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Full Length Research Article

PRODUCTION OF CELLULASE ENZYMES BY *BACILLUS* SP AND *PSEUDOMONAS* SP ISOLATED FROM ANTHILL SOIL

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ABSTRACT
Cellulase turns the most widespread biopolymer and biologically sustainable resource, 'cellulose,' into reducing sugar. The study aimed at producing cellulase enzymes by bacteria isolated from anthill soil. Cellulase-producing bacteria were isolated from anthill soil using Carboxymethyl cellulose (CMC) medium. The isolates were screened for cellulase production by cultural, morphological, biochemical and sugar fermentation tests. Optimization of the fermentation medium for maximum cellulase production was carried out by one factor at a time (OFAT). Data obtained were analysed with the analysis of variance (ANOVA) using SPSS 2007, version 16.0. The identified *Pseudomonas* sp, *Staphylococcus* sp, *E. coli* and *Bacillus* sp were isolated with highest potential of cellulase production. The culture conditions like pH, temperature, carbon sources and nitrogen sources were optimized. The optimum conditions found for cellulase production were 40^{o}C at pH 8.5 with maltose as carbon source and yeast extract as nitrogen source. The highest activity and stability of cellulase enzymes between neutral to alkaline pH and high temperature.

Keywords: *Pseudomonas* sp, *Bacillus* sp, Cellulase and Anthill Soil.

INTRODUCTION

Cellulases are enzymes formed by many micro-organisms such as bacteria, fungi and protozoans. Cellulase turns the most widespread biopolymer and biologically sustainable resource in the world, 'cellulose,' into reducing sugar (Singh *et al.,* 2021). Cellulases can efficiently hydrolyse cellulose into a glucose unit through the complementary activities of three major forms of cellulase enzymes, particularly endoglucanases (EC 3.2.1.4), exoglucanases, along with cellobiohydrolases (CBHs) (EC3.2.1.91), as well as β-glucosidase (BG) (EC 3.2.1.21) (Zhang, 2021). While cellulase productions are found in fungi, bacteria are increasingly involved in the production of cellulase because they have faster growth rate than fungi (Shaikh *et al*., 2010). Some bacterial species such as *Cellulomonas* species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* have cellulolytic property. The composition of enzyme with microbe can vary across different biomass degrading ecosystems, based on the original biomass source and environmental influences (Mahjabeen *et al*., 2016).

Cellulose is a D-glucose unit homopolymer connected by β-1, 4 links (Horn, 2012). On the basis of energy content, it is the most available renewable natural resource as well as a relatively cheap energy source (Zhang, 2021). Cellulose is also the principal and structural element of plants which is mostly degraded by the enzyme cellulase. One chain end of the cellulose structure comprises of a fragment of D-glucopyranose where in the anomeric carbon atom is included in a glycosidic interaction, whereas the remainder on the other end of the chain has a loose anomeric carbon, and therefore both ends of the chain are chemically distinct giving the molecule a polarity (Pe'rez *et al*., 2002).
Cellulases currently constitute a major product on the industrial enzyme market around the world. The growing concern about the depletion of unrefined petroleum (crude oil) as well as greenhouse gas emissions has evidently prompted the development of bioethanol from lignocellulosic materials, in particular through the enzymatic hydrolysis of lignocellulosic materials (Singh *et al.,* 2021). Taking the above points into consideration, it is crucial to gain knowledge of cellulase producing microbes, which is of significant importance with respect to nutrition, biodegradation, biotechnology and the carbon-cycle. Providing insights into the metabolism, physiology and functional enzyme systems of the cellulolytic bacteria that are responsible for the largest flow of carbon in the biosphere. Therefore, the aim of the study was to produce cellulase enzymes from *Bacillus* sp and *Pseudomonas* sp isolated from anthill soil.

MATERIALS AND METHODS

Sampling Site and Sample Collection

Soil samples used for the study were obtained from three separate anthill sites in Chikun Local Government Area of Kaduna State. Each sample was obtained at a depth of 15 cm from the sampling locations which include Juji, Sabon Gaiya and Kakau in Chikun L.G.A. of Kaduna State. The soil samples were then taken in Ziplock bags, transported into the laboratory for analyses and placed at 4^{o}C refrigerator for further use.

Isolation of Cellulase Producing Bacteria

Traditional spread plate techniques was used after serial dilution to isolate cellulolytic bacteria on agar plate. After that 0.1 mL was inoculated on the Carboxymethyl cellulose (CMC) containing: 1.0% Carboxymethyl cellulose (CMC) medium, 1.0% peptone, 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_2O$ and 0.2% gelatin and 1% agar (Gupta *et al*., 2012). Different discrete colonies were found on the agar plate and were selected further.

