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PURIFICATION AND CHARACTERIZATION OF *PSUEDOMONAS AERUGINOSA* IZ CELLULASE GROWN ON CORN COB

Itani A[1], Olama Z[2*] and Holail H[2]

[1] Department of Biological and Environmental Sciences, Faculty of Science. Beirut Arab University. Beirut, LEBANON
[2]Alexandria University –Faculty of Science

ABSTRACT: *Partial purification of crude Psuedomonas aeruginosa IZ cellulase enzyme was carried out by ammonium sulfate fractionation (85%). The specific activity of cellulase increased 493.3% (from 0.15 U/ml protein in the crude enzyme to 0.89 U/ml protein after ammonium sulfate treatment) while the unit protein was decreased 21.9% (from 48.5 mg/ml in the crude enzyme to 37.9 mg/ml after ammonium sulfate treatment) with activity preservation and a purification fold of 5.93. Further purification of the partially purified enzyme was achieved using anion exchange chromatography on DEAE sephadex A-50. The cellulase activity was enriched after this step of purification; the specific activity increased 301.1 % (from 0.89U/ml protein after ammonium sulfate treatment to 3.57 U/ml protein after anion exchange chromatography) with a purification fold of 23.8. The protein was reduced by 56.99 % (from 37.9 after ammonium sulfate treatment to 16.3 after anion exchange chromatography). The highest active cellulase fractions obtained from anion exchange chromatography on DEAE sephadex A-50 were loaded on a Sephadex G-100 column for Gel filtration chromatography. The specific activity of cellulase was further increased by 85.71 % (3.57 U/ml protein after anion exchange chromatography to 6.63 U/ml after gel filtration) with purification factor fold of 44.2 and protein decreased by 39.26 % (from 16.3mg/ml after anion exchange chromatography to 9.9 mg/ml after gel filtration chromatography). The optimum pH and incubation temperature for the purified Psuedomonas aeruginosa IZ cellulase enzyme were 6.5 and 35˚C respectively. The enzyme kinetics studies indicated that the enzyme activity is dependent on the concentration of the substrate. When the relation between the enzyme activity and the substrate concentration was treated by Lineweaver-Burk analysis, it was found that K_m and V_{max} were 4 mg/ml and 7.2 U/ml respectively.*

KEYWORDS: *Purification, characterization, Psuedomonas aeruginosa IZ, cellulase, corn cobs*

INTRODUCTION

In nature, complete cellulose hydrolysis is mediated by a combination of three main types of Cellulases: (1) Endoglucanases (EC 3.2.1.4), (2) Exoglucanases, including Cellobiohydrolases (CBHs) (EC 3.2.1.91), and (3) β -Glucosidase (BG) (EC 3.2.1.21) (Zhang & Zhang, 2013). Cellulases hydrolyze β -1,4 linkages in cellulose chains. The catalytic modules of Cellulases have

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been classified into13 families (1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 51, and 48) based on amino acid sequence similarities and crystal structures (Henrissat, 1991). Cellulase like activities have been also proposed for families 61 and 74 (Schulein, 2000). Cellulases display a variety of topologies ranging from all β-sheet proteins to β/α- barrels to all α-helical proteins (Zhang & Zhang, 2013)

Currently, Cellulases occupy almost 15% of the global industrial enzyme market, coming in third after amylase (25%) and protease (18%). (Sajith et al., 2016). The increasing concern about exhaustible energy resources and pollution have resulted in an increased interest in the usage of cellulases for enzymatic hydrolysis of lignocellulosic waste materials (Zalvidar et al., 2001 and Sun & Cheng, 2002). Degradation of lignocellulosic biomass is mainly carried out by microorganisms that utilize it as a source of carbon and nutrient/energy growth. These include species of bacteria (*Clostridium, Cellulomonas, Bacillus, Pseudomonas, Fibribacter, Ruminococcus, Butyrivibrio,* etc.,), fungi (*Aspergillus, Rhizopus, Trichoderma, Fusarium, Neurospora, Penicillium*, etc.,), and actinomycetes (*Thermomonospora, Thermoactinomyces*, etc.) (Sajith et al., 2016). Accordingly, cellulase hydrolysis can be exploited for the production of bioethanol, organic acids, free sugars, antibiotics and animal feeds (Gaur & Tiwari, 2015 and Sajith et al., 2016). Furthermore, cellulases have attracted much attention because of their application in various industrials processes, including food, textiles, laundry, pulp and paper as well as in agriculture (Kuhad et al., 2011). Since enzymes are generally recoverable, specific, low in energy requirements, and nonpolluting, enzymatic hydrolysis of cellulose is preferred to acid and alkali hydrolyses (Sajith et al., 2016).

