Original Research Article

Optimization of cellulase production from cellulose degrading microorganism isolated from cow dung

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Abstract

Cellulases are the enzymes hydrolyzing cellulosic biomass and are produced by the microorganisms that grow over cellulosic matters. The aim of the present study was isolation, identification and screening of bacteria with high cellulase activity from cow dung and optimization of cellulase production. In the present study cellulase producing bacteria were isolated from cow dung on screening media containing Carboxy Methyl Cellulose (CMC). The organisms were identified using morphological and biochemical characterization. Cellulase assay was carried out using CMC as a substrate and product liberated was determined by DNSA method. Media was optimized and the enzyme was assayed at different parameters. The enzyme was further purified using ammonium sulphate precipitation, dialysis and ion exchange chromatography and specific activity was determined. Cellulase enzyme producing microorganism was isolated from cow dung and identified as *Bacillus* sp. The media was optimized for cellulase production and the isolate produces maximum cellulase at pH 7, temperature 37 ºC and 72 hrs of incubation time. Cellulase characterization shows that, cellulase has optimum activity at pH 5, temperature 45 ºC and 15 min of incubation with CMC as substrate. The *Bacillus sp.* showed the 40.03% yield of cellulase at 8.65 fold with specific activity of 15.31 units/mg after ion exchange chromatography.

Key Words: Cellulase, *Bacillus sp.* Microorganism, Cow dung

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INTRODUCTION

Agricultural and industrial cellulosic waste is major pollutant which accumulates in environment. Cellulose accounts for approximately 50 % dry biomass of agriculture waste (Haruta *et al.*,2003). Cellulose is homopolysaccharide regarded as the most important renewable resource for bioconversion. Microorganisms including bacteria and fungi are able to carry out bioconversion of cellulose. Many cellulosic substances were hydrolyzed to simple sugar for making single cell protein, alcohol and sweetener. It has become economic interest to develop an effective method to hydrolyze the cellulosic biomass. The use of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest. Cellulose is most abundant primary product of photosynthesis and the renewable bioresource produced in the biosphere (100 billion dry tons/year) (Saraswati *et al.*, 2012 and Venkata *et al.*, 2013). Approximately 70% of plant biomass is present in 5- and 6-carbon sugars (D-xylose, D-arabinose, D- glucose, D- galactose, D-mannose) which are found in lignocellulosic biomass comprised of mainly cellulose, lesser amount of hemicelluloses and least amount of all lignin. It is possible to convert this biopolymer into monomeric molecule of glucose by both chemical and biological means. Cellulose represents a huge source of energy for microorganisms, the main agents responsible for soil organic matter decomposition (Shaikh *et al.*, 2010). It is commonly degraded by an enzyme known as Cellulase.

Cellulases have been grouped into endo-1,4-β-glucnase (Endogluconase), exo-1,4-β-glucnase (Exoglucanases) and β-glucosidase that synergistically hydrolyze cellulose into soluble sugars and glucose (Lynd *et al*., 2002). Microorganisms like bacteria and fungi are the well known sources for cellulases. Bacteria as compared to fungi are having high growth rate and seem to be potential source for cellulase production (Sonia *et al*., 2013). Some bacterial genera such as *Cellulomonas, Cellvibrio, Pseudomonas sp., Bacillus* and *Micrococcus* posses cellulolytic property (Nakamura and Kppamura K,1982., Immanual *et al*.,2006). Enzyme production is closely controlled in microorganisms and to improve its productivity, these controls can be ameliorated. Cellulase yields appear to depend upon a complex relationship involving a variety of factors like inoculums size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth (Immanual *et al*.,2006). Basic and applied research on microbial cellulases has not only generated significant scientific knowledge but has also revealed their enormous potential in biotechnology. At present, cellulases and related enzymes are used in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture and for research purposes (Bhat 2000). Indeed, the demand for these enzymes is growing more rapidly than ever before, and this demand has become the driving force for research on cellulases. The present study was focused to isolate and screen cellulolytic bacteria for the production of cellulase and optimization of process parameters for maximum cellulase production and purification from selected isolated culture.