Primary Screening of Cellulose Degrading Bacteria

Carboxymethyl cellulose (CMC) agar plates were soaked with Congo red solution for approximately 5 minutes and then allowed to stand at room temperature. The respective broth culture which showed clear zone were streaked on CMC agar media and incubate at 37°C for 72 hours (Gupta *et al*., 2012). The CMC hydrolysis clear zone and colony diameter ratio were measured and reported in millimetre (mm).

$$\text{Clear zone diameter(mm)} = \frac{\text{Clear zone}}{\text{Colony diameter}}$$

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Morphological Characteristics of Cellulase Producing Bacteria

Bacterial isolates were pre-sumptuously characterized by morphology, as well as Gram staining technique. The parameters investigated included colonial morphology (evaluated for size, pigmentation, form, margin and elevation)

Biochemical tests such as catalase, coagulase, oxidase, citrate, urease, voges proskauer and motility were carried out to confirm the isolate (Cheesebrough, 2002).

This was a test conducted to confirm the ability of bacterial isolates to utilize various sugars. The sugars included cellobiose, glucose, galactose, mannitol, fructose, lactose, xylose, DNase and sorbitol (Olutiola *et al*., 2000).

Inoculum Preparation of the Bacterial Isolates

For inoculum preparation, the two isolates coded AS1 and AS3 were each cultured in 5 mL of sterile Luria Broth medium. The flasks were taken at 200 rpm for 24 h at 37°C. The OD of cell suspension was read at 600nm and recorded as 0.5 as inoculum concentration. Inoculum of each bacteria isolate, (3%) was inoculated into 50 mL of two separate production media.

Optimization of Process Parameters for Maximum Cellulase Production

Effect of pH on Enzyme Production*:* To determine optimal pH, the bacteria was inoculated in nutrient broth and incubated for 3days and the supernatant was filtered, centrifuged and treated with different pH ranges from 5.5, 6.5, 7.5 and 8.5. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The supernatant was assayed for cellulose activities (Irfan *et al*., 2012).

Effect of Temperature on Enzyme Production: To determine optimal temperature, the bacteria was inoculated in nutrient broth and incubated for 3days and the supernatant was filtered, centrifuged at 10,000 rpm for 10 minutes and treated with varying temperatures of 37°C (room temperature), -14°C (refrigerator), 40°C (incubator) and 45°C (hot air oven). The supernatant was further used for cellulase activities (Irfan *et al*., 2012).

Effect of Carbon and Nitrogen Sources on Enzyme Production: In order to determine the optimized carbon source, various carbon sources such as dextrose, fructose, sucrose, and maltose, with different nitrogen sources both organic and inorganic such as (ammonium nitrate, ammonium chloride, urea and yeast extract) were added individually at different concentrations (ranging from 0.5, 1.5, 2.5 and 3.5% w/v) in to the broth medium. The organism was incubated and allowed to grow (3 days) until its log phase at 37°C (Irfan *et al*., 2012). Then, the supernatant was used for cellulase actitivies.

Crude Enzyme Production by Submerged Fermentation Process

The bacterial isolates A and C were used in the cellulase production under optimum conditions. Carboxymethylcellulose (CMC) broth containing Carboxymethylcellulose (CMC) 10.0g; Tryptone 2.0g%; $KH_2PO_4$1.0 g; $Na_2HPO_4$1.0 g; $MgSO_4.7H_2O$ 1.0 g%; $CaCl_2.2H_2O$ 1.0 g%; $FeSO_4$.7H2O 0.2 g. The media were inoculated with 2.5 mL (5%) of the selected bacterial isolate from the prepared inoculum and incubated at 37°C for 48hrs under shaking condition (at 150 rpm). After fermentation, the fermented broth was centrifugated to remove undesired materials at 12000 rpm for 10 minutes at 4°C (Gupta *et al*., 2012; Shaikh *et al*., 2010).

