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Molecular Characterization of Cellulolytic Bacteria Derived From Termite Gut and Optimization of Cellulase Production

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A B S T R A C T

Cellulolytic bacteria were previously isolated from the termite guts and efforts were taken to optimize parameters for cellulase production. Activities of diversity of enzymes which include, CMCase, avicelase and filterpaperase (FPase) were studied from crude cellulase extracts. CMCase activity was found optimum at different temperatures (35°C, 40°C and 45°C) at pH 7 and after 48 hrs of incubation time. Avicelase and FPase activities were found optimum at 35°C, pH 7 and after 48 hours of incubation. Results clearly indicated that there is a great impact of temperature, pH and incubation period on cellulase production. Isolate C3 and Z were found highest CMCase and FPase producers respectively. 16s rRNA gene analysis confirmed that, isolates belong to different species of *Pseudomonas, Klebsiella, Salmonella, Serratia and Enterobacter*. Molecular characterization using REP, ERIC and BOX sequences showed high-moderate level of diversity among these isolates. Growth profiles of isolates showed that, they can grow best between temperatures 30°C-40°C at pH 7.

Introduction

Lignocellulose, the major component of plant cell walls, is the most abundant and sustainable biomass on the earth, and is recognized for its potential for renewable energy production (Arakawa 2008, Scharf 2009). It is a general term which refers to a structure made up of three dominant compounds cellulose, hemicellulose and lignin. For the degradation of lignocellulose, diversity of enzymes is required which can be classified as, endo-β-1, 4-glucanase (CMCase or endoglucanase), exo-β-1, 4-glucanase (exocellulase, avicelase) and β-glucosidase (cellobiase). All of the three enzymes are involved in breaking the complex polymer into fermentable sugars. Endo-β-1, 4-glucanases are needed to break glucosidic bonds for creating free chain ends whereas, exoglucanase are required for the degradation of the same molecule by removing cellobiose units remaining at the free chain ends and β-glucosidase which hydrolyzes cellobiose into simple sugars. Insects have evolved very effective strategies to use lignocellulosic substrates as a source of energy (Willis *et al.*, 2010). This makes them an optimal source for prospect of novel cellulolytic enzymes. Termites are one of the most lignocellulose digesting insects and can act as one of the best source for cellulolytic systems as microbial

communities inside the gut are known to produce cellulase. Under optimal conditions, cellulase can convert complex polymers (cellulose) in to simple sugars (glucose).Cellulose has attracted worldwide attention as a renewable resource that can be converted into bio based products and energy (Li *et al.,* 2009). One of the limiting steps in the biomass-to-ethanol process is the degradation of cellulose to fermentable sugars (saccharification). This currently relies on the use of bacterial and/or fungal cellulases, which tend to have low activity under biorefinery conditions and are easily inhibited (Fischer *et al.,* 2013).Application of these enzymes can serve as source for lignocellulose degradation not only in industries but also at field level for decomposing lignocellulosic biomass which remains in the field after harvesting. Keeping these facts in mind, we carried out optimization of the the parameters for cellulase production and identification of these microbes at molecular level.

Materials and Methods

The bacteria used in this study were previously isolated from the gut of termites which were collected from different geographical locations of Chhattisgarh, India (Shinde *et al.,* 2017). Out of 33 isolates, 16 isolates (B1, B2, B3, C1, C2, C3, M1, A1, Z1, L, R, N, Z, B1 plate, B and C) were found potential cellulase producers by forming halo zoneson the media supplemented with CMC as a sole carbon source. The pure colonies of the isolates were maintained in NAM slants at 16°C for further studies.

Preparation of crude enzyme

Bacterial isolates were inoculated in conical flasks containing sterilized nutrient broth (25 ml) and incubated at 37°C for 48 hours. After 48 hours, bacterial cultures were centrifuged at 8000 rpm for 10 minutes. After centrifugation, supernatant was collected as a source of crude enzyme and stored at 4°C for enzyme assays.

Cellulase activity assay

Enzyme activity of crude cellulase was estimated with the help of different commercially available chemical substrates, such as carboxymethyl cellulose (CMC) avicel and filter paper. The amount of the reducing sugars released was determined according to the DNS method (Miller *et al.,* 1959) with some modifications. The amount of reducing sugar was calculated from a previous established standard curve using D-glucose as a standard. Carboxy-methylcellulase (CMCase) activity was expressed in terms of units. One unit is the amount of enzyme releasing 1 µmol of reducing sugar from carboxymethyl celluloseper ml per min. One unit of Avicelase activity was defined as the amount of enzyme released 1 µmol of reducing sugars from avicel per ml per min. Filterpaperase (FPase) activity was determined by using a method described by wood and Bhat (1988) with some modifications. One unit is the amount of enzyme in the culture filtrate releasing l µmol of reducing sugars from filter paper per min. Filter paperase (FPase) is expressed in terms of filter paper units (FPU).

Optimization of different parameters for determining maximum cellulase activity

Different parameters like pH, temperature, and incubation period were optimized for carboxymethylcellulase (CMCase), avicelase (exoglucanase) and filterpaperase (FPase) production. For the optimization of temperature, experiment was carried out at different temperatures (30°C, 35°C, 40°C, 45°C & 50°C). To study the effect of pH on cellulase enzyme activity, different buffers

such as 50mM sodium citrate (pH 5-6), 50mM potassium phosphate (pH 7), 50mMTrisHcl (pH 8)were prepared. To 0.5 ml of crude enzyme, 0.5 ml of 1% CMC, 0.5 ml of 1% avicel, Whatman filter paper strip (1 x 6 cm) in the above buffers was added. To determine optimum incubation period, bacterial cultures growing in nutrient broth were taken out at the different time intervals and the enzyme activity was determined.

DNA extraction

For the DNA extraction, bacterial isolates were inoculated in nutrient broth and incubated for 48 hrs. DNA was isolated by using Ultraclean® Microbial DNA isolation kit, *MO-BIO* Laboratories.

Identification of bacterial isolates on the basis of 16s rRNA gene amplification

The 16s rRNA gene was amplified by using a set of universal primers according to Khianngam *et al.*, 2014: 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3'). PCR was carried out in 10 µl reaction mixture containing 1X assay buffer (1mM) Tris-HCl at pH 9.0, 50mM KCl, 2.5mM MgCl2, 0.1mM each dNTP mix, 1µM both forward and reverse primers, 50-60ng of template DNA and 0.4 U Taq DNA polymerase (Axygen) in BIORAD T100^{TM}Thermocycler according to following temperature profiles: 95°C for 5 min, 30 cycles of 30 seconds at 95°C, 55°C for 1 min, 72°C for 1 min and final elongation at 72°C for 7 min.

ERIC-BOX-REP PCR based genotypic analysis

ERIC, REP, BOX primer sequences were used in PCR to detect differences in the number and distribution of these bacterial repetitive sequences in the bacterial genome. Primers sequences and temperature profiles used in the study are shown in Table 1and Table 2.

Bioinformatics analysis and phylogenetic tree construction

The purified products were sent for sequencing to DNA sequencing facility. The data obtained after sequencing was compared with the online data base at GenBank using BLAST-N search program in NCBI. (http://www.ncbi.nlm.nih.gov). The sequences were aligned and phylogenetic tree was constructed using software MEGA 6.0 with neighbor-joining method at 1000x bootstraps (Tamura *et al.*, 2011).

