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Cellulolytic Potentials of *Bacillus* Species CDS4 Isolated from Cow Dung Samples



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ABSTRACT

Laboratory study investigating the potentials of cow dung samples to harbor biotechnologically-relevant bacteria capable of elaborating cellulase was conducted. The cellulolytic bacteria were isolated from cow dung samples on 1% Carboxymethyl cellulose (CMC) agar plates for 48 hours. The plates were flooded with 0.1% Congo red solution for 15 minutes and finally washed with 1M NaCl solution to visualize zones of clearance exhibited by the cellulolytic bacteria. Results of the primary and secondary screen tests on CMC agar and broth respectively revealed that only 5 out of the 11 (45.45%) bacterial isolates demonstrated their potentials of elaborating the required enzyme. Morphological and physiological characterization of the isolates revealed that they were all species of *Bacillus*, underlying the ubiquity and versatility of this genus. Although, productivity screening revealed low tendencies of 4 of the 5 bacterial isolates to release the extracellular enzyme into the medium, only *Bacillus* species CDS4 was the most productive, demonstrating 39.53% crude cellulase productivity. The amount of cellulase elaborated by *Bacillus* species CDS4 was 128.52 U/ml using ammonium sulphate and sucrose as preferred sources of nitrogen and carbon (respectively) at an incubation time of 72 hours at 30°C and pH 6.5. The results suggest a tight control of the isolates over cellulase production and the synergistic role *Bacillus* species play in the metabolism of cellulose in the host.

1. INTRODUCTION

Municipal solid wastes partly composed of cellulosic wastes such as papers, wood, agricultural residues and cardboards is becoming a serious unsightly problem in developing countries. Celluloses are regarded as the most important renewable resource for bioconversion (Saraswati *et al.*, 2016). Thus, it has become an economic interest to develop effective methods to hydrolyse cellulosic biomass (Javalkar and Alam, 2013). Cellulose, the most abundant polymer in nature is a linear polysaccharide of up to 15000 D-glucose residues, having β -1,4- glycosidic linkages and often occurs in combination with other materials like lignin and hemicelluloses (Verma *et al.*, 2012). It may undergo either cellulolysis (incomplete break down into smaller polysaccharides called cellodextrins) or hydrolysis (complete breakdown into glucose) (Verma *et al.*, 2012). Abundant availability of the cellulose makes it an attractive raw material though most of the cellulosic wastes are often disposed off by biomass burning (Gupta *et al.*, 2012; Jurak *et al.*, 2018). Therefore, recycling of such cellulosic wastes can decrease greenhouse effect (Kazaragis, 2015) and can be used as one of the main renewable sources of energy (Zhou *et al.*, 2018). Cellulose can be degraded by cellulases which are enzymes that catalyse the hydrolysis of β -1,4-glycosidic bonds of cellulose into shorter-chain polysaccharides such as cellodextrin, cellobiose and glucose. Microbial cellulases are inducible enzymes which are synthesized by some microorganisms (mostly bacteria and fungi) either as cell-bound or extracellular enzymes during their growth on cellulosic materials (Kanokphorn *et al.*, 2011).

Cellulases have attracted much interest because of their extensive applications in various industries. Traditionally, they are useful in food and brewery production, animal feed processing, detergent production and laundry, textile processing and paper-pulp manufacturing (Shanmugapriya *et al.*, 2012; Kuhad *et al.*, 2017; Karmakar and Ray, 2018). The applications of cellulases in cellulose biorefinery for producing fermentable sugars are expected to rapidly increase in the foreseeable future due to the problems in sustainable supply of fossil fuel and the increased demand for production of biofuels and chemicals from renewable resources (Juturu and Wu, 2017). The application of cellulases in textile, food, detergent, leather and paper industries demands identification of highly stable enzymes active at extreme pH and temperature (Cherry and Fidantsef, 2013; Abdelnasser and Ahmed, 2015). The search for a novel and improved bacterial strain with a high cellulase productivity, better activity and high stability against temperature and pH might make the process more economical. Increasing demand, limited supply and rising cost of fossil fuels has led to utilization of renewable

resources like cellulose for the production of alternative energy (Taechapoempol *et al.*, 2011; Faridha *et al.*, 2013). There is an increasing interest in the production of biofuel using cellulosic biomass as a renewable source of energy by breaking them into sugars using cellulase enzymes (Demain *et al.*, 2015). The production of fuels and chemicals from cellulosic substrates using cellulases would reduce the use of fossil fuels and decrease air pollution (Fernando *et al.*, 2016; Zhou *et al.*, 2017). The hydrolysis of cellulose to glucose and soluble sugars has thus become a subject of intense research and industrial interest (Sreena *et al.*, 2015). Hence, the aim of this study was to isolate and screen for efficient cellulose- producing bacteria from cow dung and to optimize the conditions for the production of cellulase.

