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Cellulosic ethanol production by *Zymomonas mobilis* harboring an endoglucanase gene from *Enterobacter cloacae*

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ABSTRACT

Enterobacter cloacae was isolated from the gut of the wood feeding termite, *Heterotermes indicola*, and a 2.25-kb fragment conferring cellulase activity was cloned in *Escherichia coli*. The cloned fragment contained a 1083-bp ORF which could encode a protein belonging to glycosyl hydrolase family 8. The cellulase gene was introduced into *Zymomonas mobilis* strain Microbial Type Culture Collection centre (MTCC) on a plasmid and 0.134 filter paper activity unit (FPU)/ml units of cellulase activity was observed with the recombinant bacterium. Using carboxymethyl cellulose and 4% NaOH pretreated bagasse as substrates, the recombinant strain produced 5.5% and 4% (V/V) ethanol respectively, which was threefold higher than the amount obtained with the original *E. cloacae* isolate. The recombinant *Z. mobilis* strain could be improved further by simultaneous expression of cellulase cocktails before utilizing it for industrial level ethanol production.

1. Introduction

Microorganisms that possess both cellulolytic and ethanol fermentation capabilities would be very useful for the conversion of lignocellulosic biomass into bioethanol. Currently, no natural microorganisms are known that exhibit all the features desired for lignocellulosic ethanol production. Recombinant organisms including *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Escherichia coli*, *Klebsiella oxytoca* and *Erwinia herbicola* (Su et al., 1989; Fujita et al., 2004; Dien et al., 2003) have been developed that harbor cellulolytic and ethanol fermentation genes; however, these genetically engineered strains have not yet demonstrated sufficient ethanol productivity required for commercial scale production, in part because of the gene expression and stability problems (Gunasekaran and Chandraraj, 1999).

The ethanologenic bacterium, *Z. mobilis* is a potential candidate for ethanol production (Swings and Deley, 1977) because of its unique characteristics such as, high sugar uptake, high specific ethanol production, non-requirement of controlled addition of oxygen during fermentation and high ethanol tolerance. It can utilize simple sugars like glucose, fructose and sucrose as substrates for ethanol production but not cellulose (Skotnicki et al., 1982). Although the carboxy methyl cellulase (CMCase) gene of *Cellulomonas uda* (Misawa et al., 1988) and endoglucanase genes from *Bacillus subtilis* (Yoon et al., 1988), *Pseudomonas fluorescens* (Lejeune et al., 1988) and *Erwinia chrysanthemi* (Goachet et al., 1989) have been introduced into *Z. mobilis*, no endoglucanase activity was detected in the culture supernatant of the recombinant strains, and the cellulase genes were expressed only poorly. In the current study, an endoglucanase gene isolated from a cellulolytic *Enterobacter* strain inhabiting the gut of a termite was introduced into *Z. mobilis* and the ethanol production capability of the recombinant strain was determined.

2. Methods

2.1. Bacterial strains, plasmids and culture conditions

Z. mobilis subsp. *mobilis* MTCC 92, *E. coli* BL 21 and *E. coli* SK1592 were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. *E. coli* harboring pET 20b (+) (Amp^R, T7 expression vector, size of plasmid 3.7 kb) was kindly provided by Dr. S. Krishnasamy, School of Biotechnology, Madurai Kamaraj University, Madurai, India. *Z. mobilis* subsp. *mobilis* was grown on yeast extract medium containing 20% glucose, 0.5% yeast extract, 0.1% ammonium sulfate, 0.1% potassium dihydrogen ortho-phosphate, and 0.05% magnesium chloride, pH 7, at 30 °C with agitation at 100 rpm. *E. coli* SK1592 was grown on Lysogeny agar (Bertani, 1951) supplemented with kanamycin (25 μg/ml) and streptomycin (25 μg/ml) under static conditions at 37 °C. *E. coli* (pET 20b (+) was grown on Lysogeny agar with ampicillin (50 μg/ml) under static condition at 37 °C.