Statistical analysis

All the experiments were arranged in completely randomized block design with two replications in each treatment. Specific PCR amplification products (ERIC, BOX and REP) were scored as present (1) or absent (0) depending on decreasing order of their molecular weights of DNA sample. The presence or absence of bands was converted into binary data (1 for presence and 0 for absence of each band) and similarity matrices were calculated using NTSYS (Numerical Taxonomy System Biostatistics) computer program on binary data of selected groups of primers detailed. Cluster analysis was done within SAHN program by using UPGMA (unweighted pair-group method with arithmetic averages).

Effects of temperature and pH on growth of bacteria

To study the effect of pH on the growth of bacteria, isolates were inoculated in the test tubes containing nutrient broth (pH 6, pH 7, pH 8) and kept for two days. To study the effect of temperature on the growth of

bacteria, isolates were inoculated in the test tubes containing nutrient broth and the tubes were kept at various temperatures, 30°C, 35°C, 40°C and 45°C. The growth of bacteria was determined spectrophotometrically by taking the O.D at 600 nm.

Results and Discussion

Effect of temperature, pH and incubation period on CMCase activity

All the sixteen potential cellulose degrading bacteria were studied for optimization of parameters for optimum CMCase production. The enzyme activity of potential isolates at different parameters is shown in Table 3. Bacterial isolate C3 showed maximum CMCase activity at 35°C whereas, other isolates B1, B3 and C1 showed maximum enzyme activity at 40°C. Maximum activity for B2 isolate was found at 45°C. Other isolates showed efficient activity at 35°C (Fig.1). Decrease in the temperature below 35°C and increase in the temperature above 45°C resulted in low enzyme activity. The data obtained clearly suggests that bacterial cellulase has an optimum temperature between 35°C and 45°C. The data is accordance with the previous studies. Khatiwada *et al.,* 2016 reported maximum CMCase production by *Bacillus* and *pseudomonas species* at 37°C and *Serratia species* at 35°C. Haripriya *et al.,* 2017, Vimal *et al.,* 2016 obtained maximum CMCase activity at 40°C. Like temperature, pH is also an important factor that affects the enzyme yield. All the isolates showed maximum CMCase activity at pH 7. pH below or above 7 resulted in low enzyme activity (Fig.2). The data obtained clearly indicates that bacterial CMCase has optima pH 7. The results obtained are similar with the previous studies. Sharma *et al.,* 2015 found optimum pH at 7 for endoglucanase activity by *Bacillus sp*. isolated from termite. Shaikh *et al.,* 2013 obtained maximum CMCase activity at pH 7 and 7.5 by *Pseudomonas* and *Bacillus sp.* Similarly, Effect of incubation time showed variations in cellulase production. The optimum time for cellulase production with CMCase was observed at 48 hrs of incubation period for all the isolates. The cellulase activity was significantly reduced after 48 hours of incubation (Fig.3).The results of the study are in accordance with the previous studies. Vimal *et al.,* 2016 reported maximum cellulase production after 48 hrs by *Bacillus sp*. Khatiwada *et al.,* 2016 obtained maximum cellulase production after 24 hrs and 48 hrs of incubation time for *Bacillus, Serratia* and *pseudomonas sp.* From our study it was found that, maximum CMCase activity was observed at temperature between 35°C to 45°C, at pH 7 and after 48 hrs of incubation period.

Effect of temperature, pH and incubation period on avicelase activity

All the sixteen potential cellulose degrading bacteria were studied for optimization of parameters for optimum avicelase production. The enzyme activity of potential isolate at different parameters is shown in Table 4.Avicelase activity was studied between 30°C to 45°C. The data obtained showed that, all isolates have maximum avicelase activity at 35°C (Fig.4). Among all isolates, C3 isolate showed maximum enzyme activity. Avicelase activity was found low at 30°C, 40°C and 45°C as compared to 35°C. Increase or decrease in the temperature resulted in low enzyme yield. The results are accordance with the previous studies. A study conducted on isolation, production and optimization of avicelase enzyme from sawdust showed the optimum temperature for avicelase activity was at 37°C and 50°C (Fauzi *et al.,* 2013). *Rani et al.,* 2015 stated that, the optimum temperature for avicelase activity ranges between 25°C to 50°C for bacteria, fungi and

actinomycetes. Likewise, Effect of pH on avicelase activity indicated that all isolates have maximum enzyme activity at pH 7 (Fig. 5).Among all isolates, isolate C3 showed highest enzyme activity. Enzyme activity was low after increase and decrease in the pH. The results of the study are similar with the previous studies. A study conducted on isolation, production and optimization of avicelase enzyme from sawdust showed the optimum pH for avicelase activity was 7 (Fauzi *et al.,* 2013). Rani *et al.,* 2015 stated that, the optimum pH for avicelase activity ranges between 4.4 to 8 for bacteria, fungi and actinomycetes.

Incubation period for optimum avicelase production was also studied and it was found that, bacterial avicelase has maximum activity after 48 hrs of incubation time (Fig. 6). Enzyme activity was gradually decreased after 48 hrs of incubation time. Rani *et al.,* 2015 stated that, bacterial species like *Bacillus subtilis*, *Geobacillus stearothermophilus* has maximum avicelase production after 48 hrs of incubation time. From our study it was found that, maximum avicelase activity was observed at 35°C at pH 7 and after 48 hrs of incubation period.

Effect of temperature, pH and incubation period on filterpaperase (FPase) activity

All the sixteen potential cellulose degrading bacteria were studied for optimization of temperature for maximum FPase activity. The enzyme activity of the most potential isolate at different parameters is shown in Table 5. All isolates showed maximum FPase activity at 35°C. Among all isolates, maximum FPase activity was found at 35°C for bacterial isolate Z as compared to other isolates. FPase activity was decreased after increase in the temperature. Thus, results clearly indicate that bacterial FPase has an optimum temperature at 35°C(Fig. 7).The results of this study are similar with previously reported studies. Swaroopa Rani *et al.,* 2004 optimized fermentation conditions for filterpaperase and found that activity was maximum at 35°C. Cultivation at different temperatures and in presence of various carbon sources revealed that all the three strains produced more amounts of endoglucanase, β-glucosidase and filter-paperase activities at 35°C (Sohail *et al.,* 2014). In case of pH, maximum enzyme activity was found at 7 for all isolates. Among all isolates, highest enzyme activity was shown by Z isolate. FPase activity at pH 5, pH 6 and pH 8 was found low as compared with pH 7. pH below and above 7 resulted in low enzyme activity (Fig. 8). The data obtained clearly showed that, bacterial FPase has optima of pH 7. Swaroopa Rani *et al.,* 2004 optimized fermentation conditions for filterpaperase and found that activity was maximum at pH 7. However, optimization and characterization of cellulolytic enzymes produced from *Gliocladiumroseum* showed FPase has an optimum pH of 7 (Salem *et al.,* 2015).The effect of incubation period was determined and it showed variations in FPaseproduction. All the isolates showed maximum FPase enzyme production after 48 hrs of incubation period. Highest enzyme activity was shown by Z isolate as compared to others. Enzyme activity was reduced after 48 hours of incubation period (Fig. 9).The data obtained showed that bacterial FPase has optimum incubation time after 48 hrs. The results of the study are found similar with previous studies. Swaroopa Rani *et al.,* 2004 obtained highest FPase activity after 48 hrs on incubation time from *Clostridium papyrosolvens* CFR-703. Maximum FPase activity was recorded on the 2nd day of incubation irrespective of carbon sources used, and activity was gradually decreased with the incubation time increases (Yadav *et al.,* 2017).

