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Characterization of Cellulose Degrading Bacterium, *Bacillus megaterium* S3, Isolated from Indigenous Environment

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Abstract.- A cellulose degrading bacterium, identified as *Bacillus megaterium* S3 on the basis of biochemical and 16S rRNA ribotyping, was isolated from vegetable market, Lahore, Pakistan. It was screened by efficiently growing on carboxymethyl cellulose (CMC-Na), Congo red agar, and minimal salt medium containing filter paper. The most suitable temperature for the growth of *B. megaterium* S3 was found to be 45ºC while the optimum growth pH was 7. *B. megaterium* S3 had a lag phase of 4 hours in LB medium while this phase prolonged to 6 hours in CMC-Na medium. *B. megaterium* S3 had shown maximum enzyme activity in extra-cellular assay (75%) as compared to intra-cellular assay (17%). The optimum temperature and pH for the crude enzyme activity were found to be 50ºC and 8, respectively. Maximum crude enzyme extract (cellulase) activity was found in the presence of Zn ions. This alkali-thermophilic enzyme from *B. megaterium* S3 can be exploited for biotechnological and industrial applications.

Key words: Cellulose, *Bacillus megaterium*, CMC-Na, cellulase.

INTRODUCTION

The conversion of biomass into biofuel has become a major industrial challenge over the past decades because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity (Lynd *et al*., 1999). Besides this, environmental pollution, especially in densely populated areas, needs more attention and issues such as waste prevention and recycling as well as bio-product development are thus topical. The elaboration of biofuels is encouraged due to the depletion of fossil fuel reserves and increased pollution as a result of its combustion. The biodegradation of waste cellulose materials to fermentable sugars would assist in the limitation of waste as well as with the development of sustainable bioenergy (Jeffries and Schartmann, 1999) and bio-products like lactic acid (Venkatesh, 1997). The main issue in such a process concerns the degradation in fermentable sugars of the main plant cell wall polysaccharide, cellulose, with the most efficient enzymatic cocktail that is mostly manifested in fungi (Perrson *et al*., 1991) and bacteria (Gilkes *et al*., 1991).

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Cellulose, an unbranched β-1,4-linked homopolymer of glucose, is the most abundant renewable fuel resource on Earth. The enzymatic degradation of cellulose has gained much attention because this process has great economic potential and low possibility for causing environmental pollution (Bayer *et al*., 1994). Many cellulolytic microorganisms and their enzyme systems have been studied extensively for the enzymatic conversion of cellulosic substances (Ladisch *et al*., 1983; Sulman and Rehman, 2013). Microbial cellulolysis naturally occurs in the aerobic and anaerobic biotopes where cellulose accumulates and this hydrolysis of cellulose is aided by the enzymes cellulases, which produces glucose, an easily fermentable monosaccharide (Wyman *et al*., 1993; Bergeron, 1996).

Cellulases are inducible enzymes, which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). Microorganisms hydrolyze and metabolize insoluble cellulose by these extracellular cellulases that are either free or cell associated. The biochemical analysis of cellulase systems from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during the past two decades. Cellulases have attracted much interest because of the diversity of their application such as their usage in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims,

as well as in household laundry detergents for improving fabric softness and brightness (Cavaco-Pola, 1998). A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Gong *et al.*, 1999).

Many bacteria can degrade cellulose. The cellulase systems they produce usually comprise a number of distinct enzymes, which can have specific activities comparable to those of cellulolytic fungi. The cellulase systems of certain bacteria may be found as discrete multienzyme aggregates. More than 60 enzymes have been characterized at the protein level and more than 40 bacterial cellulase genes have been cloned. A number of bacterial cellulases contain distinct catalytic and cellulose-binding domains (Gilkes *et al*., 1991). The genus Bacillus represents one of the important groups of bacteria in secreting extracellular commercial enzymes such as proteolytic, amylolytic and also cellulolytic enzymes (Crueger and Crueger, 1982) especially when they are provided with suitable medium and growth condition.

