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AUGMENTING DEAMINASE ACTIVITY OF *Escherichia coli*

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Abstract

Cytosine deaminase is an important component in the arsenal for cancer treatment, specifically in the strategy of the CD-Mediated Gene - Directed Enzyme/Prodrug Cancer Therapy. It deaminates one of the chemotherapeutic prodrugs, 5-flourocytosine and converts it into 5-flourouracil, which selectively increases the sensitivity of the cancer cells to the nontoxic prodrug than its active form (5-flourouracil) alone. This enzyme is produced by a variety of bacteria such as *Klebsiella pneumoniae* and *Escherichia coli.* However, increasing the gene dosage in *Escherichia coli* would raise the activity of this enzyme at least 2- folds due to the accumulation of the expression products of two copies of the same gene. Therefore, the gene encodes for this enzymes from a clinical *Klebsiella pneumoniae* was cloned into *Escherichia coli* strain *BL21* (DE3) and its expression was optimized via Central Composite Design. The anticancer activity of the partially purified enzyme was tested against A-549 lung cancer cell line in presence of 5-flourocytosine. The purified enzyme showed a three - fold increase in cytotoxicity assay against the cancerous cells compared to traditional drug; 5-Flourouracil. In conclusion, gene dosage technology can augment the activity of natural enzymes and make it feasible to produce at large scale. This would contribute to reduce the price and enhance the effectiveness of chemotherapeutic agent such as 5-flourocytsine.

Key words: Cytosine deaminase, 5-flourocytosine, 5-flourouracil, *Klebsiella pneumoniae* and *Escherichia coli* and cytotoxicity.

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1. Introduction

Cancer is a huge health problem worldwide. In 2012, the International Agency for Research on Caner (IARC) has estimated that 8.2 million cancer deaths and the cancer burden has increased to 14.1 million new cases (Ferlay *et al*., 2015). They also reported that 32.6 million people living with cancer within 5 years of diagnosis and the overall age - standardized cancer incidence rate is almost 25 % higher in men than in women. However, there is less regional variability in mortality than in incidence. Various strategies and techniques used to treat cancer (a group of terminal diseases) such as radiotherapy, chemotherapy and surgery. Usually, radiotherapy and chemotherapy are associated with tremendous amount of pain and long term side effects, in addition to the high cost which is beyond the reach of most of patients in developing countries like Egypt (Coates *et al*., 1983). Therefore, looking for alternative and more effective therapies with no or minimum side effects is one of the ever going research and development programs around the world.

For over 100 years, some bacterial infections were noticed to elicit remission in certain forms of cancer, however, the development of wild-type or modified bacterial (e.g. *Pseudomonas aeruginosa*) and viral strains to treat

cancer was lagging (Vlieghe *et al*., 2010). A recent approach of the Cytosine deaminase (CDase)/Gene Directed Enzyme Pro-drug Therapies was proposed by (Greco and Dachs (2001); it is an emerging therapy with a lot of promises. This relatively modern approach depended on activation of specific cancer pro-drugs such as the fluorinated pyrimidine analogue, 5-fluorocytosine (5-FC), by the bacterial cytosine deaminase (CDase) into 5-flourouracil (5-FU) which targets cancer cells and helps normal cells to selectively avoid damages associated with its use (Mullen *et al*., 1994). This combination of the bacterial CDase and the prodrug 5-FC was developed further by Negroni *et al*. (2007) into a novel suicide system in gene therapy for colon cancer. Their encouraging results indicated that 5-FC treatment of CDase-engineered tumor cells induces production of 5-FU intratumoral cells only leading to highly efficient immediate death of these tumor cells. Other scientific groups adopted the cloning of the bacterial CDase gene into suitable expression vector (CMV and pGEX) and examined its efficacy in treatment of skin or colon carcinoma (Yi and Huang, 2005).

The validity of the CDase in the different strategies and models examined by different groups, as circulated in the literature, leave no doubts about the importance of using CDase. However, the availability of the enzyme depends on its production at large-scale, which was greatly lacking as indicated by the survey of literature. Moreover, the ability of a producing microbe such as *Escherichia coli* is limited by the single copy gene encodes for this enzyme and hence to overcome this limitation novel approaches such as increasing the gene dosage are encouraged. The main target of this research is to clone an extra copy of the CDase gene from *Klebsiella pneumoniae* into *Escherichia coli* strain *BL21*(DE3) which was expected to augment its native CDase by increasing expression leading to increase the amount of CDase produced by *Escherichia coli*. This step is prerequisite to produce high amounts of the enzyme at commercial scale and hence make it available with reasonable cost to the patients (Ya *et al*., 1976, Gholami *et al*., 2015).