The present study was evaluated to purify and cellulase enzyme produced from a novel bacterial isolate to be used in large scale industry.

MATERIALS AND METHODS

Microorganism

A novel cellulase-producer bacterial strain was isolated from Lebanese habitat and was identified genotypically as *Pseudomonas aeruginosa* IZ. It was maintained on nutrient agar slants and stored at 4˚C with regular transfers at monthly intervals. For long preservation, the bacterial slants were folded with 25% sterile glycerol.

Preparation of seed culture

Transfers from 18 hour old bacterial slants were used to inoculate 250 ml Erlenmeyer flasks containing 50 ml nutrient broth. Flasks were incubated at 35^0C ± 2 under shaken conditions -180 round per minute (rpm) - until optical density (O.D.) at 600 nM reaches $O.D_{600} \leq 1$. 6% (v/v) were taken as a standard inoculum unless otherwise stated.

Cultivation Techniques

Submerged fermentation (SmF) was the cultivation method used. Cultivation of *Pseudomonas aeruginosa* IZ was achieved in 250 ml Erlenmeyer flasks each containing 50 ml of the complex medium 1 (CM1) (g/L): Corn cob powder, 40; KH_2PO_4, 1; K_2HPO_4 1.45; $MgSO_4.7H_2O$, 0.4,

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$CaCl_2$, 0.05; $(NH_4)_2SO_4$, 5; $FeSO_4.7H_2O$, 0.00125; pH 7.0. The medium was sterilized by autoclaving for 20 min at 121˚C. The flasks were received 6 % (v/v) seed culture as a standard inoculum (unless otherwise stated), and then incubated at 37˚C for 5 days under shaken conditions (150 rpm).

Crude Enzyme Preparation (cell free extract)

At the end of the fermentation period, the fermented culture medium was centrifuged at 6000 rpm for 20 min at 4^0C. The obtained cell free supernatant was used as the crude enzyme.

Enzyme Assay

Enzyme assay was determined in terms of carboxymethyl cellulase activity. The reducing sugars produced were measured as glucose using a modified technique described by Miller (1959). One unit of enzyme activity is defined as the amount of protein (cellulase) required to liberate 1 µmol of reducing sugar (D- glucose) from CMC per min under the assay conditions. A calibration curve of glucose was used to convert the colorimetric readings into µmol of glucose.

Estimation of Protein Content

Each fraction of the purified enzyme was monitored for protein by reading the absorbance at 280nm.

Purification of Pseudomonas aeruginosa* IZ *Cellulase

The crude enzyme extract of 1-day old culture of *Pseudomonas aeruginosa* IZ grown under optimal fermentation conditions was subjected to several purification steps carried out at 4˚C according to Bakare et al. (2005). Briefly, fractionation was carried out using ammonium sulfate which was added slowly to the crude enzyme to achieve 85% saturation. Following fractionation, enzyme purification was conducted by the use of two chromatographic steps: firstly, anion exchange column chromatography on DEAE Sephadex A-50, followed by gel filtration column chromatography using Sephadex G-100

Kinetic Studies of Purified Enzyme under Investigation

The Effect of pH on Pseudomonas aeruginosa IZ Cellulase Activity

In order to determine the optimum pH for cellulase activity, the enzyme assay was carried out using carboxymethyl cellulose solution adjusted to different pH values using sodium citrate buffer (pH value range 5.0, 6.0, 6.5, 7, 7.5, 8 and 9). The enzyme activity was plotted against the different pH values (Bakare et al., 2005).

The Effect of Incubation Temperature on Pseudomonas aeruginosa* IZ *Cellulase Activity.

The enzyme was incubated with the substrate at different temperatures (30^0C, 35^0 C, 40^0C, 45^0C, 50^0C and 55^0C). The reaction mixtures were analyzed for cellulase activity which was measured and plotted against the different temperature values (Bakare et al., 2005).

