Cellulosic Ethanol Production by Recombinant Cellulolytic Bacteria Harbouring pdc and adh II Genes of Zymomonas mobilis

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The ethanol fermenting genes such as pyruvate decarboxylase (pdc) and alcohol dehydrogenase II (adh II) were cloned from Zymomonas mobilis and transformed into three different cellulolytic bacteria, namely Enterobacter cloacae JV, Proteus mirabilis JV and Erwinia chrysanthemi and their cellulosic ethanol production capability was studied. Recombinant E. cloacae JV was found to produce 4.5% and 3.5% (v/v) ethanol, respectively, when CMC and 4% NaOH pretreated bagasse were used as substrates, whereas recombinant P. mirabilis and E. chrysanthemi with the same substrates could only produce 4%, 3.5%, 1%, and 1.5 % of ethanol, respectively. The recombinant E. cloacae strain produced twofold higher percentage of ethanol than the wild type. The recombinant E. cloacae strain could be improved further by increasing its ethanol tolerance capability through media optimization and also by combining multigene cellulase expression for enhancing ethanol production from various types of lignocellulosic biomass so that it can be used for industrial level ethanol production.

1. Introduction

The conversion of plant cellulose biomass to fuel ethanol by microbial fermentation is the priority area of research, and the use of industrially suited microorganisms for the cost-effective biofuel production is the major technical challenge. Cellulosic ethanol would reduce our petroleum dependency, as ethanol is produced from the inexpensive and plentiful feed stocks. Efficient conversion of biomass to ethanol requires development of microorganisms capable of fermenting a wide range of carbohydrates and tolerating high concentrations of ethanol [1]. Metabolic engineering of microorganisms to utilize cellulose will be vital for improving the prospects of significant cellulosic ethanol production. Several Gram-negative bacteria such as Escherichia coli, Klebsiella oxytoca, and Zymomonas mobilis have been engineered for ethanol production [2–5].

Enteric bacteria normally produce less ethanol, because of their poor efficiency in converting pyruvate to ethanol. A suitable ethanologenic and cellulose-producing bacteria could be developed by transferring genes that encode the ethanol-fermenting enzymes [6]. Z. mobilis is one of the best ethanol producers which produces ethanol in the Entner-Doudoroff (ED) pathway, that is, homoethanol fermentation pathway with the help of two essential enzymes such as pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) encoded by pdc and adh II genes, respectively. PDC catalyzes the nonoxidative decarboxylation of pyruvate to produce acetaldehyde and carbon dioxide, whereas ADH catalyzes the reduction of acetaldehyde to ethanol during fermentation [7–9]. These two enzymes (both PDC and ADH) are sufficient to convert intracellular pool of pyruvate and NADH to ethanol [10].

The transfer of ethanol-fermenting genes (pdc and adh) from Z. mobilis to cellulolytic bacteria could definitely improve their ethanol productivity by converting pyruvate completely to ethanol. The research on the construction of recombinant ethanol fermenting bacteria by expressing
both the pdc and adh II genes was originally done by Ingram et al. [9] in E. coli to change the ethanol production ability by fermenting all sugars in the biomass. Similarly, recombinant Erwinia sp. [11] and Klebsiella oxytoca M5A1 [2] were developed to improve the ethanol production from xylose and glucose. Recombinant Gram-negative E. coli KO11 [1] and Gram-positive Clostridium cellulolyticum [12] were constructed to produce ethanol from acid hydrolysates of hemicellulose and lignocellulosic biomass, respectively. Though these reports did explain the cloning of pdc and adh genes, but the subsequent usage of the cloned genes for ethanol production was not explained clearly. The formation of additional byproducts during fermentation and tolerance to the produced ethanol are the major limitations observed in these studies. The bioethanol production from cellulolytic biomass in cellulosic microorganisms can be improved by introducing ethanol-fermenting genes under the control of an appropriate promoter [9].

In the present study, the ethanol fermenting genes such as pdc and adh II were cloned from Z. mobilis and introduced into three facultative anaerobic, Gram-negative cellulolytic bacteria. The cellulose ethanol production capability of these recombinant strains was determined through simultaneous saccharification and fermentation (SSF) process using carboxymethyl cellulose and alkali-pretreated bagasse as substrates.