Molecular identification of potential cellulolytic isolates

The amplification with 16s rRNA gene primers showed 1300 bpamplicons (Fig. 10).The 16s rRNA gene sequences thus generated were helpful in identifying the isolates at species level. The summary of the 16s rRNA gene sequences are given inTable6. The results 16s rRNA gene analysis in the present study has confirmed that the isolates belong to different strains like, *Pseudomonas, Klebsiella, Salmonella, Serratia and Enterobacter.* All the sequences were aligned and phylogenetic tree for the isolate B2 was created using Neighbor-Joining method with the help of software package Mega version 6 (Fig. 11).

Researchers have identified different types of cellulolytic bacteria from the guts of termite on the basis of 16s rRNA gene analysis.

Table.1 Sequences of primers used for characterization of bacterial isolates

Primer	Sequence
REP F	5'TCGICTTATCTGGCCTAC3'
REP R	5'TTTTCGTCGTCATCTGGC3'
BOXAIR	5'CTACGGCAAGGCCGACGCTGACG3'
ERIC F	5'AAGTAAGTGACTGGGGTGAGCG3'
ERIC R	5'TGTAAGCTCCTGGGGATTCAC3'

Table.2 Thermal profile for amplification of different pairs

Primer pair (REP)				
Steps	**Activity**	**Temperature (°C)**	**Time (min)**	**Repeats**
1.	Initial denaturation	94°C	3 minute	1
2.	Final denaturation	94°C	45 seconds	
3.	Annealing	38°C	1 minute	45 cycles
4.	Extension	72°C	1 minute	
5.	Final extension	72°C	8 minute	-
6.	storage	4°C	-	-
Primer pair (BOXAIR)				
Steps	**Activity**	**Temperature (°C)**	**Time (min)**	**Repeats**
1.	Initial denaturation	94°C	3 min.	1
2.	Final denaturation	94°C	45 seconds	
3.	Annealing	53°C	1 minute	
4.	Extension	72°C	1 minute	45 cycles
5.	Final extension	72°C	8 minute	
6.	Storage	4°C	-	
Primer pair (ERIC)				
Steps	**Activity**	**Temperature (°C)**	**Time (min)**	**Repeats**
1.	Initial denaturation	94°C	3 min.	1
2.	Final denaturation	94°C	45 seconds	
3.	Annealing	53°C	1 minute	
4.	Extension	72°C	1 minute	45 cycles
5.	Final extension	72°C	8 minute	
6.	Storage	4°C	-	

Table.3 Optimization of parameters for CMCase production

		Enzyme activity of potential isolates				
Different parameters	**Different values**	**C3 isolate (IU/ml)**	**B1 isolate (IU/ml)**	**B3 isolate (IU/ml)**	**C1 isolate (IU/ml)**	**B2 isolate (IU/ml)**
Temperature	30°C	0.20±0.01	0.09±0.01	0.11±0.00	0.12±0.00	0.12±0.01
	35°C	**0.41±0.008**	0.21±0.001	0.20±0.009	0.22±0.004	0.18±0.007
	40°C	0.36±0.003	**0.23±0.003**	**0.22±0.002**	**0.25±0.007**	0.19±0.007
	45°C	0.29±0.011	0.13±0.003	0.17±0.007	0.12±0.007	**0.21±0.002**
	50°C	0.13±0.003	0.10±0.011	0.12±0.001	0.12±0.003	0.13±0.004
pH	5	0.20±0.013	0.09±0.015	0.11±0.001	0.12±0.006	0.12±0.014
	6	0.29±0.005	0.13±0.003	0.13±0.001	0.14±0.003	0.13±0.003
	7	**0.41±0.008**	**0.24±0.021**	**0.20±0.009**	**0.33±0.011**	**0.17±0.014**
	8	0.14±0.008	0.06±0.001	0.07±0.003	0.12±0.003	0.07±0.001
Incubation period	6	0.06±0.002	0.05±0.003	0.05±0.003	0.05±0.003	0.06±0.002
	12	0.08±0.003	0.09±0.010	0.08±0.004	0.08±0.005	0.07±0.001
	24	0.16±0.005	0.13±0.001	0.13±0.003	0.14±0.009	0.12±0.003
	48	**0.21±0.003**	**0.20±0.005**	**0.21±0.000**	**0.21±0.006**	**0.21±0.006**
	60	0.16±0.003	0.18±0.004	0.17±0.003	0.18±0.001	0.18±0.007

Values after± represents standard error of two replicates

Table.4 Optimization of parameters for avicelase production

Different parameters	Different values	Enzyme activity of C3 isolate (IU/ml)
Temperature	30°C	0.10±0.001
	35°C	**0.14±0.001**
	40°C	0.12±0.004
	45°C	0.10±0.002
pH	5	0.13±0.001
	6	0.15±0.002
	7	**0.21±0.004**
	8	0.13±0.004
Incubation period	12	0.11±0.003
	24	0.14±0.003
	48	**0.17±0.001**
	60	0.12±0.001

Values after± represents standard error of two replicates

Table.5 Optimization of parameters for filterpaperase (FPase) production

Different parameters	Different values	Enzyme activity of Z isolate (IU/ml)
Temperature	30°C	0.056±0.001
	35°C	**0.15±0.004**
	40°C	0.12±0.005
pH	5	0.10±0.003
	6	0.12±0.002
	7	0.16±0.004
	8	0.12±0.003
Incubation period	12	0.15±0.001
	24	0.16±0.001
	48	**0.19±0.005**
	60	0.17±0.003

Table.6 BLASTn results of cellulolytic isolates

Bacterial isolate	Closest species	Accession No.	% identity (Blastn)
B1	*Pseudomonas putida*	KJ676537.1	92%
B2	*Klebsiella pneumoniae*	KR269873.1	84%
B3	*Serratia species*	KF261222.1	100%
C1	*Klebsiella pneumoniae*	KX010115.1	94%
C2	*Enterobacter cloacae*	EU073021.1	95%
C3	*Klebsiella pneumoniae*	KF974478.1	96%
M1	*Enterobacter cloacae DD266*	KR822277.1	93%
A1	*Pseudomonas plecoglossicida IN88*	KY511070.1	97%
Z1	*Pseudomonas plecoglossicida MHF*	GQ301534.1	95%
L	*Pseudomonas sp. V2M2*	FN794214.1	81%
R	*Pseudomonas putida 1017*	HQ324912.1	91%
N	*Salmonella entrica ST3*	JQ228520.1	92%
Z	*Klebsiella variicola ALK036*	KC456522.1	95%
B1 Plate	*Klebsiella pneumoniae NRC138*	KP313052.1	94%
B	*Enterobacter aerogens BD1*	KM503142.1	86%
C	*Enterobacter aerogens BPRIST043*	JF700492.1	98%