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The Effect of Substrate Concentration on Pseudomonas aeruginosa* IZ *Cellulase Activity

In order to test the effect of substrate concentration on cellulase activity, different concentrations of carboxymethyl cellulose solution (2 mg/ml, 4 mg/ml, 10 mg/ml and 20mg/ml) were prepared and used as substrate. The enzyme activity was measured and plotted against each concentration (Bakare et al., 2005).

RESULTS AND DISCUSSION

Purification of Pseudomonas aeruginosa* IZ *Cellulase

Partial purification of *Pseudomonas aeruginosa* IZ crude cellulase enzyme was carried out by fractional precipitation with ammonium sulfate. The supernatant and the precipitate were subjected to cellulase assay. It was found that the enzyme activity was completely concentrated in the precipitate. The specific activity after precipitation was 0.89 U/mg proteins recording 61% of the original enzyme activity of the crude extract (table 1). Ammonium sulfate had been extensively used for the precipitation of cellulase produced by different organisms (Bakare et al., 2005 and Kalaiselvi et al., 2013). The partially purified cellulase which was concentrated by precipitation with 85% saturation of ammonium sulfate was dialyzed and subjected to anion exchange chromatography using DEAE Sephadex A-50 column. The column was washed to remove all unbound proteins and a linear gradient of 0 to 0.5 M NaCl in 0.02 M sodium phosphate buffer pH 7 was used for the elution of any bound proteins. The purification profile of DEAE Sephadex A-50 column gave one peak of cellulase activity (figure 1) where the total cellulase activity was found to be 861.36 U/ml with yield of 37.3 %, and the specific activity was found to be 3.57 U/mg protein with 23.8 purification fold (table 1). The highest active cellulase fractions obtained from anion exchange chromatography on DEAE Sephadex A-50 were loaded on a Sephadex G-100 column pre-equilibrated with 0.02 M sodium phosphate buffer, pH 7.0 (figure 2). The specific activity of cellulase was further increased by 85.71 % (3.57 U/ml protein after anion exchange chromatography to 6.63 U/ml after gel filtration) with purification factor fold of 44.2 folds. On the other hand, the protein decreased through this step of purification by 39.26% (from 16.3 mg/ml after anion exchange chromatography to 9.9 mg/ml after gel filtration chromatography). These results came in accordance with Bakare et al. (2005) who purified cellulose from *Pseudomonas fluorescens* by ammonium sulphate precipitation, ion exchange chromatography on DEAE Sephadex A-50 and gel filtration on Sephadex G-100. Purification fold of about 5 was obtained by ammonium sulphate, ion exchange chromatography gave purification fold of about 24 and Gel filtration chromatography yielded a homogeneous preparation with a specific activity of 6.8, units/mg protein.

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Table 1: Purification profile of cellulase from *Pseudomonas aeruginosa* IZ cellulase

Purification step	Activity U/ml	Protein mg/ml	Total activity U	Total protein mg	Specific activity U/mg protein	Purification factor fold	Yield(%)
Culture filtrate (crude enzyme)	7.21	48.5	2307.2	15520	0.15	1	100
Precipitation by ammonium sulfate (85%)	33.5	37.9	1407	1591.8	0.89	5.93	61
DEAE Sephadex A-50 column	58.2	16.3	861.36	241.24	3.57	23.8	37.3
Sephadex G-100	65.6	9.9	295.2	44.55	6.63	44.2	12.8

-Specific activity = activity (U/ml) / protein (mg/ml)
- Purification factor = Specific activity of purified enzyme/Specific activity of the crude enzyme
- Yield (%) = (Total activity of the purified enzyme /total activity of the crude enzyme) x 100