2. MATERIALS AND METHODS

2.1 Sample collection

Cow dung and sawdust samples were collected in sterile containers using a sterile spatula from different locations within the cowshed at the University of Calabar farm. Ten different samples were collected and then later pooled into one composite sample. After collection, the samples were wrapped with black polythene bags and put in an ice-packed cooler and then transported to the laboratory for microbiological analyses.

2.2 Sample preparation and isolation of bacteria

Cow dung samples (10) were each weighed (10g) and added to 90ml sterile distilled water in 250ml Erlenmeyer flask. The diluted samples were agitated to ensure total dissolution for homogeneity and then allowed to stand for 10 minutes. For the purpose of isolation and enumeration, samples were given 10-fold dilutions. Appropriate dilutions were plated by the pour plate technique unto freshly prepared Carboxymethylcellulose (CMC) agar medium containing 1.0% peptone, 1.0% Carboxymethylcellulose (CMC), 0.2% K_2HPO_4 , 1.0% agar-agar, 0.3% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$ and 0.2% gelatin, with the pH adjusted to 7 as described by Yin *et al.*, (2010). Preparations were made in duplicates and plates incubated at room temperature ($28 \pm 2^\circ C$) for 48 hours.

2.3 Screening for cellulolytic bacteria

After 48 hours, all plates were flooded with 1% Congo red and allowed to stand for 15 minutes at room temperature. Sodium chloride (1M NaCl) was used for washing off the Congo red dye

from all the plates. Appearance of clear zones on the plates indicated cellulose hydrolysis. All cellulase-positive isolates were purified by repeated sub-culturing onto nutrient agar medium and preserved for secondary screening.

The potentials of all the cellulase-positive isolates were then evaluated for enzyme productivity. Positive isolates were each inoculated onto Carboxymethylcellulose (CMC) agar by the straight streak plate technique after reactivation on nutrient agar. All preparations were made in triplicates and plates incubated at room temperature for 48 hours. After 48 hours, plates were flooded with 1% Congo red and allowed to stand for 15 minutes. Thereafter, plates were washed with 1M NaCl to decolorize the stain and to reveal zones of cellulose hydrolysis. Total clear zone diameters as well as colonial diameters of bacteria were measured by means of a meter rule. Data obtained were used to determine cellulase-productivity potentials of each positive bacterium as described by Ariffin *et al.* (2007) and expressed as percent productivity. Productivity potential of 75% and above was set as cut-off mark and only isolates that passed same were selected for further studies.

2.4 Characterization of the selected bacteria

All pure bacterial cultures that tested positive to the cellulase production screen test were identified after morphological, biochemical and physiological characterization according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

2.5 Submerged fermentation process

For preparation of standard inoculum, selected isolates that showed a maximum zone of hydrolysis were cultured in 20ml of freshly prepared Luria Bertani broth. This was used as inoculum for the production medium. The composition of the production medium was (g/l); 0.03% MgSO₄, 0.2% K₂HPO₄, 1% glucose, 0.25% (NH₄)₂SO₄, 1.0% peptone, 1% CMC, 1% 1M NaCl and pH adjusted to 7. A volume of 50ml of production medium was dispensed into 250ml Erlenmeyer flasks. Flasks were sterilized by autoclaving and inoculated with 2% of 18 hours old broth culture of the most productive bacterium obtained after secondary screening and incubated at room temperature (28±2°C) on a rotary shaker operating at a speed of 150 rpm for 72 hours.

2.6 Protein detection and quantification by the Bradford method

Using bovine serum albumin (BSA) as standard, various concentrations (5 to 100 μ g) of the standard protein, bovine serum albumin, obtained from National Biochemical Cooperation were prepared in 0.15M NaCl as described by Bradford (1976).

