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Harbouring of bacterial cellulase gene into *Zymomonas mobilis* for cellulosic ethanol production

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Abstract
Pseudomonas aeruginosa, *Klebsiella pneumoniae*, *Pseudomonas fluorescens* and *Proteus mirabilis* were isolated from the gut of phytophagous insects. Cellulase gene from the above five bacteria was cloned separately into *E. coli* using pET20b(+) plasmid and the cellulase gene containing plasmids such as, *pET-cel-Pa*, *pET-cel-Pm, pET-cel-Pf* and *pET-cel-Kp* were developed and the cellulase genes were characterized by restriction analysis and DNA sequencing. The cloned cellulase genes were subcloned in pKT 230 plasmid, a stable vector of *Zymomonas mobilis*, an ethanol fermenting bacterium and the recombinant *pKT-cel-Pa, pKT-cel-Pm, pKT-cel-Pf* and *pKT-cel-Kp* were developed and transformed into *Z. mobilis*. These recombinant *Z. mobilis* strains expressing bacterial cellulase gene were used for ethanol production using carboxymethyl cellulose and 4% NaOH pretreated bagasse as substrates. All the recombinant *Z. mobilis* strains were found to ferment ethanol from pretreated cellulosic substrates but the recombinant *Z. mobilis* strain harboring cellulase gene cloned from *P. aeruginosa* (*pKT-cel-Pa*) produced ethanol 12% (using glucose), 5% (using CMC) and 4% (using 4% NaOH pretreated bagasse). The recombinant *Z. mobilis* strain could be improved further by simultaneous expression of additional cellulase genes and the strains could be used for industrial level ethanol production.

Keywords: Cellulolytic bacteria, pET20b (+), *Z.mobilis*, ethanol

1. Introduction
Cellulose is the major biopolymer on earth and is of tremendous economic importance globally. It has attracted worldwide attention as a renewable resource that can be converted into biobased products and bioenergy. Cellulose is assembled from glucose which is produced in the living plant cell through photosynthesis and about 7.5×10^{10} tons are annually synthesized through photosynthetic process (Monserrate *et al.,* 2002).
An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure. Cellulose is synthesized in nature as individual molecules which undergo self-assembly at the site of biosynthesis (Brown and Saxena, 2000). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibers (Atalla *et al.,* 1993).
Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). Complete enzymatic hydrolysis of cellulose requires the synergistic action of three types of enzymes, namely Cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase) and β-glucosidases or cellobiase. Endoglucanase activity hydrolyzes β-1, 4 linkages between adjacent glucose molecules within the amorphous regions of the cellulose polymer thereby breaking the chain in the middle. Exoglucanase activity degrades the nicked cellulose chains from their non-reducing ends producing glucose, cellobiose and/or cellotriose. Cellobiohydrolase, another type of exoglucanase activity removes larger polysaccharides from the non-reducing end of the cellulose molecule. Glucosidase or cellobiase, converts cellobiose to glucose (Eveleigh, 1987).
Microbial cellulase enzymes are the major source for the bioconversion of cellulose, as they act as phytopathogen to generate a battery of enzymes (Lacy and Lukazic, 2003), microbes reside in the gut of pest insects and ruminants and reportedly take part in the digestion of cellulose which is a major part of their diet. Most insects are unable to use lignocellulosic substrates as their main food sources, but some insects subsist on lignocellulosic biomass as their only foods. Insects harbor a large number of microbes in their gut and thus serve as an arsenal of microbial diversity. Cellulase-producing microbes play a key role in the carbon cycle, breaking down carbon compounds and releasing methane and CO_2 which are extremely important in maintaining the turnover of organic matter in the carbon cycle. These microbial cellulases are essential kind of enzyme for carrying out the depolymerization of cellulose into fermentable sugars. As a major resource for renewable energy and raw materials, it is widely

used in the bioconversion of renewable cellulosic biomass. Glucose, from appropriate hydrolysis of this cellulosic biomass under the treatment of advanced biotechnology can be used in different applications such as production of fuel ethanol, single cell protein, feed stock, industrially important chemicals and so on.

