EFFECTS OF CULTURAL AND NUTRITIONAL CONDITIONS FOR CARBOXYLMETHYLCELLULASE (CMCase) PRODUCTION BY CELLULOSE DEGRADING BACTERIA

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ABSTRACT

The aim of the present study is to identify cellulose degrading bacteria and the effects of cultural and nutritional conditions for their cellulase activity. Among the tested bacterial strains, the GT1 strain had the highest cellulase production yields. This strain was further characterized by biochemical and morphological tests and identified as *Bacillus subtilis*, therefore, we primarily concluded that GT1 was *Bacillus* sp., denoted as *Bacillus* sp. GT1. Different parameters: temperature, pH, nitrogen and carbon sources, and metal ions were optimized. The optimal pH and temperature for the activity of crude enzymes were 7 and 35°C, respectively. Supplementation of peptone and corn starch to the culture medium is favored for enzyme secretion. The metal profile of the enzymes indicated that the enzymes were stimulated by Mn^{2+}, Mg^{2+}, and Ca^{2+}, while Fe^{2+}, Zn^{2+}, and Cu^{2+} reduced activity of cellulase from the cellulolytic bacterial strain *Bacillus* sp. GT1. These results open up the broad application of GT1 in many fields.

Keywords: *Bacillus subtilis*, cellulose, CMCase, cultural and nutritional conditions, metal ions.

Ảnh hưởng của điều kiện nuôi cấy và dinh dưỡng tới khả năng sinh carboxylmethylcellulase (CMCase) của vi khuẩn phân giải cellulose

TÓM TẮT

Mục đích của nghiên cứu này là tuyển chọn và xác định các vi khuẩn phân giải cellulose và nghiên cứu ảnh hưởng của các điều kiện nuôi cấy và môi trường dinh dưỡng tới hoạt tính cellulase của chúng. Trong số các chủng vi khuẩn được nghiên cứu có 01 chủng biểu hiện khả năng sinh enzyme cellulase mạnh nhất. Chủng này được chọn và tiến hành đánh giá các đặc tính hóa sinh và hình thái tế bào, khuẩn lạc. Kết quả chủng này có nhiều đặc điểm tương đồng với chủng *Bacillus subtilis,* do đó được chúng tôi ký hiệu là *Bacillus* sp. GT1. Ảnh hưởng của các yếu tố nhiệt độ, pH, nguồn nitrogen và carbon, một số ion kim loại được đánh giá. Enzyme CMCase hoạt động tốt nhất tại pH và nhiệt độ tương ứng là 7 và 35^0C. Bổ sung pepton và tinh bột ngô vào môi trường nuôi cấy đã kích thích sinh enzyme. Các cation kim loại như Mn^{2+}, Mg^{2+}, Ca^{2+} tăng cường hoạt động của enzyme CMCase, trong khi đó Fe^{2+}, Zn^{2+}, Cu^{2+} giảm hoạt độ của enzyme này.

Từ khóa: CMCase, *Bacillus subtilis*, cellulose, điều kiện nuôi cấy và dinh dưỡng, ion kim loại

1. INTRODUCTION

Cellulose is a linear polysaccharide of glucose residues with β-1,4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products. However, the crystalline structure and insoluble nature of cellulose represent big challenges for hydrolysis. With the help of cellulolytic systems, cellulose can be converted to glucose, which is a multi-utility product, in a much cheaper and biologically favourable process.

Cellulolysis is basically a biological process controlled and carried out by the enzymes of

the cellulase system. The cellulase enzyme system is comprised of three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β-exoglucanase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Endoglucanase is responsible for the random cleavages of β-1,4-glycosidic bonds along a cellulose chain. Exoglucanase is necessary for cleavaging the non-reducing end of a cellulose chain and splitting the elementary fibrils from the crystalline cellulose, and β-1,4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose (Shewale, 1982; Woodward and Wiseman, 1983). Only the synergy of the above three enzymes makes the complete cellulose hydrolysis to glucose (Ryu *et al.*, 1980; Wood, 1989) or a thorough mineralization to H_2O and CO_2 possible. Cellulase, due to its massive applicability, has been used in various industrial processes, such as making biofuels like bioethanol (Ekperigin, 2007; Vaithanomsat *et al.*, 2009), the animal feed industry (Ma *et al.*, 2015), agricultural and plant waste management (Mswaka *et al.*, 1998; Lu *et al.*, 2004), and chiral separation and ligand binding studies (Nutt *et al.*, 1998). Researchers keep on working to isolate microorganisms with higher cellulase activity (Ray *et al.*, 2007). Microorganisms are important in the conversion of lignocellulose wastes into important products like biofuels that are produced by fermentation (Lynd *et al.*, 2002). Bacteria, which have a faster growth rate compared to fungi, can be used for cellulase production. The potential cellulase producing bacteria are *Cellulomonas, Pseudomonas, Thermoactinomycetes,* and *Bacillus* spp. (Rasul *et al.*, 2015). The present study is aimed to identify cellulose degrading bacteria and optimize cultural and nutritional conditions for cellulase activity. Temperature, pH, nitrogen and carbon sources, and metal ions are important parameters for the optimized production of cellulase enzymes. Additionally, the cellulolytic potential for antibacterial activity of crude enzymes against pathogenic bacteria and bioethanol production were also investigated.