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2.2. Isolation, screening and enzyme activity of cellulolytic bacteria

Termites were collected from dead and dry wood at Anna University, Tiruchirappalli campus, Tamil Nadu, India. Ten termites were surface sterilized with 70% ethanol and washed with sterile water. The termite guts were removed, pooled and homogenized with 1 ml of sterile water. The homogenate was serially diluted to 10^{10} dilution and plated on CMC agar (Luria agar supplemented with 0.6% of carboxy methyl cellulose) (Wood and Bhat, 1988). The plates were incubated at 37 °C for 24 h in both aerobic (incubator) and facultative anaerobic condition (candle jar method) (Wenzel et al., 2002; Wagner et al., 2003). Cellulase-producing colonies were detected by the Congo red overlay method (Wood, 1980). The plates were flooded with a 0.1% aqueous Congo red solution for 10 min and washed with 1 M NaCl. A clearing zone around the colony indicated hydrolysis of cellulose. Selection of the most efficient cellulose degrading bacterial colonies was done by determination of reducing sugar production by the Di Nitro Salicylic (DNS) method (Joachim et al., 2002). Crude enzymes (unpurified cellulase enzyme secreted in the culture supernatant of *Enterobacter cloacae*) were then tested for their abilities to hydrolyze 1×1 cm filter paper (Whatman No.1). Filter paper was placed in 0.2 M sodium acetate buffer (pH 5.8). Each reaction was prepared in two sets for incubation at 30 °C and 50 °C by mixing 1 ml culture supernatants with 1 ml buffer, incubated for 1 h. After incubation, reducing sugar was measured. Experiments were performed in triplicates and mean values were calculated using the formula, filter paper activity (FPU) units/ml = 0.37/(enzyme) releasing 2.0 mg of glucose (Ghose, 1987).

2.3. Identification of bacterial isolates

The isolated cellulolytic bacterium was identified using standard biochemical methods (Sneath et al., 1984) and 16S rRNA typing. The 16S rRNA gene sequence of the isolate was compared with 16S rRNA sequences in the European Molecular Biological Laboratory (EMBL) database, GenBank (GB, Germany) and the Data Base of Japan (DBJ) and analysed using the BLAST algorithm (Altschul et al.,1997) available at NCBI (National Centre for Biotechnology Information). The bacterium was identified as *E. cloacae* JV (FJ799063).

2.4. Cloning of the cellulase gene

Chromosomal DNA was isolated from *E. cloacae* as described by Sambrook and Russel (2003) and pET 20b (+) plasmid DNA was isolated by the alkaline sodium dodecyl sulfate method as described by Birnboim and Doly (1979). *E. cloacae* DNA and pET 20b (+) plasmid DNA were digested with *Bam*HI (Fermentas, USA) at 37 °C for 2 h. A total of 0.5 μg of plasmid DNA and 2.5 μg of chromosomal DNA digested with *Bam*HI were mixed and ligated with T_4 DNA ligase (Fermentas, USA) overnight at 12 °C. The ligation mixture was used to transform *E. coli* strain BL 21. Transformants were plated onto Lysogenic agar containing 0.6% of CMC and ampicillin (50 μg/ml) and grown at 37 °C. The plates were flooded with 5 ml of aqueous solution of Congo red (1 mg/ml), incubated for 30 min and washed a few times with 5 ml of 1 M NaCl (Wood, 1980). Colonies surrounded by a clear zone were considered cellulase positive. Plasmid DNA from a cellulolytic clone (pET6 Cel) was digested with *Bam*HI and the insert DNA was eluted from the gel using a DNA extraction kit (Fermentas, USA), ligated into the *Bam*HI site of pKT230 and transformed into *Z. mobilis* (Goodman et al., 1984). Transformants of *Z. mobilis* producing cellulase enzyme were screened by the Congo red overlay method (Wood, 1980). Intracellular cellulase activity was determined using the method described by Vadehra et al. (1965). Localization of enzyme activities was assayed by the sucrose/EDTA method of Willis et al. (1974). The production of reducing sugars from cellulose was measured by the DNS method (Joachim et al., 2002).

2.5. Pretreatment of cellulosic substrates for ethanol production

Bagasse (sugar cane) was cut into pieces of approximately 1 cm, ground using mortar and pestle. The ground bagasse was washed with boiling distilled water for 30 min and dried in the oven at 80 °C, powdered using mixer grinder. One to 10% NaOH was used to pretreat bagasse. The different fractions of pretreated bagasse were thoroughly washed with distilled water and dried at 60 °C for 48 h (Ram and Seenaya, 1991). The pretreated bagasse was subjected to Fourier Transform Infra Red Spectroscopy (FTIR) in order to study the lignin digestion by pretreatment along with untreated substrates as control. Two milligram of pretreated bagasse was mixed with 200 mg of spectroscopic grade KBr and FTIR spectra were recorded using a Nicolet 520P spectrometer with detector at 4 cm^{-1} resolution and 20 scans per sample (Hinterstoisser and Salmean, 2000) were carried out. Crystallinity of the cellulose was analysed by the X-ray Diffraction method (Zhao et al., 2007).