Several organisms (such as fungi and bacteria) are also known to produce small quantities of ethanol. However these organisms produce in addition to ethanol other undesirable products, hence they are not suitable for alcohol production. Fermentation is often most active under anaerobic conditions, when carbohydrate is converted into ethanol and acetaldehyde. The organisms of primary interest to industrial operations in fermentation of ethanol include *Saccharomyces cerevisiae, S. uvarum, Schizosaccharomyces pombe* and *Kluyueromyces* sp (Lynd *et al*., 2005).

Apart from fungi, certain bacteria are capable of solubilizing, transforming and minerilisation of lignocelluloses. Stability of fungi are not good in practical treatment under extreme environmental and substrate condition such as increase in pH, oxygen limitation and substrate concentration whereas bacteria are worthy of being studied for their cellulolytic potential due to their immense environmental adaptability and biochemical versatility. In addition to the cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to bacterial high growth rate. Thus, enhancing the activity of bacterial cellulase enzyme and reducing its production cost are two key issues in the enzymatic hydrolysis of cellulosic materials. Genetic engineering techniques are being used to clone the bacterial cellulase coding sequences into bacteria, yeasts, fungi, plants and animals to create new cellulase production systems with possible improvement of enzyme production and activity (Chandra *et al*., 2007).

Hence, research was carried out to study various cellulolytic bacteria from phytophagous insects, their cellulolytic capability. The present study was also focused to isolate cellulase gene from cellulolytic bacteria isolated from insect gut microflora. The characterization and structural analysis of bacterial cellulase genes were also carried out. The cloned bacterial cellulase genes from five different cellulolytic bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Pseudomonas fluorescens* and *Proteus mirabilis* were subcloned into pKT230, a *Zymomonas mobilis* vector and introduced into *Zymomonas mobilis*, in order to develop recombinant strains for ethanol production. Thus developed recombinant *Z. mobilis* strains were studied for their capability to both cellulolysis and ethanol production solely from pretreated bagasse.

2. Materials and 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 30ºC 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 37ºC. *E. coli* (pET 20b (+) was grown on Lysogeny agar with ampicillin (50 µg/ml) under static condition at 37ºC.

2.2 Collection of insects

Fifth instar larvae of *B. mori*, that vigorously feed on mulberry leaves were obtained from Central Sericulture Research Institute, Samayanallur, South India. They were reared at room temperature (32 ±1ºC) and humidity of 82-90% with mulberry leaves as diet. The adult beetle *O. rhinoceros* were collected from the coconut plantation in and around Tiruchirappalli. Tiruchirappalli is situated at the centre of the Tamil Nadu. It is located at an altitude of +78 MSL. Alive adult beetles were collected in plastic containers and taken to the laboratory for a complete microbial analysis.

Termites were obtained from the dead and dry woods in the Anna University, Tiruchirappalli campus. It is located at an altitude of + 78 meter MSL. The environmental conditions such as, temperature and humidity were 32± 2ºC and 80 to 90% respectively. The termite's specimens were identified using morphological charceteristics (Department of Entomology, St. Joseph college of Arts and science, cuddalore, Tamil nadu, India).

2.3 Isolation of cellulolytic bacteria from the gut of phytophagous insects

The adult beetles of *O. rhinoceros* and *B. mori* were removed, pooled and homogenized with 1 ml of sterile water. The homogenate was serially diluted to 1010 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 37ºC 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 cellulolytic bacteria) were then tested for their abilities to hydrolyze 1 x 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 30ºC and 50ºC 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).

2.4. 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 isolates were 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 bacteria were identified as *P. aeruginosa*

(GU213909), *K. pneumoniae* (HQ231794), *P. fluorescens* (HQ231795) and *P. mirabilis* (HQ231796).

