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# Purification and Characterization of Produced Cellulase Enzyme by Irradiated *Achromobacter spanius*

**Abeer M. Mousa [1*], Osama A. Abdel Aziz [1*] Ola E. A. Al-Hagar [2*]**

[1*] *Soil and Water Research Department, Nuclear Research Center, Atomic Energy Authority, Egypt*

[2*] *Plant Research Department, Nuclear Research Center, Atomic Energy Authority, Egypt*

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***Achromobacter spanius*** **was endophytic cellulase producing bacteria. They were irradiated by gamma rays at doses from 0.5 – 5 kGy. Irradiated *Achromobacter spanius* increase extracellular cellulase activity in comparison with non-irradiated strain. Irradiated strain at dose 3.5 kGy was identified as higher cellulase enzyme activity than those of other doses. The cellulase enzyme was purified by applying sephadex G-100 gel chromatography technique. The highest activity of cellulase was obtained at enzyme volume and incubation period of 0.2 ml and 30 minutes respectively. The optimum pH value was 7 and the optimum activity with temperature was 35 $^{o}$C. The enzyme showed the highest activity when carboxmethyl cellulose (CMC) used as substrate on reaction mixture. According to thermal stability, the purified cellulase has high activity after 5 minutes when purified cellulase was incubated at 60 $^{O}$C. The purified cellulase, with a molecular weight (M.W.) of 32.5 kDa,**

***Key words:*** Cellulase, Achromobacter spanius, gamma radiation, purification

## Introduction

Cellulose is a renewable source of energy and a major structural component of plants [1]. It is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere [2]. Cellulose, a crystalline polymer of D-glucose residues connected by β-1, 4 glucosidic linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature [3]. Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources.

Cellulases are inducible enzymes which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials [4]. The cellulase complex is comprised of three major components; carboxymethyl cellualase (CMC ases) or endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) [5]. These enzymes convert cellulose into an utilizable energy source, glucose which provides a key role in utilization of biomass. Cellulases are the enzymes that help in the conversion of cellulose substrates into fermentable sugars. There is an increasing interest in cellulase production by bacteria as it has a high growth rate when

Corresponding author: abeeraboseer@yahoo.com
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compared to fungi and has a good potential in the production of cellulase. It is used in ruminant nutrition for improving digestibility, fruit juices processing and in deinking of paper [6]. For enhancing growth of crops and controlling plant diseases, various enzyme preparations consisting of different combinations of cellulases, hemicellulases, and pectinases have potential applications in agriculture [1,7]. Improvement of soil quality was also reported by the use of cellulase which reduces dependence of mineral fertilizers [8]. Decomposition of cellulose in soil was accelerated by addition of exogenous cellulase supplementation was reported by Fontaine et al [9]. Therefore, using exogenous cellulase may be a potential means to accelerate straw decomposition and increase soil fertility [8].

Cellulases from various sources have distinctive features as they exhibit specific pH optima, thermal stability and exact substrate specificity may also vary with the origin [1,10]. Various bacterial genera reported for cellulolytic activities include *Bacillus, Clostridium, cellulomonas, Rummminococcus, Alteromonas, Acetivibrio* etc. Among bacteria, *Bacillus sp*. including *B. brevis* [11], *B. pumilus* [12], *B. amyoliquefaciens* DL-3 [13] , and *Bacillus subtilis* YJ1 [14] *Bacillus sp*. [15] ; Rawat and Tewari [16] are well recognized cellulose production under submerged condition [17, 18]. Three strains, *viz*., *Pseudomonas* sp., *Bacillus* sp. and *Achromobacter xylosoxidans* with cellulolytic activity, which are more promising than the reported cultures of related genera. Of these, *Bacillus* sp. showed reasonably high cellulolytic activity [19]. From the economic point of view, acid hydrolysis of cellulosic materials may be cheaper, but lead to the accumulation repulsive by product. Enzymatic hydrolysis of cellulosic materials gives pure product [20]. The objective of the present study is the purification of cellulase enzyme and optimal characterization by irradiated *Achromobacter spanius.*

## Materials and Methods

*Bacterial strain and gamma radiation*

*Achromobacter spanius* as cellulase producing bacteria. It was provided by El-Gamal [21] in laboratory Soil Microbiology Unit of Soil and Water Research Department- Nuclear Research Center, Atomic Energy Authority, Inshas, Egypt and maintained on carboxymethylcellulose (CMC) medium [14]. CMC media contains (g/l): Peptone 10; CMC 10; $K_2HPO_4$ 2 ; $MgSO_4.7H_2O$ 0. 3; $(NH_4)_2So_4$ 2.5 and gelatin 2; at pH 7 for 3 days of incubation in shaker with 200 rpm at 37˚C.

