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Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity

[Selülaz aktivitesine sahip bakterilerin topraktan izolasyonu, değerlendirilmesi ve selülaz üretim ve aktivitesinin optimizasyonu]*

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ABSTRACT

Objective: The aim of the present study is to demonstrate the isolation, identification and screening of bacteria with high cellulase activity from soil samples.

Materials and Methods: Cellulose degrading bacteria were isolated from soil sample using serial dilution and pour plate method. Bacteria were further identified by morphological and biochemical tests and subjected to cellulase production in 250 ml Erlenmayer flask using potato waste medium for 48h of fermentation period at 35^{o}C with agitation speed of 140rpm.

Results: Seven different bacterial strains were isolated and screened for cellulase production in submerged fermentation process. Among these seven tested bacterial strains; ASN2 showed maximum yield for cellulase production. This strain was further characterized by biochemical and morphological tests and identified as *Cellulomonas* sp. ASN2. Supplementation of glucose, peptone and cysteine to the fermentation medium are favored enzyme secretion. The optimum pH and temperature for the activity of crude enzyme was 7.5 and 60^{o}C, respectively. Metal profile of the enzyme indicated that Co^{2+} and Mn^{2+} are the strong stimulators while Hg^{2+} and Fe^{2+} inhibited the activity of cellulase from cellulolytic bacterial strain, *Cellulomonas* sp.ASN2.

Conclusion: Results indicated that favorable fermentation conditions and the selection of a suitable growth medium played a key role in the production of cellulase from newly isolated *Cellulomonas* sp. ASN2. Due to its particular characteristics this enzyme will be used in saccharification process for bioethanol production from plant biomasses.

Key Words: Cellulolytic bacteria, cellulase characterization, fermentation.

Conflict of Interest: The author declares no conflict of interest.

ÖZET

Amaç: Bu çalışmanın amacı toprak örneklerinden sağlanan yüksek selülaz aktiviteli bakterilerin izolasyon, tanımlandırma ve değerlendirmesinin yapılmasıdır.

Gereç ve Yöntemler: Toprak örneklerinden selülozu yıkan bakteriler seri dilüsyon ve dökme plak (pour plate) metodu ile izole edildi. Daha sonra bakteriler morfolojik ve biyokimyasal testler ile tanımlandırılarak, 250 ml'lik Erlenmayer kabında patates atığı vasatta 48 saatlik fermantasyon süresinde 35˚C'de 140 rpm karıştırma hızında selülaz oluşumuna bırakıldı.

Bulgular: Yedi farklı bakteri suşu izole edildi ve fermentasyon sürecinde selülaz üretimleri takip edildi. Bu test edilen yedi bakteri suşu arasından; selülaz üretimi açısından en fazla ürün ASN2'de görüldü. Bu suş daha sonra biyokimyasal ve morfolojik testler ile tanımlandırılarak *Cellulomonas* sp. ASN2 olarak belirlendi. Fermentasyon vasatına glukoz, pepton ve sistein eklenmesi enzim salınımını destekledi. Ham enzim aktivitesi için optimum pH 7.5 ve sıcaklık 60˚C'dı. Enzim metal ilişkisine bakıldığında Co^{+2} ve Mn^{2+}'nin selülaz aktivitesine sahip bakterial suşlarda, *Cellulomonas* sp.ASN2, kuvvetli simülatörken, Hg^{2+} ve Fe^{2+} selülaz aktivitesini inhibe etmektedir.

Sonuç: Elde edilen sonuçlardan da görüldüğü gibi selülaz üretiminde, yeni izole edilen *Cellulomonas* sp. ASN2, verimli fermentasyon koşullarında ve uygun büyüme vasatının seçildiği durumlarda anahtar rol oynamaktadır. Bu enzim bazı özelliklerine bağlı olarak, bitkisel biyoyakıtlardan biyoetanol oluşturmak için şekere dönüştürme işleminde kullanılabilecektir.

Anahtar Kelimeler: Selülaz aktiviteli bakteri, selülaz karakterizasyonu, fermentasyon

Çıkar Çatışması: Yazarlar herhangi bir çıkar çatışması bildirmemektedirler.

