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A Comparative Study of Different Staining Techniques for Cellulase Activity on CMC (Carboxy Methyl Cellulose) Agar

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Abstract

The present experiment was conducted at Department of Plant Pathology, College of Agriculture, Navsari Agricultural University, WaghaiduringMarch-October, 2021. cellulolytic bacterial isolates CD11, CD17, CD19, CD22 and CD35 were screened to determine the staining efficiency of various stains for cellulase activity on CMC (Carboxy Methyl Cellulose) agar and it was observed that all the isolates conferred clear zone around the colonies through different staining dyes and bacterial isolate CD35 showed the highest cellulolytic index in all the dyes. The cellulolytic index of CD35 was highest with gram's iodine (3.34) next in order of coomassie brilliant blue (2.96), safranin (2.55) and congo red (2.15). Significantly, higher cellulase activity was recorded in CD35 (0.169 U ml^{-1}) at 24 hrs after inoculation It was followed by CD17 (0.124 U ml^{-1}), CD19 (0.101 U ml^{-1}) and CD11 (0.081 U ml^{-1}), while it was lowest in CD22 (0.052 U ml^{-1}). Maximum cellulase activity was recorded at 72 hrs after inoculation by all the selected isolates except CD22 isolate, which was maximum 96 hrs after inoculation. CD35 gave significantly maximum cellulase activity (0.822 U ml^{-1}) at 72 hrs after inoculation. Next in order to cellulase activity was CD17 (0.477 U ml^{-1}) which was at par with CD19 (0.471 U ml^{-1}) followed by CD11 (0.292 U ml^{-1}) while, it was lowest in CD22 (0.199 U ml^{-1}). Cellulolytic bacteria CD35 was identified as *Bacillus subtilis* by morphological, biochemical and molecular methods and submitted to NCBI GenBank database with accession numbers MW715021.

Keywords: *Bacillus subtilis*, cellulose, cellulolytic index

1. Introduction

Cellulase is the enzyme that hydrolyzes the β-1, 4-glycosidic bonds in the polymer to release glucose units (Nishida et al., 2007). Cellulase is a multi-enzyme system composed of several enzymes with numerous isozymes, which act in synergy.Cellulases have enormous potential in industrial and agricultural applications. Glucose produced from cellulosic substrate by hydrolysis could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol (Levy et al., 2002), butanol, methane, amino acid, single-cell protein, organic acids etc. (Luo et al., 1997; Penttila et al., 2004), feed preparation, (Ishikuro, 1993) waste-water treatment, detergent formulation (Oksanen et al., 2000), textile production (Miettinen-Oinonen et al., 2004), hydrolysis of wastepaper (Van-Wyk and Mohulatsi, 2003) and as lytic enzymes, they are of also major importance is the protoplast production (Davis, 1985; Mandels, 1974; Bhat, 2000) for tissue culture and plant metabolites production.Additional potential applications include the production of fruit juice, wine and beer. However, all these uses are of rather small magnitude compared with cellulase requirements for bioconversion of lignocellulosic biomass to ethanol fuel.

Cellulases are produced by large number of microorganisms and are exoglucanases, endoglucanases, and β-glucosidase. Microorganisms produce these enzymes in diverse nature which determines their efficiency in cellulose hydrolysis (Jayasekara and Ratnayake, 2018). Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications. Cellulases are inducible enzymes synthesized by a large number of microorganisms

including both fungi and bacteria during their growth on cellulosic materials (Kubicek, 1993; Sang-Mok and Koo, 2001). A microbial enzyme isolated from natural ecosystems has unique properties that could make them good candidates for improving biomass conversion efficiency into value-added goods, chemicals, and fuels. However, research into the composition of cellulosic biomass and the natural sources of microbial enzymes that drive biomass conversion efficiency is still in its early stages (Haile and Ayele, 2022; Mukherjee et al., 2022).