*Achromobacter spanius* cells were inoculated in CMC medium and cultured at 37$^oC$ for three days. *Achromobacter spanius* was irradiated by γ rays in cobalt-60 gamma cell 220 located at Cyclotron, Nuclear Research Center, and Atomic Energy Authority. The dose rate was 1.09 kGy at the time of experiment. Agar slants of the tested strains were subjected to dose levels from 0.5 to 5.0 kGy. Different doses of irradiation were used to select the most efficient strain of high cellulase production. The cellulase activity was determined using method [22]. One of units of cellulase activity is expressed as the amount of one $ug^{-1}$ of glucose release per minute.

*Purification of cellulase produced by irradiated Achromobacter spanius*

The following steps were performed for purification of cellulase produced by irradiated *Achromobacter spanius* at 3.5 kGy as a result of its growth on CMC medium under all production optimal conditions.

*Preparation of cell-free filtrate:*

Irradiated *Achromobacter spanius* was cultivated on CMC broth medium, taking into consideration all the optimal conditions. After sterilization, the medium was inoculated with $10^8$c.f.u./ml obtained from 3 days old culture. Inoculated medium was incubated at 37$^oC$ for 3 days. At the end of the incubation period, the culture was centrifuged at 8000 X *g* for 10 min and the supernatant was then filtrated through 0.2 um pore size Millipore, to obtain the filtrate (cell free crude enzyme) [23]**.**

*Ammonium sulphate crude enzyme fractional precipitation:*

Ammonium sulphate crystals were added to the crude enzyme preparation with concentration percentages of 20, 40, 60, 80 and 100% respectively. The mixtures were stored overnight at

4 $^{O}$C. The precipitated protein was obtained by centrifugation for 15 min at 8000 X *g* under cooling. The obtained protein pellets were dissolved in 5 ml of phosphate buffer at pH 7. This precipitate protein was used for further determination of both cellulase activity and protein content. The addition of ammonium sulphate (crude enzyme) was preformed gradually with stirring at 4 $^{O}$C [24].

*Dialysis against tap water and sucrose:*

This purification step was carried out to remove the accumulated traces of ammonium sulphate. The precipitated pellets obtained from ammonium sulphate fractionation, were dissolved in phosphate buffer at pH 7 and introduced inside a special plastic bag to dialyze against distilled water for 3 hrs, and followed by the same buffer. Finally, the cellulase preparation was concentrated against pure sucrose crystals. During this process the volume decreased and the enzyme got concentrated. Cellulase activity was determined according to Miller [22] and protein content were also estimated according to Lowry [25].

*Purification of partially purified cellulase by applying sephadex G-100 Gel chromatography technique:*

The dialyzed partially purified cellulase preparation was applied onto column glass packed with Sephadex G-100. This was equilibrated with phosphate buffer at pH 7, and then eluted with the same buffer at pH 7. Preparation of the gel column and the fractionation procedure could be summarized in the following:

Five grams of Sephadex G-100 were suspended into 100 ml of distilled water and then 100 ml at phosphate buffer at pH 7 and allowed to swell overnight in the refrigerator. Sodium azide (0.2%) was added to prevent any microbial growth. A glass column (1.5 x 40 cm) was packed carefully by pouring the previously degassed slurry of gel into vertical column partially filled with the same buffer.

The dialyzed cellulase (1 ml) preparation was applied carefully to the top of the gel. It was allowed to pass into the gel by running the column. Buffer was then added without disturbing the gel surface and then the column was connected to the reservoir. Twenty fractions were collected (each contain 5 ml), both cellulase activity and protein content were determined for each separate fraction.

*Factors affecting activity of the purified cellulase enzyme :*

**Enzyme volumes:** Different purified cellulase volumes of (0.05, 0.1, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 ml) were applied into the reaction mixtures, then the enzyme activity was determined.

**pH values:** This experiment was performed to investigate the effect of different pH values on the purified cellulase activity. The purified cellulase reaction mixtures were incubated at 37 $^{O}$C at different pH values 3 and 5 using citrate buffer and pH 7 and 8 by using phosphate buffer. The enzyme activity at each particular pH value was determined.

**Incubation temperatures:** Effect of different incubation temperatures on purified cellulase activity was carried out by incubating the reaction mixture at different incubation temperatures 20, 25, 30, 35, 40, 45, 50, 55 and 60 $^{O}$C, respectively.

