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

V.S. Shinde¹*, T. Agrawal¹ and A.S. Kotasthane²

¹Department of Plant Molecular Biology and Biotechnology, IGKV, Raipur-492012, India
²Department of Plant Pathology, IGKV, Raipur-492012, India

*Corresponding author

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.

Keywords
Cellulase, DNS, Optimization, 16s rRNA identification, ERIC-REP-BOX.

Article Info
Accepted: 21 September 2017
Available Online: 10 October 2017

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

2474
communities inside the gut are known to produce cellulase. Under optimal conditions, cellulase can convert complex polymers (cellulose) into 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 a 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 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 zones on 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. Carboxymethylcellulase (CMCase) activity was expressed in terms of units. One unit is the amount of enzyme releasing 1 μmol of reducing sugar from carboxymethyl cellulose per 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 1 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), 50mM TrisHCl (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’AGAGTTTGATCCTGCTCAG3’) and 1492R (5’GGTTACCTTGTTCAGACTT3’). 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™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 1 and 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 Gliocladium roseum showed FPase has an optimum pH of 7 (Salem et al., 2015). The effect of incubation period was determined and it showed variations in FPase production. 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 bp amplicons (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 in Table 6. The results 16s rRNA gene analysis in the present study has confirmed that the isolates belong to different strains like, Pseudomonas, Klesbiella, 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'CTACGGCAAGGCGACGCTGACG3' |
| 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

| 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 ± represent 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 enterica 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.
**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
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 (Pouramezan 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
**Int.J.Curr.Microbiol.App.Sci (2017) 6(10): 2474-2492**

*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 (*Klebsiellavarigracula*) 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.

**References**

Arakawa, G., Watanabe H, Yamasaki H, Maekawa Hand Tokuda, G. 2009. Purification and molecular cloning of xylanases from the wood-feeding termite, *CoptotermesformosanusShiraki*. Biosci.Biotechnol.Biochem.73, 710-718

Balamurugan, A., Jayanthi R, Nepolean P, VidhyaPallavi R and Premkumar, R.2011.Studies on cellulose degrading bacteria in tea garden soils African J. of Plant. Sci. 5(1): 22-27.

Bholay, A. D., Gaur A, Ganeshan M and Shah, R. 2014.Exploration of cellulolytic potential of Termite gut flora for sustainable development. J. of Environ. Sci.Toxicol& Food Technol.8(2): 71-76.

Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic, palindromic and enterobacterial repetitive intergenic concensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58, 2180-2187.