2.5. Cloning of the cellulase gene

Chromosomal DNA were isolated from *P. aeruginosa, K. pneumoniae, P. fluorescens* and *P. mirabilis* 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). *P. aeruginosa, K. pneumoniae, P. fluorescens* and *P. mirabilis* 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 cellulolytic clones were 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).

2.6. 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).

2.7. 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 *O. rhinoceros* and *B. mori*

Among the 25 cellulase-producing isolates obtained from *O. rhinoceros* and *B. mori* guts, showed the highest cellulose activity as determined by the DNS method. The optimum pH and temperature were 6 to 7 and 30-45°C, respectively (Figs. 1 and 2). These results showed that near neutral pH value was more favourable to cellulase activity in *P. aeruginosa K. pneumoniae, P. fluorescens* and *P. mirabilis*. Two cellulolytic strains isolated from the gut of *O. rhinoceros* were *P. aeruginosa* JV and *K. pneumoniae* JV. *P. aeruginosa* JV showed sequence 99% similarity with *Pseudomonas* sp. GZ1, *Pseudomonas* sp. L29, *Pseudomonas* sp. NR2(2010) phylogenetically and *Klebsiella pneumoniae* JV showed 98% sequence similarity with Bacterium DX120E, *Klebsiella variicola* At-22, *Enterobacteriaceae* bacterium PB29. *Proteus mirabilis* JV and *P. fluorescens* JV were isolated from the digestive tract of *B. mori*. *Proteus mirabilis* JV showed sequence similarity with *Proteus mirabilis* strain FUA1237 (98%), *Proteus vulgaris* (97%), *Proteus mirabilis* strain MA (96%) and *P. fluorescens* JV showed 98% similarity with *Pseudomonas fluorescens* strain YUST-DW18 (98%).

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

The plasmid DNA of transformants showing higher cellulolytic activity were isolated and restricted with *Bam*HI enzyme. The sizes of the insert DNA in each cellulolytic clone in the genomic DNA library were determined by restriction analysis. The cellulase gene cloned from *P. fluorescens* contained an insert size of 2.7 kb *Bam*HI fragment. Sequencing of this cloned DNA showed the presence of a 2247-bp open reading frame (HM235919) that encoded a protein with a molecular mass of 80.160 kDa. The predicted amino acid sequence of the gene was 99% identical to that of the cellulase of *Cellvibrio japonicus celC* gene for cellodextrinase C (X61299.1). The cellulase gene cloned from *K. pneumoniae* contained in an insert 1.3-kb *Bam*HI fragment. Sequencing of this insert DNA revealed the presence of a 1107-bp open reading frame (HM174251) that potentially encoded a protein with a molecular mass of 40.88 kDa The predicted amino acid sequence of this gene was 100% identical to that of the cellulase of *K. pneumoniae* cellulase gene (HM235918.1). The cellulase gene cloned from *P. aeruginosa* contained in an insert of 3 kb *Bam*HI fragment. Sequencing of this insert showed that the presence of a 2883-bp open reading frame (GQ872426) that potentially encoded a protein with a molecular mass of 100.07 kDa. The predicted amino acid sequence was 99–100% identical to that of the glycosyl hydrolase family of *Teredinibacter turnerae* T7901 (CP001614.2). The recombinant cellulase producing *E. coli* harbored a *Proteus mirabilis* derived 1.7-kb *Bam*HI fragment.

