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Molecular characterization of cellulose-degrading *Bacillus pumilus* from the soil of tea garden, Darjeeling hills, India

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Abstract

Bio-fuel produced from ethanol is economically and environmentally advantageous in context of changing global climate. A large number of microorganisms are capable of cellulase production but most of them cannot be utilized commercially due to their low activity. In the present study, an effiecient cellulose degrading strain of *Bacillus pumilus* was obtained after thorough screening for the production of extracellular cellulases. Out of a total of 144 microbes isolated from soils of Darjeeling hills of India, nineteen were found to be cellulose degrader under *in vitro* conditions as observed by clearing zone on CMC – agar plates. Isolate #35 had high cellulolytic activity as observed by a clearing zone of 26.83 mm diameter formed on CMC – agar plate. The isolate was characterized and identified as *Bacillus pumilus*. The isolate was submitted to National Agriculturally Important Microbial Culture Collection (NAIMCC), NBAIM, Mau with Accession number NAIMCC–B-01415. Transposon (Tn5) mutants of wild type isolate *Bacillus pumilus* NAIMCC–B-01415 were generated and screened for the absence of cellulose degradation. Of 365 *B. pumilus* NAIMCC-B-01415 mutants obtained, only two were unable to degrade cellulose under *in vitro* conditions. Inverse PCR studies with *B. pumilus* NAIMCC–B-01415 :: TL5, a cellulose degradation mutant of *B. pumilus* NAIMCC –B-01415 revealed presence of Cys B (Cystein protein regulatory) gene involved in cellulose degradation. The participation of Cys B gene in cellulase degradation is reported here.

Key words

Bacillus pumilus NAIMCC –B-01415, CysB, Inverse PCR, Tn5 mutagenesis

Introduction

Depletion of fossil fuels is an issue of global concern and biofuel produced from non-conventional sources of energy are futuristic alternatives. Bioethanol not only provides a cheap and ecofriendly energy source but also in most cases effectively utilizes waste. In recent years, there has been tremendous interest in plant cellulosic biomass as a unique resource for renewable energy generation (Saxena *et al.,* 2009). The carbohydrates in agricultural lignocellulosic residues of plants can be utilized in various ways for energy purposes, among which ethanol production is one of the most promising approach (Cheng and Timilsina, 2011). The plant cellulose is efficiently hydrolyzed to glucose by the synergistic action of three types of cellulases ie endoglucanases (1,4-β-D glucan 4-glucohydrolase), exog lucanases (β-1,4-D-glucan cellobiohydrolase), and β-glucosidases (β-D-glycoside glucohydrolase). The glucose, thus produced is effectively fermented to ethanol, which can be used as fuel. The cellulases are thus the key enzymes further involved in the conversion of the world's most abundant carbon source *i.e.* cellulose to ethanol. Cellulases are mostly of fungal origin though bacterial cellulases are also known to exist but only a few have been shown to produce adequate levels for their meaningful utilization (Lynd *et al.,* 2002; Demain *et al.,* 2005). Various physical and chemical methods, including mutagenesis, have been used to develop bacterial and fungal strains that produce higher amounts of cellulase, all with limited success (Chand *et al.,* 2005). Understanding the molecular mechanism of cellulose bioconversion to a fuel resource would help in efficiently utilizing microbial capability to generate biofuel in future. Simultaneously, the search for potential sources of cellulolytic enzymes is continuing in the interest of successful bioconversion of

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lignocellulosic biomass (Kumar *et al.,* 2008).

The present study aimed at understanding the molecular basis of cellulose degradation by a cellulolytic microbe, *Bacillus pumilus* NAIMCC-B-01415. *B. pumilus* NAIMCC-B-01415 was isolated from the soil of diverse habitat of tea gardens in Darjeeling hills, India. Tn5 mutagenesis and Inverse PCR based studies led to unravelled the presence of Cys B (Cystein protein regulatory) gene and its role in cellulose degrading pathway of *B. pumilus* NAIMCC-B-01415. The role of Cys B in cellulase degradation mechanism of a *Bacillus* species is reported.