2. MATERIALS AND METHODS

2.1. Microorganisms

Bacterial strains were collected from the collection of the Microbial Laboratory, Department of Microbial Technology, Faculty of Biotechnology, Vietnam National University of Agriculture.

2.2. Screening of cellulolytic bacteria

The cellulolytic activity of the bacterial strains was tested by a modified agar-well diffusion method. The bacterial colony having the largest clear zone was selected for identification and optimization of conditions for cellulase production.

According to Narendhirakannan *et al.* (2014), the modified agar well diffusion method can be employed to measure cellulase activity of crude enzymes. Sterile agar contained 1% CMC poured in sterile Petri plates and after agar solidification, punched with eight millimeter diameter wells. Wells were filled with 100 µl of crude enzymes or sterile distilled water (blanks). The crude enzymes were exposed to a temperature of about 4°C for 30 min. The test was carried out in triplicate. The Petri dishes were incubated at 30 ± 2°C for 24 h. After incubation, culture plates were flooded with Lugol's iodine solution. A clear zone formation around the microbial colonies indicated the hydrolysis of cellulose or CMC. The highest activity was assumed by the largest clear zone.

The celluase activity was determined through the ability of cellulose hydrolysis using the formula: D - d (mm), where D = diameter of clear zone and d = diameter of agar well.

2.3. Maintenance of pure culture

Pure cultures of the selected bacterial isolate were individually maintained on CMC supplemented minimal agar slants at 4˚C until used.

2.4. Inoculum development

Pure cultures of the selected bacterial isolate were inoculated in LB broth medium at pH 7 for

24 h. After 24 h of fermentation, the vegetative cells were used as the inoculum source.

2.5. Identification of cellulolytic bacteria

Identification of the cellulolytic bacterium was performed in accordance with *Bergey's Manual of Systematic Bacteriology* (Garrity *et al.*, 2004), which was based on morphological and biochemical tests.

2.5.1. Morphology and gram characteristics

The gram characteristics and morphology of the isolates were studied by the Gram staining method according to Pepper and Gerba (2005).

2.5.2. Biochemical characterizations

According to Garrity *et al.* (2004), in order to identify the cellulolytic bacterium, the following tests were carried out:

Motility: To check the motility of the selected strain, soft agar stabbing (tube method) was used. We prepared soft agar in a test tube (without a slanted surface). Cells were stab - inoculated into the agar (the top surface was not inoculated). Non-motile bacteria will only grow where they were inoculated. Motile bacteria will grow along the stab and will also swim out away from the stabbed area. Thus, a negative result is indicated by growth in a distinct zone directly along the stab. A positive result is indicated by diffuse (cloudy growth), especially at the top and bottom of the stab.

Growth at 50°C: This characteristic was tested by suspending the bacterium in sterile LB liquid broth at 50°C. After 48 hours, the suspension was spread on sterile LB agar to check the survival of the bacteria.

Growth in 10% NaCl: The bacterium was suspended in a tube containing sterile LB broth with 10% NaCl. After 48 hours, the suspension was spread on sterile LB agar to check the survival of the bacterium.

Utilization of citrate: An inoculum from a pure culture was transferred aseptically to a sterile tube of Simmons citrate agar. The inoculated tube was incubated at 35°C for 24 hours and the results were determined. Abundant growth on the slant and a change from green to blue in the medium indicated a positive test for growth using citrate.

Casein hydrolysis: Crude enzymes of bacterial isolates were put in the wells of sterile casein agar containing 0.1% casein and incubated at 30°C for 4-6 hours. Black Amido was then poured on the plates to detect zones of casein hydrolysis around the wells.