Coomassie Brilliant Blue G-250 was prepared by dissolving 100mg of it in 50ml of 95% ethanol. To this solution, 100ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1L. Protein solution containing 5 to 100 μ g proteins in a volume up to 0.1ml was transferred into 13 x 125mm test tubes. The volume in each test tube was adjusted to 0.1ml with phosphate buffer (0.05M; pH 7.0). Five millilitre (5ml) of protein reagent (Coomassie Brilliant Blue G-250) was added to each test tube and the contents mixed by inversion. Development of blue colour was indicative of protein presence. The absorbance at 595nm was measured after 2 minutes in 3ml cuvettes against a blank reagent already prepared from 0.1ml of phosphate buffer (pH 7) and 5ml of protein reagent for intensity quantification. The weight of protein was recorded and later used to plot against the corresponding absorbance resulting in a standard curve used to determine the concentration of protein in the unknown samples. Thereafter, 0.1ml of crude enzyme solution was transferred into 13 x 125mm test tubes and the volume adjusted to 0.1ml with the phosphate buffer (pH 7). One millilitre (1ml) of protein reagent was added to the test tubes and the contents mixed as in the standard method. Development of blue colour as in the standard indicated protein presence in the crude enzyme broth. Absorbance at 595nm was measured against reagent blank prepared from 0.1ml of the appropriate buffer as in the standard method. Amount of protein in crude enzyme solution was read off the standard curve prepared as earlier described.

2.7 Selection of appropriate major nutritional sources for protease production

2.7.1 Effect of carbon source on cellulase production

To identify the suitable carbon source for cellulase production, six different carbon sources including mannitol, glucose, sucrose, fructose, lactose, and starch were used to replace the carbon source (CMC) of the production medium. The assay was carried out after 72 hours of incubation on a rotary shaker agitating at 150 rpm.

2.7.2 Effect of nitrogen source on cellulase production

The production of cellulase was optimized by screening different nitrogen sources separately. Five different nitrogen sources were sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride and ammonium sulphate. The nitrogen sources were added at 0.1% dry concentration in production medium.

2.7.3 Effect of incubation time on cellulase production

Different incubation times (6, 12, 24, 48, 72, 96, 120 hours) were employed to study their effect on the cellulase production. The culture filtrates were collected at respective time intervals and assayed.

2.7.4 Effect of temperature on cellulase production

The production was carried out at different temperatures such as 20, 25, 30, 35, 40, 45, 50°C to study their effect on cellulase production for 72 hours. The culture filtrates were then collected and assayed.

2.7.5 Effect of pH on cellulase production

The pH of the production medium was adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 with 1N NaOH and 1N HCl. The production was carried out at 30°C to study their effect on enzyme production.

3. RESULTS

Since most of the natural wastes are degraded by the native microbes present in it, the present study focus on analysing the microbes present in cow dung for their ability to produce cellulase. The result of the primary isolation and screening of bacteria for cellulase producing abilities are presented in Table 1. The result reveals that only 5 (45.45%) out of 11 morphologically distinct colonies isolated from the composite cow dung sample could pass the primary screening as cellulase producing bacteria. When subjected to secondary screening for productivity assessments as shown in Table 1, CDS4 was found to be the most productive of the isolates in terms of cellulase production where it demonstrated 39.53% productivity.

The results of the cultural, biochemical characterization and sugar fermentation profile of the cellulase-positive bacteria are presented in Table 2. The Table reveals that all the isolates were

species of the genus *Bacillus*, a bi-polarly stained Gram positive aerobic rod shaped bacterium. Table 3 presents the results for the production of cellulase in liquid media by *Bacillus* sp. CDS2, *Bacillus* sp. CDS4 and *Bacillus* sp. CDS8. The result reveals that *Bacillus* sp. CDS4 produced the highest amount of cellulase in submerged culture where 6.82µg/ml was produced. Figure 1 is the calibration curve for the estimation of crude protein in test solutions using the linear equation obtained from the plot. Figures 2 to 6 present the effects of carbon sources, nitrogen sources, incubation time, temperature and pH on cellulase production by *Bacillus* sp. CDS4. The results showed that *Bacillus* sp. CDS4 was able to achieve an optimum enzyme activity using sucrose and ammonium sulphate as sources of carbon and nitrogen at a temperature of 30°C, pH of 6.5 and an incubation period of 72 hours.

Table 1
Cellulolytic potentials of cow dung bacterial isolates

S/N	Isolate code	Total zone diameter (mm)	Colonial diameter (mm)	Productivity (%)
1	CDS1	10.0±1.4	8.7±1.2	14.94
2	CDS2	12.0±1.2	10.0±1.4	20.00
3	CDS4	12.0±1.3	8.6±1.0	39.53
4	CDS6	11.0±1.2	9.3±1.3	18.28
5	CDS8	9.0±1.2	7.5±1.2	20.00