2. Methods

2.1. Bacterial Strains, Plasmids, and Growth Conditions. Z. mobilis subsp. mobilis MTCC 92 [5] and E. coli DH5α were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. E. coli harboring pUC18 ampR, cloning vector, lac promoter, (2.7 kb) was obtained from Fermentas (USA). E. cloacae JV and P. mirabilis JV were isolated and characterized in our laboratory from the gut of termite (Heterotermes indicola) and silk worm (Bombyx mori), respectively. The 16s rDNA sequence of the organisms was submitted in National Center for Biotechnology Information (NCBI), E. cloacae JV (FJ 799063) and P. mirabilis JV (HQ231796). E. chrysanthemi was obtained from the National Institute of Agrobiological Sciences, Japan. Z. mobilis subsp. mobilis was grown on yeast extract medium supplemented with 20% glucose, 0.5% yeast extract, 0.1% ammonium sulphate, 0.1% potassium dihydrogen orthophosphate, and 0.05% magnesium chloride, pH 7, at 30°C with agitation at 100 rpm. E. coli harbouring pUC18 was grown on Luria agar with ampicillin (100 μg/mL) under static condition at 37°C and E. chrysanthemi, E. cloacae JV, P. mirabilis JV, and E. coli DH5α were also cultured on Luria agar.

2.2. Cloning of pdc and adh II Genes. Chromosomal DNA was isolated from Z. mobilis MTCC 92 as described by Sambrook and Russel [13] and the pUC18 plasmid DNA was isolated by alkaline lysis method [14]. Cloning of pdc gene was carried out by restricting both the total genomic DNA (20 μg) and plasmid DNA (7 μg) with 10 units of EcorI, 10 units of BamHI (Fermentas, USA), 2.5 μL of restriction buffer, and 9.5 μL of sterile distilled water to a total volume of 25 μL. The adh II gene cloning was done as described above using BamHI and HindIII enzymes. The reaction mixtures were incubated at 37°C for 2 h. The restriction reaction was stopped by heating the reaction mixture at 65°C for 20 min. The restricted and purified DNA samples were ligated by mixing 8 μg of digested genomic DNA, 1 μg of digested pUC18 plasmid DNA, 4 μL of T4 DNA ligase buffer, and 5 units of T4 DNA ligase enzyme (Fermentas, USA) and incubated at 16°C for 16 h [13]. The ligated mix was transformed into competent E. coli DH5α cells by CaCl2 method. The transformants were plated on Luria agar supplemented with ampicillin (50 mg/mL), IPTG (40 mg/mL), and X-gal (20 mg/mL), the white-coloured recombinant clones were selected. The pdc clones expressing pyruvate decarboxylase enzyme were further screened by plating the white colonies on Luria agar supplemented with 1% Schiff reagent, 50 mM sodium pyruvate and ampicillin (50 mg/mL) whereas the adh clones were screened on Luria agar supplemented with 1% Schiff reagent, 5% ethanol, and ampicillin (50 mg/mL) [15]. The clones showing intensive red colour on aldehyde indicator plates were selected as positive clones.

2.3. Cell Extracts Preparation. The pdc and adh positive clones were grown in 100 mL Luria broth supplemented with ampicillin (100 mg/mL) for 18 h at 37°C. After incubation, cells were harvested by centrifugation (10,000 rpm, 5 min, 4°C) and the cells were washed with 10 mM Tris buffer (pH 7.0) containing 1 mM EDTA and resuspended in 10 mL of the same buffer. Lysozyme was added at a final concentration of 1 mg/mL and the mixture was incubated for 30 min at room temperature. Cells were lysed by sonication using a Bandelin sonicator (UW2200) for three cycles at 40 W with 45 sec intervals. Cell extracts were collected by centrifugation (15,000 rpm, 30 min, 4°C) and the supernatant was used as source of enzyme [16].

3. Expression of Z. mobilis Genes in E. coli

3.1. PDC Activity. PDC activity was measured in triplicate by monitoring the pyruvic acid-dependent oxidation of NADH with ADH as a coupling enzyme. The reaction mixture consisted of 2.7 mL of 200 mM citrate buffer, 100 μL of 1 M sodium pyruvate, 50 μL of 6.4 mM β-NADH, and 100 μL of cell extract. The reaction mixture was mixed and the assay was carried out at 25°C. The enzyme activity was determined by measuring the conversion of NADH to NAD+ at 340 nm using varian spectrophotometer. The decrease in absorbance value was recorded. The rate that is rA 340 nm/mL was obtained using the maximum linear rate for both the test and the blank [17]. One unit of activity is defined as the amount of activity required for the conversion of 1 μmol of NADH to NAD+ per min.