Fig.1 Effect of Temperature on CMCase activity

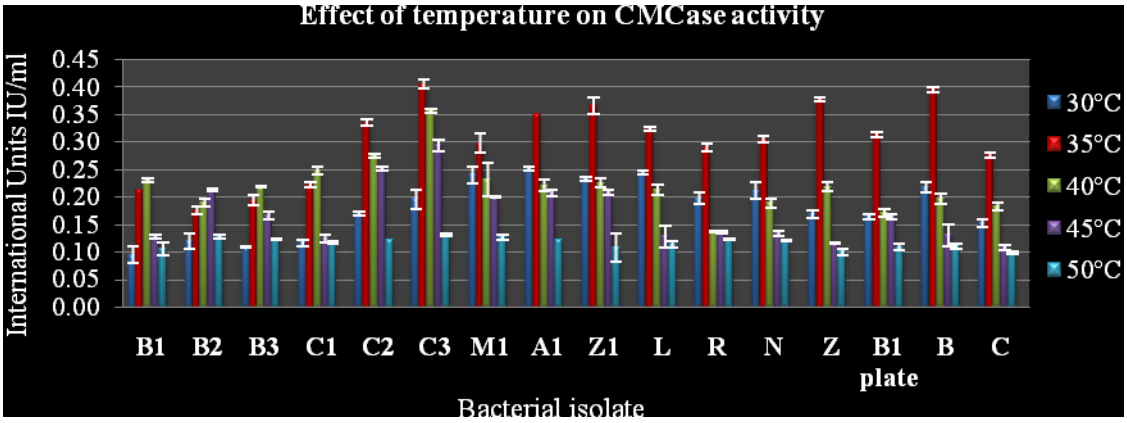


Fig.2 Effect of pH on CMCase activity

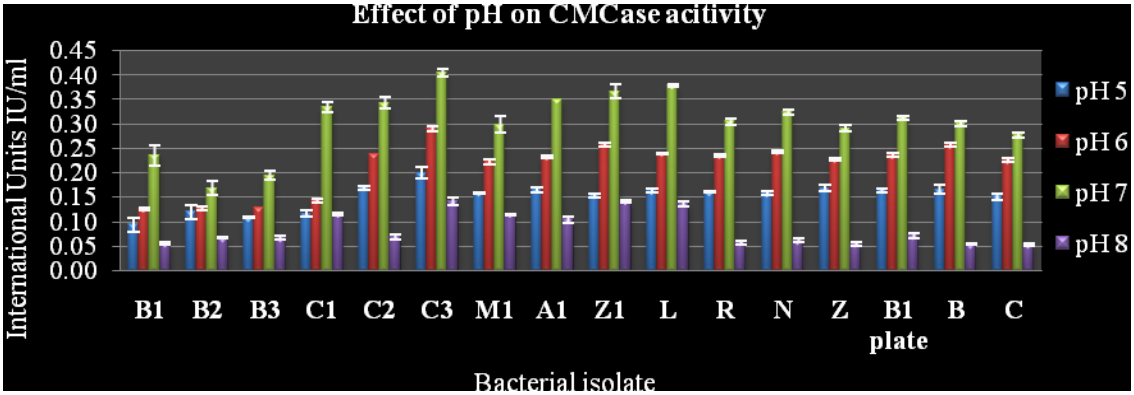


Fig.3 Effect of incubation period on CMCase activity

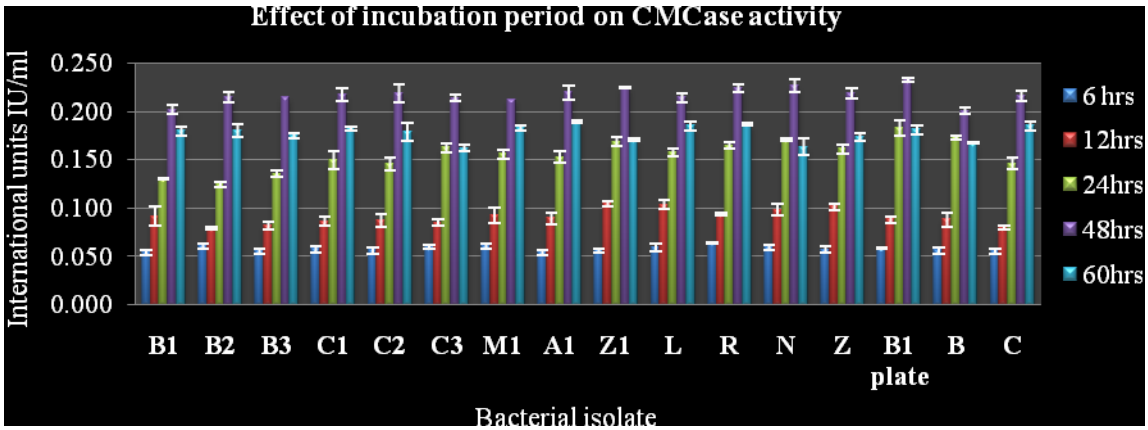


Fig.4 Effect of Temperature on avicelase activity

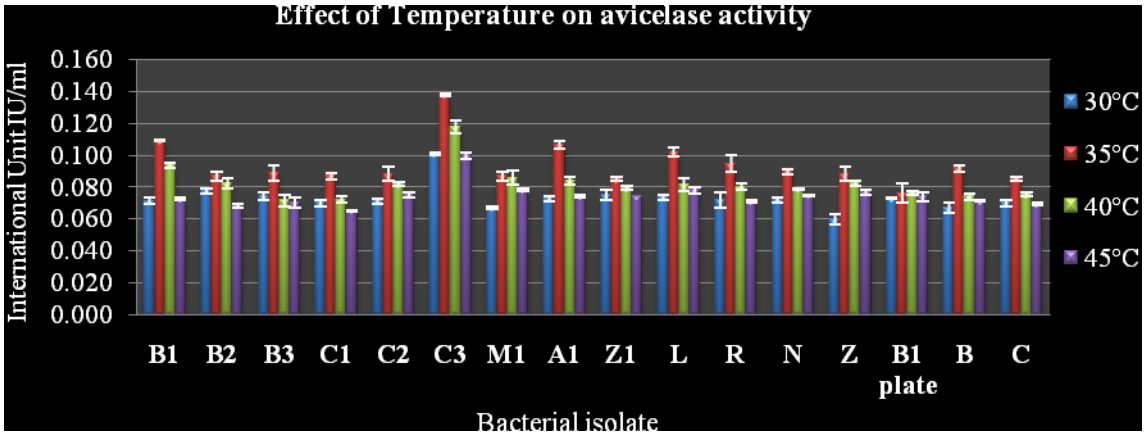


Fig.5 Effect of pH on avicelase activity

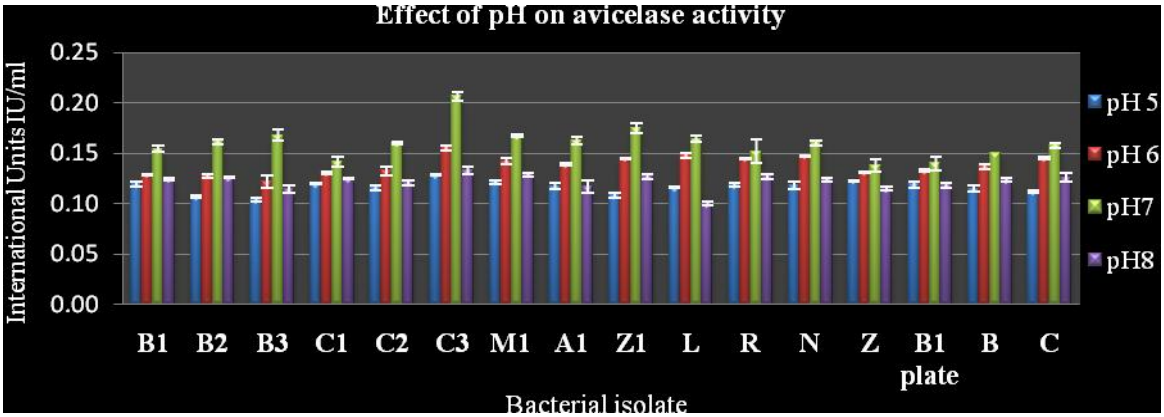


