Carboxymethyl Cellulase Production from Newly isolated *Cellulomonas* sp. in Submerged Fermentation

Yeni İzole Edilmiş *Cellulomonas* Sp.'den Batık Fermantasyonda Karboksimetil Selülaz Üretimi

Research Article

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ABSTRACT

The present study dealt with the production of cellulase from locally isolated cellulolytic bacterium *Cellulomonas* sp which was isolated from soil. Cellulase enzyme production was carried out in 250 mL Erlenmeyer flask using potato waste as a substrate in submerged fermentation. The strain produced maximum cellulase with initial medium pH of 7, inoculum size of 2% (v/v) and incubation temperature of 35°C for 48 h of fermentation period. Supplementation of 2% corn steep liquor had a profound effect on titer of enzyme production. The isolated bacterium *Cellulomonas* sp. ASN2 can be used as potential producer of effective cellulase which would be beneficial in industrial applications.

Key Words

Isolation, *Cellulomonas* sp. Cellulases, Submerged fermentation

ÖZET

Mevcut çalışma, topraktan alınmış yerel olarak izole edilen selülolitik bakteri *Cellulomonas* sp.'den selülaz üretimi ile ilgilidir. Selülaz enzim üretimi, batırılma fermantasyonunda substrat olarak patates atığı kullanılarak 250 mL erlenmeyer şişe içinde gerçekleştirilmiştir. Suş, başlangıç ortam pH'ı 7, % 2 (v/v) inoklum büyüklüğü ve 35°C inkübasyon sıcaklığında 48 saat fermantasyon süresinde üretilmiştir. 2% mısır maserasyon sıvısı takviyesi enzim üretim titresi üzerinde oldukça yoğun bir etki gösterdi. İzole edilen *Cellulomonas* sp. ASN2 bakterisi endüstriyel uygulamalarda yararlı olacak selülaz üretiminde etkili potansiyel bir üretici olarak kullanılabilir.

Anahtar Kelimeler

İzolasyon, *Cellulomonas* sp. Sellülazlar, Batık fermantasyon

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INTRODUCTION

Cellulases are inducible enzymes which can hydrolyze cellulosic materials into glucose by using synergistic action of three enzymes including endo-β-1, 4-glucanase (EC 3.2.1.4, EG; Cleave internal linkages randomly), exocellobiohydrolase (EC 3.2.1.74; hydrolyze cellobiosyl units from non-reducing ends), and β-D-glucosidase (; hydrolyzing glucosyl units from cellooligosaccharides; EC 3.2.1.21) [1]. Recently, cellulases has major applications in industries such as in textile industries, laundry detergents [2], in animal feeds, in fruit juices processing, in baking and in de-inking of paper [3]. A major role of cellulases is the bioconversion of cellulosic waste materials into biofuels [4]. Cellulases have independently folding, structurally and functionally distinct units called domains which make these enzymes modular [5]. Carhohydrate binding module (CBM) is an important module of these enzymes. Its major function is to bring catalytic domain closer to crystalline cellulose. Some CBM are preferential for noncrystalline cellulose binding. Bacteria which can produce cellulases have been exploited over the past few decades from different sources such as decaying plant materials, composts, organic matter; feces of ruminants and from extreme environments [6] and a number of cellulases have been produced and characterized. Present study is aimed to isolate cellulytic bacterium from soil and production of cellulases from newly isolated bacterium.

MATERIALS AND METHODS

Isolation and identification of cellulolytic bacteria:

A cellulase producing bacterium was isolated from soil samples of Kasur, Pakistan by using serial dilutions and pour plate technique. The strain was isolated with specific medium and identified morphologically and biochemically as described earlier [7]. The strain *Cellulomonas* sp ASN2 was maintained on nutrient agar slats and stored at 4°C and revived weekly.

Inoculum Preparation

Bacterial cells were propagated in a medium containing 1% glucose, 0.2% K_2HPO_4, 0.03% $MgSO_4$, 1% peptone, and 0.25% $(NH_4)_2SO_4$ at pH 7 and incubation temperature of 35°C for 24 h. This bacterial cell suspension was used as an inoculum source in subsequent fermentation experiments.

