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OPTIMUM CONDITIONS FOR CYTOSINE DEAMINASE PRODUCTION BY LOCAL ISOLATE OF *ESCHERICHIA COLI* E9.

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ABSTRACT

This study was aimed to isolate a higher cytosine deaminase producer *Escherichia coli* and studying the optimum condition for its production, a total of twenty-five urine samples were collected for the isolation of *E. coli* bacterium from Al-Imamein AlKadhumain medical city hospital in Baghdad. From these samples, a total of 10 bacterial isolates were obtained when subjected to morphological and microscopical tests. Cytosine deaminase enzyme activity was determined using cytosine as substrate, results indicated that 10 isolates of them are cytosine deaminase producer with different specific activity ranged between (0.122-0.35) U/mg and the isolate *Escherichia coli* E9 was the most efficient in the production of cytosine deaminase with specific activity of 0.35 U/mg protein. Therefore, it was chosen to determine the optimum conditions for cytosine deaminase production. Maximum cytosine deaminase production was achieved after supplementation of the minimal salt medium (pH 8.5) with 0.1% citric acid, 0.1% peptone and incubated at 37^{o}C for 24h. Under these conditions, the specific activity of cytosine deaminase produced in culture supernatant was sharply increased to 0.7 U/mg protein.

KEYWORDS: This study was aimed Al-Imamein AlKadhumain to 0.7 U/mg protein.

INTRODUCTION

Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) stoichiometrically catalyzes the hydrolytic deamination of cytosine and 5-fluorocytosine to uracil and 5-fluorouracil, respectively.[1] Cytosine deaminase was first identified in 1923.[2] And has been studied since in yeast, some bacteria and mold. Subsequently, cytosine deaminases from *Serratia marcescens*[3] and *Pseudomonas aureofaciens*[3] were first purified to homogeneity in 1975, Furthermore, extracellular cytosine deaminase was purified from *Chromobacterium*

violaceum YK 391 and *Salmonella typhimurium*[4] then from *Escherichia coli*[5] *and Aspergillus fumigatus.*[6] *Chromobacterium violaceum YK 391* produced not only extracellular enzyme, but also intracellular cytosine deaminase.[7] The enzyme is of wide spread interest both for antimicrobial drug design and for gene therapy application against cancer.[8] 5-fluorouracil (5-FU) is toxic, and has antitumoral activity[9] and a strong broad-range antimicrobial spectrum[10] 5-FC, after its conversion to 5-FU by cytosine deaminase, has antineoplastic activity and acts as a selective fungicide.[11,12] This antifungal activity of 5-FC has been attributed to the participation of the cytosine deaminase in the fungi themselves in which the enzyme deaminates 5-FC to 5-FU. Cytosine deaminase activity has not been found in mammalian and plant cells, therefore 5-FC is not metabolized.[13] However, a small amount of 5-FU has been detected in the blood and it has been proposed that deamination of 5-FC might be catalyzed by cytosine deaminase of intestinal microflora. It is known that enzyme prodrug therapy is being developed as treatment for cancer and other pathological conditions and cytosine deaminase / 5-flurocytosine strategy is one of most widely tested enzymes prodrug strategies in both animal models and clinical trials.[8]

MATERIALS AND METHODS

Samples collection

A total of 25 urine specimens were collected. Swab specimens were aseptically transferred under cooling conditions to the laboratory for analysis. Each specimen was inoculated on mackonky agar. All plates were incubated aerobically in incubator at 37ºC for 24 hrs.

Destruction of cells by ultrasonic

The Bacterial cell was washed twice with potassium phosphate buffer. Then the washed cells were subjected to ultrasonic probe with 19.5 pulse / sec for 30 sec. The process was repeated 8 times with stop for one or two minute to avoid high temperature of the mixture and the probe, a drop of cell was taken to examine under microscope to ensure the complete destruction of cells, then centrifuged at 9000 rpm for 15 minutes at 4^0C, supernatant was assayed for cytosine deaminase activity.

Determination of specific cytosine deaminase activity for *Escherichia coli*

Cytosine deaminase activity was determined according to katsuragi *et al.*,[5] by adding 0.4 ml of enzyme solution to 1 ml of cytosine solution prepared in and 0.6 ml of potassium phosphate solution. then incubated for 30 minutes at 37^oC in water bath. The reaction was stopped by adding 6 ml of the 0.1M HCL. The solution was centrifuged at 9000 rpm for 15

minutes. Then the absorbance was measured for the supernatant at 280 nm using UV-spectrophotometer.

