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Screening of Cellulolytic Bacteria Isolated from Garden Soil Using Different Staining Reagents

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ABSTRACT
Cellulase is any of the several enzymes produced chiefly by fungi, bacteria and protozoan that catalyze cellulolytic materials. The aim of the study was chiefly to screen cellulolytic bacteria using different staining reagents. The soil samples were collected from different garden locations around Kasarawa village in Sokoto. Cellulase producing bacteria were screened individually by transferring pure cultures of bacteria isolates in CMC agar plates and incubated for 48 hours. CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15min. at room temperature. One molar NaCl was thoroughly used for counterstaining the plates, and the plates were observed for clear zones around colonies. The same procedure was repeated when CMC plates were flooded with Iodine, Safranin, Crystal violet and lactophenol cotton blue. The result revealed the colonial characterization such as shape, color, size, elevation, and gram reactions for each of the isolates. A Total of 12 isolates were obtained from which 8 showed cellulolytic activities when screened using Congo red on carboxymethyl cellulose (CMC) agar plates most of them were gram negative rods. The isolates labeled KS_1, KS_2, KS_3, KS_4, KS_5, and KS_6 produced clearance zones when stained with crystal violet, the isolate labeled KS_2. KS_3, KS_4, and KS_7 produced clearance zones when stained with lactophenol cotton blue, the isolates KS_4, KS_6, KS_7 and KS_8 produced clear zone when stained with iodine while Safranin produced no clear zones when used to stain the CMC agar plates The study revealed that other staining reagents could be used to screen cellulolytic bacteria and this reagents are more readily available.

INTRODUCTION

Cellulose is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere (Jarvis, 2002; Zhang and Lynd, 2004). Cellulolytic microorganism play important role in the biosphere by reducing complex polymer cellulose into various economically important product like monomeric sugar, microbial biomass proteins, compost (Balamurgan *et al.*, 2012).

These microorganisms, responsible for cellulose decomposition brings about enzymatic hydrolysis of the complex polymer, i.e the enzyme system which involves group of different enzymes is collectively known as cellulase (Christopherson, 2013). Enzymatic decomposition of cellulose is carried out by using cellulase complex and consists of several stages. Initially, the endo –b-1,4-glucanase breaks glycosidic linkages within the cellulose chain that leads to the formation of fragments of relatively large free ends. Then exo –b-1,4-glucanasecatalyses the cleavage of the chain from the end of the cellobiose disaccharide. And finally, the latter is hydrolyzed to glucose by using b-glucosidase (Mayer, 2002).

Cellulose for industrial use is mainly obtained from wood pulp and cotton. Some animal, particularly ruminants and termites, can digest cellulose with the help of symbiotic microorganism that live in their guts such as *Trichonympha*. In human nutrition, cellulose acts as a hydrophilic bulking agent for faeces and is often referred to as dietary fiber (Guatam *et al.*, 2011). Hydroxyl bonding of cellulose in water produces a sprayable, moldable material as an alternative to the use of plastic and resins. The recyclable material can be made water and fire – resistant. It provides sufficient strength for use as a building material (Balbo and Laurie, 2012). Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, celluloses are widely used in textile industry and in laundry detergents. They have also been used in pulp and paper industry for various purposes and they are even used for pharmaceutical applications.

Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present. Medically, cellulose is used as a treatment for phytobezoars, a form of cellulose bezoar found in the human stomach, and it has exhibited efficacy in degrading polymicrobal bacterial biofilms by hydrolyzing, B- (1-4) glycosidic linkage within the structural ,matrix exo-polysaccahrides of the extra cellular polymeric substance (EPS) (Fleming *et al.*, 2017).

Over the years most researchers were not able to continue with screening of cellulolytic bacteria in the absence of Congo red. Therefore, this research focuses on providing alternative to Congo red for screening cellulolytic bacteria, using different staining reagents which can be readily available.

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METHODLOGY

Sample Collection

The soil samples were collected from different garden locations around Kasarawa village in Sokoto. The soil was dug to a 0-20 cm depth, scooped into sterilized polythene bags, labelled and taken to the laboratory for analysis. Fivefold serial dilution of each soil sample was prepared in sterilized distilled water and 0.1ml of the diluted sample from the fifth test tube (x 10^{-5}) was spread on nutrient agar. The plates were incubated at 37^{o}C for 24 hrs. Pure cultures of selected bacteria isolated were individually maintained on nutrients agar slants at 4^{o}C until used (Nafiu e*t al.*, 2017).

