Open Access
Baghdad Science Journal **Vol.15(3)2018**

DOI: http://dx.doi.org/10.21123/bsj.2018.15.3.0262

Characterization and Cytotoxic Activity of Cytosine Deaminase Enzyme Purified from Locally Isolated *Escherichia coli*

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Received 12/2/2018, Accepted 15/ 7/2018, Published 13/9/2018

Abstract:

This research was aimed to the purification and characterization of cytosine deaminase as a medically important enzyme from locally isolated *Escherichia coli*; then studying its cytotoxic anticancer effects against colon cancer cell line. Cytosine deaminase was subjected to three purification steps including precipitation with 90% ammonium sulfate saturation, ion exchange chromatography on DEAE-cellulose column, and gel filtration chromatography throughout Sephadex G-200 column. Specific activity of the purified enzyme was increased up to 9 U/mg with 12.85 folds of purification and 30.85% enzyme recovery.

Characterization study of purified enzyme revealed that the molecular weight of cytosine deaminase produced by *E. coli* was about 48 KDa, the highest enzyme activity at pH 8.5, and is most stable at pH 7.5 - 9, the enzyme also showed a full activity at a range of temperatures between 45-60 ^{0}C.

Enzyme activity was strongly inhibited in the presence of mercuric chloride and copper sulphate, when added individually at a constant concentration. However, calcium chloride, manganese chloride and ferric chloride caused a little increase in enzyme activity while sodium azide had no effect on enzyme activity.

Upon cytotoxic effect study through micro-cultured tetrazolium assay (MTT) against Caco-2 cell line. Purified cytosine deaminase was found to inhibit the growth of Caco-2 cancer cell line with an IC50 of 242.5 µg/ml in a comparison to an IC50 of 1864 µg/ml for crude enzyme. Besides, the enzyme didn't show significant effect on WRL normal cell line**.**

Keywords: Cytosine deaminase, *Escherichia coli*, MTT assay, purification

Introduction:

Microbes represent a rich source of enzymes in a traditional way. Enzymes have been extracted from plants and animals. However, microbial enzymes have formed the basis for enzyme production commercially (1). Also, enzymes from microbial sources have gained interest for their widespread uses in industries and medicine according to their catalytic activity, stability and ease of production, low energy input, reduced processing time, nontoxic, cost effectiveness, and eco-friendly characteristics in addition to optimization than plant and animal enzymes (2,3,4).

Cytosine deaminase (CD, EC 3.5.4.1) stoichiometrically catalyzes the hydrolytic deamination of cytosine and 5-fluorocytosine to uracil and 5-fluorouracil, respectively (5). The enzyme is gave a wide interest for antimicrobial drug design and for gene therapy application against cancer.

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It is known that enzyme prodrug therapy is being developed as a treatment for cancer and other pathological conditions and cytosine deaminase / 5-flurocytosine strategy is one of the widely tested enzymes prodrug strategies in both animal models and clinical trials (6). Cytosine deaminase has the ability to convert the non-toxic prodrug 5-fluorocytosine (5FC) into the most widely used cytotoxic chemotherapeutic agent 5-fluorouracil (7). It has been isolated and characterized from various sources by several researchers, since CD gain great attention in sucide gene therapy to treat tumor either from bacterial or yeast sources. However, from either of these sources the enzyme has limitations because yeast CD showed higher affinity for 5-FC than *E. coli* CD but less thermostable. While, *E. coli* CD has lower affinity for 5-FC but is more thermostable than yeast CD (8).

According to few studies on this enzyme in Iraq and due to its anticancer activity, new sources for CDs is needed with high efficiency and another property differs from which is used up to date in tumor treatment.

