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Production and Purification of Extracellular L-Asparginase from Mutant isolate *Geobacillus thermoleovorans.*

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ABSTRACT

The wild strain of *Geobacillus thermoleovorans* able to produce L-asparginase enzyme was subjected to strain improvement programs in order to increase L-asparginse production. The selected mutant with UV coded by UVG7 which produce about (84 U/mg) compared to parent strain which was produce about 72 U/mg. The UVG7 strain was subjected to NTG treatment. The selected mutant that show improvement in L-asparginase activity was UNG7-N12 which produce (121 U/mg) compared to parent strain.

Keywords: L-asparginase, UV, NTG, *Geobacillus thermoleovorans* and mutant.

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INTRODUCTION

L-Asparaginase (EC 3.5.1.1) is an enzyme that catalyzes the hydrolysis of asparagine to aspartic acid. The rationale behind asparaginase is that it takes advantage of the fact that Acute Lymphoblastic Leukemia (ALL) leukemic cells are unable to synthesize the non-essential amino acid asparagine whereas normal cells are able to make their own asparagine. Leukemic cells require high amount of asparagines, these leukemic cells depend on circulating asparagine. Asparaginase however catalyzes the conversion of L-asparagine to aspartic acid and ammonia. This deprives the leukemic cell of circulating asparagine. The main side effect is an allergic or hypersensitivity reaction. Asparaginase has also been associated with pancreatitis. Additionally, it can also be associated with a coagulopathy as it decreases protein synthesis, including synthesis of coagulation factors (examples progressive isolated decrease of fibrinogen) and anticoagulant factor (generally antithrombin III; sometimes protein C & S as well), leading to bleeding or thrombotic events such as stroke(1,2). *L-asparginase has attracted much attention for bulk production with low cost due to its wide range of applications (3).*

Strain improvement can reduce the costs with increased productivity and can possess some specialized desirable characteristics. Such improved strains can be achieved by inducing genetic variation in the natural strain and subsequent screening. Thus a major effort of industrial research in producing enzymes is directed towards the screening programs. Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production (4).

Various mutagenic agents such as ultraviolet rays (UV), N methyl-N''-nitro-N-nitrosoguanidine (NTG), X-rays, gamma rays, nitrous acid, ethyl methyl sulfonate (EMS) etc., are generally used for yield improvement studies. The ultra violet irradiation (UV) is the most convenient of all mutagens to use and it is also very easy to take effective safety precautions against it. The UV light is the best studied mutagenic agent in prokaryotic organisms. It gives a high proposition of pyrimidine dimers and includes all types of base pair substitutions (5,6). In this study we tried to improve L-asparginase production from *Geobacillus thermoleovorans* by using different physical and chemical mutagen.

MATERIALS AND METHODS

Microorganisms, Cultivation medium and cultural conditions

The bacteria Geob*acillus thermoleovorans* isolated from soil was employed in the present study. The strain was grown and maintained on LB medium at 37°C for two days and stored at 4°C for the study. This bacteria was identified by Vitec system. The slants were sub cultured at monthly intervals and stored at 4°C in the refrigerator.

The production of L-asparginase was performed in modified M9 medium containing (g/l): glucose – 3.0, $Na_2HPO_4.2H_2O$ - 6.0, KH_2PO_4 - 3.0, NaCl - 0.5, $MgSO_4.7H_2O$ - 0.5, $CaCl_2.2H_2O$ -0.015, aspargine 3.0 and initial pH was maintained at 7.0 (7) ,with incubation temperature 37°C. The flasks were agitated at 150 rpm and incubated at 37°C for 24 h. After incubation the cells were removed by centrifugation at 6000 × g for 10 min at 4°C. Then the collected supernatant was subjected to assay of extracellular L-asparaginase activity.

Enzymatic Assay

L-asparaginase activity was measured by direct Nesslerization of ammonia. The activity of Lasparaginase was measured employing the modified method of Wriston (8).

UV survival curves of parent strain and selection of mutants

Strain improvement for the selected parent strain was done by mutation and selection. The wild strain (*G. thermoleovorans*) was subjected to UV irradiation. The dose survival curve was plotted for selecting the mutants between 10 and 0.1% rate. Mutation frequency was mentioned to be high when the survival rates were between 10 and 0.1% (9).

The cell suspension of wild strain was prepared in phosphate buffer saline (PBS), pH 8.0 and 4 ml quantities were pipetted aseptically into sterile flat-bottomed petridishes of 100 mm diameter. The exposure to UV source was carried out by "UV-Transilluminator – Cross Linker". The distance between the UV source and irradiated suspension was 11cm. Four ml samples of bacterial suspension in PBS were irradiated in sterilized petridishes (9 cm in diameter) for the following doses: 0, 3, 6, 9, 12, 15 and 18 J/m^2. During the exposure, the lid of the petridish was removed.