Materials and Methods

Bacillus pumilus NAIMCC-B-01415, a wild type of cellulose degrading bacteria, isolated from the rhizospheric soil of tea plants growing in Darjeeling, West Bengal (India) was used in the present study. Rifampicin resistance was introduced in the wild type isolate, *B. pumilus* NAIMCC-B-01415, which resulted in the development of strain *B. pumilus* NAIMCC-B-01415r. *B. pumilus* NAIMCC-B-01415r was used in conjugational mating studies for the generation of insertional mutants of the wild type isolate. Transposon (Tn5) mutagenesis of *B. pumilus* NAIMCC-B-01415 was carried out and *B. pumilus* NAIMCC-B-01415::TL 5 was isolated as a mutant which had lost the cellulose degrading property of the wild type parent, *B. pumilus* NAIMCC-B-01415. *Escherichia coli* S17 (pSU5011::Tn5) (Simon, 1984), a neomycin resistant (Neo R) strain of *Escherichia coli* was used as Tn5 donor for carrying out insertional mutagenesis. The transposon Tn5 carries a neomycin resistance gene. *B. pumilus* NAIMCC-B-01415 and its derivatives were grown on nutrient agar medium (NA) (Himedia, India) while *Escherichia coli* strains and their derivatives were grown on Luria agar medium (LA) (Himedia, India).

Assay for *in vitro* cellulase production : Qualitative cellulase production assay of the isolated cultures was done on carboxymethyl cellulose–agar (CMC-agar) (Sodium carboxy methylcellulose - 5 g l^{-1}, $NaNO_3$ - 1 g l^{-1}, K_2HPO_4 - 1 g l^{-1}, KCl - 1 g l^{-1}, $MgSO_4$ - 0.5 g l^{-1}, yeast extract - 0.5 g l^{-1}, glucose – 1 g l^{-1} and agar – 1.8%) The bacterial isolates were spotted on CMC-agar plate and incubated for 2 days at 30°C. After incubation, the plates were first treated with 0.1% Congo red and allowed to stand for 15 min followed by 1M NaCl for 15 mins. The cellulose degraders formed a clear zone around the colonies (Gomashe *et al*., 2013) Bacteria lacking clear zone formation were discarded while those with the property of cellulose degradation were taken for further studies and stored in tubes containing nutrient broth plus 40% glycerol at -70°C.

Total cellulase activity was determined by measuring the amount of reducing sugar formed from a 1 × 6-cm strip of Whatman no. 1, standard sized filter paper. The enzyme activity was determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Biotechnology (Ghose, 1987). Reducing sugar was estimated as glucose following the method of Miller (1959). This assay was performed so that 0.5 ml of diluted enzymes released about 2.0 mg of glucose equivalents in 60 min, as determined by 3, 5-dinitrosalicylic acid (DNS) assay. Endoglucanase activity was determined by incubating 0.5ml of supernatant with 0.5ml of 2% CMC in 0.05M sodium citrate buffer (pH 4.8) at 50ºC for 30 min. Reducing sugars were estimated spectrophotometrically at 540nm with 3, 5-dinitrosalicylic acid (Miller, 1959) using glucose as standards. The enzyme activities were determined as International Unit (IU), which is defined as the micro mole of glucose equivalent liberated per minute of culture filtrate under assay conditions viz., pH and temperature.

Isolation and characterization of *Bacillus pumilus* NAIMCC-L01415 : The potential cellulose degraders were isolated from rhizospheric soil of tea gardens of Darjeeling (Bengal) by random soil collection method. Soil free of stones and large plant parts were collected from different locations, pooled together and stored at 4°C until further use. Ten gram of soil was suspended in 10ml of sterile water kept on shaker for 2hrs at 160rpm and then made to stand for 2-3 hrs at room temperature. The soil samples were centrifuged and the pellet were discarded. Supernatant was spread plated on nutrient agar medium by serial dilution (upto109). The colonies obtained were further purified by single colony isolation method.

Genomic DNA was isolated from *B. pumilus* NAIMCC-B-01415 and its mutants using Quick–gDNATM Mini Prep extraction kit as per manufacturer's instructions (Zymo Research, USA).