Fig.6 Effect of incubation period on avicelase activity

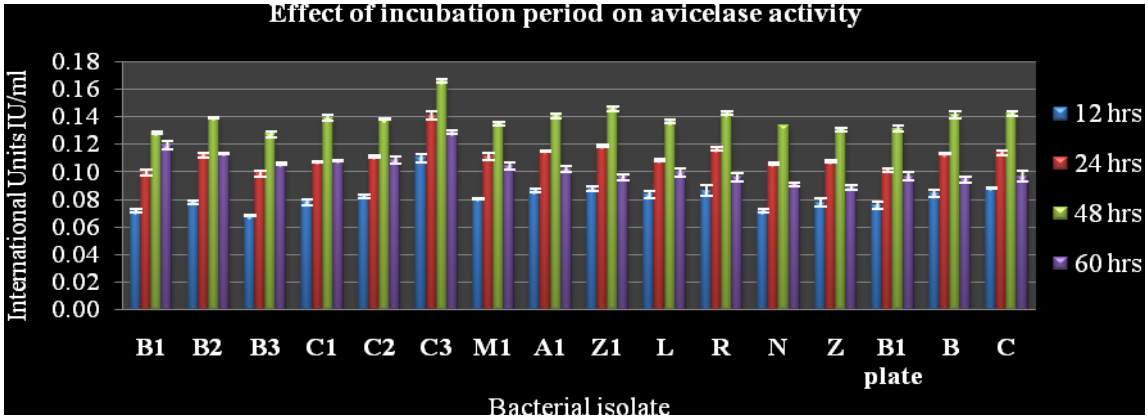


Fig.7 Effect of Temperature on Filterpaperase activity

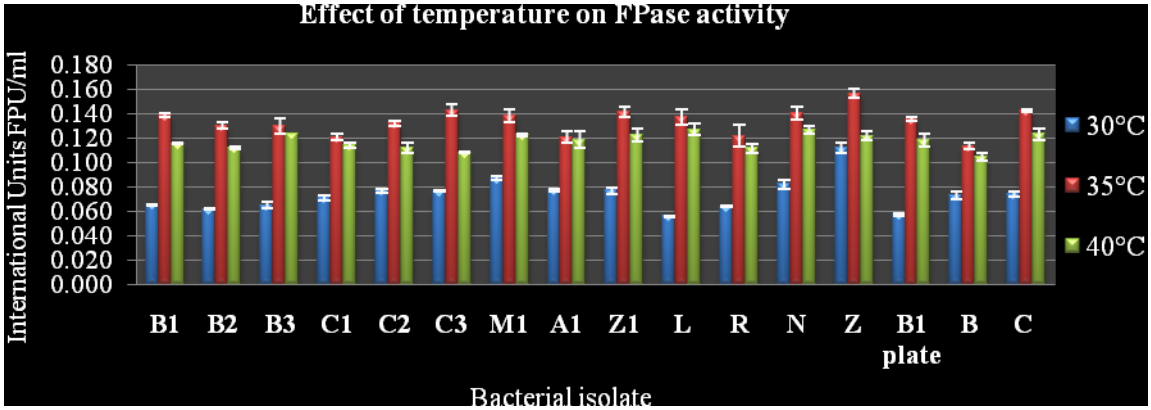


Fig.8 Effect of pH on Filterpaperase activity

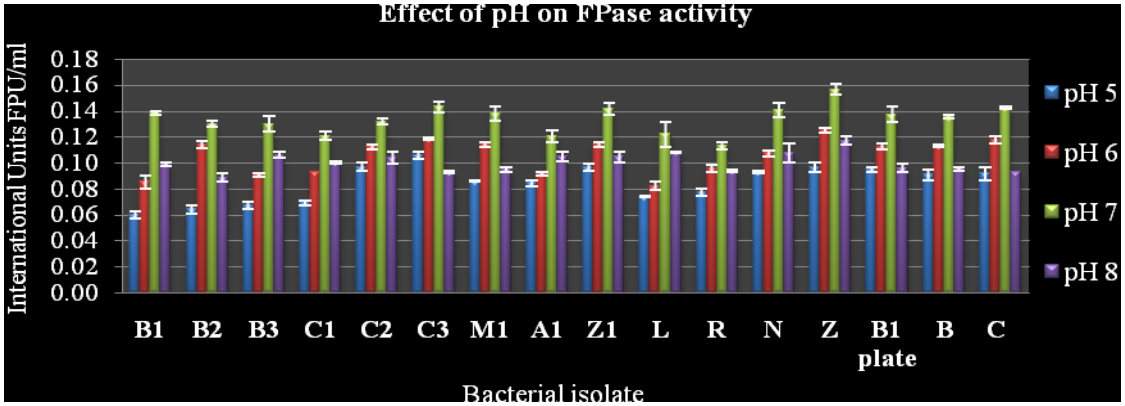


Fig.9 Effect of incubation period on Filterpaperase activity

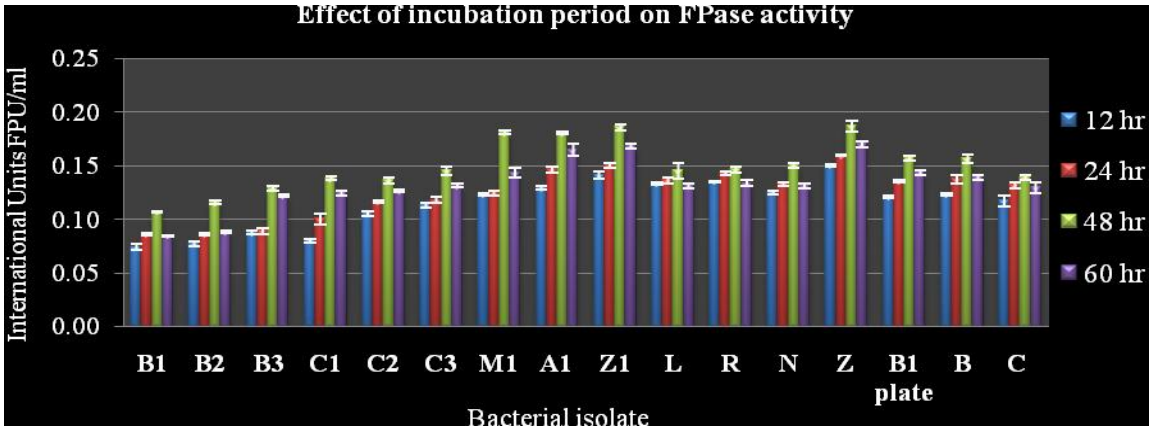
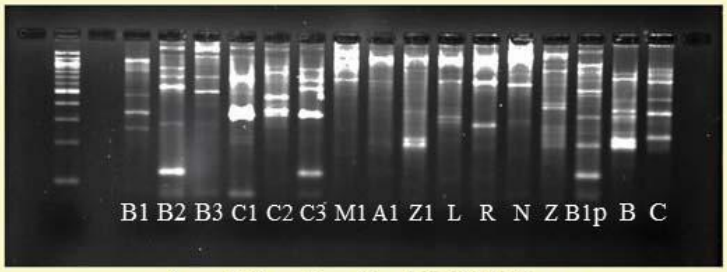
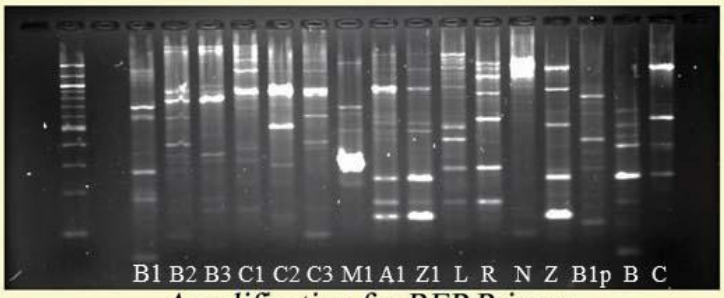