Production of CMCase

Twenty five milliliter of fermentation medium (1% potato waste (substrate), 0.2% K_2HPO_4, 0.03% $MgSO_4$, 1% peptone, 0.25% $(NH_4)_2SO_4$) was taken in 250 mL Erlenmeyer flask which was cotton plugged and autoclaved at 121°C for 15 min. After sterilization, the medium was allowed to cool at room temperature. The medium was inoculated with 1 mL of *Cellulomonas* sp. ASN2 and incubated in a water bath shaker (Eyela NTS- 331) at 35°C for 48 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 supernatant thus obtained after centrifugation served as crude enzyme source.

Estimation of CMCase activity

Endoglucanase (CMCase) activity was determined as described earlier [7]. 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 1.5 mL of DNS reagent and boiled at 100°C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. One unit (U) of enzyme activity is expressed as the quantity of enzyme which is required to release 1 μmol of glucose per minute under standard assay conditions.

Effect of incubation period on production of CMCase:

To check the optimum incubation period a set of experiment was conducted and incubated at various incubation periods like 24, 48, 72, 96 and 120 h. After the termination of fermentation the enzyme was harvested and assayed using standard procedures.

Effect of incubation temperature on production of CMCase:

Various incubation temperatures such as 20, 25, 30, 35, 40 and 45°C were tested for optimum production of CMCase by newly isolated *Cellulomonas* sp in submerged fermentation.

Effect of initial medium pH on production of CMCase:

To study the effect of initial medium pH on CMCase production, experiment was performed by changing the pH of the medium like 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. pH of the medium was adjusted before sterilization.

Effect of different inoculum sizes on production of CMCase:

Different inoculum sizes like 0.5%, 1.0%, 1.5%, 2%, 2.5% were used for optimized production of CMCase from *Cellulomonas* sp. ASN2 in submerged fermentation process.

Effect of different concentrations of corn steep liquor on enzyme production:

Various concentrations of corn steep liquor such as 0.5%, 1.0%, 1.5%, 2%, 2.5% and 3% were supplemented to the fermentation medium to optimize the cellulase production from newly isolated *Cellulomonas* sp. ASN2 in submerged fermentation.

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

Total 42 bacterial isolates were obtained of which seven strains ASN1, ASN2, ASN3, ASN4, ASN5, ASN6 and ASN7 were cellulytic as they can grow on CMC agar plates at 35°C (Figure 1). Two cellulolytic bacterial isolates ASN2 and ASN3 with highest hydrolytic activity and CMCase production were subjected to identification, optimization and CMCase characterization.

Colony morphological characteristics of these seven cellulytic bacterial isolates are shown in Table 1. Of these seven strains, ASN2 was selected and identified [7] used for the production of carboxymethyl cellulose in submerged fermentation using potato waste as a medium.

The newly isolated bacterium designated as *Cellulomonas* sp.ASN2 was incubated at 35°C for various time intervals such as 24, 48, 72, 96 and 120 h to check the optimum time for cellulase secretion. Figure 2 illustrated that the bacterium gave better yield after 48 h of incubation period. As the incubation period increased a decline in enzyme production was observed. Selvankumar et al. [8] produced endoglucanase from *B. amyloliquifaciens* using coffee pulp as a substrate in solid state fermentation and obtained maximum yield of enzyme after 72 h of incubation period. Shabeb et al. [9] produced cellulase from *B. subtilis* KO using low cast medium and obtained maximum yield (32 IU through CMCZ) after 24 h of fermentation using molasses as a carbon source. Heck et al. [10] also reported peak CMCase activity (1.08 UI/mg protein) at 24 h fermentation period.

Figure 3 represents the effect of initial medium pH on cellulase enzyme production in submerged fermentation process. Results indicated that increase in initial pH of the medium up to 7 caused an increase in cellulolytic activity and maximum activity in the supernatant (0.45 ± 0.13 IU) was observed at initial pH of 7. Cellulase activity decreased when *Cellulomonas* sp. ASN2 was grown at initial medium pH above 7. Das et al. [11] isolated a *Bacillus* strain from cow dung and reported that this strain was well grown on initial pH 7 and produce maximum amount of cellulase enzyme. Immanuel et al. [12] isolated three bacterial strains from coir waste and identified them as *Cellulomonas* sp. *Bacillus* sp. and *Micrococcus* sp. In their study, the *Cellulomonas* sp showed maximum cellulase activity in its supernatant at pH 7 with 1.5% substrate concentration. Two bacterial strains belonging to *Bacillus* species, i.e. *B. subtilis* CY5 and *B.circulans* TP3, also had maximum cellulase activity when they were grown at pH 7.5 [13]. Some studies [14,15] reported that pH 7 is very effective pH for the degradation of cellulose.