PCR amplification of 16SrDNA region of *B. pumilus* NAIMCC-B-01415 was carried out in 50ml reaction volume containing 100ng of template; 16SrDNA universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1520 r (5'-AAGGAGCTGATCCAGCCGCA- 3') (Kuhnert *et al.,* 1996) at 0.1mM each; 1.5 mM MgCl2; dNTP mix at 200mM and 2U of Taq DNA polymerase. The reaction mixture was heated to 94°C for 5 min in DNA thermal cycler (icycler, Bio-Rad) and programmed for 30 cycles of amplification as denaturation at 94 °C for 1 min, annealing at 54 °C for1 min and extension at 72 °C for 1.5 min. The final extension was performed at 72°C for 7 min. The amplified product (5 µl) was analyzed by agarose gel electrophoresis (1.5% agarose), and the products were stained with ethidium bromide and visualized under UV light. The amplified PCR product was purified after electrophoresis in 1.5 % (w/v) agarose gel. The purified PCR product was sequenced (both strands) in a commercial sequencing laboratory (Chromous Biotech). The high quality sequences obtained were BLAST analysed to assess the homology with other microbes.

Antibiotic sensitivity test : Resistance of *B. pumilus* NAIMCC-B-01415 to series of antibiotics were determined on nutrient agar

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medium containing filter sterilized antibiotic discs at different concentrations in mg ml^{-1}: kanamycin (5,20,50), chloramphenicol (10,25,50), gentamycin (10,30,50), rifampicin (2,5), naldixic acid (10, 20, 30), streptomycin (10, 25), ampicillin (10, 25, 50), penicillin (10, 20) and tetracycline (10,30). 100 µl of overnight grown culture of *Bacillus pumilus* NAIMCC-B-01415 was spread on NA plates and allowed to dry for 10 min; the antibiotic impregnated discs were placed on the surface of agar plates gently with the help of sterile forceps. The plates were incubated at 30°C for 24hr. Isolates were considered resistant when bacterial growth occurred around the discs and sensitive when no growth occurred.

Isolation of rifampicin resistant cultures of isolated *Bacillus pumilus* NAIMCC-B-01415 : Rifampicin resistant cells of *B. pumilus* NAIMCC-B-01415 (Neomycin sensitive) were isolated using the standard protocol (Glover, 1962). Rifampicin resistant culture of *Bacillus pumilus* was designated as *B. pumilus* NAIMCC-B-01415r.

Tn5 mutagenesis : Transposon (Tn5) mutagenesis of *B. pumilus* NAIMCC-B-01415r (rifampicin resistant) was carried out using *Escherichia coli* S17 (pSUP5011::Tn5) (Neomycin resistant) as the Tn5 donor (Lupski, 1990). Tn5 mutants of *B. pumilus* NAIMCC-B-01415r (rifampicin resistant) were isolated on medium containing antibiotics neomycin @ 100µg ml^{-1} and rifampicin @ 100µg ml^{-1}. *In vitro* cellulase production assay method was used to screen the isolated Tn5 mutants of *B. pumilus* NAIMCC-B-01415r for loss of cellulose degradation. The selected Tn5 mutant was designated as *B. pumilus* NAIMCC-B-01415:: TL5.

The presence of Tn5 in the genomic DNA of *B. pumilus* NAIMCC-B-01415:: TL5, the Tn5 mutant of *B. pumilus* NAIMCC-B-01415-r which had lost its cellulose degradation property was confirmed by colony PCR. Single colony of *B. pumilus* NAIMCC-B-01415:: TL5 was used as template DNA. The neomycin specific primer pair (Reiznikoff 1993) (F5'-TAGACTGGGCGGTTTTAT GGACAG -3') and (R5'- AACTCCGCGAGGTCGTCCAGCCTC -3') were used to amplify a 1.2kb region in the genomic DNA of the mutant *B. pumilus* NAIMCC-B-01415 TL5. Reaction mixture (25µl), consisting of 1X PCR buffer; primers at 0.1mM each; 1.5 mM MgCl2; dNTP mix at 200mM and 2U of Taq DNA polymerase were used to perform amplification in thermal cycler (icycler, Bio-Rad). After initial denaturation at 94°C for 5 min, the reaction mixture was subjected to 30 cycles of amplification as denaturation at 94 °C for 1 min, annealing at 58.7°C for 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. An aliquot of the reaction mixture (5 µl) was analyzed by agarose gel electrophoresis (1.5% agarose), and the products were stained with ethidium bromide and visualized under UV light.