**Different substrate:** To investigate the effect of different substrate on purified cellulase enzyme activity. The substrate was applied into the reaction mixture cellulose powder, filter paper and carboxmethyl cellulose. Enzyme activity was determined as mentioned before.

**Incubation times:** This experiment was designed to determine the effect of different incubation time on the activity of the purified cellulase. The experiment was carried out by incubating the purified cellulase with best substrate at 35 $^{o}$C for different incubation period 15, 30, 45 and 60 minutes, respectively.

**Thermostability of the purified cellulase:** Thermostability of the purified cellulase preparation was studied under all optimal reaction conditions. The temperature range within the purified cellulase remains active. It was of interest to study the stability of cellulase when exposed to

a temperature higher than optimum. The purified cellulase was incubated at 60 $^{O}$C in phosphate buffer at pH 7 for time intervals and assayed for remaining activity was determined by adding the substrate and carrying out the enzyme assay.

**Determination molecular weight:** Purified cellulase from *Achromobacter spanius* was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme run at phosphate buffer at pH 7 on gel with Brilliant stain and markers as albumin (66 kDa), carbonic anhydrase (30 KDa), chymotrypsinogen A (25 KDa) ovalbumin (45 kDa) and ribonuclease A (13 KDa).

*Statistical analysis:*

The data were compared according to Duncan multiple range test [26].

## Results

Since *Achromobacter spanius* proved to be the most potent cellulase bacterial isolate. It was chosen to study its cellulase production under all optimum environmental and nutritional conditions.

The use of broth CMC media to screen irradiated Achromobacter spanius produced an increased cellulase enzyme by quantify assay. The increase specific activity for cellulase enzyme on the dose 3.5 kGy was 12.3 Units/mg protein), Table (1).

The increasing concentration of ammonium sulphate up to 60 % resulted in an increase in the specific activity of cellulase (6.03 Units/mg protein). Ammonium sulphate at 80 % saturation resulted in a decrease in specific activity of cellulase (5.41 Units/mg protein). The result indicated that, ammonium sulphate 60(%) is suitable for enzyme purification Table (2).

The precipitated protein at concentration of (60 %) saturation of ammonium sulphate was dissolved in the least amount of phosphate buffer at pH 7. The concentrated enzyme was applied to sephadex G-100 column chromatography. Elution of enzyme was carried out by phosphate buffer pH 7. The obtained results presented in Fig. (1) showed that, there was active peak obtained in fraction number (8), which may be cellulase enzyme. It was found that, the active fraction (No. 8) had the highest specificity (3.5 Units/mg protein).

*Characterization of cellulase produced by Achromobacter spanius strain*

The following characters were investigated for their effect on the purified cellulase produced by irradiated *Achromobacter spanius*

*Effect of purified enzyme volume, pH and temperature on purified cellulase activity*

Cellulase activity was found to be gradually increased with increasing the cellulase volume up to fixed concentration (0.2 ml) above which there was a sharp decrease in cellulase activity. The highest activity of cellulase (1.335 Units) was obtained at enzyme volume 0.2 ml (Table 3). The highest activity of cellulase (1.790 Units) was obtained at pH 7 by using phosphate buffer Table (3). The enzyme activity was determined at different incubation temperatures ranged from 20-60 $^{O}$C. The maximum activity of cellulase obtained at 35$^{O}$C and above this there was an inhibition in enzyme activity Table (3).

**Effect of thermal stability, substrate specificity and incubation time on purified cellulase activity**

Data identify the cellulase activity, when the enzyme was exposed to 60 $^{O}$C for different times, just before performing the enzyme assay. The activity of cellulase was stable with the temperature 60$^{O}$C for 5 minutes, and above that there was reduction in enzyme activity (Table 4). The purified cellulase enzyme showed highest activity against CMC Table (3) while cellulose powder and filter paper were used in the assay, the enzyme activity depressed. The cellulase activity of the purified enzyme at different incubation period. It appeared that the maximum activity was obtained after 30 minutes for cellulase, while after 45 minutes (0.430 Units) the enzyme activity decreased (Table 4).

*Molecular weight of purified cellulase produced by Achromobacter spanius strain*

The purified cellulase enzyme was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis to obtain protein profile. The molecular weight of the cellulase enzyme was 32.5 KDa by comparing with marker.