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 the method described by Bradford.[14]

Optimum carbon and nitrogen source for cytosine deaminase production

Various carbon (glycerol, lactose, starch, glucose, raffinose, sucrose and citric acid) and nitrogen sources (peptone, beef extract, yeast extract, NH_4Cl, $(NH_4)_2Cl$ and urea) at initial concentration of 0.1% (w/v) were added individually in minimal salt medium[15] which composted of g/L {Yeast extract[8], Na2HPO4 (10.75), K2HPO4 (3.55), MgSO4 (0.025), MnCl2.4H2O (0.0025), FeSO4.7H2O (0.0027), CaCl2.6H2O (0.015)} the initial pH of the medium was set at 7.5. Cytosine deaminase activity was determined in the supernatants after inoculation of the medium with the locally isolated *Escherichia coli* culture, and incubated at 37°C.

Optimum pH for L-asparginase production

Optimal pH for production of cytosine deaminase was determined by preparing the medium with different pH values (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9). Cytosine deaminase activity was measured in the supernatant after inoculation of the production medium with *Escherichia coli* log phase culture, and incubation at 37°C.

Optimum temperature for cytosine deaminase production

Escherichia coli was grown in the production medium and incubated at different temperatures (25, 30, 37, 40, 45 and 50) °C. The cytosine deaminase activity was determined.

Optimum incubation period

Effect of the incubation period on cytosine deaminase production by *Escherichia coli* was studied by incubating at different periods of time (12, 24, 36, 48 and 60) hr.

RESULTS AND DISCUSSION

Screening ability of *Escherichia coli* for cytosine deamiase production

The ability of local *Escherichia coli* isolates for cytosine deaminase production was screened by determining the Enzyme activity katsuragi *et al.*,[5] Ten of twenty-five isolates are cytosine

deaminase producing with different specific activities (Table 2). Depending on these results, the isolate named E9 was found to be the most efficient in the production of cytosine deaminase with specific activity about 0.350 U/mg protein, therefore it was chosen for further study.

Table (2) Specific activity of cytosine deaminase produced by 10 local isolates of *Escherichia coli.*

Isolate number	Specific activity (U/mg)
E6	0.150
E7	0.173
E8	0.333
E9	0.350
E11	0.339
E15	0.157
E16	0.122
E17	0.113
E18	0.338
E21	0.163

Optimum carbon and nitrogen sources

Escherichia coli E9 was cultivated in a minimal salt media containing 0.1% from each of the various carbon and nitrogen sources. Results in Figure (2) and Figure (3) shows that, this isolate was capable of utilizing different carbon sources as a sole source for carbon and energy, while production of cytosine deaminase was varied according to the type of the carbon source. Since, citric acid was the best carbon source for cytosine deaminase production, while glucose, sucrose and raffinose were the less effectives. The type of nitrogen source also affected enzyme production, among the various nitrogen sources, maximum cytosine deamiase specific activity was obtained when peptone was added to the medium. Also, good level of enzyme activity was obtained with other nitrogen sources.

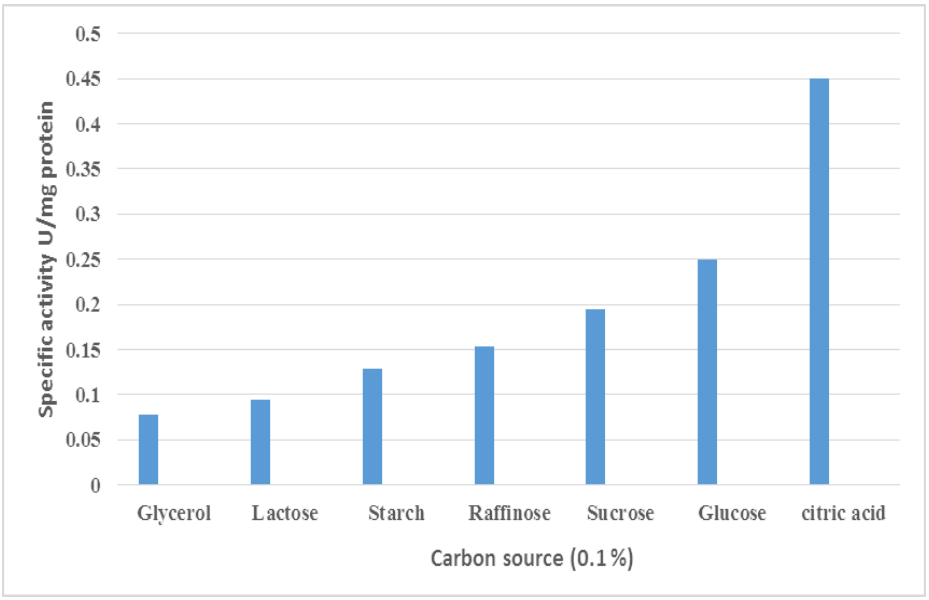

Figure (2) Effect of carbon source on cytosine deaminase production by *Escherichia coli* E9.