Inverse PCR to identify the gene involved in cellulose degradation : Inverse PCR (Vincent *et al.,* 1999) was carried with the isolated genomic DNA of *B. pumilus* NAIMCC-B-01415:: TL5. The genomic DNA was double digested sequentially with EcoRI (Promega) and then with Bam H I (Promega). The restricted DNA was then self ligated with T4 DNA ligase (Promega). Using the self ligated DNA as template, Inverse PCR was carried out with primer pair (primer I 5'- GCAGAAGTTATCATGAACGTTACC-3'and primer II 5'-GACCTTGCACAGATAGCGTGGTCC -3') designed based on the sequence of the IS50 regions of Tn5 (Reznikoff, 1993). Primers were designed to amplify DNA in an outward direction with respect to Tn5. Reaction mixture (25µl) consisting of consisting of 1X PCR buffer, 1.5mM MgCl2, 200µM dNTP mixture, 2U of Taq DNA polymerase (Genei), 25pmol each primer and 10µl restricted- self ligated template DNA were subjected to 30 cycles of amplification in a PCR thermal cycler (icycler, Bio-Rad). The amplification cycle was as follows: preheating at 94°C for 5 mins, denaturation at 94°C for 1min, annealing at 58.7°C for 1min and extention at 72°C for 1min. The final extension was performed at 72°C for 10 min. The amplified products were electrophoresed in 1% (W/V) agarose gel.

Cellulose degradation as a function of cystein : The ability of degradation of CMC of the Tn5 mutant *B. pumilus* NAIMCC-B-01415:: TL5 was executed on Davis minimal agar media amended with CMC and cystein. *B. pumilus* NAIMCC-B-01415:: TL5 mutant was grown on Davis minimal media (Sigma-Aldrich) (g l^{-1} Dextrose 1.0, Dipotassiumphosphate 7.0, Monopotassium phosphate 2.0, Sodium citrate 0.5, Magnesium sulfate 0.1, Ammonium sulfate 1.0, Agar 15.0) amended with CMC @ 5g l^{-1} as a source of carbon. Two sets of plates were prepared such as, Davis minimal media with CMC and Davis minimal media with CMC and L-cysteine @ 25uM conc. B. pumilus NAIMCC-B-01415:: TL5 was spotted on the plates and uninoculated plates were served as control. Plates were incubated at 30ºC for 4 days. After incubation plates were treated with 0.1% congo red followed by 1M NaCl for detection of decomposition of CMC.

Results and Discussion

One hundred and forty four isolates were were obtained from the tea gardens, Darjeeling hills, of which nine were found to have distinct cellulose degrading ability as observed by the visible clearing zone formed by the isolates on carboxy methyl cellulose-agar media (Table1). An isolate (#35) from soil of Darjeeling hills later identified as *B. pumilus* NAIMCC-B-01415 was observed to be most effective in degrading cellulose under *in vitro* conditions as it formed a clear zone of 26.83mm (average of three replicates ie R1, R2, R3) on carboxy methyl cellulose-agar media. Total enzyme assay for cellulase activity for *B. pumilus* NAIMCC-B-01415 was found to be 0.189 IU ml^{-1}, whereas, endoglucanase activity was recorded to be 0.386 IU ml^{-1}.

Bacterial isolate #35 was identified to its nearest species based on 16s rDNA sequence data. The ~1.5kb rDNA fragment of genomic DNA of the isolate was amplified using 16 S rDNA