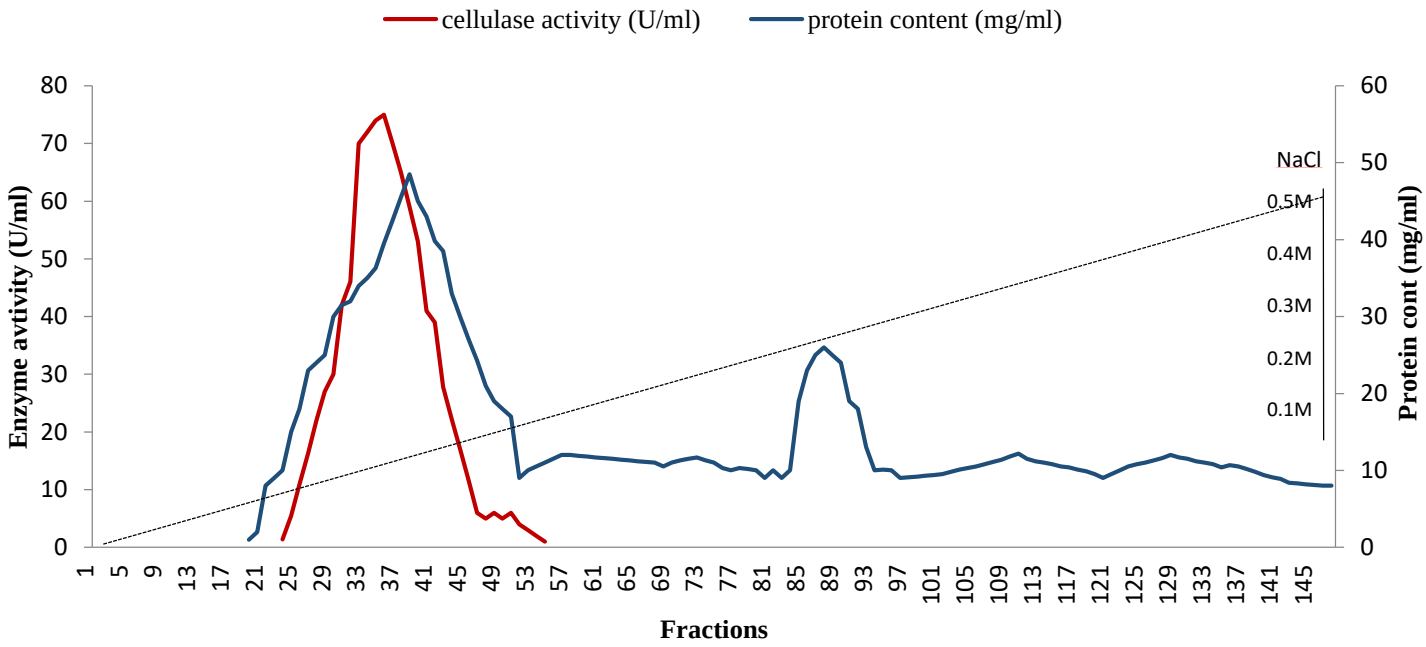

Figure 1: DEAE-Sephadex A-50 of the 85% ammonium sulphate fraction of *Pseudomonas aeruginosa* IZ cellulase. Protein was eluted with a linear gradient of 0.1-0.5 M NaCl. Total protein was monitored at 280 nM and the fractions were assayed for cellulase activity.

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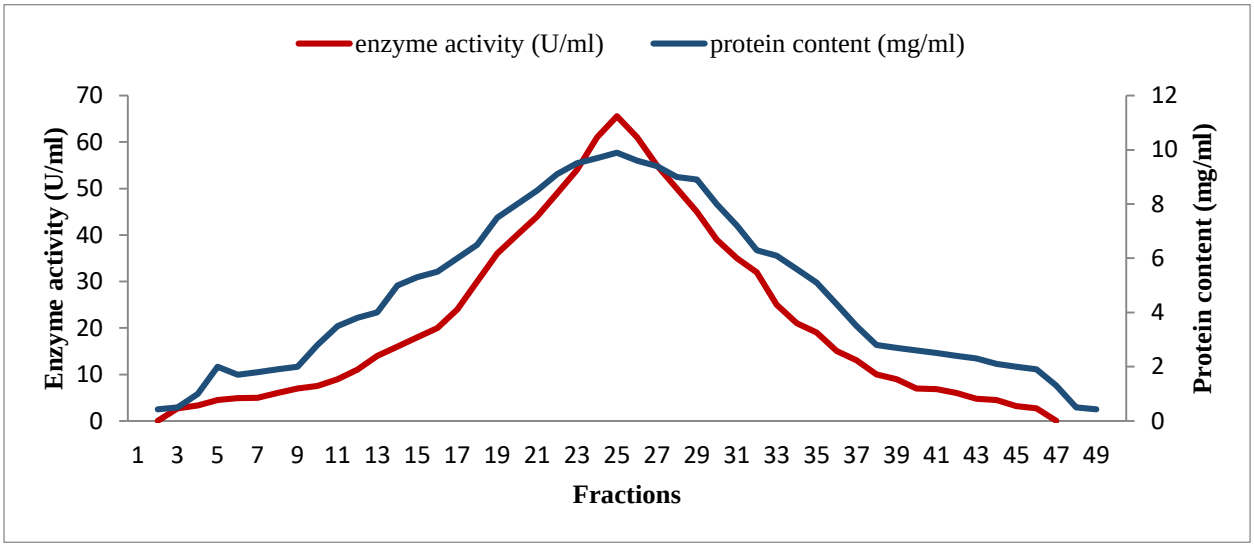