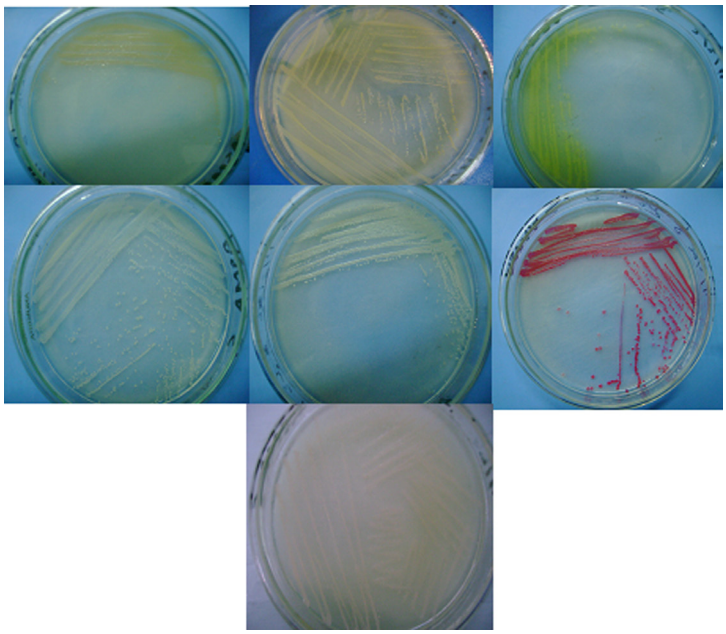

Figure 1. Growth of seven isolated bacterial strains on CMC-agar plates.

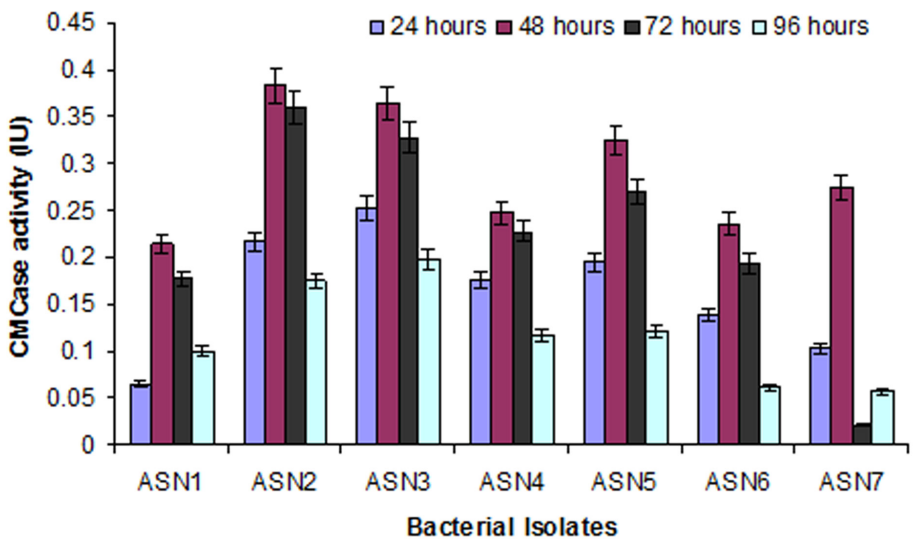

Figure 2. Effect of incubation period on cellulase activity from newly isolated cellulolytic strains *Cellulomonas* sp.ASN2 in submerged fermentation.

Table 1. Colony characteristics of cellulolytic bacterial isolates.