Charan, R.A., Reddy P. V, Reddy N. P, Reddy S. S and Sivaramakrishnan, S. 2010. Assesment of Genetic diversity in *Pseudomonas fluorescens* using PCR-based methods. Biremediation
Biodiversity and Bioavailability. Pp.11-16.
Fauzi, N. A., and Makky, E. A. 2013. Avicelase Enzyme from Sawdust: Isolation, Production and Optimization. Int. J. of Biosci.Biochem.and Bioinformatics. 3(5): 501-504.
Fischer, R., Ostafe R. and Twyman, R. M. 2013. Cellulases from insects. Adv.Biochem.Eng.Biotechnol.136, 51-64.
Haripriya, R., and Thirumalaivasan, P. 2017. Isolation of cellulolytic bacteria and production of cellulase from coir pith. Int. J. Res. Ins.4(1): 13-23.
Hethener, P., Brauman A. and Garcia, J. 1992. Clostridium termitidis sp. nov., a Cellulolytic Bacterium from the Gut of the Wood-feeding Termite, Nasutitermes lujae. Appl.Microbiol.15, 52–58.
Kavitha, D., Vijayarani K. and Kumanan, K. 2014. 16S rRNA typing of cellulolytic bacteria from the termite Odontotermes formosanus. Ind. J. Vet. & Anim. Sci. Res.43(5): 359 – 368.
Khatiwada, P., Ahmed J, Sohag M. H, Islam K. and Azad, A. K. 2016. Isolation, Screening and Characterization of Cellulase Producing Bacterial Isolates from Municipal Solid Wastes and Rice Straw Wastes. J. of Bioprocess.& Biotech.6(4): 1-5.
Khianngam, S., Pootaeng-on Y, Techakriengkrai T. and Tanasupawat, S. 2014. Screening and identification of cellulase producing bacteria isolated from oil palm meal. J. of Appl. Pharma.Sci.4(4): 90–96.
Li Xi, Yang H, Roy B, Wang D. Y, Wan F, Jiang Li, Park E. Y. and Miao, Y. 2009. The most stirring technology in future: Cellulase enzyme and biomass utilization. Afr. J.Biotechnol.8, 2418-22.
Maruthamalai, R. P., and Mahalingam, P. U. 2012. Screening and Partial Characterization of Cellulose Degrading Bacteria from Decayed Sawdust. Int. J of Sci. and Res. 3(8): 328-331.
Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426-428.
Pourramezan, Z., Ghezelbash G. R, Romani B, Ziae S. and Hedayatkhah, A. 2012. Screening and Identification of Newly Isolated Cellulose Degrading Bacteria from the Gut of Xylophagous Termite Microcoterotermes diversus (Silvestri). Microbiol.81(6):736–742.
Ramin, M., Alimon A. R and Abdullah N. 2008. Isolation and identification of bacteria from the termite Coptotermes curvignathus (Holmgren) present in the vicinity of University Putra Malaysia. Res. J. of Microbiol. 3(4): 288-292.
Rani, R. R. 2015. Microbial Avicelase: an Overview. Bull.Env.Phamacol. Life Sci. 4(4): 03-13.
Salem, A. A. and Rehman, A. H. M. 2015. Optimization and characterization of cellulolytic enzymes produced from Gliocladium roseum. J. Agri. Chem.& Biotech.6(11): 473-488.
Scharf, M. E. and Tartar, A. 2008. Termite digestomes as sources for novel lignocellulases. Biofuels Bioproducts & Biorefining 2, 540-552.
Shaikh, N. M., Patel A. A, Mehta S. A. and Patel, N. D. 2013. Isolation and Screening of Cellulolytic Bacteria Inhabiting Different Environment and Optimization of Cellulase Production. Uni. J. of Environ. Res. and Technol.3(1): 39–49.
Sharma, D. 2015. Isolation of Cellulolytic Organisms from the Gut Contents of Termites Native to Nepal and Their Utility in Saccharification and Fermentation of Lignocellulosic
Biomass. J. of Biomass to Biofuel2,2368-5964.

Shinde, V. S., Shinde R. M, Agrawal T. and Kotasthane, A. S. 2017. Extracellular cellulose activity of termite gut bacteria isolated from different geographical locations of Chhattisgarh (C.G.), India. Plant Archives. 17(1): 601-607.

Sohail, M., Ahmad A. and Khan, S. A. 2014. Comparative studies on production of cellulases from three strains of Aspergillus niger. Pak J. Bot.46 (5): 1911-1914.

Swaroopa, R. D., Thirumale S and Nand, K. 2004. Production of cellulase by Clostridium papyrosolvens CFR-703. World J. of Microbiol. & Biotechnol. 20, 629-632.

Tamura, K., Peterson D, Peterson N, Stecher G, Nei M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol 10, 2731-9.

Vimal, J., Venu A. and Joseph, J. 2016. Isolation and identification of cellulose degrading bacteria and optimization of the cellulase production. Int. J. Res.Biosci.5 (3): 58-67.

Willis, J. D., Oppert C. and Jurat-Fuentes, J. L. 2010. Methods for discovery and characterization of cellulytic enzymes from insects. Insect Sci.17, 184-98.

Wood, T. M. and Bhat, K. M. 1988. Methods for measuring cellulase activities. Methods in Enzymol. 160: 87-112.

Yadav, S. P., Shruthi K, Siva Prasad B. V. and Chandra, S. M. 2017. Isolation and Identification of Aspergillus protuberus from Mahanandi Forest Sample and Investigation of Its Cellulase Production. Indian J. of Adv. In Chem. Sci. 5(1): 8-15.

How to cite this article:

Shinde, V.S., T. Agrawal and Kotasthane, A.S. 2017. Molecular Characterization of Cellulolytic Bacteria Derived From Termite Gut and Optimization of Cellulase Production. Int.J.Curr.Microbiol.App.Sci. 6(10): 2474-2492. doi: https://doi.org/10.20546/ijcmas.2017.610.292