Figure 2: Sephadex G-100 chromatography of the DEAE-Sephadex A-50 fractions of Pseudomonas aeruginosa IZ cellulase

Enzyme characterization

The kinetic properties of the purified *Pseudomonas aeruginosa* IZ enzyme were studied. The purified enzyme proved to be active over a broad range of pH values. Cellulase was active at neutral and acidic conditions, with optimum pH 6.5 (figure 3). In agreement with these findings, Bakare et al. (2005) reported that the optimum pH for *Pseudomonas fluorescens* cellulase was 6.5. Temperature profile showed that *Pseudomonas aeruginosa* IZ cellulase had an optimum activity at incubation temperature of 35˚C (figure 4). This optimum enzyme temperature is similar to those recorded for 3 different strains of *Pseudomonas fluorescens* (Bakare et al., 2005) where the optimum temperature was 35˚C.

The activity of the enzyme increased progressively in a substrate concentration dependent manner up to rather high substrate concentration (20 mg/ ml) (figure 5). The enzyme was subjected to line Weaver-Burk analysis using the tested substrate concentration ranges; the apparent K_m and V_{max} were 4 mg/ml and 7.2 U/ml/min respectively, indicating high affinity of the enzyme to the substrate (figure 6). Similarly, Bakare et al. (2005) reported that the K_m and V_{max} values of *Pseudomonas fluorescens* cellulase were 3.6 mg/ml and 3.3 U/ml, respectively.

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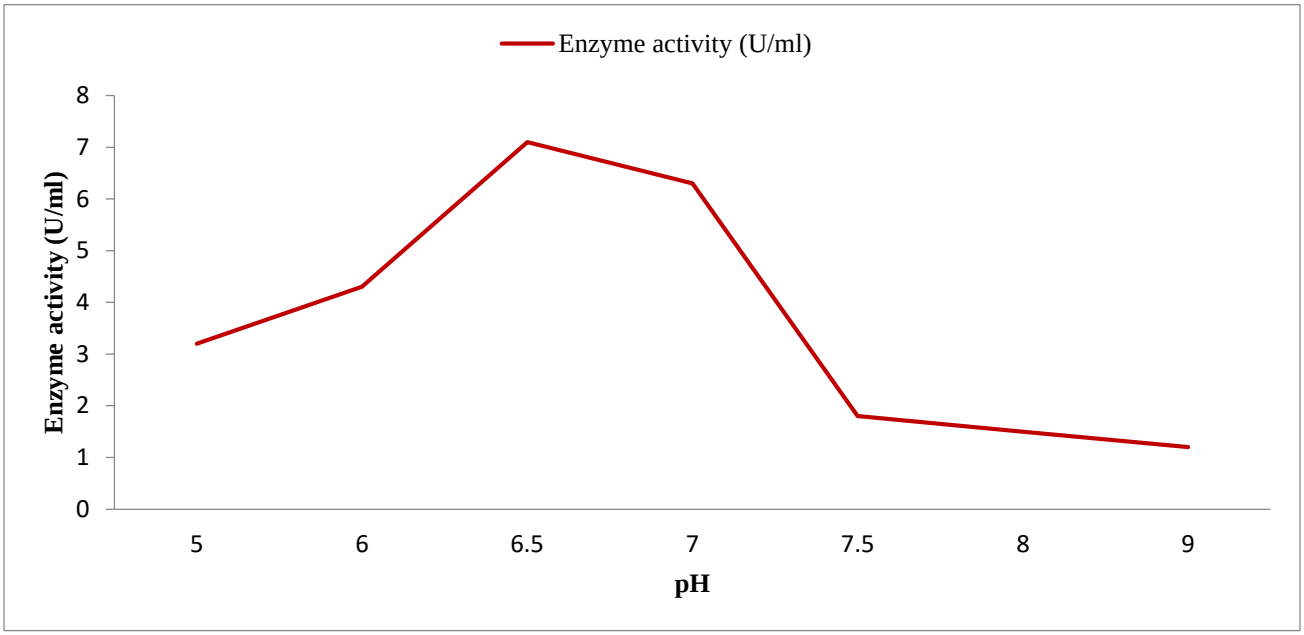