Characterization of Isolates

Characterization of isolates was carried out, which was based on morphological and biochemical tests After incubation for 24 hrs, growth of bacteria colonies were observed on nutrient agar plates, the morphological features of colonies were identified and also gram staining test was carried out to determine the gram reactions of bacteria colonies. The biochemical tests employ in the study were citrate, catalase, coagulase, glucose, gas hydrogen sulphide, lactose methyl red sucrose, urease and voges proskauer and were conducted as described by (Cheesbrough,2006:Oyeleke and Manga 2008).

Screening of Cellulolytic Bacteria

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48hours, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min. at room temperature. One molar Nacl was thoroughly used for counterstaining the plates. And the plates were observed for clear zones around colonies. (Palavesam, 2007). Also the isolates for which the clear zones were observed were also flooded with crystal violet, and counterstained with 1 molar NaCl, allowed to stand for 15minute and then were observed for clear zones around colonies. This procedure was repeated using Lactophenol cotton blue, Safranin and iodine as staining reagents.

RESULTS AND DISCUSSION

The study was conducted in different soil samples from different garden locations of Kasarawa village around the Sokoto state university. the isolates were screened using CMC media and those exhibited cellulolytic activity after flooding with 1% Congo red and counterstained with one molar NaCl were selected for morphological and biochemical characterization and for screening using different staining reagents. The selected isolates were: KS_1, KS_2, KS_3, KS_4, KS_5, KS_6, KS_7, and KS_8.

Morphological Characterization

The morphological features of isolates were determined from parent plates. The results of morphological characteristics of isolates revealed different shape, size, color and elevation for each of the isolates as well as gram reactions were recorded. This was done as a primary method of identification of the given isolates. A total of eight isolates were isolated, six out of the entire total were gram negative organism while the other two are gram positive organisms.

Eight (8) cellulolytic bacteria isolates were isolated in soil excavated from different garden locations of the Kassarawa village around Sokoto State University. Many researchers had reported different morphological characteristics of isolates. This finding is in agreement with that of Nafiu *et al.* (2017), Khiangam *et al*. (2014) who revealed some morphological features of cellulose producers. Also, Kasana *et al*. (2008) reported similar morphological characteristics to those in the finding of this research.

Screening of Cellulolytic Bacteria with Different Staining Reagents

The result of screening of isolates using different staining reagents is shown in table (3) below. In similar study of Kushwaha *et al*. (2012) revealed that *Bacillus subtilis* was the most frequent cellulolytic bacteria found in soils after flooding with Congo red.

Table 1. Morphological Characterization of Isolates from Parent plates.

S/N	Isolate	Shape	Size (mm)	Color	Elevation	Gram reaction
1	KS1	Circular	2.0	Milky	Flat	-ve rod
2	KS2	Circular	2.0	Pale yellow	Raised	-ve rod
3	KS3	Irregular	3.0	Pale yellow	Raised	-ve rod
4	KS4	Circular	1.5	Milky	Raised	+ve rod
5	KS5	Rhizoid	2.0	Milky	Flat	-ve rod
6	KS6	Circular	5.0	Milky	Flat	+ve rod
7	KS7	Circular	2.0	Milky	Raised	-ve rod
8	KS8	Rhizoid	2.0	Pale yellow	Flat	-ve rod

Key: +ve = Positive
-ve = Negative

Biochemical Characterization

The results of biochemical characteristics of given isolates are presented below.

Table 2.

Tests	KS 1	KS 2	KS 3	KS 4	KS 5	KS 6	KS 7	KS 8
Gram Reaction	Gram negative	Gram negative	Gram negative	Gram Positive	Gram negative	Gram Positive	Gram negative	Gram negative
Catalase	+	+	-	+	-	+	+	+
Citrate	-	+	+	+	-	+	+	+
Coagulase	+	-	+	-	+	+	-	-
Glucose	+	+	+	+	+	+	+	+
Gas	-	+	+	+	+	-	+	+
H_2S	-	+	+	-	+	+	+	+
Lactose	+	+	-	-	-	-	-	-
Methyl red	-	-	+	-	-	-	-	+
Sucrose	+	+	+	+	+	+	-	+
Urease	-	-	+	+d	+d	+d	-	+d
Voges Proskauer	+	+	-	+	+	+	+	-

Table 3. Diameter of Clearances Zone (mm) stained with different staining reagents.

