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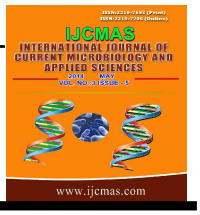


Original Research Article

A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates

Hardik R. Gohel[1]*, Chintan N. Contractor[2], Sandip K. Ghosh[1], Vincent J. Braganza[1]

[1]Loyola Centre for Research and Development, Navarangpura, Ahmedabad, India
[2]Amity Institute of Biotechnology, Jaipur, India
Corresponding author

A B S T R A C T

Keywords

Cellulase, staining methods, comparative study

Microbes are known to produce many industrially important extracellular enzymes. Cellulase is one of them. Screening of these microbes is a very important and critical step. Several methods are in use for the selective screening. Stain the CMC containing plate with congo red is one of the most popular method. But we found that, staining with congo red has less efficiency and it also deactivate the microbes. So, In the present work, an attempt was made to compare various staining methods for their staining efficiency. In the result, it was found that gram's iodine gives the best results followed by congo red staining.

Introduction

Cellulose is one of the most abundant forms of biomass present on the earth and is considered an inexhaustible source of raw material for various products (Rampersad et al. 1998; Angsana et al. 2009). A sure way of utilizing it starts with its breakdown into its smaller oligosaccharides or monosaccharides. Cellulases are a class of enzymes that catalyze these reactions(Mingardon et al. 2011; Angsana et al. 2009). Based on the cleavage the enzyme performs they are divided into three types: i) Endo which cleaves at random sites of the biopolymer ii) Exo which cleave the 2 or 4 units from the edge of the reduced cellulase chain that is formed by the Endo cellulose and iii) β-Glucosidase that hydrolyzes extra cellular products to individual monosaccharide (Sazci & Erenler 1986; Mingardon et al. 2011).

Several previous reports have shown that certain microbes are able to utilize cellulose as a source of energy. These microbes produces extracellular cellulase and hence known as cellulolytic microorganisms. *Bacilli* and fungi are most popular class for commercial production as these cellulases have very high economic value (Angsana et al. 2009; Gerardi 2003; Karan et al. 2012; Gopinath et al. 2005; Sazci & Erenler 1986). It is very essential to find out a rapid and easy screening method to differentiate between cellulolytic and non cellulolytic microbes.

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 & Erenler 1986; Angsana 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 methods for cellulase activity on solid media. We have used congo red, grams iodine, coomassie brilliant blue and safranin.

Materials and Methods

Isolation and Screening of microbes

1.0 g of regular garden soil was collected and serial dilutions up to 10^{-7} were performed. Spread plate method was performed on suitable media containing carboxy methyl cellulose (1.0%) as a sole carbon source. Plated were incubated at 37ºC until the visible colonies form.

Preparation of plates for various staining

Selected colonies were selected based on their enzyme activity for comparative analysis. For the experiment microbes were grown on CMC containing plates for 48 hours and then treated with different staining procedures.

Preparation of Stains

Congo Red Stain

0.1 % aqueous dye was prepared along with 1 M NaCl solution for washing off

Coomassie Brilliant Blue R 250 Stain

0.1 % Coomassie Brilliant Blue solution prepared in 8 ml Methanol. 10 ml of distilled water was added and 2 ml of Glacial acetic acid is added.

Safranin Stain

2.5 % Saffranine dissolved in distilled water.

Grams Iodine Stain

0.133 g KI and 0.067 g Iodine dissolved in 20 ml distilled water.

Staining Method

Colonies were plated on 1% CMC agar plates and the plates were flooded with the respective dyes and were incubated with the dye for 10-12 minutes and then washed with water except congo red and safranin. These stains were washed with 1 M NaCl solution and other plates were washed with distilled water.

The inoculation volume was kept constant at 2 µl and incubation time was kept at 48 hours which is known to be the optimum time for cellulolytic enzyme production.

Results and Discussion

Cellulases are known to convert cellulose into monomeric or dimeric structure hence carboxymethyl cellulose (CMC) was used as a carbon source which is a soluble form of cellulose (Angsana et al. 2009; Sissons et al. 1987; Sazci & Erenler 1986). The above mentioned dyes were used to observe the zone of clearance produced by activity of cellulase. It is widely accepted that the diameter of the zone of clearance indicates the ability of the bacteria to hydrolyze cellulose. Initially it was presumed that in each plate uniform zone of clearance will be obtained but results were different from our expectation. From

Table.1 Comparison of Zone of Clearance

Colony name	Clearing zone diameter on CMC agar plates (mm)			
	Grams Iodine	Congo Red	Safranin	Coomassie Brilliant Blue
DC1	22	17	15	16
Y1	23	15	15	3.5
PP1	19	11	8	ND
O1	21	12	11	ND
DY1	22.5	8.5	10	ND

Zone of clearance indicated the efficiency of grams iodine and congo red are very high as compare to safranin and coomassie brilliant blue.