The present study is concerned with the isolation and characterization of *B. megaterium* S3 from native environment. Optimum growth conditions and effect of CMC-Na on the growth of the organism are ascertained. Some enzymatic characteristics *i.e.*, pH, temperature, and effect of metal ions on the cellulase activity are also determined.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

A total of 9 samples were collected from vegetable market, Kot Lakhpat, and Gulshan Iqbal Park, Lahore and proceed for the isolation of cellulose degrading bacteria. Temperature and pH of the samples were also noted at the time of collection. All samples were serially diluted up to 10^{-3} in 10 ml of sterilized water. An aliquot of 100 µl of each dilution was spread on LB agar plates and incubated at 37°C for 24 hours. Morphologically 30 different bacterial colonies were observed and purified on LB agar plates by streak plate method.

Screening of cellulose degrading bacteria

Growth of isolated and purified colonies was checked on carboxymethyl cellulose-Na (CMC-Na) and well grown colonies were selected for Congo red dye test. Bacterial isolates selected from CMC-Na (10 mg/ml) medium were streaked on CMC-Na Congo red agar plates and incubated at 37°C for 3-5 days to observe hallow zones around the colonies.

Growth on salt medium with filter paper

Minimal salt medium ($NaNO_3$ 2.5g, KH_2PO_4 1.0g, $MgSO_4.7H_2O$ 0.6g, NaCl 0.1g, $CaCl_2$.$6H_2O$ 0.1g, $FeCl_3$ 0.01g, and gelatin 2.0g, in 900 ml of distilled water) was supplemented with filter paper strips 10 g/l (1%) as a sole carbon source and pH of the medium was adjusted to 7. The final volume of the medium was made up to 1000 ml. The medium was inoculated with 16 hours old bacterial culture and incubated at 37°C on shaking incubator for 15 days. After 15 days the results were noted for the change in texture of the filter paper.

Determination of cellulase activity

Three bacterial isolates were grown in mineral salt medium containing 1% CMC-Na medium as a sole carbon source. The flasks were inoculated with 16 hours old bacterial culture and were incubated at 37°C for 5 days. Bacterial cells were harvested by centrifugation at 4000 rpm for 10 minutes. Cells were disrupted by sonication (Heilscher Ultrasonic Processors UP 400, S) at 4°C for 1 time for 15 s and centrifuged at 10000 rpm for 10 minutes at 4°C. The cell lysate was used for measurement of intra-cellular enzyme activity. The culture supernatant (for extra-cellular enzyme activity) was saturated with 60% ammonium sulfate to precipitate the enzyme. After 24 hours, the precipitated solution was centrifuged at 10000 rpm for 10 minutes at 4°C. Ammonium sulfate precipitated samples were desalted and cellulase activity was measured according to Yang *et al*. (2003) by using 1% w/v CMC solution into 0.1 mol/l phosphate buffer (pH 7) as a substrate. The reaction mixture contained 0.5 ml of supernatant as an enzyme. The mixture was incubated at 50°C for 30 minutes. The CMC enzyme-mixed solution reaction was stopped by adding 1 ml of DNS reagent followed by boiling at 90°C for 10 minutes. The developed color was read at 540 nm using spectrophotometer. One unit of enzyme activity was

defined as the amount of enzyme releasing reducing sugar equivalent to 1 µmol glucose per minute under the assay condition. On the basis of CMC-Na-Congo red agar plate clearing zone, change in texture of the filter paper and enzyme assay results one bacterial isolate (S3) was selected for further study.

Morphological, biochemical, and molecular characterization

The isolate was Gram stained. For biochemical characterization the isolate was tested for catalase activity, oxidase activity, nitrate reduction, and Voges-Proskauer test. Some specific tests were performed for further characterization of isolates such as sporulation test, phenylalanine deaminase test, and utilization of different sugars. The procedures adopted for all the above physical and biochemical tests were taken from Benson (1994). For molecular characterization genomic DNA was extracted as described by Carozzi *et al*. (1991) and the 16S rRNA gene was amplified by PCR using 16S rRNA primers (RS-1; 5′-AAACTC-AAATGAATTGACGG-3′, and RS-3; 5′-ACGGGCGGTGTGTAC-3′) (Rehman *et al*., 2007). PCR was performed by initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The PCR product of 0.5kb was purified with a Fermentas purification kit (#K0513) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. Nucleotide sequence similarities were determined using BLAST (NCBI database; http://www.ncbi.nlm.nih.gov/BLAST).