Starch hydrolysis: Crude enzymes of bacterial isolates were put in the wells of sterile starch agar containing 1% starch and incubated at 30°C for 4-6 hours. Lugol's iodine was then poured on the plates to detect zones of starch hydrolysis around the wells.

Catalase: A loop full of growth of each bacterial isolate from a nutrient agar dish was stirred in 30.0 v/v hydrogen peroxide and observed for evolution of gas.

Ammonia production: Ammonia production was tested by inoculating bacterial isolates in tubes containing sterile peptone nitrate broth and detected by the Nessler indicator.

Voges - Proskauer test: Inoculum from a pure culture was transferred aseptically to a sterile tube of MR-VP broth. The inoculated tube was incubated at 35° - 37°C for 24 hours. The test was performed by adding alpha-naphthol and potassium hydroxide. A cherry red color indicated a positive result, while a yellow-brown color indicated a negative result.

2.6. Effects of cultural and nutritional conditions on cellulase production

2.6.1. Effect of pH

The selected bacterial strain was cultured in LB broth with 0.1% CMC at various pHs ranging from 3 to 12 at 30°C. After 48 hours, the cellulolytic activity was tested by the modified agar-well diffusion method (Narendhirakannan *et al.*, 2014).

2.6.2. Effect of temperature

The effect of temperature on the activity of cellulase was determined by culturing the bacterium at different temperatures between 25

to 75°C. Enzyme activity was assayed by the modified agar-well diffusion method (Narendhirakannan *et al.*, 2014).

2.6.3. Nitrogen sources

The selected strain was cultured in basal salt medium containing 0.5% nitrogen sources such as beef extract, $(NH_4)_2SO_4$, KNO_3, $(NH_4)_3C_6H_5O_7$, NH_4Cl, NH_4NO_3, $NaNO_3$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, peptone, and yeast extract. After 48 hours, the crude enzymes were extracted to check cellulolytic activity.

2.6.4. Carbon sources

1% carbon sources (α-lactose, CMC, D-glucose, D-sobitol, D-(+)-xylose, dextrin, mannitol, saccarose, maltose, starch, corn starch, arrowroot powder, and tapioca starch) were added into the cultural medium of the selected bacterium.

2.6.5. Metal ions

Various divalent metal ions, including Ca^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, were applied to check the optimum activity of enzymes. Each metal ion was used at a concentration of 5 mM.

2.7. Statistical analysis

All data were statistically analyzed using the Microsoft Excel program. Three replicates were measured for each condition.

3. RESULTS AND DISCUSSION

3.1. Screening of cellulolytic bacteria

Cellulose is one of the most widely used natural substances. However, the crystalline structure and insoluble nature of cellulose represent big challenges for enzymatic hydrolysis. Therefore, microorganisms, especially bacteria, are important in the conversion of lignocellulose components into valuable products.

Eight cellulolytic bacteria were collected for analysis of cellulolytic characteristics. Among all these tested bacterial strains, all eight bacterial isolates were found to be positive for cellulase production on screening media as they each produced a clear zone (as shown in Figure 1) during aerobic incubation.

GT1 produced the largest clear zone diameter as shown in Figure 1. The GT1 strain was further identified using morphological and biochemical methods. The diameters of the clear zones of the cellulose degrading strains isolated by Gupta *et al.* (2012) ranged between 28.0 to 50.0 mm. In this study, results showed that the cellulose hydrolytic ability of the GT1 strain is at a medium level, and slightly higher than the results obtained by Rasul *et al.* (2015).

3.2. Identification of cellulolytic bacterium

Morphology and Biochemical characterizations Colonies of GT1 on LB medium containing a percentage of CMC had a whitish color, and margins were irregular and 3-4 mm in diameter at 30°C. Fresh cultures of this isolate consisted of gram positive, slender, and rod shaped cells (Fig. 2 and Fig. 3).

According to *Cowan and Steel's Manual for the Identification of Medical Bacteria* (Barrow and Feltham, 1993), to identify bacteria, we had to carry out several biochemical tests. The results of all these tests are listed in detail in Table 1.