Selected bacterial isolates	Colony pigment	Colony size (mm)	Margin	Elevation	Surface texture
ASN1	Orange	1-2 mm	Round	Convex	Smooth
ASN2	Yellow- white	1 mm	Round	Convex	Smooth
ASN3	Yellow	1-2 mm	Round	Convex	Smooth
ASN4	White	1 mm	Round	Convex	Smooth
ASN5	White	1 mm	Round	Convex	Smooth
ASN6	Red	2 mm	Round	Convex	Mucoid
ASN7	Off white	1-2 mm	Round	Convex	Mucoid

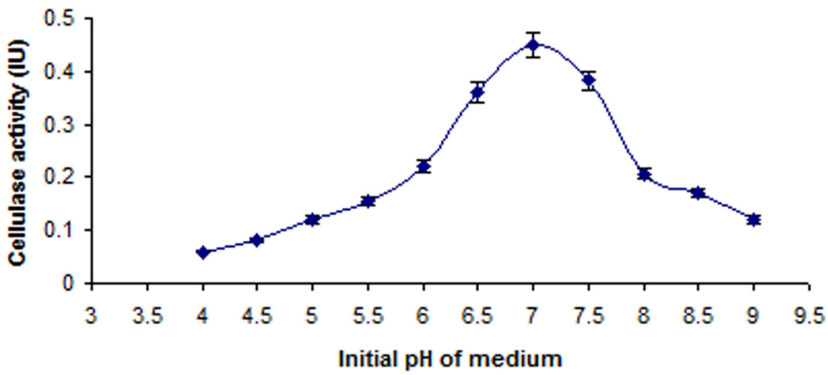

Figure 3. Effect of initial medium pH on cellulase activity from newly isolated cellulolytic strain ***Cellulomonas*** sp.ASN2 in submerged fermentation.

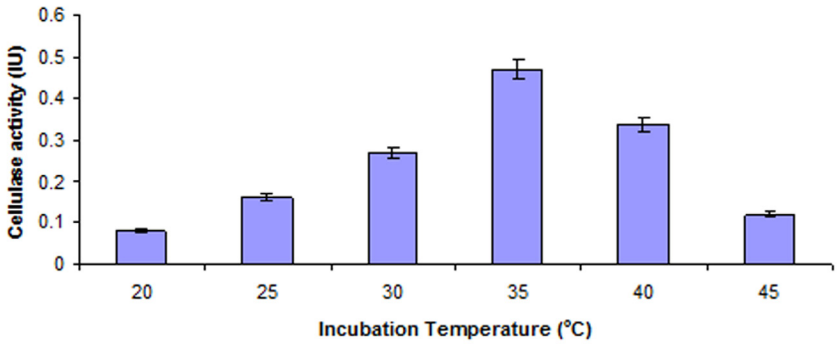

Figure 4. Effect of incubation temperature of ***Cellulomonas*** sp.ASN2 on cellulase activity in submerged fermentation.

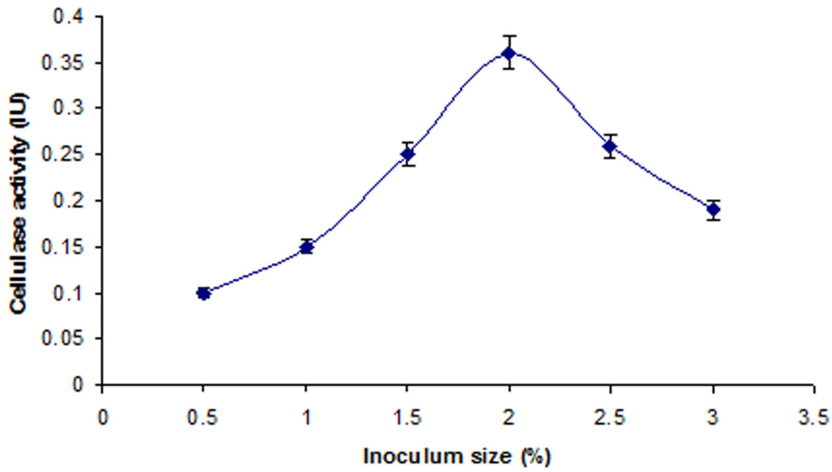

Figure 5. Effect of different inoculum sizes on cellulase activity from newly isolated cellulolytic strain *Cellulomonas* sp. ASN2 in submerged fermentation.

