ethanol when grown anaerobically under alkaline conditions in the absence of nitrate. Cell extracts from aerobically and anaerobically grown *E. coli* HB101 (pCADH100) and HB101-adhl (pCADH100) cells exhibited increased levels of NAD-dependent ADHII activity with either ethanol or butanol as the substrate.

3. PROSPECTS

Cellulose is one of the most abundant renewable resources on the planet. Conversion of cellulose hydrolysates to ethanol by microorganisms is an important method for the rational exploitation of this resource. *Pdc* and *adhII* genes play an important role in the ethanol fermentation process based on cellulose hydrolysates since the ethanol yield and efficiency of the fermentation are directly affected by the expression of these two genes. Therefore, it is essential to further investigate the application of these two genes in constructing genetically engineered strains. Screening microorganisms and constructing the high-performance plasmids in which the genes are in high-level expression should be the main direction of any related research.

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