Cellulase Enzyme Assay

Reducing Sugar Estimation by Dinitrosalicylic acid (DNSA) Method

Initially, 0.2- 1.2mg/mL concentrations of glucose solutions were prepared at different ranges of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2. One (1) mL of 0.05M citrate buffer (pH 5.5) was added in all the test tubes followed by 3 mL of Dinitrosalicylic acid (DNS) reagent (Miller *et al*., 2010; Shaikh *et al.,* 2010). Glucose liberation was estimated using glucose calibration curve (Shoham *et al*., 2019) and enzymatic activity was determined by the following equation (Islam *et al*., 2014):

$$\text{Enzyme activity (U/mL)} = \frac{\text{Reducing sugar (product concentration) X 1000 X dilution factor}}{\text{Molecular weight of glucose X incubation time (minute)}}$$

Characterization of Purified Cellulase Enzymes

Effect of pH on Activity of Crude Cellulase: The optimum pH for the crude enzyme was determined by incubating crude enzyme with substrate (1% CMC) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0 to 4.0), 0.05 M sodium phosphate buffer (pH 5.0 to 6.0), 0.05 M Tris-HCl (pH 7.0 to 8.0) and 0.05 M glycine-NaOH (pH 9.0 to 10.0) (Miller *et al*., 2010; Yin *et al*., 2017). The optical density (OD) was measured at 600nm in a spectrophotometer.

Effect of Temperature on Activity of Crude Cellulases: The effect of temperature on activity of cellulase was determined by incubating crude enzyme with 1 %CMC in 0.05 M phosphate buffer (pH 7.0) at temperatures including 20 °C,30 °C,40 °C,50 °C,60 °C,70 °C and 80 °C. Cellulase activity was assayed by DNS method as described above according to Miller *et al*. (2010). The optical density (OD) was measured at 600 nm in spectrophotometer.

Data Analysis

After the study has been completed, experimental data were evaluated using the Statistical Package for Social Sciences (SPSS, 2007).

RESULTS

Isolation and Screening for CMCase Activity

The isolate appeared as white colonies on CMC agar. After staining with 0.3% Congo red and 1N sodium chloride, halo zone was detected on CMC plate (**Figure 1**). Isolate from this source was selected on screening media by observing zone of clearance after spraying Congo red stain.

In the five isolate, the cellulolytic activity of organism was determined with diameter of hydrolytic zone around growing colony on CMC agar, measured and recorded on mm **Table 1).** Isolate code AS1, AS2, AS3, AS4 and AS5 were found to give maximum zones of cellulose hydrolysis of 35.0mm, 20.0mm, 40.0mm, 20.0mm and 0.0mm. Biochemical characteristics of the isolated bacteria were presented in **Table 2.** The isolated bacteria exhibited` different reactions to various biochemical test reagents and sugars fermentation test. They were tested for gram reaction, catalase test, indole, citrate, motility, vp, oxidase test and different sugar fermentation.

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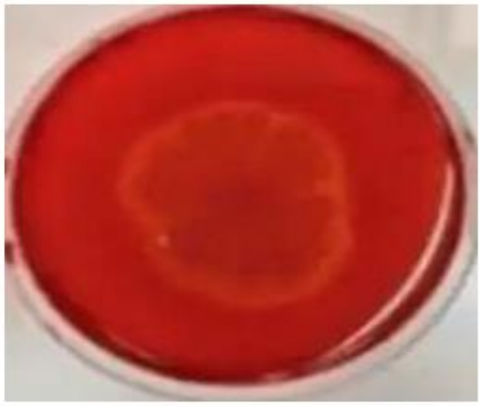
AS1



AS3

Figure 1: Zone of Clearance on Screening Media after Congo Red Staining due to Hydrolysis of Cellulose by Bacterial Isolates containing 1% CMC

Table 1: Mean Zone Diameter of Hydrolyses Produced by Cellulase-Producing Bacteria on CMC Agar from Anthill Soil

Isolate Code	Mean Zone Diameter of Hydrolysis on CMC
AS1	35.0
AS2	20.0
AS3	40.0
AS4	20.0
AS5	0.0

Key: CMC = Carboxymethylcellulose

Table 2: Biochemical characterization of the Bacteria Isolated from the Anthill Soil

S/N	IC	Gram	Cell Morphology	Citrate	Catalase	Oxidase	Methyl Red	Voges Proskauer	Motility	Urease	Cellobiose	Fructose	Glucose	Lactose	Galactose	Xylose	DNase	Probable organisms
1	AS1	+	Rod	+	+	+	-	+	+	-	+	+	+	+	+	+	+	*Bacillus sp*
2	AS2	-	Rod	-	+	+	+	-	+	-	-	-	+	+	+	+	-	*E. coli*
3	AS3	-	Rod	+	+	+	-	+	+	-	-	-	-	-	-	-	-	*Pseudomonas sp*
4	AS4	+	Cocci	+	+	+	+	+	-	+	-	+	+	+	+	-	+	*Staph. Sp*
5	AS5	-	Rod	+	+	-	-	+	-	+	+	+	+	+	+	+	-	*Klebsiella sp*

Key: S/N = Serial number**, IC =** Isolate code**, + =** Positive, **- =** Negative**, sp =** specie

Optimization of Culture Conditions for Cellulase Production

The optimum conditions for isolate AS1 and AS3 were observed in the substrate. The maximum enzyme activity was recorded at a pH-7.5-8.5with yeast extract as nitrogen source and maltose as carbon source at 40ºC (**Table 3**). The optimized culture condition was used for the bulk production process.