Figure 3: Activity of the purified *Pseudomonas aeruginosa* IZ cellulase as affected by pH

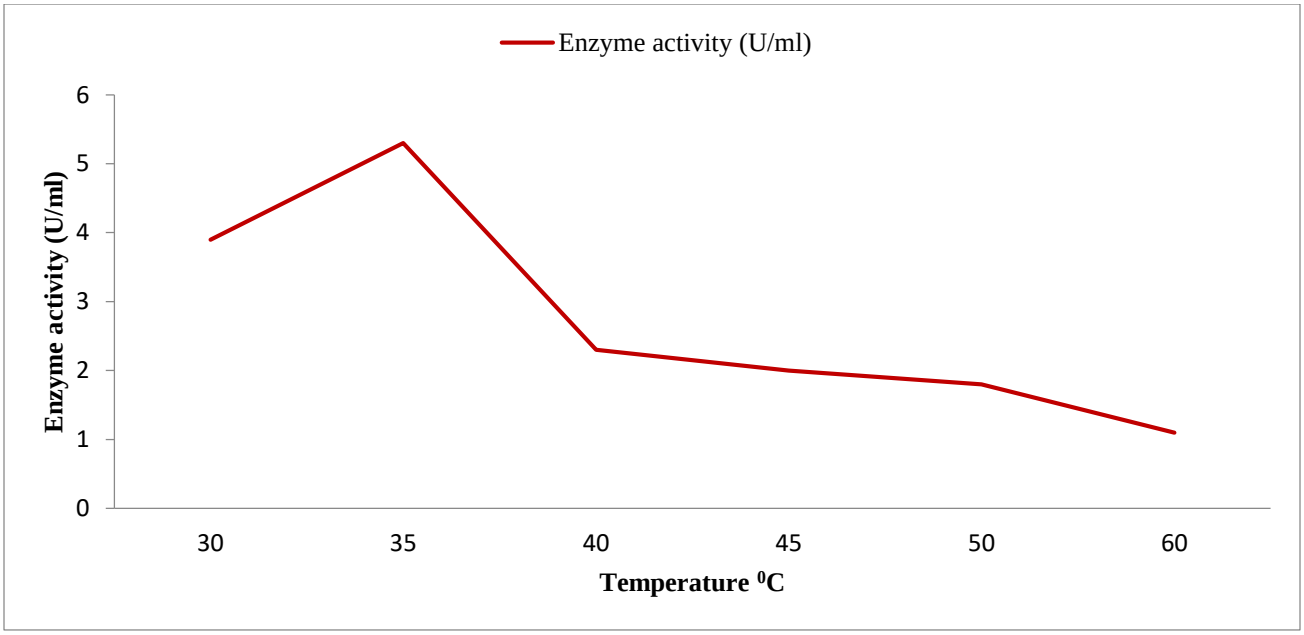

Figure 4: Activity of the purified *Pseudomonas aeruginosa* IZ cellulase as affected by incubation temperature (^{0}C)