Optimization of temperature and pH

To ascertain the effect of temperature on the bacterial isolate, LB broth medium was inoculated with 10 µl from 16 hours grown bacterial culture and incubated at 30°C, 37°C, 45°C, and 55°C for 24 hours. Three replicates for each bacterial isolate were used to maintain the accuracy. Similarly LB broth medium with pH of 5, 6, 7, 8, and 9 was inoculated with 10 µl from 16 hours grown bacterial culture and incubated for 24 hours at 45°C. Growth was assessed by measuring the optical density at 600 nm after 24 hours of incubation at respective temperatures by using a LAMBDA 650 UV/Vis Spectrophotometer (PerkinElmer, USA).

Effect of CMC-Na on bacterial growth

The growth behavior of the bacterial isolate was checked over a time period of 24 hours. *B. megaterium* S3 was grown in LB medium (control) and mineral salt medium with 1% CMC-Na as a sole carbon source at 37°C and pH of 7. The flasks were inoculated with 16 hours old bacterial culture and incubated at 37°C. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, and 24 hours. Absorbance was taken at 600 nm.

Effect of pH, temperature, and metal ions on crude enzyme extract (cellulase) activity

The effect of pH on the activity of cellulase (crude enzyme extract) was measured by incubating 0.5 ml of the diluted enzyme and 0.5 ml of buffers of different pH [sodium acetate buffer (pH 5-6), sodium phosphate buffer (pH7-8) and tris-HCl buffer (pH 9)] ranging pH from 5 to 9, containing 1% CMC-Na. The crude enzyme activity was determined by performing the standard assay procedure (Yang *et al*., 2003) at pH 8 within a temperature range of 30-90°C. After treatment the residual enzyme activity was assayed. The metal ion effect on enzyme activity was examined by chloride salts of various metals. Each metal was added in the reaction mixture at a final concentration of 0.1 mM and crude enzyme extract (cellulase) activity was determined at 50°C and pH 8. No metal ions were added in the control assay.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Cellulose degrading bacteria

A total of 9 samples were collected for the

isolation of cellulose degrading bacteria. Purified 30 bacterial isolates were screened for cellulose degradation on 1% CMC-Na medium and confirmed by the Congo red dye test where only three isolates showed best hallow zones (Fig. 1). Among the three bacterial isolates, *B. megaterium* S3 showed the largest zone and was selected for further study. Lu *et al*. (2005) reported fifteen bacteria that grew vigorously and showed the ability to develop clearing zones around their colonies on cellulose Congo-red agar during aerobic incubation. The clearing zone size and colony diameter of the isolate were indicating that the isolate has high ability of cellulase production. Isolation and characterization of cellulolytic bacteria have been reported by many workers (Maki *et al.*, 2011; Irfan *et al*., 2012).

Growth on salt medium with filter paper

The selected cellulose degrading bacterium exhibited high growth in CMC-Na and in minimal salt medium with filter paper. The changed texture of the filter paper confirmed its utilization as a sole carbon source by the bacterium (Fig. 2). The activity of the cellulase isolated from *Bacillus* species was analyzed against CMC-Na. Han *et al*. (1995) reported in a study the activity was found against carboxymethyl cellulose (Cm-cellulose), p-nitrophenyl-β-D-cellobioside (pNPC), 4-methylumbelliferyl-,3-D-cellobioside (MUC), and avicel PH101. However, the specific activity toward avicel was much lower than that of the soluble substrates. Neither MUG nor pNPG was hydrolyzed. In the present investigation *B. megaterium* S3 showed growth in both media containing 1% CMC-Na and filter paper separately. These results depict that this bacterium can be used for the degradation of a variety of cellulosic materials which are industrially important and economical.