MATERIALS AND METHODS

Isolation and Characterization of Cellulase Producing Microorganisms: The cow dung was collected from the farming area of Ahmednagar (M.S.), India. The samples were serially diluted using sterile saline. The diluted samples were plated on nutrient agar by spread plate method. The isolated colonies were further purified using streak plate technique and screened for Cellulase production. The screening was done by streaking the isolated colonies on screening medium (carboxymethlycellulose - 0.5g, $NaNO_3$ - 0.1g, K_2HPO_4 - 0.1g, KCl - 0.1 g, $MgSO_4$ - 0.05g,Yeast extract - 0.05g, Agar - 1.6 g, Distilled Water - 100ml). After 24 hours of incubation the plates were flooded with 0.1% Congo red solution and left undisturbed for15 minutes. Clear zones formed by cellulase positive strains were visualized by distaining of the plates using 1M NaCl solution. Positive and better zone producing strain were chosen and continued for further studies. The isolates were characterized with the help of morphological and biochemical tests. The morphological test viz. Gram staining, Colony morphology, Endospore staining, Capsule staining, Motility test and biochemical test viz. Catalase test, Indole test, Vogus-Proskauer test and Starch hydrolysis test were performed (Buchanan *et al*., 1974 and Apun *et al*., 2000).

Cellulase Assay and Protein Estimation: Three production media were centrifuged at 6000 rpm for 10 min at 4°C and supernatant was used as crude enzyme. The crude enzyme was purified by following methods. Cellulase activity was estimated using a 1% solution of carboxymethlycellulose (CMC) in 0.05M sodium acetate buffer (pH 4.8) as substrate. The reaction mixture contained 1 ml sodium acetate buffer, 1 ml of substrate solution and 0.5 ml of crude enzyme solution. The reaction was carried out at 45°C for 15 min. The amount of reducing sugar released in the hydrolysis was measured by DNSA method (Miller, 1959). One unit of Enzyme activity was determine as the amount of Cellulase required to release 1µM of reducing sugar per ml per minute under above assay condition. Protein concentrations in a crude sample were determined by Folin Lowry method (Lowry *et.al.,* 1951) using bovine serum albumin (BSA) as a standard.

Media Optimization: The optimum conditions for cellulase production were determined for the selected isolates. The cellulase fermentation was carried out at different pH, temperature, and incubation time and the crude enzyme was collected from each set to check the enzyme activity.

Effect of incubation period on cellulase production: Different incubation times (24, 48, 72 and 96 hours) were employed to study effect of time on cellulase production. The culture filtrates were collected at respective time interval and crude enzymes assay carried out using CMC as substrate at 45 ºC.

Effect of pH on cellulase production: For determination of optimum pH for cellulase production, the isolate was inoculated in the production media of different pH ranging from 5, 6, 7, 8, 9 and 10 for 72 hrs at 37 ºC. The crude enzymes assay carried out using CMC as substrate at 45ºC.

Effect of Temperature on cellulase production: For the determination of optimum temperature for production of cellulase, the isolate was inoculated in production media and fermentation was carried out at various temperatures in the range of 5 ºC, 15 ºC, 25 ºC, 35 ºC, 45 ºC at pH 7 for 72 hrs. The crude enzymes assay was carried out using CMC as substrate at 45 ºC (Shaikh *et al*., 2010, Saraswati *et al*. 2012, and Abubakar *et al*., 2013).

Purification of cellulose Ammonium sulphate precipitation: Crude cellulase extracts were subjected to 70% salt precipitation by gradual addition of solid

ammonium sulphate with continuous stirring using glass rod at 4 ºC for 10-20 minutes. The precipitated enzymes were collected by centrifugation at 10,000 rpm for 15 minute. The supernatant was discarded and pellet was dissolved in 10 ml sodium acetate buffer (pH 5.5). The precipitated enzyme was assayed for enzyme activity and protein content (Muhammad *et al*., 2012).

Dialysis: For partial purification and removal of excess salts, enzyme collected after ammonium sulfate precipitation was dialyzed against 0.05 M sodium acetate buffer (pH-4.8) at 4 ºC. The partially purified sample was assayed for enzyme activity and protein content.

Ion Exchange Chromatography: The precipitated enzyme was then subjected to ion exchange chromatography with DEAE cellulose column. One ml of dialyzed sample was loaded on column and elutions were collected by using four sodium acetate elution buffers of pH 4.8 with 25mM NaCl, 50mM NaCl, 75mM NaCl 100mM NaCl concentration.