2.6. Ethanol production from cellulosic substrates

One millilitre of recombinant *Z. mobilis* was inoculated into medium containing 1 g of 4% NaOH-pretreated bagasse, 0.5% yeast extract, 0.1% ammonium sulfate, 0.1% potassium dihydrogen ortho-phosphate, and 0.05% magnesium sulfate. The cultivation was done in a round bottom flask connected with a U-tube. The outlet was fitted with a test tube containing $Ca(OH)_2$ to maintain anaerobic conditions and pH of the fermentation medium (Fogel et al., 1982; Jeffers, 2000). The culture was allowed to grow for three days at 30 °C, pH 7 with agitation at 100 rpm. The ethanol was distillated at 78.5 °C (Jeffers, 2000). A 0.5-ml aliquot of distillate was added to 4.5 ml of potassium dichromate solution (3.4 g of $K_2Cr_2O_7$, 19 ml of concentrated H_2SO_4 and 81 ml of H_2O) and mixed well in test tubes. The test tubes were incubated at 60 °C for 15 min, the mixture was filtered and the optical density was measured at 600 nm. Ethanol production was calculated using a standard graph as described by Kiransree et al. (2000). Experiments were performed in triplicates and mean values were calculated.

3. Results and discussion

3.1. Isolation and characterization of cellulolytic bacteria from termite

Among the 12 cellulase-producing isolates obtained from termite guts, the isolate forming large mucoid pink colonies on eosin methylene blue (EMB) agar plate showed the highest cellulase activity as determined by the DNS method. The optimum pH and

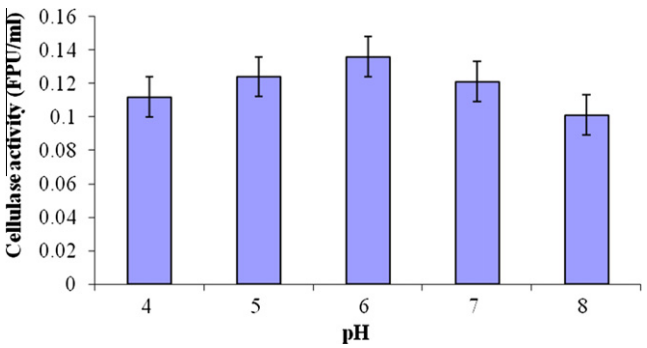

Fig. 1. Filter paper (total cellulase) activity of *Enterobacter cloacae* from pH 4–8.

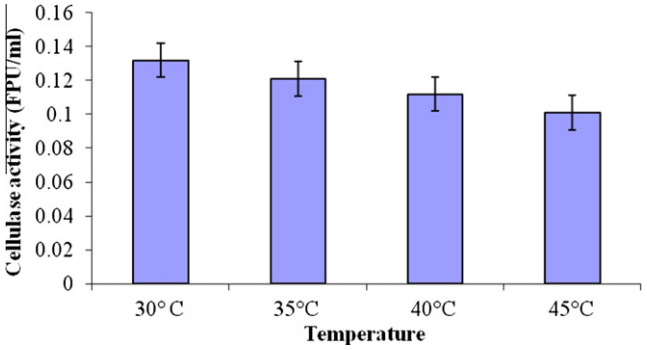

Fig. 2. Filter paper (total cellulase) activity of *Enterobacter cloacae* from temperatures 30 °C to 45 °C.

temperature were 6 and 30 °C, respectively (Figs. 1 and 2). These results showed that near neutral pH value was more favourable to cellulase activity in *E. cloacae*. The 16S rRNA gene sequence of this isolate (FJ799063) was 99% identical to that of *E. cloacae* (GQ418145.1). *E. cloacae* isolated from the blue pumpkin beetle, *Aulacophora atripenis*, has previously been described as a cellulase (endo-1,4-D-glucanase) producer (Sami and Shakoori, 2008; Sami et al., 2008).

3.2. Cloning of cellulase gene in pET 20b (+) vector

The recombinant cellulase-producing *E. coli* harbored an *E. cloacae* derived 2.25-kb *Bam*HI fragment. Sequencing of this insert (GQ368735) demonstrated the presence of a 1083-bp open reading frame potentially encoding a protein with a molecular mass of 40,327 Da. The 13 N-terminal amino acids likely encode a signal peptide. The predicted amino acid sequence was 99–100% identical to that of the endo-1,4-D-glucanase of *Citrobacter rodentium* ICC168 (FN543502.1) and *Enterobacter* sp. (CP000653.1). The ORF region of the insert shows 99% similarity to the cellulase synthase operon C domain protein of glycosyl hydrolase family 8 in *Enterobacter* sp. and 80% similarity to the endo-1,4-beta-glucanase of *C. rodentium* ICC168.