3.2. ADH Activity. Assay of ADH was measured in triplicate by monitoring the ethanol-dependent reduction of NAD, in which the conversion of NAD to NADH was determined-spectrophotometrically. The reaction mixture containing
0.1 mL of 15 mM NADP, 2.4 mL of 100 mM Tris- HCl, 0.3 mL of propane 2-ol (100%), and 0.2 mL of cell extract and incubated at 40°C for 5 min. The alcohol-dependent reduction of NAD⁺ using propane-2-ol was measured at 340 nm [18]. One unit of ADH activity is defined as the amount that reduces 1 μmol of NAD⁺/min.

Both enzyme activities were calculated using the following formula:

$$\text{Unit/mL extract} = \frac{(\Delta A_{340\text{nm/min test}} - \Delta A_{340\text{nm/min blank}})}{(6.22) \times \text{(reaction volume) \times DF}}$$  

6.22 is the millimolar extinction coefficient of β-NADH at 340 nm and DF is the dilution factor.

3.3. DNA Sequencing. The clones which showed higher PDC and ADH activity were selected for sequencing. The cycle sequencing reaction was performed using BigDye Terminator V3.1 Cycle Sequencing Kit containing AmpliTaq DNA polymerase (from Applied Biosystems, PN: 4337457). The sequencing reaction mix was prepared by adding 1 μL of BigDye v3.1, 2 μL of 5x sequencing buffer, and 1 μL of 50% Dimethyl sulfoxide (DMSO). Four microliters of sequencing reaction mixture, 4 Pico moles of primer (2 μL), and sufficient amount of plasmid DNA were added. The constituted reaction was denatured at 95°C for 5 min. Cycling began with denaturing at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension for 4 min at 60°C and cycle repeated for 30 cycles in a MWG thermocycler. The reaction content was then purified on sephadex plate (Edge Biosystems) by centrifugation to remove unbound labeled, and unlabeled nucleotides and salts. The purified reaction product was loaded on to the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 h. The nucleotide sequences of both pdc and adh II genes were analysed, confirmed, and submitted to the National Center for Biotechnology Information (NCBI).

3.4. Cloning of pdc and adh II Genes. Both pdc and adh II genes were cloned together by digesting pUC18-adh with BamHI and HindIII enzymes and eluted from the gel to ligate with BamHI- and HindIII-digested pUC18-pdc clone in such a way that the adh II fragment was at the downstream of pdc gene. The ligated mix was transformed into competent E. coli DH5α cells by calcium chloride method. The transformants having both ADH and PDC activity were screened further on selective aldehyde indicator plates, enzyme assays, and by restriction analysis [13].

3.5. Ethanol Tolerance Assay. Single colony of each cellulolytic bacteria such as E. chrysanthemi, E. cloacae JV, and P. mirabilis JV were inoculated separately in 5 mL of Luria broth and incubated at 37°C in a shaker at 200 rpm. Five hundred microlitres of the overnight cultures were subcultured to 50 mL Luria broth (supplemented with 0, 2, 4, 6, and 10% ethanol) in closed culture tubes to prevent ethanol volatilization and were incubated at 37°C on a rotary shaker with an aeration speed of 200 rpm and the density of bacterial culture was measured at 600 nm [19].

3.6. Transformation of Cellulolytic Bacteria. The pUC18-pdc-adh II plasmid was purified from E. coli and transformed into cellulolytic bacteria such as E. chrysanthemi, E. cloacae JV, and P. mirabilis JV by electroporation (single pulse at 6.25 KV using 25 mF capacitor at a resistance of 200 Ohm in cooled 0.2 cm cuvette which contained 50 ng of plasmid DNA, 40 μL of P. mirabilis JV, E. cloacae JV, and E. chrysanthemi competent cells in separate cuvettes using Biorad electroporator). After electroporation, cells were incubated for one hour in SOC medium and then plated on selective agar supplemented with ampicillin (100 μg/mL) [20]. Those clones that developed intensive red color on aldehyde indicator plates were selected. The clones showing higher enzymatic activity were further confirmed by PDC/ADH assay.