Sequencing of this insert revealed the presence of a 1053-bp open reading frame (HM235922) that potentially encoded a protein with a molecular mass of 40.54 kDa. The predicted amino acid sequence was 100% identical to that of the putative cellulase family of *P. mirabilis* (AM942759.1). The *E. coli* cells containing the cellulase genes from different cellulolytic bacteria exhibited cellulase activity comparable to that of wild type cellulolytic bacteria from which the cellulase genes were isolated. Clearing zone around the colonies containing cellulase gene was larger than the clearing zone found in wild type colonies of wild type of cellulolytic bacteria. The *E. coli* cells containing pET-cel exhibited cellulase activity comparable to that of *P. aeruginosa, K. pneumoniae, P. fluorescens* and *P. mirabilis*. (Table 1). The clearing zones around the colonies containing pET-Cel were larger than the clearing zones found in wild type colonies of *P. aeruginosa, K. pneumoniae, P. fluorescens* and *P. mirabilis*.. 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-Pa recorded, with CMC as a substrate was 0.207 FPU/ml at pH 6.0, Temperature 40ºC (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 hemicelluloses (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).

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

The cellulase gene-containing DNA fragments were 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)-b-D-glucans and (1,3)-b-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* harbouring 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 *P. aeruginosa, K. pneumoniae, P. fluorescens* and *P. mirabilis* were correctly recognized in *Z. mobilis*. A similar situation was observed with Fig. 2. Filter paper (total cellulase) activity of *Enterobacter cloacae* from temperatures 30 °C to 45 °C. the *Bacillus licheniformis* a-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 b-glucosidase gene was also reported to produce a little amount of ethanol from cellulosic substrates (Su et al., 1989). *Z. mobilis* harboring a plasmid with a cellulase-encoding DNA from *Enterobacter cloacae* JV was able to utilize CMC and NaOH-treated bagasse as substrates for ethanol production (Vasan et al., 2011). 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 yeast extract supplemented glucose as a substrate and no ethanol production was found when only CMC, and pretreated bagasse were used as substrates. But the recombinant *Z. mobilis* (harbouring *P. aeruginosa* cellulase gene) produced 12% (V/V), 5% (V/V) and 4% (V/V) ethanol with yeast extract supplemented with glucose, CMC and 4% NaOH pretreated bagasse respectively as substrates at 72hr. After 72hr the ethanol production decreased. This could be due to the oxidation of alcohol to acetic acid (Swings and De Ley, 1977). 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.

4. Conclusions

Z. mobilis harboring a plasmid with a cellulase-encoding DNA from *P. aeruginosa, K. pneumoniae, P. fluorescens* and *P. mirabilis* were 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.

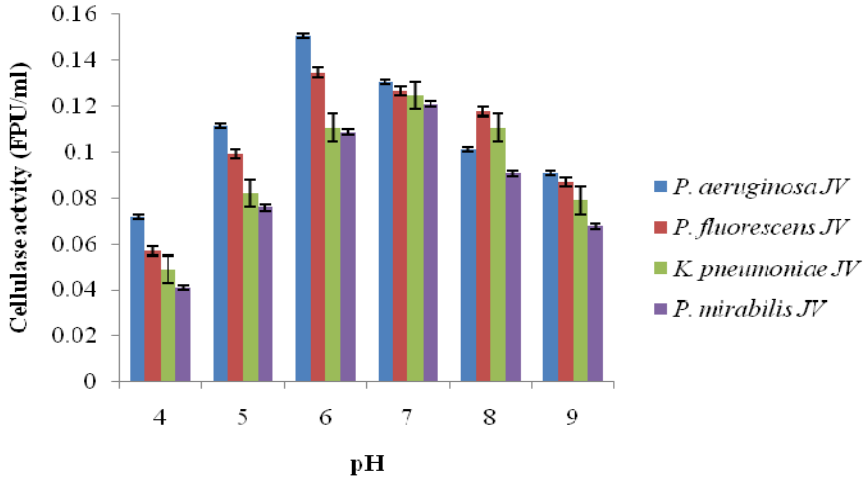

Fig 1: Filter paper (total cellulase) activity of cellulolytic bacteria from pH 4–9.