Characterization of Cellulase: The cellulase enzyme activity was determined at different pH, temperature and incubation period (Saraswati *et al*., 2012; Shaikh *et al*., 2010).

Effect of pH on cellulase activity: To determine optimum pH, cellulase assay was carried out at pH 4.0 to 6.0 (0.05 M sodium acetate buffer) and pH 7.0 and 8.0 (0.05 M sodium phosphate buffer) using CMC as a substrate and product liberated was determined by DNSA method.

Effect of temperature on cellulase activity: To determine optimum temperature, the enzyme assay was carried out at pH 4.8 and various temperatures viz 5 ºC, 15 ºC, 25 ºC, 35 ºC, 45 ºC, 55 ºC using CMC as a substrate and product liberated was determined by DNSA method.

Effect of Incubation period on cellulase activity: To determine optimum incubation time, enzyme assay was carried out at pH 4.8 and various incubation time period viz. 5, 10, 15, 20 and 25 min using CMC as a substrate and product liberated was determined by DNSA method (Saraswati *et.al.*, 2012; Abubakar *et.al*., 2013).

RESULTS AND DISCUSSION

Isolation and Characterization of Cellulase Producing Microorganisms: Three cellulase producing bacteria were isolated from the cow dung sample by spread plate technique on screening media containing CMC. The substrate CMC is commonly used for cellulose assay due to its simplicity and easy digestion by the microbes (Shanmugapriya *et al*., 2012). Observations depicted in Fig. No.1 indicates growth of organisms on screening media. One isolate from this source was selected on screening media by observing zone of clearance after spraying congo red stain (Fig. 2).To indicate the cellulolytic activity of organism, diameter of hydrolytic zone around growing colony on CMC agar was measured. The isolates No.1, 2, and 3 qualifying for cellulase production was then used for quantification of cellulase activity in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a di-nitrosalicylic acid (DNSA). The bacterial isolates no.1 exhibiting relatively high enzyme activity was selected for process optimization.

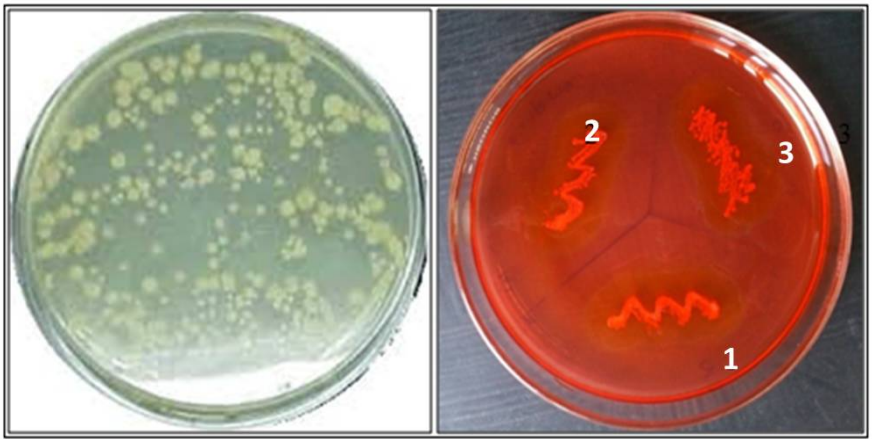

Figure 1: Isolation of microorganisms on screening media **Figure 2:** Zone of clearance on screening media

Characterization of Isolates: Selected isolate was presumptively identified by the means of morphological examination and biochemical characterization. The parameters investigated included colonial morphology,

Gram's reaction, endospore formation, catalase production, Voges Proskauer (VP) reaction, indole production, starch hydrolysis, citrate utilization and gelatine hydrolysis ((Table-1). The results were compared with Bergey's Manual of Determinative Bacteria (Buchanan *et al*., 1974). Based on morphological, cultural and biochemical characters, the isolates no.1 obtained from cow dung were found to be of *Bacillus* sp.