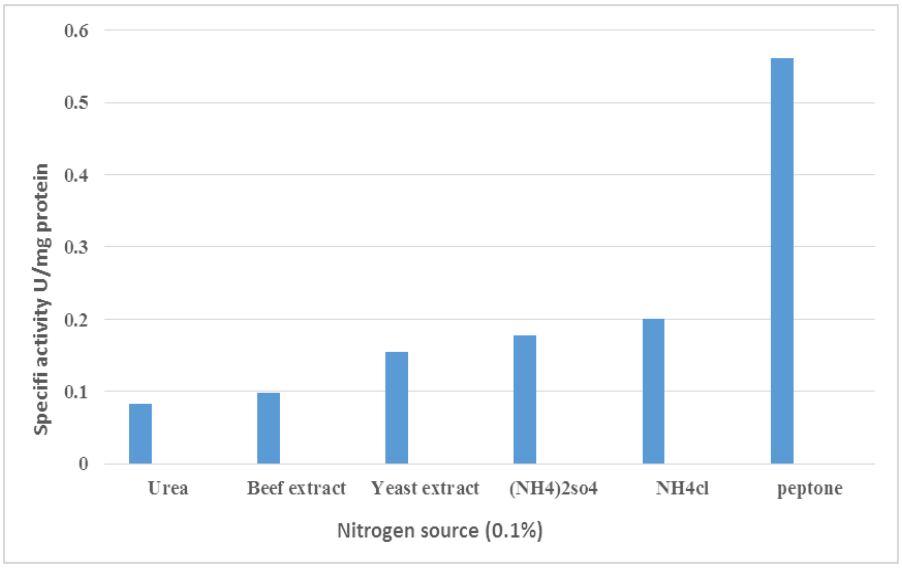

Figure (3) Effect of nitrogen source on cytosine deaminase production by *Escherichia coli* E9.

Optimum incubation period

Incubation period affects the enzyme production significantly and it varies from 24 h to a week depending upon type of microorganism and other culture conditions such as inoculum size, metabolic state of cell, pH and temperature Sharma *et al.*,[16] *E. coli* E9 had cytosine deaminase specific activity of 0.07 U/mg protein at 12 hr., this was followed by an increase in cytosine deaminase specific activity at 24 hr. to 0.7 U/mg protein, which recorded

maximum enzyme activity. After this hour, there was a decline in the activity gradually until reached 0.065 U/mg protein at 60 hr. Figure (4).

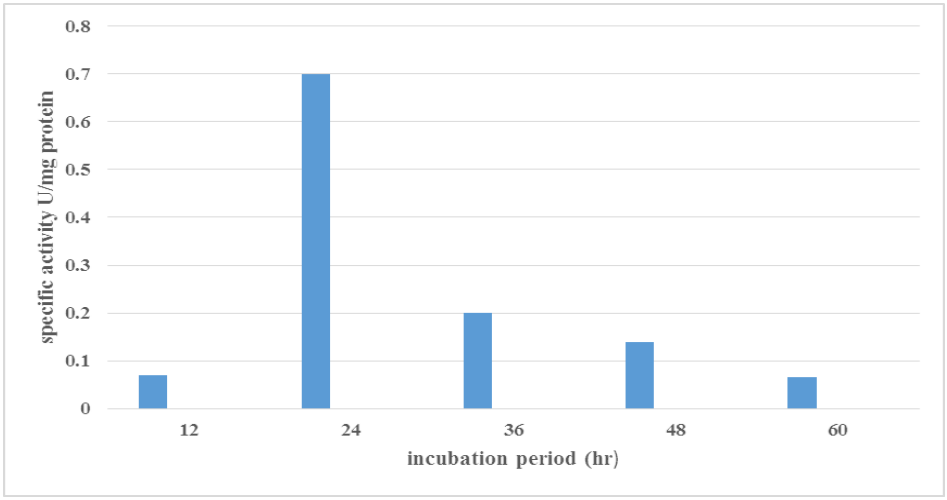

Figure (4): Optimum incubation period for cytosine deaminase production by locally *E. coli E9* after incubation at 37°C.