Materials and Methods:

Inoculation

E. coli was isolated from urine samples obtained from Al-Imamein Al-Kadhumain Medical City Hospital patients in Baghdad (9) and the isolated colony was used to inoculate the mineral salt medium optimized in a previous study (10) containing: distilled water 1.0 L, citric acid 1.0g as a best carbon source, peptone 1.0g as a best nitrogen source, KH_2PO_4 3.5g, $MgSO_4 \cdot 7H_2O$ 0.025 g, $Na_2HPO_4$10.75g adjusted at pH 8.5 and incubated at 37 ^oC for 24 hr. , pH adjusted to 8.5 with 0.1 M HCl/NaOH.

Enzyme Extraction

The enzyme was prepared by destruction of the cell pellet using Branson/USA ultra-sonication with 19.5 pulse / sec for 30 sec, then centrifuged at 6000 g for 5 minutes and the cell free supernatant (CFS) was used as a crude enzyme.

Enzyme Assay

Cytosine deaminase activity was assayed as described by (11); the reaction mixture containing 0.4 ml of enzyme solution, 1 ml of 133 g/L cytosine solution and 0.6 ml of 0.2M potassium phosphate solution incubated for 30 minutes at 37 ^oC in water bath. The reaction was stopped by the addition of 6 ml of 0.1M HCL. The solution was centrifuged at 6000 rpm for 15 minutes. The absorbance was measured at 280 nm by UV160 spectrometer (Shimadzu). The blank was prepared using the same steps except the addition of stop solution into cytosine before the addition of enzyme solution. Protein concentration was determined according to (12).

Purification of Cytosine Deaminase

Ammonium sulfate precipitation

Ammonium sulfate was added to the cell free supernatant with saturation ratio of 90%. The mixture was mixed gently on magnetic stirrer at 4°C for 20 minutes. Then centrifuged at 9,000 rpm for 15 minutes at 4°C. The precipitated proteins were dissolved in a suitable volume of 0.05 M Tris- HCl buffer at pH8.

Purification by column chromatography

DEAE-Cellulose column was prepared according to Healthcare, and sephadex G-200 was prepared as recommended by Pharmacia Fine Chemicals Company.

Determination of enzyme purity and its molecular weight

Purity of cytosine deaminase was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using 10% running gel and 3% stacking gel according to the method of (13) in the presence of ready to use standard proteins marker (hen egg-white lysozyme, soybean trypsin inhibitor, bovine erythrocyte carbonic anhydrase, ovalbumin, bovine serum albumin, and human erythrocyte phosphorylase B). Distances of protein migrated to anode were measured after electrophoresis on polyacrylamide gel to calculate the enzyme molecular weight.

Cytosine deaminase characterization

Enzyme characterization study was done according to (14) with slight modifications.

Determination of pH effects on cytosine deaminase activity

Purified enzyme was added to 0.1 mM cytosine and 0.05M buffers of PH ranging from 4 to 9. The buffers used were sodium acetate buffer with pH (4.5, 5, 5.5, 6 and 6.5), tris buffer for pH values (7, 7.5, 8, 8.5 and 9).

Enzymatic activity in each one was measured as described above and the relationship between different pH and enzyme activity was plotted.

Determination of pH effects on cytosine deaminase stability

The enzyme was pre-incubated with buffers of various pH (4–9) for 30 min. at 37^oC. After that the tubes were cooled in an ice bath. .

Determination of temperature effects on activity and thermal stability for cytosine deaminase

The cytosine deaminase activity was measured at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80) °C. While for thermal stability, partially purified cytosine deaminase was pre-incubated in water bath at (30, 35, 40, 45, 50, 55, 60, 65, 70, 75and 80) °C for 30 min., and immediately transferred into an ice bath. Enzymatic activity was determined and the remaining activity (%) was plotted against the temperature.

Determination of ions and inhibitors effect on cytosine deaminase activity

The effect of different inorganic ions on enzyme activity was determined by pre-incubating the enzyme with different salts ($MgCl_2$, NaCl, $CaCl_2$, KCl, $MnCl_2$ and $FeCl_3$) prepared at the concentrations of (1mM) for 30 min. at 37°C.Also, the effect of inhibitors and chelating agents (Sodium azide, $HgCl_2$ and $CuSo_4$) prepared at (1 mM) concentration was determined by incubating with the enzyme at 37°C for 30 min.