CDS: Cow dung samples

Table 2
Characterization of cellulase-producing bacterial isolates

Isolate code	Pigmentation	Consistency	Margin	Form	Gram reaction	Bacterial shape	Endospore	Spore location	VP	IMR	Nitrate	Starch	Catalase	Indole	Citrate	Motility	Glucose	Lactose	Sucrose	Probable isolates	
CDS1	Cream	Mucoid	Entire	Round	+	Rods in chains	+	Sub-terminal	+	-	+	+	+	-	+	+	+	+	+	+	<i>Bacillus</i> sp
CDS2	Cream	Moist	Entire	Round	+	Rods	+	Sub-terminal	+	-	+	+	+	-	+	+	+	-	+	+	<i>Bacillus</i> sp
CDS4	Cream	Moist	Entire	Round	+	Rods	+	Central	+	-	+	+	+	-	+	+	+	-	+	+	<i>Bacillus</i> sp
CDS6	Cream	Mucoid	Entire	Round	+	Long rods	+	Central	+	-	-	+	+	-	+	+	+	+	+	+	<i>Bacillus</i> sp
CDS8	Cream	Mucoid	Entire	Round	+	Long rods	+	Central	-	+	-	+	+	-	+	+	+	+	+	+	<i>Bacillus</i> sp

+: Positive - : Negative ±: Variable

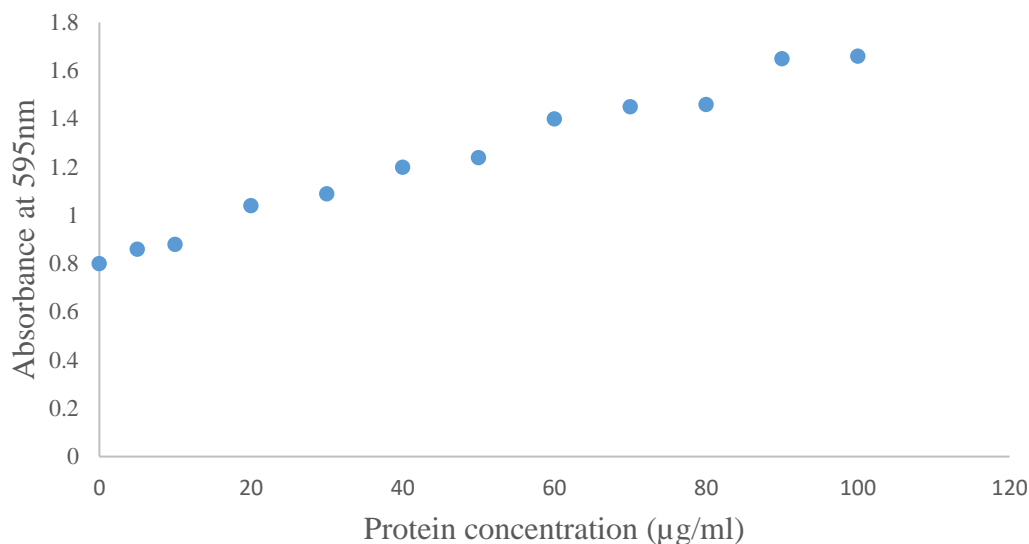


Figure 1: Calibration curve for enzyme quantification in crude cellulase solution using bovine serum albumin as standard

Table 3

Cellulase production potentials of isolates in submerged culture

S/N	Isolates	OD ₅₉₅ /Amount of cellulase
1	CDS2	0.871/0.47±0.4
2	CDS4	0.926/6.82±0.3
3	CDS8	0.884/1.99±0.6