2. Materials and Methods

Bacterial strains, plasmid and growth condition

Thirty Gram negative, clinical isolates were obtained from Clinical Microbiology Laboratories, Clinical Pathology Department, Faculty of Medicine, Mansoura University, Egypt. Standard *Klebsiella pneumoniae* ATCC 10031 was obtained from Microbiological Resources Center (MIRCEN), Cairo, Egypt. While, *Escherichia coli JM109*, *Escherichia coli BL21* and pET 29 a (+) plasmid were available in our laboratory. All bacterial strains were grown on Luria Britani medium (LB), composed of g/L: 10.0 peptone, 5.0 yeast extract and 10.0 sodium chloride and incubated at 37 ^{o}C (Kim and Yu, 1998). Tryptone Bile X-Glucuronide medium (TBX) (36 g per liter) was used to verify the identity of *Escherichia coli* (Taormina *et al*., 1998).

Screening for cytosine deaminase-producing bacteria

Clinical and standard bacterial isolates were screened for cytosine deaminase activity using 5-flourocytosine (5-FC) as substrate, according to the methods described by Mahan *et al*. (2004). The reaction mixture contained 30 mM 5-FC, 50 mM Tris-HCl, pH 7.5, incubated at 37 ^{o}C for 15 min, then 0.1 N HCl 5 µl was added to stop the reaction and residual concentration of 5-FC was measured spectroscopically at 290 nm.

Identification of the cytosine deaminase most active isolate

The most active local bacterial isolates producing the CDase enzyme was identified automatically with the MicroScane Walkaway 90 system located at the Pediatrics Hospital, Microbiology Laboratory, Mansoura University (Burns *et al*., 2001).

Cloning and sequencing of cytosine deaminase encoding gene

Isolate number (18), a local clinical isolate of *Klebsiella pneumoniae*, which was identified as the most active strain in terms of CDase activity and this isolate was used as a source of the gene to be cloned. Two specific oligonucleotide primers were designed to amplify the whole gene encodes for CDase with the following sequences (F: 5′-AAGAATTCATGACAGCAGCGCCGCTTT - 3′ & R: 5′ - AAAAGCTT AGCCTTCGGCCCCTTT - 3′). The gene was amplified by polymerase chain reaction using 35 cycles, preceded by denaturation of template DNA at 94 °C for 5 min. Each of the 35 cycles consisted of denaturation at 94 °C for 1min, annealing at 58 °C for 45 sec, and extension at 72 °C for 1min, followed by an additional extension step at 72 °C for 10 min at the end of the 35 cycles. The PCR product of 1.323 kbp was purified using QIA quick Gel Extraction Kit digested with EcoR1 and Hind111 restriction enzymes (Thermo Scientific) and directionally ligated into pET 29 a (+) vector which was previously digested with the same restriction enzymes. The recombinant plasmid was introduced into two competent *Escherichia coli* strains *JM109* and *BL21 DE3* by transformation and successful transformants were selected on LB and TBX kanamycin plate (10 µg/ml) (Tsuchizaki *et al*., 2000, Woodman 2008). Nucleotide sequencing of the cloned gene was done in the autosequencing unit of lab technology (Cairo, Egypt). The sequence was analyzed by the BLAST service and compared to its databases (Kumar *et al*., 2004, Osman *et al*., 2013).

Optimizing expression of cytosine deaminase

In order to significantly increase the amount of the produced enzyme, at shake flask level, a Response Surface Methodology (RSM) and Central Composite Design (CCD) was applied to estimate the optimum level of each of the tested variables including: temperature (T °C), Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, pH and agitation speed. The tested pH range was (5.7 - 8.3), temperature range was (28.5 - 38.5 °C), IPTG concentration was between (0.05 - 0.5 mM) and agitation speed was (155.5-194.5 rpm). The exact levels and the proposed center points are displayed in Table - 1. Thirty runs were proposed by this experimental design, each with different combination of the tested variables were performed as shown in Table - 4. The experiments were performed in 300 ml flaks kept in shaker incubator for 24 hrs. Cells were harvested by centrifugation and the enzyme activity (target responses) was measured (Moradian *et al*., 2013). The data were analyzed by the Minitab program to establish the optimum cultivation condition to achieve the maximum level of gene expression.