The optimum conditions suggested by OFAT using *Bacillus* sp. shows the maximum cellulose activities to be 5.73 IU/mL under 8.5 /pH, 40 ^{O}C temperature with maltose and yeast extract as carbon and nitrogen source (**Table 4**).

The optimum conditions suggested by OFAT using *Pseudomonas* sp. shows the maximum cellulose activities to be 6.71 IU/mL under

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7.5 (pH) 40 ^{0}C (temperature) with maltose and yeast extract as carbon and nitrogen source (**Table 5**).

The un-optimum conditions base *Bacillus* sp. and *Pseudomonas* sp. shows the maximum cellulase activities w`ere 4.71 IU/mL (*Bacillus* sp) and 5.21 UI/mL (*Pseudomonas* sp) under 8.5 (pH), 40^{o}C (temperature) with sucrose and ammonium nitrate as carbon and nitrogen source (**Table 6**).

Characterization of Purified Cellulase Enzymes

Effect of pH on enzyme activity was studied by measuring enzyme activity at varying pH values using suitable buffers. It is evident from **Figure 2** that the optimum pH for maximum activity of the purified enzyme was pH 7.5- 8. The enzyme was stable over a broad range of pH values, optimum being pH 8.0.

Effect of temperature on the activity of the purified enzyme is shown in **Figure 3**. The maximum activity was recorded at 50°C in both isolate AS1 and AS3 which declined thereafter. An earlier study has reported optimum cellulase activity from isolate AS1 and AS3 at 30°C to 35°C.

Table 3: Optimized Culture Condition Base on Different Parameters

Parameter	Optimization Level	Cellulase Activities UI/ mL	
		Bacillus sp.	*Pseudomonas* sp.
pH	5.5	3.67	3.47
	6.5	4.11	4.46
	7.5	6.67	**6.87**
	8.5	**6.70**	6.79
Temperature			
	14	4.19	4.87
	37	4.89	5.21
	40	**5.72**	**6.48**
	45	4.39	5.27
Carbon Source			
	Dextrose	4.15	5.72
	Fructose	4.55	5.32
	Maltose	**5.39**	**6.39**
	Sucrose	4.35	5.52
Nitrogen Source			
	Yeast Extract	**5.54**	**6.65**
	Ammonium Chloride	4.25	5.34
	Ammonium Nitrate	3.42	5.81
	Urea	3.23	5.47

Table 4: Optimum Cellulase Activities at Optimized Level for *Bacillus* sp

Parameter	Optimum Level	Activities IU/Ml
Ph	8.5	
Temperature	40 °C	
Carbon Source	Maltose	5.73
Nitrogen Source	Yeast Extract	

Table 5: Optimum Cellulase Activities at Optimized Level for *Pseudomonas* sp

Parameter	Optimum Level	Activities IU/ mL
pH	7.5	
Temperature	40 °C	
Carbon Source	Maltose	6.71
Nitrogen Source	Yeast Extract	

Table 6: Cellulase Activities at Unoptimized Levels for *Bacillus* sp and *Pseudomonas* sp

Parameter	Optimum Level	Activities IU/mL	
		Bacillus sp	*Pseudomonas* sp
pH	8.5		
Temperature	40 °C		
Carbon Source	Sucrose	4.71	5.21
Nitrogen Source	Ammonium Nitrate		