3.7. Analysis of Plasmid Profile and Restriction Mapping. The transformants of cellulolytic bacteria were confirmed by analyzing their plasmid profile and by restriction analysis. The transformation of plasmid pUC18-pdc-adh into the cellulolytic bacteria was confirmed through horizontal slot lysis electrophoresis as described by Vennison [21]. The transformed colonies on agar plates were resuspended in protoplasting buffer (15 μL) to a density of 10⁵ cell/mL. Bacterial cells were mixed thoroughly by vigorous vortexing. The mixtures were incubated at 37°C for 15 min for the formation of protoplasts. Agarose gel (0.7%) was prepared with 1X Tris-Boric-acid-EDTA buffer with 0.05% SDS. The gel slots were preloaded with 20 μL of lysis buffer and allowed to stand for 20 min. Then 10 μL of protoplast suspension was loaded into each slot and the electrophoresis was carried out initially with 50 volts and then to 100 volts till the completion of the run. After the completion of electrophoresis, the gel was stained with 0.05 μg/mL of ethidium bromide. The size of plasmids such as pUC18-pdc, pUC18-adh, and pUC18-pdc-adh was determined by linearizing the plasmids with BamHI enzyme and electrophoresed on 0.7% of agarose gel along with the DNA molecular weight marker. The DNA bands were visualized under UV transilluminator and photographed using Alpha gel documentation system (USA).

3.8. Cellulosic Ethanol Production. Ethanol fermentation experiments were carried out independently with 0.6% carboxyl methyl cellulose and 1 g of 4% NaOH-treated bagasse [5] in the luria broth supplemented with 0.1% ammonium sulfate, 0.1% potassium dihydrogen orthophosphate, and 0.05% magnesium sulphate at 37°C and pH 7.0 with an agitation speed of 150 rpm agitation as described by Jeffers [22]. The fermentation was performed in a round bottom flask connected with an U-tube. The outlet was fitted with a test tube containing Ca(OH)₂ to maintain anaerobic conditions and pH of the fermentation medium [23]. After 48 h, the ethanol was distilled at 78.5°C and ethanol concentrations in the distillate were determined by potassium dichromate method [24].
4. Results

4.1. Cloning of pdc and adh II Genes and Their Expression in E. coli. Z. mobilis genes encoding PDC and ADH II enzymes were expressed in E. coli using a vector pUC18 (Figure 1) and the transformants expressing the gene were screened by red spots on aldehyde indicator plates supplemented with ampicillin (100 mg/mL). The PDC and ADH activity was further confirmed by direct spectrophotometric assay of cell lysates (Figure 2). A PDC clone showed a higher activity of 0.6582 (U/mL) named as pUC18-pdc, whereas an ADH clone showed an activity of 0.117 (U/mL) was named as pUC18-adh II. The experiment was repeated for six times and the enzyme activity data were statistically analyzed by Student’s t-test by comparing the enzyme activity of the clone with control E. coli strain. The statistical analysis predicted that the calculated value for both enzyme activities was greater than the tabulated value (10.1 > 2.36) at \( P < 0.05 \). These analyses showed that there was a significant difference between the enzyme activities of the clones and the control E. coli strain. The nucleotide sequences of both pdc and adh II genes cloned from Z. mobilis were deposited in NCBI (the accession number for pdc gene is HM235920 and for adh gene is HM235921). The pdc gene sequence contained an open-reading frame of 1707 bp and the adh II gene contained an open-reading frame of 1152 bp. Both pdc and adh II gene sequences showed a maximum of 99% similarity when compared to the sequence of pdc and adh II gene of Z. mobilis already available in NCBI (AB359062.1 and AB359063.1). These clones of pdc and adh genes were found to contain insert DNA of 3 and 4 kb, respectively (Figure 3).

4.2. Construction of pUC18-pdc-adh for Ethanol Production. The pUC18-adh II and pUC18-pdc were digested with the restriction enzymes BamHI and HindIII, ligated and transformed into E. coli. Clones expressing both pdc and adh II genes grew poorly on Luria agar plates but grew at higher densities than the individual pdc and adh II clone on agar plates supplemented with 2% glucose. The colony size and opacity had proven as useful markers for the identification of recombinants which harboured both pdc and adh II genes. Several colonies were selected and their intracellular enzyme activities were determined. The efficient clone with higher enzyme activity was designated as pUC18-pdc-adh and selected for further studies.