Table - 1: Variables and their levels used for the CCD experiments

Variables	Levels				
	+2	**-1**	**0**	**+1**	**-2**
pH	5.7	6	7	8	8.3
Temperature (°C)	28.5	30	33.5	37	38.5
Agitation Speed (rpm)	155.5	160	175	190	194.5
IPTG (mM)	0.50	0.56	0.3	0.1	0.05

Enzyme assay and expression level

CDase activity of expressed enzyme was monitored by the methods of Mahan *et al*. (2004), where 5-flourocytosine was used as the enzymes substrate. The activity was recorded and compared to the CDase level of both native hosts: *Klebsiella pneumoniae* and *Escherichia coli*. The concentration and rate of CDase production was detected by denatured gel electrophoresis (SDS-PAGE) as described by Lamelli (1972).

Partial purification of CDase enzyme

The recombinant *Escherichia coli* was grown in CDase- producing medium (Sezonov *et al*., 2007) for 20 hrs at 28 °C. The cell pellets

were harvested by centrifugation at 4 °C, sonicated at 60 volt for 10 min at 4 ˚C, then the supernatant was collected. All soluble cellular proteins were precipitated from the supernatant by 60 % ammonium sulfate as described by Isenberg (1995). The precipitated proteins were dialyzed (dialysis bags with a molecular weight cutoff 12 kDa) against cooled 5 mM phosphate buffer, pH 7.0 (Ing *et al*., 2012). The dialyzed fraction was further purified by gel filtration *via* Sephadex G-100 Column chromatography (Porath and Flodin 1959, Donnelly *et al*., 1988) and eluted with 0.2 M Tris-Cl, and the collected fractions were assayed for CDase activity Katsuragi *et al*. (1986). The degree and folds of purification were checked by denatured Polyacrylamide gel electrophoresis; SDS-PAGE (Sambrook *et al*., 1989 and Lamelli, 1972)

Cytotoxicity test of combined cytosine deaminase and 5-flourocytosine

Cytotoxicity tests of cytosine deaminase enzyme, 5-flourocytosine, 5-flourouracil were done at Regional Center of fungi at Al-Azhar University according to method of Kuzmin *et al*. (2010) . Human lung cancer cell line A-549 obtained from VACSERA tissue culture unit was propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg streptomycin, and 0.25 µg/ml amphotericin. All cells were maintained at 37 ^{o}C in humidified atmosphere with 5 % CO_2 and were sub cultured two times a week. Cell toxicity was monitored by determining the effect of the test samples (CDase, 5-FC, 5-FU) on cell morphology and cell viability. For cytotoxicity assay, the cells were seeds in 96-well plate at a cell concentration of 1×10^4 cell per well in 100 µl of growth medium. Partial pure enzyme was added to the medium then different concentration of the 5 flourocytosine was added after 24 hrs of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayer's dispensed into 96-well, flat-bottomed micro titer plates (Falcon, NJ, USA) using a multichannel pipette. At the ends of the incubation period, media were aspirated and the crystal violet solution (1 %) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30 %) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on micro plate reader (TECAN, Inc.), at wavelength of 490 nm. The cell cytotoxic effect of 5FC and cytosine deaminase was calculated according to LI *et al*. (2009).

3. Results and Discussion

Klebseilla pneumoniae is one of pathogenic organisms which produce economically beneficial enzymes like cytosine deaminase. A local isolate 18 of this bacterium was used throughout this study.

Screening for the most active isolate

Two levels of screening local isolates were performed: 1) Direct CDase activity measurement and 2) Detection of gene encodes for CDase by PCR.

Screening for the most active organism

CDase enzyme assay showed that only one strain possesses high potential to produce CDase enzyme (isolate number 18) with an activity of 5.9 mg/min. The recorded CDase activities for isolates 1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 13, 14, 16, 17, 19, 20, 21, 22, 23, 24, 25, 28 and 29 were low while, isolates number 5, 11, 15, 26, 27 and 30 showed moderate CDase activity. The most active isolate (18) was identified as *Klebseilla pneumoniae* by the MicroScane Walkaway 90.

Screening for CDase encoding gene

PCR products (amplified DNA fragments) were analyzed by agarose gel electrophoresis (Fig. 1). A single DNA band with a calculated electrophoretic mobility equal to 1323 bp was appeared in all lanes for the four most active clinical isolates of local *Klebsiella pneumoniae*. This band size matched the estimated molecular size of the gene codes for CDase enzyme in other bacteria.