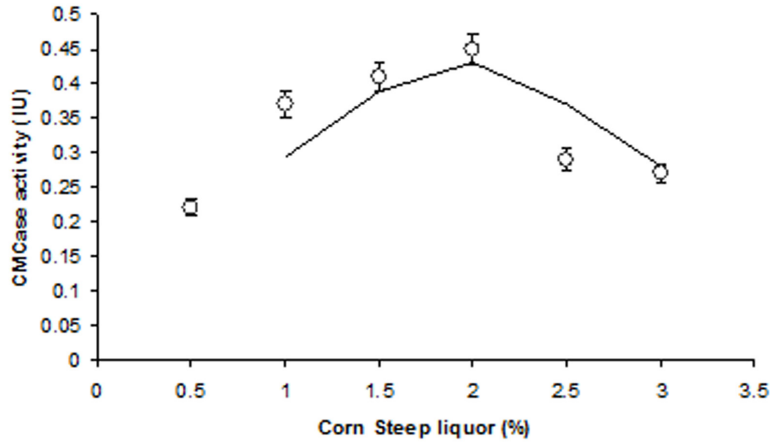

Figure 6. Effect of different concentrations of corn steep liquor on production of CMCase in submerged fermentation.

Figure 4 indicated the effect of incubation temperature on the amount or the activity of cellulase enzymes in the culture in submerged fermentation. Results revealed that incubation temperature of 35°C was optimum for maximum activity. The higher cellulase activities might be caused by high amount of enzyme production and increased extracellular secretion or increased enzyme activity at specific incubation condition. Temperature other than 35 °C resulted in decline in enzyme production. *Cellulomonas* sp can produce maximum amount of cellulase enzyme at incubation temperature of 40°C [12]. Some strains of *Bacillus* sp can grow maximum at 42°C and produce greater yield of cellulase enzyme [11]. *B. subtilis* CY5 and *B. circulans* TP3 produce maximum amount of cellulase enzyme at incubation temperature of 40°C [13].

Enzyme production was also found affected by the amount of inoculum size. In the present study, it was observed that 2% (v/v) inoculum size resulted in significant ($P < 0.05$) increase in the activity in the culture supernatant of *Cellulomonas* sp. ASN2 (0.338 ± 0.005 IU/mL/min). Lower cellulase activity was measured in the culture supernatant when higher or lower inoculum size was used (Figure 5). Effective uptake of nutrients and improved distribution of dissolved oxygen causes high enzyme production [16]. When inoculum is small reduced amount of cellulase is produced by insufficient number of bacteria [17]. Das *et al.* [11] reported 7% inoculum level was optimum for cellulase production by *Bacillus* sp in submerged fermentation and high inoculum size results in reduction of dissolved oxygen. Ray *et al.* [13] isolated two bacterial strains i.e. *B. subtilis* CY5 and *B. circulans* TP3 from fish gut which have optimum cellulase production with 3 and 4% inoculum level in solid state fermentation respectively.

Effect of different concentrations of corn steep liquor on production of CMCase:

Corn steep liquor is a mixture of amino acids, organic acids, proteins, carbohydrates, minerals and vitamins and used as a best nutritional source for microorganisms in production of fermentation products like enzymes and antibiotics. Effect of different concentrations of corn steep liquor 0.5%, 1%, 1.5%, 2%, 2.5% and 3% on enzyme production was examined. In this study, it was observed that production of cellulase was significantly higher ($P<0.05$) at 2% (v/v) concentration of corn steep liquor (0.454 IU/mL/min). When the concentration of corn steep liquor is less i.e. 0.5% (v/v), 1% (v/v) and 1.5% (v/v), the enzyme was produced in reduced amount. At 2.5% (v/v) and 3% (v/v) of corn steep liquor concentration, production of cellulase was also found less (Figure 6). Our results are in accordance with Nascimento et al. [18] who also reported 2% corn steep liquor as a basic substrate for cellulolytic enzyme production by *Streptomyces malaysiensis* in submerged fermentation. Similar findings were also reported by Farid and Shahed, [19] for *Trichoderma reesei*

as they obtain maximum enzyme production at 2 % CSL concentration.

CONCLUSION

Results of this study revealed that *Cellulomonas* sp could be used for cellulase enzyme production which have potential role in industrial applications with particular emphases on saccharification of lignocellulosic biomasses for bioethanol production. For enhanced production of cellulase enzyme, optimization of the process parameters play a pivotal role to make the process cost effective at large scale.

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