The *E. coli* cells containing pET-Cel exhibited cellulase activity comparable to that of *E. cloacae* (Table 1). The clearing zones around the colonies containing pET-Cel were larger than the clearing zones found in wild type colonies of *E. cloacae*. High copy number (~40) of pET 20b (+) might be the reason for the higher expression of the cellulase in *E. coli* (Gunasekeran and Kemp, 1999). The maximum endoglucanase activity of pET-Cel recorded, with CMC as a substrate was 6.75 IU/g at pH 6.0, Temperature 30 °C (Table 1).

3.3. Pretreatment of cellulosic substrates

The digestibility of NaOH-treated lignocellulosic materials increased from 14% to 55% with the decrease of lignin content from 55% to 20%. However, no effect of dilute NaOH pretreatment was observed for lignocellulose with lignin content greater than 26%. Dilute NaOH pretreatment was also effective for the hydrolysis of straw with relatively low 10–18% lignin content (Bjerre et al., 1996). Treatment with 4% NaOH released the maximum cellulose content from bagasse (Table 2). FTIR spectroscopy is an ideal technique to find out the impact of variations in the pretreatments on the chemical structure of the cellulose and it can also be used for determining the chemical components in a mixture (Proniewicz et al., 2001). FTIR spectra were dominated by the peaks at 3356 and 1058 cm^{-1} that correspond to the stretching vibration of O–H and C–O in cellulose. Lignin (1500–1599 cm^{-1}) and hemicellulose (1642 cm^{-1}) peaks (Sun et al., 2004) were absent in pure cellulose powder and in 4% NaOH pretreated bagasse (data not shown). The crystallinity index of 4% NaOH-treated bagasse was 0.52 ± 0.13. The NaOH-treated sample exhibited a higher crystallinity as the alkaline treatment removed the non-cellulosic polysaccharides more efficiently and also hydrolyzed the amorphous domains in the microfibrils (Zuluaga et al., 2007).

Table 1
Endoglucanase, filter paper and ethanol activities of bacterial strains.

Strain name	Endoglucanase activity (IU/ml)	Filter paper activity (FPU/ml)	Ethanol production using 4% NaOH treated bagasse (%) (V/V)
Enterobacter cloacae	6.09	0.197	1.2
pET-Cel	6.75	0.201	–
Z. mobilis	0.000	0.000	0
Recombinant Z. mobilis	5.79	0.134	4

Table 2
Cellulose content for NaOH pretreatment of bagasse.

Types of pretreatment	% of Cellulose retained
2% NaOH	73.81
4% NaOH	78.3333
6% NaOH	73.5185
8% NaOH	67.5926
10% NaOH	61.1111

3.4. Transforming cellulase gene containing DNA fragment into Z. mobilis

The cellulase gene-containing DNA fragment was cloned into vector pKT230 and transformed into *Z. mobilis*. A small but distinctly clear halo appeared around the clones indicating endoglucanase production. Congo red interacts with (1,4)-β-D-glucans and (1,3)-β-D-glucans and a clearing zone around the colony on the agar medium indicates the hydrolysis of cellulose. The cellulase activity of the recombinant *Z. mobilis* (5.79 IU/g) was lower than that of *E. coli* pET-Cel. This might be due to a lower copy number (12) of pKT230 in *Z. mobilis* when compared to pET 20b (+) vector (40 copies in *E. coli*) (Bagdasarian et al., 1981). As observed with the *E. chrysanthemi* endoglucanase expressed in *Z. mobilis* harboring the recombinant pKT230 (Goachet et al., 1989), most of the endoglucanase activity was detected in the cellular fraction of recombinant *Z. mobilis* (Fig. 3). Present results favoured the hypothesis of a periplasmic location of the enzyme. Consequently, it is assumed that the excretion signals from *E. cloacae* were correctly recognized in *Z. mobilis*. A similar situation was observed with