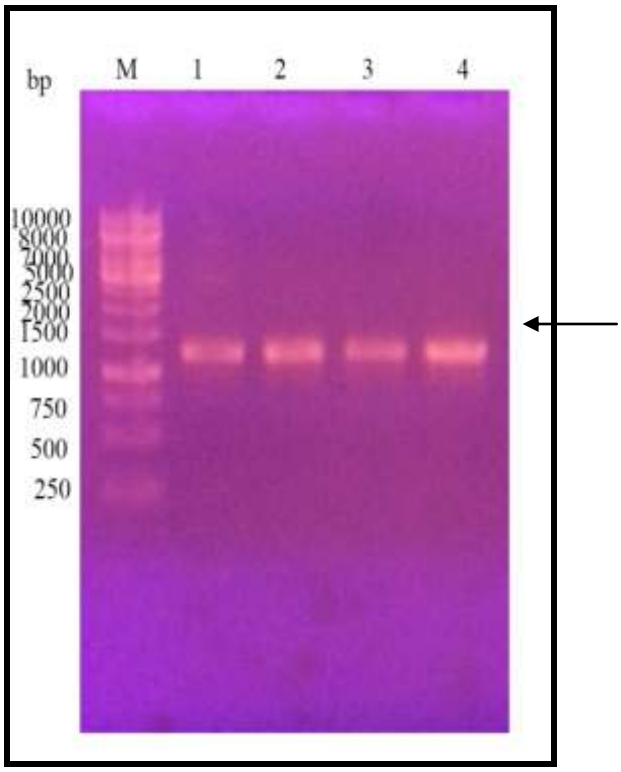


Fig - 1: PCR product analysis of amplified CDase encoding gene (lanes 1-4). Lane M: DNA molecular weight standard 10kbp

Cloning and sequencing cytosine deaminase gene into *Escherichia coli BL21* (DE3)

Cloning of cytosine deaminase gene was performed by colony PCR as described in previous section of materials and methods. Clones were screened and selected the best CDase producer. The CDase producers were analyzed not only by activity assay (Table - 3) but also by protein analysis of the clone in comparison to host cells (Fig - 3). The cloned gene was sequenced and analyzed by BLAST which compared it with the sequences deposited in the Gene Bank database. The analysis confirmed the identity of the local cloned gene to be CDase gene with 96 % similarity with *Klebsiella pneumoniae* known sequences (Fig - 2).

Expression of the cloned CDase enzyme

Comparing the intensity of the CDase protein band with electrophoretic mobility which equal to 48.4 kDa to reference strain revealed gradual increase in the intensity of a protein band with an apparent molecular weight of 48.4 KDa (Fig - 3). Lanes: 1, 2, 3, 4 and 5. The sharpest and most dense band appeared at lane 5, where the conditions of growth were optimized.

Optimization of the CDase gene expression by CCD

Bacterial growth and enzyme expression are greatly affected by pH, temperature, IPTG concentration and agitation speed. CCD matrix experimental design detected the optimum condition of all of the tested variables affecting enzyme expression and activity as shown in (Table - 2).

ATGACAGCAGCGCCGCTTTGGTTGATTCAGAACGTACGCCTGGCAGACCGCGACGGCCTGTGGCAAATAGCCATTGATAAGG
GCCGCTTTGGCGCGATCACGCCGATGGGGGAGGCGCACGATGAAAGCTATGAGGTATTGAACGCTCGCGGCGGGCTAGCGA
TCCCGCCTTTTATCGAGCCGCATATCCATCTGGATACCACCCAGACGGCCGGCGAGCCGAACTGGAATCAGTCCGGTACTCTGT
TCGAGGGGATTGAGCGCTGGGCGGAGCGGAAAGCGCTGCTCAGCCATGAGGACGTCAAAGCCCGGGCGTGGAAAACCCTGA
AGTGGCAGATCGCCAATGGCGTGCAGTTTGTCCGCACTCATGTCGATGTTTCCGATCCCACGTTAACGGCGCTGAAGGCGATG
CTGGAGGTCAAACAGGAGGTGGCGCCATGGGTCGAGTTACAGATTGTCGCTTTTCCACAGGAGGGGATCCTCTCTTACCCAAC
GGCGAGGCGCTGCTGGAGGAGGCCCTCAAGCTGGGGGCGGACGTCGTCGGGGCGATCCCGCATTTTGAGTTCACCCGTGAA
TATGGTGTGGAATCGCTGCATATCGCCTTCCGTCTGGCGCAGCAATATGACCGCCCACTGGATATTCACTGCGATGAAATTGAC
GATGAGCAGTCGCGCTTTGTTGAAACCGTTGCCGCGCTGGCGCTGAAAGCGGGGATAGGGCCGCGGGTAACGGCCAGCCAT
ACCACCGCTATGCACTCCTATAATGGCGCTTATACCTCGCGTCTGTTCCGCTTGCTTAAGCTCTCCGGCATCAACTTTGTCGCCA
ATCCGCTGGTGAATATTCATCTGCAGGGCCGGTTTGACGACTACCCGAAGCGACGGGGCATTACCCGGGTGAAGGAGCTGTT
GGCGGCGGGGATCAACGTTTGCTTTGGTCATGATGACGTCTTTGACCCGTGGTACCCGCTGGGGACCGGCAATATGCTGCAG
GTCCTGCATATGGGGCTCCATGTTTGCCAGCTGATGGGCTACCAGCAGATCAACGACGGCCTGCAGCTTATCACCGACAACAG
CACCCGCACGTTTGGCCTCGAGGATTACGGCATTGTCAGCGGCAACCCGGCTAACCTGATCATTCTCCCGGCGGAGAATGGCT
TTGAGGCCGTCCGCTGCCAGGTGCCGGTGCGCTGGTCAATTCGCCAGGGAAGGGTGATCGCGACCACCCAGCCGGCGCAAAG
CTGGATCCAAACTGACCGCGGTGGCGAGGAGCTTTCTTTTATGAGGAACAGCCCCCTTGCGGATGCAAAGGGGCCGAAGGCT
TAG