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universal primers (Fig. 1). The size of the obtained amplicon corresponded well to the expected size of 16S rDNA genes of most members of phylum Bacteria. The PCR product obtained was sequenced bi-directionally using the forward, reverse and internal primer (Chromous Biotech Pvt. Ltd.) and the sequence data was aligned and analyzed to identify the bacterium and its closest neighbours. The isolate #35 was identified as *Bacillus pumilus* based on 16S rDNA conserved domain homology. The 1283 bp 16S rDNA conserved domain sequences showing maximum homology to *Bacillus pumilus* was submitted to NCBI genbank (Acc. No: JF806516.1). The isolate was submitted to National Agriculturally Important Culture Collection repository at National Bureau of Agriculturally Important Microorganisms (ICAR), Mau, India with Accesssion number NAIMCC-B-01415. The isolate #35 was named as *Bacillus pumilus* NAIMCC-B-01415. 16S rDNA sequences are highly conserved sequences and have been demonstrated to be useful for phylogenetic analysis of different taxa as well as identification of organisms. Comparative analysis of partial 16S rDNA sequences from unidentified bacterial isolates with extensive database available is used to assign the isolate to a genus or species within higher taxa of domains of Bacteria or Archaea (Cathy, 2007).

Cellulases are indispensable reagents for various industries like the pulp and paper, detergent and clothing. In recent years, role of cellulases in production of biofuel has generated a lot of interest in understanding the expression of cellulase genes and their optimum utilization for mankind. A number of microorganisms are known to produce cellulase enzyme yet *Trichoderma longibrachiatum* (formerly *Trichoderma reesei* or *T. viride*) is the only source of commercially available cellulase for native cellulose (Schwarz, 2001). Bacteria like *Clostridium thermocellum*, *Ruminococcus albus* and *Streptomyces* sp. have been reported to produce cellulase (Ohara *et al.,* 2000), however pure and commercial preparation of bacterial cellulases are not yet available (Kotchoni *et al.*, 2003).

Table 1 : Zone of clearance (in mm) on CMS agar media by the cellulose degrading microbes isolated from soils of Darjeeling hills, India

Isolate No.	Zone of clearance (mm)			
	R1#	R2#	R3#	Average
35*	22.5	23	23	26.83
36	16.5	17	19	17.5
37	7	8	9.5	8.16
40	16.5	16.5	17	16.66
41	16.5	16	21	17.83
42	25	26	25.5	25.5
43	27.5	28	25	22.83
45	17.5	17.5	18	17.66
48	18	19	18	18.33

*Isolate #35 identified as *Bacillus pumilus* NAIMCC-B-01415 has shown maximum cellulose degradation activity; # : R1,R2,R3 denote different replicates of the experiment

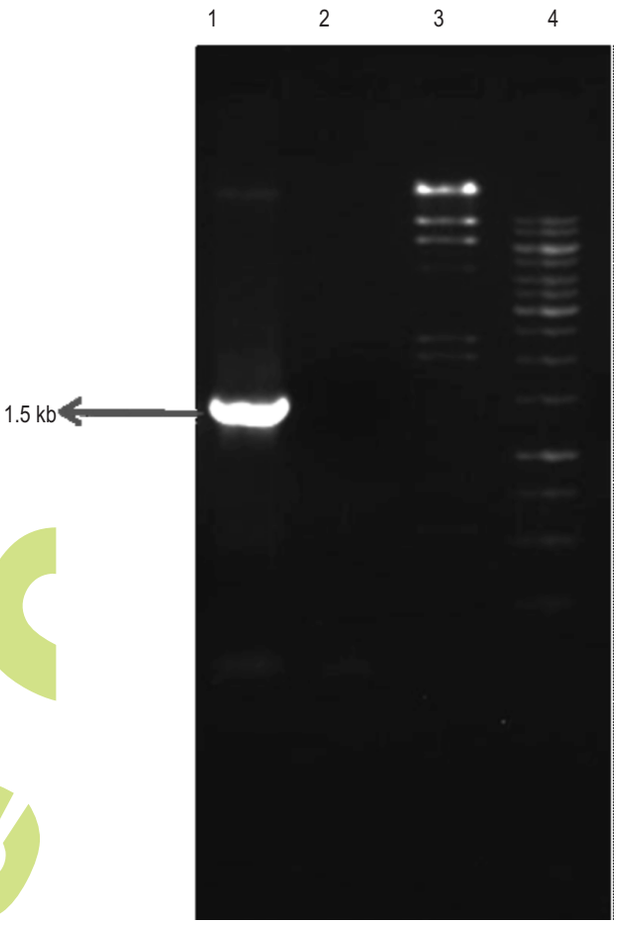

Fig. 1 : Amplification of 16s rDNA fragment of *B. pumilus* NAIMCC-B-01415. 1- 1.5kb 16s rDNA fragment of *B. pumilus* NAIMCC-B-01415, 2- negative control, 4- Lambda Hind III molecular size marker of DNA, 5- 1kb molecular size marker of DNA.