Introduction

Sustainable resources, which are in need of human beings, are derived from plant biomass. Cellulose is the major component of plant biomass [1]. Plants produce 4×10^9 tons of cellulose annually [2]. It is a polymer of β-1,4 linked glucose units. Its crystalline structure and insoluble nature represents a big challenge for enzymatic hydrolysis. Microorganisms are important in conversion of lignocellulose wastes into valuable products like biofuels produced by fermentation [3]. Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes [4]. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes [5]. For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts [6]. Researchers keep on working to isolate microorganisms with higher cellulase activity [7]. Present study is aimed to isolate bacteria, which can produce more efficient cellulase enzymes in submerged fermentation.

Materials and Methods

Isolation of cellulolytic bacteria

Cellulolytic bacterial strains were isolated from soil by using serial dilutions and pour plate technique. The medium used for isolation of cellulolytic bacteria contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin at pH 7 for 48 hours of incubation at 30˚C [8]. Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at 4°C for further identification and screening for cellulase production.

Screening of cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1 % congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis [9]. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.

Identification of cellulolytic bacteria

Identification of cellulolytic bacteria was carried out by method as described by Cowen and Steel [10] and Cullimore [11], which was based on morphological and biochemical tests.

Inoculum development

Pure cultures of selected bacterial isolates were individually maintained on CMC supplemented minimal agar slants at 4˚C, until used. Pure cultures of selected bacterial isolates were inoculated in broth medium containing 0.03 % $MgSO_4$, 0.2 % K_2HPO_4, 1 % glucose, 0.25 % $(NH_4)_2SO_4$ and 1 % peptone at pH 7 for 24h of fermentation period. After 24h of fermentation period these vegetative cells were used as inoculum source.

Cellulase enzyme production

Newly isolated strains were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium was prepared using 1% potato waste (as cellulose substrate), 0.2 % K_2HPO_4, 0.03 % $MgSO_4$, 1 % peptone, 0.25 % $(NH_4)_2SO_4$ and autoclaved at 121°C for 15min. After sterilization, the medium was allowed to cool at room temperature. The medium was inoculated with 1 ml of selected bacterial isolates and incubated in a shaker (Eyela NTS- 331) at 35˚C for 24 h of fermentation period with agitation speed of 140 rpm. After termination of the fermentation period the fermented broth was centrifuged at 14000 × g for 10 min at 4˚C to remove the unwanted material. The clear supernanant thus obtained after centrifugation served as crude enzyme source.

Estimation of cellulase activity

Cellulase activity was assayed using dinitrosalisic acid (DNS) reagent [12] by estimation of reducing sugars released from CMC solubilized in 0.05 M phosphate buffer at pH 8 [13]. The culture broth was centrifuged at 14000 × g for 10 min at 4˚C and the clear supernanant served as crude enzyme source. Crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M phosphate buffer and incubated at 50˚C for 30 min. After incubation, reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100˚C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve [14]. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1μmol of glucose per minute under standard assay conditions.

Optimization of nutritional conditions for cellulase production

Different nutritional conditions such as; additional 0.5% carbon sources (CMC, starch, maltose, glucose, sucrose, cellulose powder), amino acids at concentration of 0.01% (alanine, glutamic acid, cystine, lysine, tyrosine), various nitrogen sources $(NH_4)_2SO_4$, KNO_3, ammonium citrate, NH_4Cl, $NaNO_3$, peptone, tryptone, yeast extract) and urea at concentration of 0.25% were optimized for cellulase production by newly isolated *Cellulomonas* sp. ASN2 in submerged fermentation process.

Characterization of the crude cellulases of newly isolated cellulolytic strain:

Effect of pH on activity and stability of crude cellulases

The optimum pH for the crude enzyme was determined by incubating crude enzyme with substrate (1% CMC) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0 to 6.0), 0.05 M sodium phosphate buffer (pH 6.0 to 8.0), 0.05 M Tris-HCl (pH 8.0 to 9.0) and 0.05 M glycine-NaOH (pH 9.0 to 11.0). Crude enzyme mixture in those buffers was incubated for 30 min at 50˚C. Cellulase activity was assayed by DNS method. The pH stability was determined by incubating crude enzyme mixture in above-mentioned buffers at room temperature for 30min and enzyme stability was determined by using DNS method.