Fig - 2: Nucleotide sequence of the local isolate of *Klebsiella pneumoniae* cytosine deaminase gene

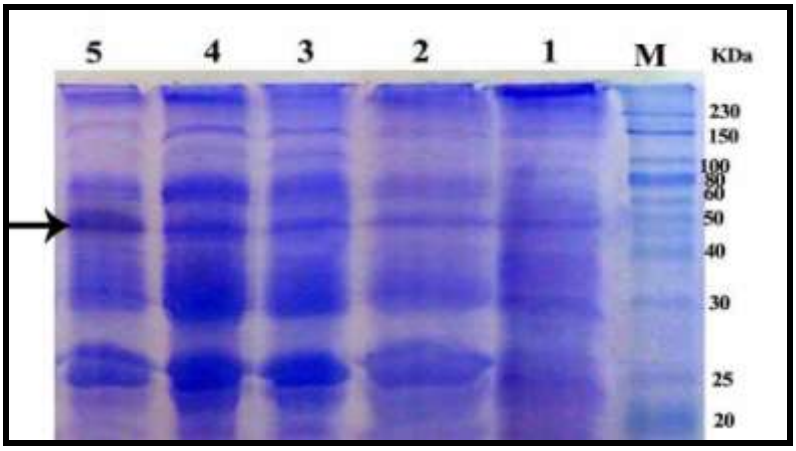


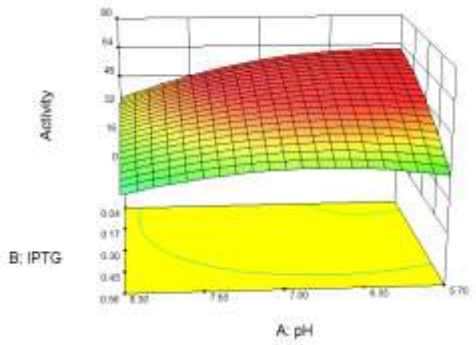
Fig - 3: Total cellular protein analysis of: lane 1: *Klebsiella pneumoniae* SDS- PAGE. Lane 2: *Escherichia coli BL21* (DE3). Lane 3: *Escherichia coli BL21* (DE3) with empty plasmid. Lane 4: *Escherichia coli BL21* (DE3) with recombinant plasmid at 0 induction time. Lane 5: *Escherichia coli BL21* (DE3) with recombinant plasmid after 20 hr of induction with IPTG. The arrow indicated the CDase protein band

Table - 2: Variables and their responses at CCD design

Run	Variables				Responses
	pH	Temperature (°C)	IPTG (mM)	Agitation (rpm)	Specific activity (µg/min/mg)
1	7	33.5	0.3	175	0.311
2	8.3	33.5	0.3	175	0.184
3	6	37	0.1	160	0.22
4	7	33.5	0.3	155	0.39
5	8	30	0.5	190	0.052
6	7	33.5	0.3	175	0.211
7	7	33.5	0.3	175	0.190
8	6	30	0.5	160	0.015
9	8	37	0.1	160	0.38
10	7	33.5	0.56	175	0.012
11	8	37	0.5	160	0.12
12	7	33.5	0.04	175	0.092
13	7	33.5	0.3	175	0.33
14	5.7	33.5	0.3	175	0.51
15	6	30	0.1	160	0.77
16	6	30	0.1	190	0.69
17	8	37	0.1	155	0.53
18	7	33.5	0.1	194	0.61
19	6	37	0.5	190	0.09
20	6	30	0.5	190	0.114
21	7	33.5	0.3	175	0.230
22	7	33.5	0.04	175	0.421
23	8	37	0.5	190	0.032
24	7	33.5	0.3	175	0.290
25	5.7	37	0.3	190	0.38
26	8	38.5	0.3	175	0.0086
27	7	33.5	0.3	175	0.250
28	8	30	0.5	175	0.009
29	8	28	0.04	160	1.2
30	5.7	28	0.3	175	0.55