OD₅₉₅: Optical density at a wavelength of 595nm

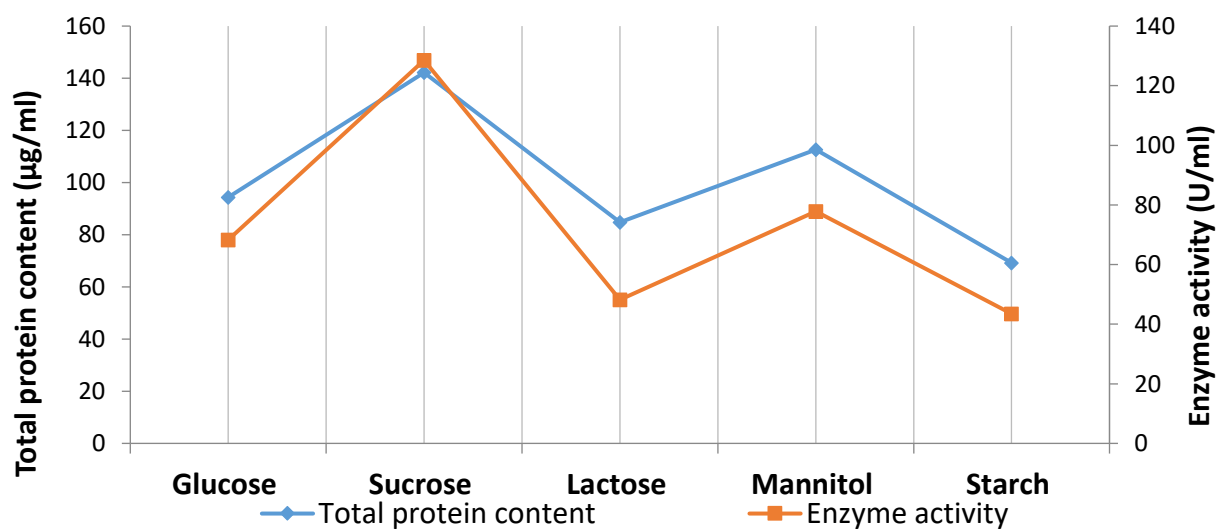


Figure 2: Effect of different carbon sources on cellulase production by *Bacillus* sp. CDS4

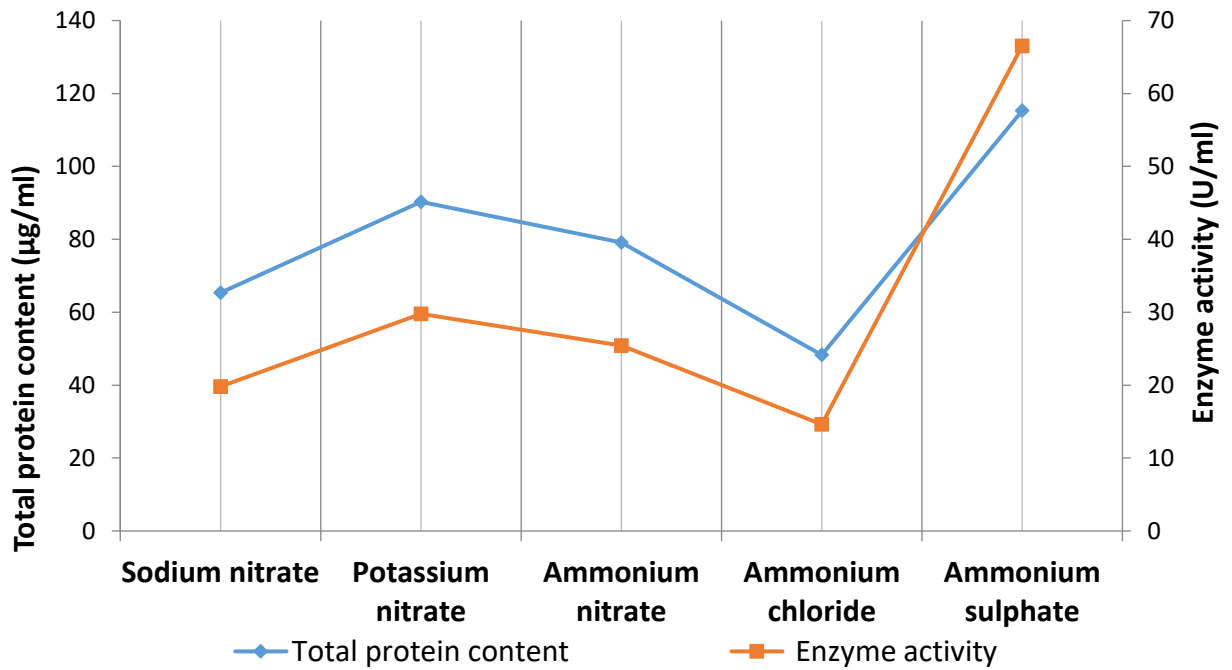


Figure 3: Effect of different nitrogen sources on cellulase production by *Bacillus* sp. CDS4