For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot springs, organic matters, faeces of ruminants and composts (Doi, 2008). Cellulases are produced by bacteria, fungi, protozoans, plantsand animals (Zhang and Zhang, 2013). It is very essential to find out a rapid and easy screening method to differentiate between cellulolytic and non-cellulolytic microbes. The microorganisms to be inspected are cultured on a gel-like medium containing CMC, leaving sufficient time for hydrolysis activity to occur. The medium is then dyed with a dye that interacts only with either CMC or the hydrolysis products, differentiating the hydrolysis zone by a changed color, enabling visualization of the hydrolysis that occurred (Yeoh et al., 1985). In the past, a variety of dyes have been introduced for post hydrolysis plate staining, the most common of which are Gram's iodine and Congo red (Kasana et al., 2008; Johnsen and Krause, 2014). Previous studies have shown that congo red has better efficiency to differentiate on solid media whereas DNS is the most preferred assay for liquid culture (Sazci and Erenler, 1986; Romsaiyud et al., 2009). However, there are many other methods available but are less popular. In this study, an attempt was made to determine the staining efficiency of various stains for cellulase activity on CMC agar and identification of potent cellulolytic isolate.

2. Materials and Methods

Assessment of different staining methods for cellulase activity on CMC (Carboxy Methyl Cellulose) agar plates was conducted at Department of Plant Pathology, College of Agriculture, Navsari Agricultural University, Waghai in 2021–22.

2.1. Microorganism and culture condition

A total of 5 out of 56 previously isolated cellulolytic bacteria (CD11, CD17, CD19, CD22 and CD35) from the different locations of the Dangs (Gujarat) were selected based on primary screening for the study. The culture was maintained on nutrient agar slant at 4°C and sub-cultured regularly.

2.2. Screening of cellulolytic bacteria through various staining methods

Cellulolytic bacterial isolates CD11, CD17, CD19, CD22 and CD35were further compared for cellulolytic index on CMC agar plates with different staining dyes *i.e.* 0.1% Congo red, 2.5% Safranin, Gram's Iodine solution and 0.1% Coomassie Brilliant Blue R 250 solution (Gohel et al., 2014). The colonies plated on CMC agar were flooded with the respective dyes for 10–12 min then washed with distilled water (Gram's Stain and Coomassie Brilliant Blue) and 1M NaCl (Congo Red and Saferenin). A clear zone appeared around growing microbial colonies indicated cellulose hydrolysis. The cellulolytic index was calculated by using the following equation 1 (Ferbiyanto et al., 2015).

Cellulolytic Index=(Diameter of Zone-Diameter of Bacterial colony)/(Diameter of Bacterial colony)

2.3. Quantitative screening of cellulase enzyme activity of bacterial isolates

High cellulolytic index producing isolates were screened for quantitative cellulase enzyme production in Basal Salt Medium (BSM) ((g l^{-1} of yeast extract, 5.0; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 5.0; NaCl, 10.0; pH, 7) containing 1% CMC inoculated with 1 ml of $2.0x10^6$ cf U ml^{-1} of bacterial isolates. The flasks were incubated at 37±2°C at 120 rpm (Shaikh et al., 2013) and enzyme activity was monitored up to 120 hrs at 24 hrs of interval. Cellulase production was determined by Dinitro Salicyclic Acid (DNSA) method proposed by Miller (1959).

2.4. Characterization of potential cellulolytic isolates

Potent cellulolytic bacterial isolate was identified based on colony characteristics (size, form, margin, elevation, surface, consistency, pigmentation, opacity, etc.) on Nutrient Agar (NA), morphological characteristics (cell shape, arrangement, Gram's reaction, etc) by microscopic examination and biochemical characteristics (catalase, indole, methyl red, Voges Proskauer's, citrate utilization, carbohydrate utilization, etc.) by using HiMedia kit (KB001 HiIMViC™ biochemical test kit) and observations were recorded.

2.5. Identification of potential cellulolytic isolates

Most potential cellulolytic bacteria were identified using 16S rRNA sequencing (Reller et al., 2007). For the identification of cellulolytic bacteria, DNA was isolated from the culture by the CTAB method and its quality was evaluated on 1.0% agarose gel. The bacterial 16S rRNA gene was amplified from the total genomic DNA using universal eubacteria specific primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3') by PCR followed by purification by column purification to remove contamination. DNA sequencing was done at Saffron Life Sciences, Surat. Briefly, sequencing was carried out with 27F primer using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. Quarry sequence was searched for homology on NCBI using the BLAST tool. Based on maximum identity score and homology with available sequences, a phylogenetic tree was constructed using NCBI web tools and the sequence was submitted to NCB1-GeneBank to obtain accession number.