The data showed the top five fermentation runs (shake flask levels) that gave the highest responses in terms of CDase activities can be arranged in the descending order: 29 > 15 > 16 > 18 > 30. Moreover, those responses were analyzed and the optimized conditions for expression of CDase enzyme was attained at pH 6.0, 28˚C, 0.04mM IPTG and 155 rpm. The highest specific activity of the CDase at the optimized variables was 1.4/µg/min/mg. The interaction between the variables is illustrated in 3D structure which follows the peaking at each run (Fig - 4).

(a)



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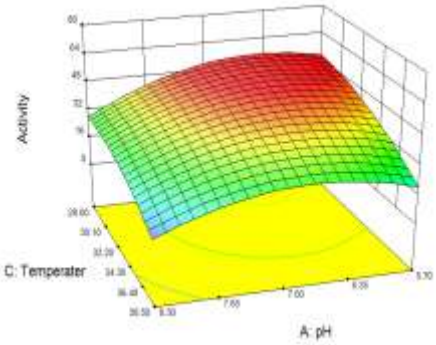

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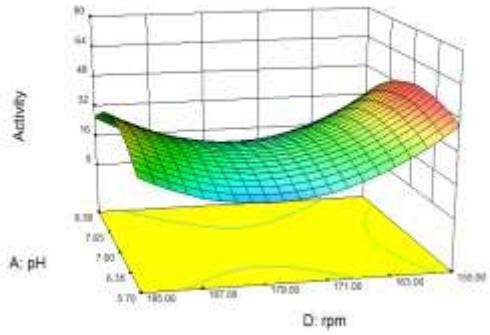
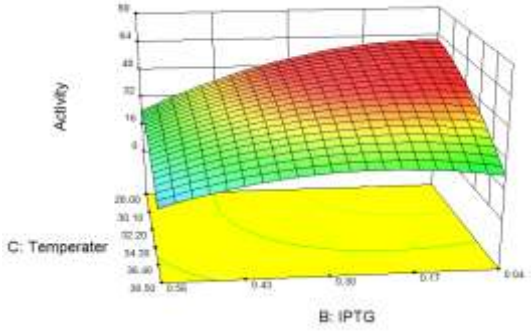
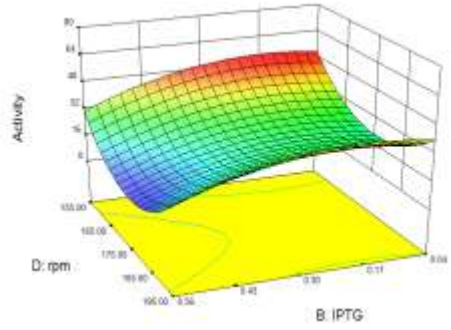


(d)



(e)



(f)

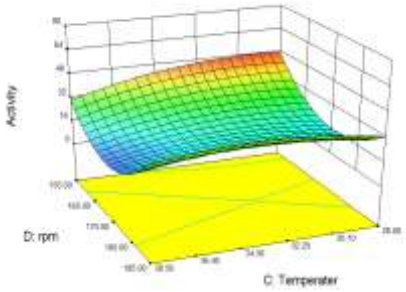


Fig (4): The analysis by CCD design and 3D structure which indicate the interaction between different factors. The red color indicated the maximum CDase activity. Panel (a) Shows the interaction between IPTG and pH. Panel (b) Shows the interaction between temperature and pH. Panel (c) Shows the interaction between rpm and pH. Panel (d) Shows the interaction between temperature and IPTG. (e) Shows the interaction between IPTG and rpm. Panel (f) Shows the interaction between temperature and rpm

Table - 3: Clone augmentation of CDase activity in *E.coli BL21DE3*

Organism	Specific activity (µg/min/mg)
Klebsiella pneumoniae	0.624
Escherichia coli BL21DE3 without plasmid	0.4
Escherichia coli BL21DE3 with plasmid	0.409
Escherichia coli BL21DE3 with recombinant plasmid	1.44

Enzyme activity of transformed strain (*Escherichia coli BL21DE3* with recombinant plasmid) increased by 2.3 fold than *Klebsiella pneumoniae*, 3.4 fold than *Escherichia coli BL21 DE3* without plasmid and by 3.5 fold than *Escherichia coli BL21 DE3* with pET29 plasmid alone.