Bacillus pumilus is a gram-positive bacterium which has gained importance only in the recent past due to production of its antimicrobial or surfactant compounds and enzymes as lipase (Fritze, 1993). *Bacillus pumilus* strains GB34 and strain QST 2808 have been registered with US Environmental Protection Agency as a biopesticide and are being marketed under different names for controling various foliar and soil-borne diseases (Fravel, 2005). Studies have also shown that *Bacillus pumilus* MTCC7615, an isolate from rice field as antagonistic against *Rhizoctonia solani* under *in vitro* conditions (Padaria and Singh, 2009; Padaria *et al.,* 2008) and *B. pumilus* NAIMCC-B-01415 isolated in the present study is a cellulose degrading microbe.

The isolated *B. pumilus* NAIMCC-B-01415 was wild type and found to be sensitive to all the antibiotics used whereas E. coli S17 strain was found sensitive to different concentrations of chloramphenicol, ampicilin, rifampicin, tetracyclin, streptomycin and gentamycin and resistant to different concentrations of kanamycin and neomycin (Table2).

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Table 2 : Intrinsic level of antibiotic sensitivity of *B. pumilus* NAIMCC-B-01415 and *E. coli* S17

Antibiotic	Concentration (µg disc^{-1})	Growth of microbes	
		B. pumilus NAIMCC -B-01415	E coli S17
Streptomycin	10,25,50	-	-
Gentamycin	10,30,50,	-	-
Ampicilin	2,10,25	-	-
Chloramphenicol	10,25,30,50	-	-
Neomycin	30	-	+
Nalidixic acid	30	-	-
Rifampicin	2,5,15,30	-	-
Kanamycin	5,30	-	+
Tetracyclin	10,30	-	-

Antibiotic sensitivity test with isolate identified as *B. pumilus* NAIMCC-B-01415 and with *E. coli* S17 (pSUP5011 :: Tn5) was essential to plan the conjugational mating experiment for Tn5 mutagenesis. Since *B. pumilus* NAIMCC-B-01415 did not carry any distinct antibiotic resistant marker, rifampicin resistance was introduced in the isolate so as to follow the transfer of transposon (Tn5) from *E. coli* S17 to *B. pumilus* NAIMCC-B-01415. The 79 rifampicin resistant colonies obtained, were purified by re-streaking and the rifampicin resistant *Bacillus pumilus* NAIMCC-B-01415 culture, taken up for further studies was named as *B. pumilus* NAIMCC-B-01415r.

Transposon Tn5 induced mutants of *B. pumilus* NAIMCC-B-01415r were isolated. *E. coli* S17 (pSUP5011::Tn5) with neomycin resistance was used as Tn5 donor. A total of 365 mutants were isolated based on the resistance to rifampicin and neomycin. Out of 365 mutants, two mutants were characterized to lack the property of *in vitro* cellulose degradation. Thus, the frequency of obtaining cellulose degrading mutant was 0.5%.

Random Tn5 mutagenesis approach has been effectively used in microbes for identifying and studying the function of genes involved in various metabolic pathways. Inactivation of genes occurs due to the insertion of transposable element and the mutants isolated can then be screened for loss of function (Neeraj *et al.* 2010). The frequency for getting a mutant of a desired character is generally low and comparable to spontaneous mutation rates. Low frequency is attributed to reasons such as tight regulation, inefficient transcription and translation signalling, or due to intrinsic inefficiencies in the transposition process itself. *B. pumilus* NAIMCC-B-01415r Tn5, mutant for cellulose degradation, used for further studies, was named as *B. pumilus* NAIMCC-B-01415::TL5 (Fig. 2).