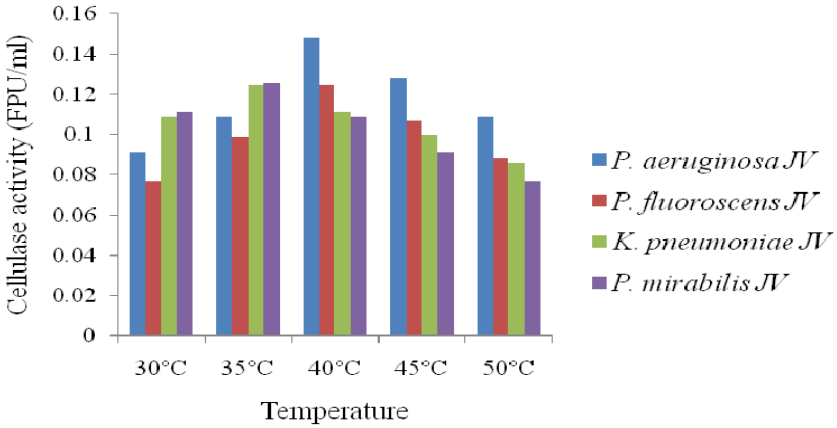

Fig 2: Filter paper (total cellulase) activity of cellulolytic bacteria from temperatures 30ºC to 50ºC.

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)
Pseudomonas aeruginosa JV	3.01±0.17	0.207±0.11	1.1
pET-cel-Pa	3.49±0.01	0.171±0.02	0.000
pKT-cel-Pa	3.01±0.03	0.152±0.07	3.5
Pseudomonas fluoroscens JV	2.32±0.23	0.179±0.09	1.1
pET-cel-Pf	5.77±-0.03	0.146±0.01	0.000
pKT-cel-Pf	4.90±-0.06	0.135±0.08	3.0
Klebsiella pneumoniae JV	4.22±0.16	0.154±0.13	0.9
pET-cel-Kp	4.3±0.04	0.191±0.03	0.000
pKT-cel-Kp	4.1±0.08	0.172±0.01	3.0
Proteus mirabilis JV	3.09±0.15	0.148±0.23	0.85
pET-cel-Pm	3.53±0.06	0.159±0.05	0.000
pKT-cel-Pm	3.01±0.02	0.139±0.04	2.5
Z. mobilis	0.000	0.000	0.000
pET 20 b(+)	0.000	0.000	0.000

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	73.51
8% NaOH	67.59
10% NaOH	61.11

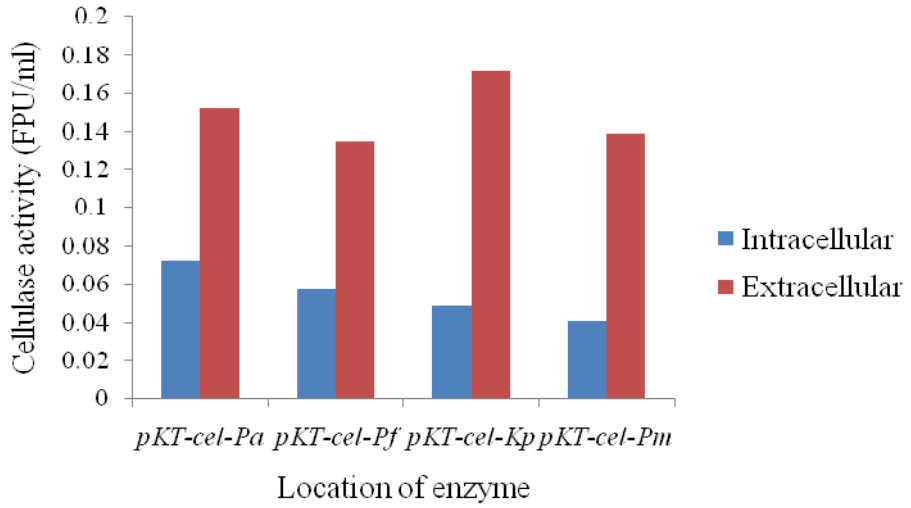

Fig 3: Localization of cellulase enzyme activity in recombinant *Z. mobilis*.

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