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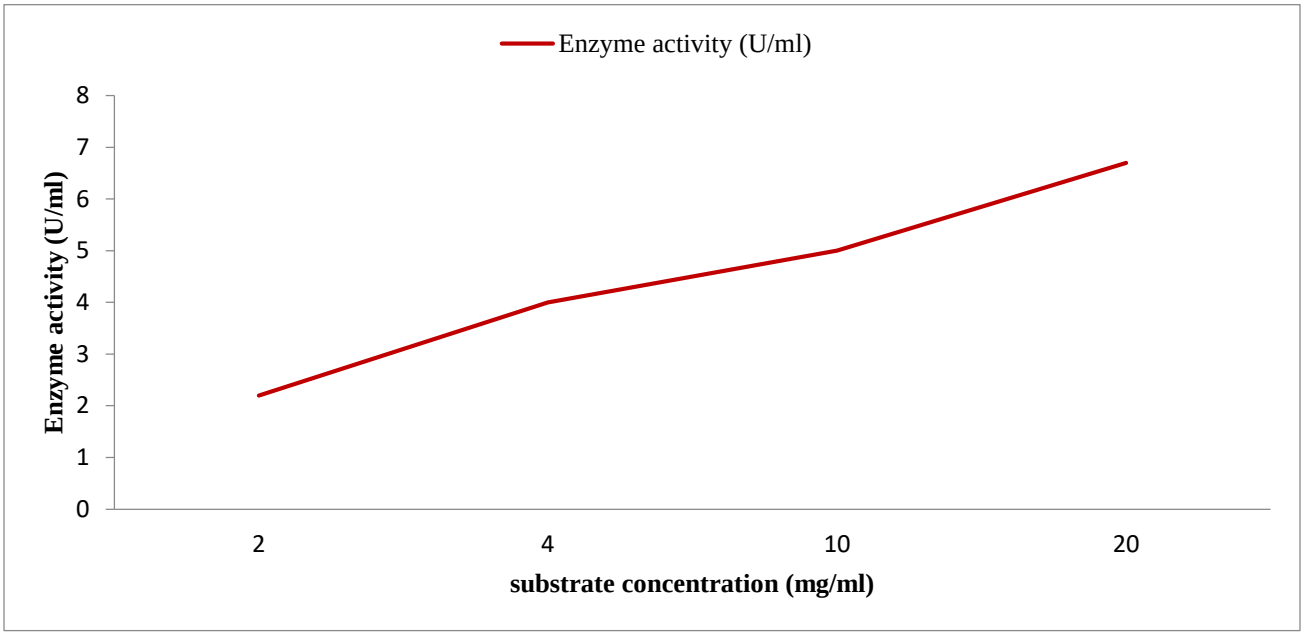

Figure 5: The effect of substrate concentration on *Pseudomonas aeruginosa* IZ cellulase activity

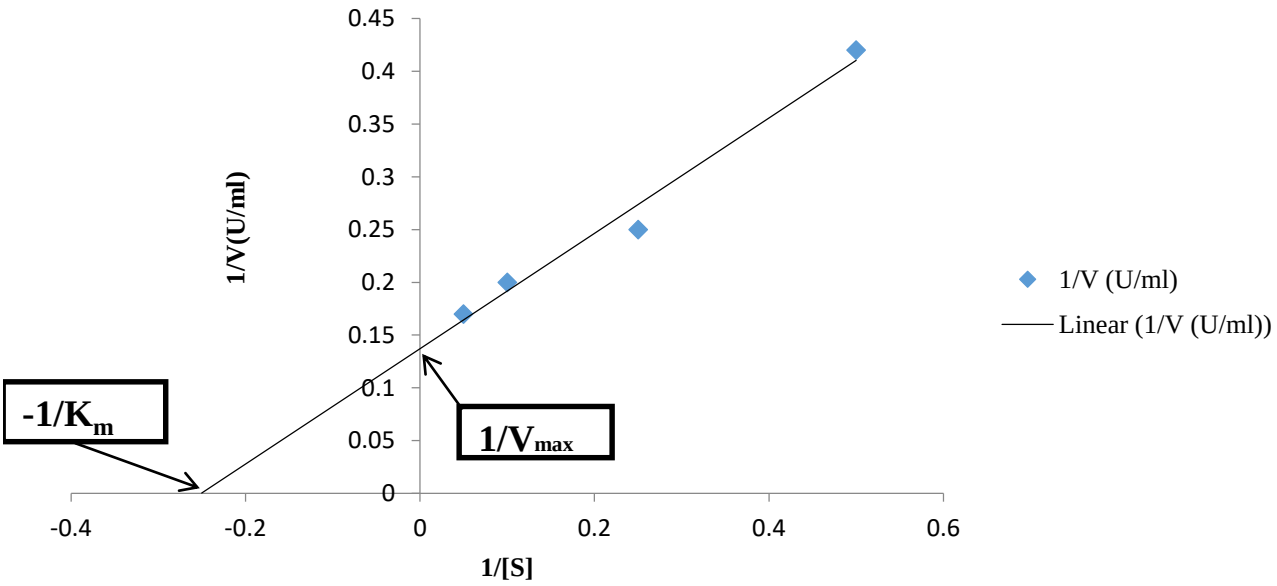

Figure 6: Line weaver-Burk plot of *Pseudomonas aeruginosa* IZ cellulase activity

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