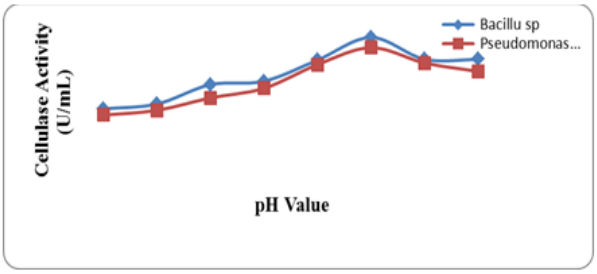

Figure 2: Effect of pH on Cellulase Activity *Bacillus*sp and *Pseudomonas*sp

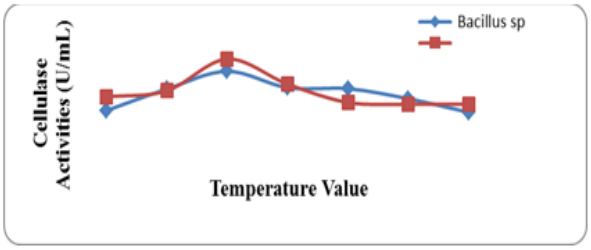

Figure 3: Effect of Temperature on Cellulase Activity from *Bacillus* sp and *Pseudomonas* sp

DISCUSSION

The present study aimed at isolating, identification and producing cellulase producing bacteria that can hold a high demand in the environmental clean-up to overcome the limitation of cellulosic waste management and reduce the cost of waste control. Out of 5 colonies that were isolated from the anthill soil sample, 2 colonies coded AS1 and AS3 had the highest capability of cellulose hydrolysis (Shanmugapriya *et al*., 2012). Two isolate from this source was selected on screening media by observing zone of clearance after spraying Congo red stain to indicate the cellulolytic activity of organism, diameter of hydrolytic zone around growing colony on CMC agar was measured. The formation of clear zone around the colony may be due to the ability of the isolate to degrade carboxymethyl (CMC) present in the media for cellular development (Hatami *et al*., 2008). This highlights the viability of utilizing anthill soil as a source for isolating bacteria with cellulase production capabilities, specifically *Bacillus* sp, and *Pseudomonas* sp. which have demonstrated high ability for cellulase production (Shanmugapriya *et al*., 2012).

The best cellulase producing isolates were tentatively identified as *Bacillus* sp. and *Pseudomonas* sp. The media and culture conditions like carbon source, nitrogen source, temperature and pH were further optimized for the maximum production of cellulase in a stepwise manner. The maximum cellulase activity for *Bacillus* sp. and *Pseudomonas* sp. was found when medium was supplemented with different carbon and nitrogen source in an optimum condition. Hence it can be concluded that that cellulase is an inducible enzyme, whose production depends upon the presence of substrate which also acts as its inducer. The effect of different nitrogen sources like ammonium chloride, yeast extract, ammonium nitrate and urea each with 1% (w/v) concentration in media was observed on cellulase production after 72 hours of incubation. The maximum cellulase activities was within pH 7-8 at 40-45^{o}C respectively, with maltose and yeast extract as a major source of carbon and nitrogen. This could be as a result of the utilization of variety of other carbohydrates in addition to cellulose (Rajoka and Malik, 2017). The presence of external nitrogen source is essential in the fermentation media during extracellular enzyme production for effective utilization of soluble carbohydrates. The use of organic nitrogen sources as compared to inorganic sources for maximum cellulase production was found to be more suitable for maximum cellulase production (Ariffin *et al*., 2008).

The maximum cellulose activities for optimum yield was observed at 40-45°C respectively. There was reduction in cellular activity, with further increase in temperature. The increase in temperature, affect cellulase activity due to thermal denaturation of enzymes. These results clearly indicate that temperature 45°C is optimum temperature for cellulase activity (Pachuri *et al*., 2020). Similar results of maximum cellulase production were achieved after 72 hours of incubation at 37°C from *Bacillus* as reported by Shankar and Isa, (2011). The optimum pH for cellulase production was observed at pH 8.5 for *Bacillus* sp. with maximum activity of 6.70 and 6,87 IU/mL for *Pseudomonas* sp. respectively. The optimum pH for cellulase production was observed at pH 8.5 for *Bacillus* sp with maximum activity of 6.70 and 7.67IU/mL for *Pseudomonas* sp respectively. The cellulase showed optimum activity at pH 7.5-8. These results indicated that pH 7.5-8 is optimum pH for cellulase activity as reported by Ali *et al.* (2018) who reported maximum yield of cellulase from *Aspergillus niger* at 40ºC, respectively.

Conclusion

Cellulase enzyme producing *Bacillus* sp. and *Pseudomonas* sp. with putative cellulase activity were successfully isolated and screened from anthill soil sample. The media was optimized for cellulase production and the *Bacillus* sp. and *Pseudomonas* sp. produces maximum cellulase at pH 7, temperature 35ºC with maltose and yeast extract as the major carbon and nitrogen sources. Cellulase characterization showed an optimum activity at pH 5, temperature 45ºC. The optimization of production medium is important for increasing productivity and reducing costs strategy.

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