MTT (Cytotoxic Assay)

MTT assay is a non-radioactive colorimetric assay which is used to measure the cell viability in response to a variety of cytotoxic stimuli. The assay is based on the reduction of yellow, water soluble tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) within metabolically active cells. The reduction of the tetrazolium salt occurs by the action of mitochondrial dehydrogenases present only in viable cells, yields a purple formazan

product which can be quantified spectrophotometrically. The percentage viability of the treated cells was calculated by comparison with normal cell line WRL (15). Colorectal cancer Caco-2 cell line was supplied by Pharmacology Department/Medicine College/Malaya University.

Cell Viability %= [Absorbance of treated sample/Absorbance of non-treated sample] × 100 (Non-treated cultures in all experiments contained the medium only).

A dose response curve was plotted to enable the calculation of the concentrations that kill 50 % of Caco-2 / WRL cells (IC50).

Results and Discussion:

Purification of cytosine deaminase

The Purification profile employed using different techniques were summarized in Table 1. The precipitated enzyme obtained by 90% ammonium sulfate saturation was partially purified using DEAE-cellulose ion exchange chromatography Fig.1. In this step, the eluted proteins (Fractions 79 to 85) contained most of the cytosine deaminase activity.

Table 1. Purification steps for cytosine deaminase produced by *E. coli*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme(CFS)	100	0.875	1.25	0.7	87.5	1	100
Ammonium sulfate precipitation (90%)	25	1	0.7	1.42	25	2.02	28.57
DEAE-cellulose	15	2	0.5	4	30	5.71	34.28
Sephadex G-200	15	1.8	0.2	9	27	12.85	30.85

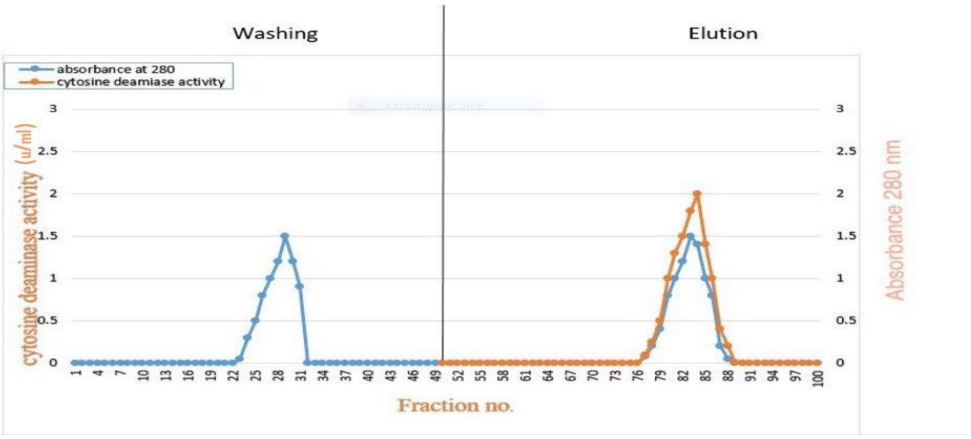

Figure 1. Ion exchange chromatography of cytosine deaminase produced by locally isolated *E. coli* using DEAE-Cellulose column (2x20cm) with a flow rate of 30ml/hr.

Cytosine deaminase from *E. coli* was finally purified by applying the active fractions obtained from the previous step onto Sephadex G-200 column. The elution pattern shown in Fig. 2 yielded a single protein peak and the enzyme activity was entirely associated with this peak and the purified enzyme had a specific activity of 9 U/mg with purification fold of 12.85 and the cytosine deaminase yield 30.85%.