Effect of temperature on activity of crude cellulases

The effect of temperature on activity of endoglucanase was determined by incubating crude enzyme with 1 % CMC in 10mM phosphate buffer (pH 8.0) at temperatures between 20 to 90°C. Enzyme activity was assayed by DNS method at different temperatures as described above.

Effect of various metal ions on activity of crude cellulases

Various divalent metal ions including Co^{2+}, Ni^{2+}, EDTA, Hg^{2+}, Ca^{2+}, Mn^{2+}, $Fe^{2+}$and Mg^{2+} was applied to check the optimum activity of enzyme. Each metal ions and EDTA were used at concentration of 5mM.

Statistical analysis

One way analysis of variance (ANOVA) was done using Statistical Package for the Social Sciences (SPSS) for the determination of significant differences within different conditions, Tukey test was applied. Three replicates were determined for each condition. A significant difference was found when $p< 0.05$.

Results and Discussion

Cellulose is the main building block of plants and have major fraction of organic carbon in soil. Microorganisms, which live in soil, are accountable for recycling of this organic carbon to the environment [15]. Degredation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms [16]. About one fifth of fresh water and soil samples yield cellulose degrading bacteria after enrichment but some samples did not bear such kind of bacteria [17]. This is due to existence of microenvironments where different growth conditions for cellulose degrading bacteria are present. These bacteria are generally found in well manure soils [18]. Several microorganisms have been discovered for decades which have capacity to convert cellulose into simple sugars [19] but the need for newly isolated cellulose degrading microorganism still continues [20].

In the present study, seven cellulolytic bacteria were isolated from soil samples. These seven bacteria were screened for cellulase production in submerged fermentation process using suitable medium. Among all these seven tested bacterial strains; ASN2 gave better yield as shown in Figure 1. ASN2 strain was further identified by using morphological and biochemical tests (Table 1 and Figure 2). Colonies of ASN2 on CMC agar were yellow white color, glistening; margins were entire, convex and circular and 1-2 mm in diameter at 30˚C. When scraped with a loop, colonies were slimy or viscous and tend to clump. Fresh culture of this isolate consists of Gram negative, slender and rod shaped cells but the older cultures contain coccoid cells. Microscopic examination of this isolate revealed that it was gram negative, non-spore forming and motile. It was negative for indole production, Voges Proskauer test and citrate utilization and positive for catalase and nitrate reduction. It could ferment glucose, cellulose, lactose and sucrose. From these characteristics, isolate ASN2 was identified as *Cellulomonas* sp.

Researchers studying on cellulolytic activity have isolated various bacteria from different environmental sources. Hatami et al. [21] isolated aerobic cellulolytic bacteria from forest and farming soils and determined their ability to decompose cellulose. Yin et al. [8], isolated *Cellulomonas* sp. YJ5 from soil samples and cellulase activity was determined after 48 hours of incubation. Das et al. [16] isolated eight bacterial strains from cow dung samples and they found that maximum amount of cellulase producing bacterial isolate was *Bacillus* sp. In another study, Otajevwo and Auyi [22] isolated *Pseudomonas* sp. and *Serratia* sp. from soil samples, which have greater ability to produce cellulase enzyme.

Optimization of cultural and nutritional conditions for cellulase production by cellulolytic bacterial isolate Cellulomonas sp.ASN2

The isolated bacterial strain *Cellulomonas* sp.ASN2 had the ability to utilize various additional carbon sources, which are available in soil environment. It was observed that glucose significantly ($p<0.05$) increased the cellulase activity in the supernatant (0.475 ± 0.019 IU/ml/min) when compared to other carbon sources (Table 2). Similar findings were also reported by Jaradat et al. [23] using glucose as a basic carbon source for cellulase production by *Streptomyces* sp. Utilization of CMC as carbon source is best for cellulase production as reported by Das et al. [16] for *Bacillus* sp (3.028 µg/mg/min). Ojumu et al. [24] reported about some lignocellulosics, which serves as carbon source for the production of cellulase. Some investigators [25-28] showed that addition of cellulose, filter paper, CMC, starch or cellobiose to the fermentation medium favored cellulase production by *Cellulomonas* sp, *Clostridium* and *Bacillus* sp.