Figure.1 Zone of Clearance of cellulose on agar plate after staining with coomassie brilliant blue

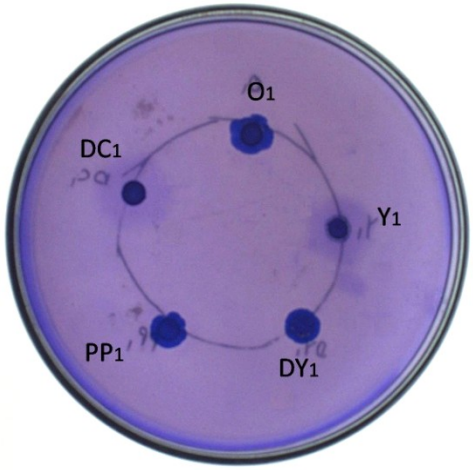


Figure.2 Zone of Clearance of cellulose on agar plate after staining with safranin

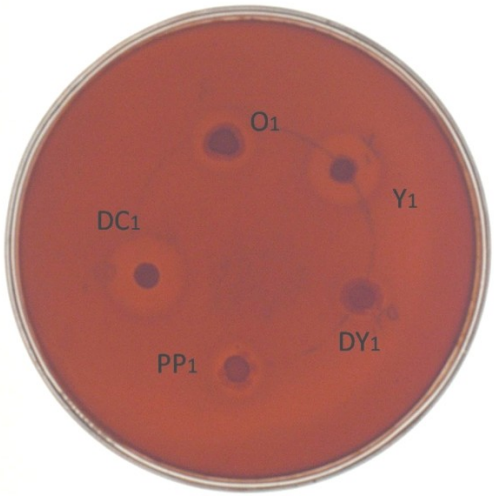


Figure.3 Zone of Clearance of cellulose on agar plate after staining with gram's iodine

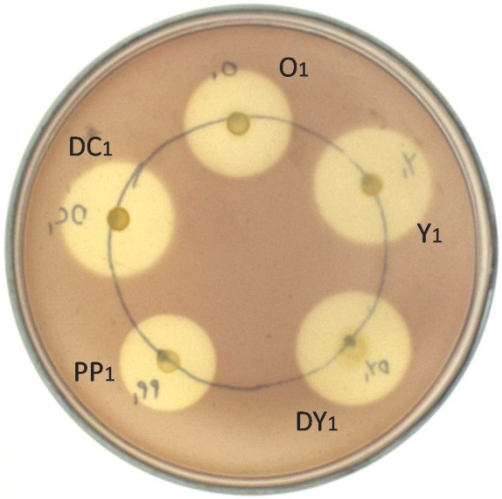
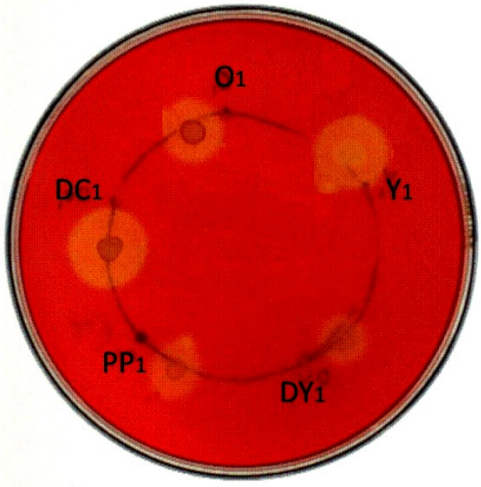


Figure.4 Zone of Clearance of cellulose on agar plate after staining with congo red



Figures clearly indicate that grams iodine CMC agar and congo red are very efficient method for determination of cellulase activity on agar plates as compare to brilliant blue and safranin.

this, it was noted that staining efficiency is also dependent on the degree of cellulase degradation (Kasana et al. 2008; Maki et al. 2011). Area were the degree of degradation was lower stains like coomassie brilliant blue and safranin get retained in the polymer and giving smallest zone of clearance (Srebotnik & Messner 1994; Doğu & Grabner 2010; Maki et al. 2011) (Figure 1 & 2). While in case of grams iodine and congo red retention is very less because of higher degradation activity, resulting into significantly higher zone of clearance(Kasana et al. 2008; Kera et al. 2012; Florencio et al. 2012; Dashtban et al. 2010; Fujimoto et al. 2011) (Figure 3 & 4).

The diameter of the zone of clearance for each dye is represented in Table 1.

Reason behind formation of zone lyse in the dyes which bind with the polysaccharide and forms a visible complex. Cellulase produced by celluloytic bacteria in the plate breaks down the polysaccharide as a result of which area surrounded by colony were exhausted with polysaccharides and replaced with smaller monosaccharides and disaccharides. To these mono and disaccharide dyes cannot be bind efficiently and resulted into a visible clear zone. Although it is clear from the figure the Coomassie Brilliant blue did not produce clear zones but there was a presence of a dark bluish halo around some colonies where as no such halo was observed in other colonies.

Apart from this Safranin which is not previously used for a celluloytic determination assay was used yielding fruitful results. Breakdown of the carbohydrates result in poor binding of the dye to the agar which results in formation of halos with less intensity suggesting celluloytic activity. Congo red, a well established and widely method has shown better efficiency as compare to safranin and brilliant blue. It was also found that Congo red stain inactivate the microbe hence they cannot be used further for any study (Florencio et al. 2012; Fujimoto et al. 2011). In our study we have found that grams iodine gives the best results with prominent and distinct clear zones and within 2-3 minutes. Furthermore it is not toxic to the cells. Cells could be reused for other study.

It is clear from the results that grams iodine is the best plate assay method for determining cellulase activity. Also safranin which has not been previously could be used as one of the staining method, however it is less efficient.

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