The results of the morphological properties and biochemical characteristics were compared to known species. The GT1 strain possessed the properties and characteristics most like *Bacillus subtilis*. Therefore, based on morphological and biochemical characteristics primarily, GT1 was *Bacillus sp.*, denoted as *Bacillus sp. GT1.*

3.3. Process of optimization for maximum cellulase production

The isolated bacterial strain Bacillus sp. GT1 requires optimization of cultural and nutritional conditions for growth and better cellulose production. These conditions include pH, temperature, nitrogen and carbon sources, and metal ions.

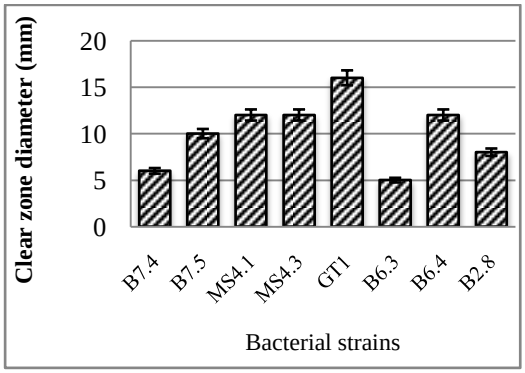

Figure 1. Cellulase production of collected bacterial strains

Figure 2. Colony of GT1 on LB medium containing 1% CMC

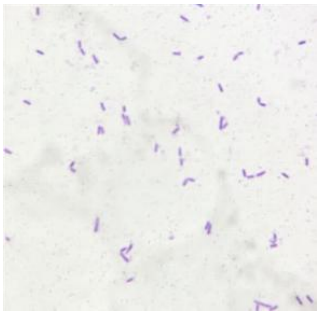

Figure 3. Gram staining of strain GT1 after 24 hours of incubation

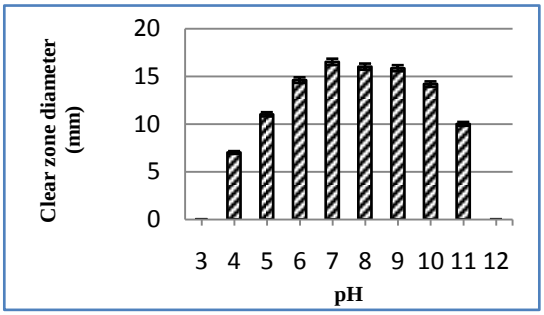

Figure 4. Effect of pH on the cellulase production from the GT1 strain

Table 1. Biochemical reactions and characteristics of the cellulolytic bacterial strain GT1

Characteristics /biochemical test	GT1 strain
Motility	+
Growth at 50°C	+
Growth in 10% NaCl	+
Utilization of citrate	+
Casein hydrolysis	+
Starch hydrolysis	+
Catalase	+
Ammonia production	+
Urease	-
VP test	+

3.3.1. Effect of pH on cellulase production

Enzymes are affected by changes in pH. Any change in pH causes changes in the enzyme active site. The most favorable pH value, the point at which the enzyme is most active, is known as the optimal pH. An increase or decrease in pH also causes denaturation of enzymes, thereby affecting their activity. The

range of pHs at which the bacterial strain had good activity was from 6 to 10. It was found that the cellulolytic strain GT1 was capable of producing enzymes at a broad pH range (Fig. 4). This made it easy to adapt the environmental conditions. For the GT1 strain, the highest cellulase production was found at pH 7. At pH 3 and 12, it didn't show any activity. This can easily be explained since at pHs that too high or too low, bacterial strains cannot grow. If it could survive, the enzymes it secreted would be inhibited by the extreme pH and lose cellulolytic activity.

A similar finding was also reported by Shaikh *et al.* (2013). They showed that the isolate CDB27 reached its maximal cellulase productivity at a pH of 7. Immanuel *et al.* (2006) reported that enzymes hydrolyzed substrates in the pH range of 4.0 to 9.0, with maximal production occurring at pH 7. Yin *et al.* (2010) isolated *Cellulomonas* sp. YJ5 showing its optimal pH was 7 and its pH stability range was 7.5-10.5. According to the research reported by Balamurugan *et al.* (2011), who tested various pHs within the range of 4.0 to 8.0, the maximal enzyme secretion of cellulose degradation bacteria was recorded at pH 7.0, even though all the strains grew from pH 4.0 to 8.0.

3.3.2. Effect of temperature on activity of cellulase

The cultivation temperature has marked influence on the growth rate as well as on the level of cellulose production. Each enzyme has an optimum temperature at which it performs best. Below or above this temperature, the enzyme loses its functionality.