Table 1: Colony Characterization of Isolate 1

Colony character	Cow dung (Isolate 1)
Size	3mm
Shape	Circular
Colour	Creamy
Margin	Entire
Elevation	Convex
Opacity	Opaque
Consistency	Dry
Gram character	Gram positive rod
Endospore formation	Positive
Capsule staining	Negative
Motility	Motile

Table 2: Biochemical Characterizations of Isolate 1

Biochemical Test	Cow dung (Isolate1)
Catalase test	Positive
Indole Test	Negative
Vogus-prokauer	Negative
Starch Hydrolysis	Positive

Production and Purification of Cellulase: Cellulase was produced by using organisms isolated from cow dung. From graph it is clear that the activity of cellulase increases as enzyme gets concentrated by ammonium sulphate precipitation and then by DEAE cellulose anion exchange chromatography.

The results showed that specific activity of cellulase enzyme isolated from the isolate increases as fold purification increases (Table 3). Enzyme isolated from *Bacillus* sp. showed the 40.03% yield of cellulase at 8.65 fold with specific activity of 15. 31 units/mg after anion exchange chromatography. These observations were in agreement with earlier report on *Bacillus subtilis* by Jansova *et al*. (1993) and Das *et al*., 2016 and *Bacillus sp* C1AC55.07 (Dies *et al*.,2014). Roop *et al* (2017) also noted similar results for cellulase production by using *Bacillus sp.*

Table 3: Steps for Cellulase purification with Specific activity of isolate 1

Purification step	Volume (ml)	Protein (mg)	Activity (units)	Total activity (unit*ml)	Specific activity (units/ mg)	Fold purification	% yield
Crude	100	224.35	0.4012	401.2	1.78	1	100
Ammonium sulphate	10	94.65	0.4296	203.96	2.15	1.20	50.83
Dialysis	2	31.67	0.4566	155.74	4.91	2.76	38.81
Ion exchange chromatography	1	9.8	0.4962	128.53	15.31	8.65	40.03

Media Optimization

Effect of pH on cellulase production: It is evident from the Figure 3 that the isolate showed optimum cellulase production at pH 7 incubated for 72 hrs at 37 °C. These results clearly indicate that pH 7 is optimum for cellulase production. Our findings were in accordance with earlier reports (Hongzhi *et al*.2017). The obtained results coincide with Yang and his group in 1995 who reported that cellulase production was high between pH 7-9 for *Bacillus spp*.

Kumar and his group in 2012 reported maximum cellulase production at pH 8.0 (63U/ml) by *Bacillus cereus.*

Effect of temperature on cellulase production: Figure 4 shows the optimum cellulase production at temperature 35°C incubated for 72 hrs at pH 7. These results clearly indicated that temperature of 35 °C is optimum for cellulase production. The obtained results coincide with Yang and his group in 1995 who reported that many *Bacillus spp*. needed 32-37 °C for better production of cellulase (Yang *et al*.,1995). Whereas very high cellulase production was reported with *Bacillus* sp. BSS3 at pH 9, 37 °C with 1% CMC (Sreedevi *et al*., 2013).

Effect of Incubation period on cellulase production: Figure 5 showed that, the isolate showed optimum cellulase production at 72 hrs of incubation at temperature 37 °C and pH 7. These results indicated that 72 hrs of incubation is optimum for cellulase production. Shankar and Issairasu in 2011 reported the similar results of cellulase production after 72 hours of incubation at 37 °C from *Bacillus pumilus* EWBCM1 (Shankar and Isaiarasu 2011).