3. Results and Discussion

3.1. Screening of cellulolytic isolates through various staining methods

The cellulolytic bacterial isolates *i.e.* CD11, CD17, CD19, CD22

and CD35 were compared for cellulolytic index on CMC agar plates with different staining reagents (Table 1 and Figure 1). Isolate CD35 showed significantly highest zone to colony ratio (3.15) and cellulolytic index (2.15) with congo red staining reagent. Next in order of zone to colony ratio and the cellulolytic index was CD19 and it was at par with CD22 and CD11 while, it was lowest in CD17 (2.28 and 1.28, respectively).

In Safranin stain, isolate CD35 showed significantly highest zone to colony ratio (3.55) and cellulolytic index (2.55) followed by CD11, CD11 and CD17 was at par while, zone to colony ratio and the cellulolytic index was lowest in CD22 (2.24 and 1.24). Isolate CD35 showed significantly highest zone to colony ratio (4.34) and cellulolytic index (3.34) through gram's iodine stain, then in order of zone to colony ratio and the cellulolytic index was CD17 followed by CD11, while CD11 was at par with CD22 and lowest zone to colony ratio (2.00) and cellulolytic index (1.00) was observed in CD19. Significantly highest zone to colony ratio (3.96) and cellulolytic index (2.96) was observed in CD35 by coomassie brilliant blue staining

Table 1: Zone to colony ratio and cellulolytic index of selected bacterial isolates on CMC agar with different staining reagents

Sl. No.	Strain No.	Congored		Safranin		Grams Iodine		Coomassie Brilliant Blue	
		Zd Cd^{-1}	Cellulolytic index	Zd Cd^{-1}	Cellulolytic index	Zd Cd^{-1}	Cellulolytic index	Zd Cd^{-1}	Cellulolytic index
1	CD11	2.49	1.49^{b}	2.72	1.72^{b}	2.30	1.30^{c}	1.83	0.83^{d}
2	CD17	2.28	1.28^{c}	2.70	1.70^{b}	3.90	2.90^{b}	1.93	0.93^{d}
3	CD19	2.55	1.55^{b}	2.47	1.47^{c}	2.00	1.00^{d}	2.33	1.33^{b}
4	CD22	2.53	1.53^{b}	2.24	1.24^{d}	2.16	1.16^{cd}	2.10	1.10^{c}
5	CD35	3.15	2.15^{a}	3.55	2.55^{a}	4.34	3.34^{a}	3.96	2.96^{a}
SEm±	0.06	0.06	0.04	0.04	0.07	0.07	0.04	0.04	0.04
CD (p=0.05)	0.19	0.19	0.13	0.13	0.21	0.21	0.13	0.13	0.13
CV %	4.73	7.70	3.26	5.14	4.80	7.27	3.50	5.95	5.95

Zd: Zone diameter (mm); Cd: Colony diameter (mm); Note: Different letters in the same column indicate significant differences

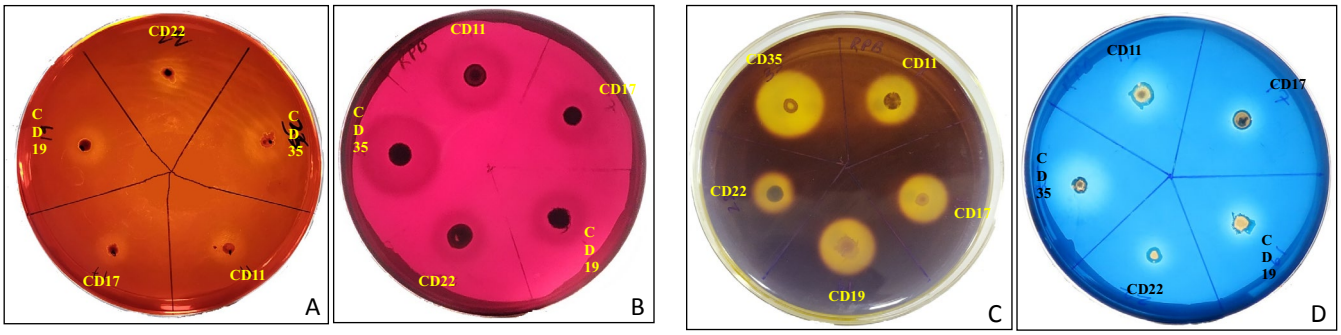

Figure 1: Zone of cellulose degradation by isolates on CMC agar with different stains; A: Congo red; B: Safranin; C: Gram's Iodine; D: Coomassie Brilliant Blue

reagents and it was followed by CD19, CD22 and CD17, while it was lowest in CD11.