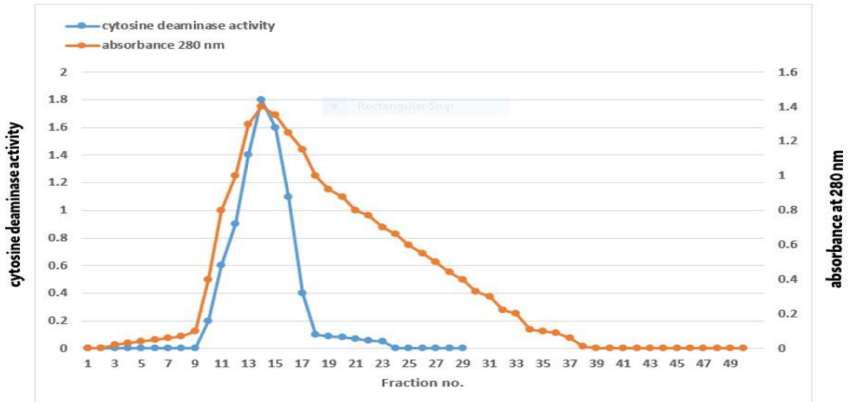

Figure 2. Gel filtration chromatography of cytosine deaminase produced by locally isolated

E. coli **using SephadexG-200 column (1.5cmx35cm) equilibrated with 0.01 M Tris-HCL buffer pH8**

In another study, *Saccharomyces cerevisiae* cytosine deaminase was purified by sephadex G-200 as third step of purification (after ammonium sulfate precipitation and DEAE-cellulose) to get enzyme with specific activity of 4.0 U/mg and 1.190% yield (16).

Determination of cytosine deaminase molecular weight

The homogeneity of the purified cytosine deaminase was investigated by 10%SDS-PAGE. Analysis of the protein profile Fig. 3 shows that the enzyme gave a single band which indicates the purity of the final preparation. Also, depending on the interpolation from the linear molecular mass versus the Rm value (relative mobility). the molecular weight of the protein band was estimated to be 48kDa Fig. 4.

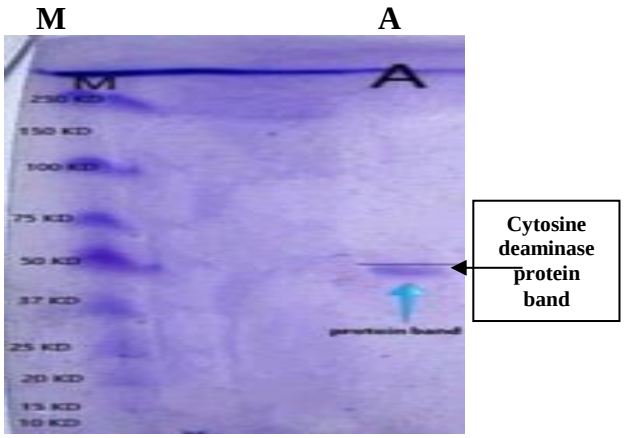

Figure 3. SDS- PAGE (10%) of cytosine deaminase purified from *E. coli* where:
M: Proteins marker A: Proteins band (cytosine deaminase) after gel filtration step

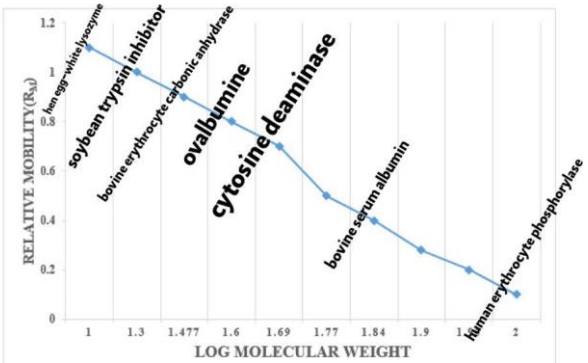

Figure 4. Log molecular weight of cytosine deaminase produced by *E. coli* after electrophoresis

Cytosine deaminase characteristics

PH effect on enzyme activity and stability

Microbial enzyme activity is greatly influenced by the surface charges present on the amino acids. Enzyme activity can be either enhanced or inhibited depending on the change in the pH, and hence can influence the growth of microorganisms (17).