As the temperature increased from 25°C, enzyme production increased but it started to decline when the temperature increased above 45oC, and enzyme activity was lost at 75°C. The cellulase enzymes act well at temperatures ranging from 35°C to 45°C. The optimum temperature of cellulase was achieved at 35°C (Fig. 5). A similar finding was also reported by Balamurugan et al. (2011). In that study, temperatures ranging between 20 and 40°C were tested for activity of cellulose degradation bacteria (CDB), while the maximal cellulase production of CDB was observed at 35°C. In a previous study, P. curdlanolyticus B-6 was cultivated for enzyme production at pH 7.0 and 37°C (Waeonukul et al., 2009). Maruthamalai Rasi and Mahalingam (2012) indicated that bacterial isolates such as Bacillus spp. 1, Bacillus spp. 2, Micrococcus spp., Pseudomonas spp. 1, and Acinetobacter spp. showed better growth performance at 37°C with 1.5% CMC in a medium of acidic and neutral pH conditions than alkaline pH conditions.

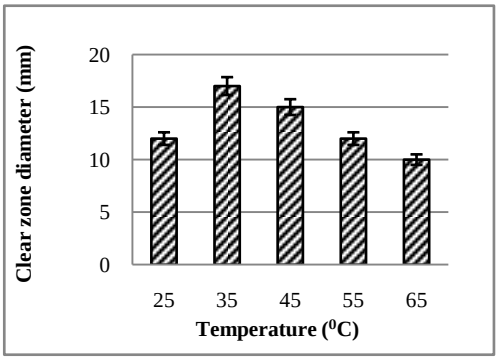

Figure 5. Effect of temperature on cellulase production from the strain GT1

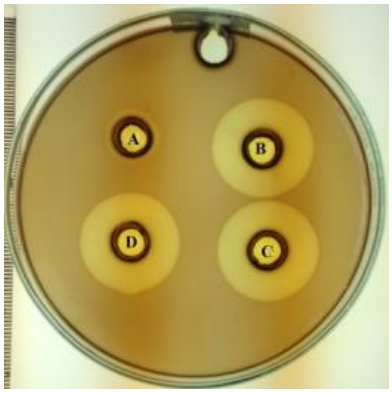

Figure 6. Cellulase production of *Bacillus* sp. GT1 in the medium containing peptone

Note: A: control well (the medium didn't contain any nitrogen sources); B, C, D: wells of crude enzymes (the medium contained peptone)

Table 2. Effects of nitrogen and carbon supplementation on cellulase production of the GT1 strain

No	Nitrogen sources	Clear zone diameters (mm)	No	Carbon sources	Clear zone diameters (mm)
0	Control	7	0	Control	7
1	Peptone	19	1	Corn starch	16.5
2	$(NH_4)_3C_6H_5O_7$	18.5	2	Arrowroot powder	13
3	Yeast extract	18	3	Dextrin	10
4	Beef extract	17	4	Tapioca starch	9
5	$(NH_4)_2HPO_4$	15	5	Starch	9
6	$(NH_4)_2SO_4$	13.5	6	D-sobitol	8.5
7	$NH_4H_2PO_4$	13.5	7	Mannitol	7
8	NH_4NO_3	13	8	Saccarose	7
9	KNO_3	11	9	CMC	5
10	$NaNO_3$	11	10	α-lactose	2
11	NH_4Cl	7.5	11	D-glucose	0
			12	Maltose	0
			13	D-(+)-xylose	0

3.3.3. Effects of nitrogen sources

Nitrogen is the main building block of proteins and is one of the main constituents of protoplasm. It was found that all the nitrogen sources, which were used in the present study, significantly supported cellulase enzyme production.

Including peptone in the medium resulted in the highest cellulase production, which was calculated as having a 19 mm diameter clear zone at 35°C after 48 hours of incubation. It was followed by $(NH_4)_3C_6H_5O_7$ and yeast extract (Table 2).

Our findings are in accordance with Das *et al.* (2010) who achieved maximal cellulase production in a medium containing peptone as a nitrogen source. Another study (Doi, 2008) also reported that peptone was a good nitrogen source for cellulase production.

3.3.4. Effect of carbon sources

Because cellulases are inducible enzymes, the medium for cellulase production in fermentation usually contains cellulose-rich substrates as a carbon source (Yang *et al.*, 2014). In the present study, different carbon sources at various concentrations were examined to study their effects on GT1 celulase production under identical conditions.