PCR studies confirmed the presence of Tn5 insertion in genomic DNA of cellulose degradation defective Tn5 mutant *Bacillus pumilus* NAIMCC-B-01415:: TL5. An expected amplicon of 1.2kb was obtained with neomycin (Tn5) specific primers whereas no amplicon was obtained when genomic DNA of *Bacillus pumilus* NAIMCC-B-01415 was the template in the same reaction (Fig. 3).

B. pumilus NAIMCC-B-01415 TL5 was taken up for further molecular analysis to identify the gene having role in cellulose degrading ability. Inverse PCR reaction using the genomic DNA of *B. pumilus* NAIMCC-B-01415 TL5 yielded an amplicon of ~600 bp (Fig. 4). The amplicon was sequenced and nucleotide BLAST analysis of the obtained sequence data showed homology with cysteine regulatory protein (cys B) gene. The partial gene sequence was submitted in the NCBI (National Centre for Biotechnology Information, USA) genebank with Accession number: JQ257500.

Elucidating the mechanisms of cellulase gene expression in various cellulose producing microbes has been a subject of

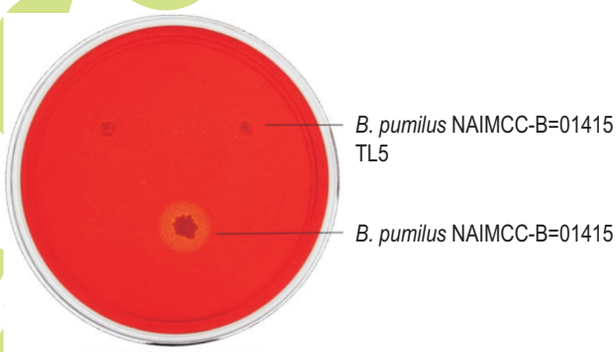

Fig. 2 : Loss of cellulose degrading property of Tn5 mutant *B. pumilus* NAIMCC-B-01415 TL5 as compared to wild type *B. pumilus* NAIMCC-B-01415

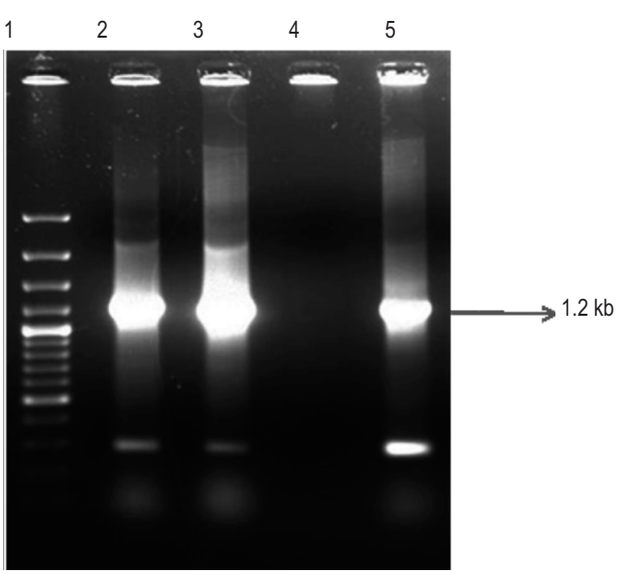

Fig. 3 : PCR amplification of 1.2kb neomycin gene of Tn5. 1-100bp DNA ladder; 2-*B. pumilus* NAIMCC-B-01415 TL5, 3- plasmid DNA of *E.coli* S17 as template (positive control, 4-*B. pumilus* NAIMCC-B-01415r as template (negative control)