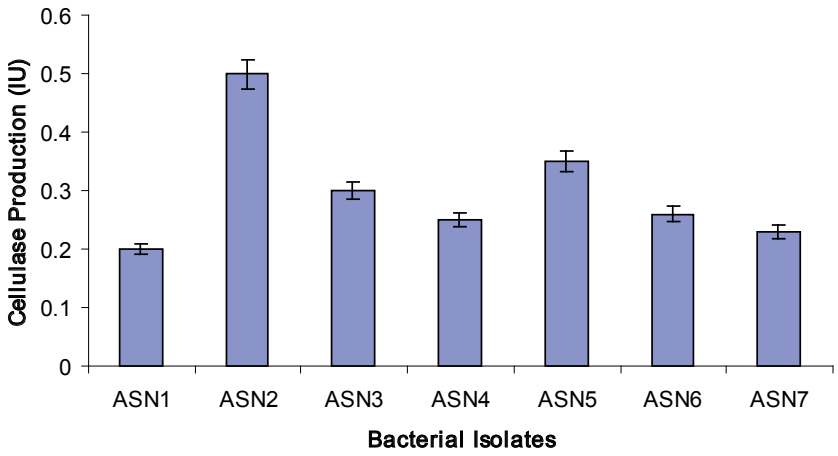

Figure 1. Cellulase activity of isolated bacterial strains in submerged fermentation at 35°C.

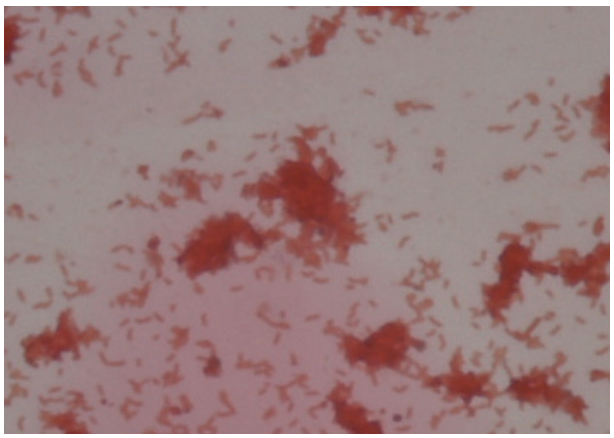

Figure 2. Grams staining of isolate *Cellulomonas* sp.ASN2 after 24 hours of incubation.

Table 1. Biochemical reaction and characteristics of the cellulolytic bacterial isolate, *Cellulomonas* sp.ASN2.

Characteristics /biochemical test	ASN2
Cell shape	Short rods
Gram's reaction	-
Spores formation	-
Indole production	-
Citrate utilization	-
Voges proskauer	-
Catalase	+
Nitrate reduction	+
Motility	+
Growth on	
Microcrystalline cellulose	+
Carboxymethylcellulose (CMC)	+
Nutrient broth	+
Fermentation of	
Glucose	+
Fructose	+
Maltose	+
Starch	+
Sucrose	+

(-) negative, (+) positive

Table 2. Effect of nitrogen, carbon and amino acid supplementation on cellulase activity of newly isolated Cellulomonas sp.ASN2.

Nitrogen Sources	Cellulase activity (IU)	Carbon sources	Cellulase activity (IU)	Amino acids	Cellulase activity (IU)
Control	0.20 ± 0.01	Control	0.19 ± 0.001	Control	0.34 ± 0.02
$NaNO_3$	0.42 ± 0.03	Glucose	0.54 ± 0.03	Alanine	0.30 ± 0.02
NH_4Cl	0.47 ± 0.03	CMC	0.40 ± 0.03	Glutamic acid	0.35 ± 0.03
$(NH_4)_2SO_4$	0.41 ± 0.03	Starch	0.38 ± 0.02	Cysteine	0.52 ± 0.03
$(NH_4)_2C_6H_6O_7$	0.34 ± 0.02	Maltose	0.34 ± 0.02	Lysine	0.46 ± 0.03
KNO_3	0.40 ± 0.03	Cellulose	0.41 ± 0.03	Tyrosine	0.32 ± 0.02
Yeast extract	0.35 ± 0.03	Sucrose	0.43 ± 0.03		
Peptone	0.54 ± 0.03				
Tryptone	0.41 ± 0.03				
Urea	0.31 ± 0.02				