4.3. Ethanol Tolerance Assay. The ethanol tolerance assay of cellulolytic bacteria was carried out by culturing the bacterial strains in the luria broth supplemented with ethanol at different concentration (0–10%) and the culture densities were measured at 600 nm (Figure 4). The turbidity of E. cloacae was clearly visible till 4%, whereas the turbidity of the other strains was visible only till 2% of ethanol. The optical density of these cellulolytic bacteria in different concentration of ethanol medium revealed that the E. cloacae growth rate was decreased slowly till 4%, but the growth rates of other strains were rapidly decreased at 1-2%.

4.4. Transformation of pUC18-pdc-adh into Cellulolytic Bacteria. The pUC18-pdc-adh clone was transformed into E. chrysanthemi, E. cloacae JV, and P. mirabilis JV through electroporation. The transformants were selected on the aldehyde indicator plates supplemented with ampicillin. On agar medium, the recombinant ethanologenic clones were readily apparent as large, raised colonies. The efficient strains that are able to convert glucose to ethanol were recognized by the production of red spots on aldehyde indicator plates. Efficient clones from each cellulolytic strain with efficient enzyme activity were selected for cellulose ethanol production. Plasmid DNA profile from all the three recombinant cellulolytic bacteria was examined through slot lysis electrophoresis which was found identical to that of pUC18-pdc-adh.

4.5. Fermentation of Cellulose to Ethanol. The optimum temperature and pH for the ethanol production was 37°C and 7.0. The fermentation was carried out under anaerobic conditions for 48 h with agitation of 150 rpm. The cellulose ethanol production capability of recombinant cellulolytic bacteria harbouring both pdc and adh II genes was studied with carboxymethyl cellulose and pretreated bagasse as substrates (Figure 5). The recombinant strains produced ethanol more rapidly and efficiently when compared to their respective parental strains. Recombinant E. chrysanthemi could produce ethanol from CMC and pretreated bagasse slightly higher than the wild type. The ethanol production by recombinant P. mirabilis from 4% NaOH-treated bagasse did not show any significant increase when compared to the wild type. The recombinant E. cloacae JV had shown twofold increase in ethanol production than the wild type. Among the recombinants E. cloacae JV harbouring pUC18-pdc-adh, plasmid construct was identified as the best strain for ethanol production, with a maximum of 4.5% and 3.5% of ethanol with carboxyl methyl cellulose and 4% NaOH treated bagasse, respectively. Experiments were performed six times and statistical analyses of the data were performed using the Student’s t-test. The statistical analyses showed a significant difference in the cellulose ethanol production between the wild type E. cloacae and recombinant E. cloacae at \( P < 0.05 \).

5. Discussion

Cellulosic ethanol production from lignocellulosic biomass is a globally developing technology. One of the major issues for cellulosic ethanol production is enzyme hydrolysis by the naturally available strains to convert cellulose to glucose. Developing a single strain for efficient cellulosic ethanol production is the technical challenge. The present work has taken up the challenge by improving the ethanol fermenting capabilities of cellulolytic...
Genomic DNA of *Z. mobilis* BamHI and EcoRI digestion

Purification and ligation

BamHI and HindIII digestion, purification, and ligation

Figure 1: Cloning of *pdc* and *adh* II genes in pUC18 plasmid.
bacteria through cloning of pdc and adh genes from Z. mobilis.

There are several reports on the construction of an artificial, pet operon, for the production of ethanol by combining both pdc and adhII genes. The first successfully constructed recombinant organism was E. coli KO11 which had the ability to ferment a wide spectrum of sugars but the ethanol yield was 4.3% from glucose as a substrate [9], but the cells could tolerate only 2% ethanol [25]. Other Gram-negative bacteria such as Klebsiella oxytoca and E. chrysanthemi were also transformed with pet operon but these strains have lower ethanol yield than E. coli KO11 [26]. The K. oxytoca was further improved to enhance the ethanol yield by overcoming its limitations, but the yield was increased to 40 g/L using raw sugarcane but the process took 13 days time for the overall production [27]. The expression of this pet operon in other Gram-positive microorganisms also had shown very less ethanol yield [28]. The engineered cellulolytic bacterium, Clostridium cellulolyticum with pdc and adh II of Z. mobilis showed 150% increase in cellulose consumption and the concentrations of acetate and ethanol

Figure 2: The intracellular PDC and ADH activities of the recombinant bacteria.