Identification of the bacterial isolate

The biochemical characteristics of the bacterial isolate are shown in Table I. The partially amplified (540bp) and sequenced 16S rRNA gene from local isolate (S3) was uploaded to the NCBI (National Center for Biotechnology Information) website to search for similarity to known DNA sequences in order to confirm the species of the

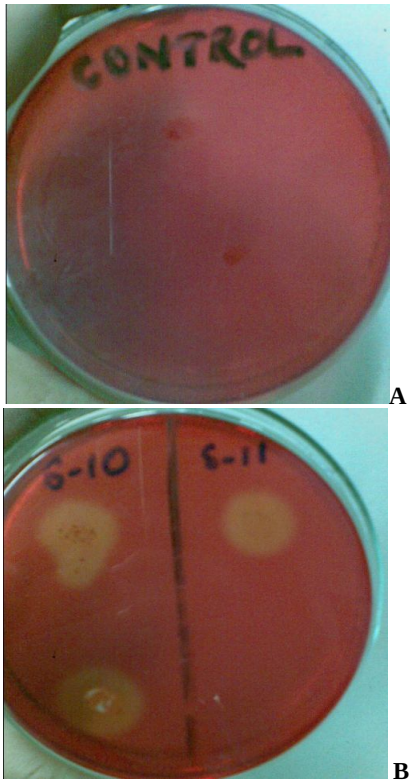

Fig. 1. Screening of cellulose degrading bacteria by 1% Congo red test (A). Bacterial isolates (S-3, S-10 and S-11) with cellulase activity exhibited clear zone on Congo red agar plate (B).

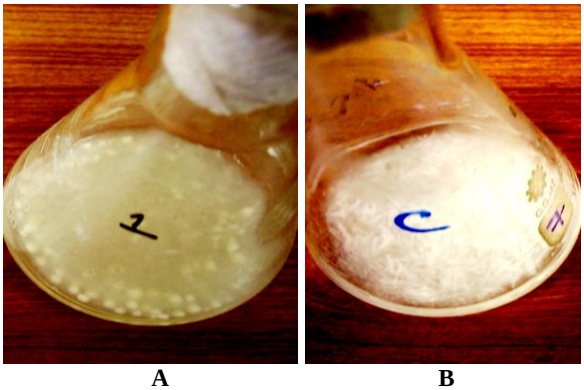

Fig. 2. Growth of *B. megaterium* S3 in minimal salt medium containing 1% filter paper. Texture of the filter paper has been clearly changed in the presence of S3 isolate (B) as compared to control (A) containing no bacterial isolate after 15 days of incubation at 37ºC.

locally isolated yeast. The BLAST query revealed that this gene is 97% homologous to already reported gene of *Bacillus megaterium*. The nucleotide sequences coding for the 16S rRNA gene of *B. megaterium* S3 have been submitted to the GenBank database under accession number JQ422055.

Table I.- Morphological and biochemical characteristics of *B. megaterium* S3.

Characters	*B. megaterium* S3
Colony shape	Round
Colour	White
Appearance	Rough
Margin	Lobate
Elevation	Raised
Spore formation	+
Oxidase	+
Catalase	-
Nitrate reduction	+
Voges-Proskeauer	+
Phenylalanine Deaminase	-
Glucose	+
Sucrose	+
Maltose)	+

Positive +; Negative -

According to a study carried out by Pason *et al*. (2006) the molecular characterization of a bacterium strain B-6 was done and this strain was a facultative, spore-forming, Gram-positive, motile, rod-shaped organism and produced catalase. Thus, this bacterium was identified as a member of the genus Bacillus according to Bergey's Manual of Systematic Bacteriology (Garrity, 2001).

Cellulase assay

Both extra- and intra-cellular enzyme activities were determined by DNS method. Results were ascertained for extra- and intra-cellular enzyme activities by the change of color from yellow to reddish brown due to the presence of sugars, which are produced by the breakdown of CMC. *B. megaterium* S3 showed high extra-cellular enzyme activity (75%) as compared to intra-cellular enzyme activity (17%). So the predominant form of the enzyme is extra-cellular (Fig. 3).

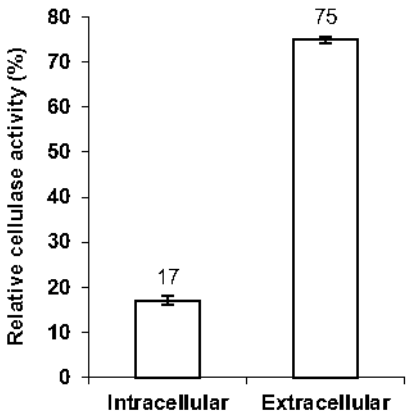

Fig. 3. Comparison between extra- and intra-cellular crude enzyme extract (cellulase) activity of *B. megaterium* S3 using CMC-Na as a substrate.