		Diameter of clearance zone(mm)				
S/N	Isolates	Congo red	Safranin	Lacto phenol cotton blue	Crystal violet	Iodine
1	KS1	10.0	ND	ND	8.0	ND
2	KS2	14.0	ND	3.0	11.0	ND
3	KS3	10.0	ND	3.0	7.0	ND
4	KS4	21.0	ND	13.0	10.0	5.0
5	KS5	14.0	ND	ND	9.0	ND
6	KS6	17.0	ND	ND	12.0	7.0
7	KS7	6.0	ND	10.0	ND	4 .0
8	KS8	6.0	ND	ND	ND	5.0

Key: ND= No Detected

This means they utilize CMC media as source of nutrient that enable them to degrade cellulase. In this research, six gram negative and two gram positive cellulolytic bacteria were isolated. Likewise Nafi'u *et al*. (2017) reported *Bacillus* species as the predominant bacteria in rumen of camel capable of producing cellulase after staining with Congo red.

Congo red was the staining reagent used by many researchers for screening of isolates capable of producing cellulase. Attempts were made in testing other staining reagents for screening of cellulase producers. In this research, different staining reagents were flooded on CMC media after 48 hours of incubation as substitute to Congo red. wsThe finding of this research revealed that crystal violet, iodine and lactophenol cotton blue can be used for screening of cellulase producers as an alternative to Congo red. This study is consistent with the finding of Hardik *et al*. (2014) used different staining reagents (Iodine, Safranin and Congo red) for determination of extra cellular cellulose activity on CMC agar plates. The results of the study revealed iodine as a good reagent for staining of cellulolytic bacteria. It was noted that staining efficiency is also dependent on the degree of cellulose degradation (Kasana *et al*. 2008; Maki *et al*., 2011). 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)

Also, the finding of Kasana *et al*. (2008) revealed that iodine could be used as a substitute to Congo red for staining cellulolytic bacteria. From Table.3 the result showed that out of 8 cellulolytic bacteria isolated from four different garden soil samples after stained with Congo red, only six of the bacteria isolates produced clearance zones when stained with crystal violet, four produced clearance zones when stained with iodine, four produced clearance zones when stained with lacto phenol cotton blue, and Safranin produced no clearance zones when used to stain the given isolates. This study shows that although Congo red showed stronger ability to stain Cellulolytic bacteria, the reagents: lactophenol cotton blue, iodine and crystal violet can be used as substitute to stain cellulolytic bacteria, and they have been found to be less expensive and more readily available than Congo red and so can be easily used in place of Congo red for the staining of cellulolytic bacteria.

The possible explanation to this finding is that bacteria capable of producing cellulose in the plate can degrade complex polysaccharide like cellulose to smaller unit. Bacteria exhibited halo zones around the colony were degraded polysaccharides and replace with smaller units of carbohydrates compounds. Hardik *et al*. (2014) reported mono and disaccharide dyes cannot be bind efficiently and resulted into a visible clear zone. 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 other reagents. It was also found that Congo red stain inactivates the microbe hence they cannot be used further for any study (Florencio *et al*. 2012; Fujimoto *et al*., 2011).

CONCLUSION

The finding of the present study revealed eight cellulolytic bacteria isolates were isolated from the four soil samples collected from different garden locations. The isolates were further screened for cellulolytic activity using iodine, crystal violet, safranin, and lactophenol cotton blue. Crystal violet produced clearance zones on six of the isolates Lacto phenol cotton produces clearance zones on four of the isolates Iodine produced clearance zones on four of the isolates, Safranin produced no clearance zones on any of the isolates. This study revealed that crystal violet, iodine and lactophenol cotton blue can be used to stain cellulolytic bacteria in the absence of Congo red, thereby resolving the limitation of using Congo red as the only staining reagent for staining cellulolytic bacteria.

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