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, supported cellulase enzyme production. Including NH_4Cl and peptone in the medium resulted in high cellulase activity in the culture supernatant which is calculated as 0.450 ± 0.012 and 0.474 IU/ml/min at 35˚C for 48 hours of incubation respectively ($p<0.05$) (Table 2). Our findings are in accordance with Jaradat et al. [23] who also achieved maximum cellulase production in a medium containing NH_4Cl as a nitrogen source. Vyas et al. [29] reported that the best inorganic nitrogen source for exoglucanase as well as endoglucanase activity is $(NH_4)_2SO_4$. Utilization of different inorganic nitrogen sources in this experiment revealed that this isolate could obtain nitrogen from naturally available nitrogen sources in soil and from fertilizers. Similar findings were also obtained by Balamurugan et al. [30]. Addition of $(NH_4)_2PO_4$ or tryptone to the molasses medium enhances the cellulase production by *B.subtilis* KO [25]. Different researchers [16, 6] have reported that peptone is a good nitrogen source for cellulase production.

Amino acids have profound effects on cellulase production as they are the building blocks of proteins [31]. In this study, it was seen that cellulase activity in the culture was significantly high ($p<0.05$) with cysteine (Table 2). In the presence of amino acids, cellulase synthesis might be increased, which resulted in increase in enzyme activity. Sharma et al. [32] found that alanine is suppressive for cellulase production. L-asparagine was found to be a good stimulator of cellulase production by *B. subtilis* CY5 in the solid-state fermentation [7].

Characterization of crude enzyme from cellulolytic bacterial isolate ASN2

Effect of pH on activity and stability of cellulase

The optimum pH for endoglucanase activity was found to be 7.5 (0.451± 0.011 IU/ml/min) and stable at pH 6.5 to 9.5 (Figure 3). Increasing or decreasing pH beyond this resulted in decline in enzyme activity. Any change in pH caused changes in the enzyme active site. Yin et al. [8] isolated *Cellulomonas* sp. YJ5 showing its optimum pH of 7 and pH stability range of 7.5-10.5. Cellulase enzyme of *B. subtilis subsp subtilis* A-54 has optimum pH of 6.5 and stable in pH range of 6.5-8 [33]. According to previous studies, cellulases are active at the pH range of 6.0 to 7.0 from *A. Niger* [34], 5.0 to 7.0 from *Lysobacter* sp. [35] and 5.0 to 6.5 from *Bacillus* strains [36]. Present findings were significant from *M. circinelloides* (pH 4-7) [379 and *B. circulans* (4.5-7.0) [38]. This range of pH is important for this enzyme, which can be used, in alkaline environments such as in processing of paper pulp.

Effect of temperature on activity of cellulase

Cellulomonas sp.ASN2 cellulases showed optimum activity at 60˚C (Figure 4). As the temperature increased

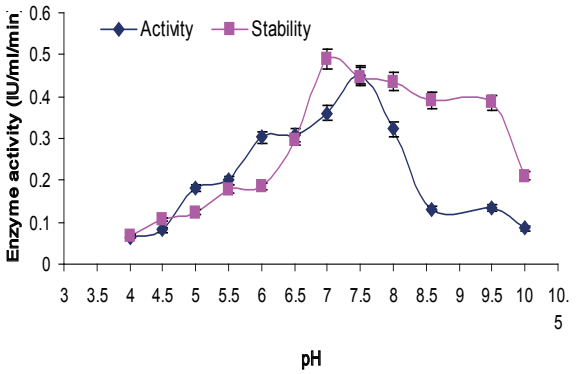

Figure 3. Effect of pH on the activity of cellulase from newly isolated cellulolytic strain *Cellulomonas* sp.ASN2.

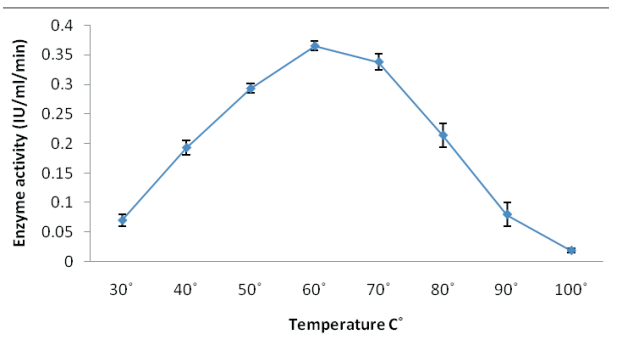

Figure 4. Effect of temperature on the activity of cellulases from newly isolated cellulolytic strain *Cellulomonas* sp. ASN2.