Purification of cytosine deaminase

The partial purification of cytosine deaminase enzyme was monitored by enzyme assay for each step (Table - 4).

Table – 4: Activity of CDase purification steps

Steps of purification / Activity	Activity of CDase (mg/min)	Specific activity (mg/min/mg)	Purification fold	Yield %
Crud enzyme extract	0.723	1.446	1.00	100
Ammonium sulfate precipitation fraction	1.402	2.8	1.936	194
Dialyzed fraction	2.02	4.044	2.795	279
Gel filtration fraction	2.6	5.3	3.66	366

Specific enzyme activity increased with each purification step. The specific activity increased from 1, 2.8, 4.044 and 5.3 mg/min/mg, for crude, ammonium sulphate precipitate, dialyzed fraction and the gel filtration fraction, respectively. Purification folds increased from 0 in crude extract to 3.66 after sephadex column and the percentage yield followed the same pattern, as well. Moreover, SDS-PAGE of purification steps (Fig - 5) showed a single band in lane (5), the fraction collected from the gel filtration column (sephadex G-100) indicated a high degree of purity.

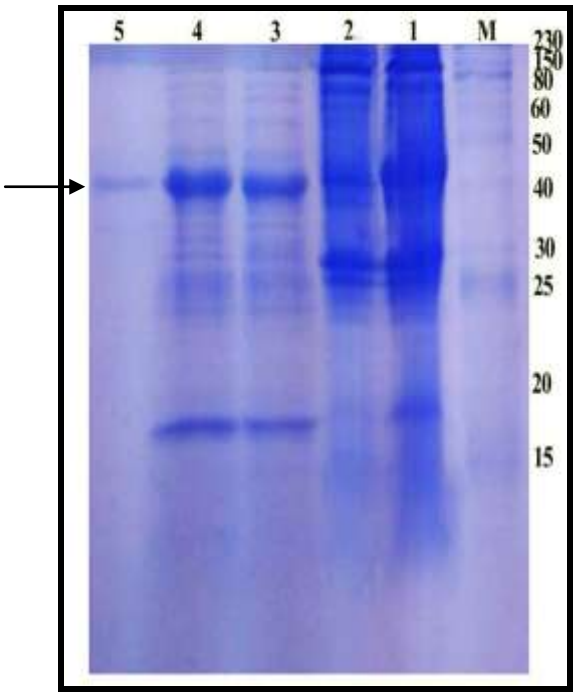


Figure - 5: M) protein ladder with molecular weight 230 kDa. 1) Crude enzyme. 2) Ammonium sulfate fraction. 3) Enzyme after dialysis bag. 4) Enzyme after Millipore filter and 5) Partial Purified enzyme from sephadex G-100 column

Anticancer activity of CDase

Cytotoxicity results of cytosine deaminase/5 - flourocytosine and traditional 5 -flourouracil against A - 549 lung cancer cell line and MRC - 5 human normal lung cells line are presented in Table – 5.

Table - 5: Cytotoxicity of cytosine deaminase, 5 - flourocytosine and 5 - flourouracil

Treatment	IC_{50} (µg/ml)	
	A-549 lung cancer cell line	MRC-5 normal cell line
Pure enzyme	186	338
5-flourocytosine	489	>500
Pure enzyme /5-flourocytosine	89.4	50.2
5-flourouracil (traditional cancer drug)	249	401

Cytotoxicity test performed against MRC-5 normal cell line and A-549 lung cancer cell line results showed that IC_{50} of pure enzyme and inactive drug 5-FC on A-549 cancer cell 89.4 which better than 249 of traditional drug 5-FU, IC_{50} of pure enzyme and 5-FC on MRC-5 normal cell 50.2 which more danger than traditional drug 5-FU of IC_{50} 401 if combination reached to normal cells, 5-FC alone on cancer cells has IC_{50} 489 and IC_{50} >500 on normal cells which indicated that it is less effective in absence of enzyme CDase of less damage on normal cells, finally enzyme alone on cancer cell line has IC_{50} 186 and IC_{50} 338 on normal cells which indication of its cytotoxicity effect yet, in absence of prodrug.

This study aimed to augment cytosine deaminase productivity from *Escherichia coli* by cloning CDase gene from a local *Klebsiella pneumoniae* strain which showed higher activity of this enzyme. The cloned gene expression was optimized using modern statistic approaches such as central composite design (CCD), which significantly increased the level of gene expression 3 folds. Upon obtaining of relatively high amount of this enzyme, a partial purification process was applied which has led to a substantial increase in its biological activity. The optimum condition to produce the CDase highest activity was found to be: IPTG concentration ranged from 0.04 -0.3 and its optimum was 0.04 mM, pH ranged was 5.7 - 7 with optimum at 6, temperature range was 28 - 33 ^{o}C and expression increase at optimum 28 ^{o}C, and rpm rang was 155 - 160 and most effect at 155 rpm.