Overall, it was observed that all selected isolates conferred clear zone around the colonies through different staining dyes and bacterial isolate CD35 showed the highest cellulolytic index in all the dyes. However, the cellulolytic index of CD35 was highest with gram's iodine (3.34) next in order of coomassie brilliant blue (2.96), Safranin (2.55) and congo red (2.15). Goel et al.(2019) also used congo red solution for staining and primary screening of cellulose degrading bacteria. Gohel et al. (2014) also compared various staining methods *viz*. congo red, coomassie brilliant blue R-250, safranin and gram's iodine for their staining efficiency of cellulolytic determination assay and reported that gram's iodine staining was the best staining method for determining cellulase activity.

3.2. Quantitative screening of cellulase enzyme activity of isolates

Selected bacterial isolates were further screened for quantitative cellulase enzyme activity through submerged fermentation using 1% CMC as carbon source up to 120 hrs at 24 hrs intervals (Figure 2). Cellulase enzyme is an important parameter for the degradation of cellulosic waste and bacteria with higher cellulase activity can effectively degrade cellulosic material.

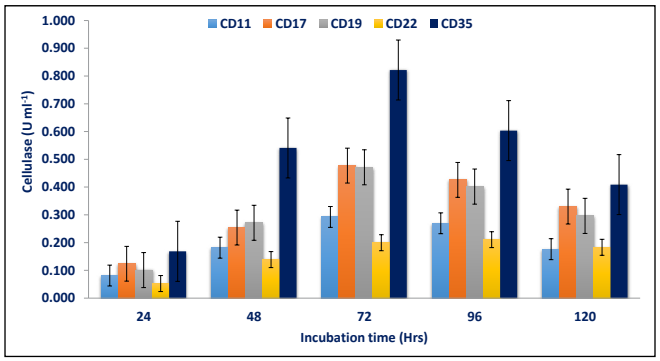

Figure 2: Cellulase enzyme activity of cellulolytic bacterial isolates at different incubation periods

Significantly, higher cellulase activity was recorded in CD35 (0.169 U ml^{-1}) at 24 hrs after inoculation, it was followed by CD17 (0.124 U ml^{-1}), CD19 (0.101 U ml^{-1}) and CD11 (0.081 U ml^{-1}), while it was lowest in CD22 (0.052 U ml^{-1}). Maximum cellulase activity was recorded at 72 hrs after inoculation by all the selected isolates except CD22 isolate, which was maximum at 96 hrs after inoculation. CD35 gave significantly maximum cellulase activity (0.822 U ml^{-1}) at 72 hrs after inoculation. Next in order to cellulase activity was in CD17 (0.477 U ml^{-1}) which was at par with CD19 (0.471 U ml^{-1}) followed by CD11 (0.292 Uml^{-1}) while, it was lowest in CD22 (0.199 U ml^{-1}).

Overall, among all the tested bacterial strains CD35 was given higher cellulase activity at all the intervals and it was increased up to 72 hrs then after it was decreasing. Verma et al. (2012) screened thermophilic *Bacillus* species and reported that maximal cellulase production was obtained after 48 hours of incubation at 45°C in a medium containing 1.5% carboxymethyl cellulose (CMC) as substrate. Lokhande and Pethe (2017) reported that among all the isolated organisms one cellulolytic bacterial isolate shows maximum enzyme activity (1.41 IU ml^{-1}) after the third day of incubation period and was identified as *Bacillus thuringiensis*. Urgessa et al. (2020) also reported that *Pseudomonas* sp. isolated from goat rumen produced significantly highest carboxymethyl-cellulase (CMCase) and filter-paperase (FPase) of 1.54 ± 0.10 U ml^{-1}, 1.46 ± 0.09 U ml^{-1} from the fermentation of 1% w/v carboxymethyl cellulose used as standard and 1% wheat straw, respectively.

3.3. *Characterization and identification of potent cellulolytic bacteria*

Potent cellulolytic bacterial isolates were characterized in terms of colony characteristics and morphological characteristics (Table 2). The experimental data revealed that all the isolates were varied in terms of colony characters such as size, form, margin, elevation, surface, consistency, pigmentation and opacity. Microscopic observation of CD35 revealed that it was rod-shaped, diplobacillus, Gram-positive and motile.