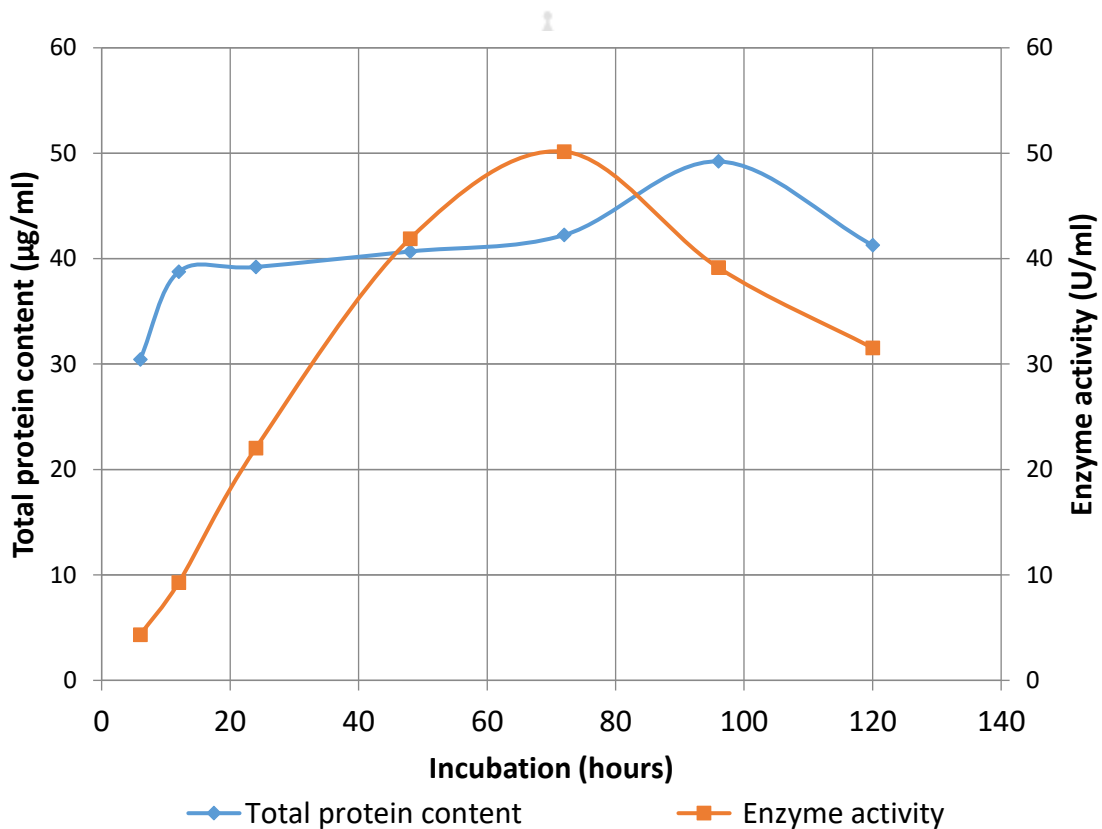


Figure 4: Effect of incubation time on cellulase production by *Bacillus* sp. CDS4