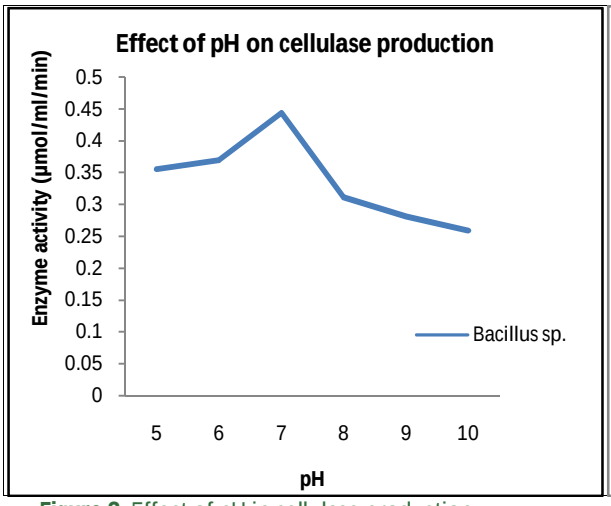

Figure 3: Effect of pH in cellulase production

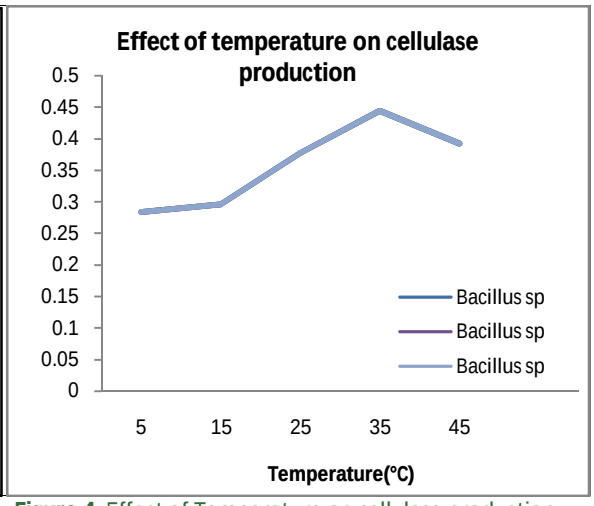

Figure 4: Effect of Temperature on cellulase production

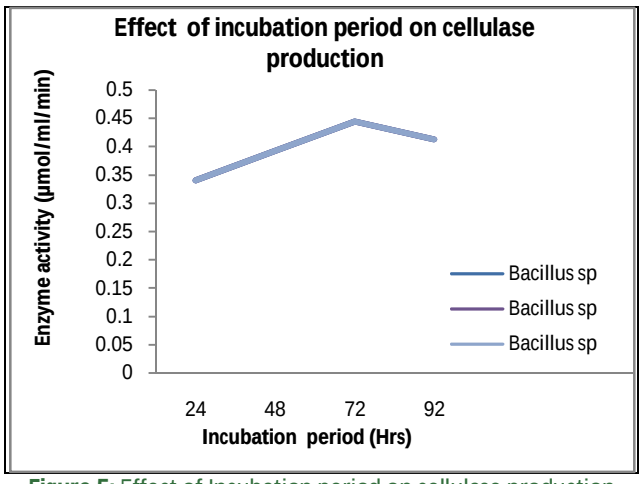

Figure 5: Effect of Incubation period on cellulase production

Characterization of cellulase

Effect of pH on cellulase activity: The cellulase from the isolate showed optimum activity at pH 5 (Fig. 6). It is evident from graph that it showed sharp peak at pH 5. These results indicated that pH 5 is optimum pH for cellulase activity. The results obtained in this study were in accordance with results reported by other research groups (Chantawannakul *et al*., 2002; Nadhimath *et al*., 2016). Some research group documented that the optimum pH of 5.0 and 5.5 for cellulase produced from *Penicillium artrovenetum* (Adeleke *et al*., 2012). In another related study, it was established that a cellulase was obtained at pH 7 and 8 (Roopa *et al*., 2017).

Effect of Temperature on cellulase activity: The cellulase from the isolate showed optimum activity at temperature 45 °C. Graph showed sharp peak at temperature 45 °C (Fig.7). These results clearly indicate that temperature 45°C is optimum temperature for cellulase activity (Saraswati *et al*., 2012; Abubakar *et al*., 2013, Pachuri *et al*., 2017). Our findings are contrary to the finding of Ray and his group (2007)**.** They noted that minimum and maximum activity of 45˚C and 40 ˚C respectively of cellulase produced by *Bacillus subtilis*.Whereas for cellulase activity of *Bacillus* species (Strain B223) cellulase were optimum at 30 ˚C and 40 ˚C (Orji *et al*., 2016).

Effect of incubation period on cellulase activity: Cellulase from isolate 1 showed optimum activity at 15 min incubation period assayed at temperature 45°C and pH 5. It is indicative from the graph as it showed sharp peak at 15 min incubation period. These results indicated that 15 min incubation period is optimum for cellulase activity at 45 °C and pH 5 as shown in Fig 8. When raw substrates like Hay, Fibre waste, waste of palmyra palm and banana bracts were used as substrate, it requires 48 hrs of incubation for cellulase activity (Roopa *et al*.,2017).