Fig.10 Molecular characterization of potential isolates based on 16s rRNA gene amplification and REP, BOX AND ERIC-PCR genotyping

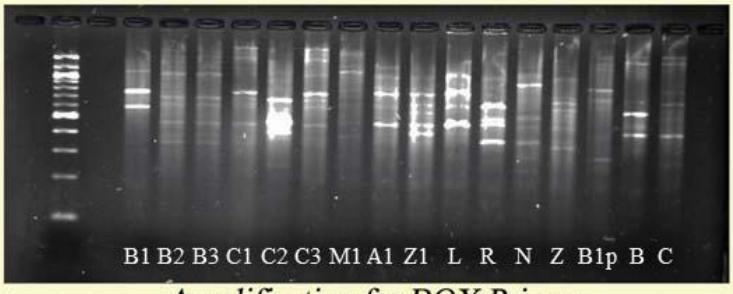
Amplification of 16s rRNA



Amplification for ERIC Primer



Amplification for REP Primer



Amplification for BOX Primer

Fig.11 Neighbor-joining tree of B2 isolate based on 16s rRNA gene sequences showing phylogenetic position of isolates and related species

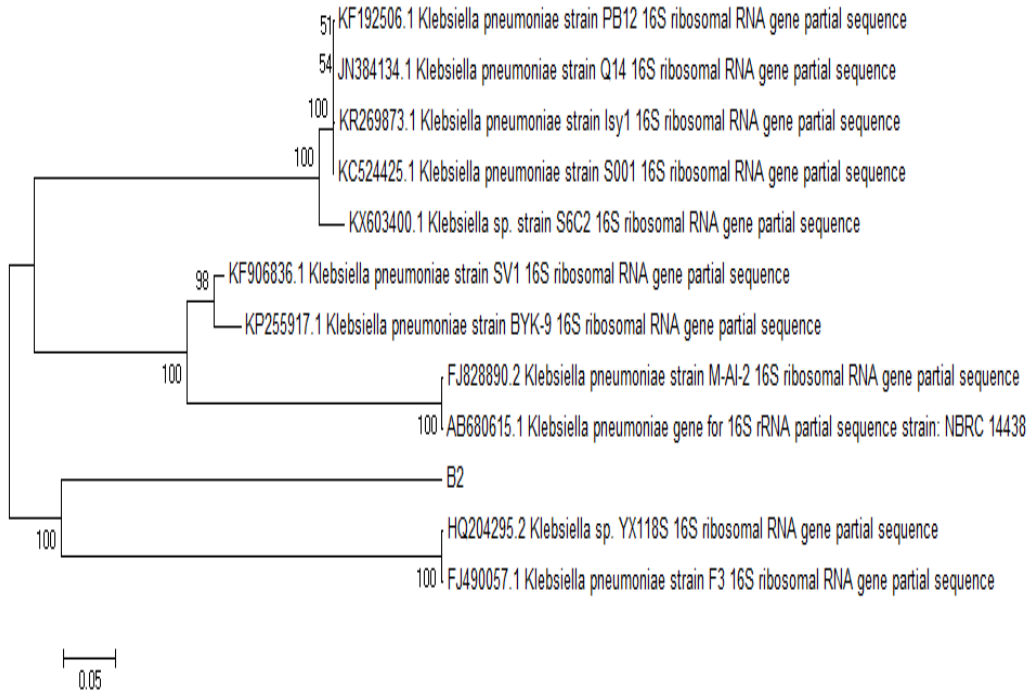


Fig.12 Dendogram of sixteen cellulose degrading isolates generated by binary matrix derived from REP amplicons

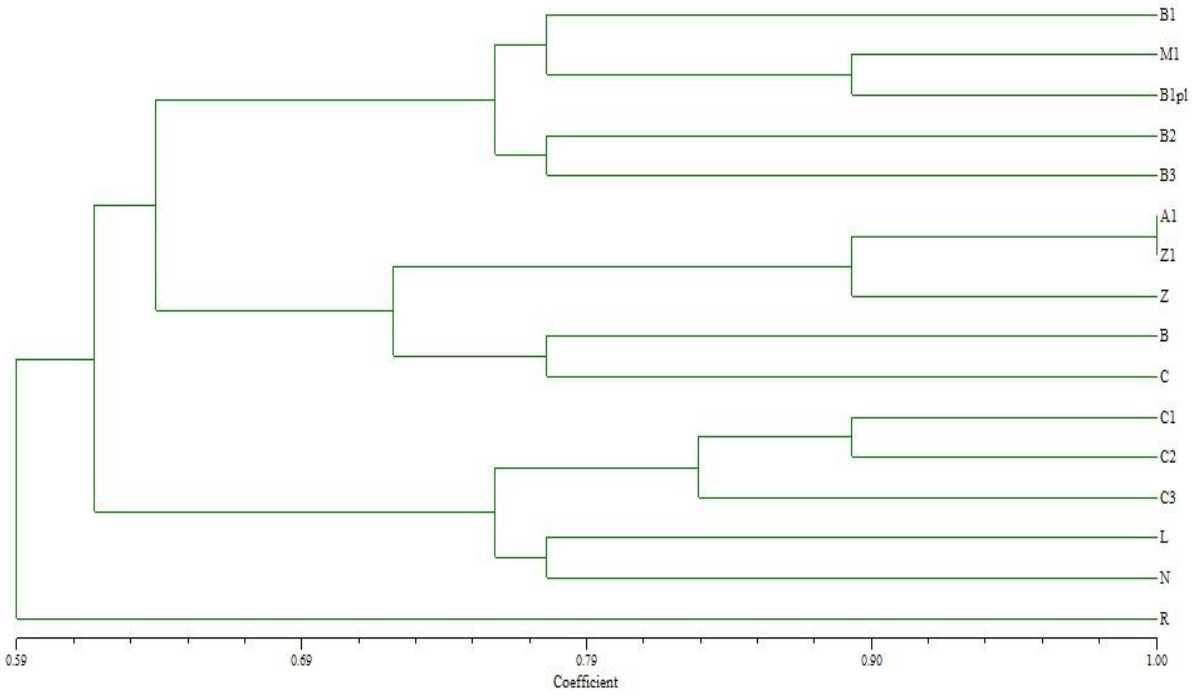


Fig.13 Dendogram of sixteen cellulose degrading isolates generated by binary matrix derived from BOX amplicons