**Table (1): Specific activity of *Achromobacter spanius* strain cellulase production after gamma irradiation**

| Radiation doses kGy | Cellulase ($ug^{-1}$) | Total enzyme activity (Units) | Total protein ($mg^{-1}$) | Specific activity (Units/mg protein) |
|---|---|---|---|---|
| 0.5 | 57.30 | 0.318 | 0.05 | 6.36 F |
| 1 | 105.6 | 0.590 | 0.09 | 6.52 E |
| 1.5 | 140.2 | 0.780 | 0.12 | 6.49 E |
| 2 | 152.9 | 0.850 | 0.11 | 7.70 D |
| 2.5 | 188.1 | 1.040 | 0.12 | 8.71 C |
| 3 | 186.8 | 1.040 | 0.10 | 10.4 B |
| 3.5 | 221.8 | 1.230 | 0.11 | 12.3 A |
| 4 | 26.40 | 0.150 | 0.10 | 1.52 H |
| 4.5 | 29.60 | 0.160 | 0.11 | 1.41 I |
| 5 | 18.70 | 0.100 | 0.12 | 0.38 J |
| Irradiated | 110.4 | 0.610 | 0.14 | 4.35 G |

**Table (2): Ammonium sulphate precipitation pattern of crude cellulase from irradiated *Achromobacter spanius* strain**

| Conc. of ammonium Sulphate (%) | Total enzyme activity (Units) | Total protein (mg/ml) | Specific activity (Units/mg protein) |
|---|---|---|---|
| 20 | 0.683 | 0.167 | 4.07 C |
| 40 | 0.785 | 0.200 | 3.92 D |
| 60 | 1.026 | 0.170 | 6.03 A |
| 80 | 1.57 | 0.29 | 5.41 B |
| 100 | 0.549 | 0.19 | 2.88 E |

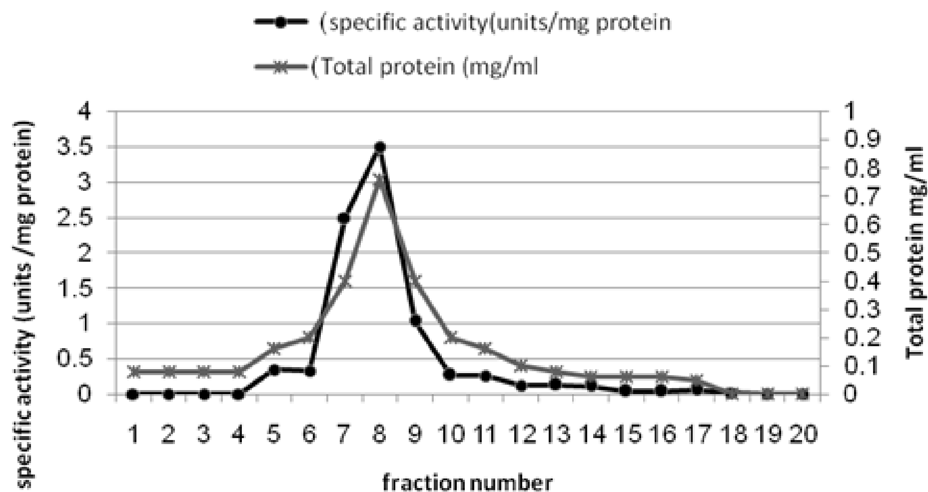


**Fig (1) Elution profile of cellulase produce by *Achromobacter spanius* on sephadex G -100**

**Table (3): Effect of different purified cellulase volume, pH and incubation temperature on purified cellulase activity**

| Enzyme volume (ml) | Enzyme activity (Units) | pH | Enzyme activity (Units) | Incubation temperature (°C) | Enzyme activity ( Units) |
|---|---|---|---|---|---|
| 0.05 | 0.005 F | 3 | 0.343 D | 20 | 0.160 H |
| 0.1 | 0.544 D | 5 | 1.217 B | 25 | 0.320 F |
| 0.15 | 0.592 C | 7 | 1.790 A | 30 | 0.530 D |
| 0.20 | 1.335 A | 8 | 0.413 C | 35 | 1.030 A |
| 0.25 | 1.075 B | | | 40 | 0.680 B |
| 0.30 | 0.227 E | | | 45 | 0.540 C |
| 0.35 | 0.004 F | | | 50 | 0.420 E |
| 0.40 | 0.001 F | | | 55 | 0.212 G |
| | | | | 60 | 0.050 I |

**Table (4): Effect of thermal stability, different substrate and incubation period on purified cellulase activity**

| Thermal stability (minutes) | Enzyme activity (Units) | Substrate | Enzyme activity (Units) | Incubation period (minutes) | Enzyme activity (Units) |
|---|---|---|---|---|---|
| 5 | 1.49 A | CMC | 1.50 A | 15 | 0.120 D |
| 10 | 1.19B | | | 30 | 0.920 A |
| 15 | 0.59 C | Filter paper | 1.31 B | 45 | 0.430 B |
| 20 | 0.28 C | | | 60 | 0.260 C |
| 25 | 0.23 CD | Cellulose powder | 0.360 C | | |
| 30 | 0.00 D | | | | |