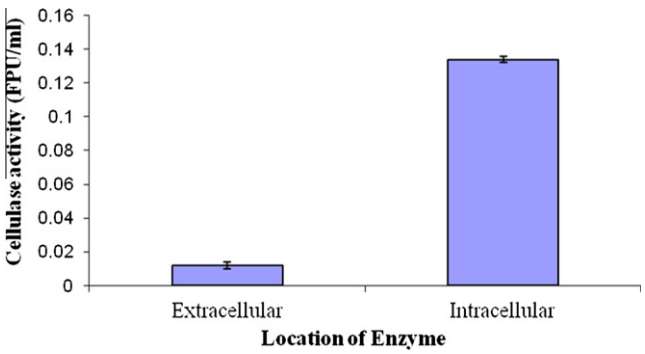

Fig. 3. Localization of cellulase enzyme activity in recombinant *Z. mobilis*.

the *Bacillus licheniformis* α-amylase (Goachet et al., 1990), which was also exported through the inner membrane of *Z. mobilis*. It is not known whether the release of cellulase into the culture medium is due to an active secretion mechanism or to an increased leakiness of the outer membrane at the end of the growth phase, possibly caused by the production of ethanol (Goachet et al., 1990).

The cellulase genes from *C. uda* (Misawa et al., 1988), *B. subtilis* (Yoon et al., 1988), *P. fluorescens* (Lejeune et al., 1988) and *Ruminococcus albus* (Yanase et al., 2005) were cloned into *Z. mobilis*. No attempt was made for ethanol production from cellulosic materials using the above constructed *Z. mobilis* strains due to low level expression of the cellulase. Recombinant *Z. mobilis* with β-glucosidase gene was also reported to produce a little amount of ethanol from cellulosic substrates (Su et al., 1989). In the present work, the recombinant *Z. mobilis* strain was found to have and was able to utilize CMC and pretreated bagasse under anaerobic conditions. The wild type *Z. mobilis* produced 12% (V/V) ethanol using glucose as a substrate and no ethanol production was found when only CMC and pretreated bagasse were used as substrates (Fig. 4). But the recombinant *Z. mobilis* produced 12% (V/V), 5.5% (V/V) and 4% (V/V) ethanol with glucose, CMC and 4% NaOH pretreated bagasse respectively as substrates (Fig. 5). Ethanol production using the recombinant *Z. mobilis* is advantageous because, it is capable of both cellulose degradation and ethanol fermentation and hence it could be cultured in a single bioreactor. But in the already available methods, cellulose degradation and ethanol fermentation are carried out separately with two different strains in a sequential manner.

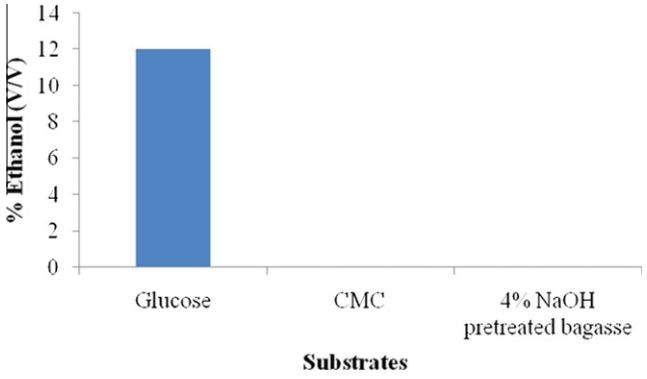

Fig. 4. Ethanol production ability of wild type *Z. mobilis* using glucose, carboxymethyl cellulose and 4% NaOH pretreated bagasse.

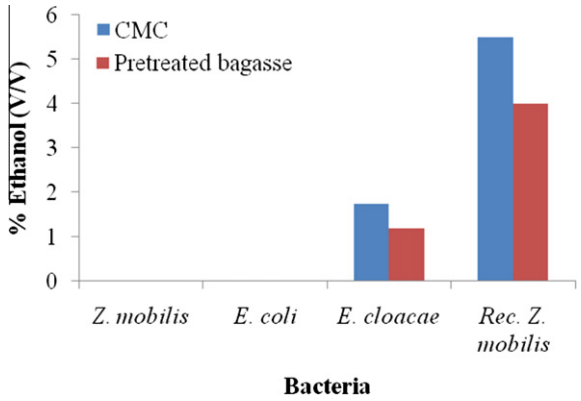

Fig. 5. Ethanol production ability of wild type and recombinant bacteria with two different cellulosic substrates (CMC and 4% NaOH pretreated bagasse).

4. Conclusions

Z. mobilis harboring a plasmid with a cellulase-encoding DNA from *E. cloacae* was able to utilize CMC and NaOH-treated bagasse as substrates for ethanol production under anaerobic conditions. Further improvement in ethanol yield could be achieved by simultaneous expression of a few cellulase genes into the same organism.

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