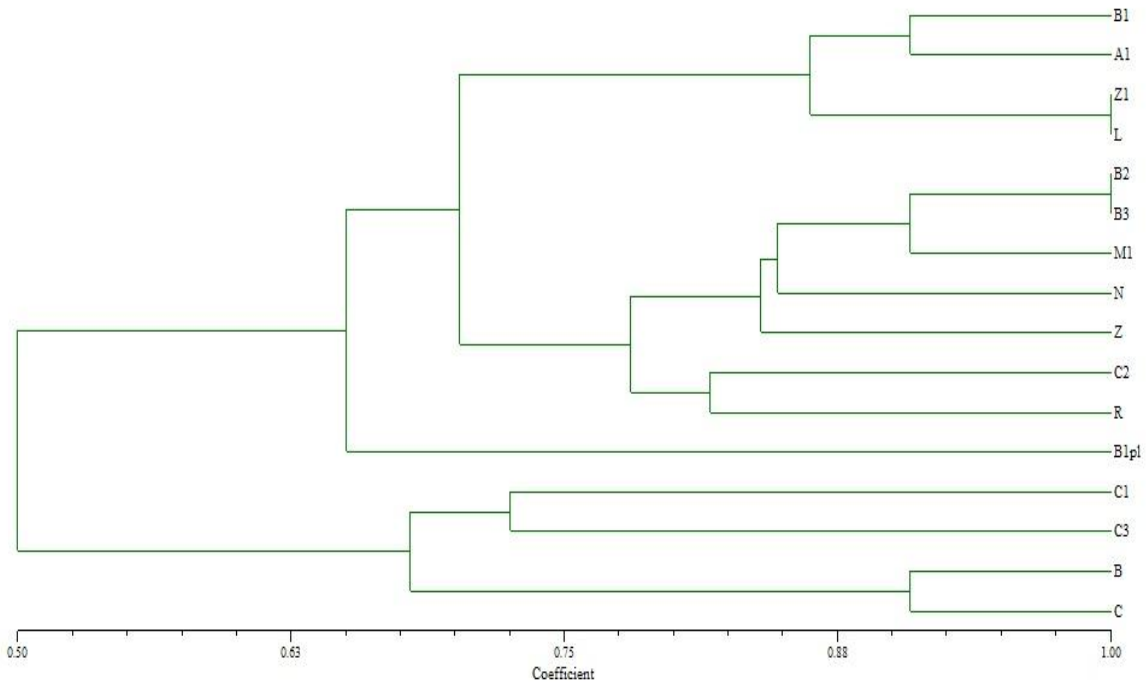


Fig.14 Dendogram of sixteen cellulose degrading isolates generated by binary matrix derived from ERIC amplicons

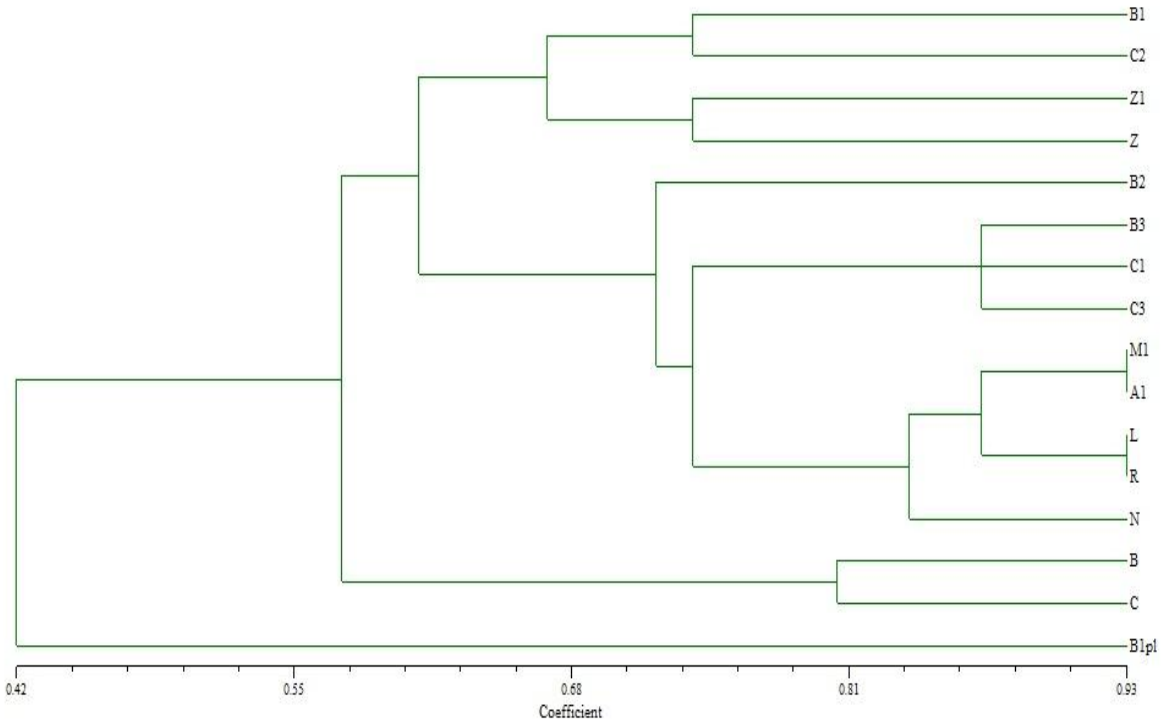


Fig.15 Effect of pH on growth of bacteria

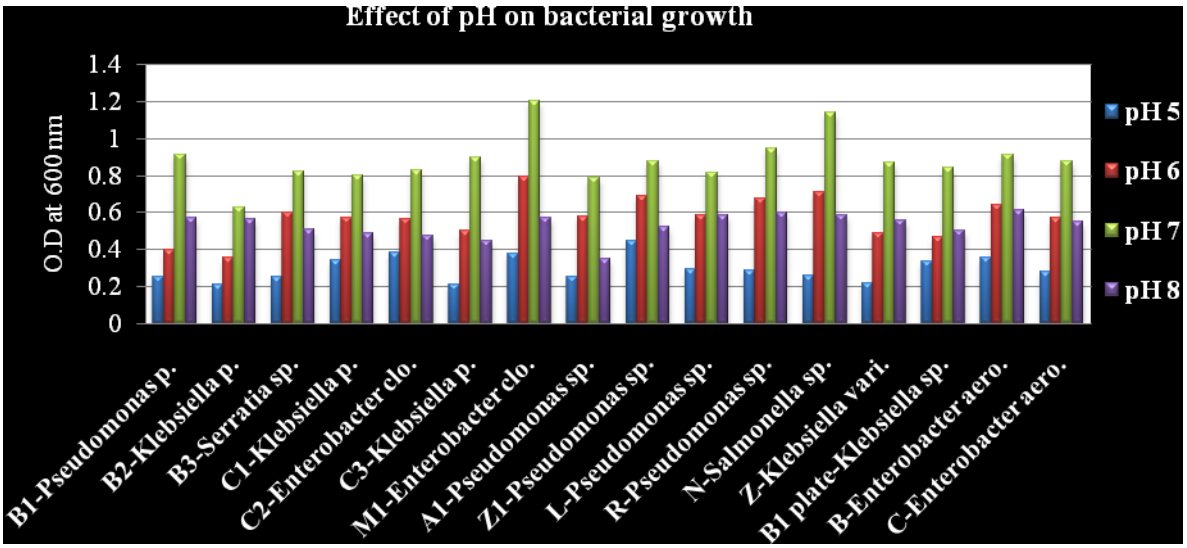
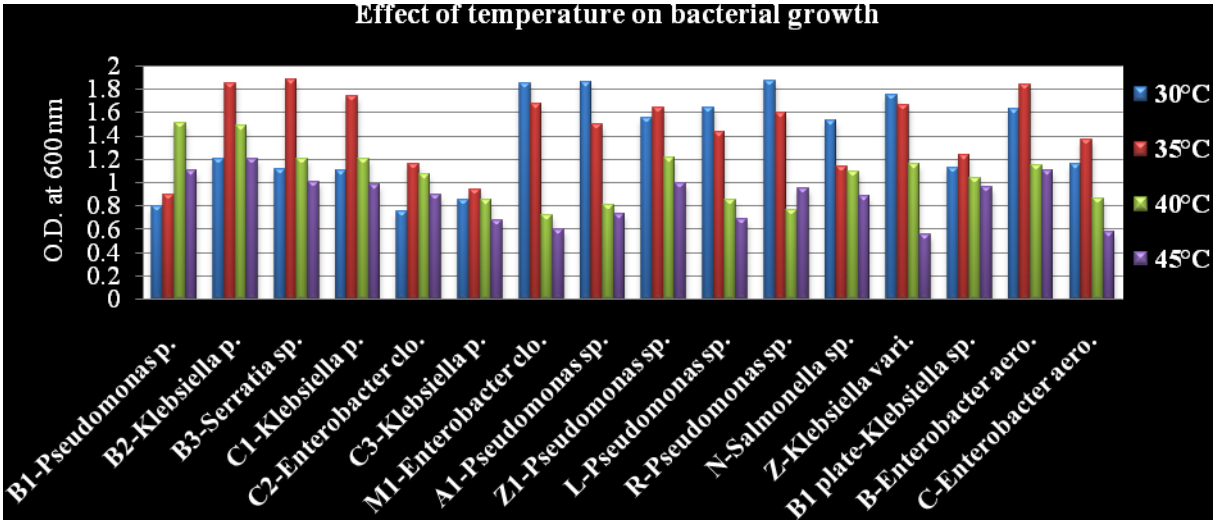