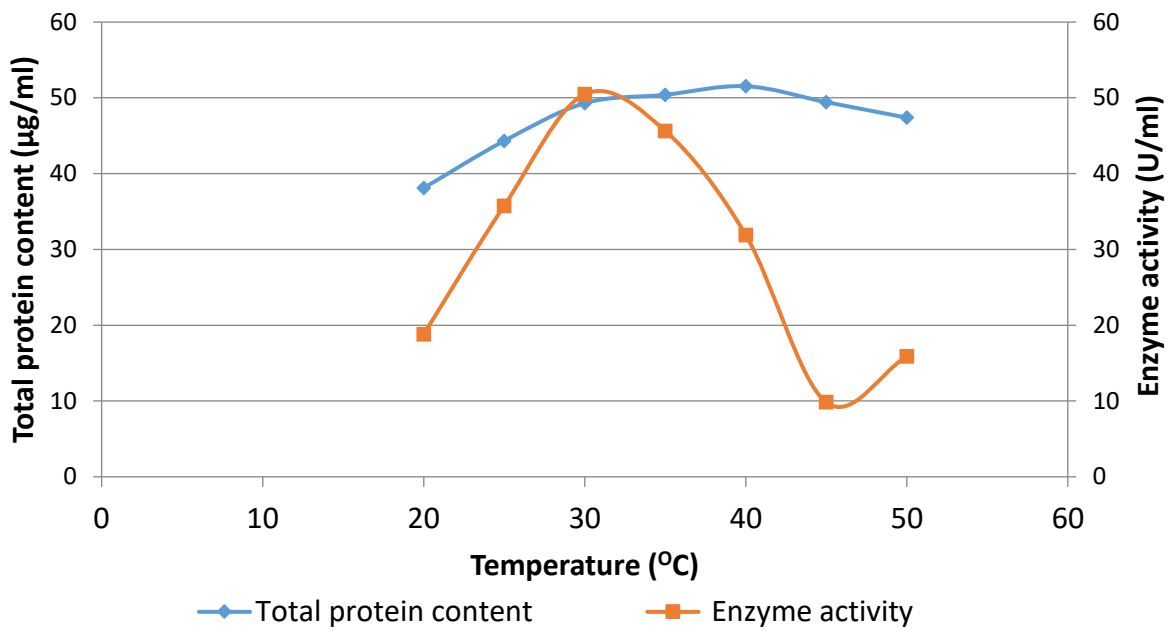


Figure. 5: Effect of temperature on cellulase production by *Bacillus* sp. CDS4

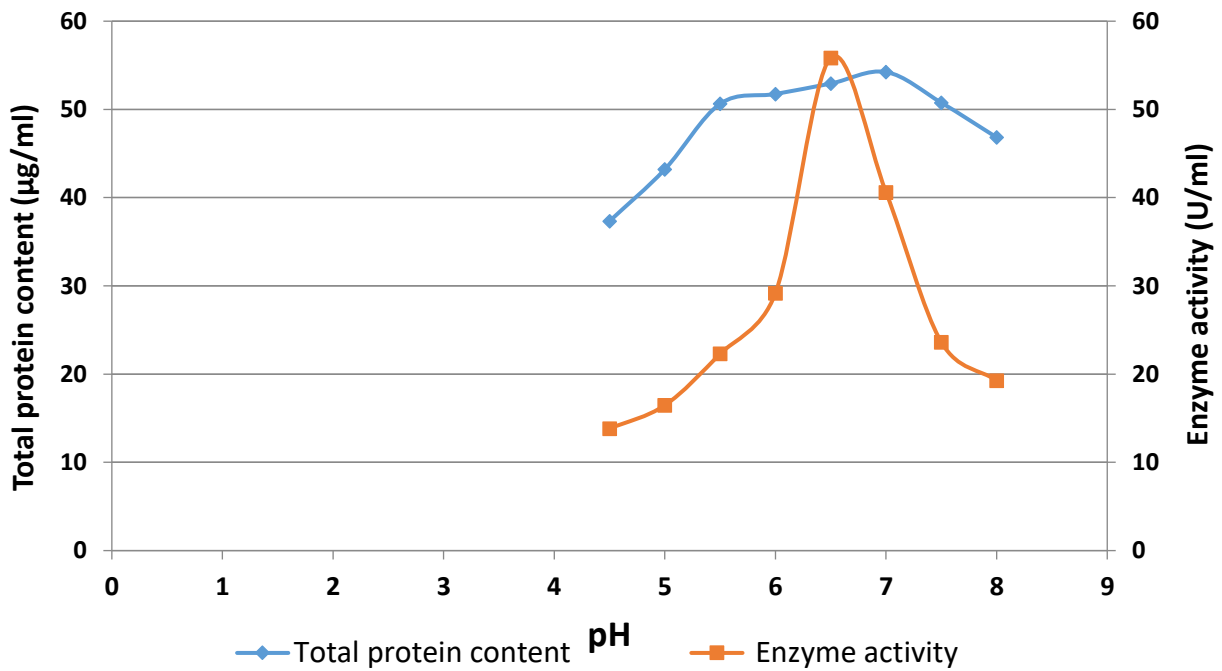


Figure 6: Effect of pH time on cellulase production by *Bacillus* sp. CDS4

DISCUSSION

The application of cellulases are myriad and present opportunities for the establishment and development of small-scale industries in developing countries like Nigeria. Degradation of cellulosic materials is a complex process requiring participation by a number of microbial

enzymes. Habitats that contain these substrates are the best sources in which to find these microorganisms (Haung and Monk, 2014). Cow dung was selected as a source for obtaining desirable cellulose producing organisms, because it is a rich source of diverse group of cellulolytic microorganisms owing to diet of the ruminants which consists of high amounts of cellulosic matter. Further, its wide availability, ease of processing and cost effectiveness also plays an important role for its selection (Haung and Monk, 2014).

The results from this study showed that only five isolates were capable of producing cellulases out of the eleven morphologically distinct colonies that were isolated and screened. This implies that cow dung bacteria are not predominantly cellulolytic but that a synergistic kind of association is obtainable in the rumen of cattle. This is similar with the work of Saraswati *et al.*, (2016) in which 21 bacterial strains were isolated from cow dung and screened for cellulolytic activity from which only 9 strains were found to be cellulase producer. Faiz *et al.* (2015) reported that cellulase can also be isolated from cattle wastes, woody biomass, cow manure and compost. Cellulase-producing bacteria were found commonly in all environments which enables them to degrade the cellulose found in waste materials (Illavarasi, 2014).