from 30˚C enzyme activity increased but activity started to decline as temperature increased above 60˚C and became completely denatured at 100˚C. Similar findings were also reported by Yin et al. [8]. They showed that optimum temperature for *Cellulumonas* sp. YJ5 cellulase was 60˚C. Cellulases from some species of *Bacillus subtilis subsp subtilis* A-53, *B. subtilis* YJ1 and *Bacillus* strains RH68 and CH43 have optimum temperature of 50˚C [39], 60˚C [39] , 70˚C (RH68) and 65˚C [36] respectively. The optimum temperature of crude cellulase of the present isolate was higher than that of *M. circinelloides* i.e. 55˚C [37].

Effect of various metal ions on activity of cellulase

In this study it was revealed that metal ions Co^{2+} and Mn^{2+} activated cellulases of *Cellulomonas* sp.ASN2, which was moderately inhibited by Mg^{2+} and strongly inhibited by Hg^{2+} and Fe^{2+} (Figure 5). The major action of these metal ions is to work as cofactor of the enzyme. Activation of cellulase occurs when storage or purification results in aggregation and autoxidation by reducing agents which causes the reduction of disulfide bonds [38]. The present study revealed that enzyme is inactivated by Hg^{2+} and Fe^{2+}, similar to the findings of Smriti and Sanwal [40] for *Catharanthus roseus* and Lu-

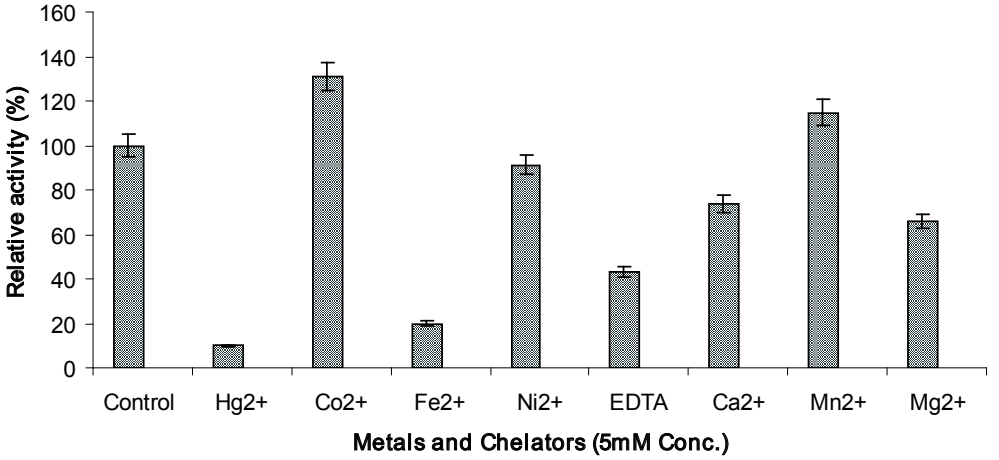

Figure 5. Effect of EDTA and different metals on the activity of cellulases from newly isolated cellulolytic strain *Cellulomonas* sp. ASN2.

cas et al. [41] for *Chalara paradoxa*. This is possibly due to binding of Hg^{2+} with thiol groups and interaction with carboxyl or imidazol groups of amino acids [41]. Activation by Co^{2+} and Mn^{2+} is similar to results of Saha [37] from *M. circineloides*. Mn^{2+} and Hg^{2+} were also found to be the strong activator and the strong inhibitor of cellulase produced from *B. subtilis* YJ1, respectively [3In this study *Cellulomonas* sp. ASN2, was successfully isolated which can utilize glucose and peptone as an additional carbon and nitrogen sources, respectively. Further supplementation of cysteine to the fermentation medium improved the enzyme production. The crude cellulases of this isolate exhibited its optimum activity at pH of 7.5 and temperature of 60˚C. The enzyme was stimulated by Co^{2+} and Mn^{2+} while Hg^{2+} and Fe^{2+} reduced its activity. Because of its activity at high temperatures, cellulase enzyme of *Cellulomonas* ASN2 isolated in this study will be beneficial for industrial applications.

Conflict of Interest: The author declares no conflict of interest.

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