Cytosine deaminase showed lower activity at acidic pH from 4-6 with an appreciable increase in activity from pH 6.5 to 8, however, maximum enzyme activity was observed at pH 8.5 Fig. 5.

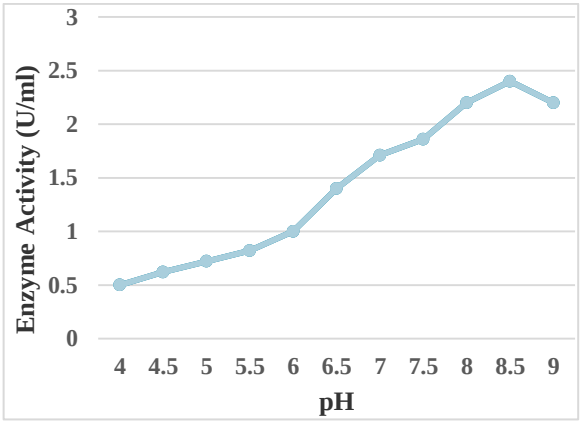

Figure 5. Effect of pH on activity of purified cytosine deaminase produced by *E. coli*

After incubation of cytosine deaminase for 30 min. the enzyme showed maximum stability in a range between pH 7.5-9, since at this pH, the enzyme gave maximum remaining activity (100 %). It retains 80% of activity at pH 7 and 10 respectively Fig.6. Most enzymes may undergo irreversible denaturation in high acidic or basic solution (18), on the other hand, If the pH is not appropriate, microbial metabolism would be disturbed and its growth would be stopped (19).

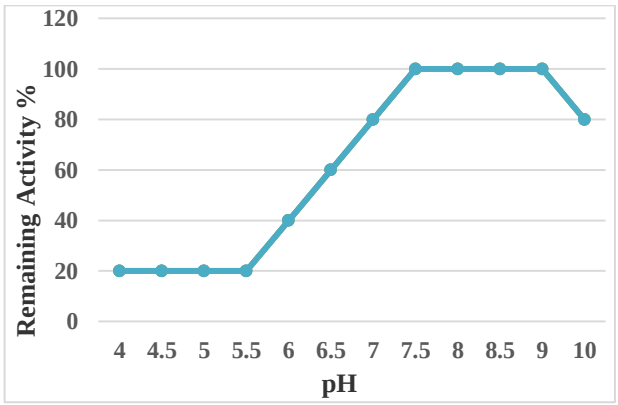

Figure 6. PH stability of purified *E. coli* cytosine deaminase

Another observation regarding the pH stability showed that *Chromobacterium violaceum* YK 391 cytosine deaminase retains 70% of its activity in the pH 7.5 (20).

Temperature effect on cytosine deaminase activity and its thermal stability

Temperature is one of the environmental factors that can affect enzyme activity (21). And can be considered as critical variable that determine the rate of any reaction, however, for biological systems; the effects of this factor are convoluted with contributions from protein stability and enzyme catalysis (22).

The highest enzyme activity was recorded at 50°C with an observed decrease in activity at less or a higher temperature of incubation. The results showed an increase in enzyme reaction activity until it reached 50 °C then it began to decline Fig.7.

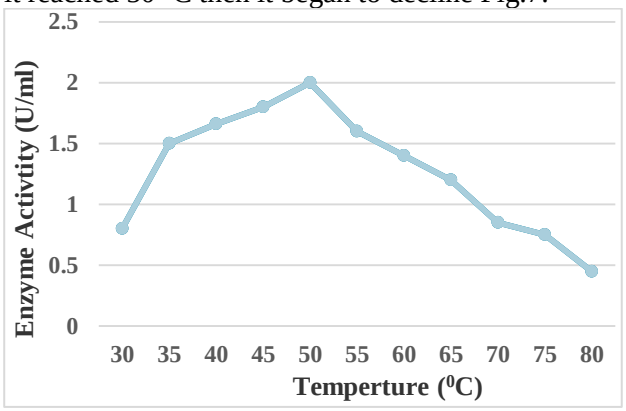

Figure 7. Effect of temperature on activity of purified cytosine deaminase produced by *E. coli* E9