The results showed that the GT1 strain could utilize various carbon sources, and the maximal CMCase production (16.5 mm) was observed when corn powder was used as the sole carbon source (Table 2). Yang *et al.* (2014) also reported that the best carbon source for exoglucanase as well as endoglucanase activity was corn powder.

3.3.5. Effects of metal ions

Metal ions play important roles in the biological function of many enzymes. The various modes of metal-protein interactions include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids, or structural regulators.

Enzyme production was stimulated by Mn^{2+}, Mg^{2+}, and Ca^{2+}, while Fe^{2+}, Zn^{2+}, and Cu^{2+} reduced their activity. According to the study by Yin *et al.* (2010), Mn^{2+} greatly activated the purified cellulase but Fe^{2+} inactivated the purified cellulase activity.

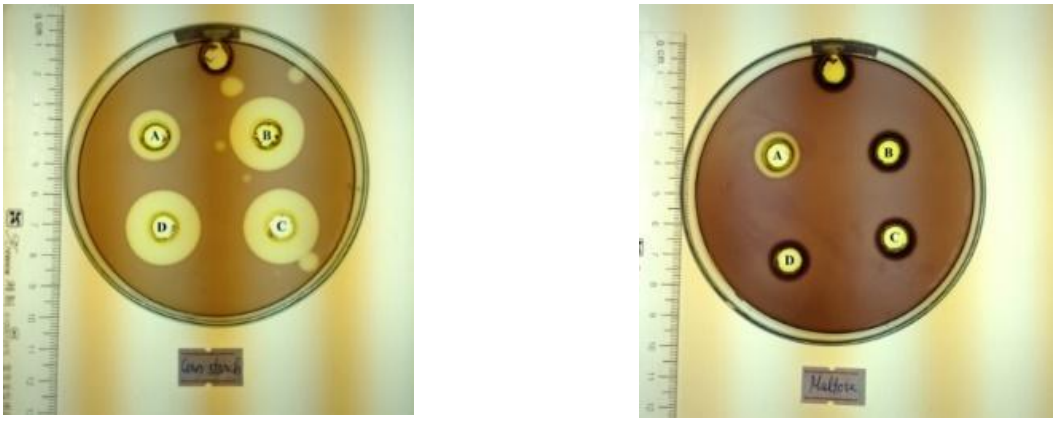

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Figure 7. Cellulase production of *Bacillus* sp. GT1 in mediums containing corn starch (1) and maltose (2)

Note: A: control well (the medium didn't contain any carbon sources);B, C, D: crude enzyme wells (the medium contained corn starch)

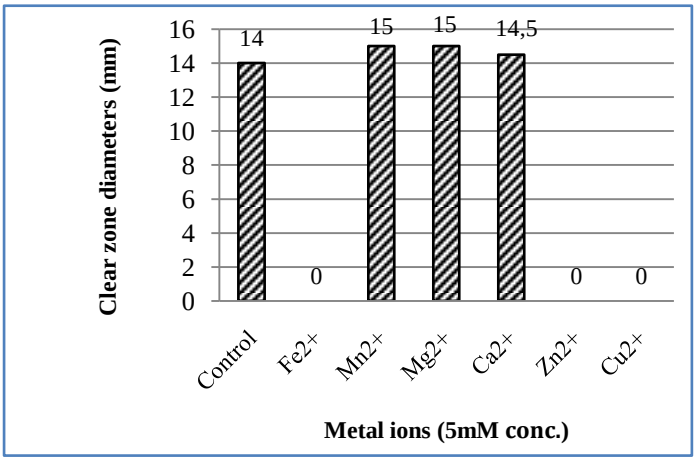

Figure 8. Effect of different metal ions on cellulase production of *Bacillus* sp. GT1 strain

4. CONCLUSIONS

The strain GT1, identified to be *Bacillus* sp., showed the highest CMCase production among the 8 strains selected. After evaluation of the effects of the cultivation conditions on cellulase production in the GT1 strain, the results indicated that the bacterial strain reached the highest cellulose yield at pH 7 and 35°C. Peptone and corn starch were favorable nitrogen and carbon sources, respectively, for enzyme activity. The enzymes were stimulated by Mn^{2+}, Mg^{2+}, and Ca^{2+}, while Fe^{2+}, Zn^{2+}, and Cu^{2+} reduced their activity. In addition, the GT1 strain expressed high cellulolytic activity for decomposition of cellulose to produce ethanol. It was also able to produce antibacterial enzymes against several bacterial pathogens.

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