The cytotoxicity of partially purified cytosine deaminase/5-florocytosin with A-549 lung cancer cell line resulted in decreasing the inhibition concentration needed to kill 50 % of the tested cancers to IC_{50} = 89.4 µg/ml in comparison with traditional drug 5-flourouracil with IC_{50} = 249 µg/ml. The apparent significant enhancement of the purified enzyme was 2.7 times.

No doubts, cancer are the most devastating disease of modern ages. It is a highly mysterious disease or a diverse group of diseases with many causes. The cancer cells go through uncontrolled and invasive cell division and at late stages these cells dislodge and spread allovers body causing the dangerous stage of metastasis which makes it harder to remedy. Moreover, most cancers showed great resistance to available drugs and chemotherapeutic chemicals. This created the urgency to find alternative therapies with limited or no toxicity to normal tissues and highly or exclusively selective for cancer cells. Treatment of cancer is not only big business to pharmaceutical companies but also constitute great challenge to scientific community. A number of strategies have been proposed, exercised and developed to satisfy this great humane objective, however, cancer prevails. Enzyme/prodrug strategy was introduced to this field to avoid the toxicity and side effects of chemotherapeutic agents on patients. In this strategy, a combination of the non-toxic prodrug 5-FC was used instead of the toxic drug 5-FU. 5-Flourocytosine (5-FC) permeates through living cell membranes with the help a cell membrane-associated permease. Once inside the cell, it was converted into 5-Flourouracil (5-FU) by cytosine deaminase (CDase). This last compound then acquires one phosphate group forming 5-FUMP which either inhibits DNA and/or protein synthesis leading to cell cancer death. In doing so, the intratumoral concentration of 5-FUMP will selectively kill cancer cells and saves normal cells (Lv *et al*., 2008).

CDase enzyme (CD; EC 3.5.4.1) was proven to be one of the important compounds to be used in combination with known drugs as part of the anticancer arsenal. It has been used therapeutically for the conversion of the prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU) (Nishiyama *et al*., 1985; Katsuragi *et al*., 1986). This enzyme is a highly selective one that does not exist in mammalian cells but essential for microbial pyrimidine metabolism specifically in pyrimidine salvage pathway where it deaminates cytosine to uracil and ammonia (O'Donovan and Neuhard, 1970). This enzyme has been isolated and purified from several microbes such as from bacteria (*Salmonella typhimurium* and *Escherichia coli*) and yeast (*Saccharomyces cerevisiae*). While, the bacterial CDases are highly thermostable and maintain high activity at 55 °C, those from yeast suffer thermal instability which makes them less desirable. However, bacterial sources of CDase are impractical for such use, requiring large-scale cultivation in order to obtain adequate activity (Sakai *et al*., 1970).

The current data of cloning and production of CDase according to the Design approach offered a way of producing large quantities of this enzyme that can be used side by side with other traditional chemotherapeutic agents. Cloning extra copy of the CDase into a bacterial expression vector pET29a (+) and used to transform *E. coli* strain *BL21*(DE3) Although, *E. coli* produces its own CDase this extra copy of the gene will exercise a dosage effect leading to overexpression of this enzyme. Not only, the gene dosage will enhance productivity of the enzyme, but also the use of modern statistical approach such as the CCD optimized its production, as well. This augmentation process is needed to produce economical amounts of this potential component for cancer therapy. A great promise was demonstrated through the combination of cytosine deaminase/5-fluorocytosine (CDase/5-Fc) in treatment of lung cancer cell line A-549. This is in accordance with the promotional use of enzyme/prodrug therapy. The added value of this strategy has been demonstrated for most of the common studied gene therapy strategies (Rehemtulla *et al*., 2004).

Administration of this enzymes directly to solid tumors together with the prodrug 5-FC will preferentially convert it to 5-FU inside the cancer cell was studied by Trinh *et al*. (1995). Also the CDase selectively caused 300 fold increase in toxicity of 5-FC when combined with antibody which made it an important player in the Antibody-directed enzyme-prodrug therapy (ADEPT) strategy (Deckert *et al*., 2003). Moreover, 5-FC completely eradicated peritoneal carcinomatosis resulted from CDase gene engineered colorectal carcinoma cells (Bentires *et al*., 2000). This study concluded that gene dosage plays important role in augmenting the productivity and hence activity of bacterial CDase enzyme. This is an important thing for scaling up production of this important enzyme.

5. References

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