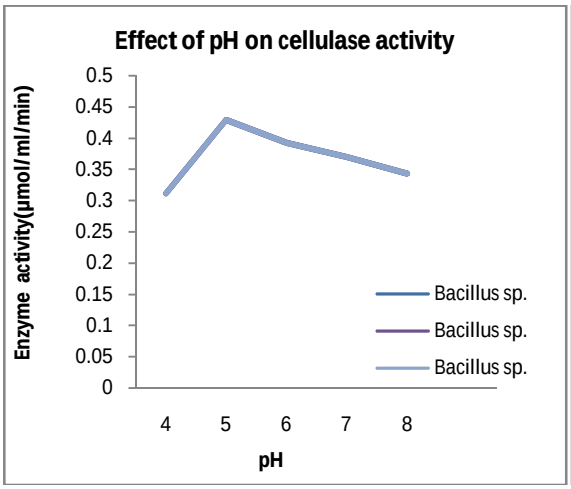

Figure 6: Effect of pH on Cellulase activity

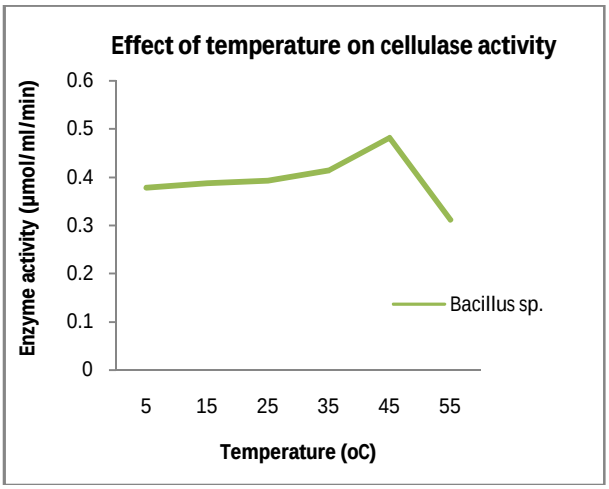

Figure 7: Effect of temperature on cellulase activity

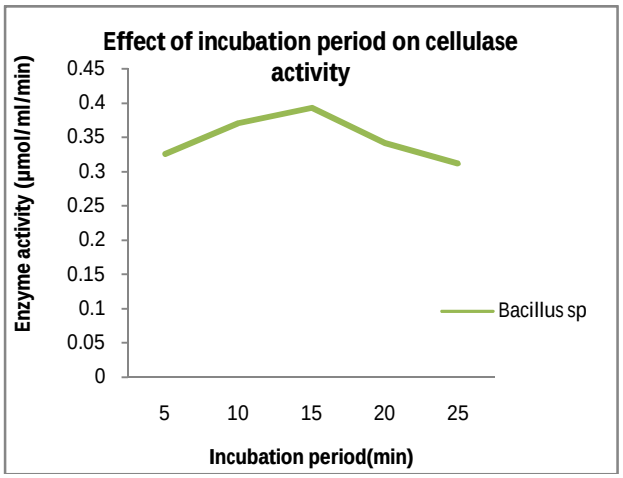

Figure 8: Effect of Incubation period on cellulase activity

CONCLUSION

The present study was aimed at screening of microbial source with putative cellulase activity. Cellulase enzyme producing microorganism *Bacillus* sp. was successfully isolated from cow dung sample. The media was optimized for cellulase production and the isolate produces maximum cellulase at pH 7, temperature 35 ºC and 72 hrs of incubation time. Cellulase characterization showed that, cellulase has optimum activity at pH 5, temperature 45 ºC and 15 min of incubation with CMC as substrate. The *Bacillus sp.* showed the 40.03% yield of cellulase at 8.65 fold with specific activity of 15.31units/mg after ion exchange chromatography purification. Further studies are needed for optimization of carbon sources and application of cellulose enzyme in various commercial fields. The optimization of the production of enzymes is important for increasing productivity and reducing costs strategy. The purified cellulase enzyme can be used for various purposes in detergent industries, food industries, and pharmaceutical industries (Sethi *et al.*, 2013: Hmad *et al.*, 2017), bioethenol production Industry (Madadi *et al* 2017). The cellulose enzyme isolated from *bacillus sp.* has shown high activity and stability in terms of pH and high temperature will be of use in various industrial and biotechnological applications.

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