The productivity result as shown in Table 1 revealed that most bacteria do not naturally produce quantities of value added metabolites but only in amounts required to perform some essential metabolic function(s). This is demonstrated by the large colonial diameters of the isolates and a corresponding small total clear zone diameter. The results from secondary screening of bacteria for cellulase production on solid medium were incidentally found to agree with result of such screening in liquid medium. It was observed that in liquid medium majority of the isolates tested could not produce sufficient quantities of the enzymes to turn the Commaisse brilliant blue dye blue. The few that did as presented in Table 3 suggested no hope for further studies with such isolates since the highest amount of total protein released into the crude enzyme solution was only about 7 µg/ml.

Table 2 of our study reveals the tentative identities of the bacteria encountered in the cow dung samples. The isolates were all species of the bi-polarly stained aerobic Gram-positive rod, *Bacillus* suggesting that these aerobic spore-formers might not be genetically endowed for copious cellulase production but that they play a role in the normal metabolism of the host animal. Going by the results on this table, it is possible that these bacteria might well be more amyolytic than cellulose degrading. Juturu and Wu (2017) were able to isolate different species of *Bacillus* capable of producing cellulase from oil palm.

Nutrient sources were found to be very important factor for cellulase production. Since carbon is considered as the primary nutrient for bacterial growth, different carbon sources were analyzed for cellulase production. Maximum production of cellulase of 128.52 U/ml was observed when sucrose was utilized as the carbon source. In a similar work done by Teodoro *et al.* (2016), maltose was reported as the best carbon source for *Bacillus* species. Also, Saraswati *et al.* (2016) reported lactose (23.96 U/ml) to be the best carbon source for the production of cellulase.

Nitrogen is required as a major nutrient by microorganisms for the synthesis of amino acids, nitrogenous compounds, nucleic acids and the energy currency, ATP to name a few. These compounds are the major drivers of cellular metabolism leading to biomass accumulation. Oftentimes, in a biotechnological production venture, the nitrogen source adopted for cellular metabolism by an organism differs from the one required for secondary metabolite synthesis (Ekpenyong *et al.*, 2017). Hence, different nitrogen sources (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate) were screened for cellulase production. Ammonium sulphate was found to be the best nitrogen source as it increased the production of cellulase up to 66.54 U/ml. Earlier in a study by Saraswati *et al.* (2016), peptone was found to be the best carbon source. However, the source may vary depending on the strain and culture conditions (Ashwini *et al.*, 2017). For example, *Bacillus subtilis* strain isolated from earthworm gut showed better production of cellulases with Malt extract as carbon source and ammonium nitrate as nitrogen source (Shankar and Isaiarasu, 2011).

As environmental parameters are essential for the production of cellulase, they were optimized by shake flask fermentation method as described by Al-Kharousi *et al.*, (2018). It was observed from the results that there was a gradual increase in cellulase production from 12th hour and the maximum production occurred at 72 hours with the enzyme activity of 50.15 U/ml. This shows that incubation time depends solely on the available nutrients present in the medium and the cultural conditions of the organism. This is in agreement with the work of Saraswati *et al.*, (2016). However, Wanderley *et al.*, (2017) reported the maximum production occurred at 72 hours with the enzyme activity of 33.93 U/ml.

Vital parameters like temperature and pH are very essential parameters to be consider during cellulase production. In order to optimize the optimum temperature for the better cellulase production, various temperatures were set for the production. The highest cellulase activity was found as 50.48 U/ml at 30°C for the cellulase production. The temperature requirement of the

organism is based on the nature of organisms. A study by Abdelnasser and Ahmed (2015), 35°C was found to be the optimum temperature for *Bacillus* sp. Also, Ashwini *et al*, (2017) obtained 75°C to be the optimum temperature for the production of cellulase by some *Bacillus* sp. Maximum production of the enzyme (55.61 U/ml) was obtained at the pH 6.5.0. The pH of the selected organism was closely related to the optimum pH values of most of the *Bacillus* spp. As an evidence to the dependence of culture condition, this work agrees with the study by Magdi *et al*, (2017) where they isolated *Bacillus subtilis* KO from Egyptian soil possessing an optimal pH range from 6.5 to 7.0.

CONCLUSION

This study provides the evidence for the production and optimization of cellulase production using *Bacillus* sp. CDS4. The results revealed that cow dung served as a good isolation source for cellulase producing bacteria since it is rich in cellulose. The maximum cellulase production with *Bacillus* sp. CDS4 was obtained at incubation time of 72 hours at a temperature of 30°C and pH of 6.5, utilizing sucrose and ammonium sulphate as sole sources of carbon and nitrogen respectively. The cellulase produced by *Bacillus* sp. CDS4 can have great applications in various industries including jeans processing and paper industries. An optimization study would further increase the production of cellulase by this organism.

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