Similar results were recorded for cytosine deaminase from *Serratia marcescenst* (16). Also, fungal cytosine deaminase showed a maximum activity at 40-45 °C (14). Polymeric enzymes with large molecular weight are seems to be less heat-stable than single poly-peptide with low molecular weight enzyme, An optimum temperature of 40°C and 50°C were recorded for yeast and *E. coli* CD, respectively (23).

Studies on thermal stability Fig.8 revealed that the enzyme gave (100%) stability upon incubation in temperatures between 45 and 60°C. on the other hand, the enzyme start to lose its activity after this temperature where the remaining activity became 40% at 80 °C.

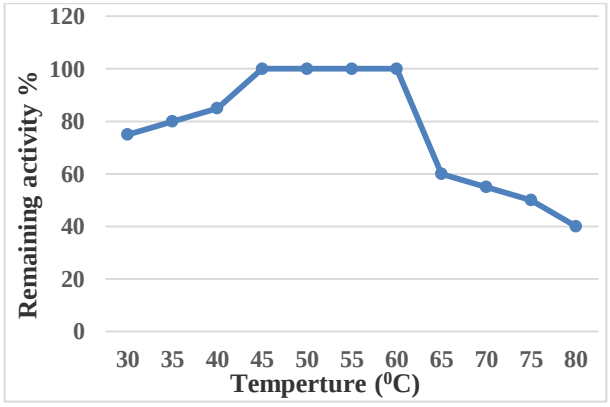

Figure 8. Temperature stability of cytosine deaminase produced by *E. coli*

Effect of ions and inhibitors on cytosine deaminase activity

Table 2 represents the effect of some inorganic ions on cytosine deaminase activity. $MgCl_2$, NaCl and KCl with (1mM) concentration had not significantly affects enzyme activity. Also, HgCl2 and $CuSo_4$ (1 mM) were found to inhibit enzyme activity by 95% and 96% respectively. On the other hand, the enzyme activity increased when $FeCl_3$, $CaCl_2$ and $MnCl_2$ were applied. Also, the enzyme showed 100% resistance to sodium azide at 1mM. The inhibition of *E. coli* cytosine deaminase by Cu^{+2} and Hg^{+2} ions suggests that this enzyme is an SH enzyme. In most enzyme action, metallic cofactors are important due to their presence or absence which regulates enzyme activity. Inhibitors and metal chelators can reduce the hydrolysis of substrate, it can also aid in characterization of a novel enzymes (24).

Table 2. Effect of metal ions and inhibitors on *E. coli* cytosine deaminase

Ions and inhibitors	Concentration (mM)	Remaining activity (%)
$MgCl_2$	1	99
NaCl	1	98
KCl	1	99
$CaCl_2$	1	108
$MnCl_2$	1	105
$FeCl_3$	1	111
$HgCl_2$	1	5
$CuSo_4$	1	4
Sodium azide	1	100

Cytotoxic activity of cytosine deaminase using MTT assay.

The purified enzyme showed a cytotoxic effect on Caco-2 cell line in a comparison to crude enzyme and this effect increased with the increasing in concentration Table 3. Also, crude cytosine deaminase showed IC50 1864 µg/ml, whereas an IC50 of 242.5 µg/ml was obtained from the purified cytosine deaminase against Caco-2 cell line.