Fig.16 Effect of Temperature on growth of bacteria



Ramin *et al.,* 2008 isolated bacteria from termite guts and later, bacteria were identified as *Bacillus cereus*strain Razmin A, *Enterobacteraerogenes* strain Razmin B, *Enterobacter cloacae*strain Razmin C, *Chryseobacteriumkwangyangense* strain Cb and *Acinetobacter* strain Raminalimon by 16s rRNA sequencing. Three cellulose degrading bacteria isolated from local termite guts belonged to the genera *Acinetobacter*, *Pseudomonas* and *Staphylococcus* and four cellulose degrading bacteria belonged to *Enterobacteriaceae* and *Bacillaceae* families (Pourramezan *et al.*, 2012).16s rRNA gene of

cellulolytic bacteria from the termite *Odontotermesformosanus* was amplified and homology analysis of the sequences showed 90-100% homology with the *Bacillus cereus, Serratiamarcescens, Pseudomonas aeruginosa, Citrobacterfreundii, Enterococcuscasseliflavus,* and *Salmonella entrica* (Kavitha *et al.,* 2014).

REP, BOX AND ERIC-PCR based genotypic analysis

Rep-PCR fingerprinting (with REP, BOX and ERIC primers) is a highly reproducible and simple method to distinguish closely related microbial strains, to deduce phylogenetic relationships and to study their diversity in different ecosystems (de Bruijn *et al.,* 1992).In the present investigation All REP based fingerprinting primers showed good variability among all isolates as almost polymorphic banding pattern was observed in all 16 isolates. Genetic variability for REP primers ranged from 0.59 to 1.00. In case of REP clustering, R isolate did not fall into any group (Fig. 12). Amplification with BOX primer resulted in amplicons of molecular size between 300 to 1300 bp respectively (Fig. 10). Moderate level of variability was observed with BOX based PCR amplification and clustering (Fig. 13). However, a high level of polymorphism was seen in PCR of 16 isolates with ERIC primer. The molecular weights of amplicons after PCR amplification of isolates with ERIC primer ranged between 50 to 1300 bp (Fig. 10). B1 plate isolate did not fall in any group in case of ERIC clustering (Fig. 14). All the isolates exhibited their high degree of genetic variability and distributed in to different clusters with ERIC, BOX and REP primers. This resulted in resolving micro diversity among cellulose degrading isolates and significant levels of genomic heterogeneity between strains within and between sites, respectively. Grouping does not appear to be based on geographic origin. The fingerprints showed wide variations due to high degree of DNA heterogeneity over all the 16 potential isolates. Similarly, work has been reported by other workers such as Charan *et al.,* (2010) who assessed the genetic diversity among *Pseudomonas* isolates using Rep-PCR.

Effect of pH on growth profile of isolates

All potential isolates were studied to determine optimum pH for their growth at various pH values ranging from pH 5 to 8. The results clearly indicate that, the optimum pH for the growth of cellulose degradation bacteria (CDB) was found to be 7.0. However, all the isolates showed moderate growth between pH 5 to 8 (Fig. 15). The isolates, *pseudomonas species, Klebsiella species, Serratia species, Salmonella species and Enterobacter species* were able to survive both acidic and alkaline environments. Results indicated that increase or decrease in the pH can affect the growth of cellulolytic bacteria. The effect of pH on the growth of cellulolytic bacteria was studied by several scientists and optimum pH for the growth was found between 7 to 7.5 (Hethener *et al.,* 1992, Balamurugan *et al.,* 2011, Maruthamalai *et al.,* 2012 and Bholay *et al.,* 2014). This is in consistence with the results obtained in present investigation.

Effect of Temperature on growth profile of isolates

All potential isolates were studied to determine optimum temperature for their growth at different temperature ranges (30°C to 45°C). The temperature specificity of isolates showed that most of them grew well between 30°C to 40°C. Maximum growth for *Pseudomonas putida* (B1 isolate) was observed at 40°C whereas; other *Pseudomonas* species (isolates A1, L and R) *Salmonella entrica* (isolate N) and

Klebsiellavariicola (isolate Z) showed luxuriant growth at 30°C. Other isolates such as, *Klebsiellapneumoniae* (isolates B2, C1, C3 and B1 plate), *Serratia species* (isolate B3), *Enterobacter cloacae* (isolates C2 and M1) and *Enterobacteraerogens* (isolates B and C) showed optimum growth at 35°C. Increasing temperature beyond 40°C resulted in growth reduction (Fig. 16). The effect of temperature on the growth of bacteria was studied by several scientists and optimum temperature for the growth was found 30°C to 40°C (Hethener *et al.,* 1991, Balamurugan *et al.,* 2011, Maruthamalai *et al.,* 2012 and Bholay *et al.,* 2014). This is in consistence with the results obtained in present investigation.

The present work provides information about different parameters for optimum cellulase production, growth profiles, molecular identification, and molecular diversity of sixteen cellulolytic isolates. The potential isolates used in this study were previously isolated from termite guts and their cellulolytic efficiency has already been determined (Shinde *et al.,* 2017). To investigate optimum parameters for cellulase production by these isolates, three substrates namely CMC, avicel and filter paper were used. It was found that, CMCase activity was observed optimum at three different temperatures (35°C, 40°C and 45°C) at pH 7 and after 48 hrs of incubation period whereas, avicelase activity was observed maximum at 35°C, pH 7 and after 48 hrs of incubation. Similarly, filterpaperase activity was optimum at 35°C, pH 7 and after 48 hrs of incubation. C3 isolate was found highest cellulase producer which was later identified as *Klebsiellapneumoniae* and Z isolate (*Klebsiellavariicola*) showed maximum filterpaperase (FPase) activity. Later, these isolates were subjected to molecular identification by sequencing 16s rRNA gene. After BLAST results, isolates were identified as *Pseudomonas, Klebsiella, Salmonella, Serratia and Enterobacter species.* Molecular characterization by using ERIC, BOX and REP primers showed variability among these isolates. The temperature and pH specificity of isolates showed that most of them grew well between 30°C to 40°C at pH 7. Further studies are in progress to investigate cellulase activity on pretreated rice straw and degradation of lignocellulosic biomass (rice straw) using most potential isolate.

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