Optimum pH

Figure (5) shows that maximum cytosine deaminase production was obtained when the pH value of the production medium was adjusted to 8.5, at this value the enzyme specific activity in culture filtrate was 0.644 U/mg protein. A decrease or increase in hydrogen ions (H+) concentration causes pH changes in the culture medium which may lead to drastic changes in the three-dimensional structure of proteins because H+ and/or OH- compete with hydrogen bonds and ionic bonds in an enzyme, resulting in enzymes denaturation.[17]

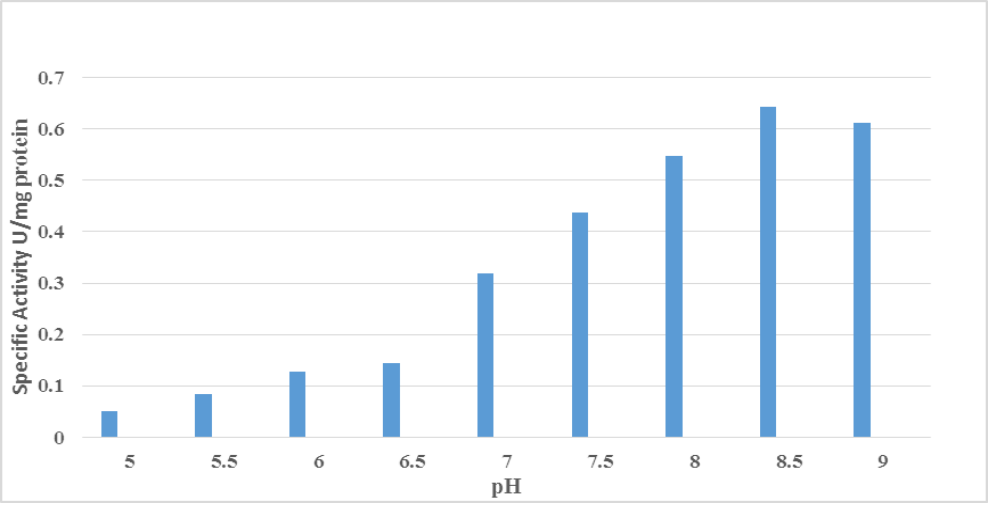

Figure (5): Effect of medium pH on cytosine deaminase production by *E. coli* E8 after incubation at 37°C for 24 hrs.

Optimum incubation temperatures

Cytosine deaminase specific activity is increased with increasing temperature for 37℃, since the specific activity was 0.2 U/mg protein at 30℃ has increased to 0.68U/mg protein at 37℃, but decreased at higher temperatures (Fig 6). Generally, for any enzymatic reaction, temperature below or above the optimal temperature will drastically reduce the rate of reaction. This may be due to the enzyme denaturation, or to losing its characteristics of three-dimensional structure. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds.[17] According to Majeed *et al.*,[18] the bacterial cytosine deaminase showed maximum activity at 37°C under optimized conditions.

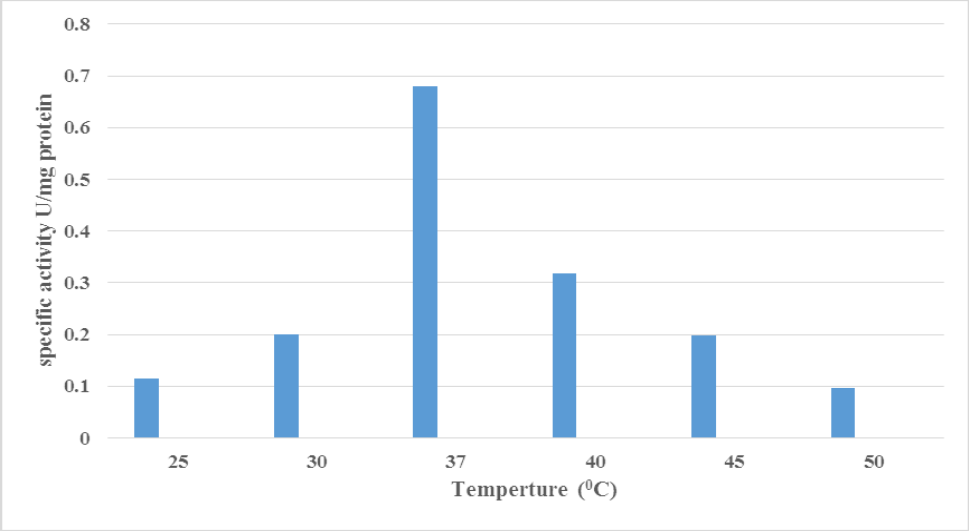

Figure (6): Effect of incubation temperature on cytosine deaminase production by *E. coli* E9.

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