Table 3. Inhibition ratio for Caco-2cancer cell line by crude and purified cytosine deaminase at different concentrations

Concentration (ɱg/ml)	Cell inhibition (%)by crude enzyme	Cell inhibition (%) by purified enzyme
400	25.66±4.231	47.99±3.653
200	5.59±2.456	36.5±5.023
100	3.59±4.546	25.56±0.1155
50	3.09±3.191	14.75±0.8105
25	6.29±1.400	9.57±3.861
12.5	4.86±2.463	7.05±3.139
6.25	4.36±3.646	6.71±1.913

At 25 ɱg/ml concentration, 93.71% and 90.43% cell viability were observed after treatment with crude and purified cytosine deaminase, respectively. However, viability reached to 52.01% using 400 Mg/ml concentration of purified enzyme this finding suggest the sensitivity of Caco-2 cell line to crude and purified cytosine deaminase in a dose dependent manner which is due to cell death Fig. 9 and 10.

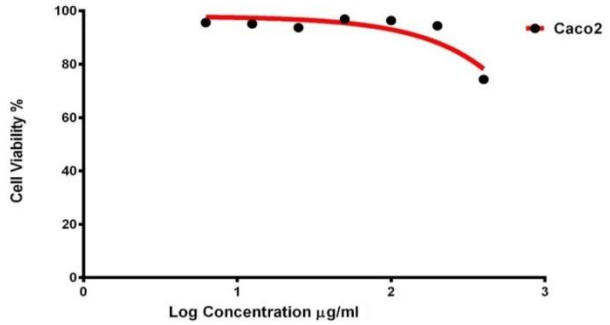

Figure 9. Cytotoxic activity of Crude cytosine deaminase produced from *E. coli* on Caco-2 colon cancer cell line using MTT test after 24h

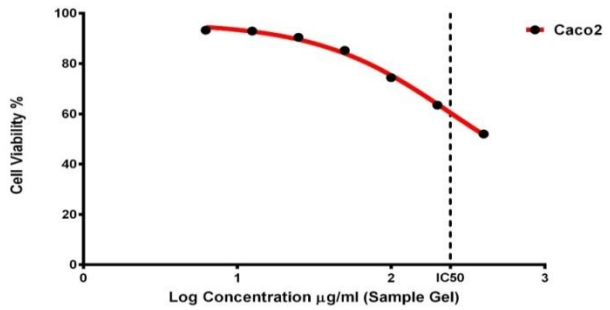

Figure 10. Cytotoxic activity of purified cytosine deaminase produced from *E. coli* on Caco-2 colon cancer cell line using MTT test after 24 h

On the other hand, *E. coli* cytosine deaminase did not show a significant toxicity on normal WRL cell line Fig.11 and 12.

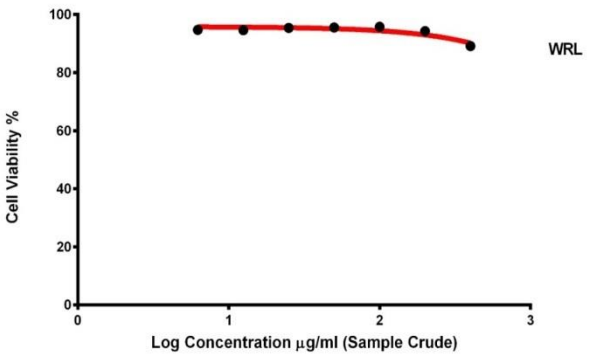

Figure 11. Cytotoxic activity of crude cytosine deaminase produced from *E. coli* on WRL normal cell line using MTT test after 24 h.

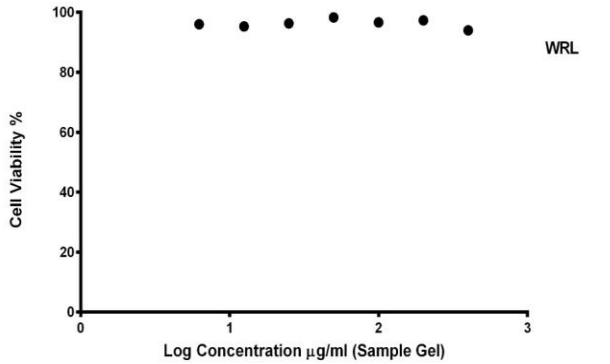

Figure 12. Cytotoxic activity of purified cytosine deaminase produced from *E. coli* on WRL normal cell line using MTT test after 24 h.