**Discussion**

Cellulose is commonly degraded by an enzyme called cellulase. Biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria, have been isolated and characterized [27]. Complete enzymatic hydrolysis of enzyme requires synergistic action of three types of enzymes, namely cellobiohydrolase, endoglucanase or carboxy methyl cellulose (CMCase) and α-glucosidases [1]. The present study evaluated the purification of cellulase enzyme and optimal characterization by irradiated *Achromobacter spanius*. The data presented indicate that increasing concentration of ammonium sulphate up to 60 %, resulted in an increase in the specific activity of cellulase (6.03 Units/mg protein). Ammonium sulphate at 80 (%) saturation resulted in a decrease in specific activity of cellulase (5.41 Units/mg protein). The result indicated that, ammonium sulphate (60 %) is suitable for enzyme purification. These results are in agreement with those of Yin *et al* [14] who found that the maximum activity of cellulase was observed in fraction (60-80 %) saturation of ammonium sulphate. The cellulase enzyme produced by the *Bacillus* sp. was purified by $(NH_4)SO_4$ precipitation, ion exchange and gel filtration chromatography, with overall recovery of 28.8 (%) [28]. Also, the obtained results presented in Fig. (1) showed that there was an active peak obtained in fraction number (8), which may be cellulase enzyme. It was found that, the active fraction (no 8) had the highest specificity (3.5Units/mg protein). The effect of pH on cellulase enzyme activity is achieved with our data where the highest activity of cellulase (1.790 Units) was obtained at pH 7 by using phosphate buffer Table (3). In some previous studies, it was shown that cellulase activity was active in range of pH (6-6.5) and stable between range 6.5 -7.5 for *Bacillus subtlilis* [14] Also, Kim reported that, the optimal pH of the enzyme was from 4.5- 7, from *Bcillus circulans* [29]. These differences may be related to their organism. Some previous studies indicated that the optimal pH was 5.0-6.5 for those from *Bacillus strains* [30] 6.0-7.0 from *Aspergillus niger* [31], and 5.0-7.0 from *Lysobacter sp*. [32]. In the same

context, the maximum activity of cellulase enzyme was showed at 35$^{O}$C and above this there was an inhibition in enzyme activity (Table 3). Yin *et al* reported that, *Bacillus YJ1* isolate the purified cellulase has optimal activity was detected at 60 $^{O}$C [14]. Bakare *et al* reported that**,** the optimum temperature of the enzyme from *Pseudomonas fluorescens* at 35 $^{O}$C [33].
On the other hand, the obtained data revealed that the cellulase activity is stable at temperature of 60$^{O}$C for 5 minutes, and above which there was a reduction in enzyme activity (Table 4). In this regard, Mayende found that purified cellulase enzyme from *Bacillus* thermal stability range between 65 and 70$^{O}$C [18]. When it was pre-incubated at 60 $^{O}$C, lost 62 (%) of its activity within 15 min and no activity was detected after 30 min.

In the present study an optimization of cellulase activity of this bacterial isolates due to the best substrate and incubation time, showed that the highest activity is recorded against CMC Table (3) while cellulose powder and filter paper were used in the assay, the enzyme activity depressed. In this regard, the same finding was showed by Yin [14]. Begum and Absar [34] they found that, optimal substrate for highest activity of purified cellulase enzyme was CMC. On the other hand, Hareesh [35] showed that, Corn bran was a good substrate for the production of cellulase by *Achromobacter xylosoxidans* BSS4 under Solid state fermentation . Also, it appeared that the maximum activity was obtained after 30 minutes incubation time for cellulase, while after 45 minutes (0.430 Units) the enzyme activity decreased (Table 4). In this work, the present data on molecular weight of purified cellulase enzyme revealed that the molecular weight of the cellulase enzyme was 32.5 KDa by comparing with marker. Yassien [36] reported that, the produced cellulase enzyme from *Streptomyces sp* has molecular weight 42 kDa.

## Conclusion

From the previously mentioned results in this study, it can be concluded that the irradiated *Achromobacter spanius* have ability to use cellulose as a substrate and it can produce high levels of cellulase. Pure enzyme under optimization may be used on the commercial scale for production cellulase. This process may have an economical impact on reduction of agricultural production costs and industrial application.

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