Figure 3: Restriction analysis of pdc and adhII clones.

Figure 4: Ethanol tolerance assay of cellulolytic bacteria.
increased by 93 and 53%, respectively, [12] but the drawback of this strain was its slower growth rate than the wild type. The major limitations in all those recombinants for cellulosic ethanol production were intracellular cellulase enzyme activity, low ethanol yield, and their inability to tolerate higher percentage of ethanol. The present work focused on the cellulose hydrolysis by the microbial enzymes and fermentation of the hydrolyzed products into ethanol. The \( pdc \) and \( adh \) clones of cellulolytic bacteria were screened on aldehyde indicator plates by adding acetaldehyde as a substrate for \( adh \) and ethanol for \( pdc \). The \( pdc \) clones had showed a PDC activity of 0.6582 U/mL and the \( adh \) II clones showed an ADH activity of 0.117 U/mL.

The cellulolytic capability of insect gut-inhabiting bacteria was higher because they naturally involved in the digestion of lignocellulosic substrates which is the diet of insects. The selected cellulolytic bacteria used in the present study were isolated from various phytophagous insects so that they were efficient in cellulolytic activity. The microorganisms such as, \( E. chrysanthemi \), \( E. cloacae \), and \( P. mirabilis \) were already known for their cellulolytic activity [20, 29]. Cloning of cellulase gene into ethanologenic bacteria had also already been reported [5]. The ethanol tolerance capability was studied to detect the effect of ethanol on the growth of microorganisms. \( E. cloacae \) exhibited the growth up to 4\% (v/v) supplemented ethanol and the other strains such as \( E. chrysanthemi \) and \( P. mirabilis \) were found to grow less rapidly in all ethanol concentrations, whereas there was no growth observed in 4\%. Addition of zinc in fermentation medium was found to increase the tolerance towards ethanol was reported [30] and over expression of genes involved in tryptophan biosynthesis and/or supplementation of tryptophan in the fermentation medium could also reportedly improve ethanol tolerance in yeast [31].

The ethanol production capability of the recombinant microorganisms was studied using fermentation medium under anaerobic conditions with carboxymethyl cellulose and 4\% NaOH pretreated bagasse as substrates. Production of high levels of PDC and ADH enzymes metabolically diverts pyruvate to ethanol as the primary product of fermentation. Expression of these enzymes for ethanol production was simultaneously increased, as evidenced by the increase in PDC activity, stronger reaction on aldehyde indicator plates (ADH II), decreased acetate, and more efficient ethanol production. As the sugar released by the cellulase action was subsequently utilized for ethanol production, as the feedback inhibition was minimum. The ethanol production of recombinant \( E. chrysanthemi \) and \( P. mirabilis \) using CMC was 3.5\% and 3\%, respectively, whereas using 4\% NaOH-treated bagasse ethanol production was less than 2\%. The ethanol production of recombinant \( E. chrysanthemi \) and \( P. mirabilis \) from 4\% NaOH-pretreated bagasse has no significant difference from that of the wild type. The ethanol production of recombinant \( E. cloacae \) from CMC and 4\% NaOH treated bagasse were 4.5 and 3.5\%, which are higher than the other cellulolytic bacterial strains studied. This might be due to its superior ethanol tolerance, wide substrate utilization, and higher cellulolytic activity. The recombinant \( E. cloacae \) can be improved further by studying sugar catabolism and nutrient requirements to increase its ethanol production.

### 6. Conclusion

Three recombinant cellulolytic bacterial strains such as \( E. cloacae \) JV, \( E. chrysanthemi \), and \( P. mirabilis \) harbouring both \( pdc \) and \( adh \) II genes from \( Z. mobilis \) have showed an increase in cellulose ethanol production capabilities when compared to their respective wild-type strains. Recombinant \( E. cloacae \) JV harboring both \( pdc \) and \( adh \) II genes produced 4.5 and 3.5\% of ethanol when CMC and 4\% NaOH-treated bagasse were used as substrates, but recombinant \( E. chrysanthemi \) produced 4 and 1.5\% and recombinant \( P. mirabilis \) produced 3.5 and 1\% ethanol using the same substrates, respectively. The cellulose ethanol production could be increased by over expressing the genes and optimizing the fermentation conditions for altering cellular metabolism for higher ethanol tolerance.

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