Meyers *et al*. (25) discussed the conversion of 5-fluorocytosine (5-FC) to the toxic metabolite, 5-fluorouracil (5-FU), then this metabolite is converted by cellular enzymes into 5-FdUMP, which inhibits the synthesis of DNA by blocking thymidylate synthase activity, 5-FUTP and 5-FdUTP, which are incorporated into DNA and RNA, respectively, so leading to cell death . In the presence of higher concentrations of fluorocytosine, high concentrations of fluorouracil in cytosine deaminase cells induce their apoptosis (26). The anticancer activity of CD expressing murine stem cell virus tested against glioma C6 /lacZ7 cell was studied by (27) using MTT assay after 7 days of incubation, this research showed that CD-expressing MSCs had a bystander anticancer affected on C6 glioma cells in proportion to the level of CD enzyme.

Conclusion:

Cytosine deaminase purified from locally isolated *E. coli* was stable at a wide range of pH and tempaerature, also the reduction observed in the viability of cell line following treatment with the enzyme provides preliminary data to have potent cytotoxic activity against cancer cell line enabling

its application in cancer therapy or suicide gene therapy.

Conflicts of Interest: None.

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التوصيف والفعالية السمية الخلوية لانزيم السايتوسين دي امنيز المنقى من العزلة المحلية اشيريشيا كولاي

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قسم التقنيات الحيوية الجزيئية والطبية، كلية التقنيات الاحيائية، جامعة النهرين، العراق.

الخلاصة:

هدف هذا البحث الى تنقية وتوصيف السايتوسين دي امينيز كأنزيم مهم طبيا من العزلة المحلية اشيريشيا كولاي، ثم دراسة التأثير السمي والضد سرطاني على خط خلايا سرطان القولون. نقي انزيم السايتوسين دي امينيز بثلاث خطوات شملت الترسيب بكبريتات الامونيوم بنسبة اشباع 90%، ثم التبادل الايوني باستخدام المبادل الايوني الموجب DEAE-cellulose، ثم خطوة التنقية باستخدام هلام الترشيح سيفادكس G-200 وقد ازدادت الفعالية النوعية للسايتوسين دي امينيز المنقى الى 9 وحدة / ملغم بروتين بعدد مرات تنقية 12.85 وحصيلة انزيمية مقدارها 30.85%.

أظهرت دراسة التوصيف للإنزيم المنقى ان الوزن الجزيئي للسسايتوسين دي امينيز المنتج من عزلة الاشيرشيا كولاي هو 48 كيلودالتون، وقد اعطى الانزيم اعلى فاعلية برقم هيدروجيني 8.5 وكان ثبات الانزيم ضمن حدود 7,5-9، وكان الانزيم ثابتا بأعلى فعالية بدرجات الحرارة تراوحت ما بين (45 - 60) م^0.

ان الفعالية الانزيمية قد ثبطت وبقوة بوجود كل من كلوريد الزئبق وكبريتات النحاس التي تم اضافتها بكميات ثابتة، على كل حال، تسبب كل من كلوريد الكالسيوم وكلوريد المنغنيز وكلوريد الحديد بزيادة طفيفة لفعالية الانزيم بينما لم تظهر مادة ازيد الصوديوم أي تأثير على فعالية الانزيم.

من خلال دراسة التأثير السمي باستخدام اختبار MTT ضد خط خلايا $Caco_2$، فقد وجد ان انزيم السايتوسين دي امينيز النقي ثبط نمو خلايا Caco-2 السرطانية وبتركيز مثبط وسطي بلغ 242.5 مايكرو غرام/مل مقارنة مع 1864 مايكروغرام/مل للإنزيم الخام. كما ان الانزيم لم يظهر أي تأثير سمي على الخلايا الطبيعية WRL.

الكلمات المفتاحية: سايتوسين دي امينيز، اشيريشيا كولاي، فحص MTT، تنقية.