Table 2: Colony and morphological characters of cellulolytic bacterial isolates

Sl. No.		CD11	CD17	CD19	CD22	CD35
Colony characters						
1	Size	Medium	Small	Medium	Medium	Medium
2	Form	Irregular	Irregular	Irregular	Irregular	Round
3	Margin	Undulate	Undulate	Undulate	Lobate	Entire
4	Elevation	Pulvinate	Flate	Umbonate	Pulvinate	Raised
5	Surface	Wrinkled	Wrinkled	Rough	Rough	Smooth
6	Consistency	Brittle	Viscid	Viscid	Butyrous	Mucoid
7	Pigmentation	White	White	White	White	Fuzzy white
8	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Morphological characters						
1	Cell shape	Rod	Rod	Rod	Rod	Rod
2	Cell arrangement	Diplobacilli	Chain	Single	Chain	Diplobacilli
3	Gram's reaction	Positive	Positive	Positive	Positive	Positive
4	Motility	Motile	Motile	Non-motile	Motile	Motile

Biochemical characterization data revealed that isolate CD35 was catalase-positive, Voges Proskauer's-positive and citrate utilization-positive while, it was negative for indole and methyl red test. Moreover, isolate CD35 also positive for carbohydrate utilization (Glucose, Arabinose, Sorbitol, Mannitol and Sucrose) while, it was negative for adonitol, lactose and rhamnose (Table 3). Identification of isolate CD35 using universal primer revealed that sequence showed 100% homology with *Bacillus subtilis* when searched for homology using BLAST tool. Phylogenetic tree of the isolate constructed on NCBI website (Figure 3). Gene sequence of the isolate was submitted to NCBI GenBank database with accession

Table 3: Biochemical characteristics of cellulolytic bacterial isolates

Sl. No.	Name of Tests	CD11	CD17	CD19	CD22	CD35
1	Catalase	+	+	+	+	+
2	Indole	-	-	-	-	-
3	Methyl Red	+	+	-	-	-
4	Voges Proskauer's	+	+	+	+	+
5	Citrate utilization	-	-	+	-	+
Carbohydrate utilization						
6	Glucose	+	+	+	+	+
7	Adonitol	-	-	-	-	-
8	Arabinose	+	-	-	+	+
9	Lactose	-	-	-	-	-
10	Sorbitol	+	+	+	+	+
11	Mannitol	+	+	+	+	+
12	Rhamnose	-	-	-	-	-
13	Sucrose	+	+	+	+	+

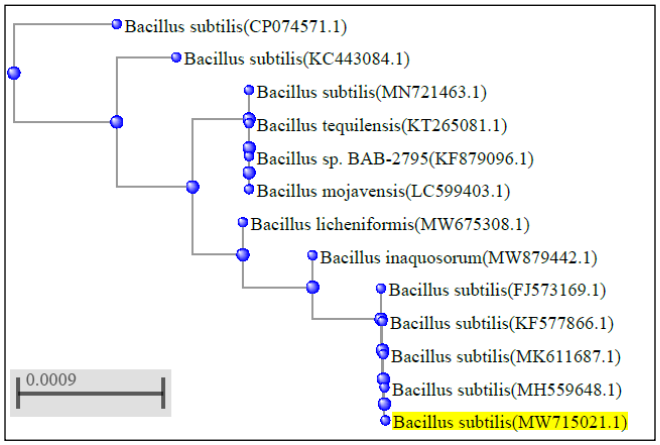

Figure 3: Phylogenetic tree of CD35 (*Bacillus subtilis*)

number MW715021. Based on morphological, biochemical and molecular characterization cellulose-degrading bacterial isolate (CD35) was identified as *Bacillus subtilis*

Rawway et al. (2018) also isolated and characterized ten cellulose-degrading bacteria (CDB1-10). Based on morphological and biochemical characteristics and 16S rRNA gene sequence, isolates were identified as *Bacillus subtilis* (CDB6, CDB8, CDB9 and CDB10), *Bacillus thuringiensis* (CDB1 and CDB5), *Brevibacillus brevis* (CDB2 and CDB4), *Brevibacillus parabrevis* (CDB7)and *Bacillus pumilus* (CDB3). Similarly, Meng et al. (2014) also reported that cellulolytic bacterial strain BY-3 formed a clade with *B. subtilis* strain K21 supported by 100 bootstrap value.

4. Conclusion

Based on the results of the current study, among all the stains gramsiodine is the best plate assay method fordetermining cellulase activity. Potential cellulolytic isolates CD35 showed a higher zone to colony ratio as well as higher cellulase activity and identified as *Bacillus subtilis* by morphological, biochemical and molecular characterization.

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