Optimization of temperature and pH

The most suitable temperature for bacterial isolate was found to be 45°C while the optimum pH was observed at 7. Lu *et al*. (2005) isolated 15 cellulose degrading bacterial isolates and a temperature stability test of these isolates showed that most of them grow between 30 and 40°C; only five of them can survive up to 50°C, indicating that all of the isolates were mesophilic bacteria. In a study a group I *Bacillus* strain, DLG, was isolated and characterized as being most closely related to *Bacillus subtilis*. When grown on trinitrophenyl-carboxymethyl cellulose medium, it was found to possess cellulolytic activity. When growth conditions were optimized the maximum cellulolytic activity in assays was observed at pH 4.8 (Robson and Chambliss, 1984). The results of this study are not consistent with our study as our bacterial isolate showed good growth at neutral pH *i.e.*, 7. The reason may be the change of environment and difference in soil conditions. Shobharani *et al*. (2013) reported the cellulase from *B. megaterium* which was optimally active at pH 6.0 and temperature of 60°C.

Effect of CMC-Na on bacterial growth

Growth curves of *B. megaterium* S3 were determined in LB and CMC-Na media. The lag phase of about 4 hours was determined in LB

medium while it was about 6 hours of duration in CMC-Na medium. Bacterial growth rate was slowed down in the medium containing CMC-N when no other carbon source except cellulose was provided in the culture medium. This extension of bacterial lag phase in CMC-Na medium as compared to LB medium may be due to metabolic shifting to utilize cellulose which usually slows down the growth rate of the bacterial cells (Fig. 4).

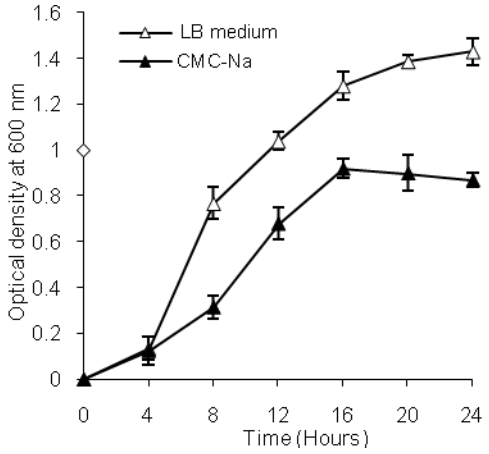

Fig. 4. Growth curves of *B. megaterium* S3 in LB (Control) and mineral salt medium containing 1% CMC-Na as a sole source of carbon. Optical density was measured after regular interval of every 4 hours at 600 nm.

Cellulase characteristics

Effect of temperature

As temperature is one of the most important variables, when *B. megaterium* S3 was checked for the effect of temperature on its enzymatic activity, maximum extra-cellulase activity (205 U/ml) was observed at 50°C (Fig. 5). Enzyme had retained 85% and 72% activity at 70°C and 90°C, respectively. Ibrahim and El-diwany (2007) studied the effect of temperature on the activity of crude cellulases ranging from 30°C to 85°C at pH of 7. The enzyme showed a good activity between 65°C to 75°C with maximum activity at 75°C. Optimum temperature range (80-100°C) of cellulase activity isolated from several archaebacteria has been reported by Bragger *et al*. (1989).

Effect of pH

The effect of pH on crude enzyme extract (cellulase) activity was observed by incubating enzyme with buffers of different pH. The bacterial isolate showed maximum enzyme activity (200 U/ml) at pH 8 (Fig. 6) and only 19% loss of enzyme activity was determined at pH of 9. Production of cellulase by *B. megaterium* S3 was found to be dependent on pH and *B. megaterium* S3 cellulase showed maximum activity in alkaline range *i.e.*, pH 8. Khan and Husaini (2006) checked the effect of pH on cellulase activity of *Bacillus* species and observed the highest yield at pH of 6. A variety of optimum pH for enzyme activity such as *B. lichenifolrmis* at pH 6.1 (Dhillon *et al*., 1985), *B. pumilis* at pH 10 (Kawai *et al*., 1988), and *B. subtilis* at pH 7 (Chundakkadu, 1998) has been reported by researchers.