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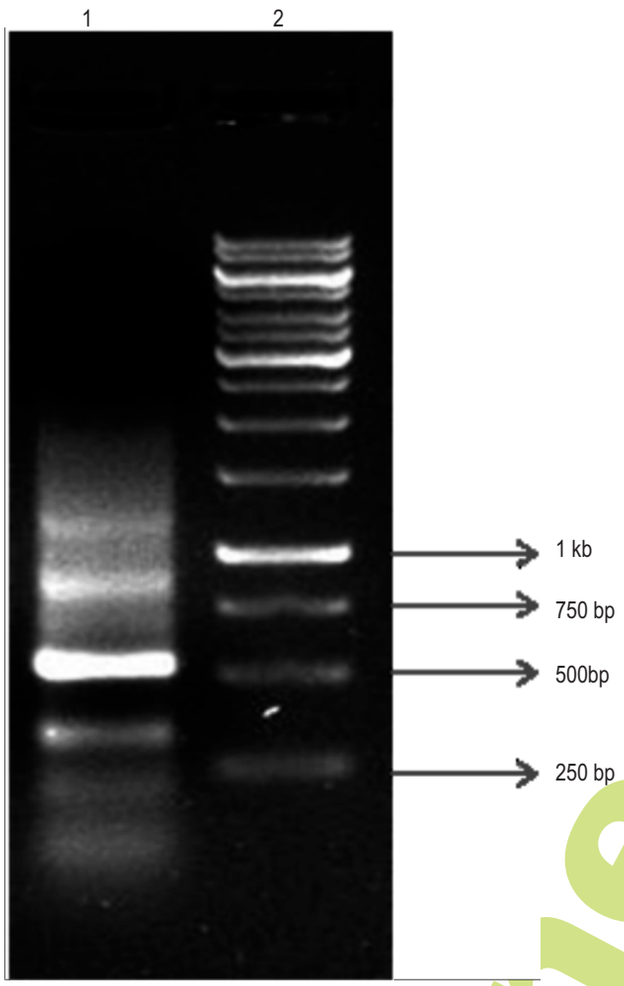

Fig. 4 : Inverse PCR of restriction digested and sef-ligated gDNA of *B. pumilus* NAIMCC-B-01415 TL5. 1- IPCR product; 2- 1kb DNA ladder

extensive research, however, still little is known about how the cellulose signal from outside the cell is transduced to initiate production of appropriate hydrolytic enzymes (Gilberg and Hazlewood, 1993, Bayer *et al.,* 1998, Lee *et al*., 2011). Molecular studies with *B. pumilus* NAIMCC-B-01415 showed involvement of a Cys B gene in cellulose degradation. CysB is known to be a transcriptional regulator that acts as an activator of transcription for synthesis of L-cysteine from inorganic sulfate and in L-cysteine transport. Purified CysB protein has been shown *in vitro* to bind to sequences immediately upstream of the -35 regions of *S.typhimurium* CysJIH and cysK promoters and to activate transcription from these promoters in the presence of acetyl-L-serine (Monroe *et al.,*1990). CysB is also involved in sulfur metabolism (Iwanicka-Nowicka and Hryniewicz ,1995).

Cellulase gene expression is influenced by both quality and quantity of the available sulphur source as observed in *Hypocrea jecorina*, a cellulase producing fungi. Regulation of uptake and sulphur metabolism in *H. jecorina* is linked with cellulase transcription. A putative E3 ubiquitin ligase, homologues of which are involved in regulation of sulphur metabolism was found in *H. jecorina* to bind to regulatory sequence of cellulase gene cbh2 promotor indicating that the presence of sulphur has a specific function in regulation of cellulase gene expression (Gremel *et al.,* 2008) . The results of the present study which clearly proves involvement of cysB gene in cellulose degradation in the microbial isolate B. pumilus NAIMCC-B-01415, falls in line with the hypothesis that sulphur metabolism and cellulase gene expression could be interconnected processes. Insertion mutantion in cysB led to the isolate *B. pumilus* NAIMCC-B-01415 becoming a mutant for cellulose degradation further confirmed the role of cysB in cellulose degradation

In order to assess the influence of cystein in the degradation of CMC, two sets of plates were prepared one having cystein and other without cystein. After 4 days of incubation, the mutant isolate *Bacillus pumilus*TL5 resulted in decomposition of CMC in the plate supplemented with cystein by exhibiting a halo zone around, while for the plate without cystein no halo zone appeared i.e. no degradation of cellulose was observed. Thus the role of cystein in cellulose degradation in *B. pumilus* NAIMCC-B-01415 was confirmed.

The present study isolated cellulose degrading *Bacillus pumilus* and further showed the involvement of a cysteine regulatory gene in cellulose degradation metabolism of *Bacillus pumilus*. Further studies on isolation and characterization of various genes involved in cellulase enzyme production shall unravel the complexity of this process so as to manipulate it for the benefit of mankind.

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