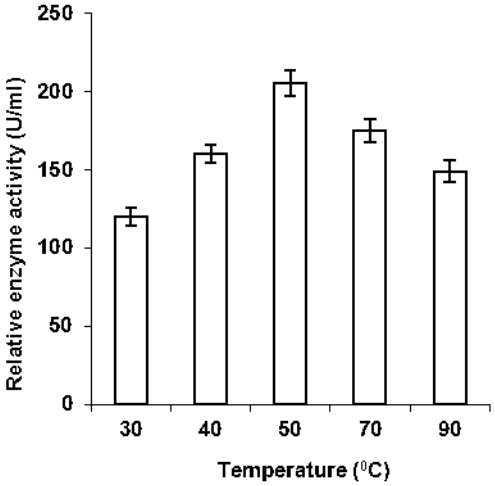

Fig. 5. Effect of temperature on the crude enzyme extract (cellulase) activity of *B. megaterium* S3 at pH 8 within a temperature range of 30-90°C.

Effect metal ions

Cellulase (crude enzyme extract) activity was also checked in the presence of various metal ions. The *B. megaterium* S3 showed increase in enzyme activity in the presence of all metal ions except Fe^{+2}. The maximum increase (17% greater than the normal enzyme reaction containing no Zn ions) of cellulase was found in the presence of Zn^{+2} (Fig. 7). The increase of crude enzyme extract (cellulose) activity in the presence of other metal ions was in

the following manner; Mn^{+2} (4%), Ca^{+2} (4%), Mg^{+2} (7%), Na^{+} (6%) and K^{+} (1%).

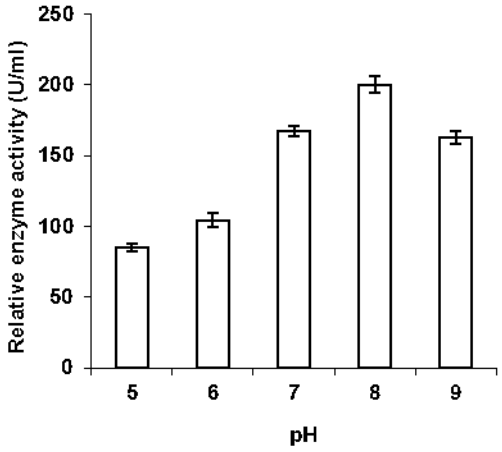

Fig. 6. Effect of pH on the crude enzyme extract (cellulase) activity of *B. megaterium* S3 in buffers of different pH [sodium acetate buffer (pH 5-6), sodium phosphate buffer (pH 7-8), and tris-HCl buffer (pH 9)] containing 1% CMC-Na.

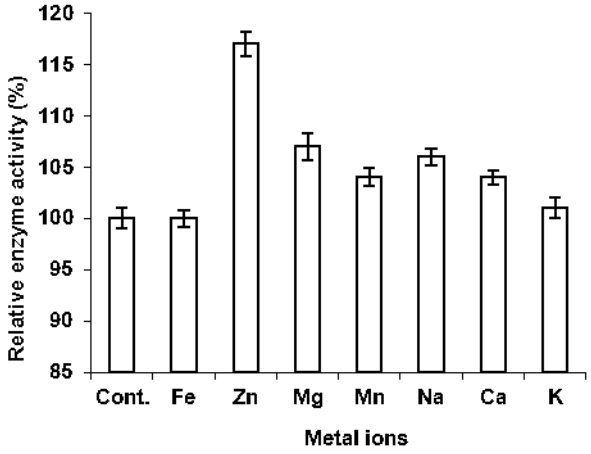

Fig. 7. Effect of metal ions on the crude enzyme extract (cellulase) activity of *B. megaterium* S3. The crude enzyme activity was determined in chloride salts of metals (0.1 mM) at 50°C and pH 8. No metal ions were added in the control assay.

In the present investigation a bacterium, isolated from vegetable market, was identified as *B. megaterium* S3 with maximum cellulase activity of 216 U/ml. The optimal pH and temperature of the organism were 7.0 and 45ºC, respectively. The predominant form of the enzyme was found to be extr-acellular (75%) as compared to intra-cellular (17%). The most suitable temperature and pH for the crude enzyme extract (cellulase) activity were found to be 50ºC and 8, respectively. Maximum enzyme activity was determined in the presence of Zn ions. *B. megaterium* S3 may find some potential applications in industrial biotechnology.

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