Each irradiated cell suspension was serially diluted with sterile PBS. The suspensions after suitably diluting with phosphate buffer (PBS) of pH 8 were plated onto LB agar medium and incubated for 24 h at 37°C. The number of colonies in each plate was counted**.** The UV survival curve is plotted and plates having less than 1% survival rate (15 and 18 min.) were selected for the isolation of mutants. The isolates were selected on the basis of microscopic differential characteristics.

NTG treatment and selection of mutants

The best UV-mutant of *G. thermoleovorans* was selected for NTG treatment. The NTG (150 mg) was accurately weighted and dissolved in 25 ml of phosphate buffer (pH 7) at a temperature of 4ºC to minimize decomposition and sterilized by passing through sterile bacterial filter (0.22 µm). 10 ml of the cell suspension was added to 25 ml of NTG solution and immediately incubated at 30ºC in a water bath. At appropriate time intervals, 5 ml samples were withdrawn and centrifuged immediately. The supernatant was decanted; the cell mass obtained was washed with sterile distilled water and finally resuspended in 5 ml of sterile distilled water. A total of seven samples were withdrawn from the cell suspension after exposed to NTG for 30 to 180 min. respectively. A control was included without exposure to NTG. The above treated samples were serially diluted; one ml quantities of the appropriate dilutions from each sample were plated on LB agar medium incubated at 37ºC for 24 h. The treated samples were diluted, plated, colony counts were made, and the percentage of survival was calculated.

Optimization of culture conditions

Effect of carbon source on L-asparaginase production

Various carbon sources such as glucose, sucrose, fructose, lactose, and starch at 0.1% concentration were supplemented to the production medium, then the culture was introduced and incubated at 37^{o}C for 24 hours. After that, the enzyme activity and protein concentration were detected by extracting sample from the fermented medium.

Effect of nitrogen source on L-asparaginase production

To study the effect of various nitrogen sources on L-asparaginase production was determined with 0.5% of nitrogen sources such as gelatine, tryptone, casiene, yeast extract, urea and peptone were incorporated in the production medium and incubated at 40^{o}C for 37 hours. After incubation, the L-asparaginase activity and protein concentration were determined.

Effect of temperature on L-asparaginase production

The effect of temperature was studied by cultivation of *G. thermoleovorans* mutant strains in M9 medium and incubated at different temperature such as 25, 30, 35, 40, and 45^{o}C for 24 h incubation. After incubation, the L-asparaginase activity and protein concentration were determined.

Effect of pH on L-asparaginase production

To determine the pH influence L-asparaginase production was studied with varying medium pH such as 5, 6, 7, 8, and 9 at 37^{o}C for 24h incubation period. After incubation, the sample was collected by centrifugation for the determination of L-asparaginase activity and protein concentration.

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Effect of amino acids on L-asparaginase production

The effect of various amino acids (L-form) (histidine, lysine, asparagine, glutamic acid, and arginine) on asparaginase activity, various amino acids (0.3%) were substitute individually in place of asparagine. Flasks were inoculated and incubated at mentioned optimized conditions and asparaginase activity was analyzed.

Purification of L-asparaginase

All steps of purification of produced enzyme were carried out at 4°C on the crude extract according to the modified method of (10).

Ammonium Sulfate Precipitation and DEAE Cellulose Chromatography

Finally powdered ammonium sulfate was added to the clear supernatant with constant stirring and incubated overnight. Maximum L-asparaginase precipitation observed at 40-60% saturation. Centrifugation was performed at 6000 rpm for 30 min to collect the precipitate. The precipitate was then dissolved in a minimal amount of 50 mM Tris-HCl buffer (pH 9) and the enzyme activity and protein concentration were measured.

The sample was applied to a DEAE-cellulose (4×60 cm) previously equilibrated with 50mm Tris- HCl buffer pH9. The column was washed with two column volume of the equilibrium buffer and the protein was eluted using a gradient of NaCl (50-1000mM) in 50 mM Tris-HCl (pH 9). The optical density was measured for each fraction at 280nm, decline of reading indicated that the washing process of column was completed.

RESULTS AND DISCUSSION

Screening of L-asparaginase producing microorganism

The selected mutants resulted from strain improvement program were plotted as shown in figure (1). Theses mutants were coded by UVG1to UVG12 and tested for their ability to produce L-asparginase as shown in Table (1). The best mutant was UVG7 which produce about (84 U/mg) compared to parent strain which was (72 U/mg). The UVG7 strain was subjected to NTG treatment as shown in figure (2). A total of twelve mutants were selected and labeled as UVG7-N1 to UVG7-N12. The results shown in Table (2) indicated that UVG7-N12 was the highest enzyme production (121 U/mg) compared to parent strain.

Form these results we could find that UV increased the production to about 1.17 times while NTG increased the production to about 1.7 times.

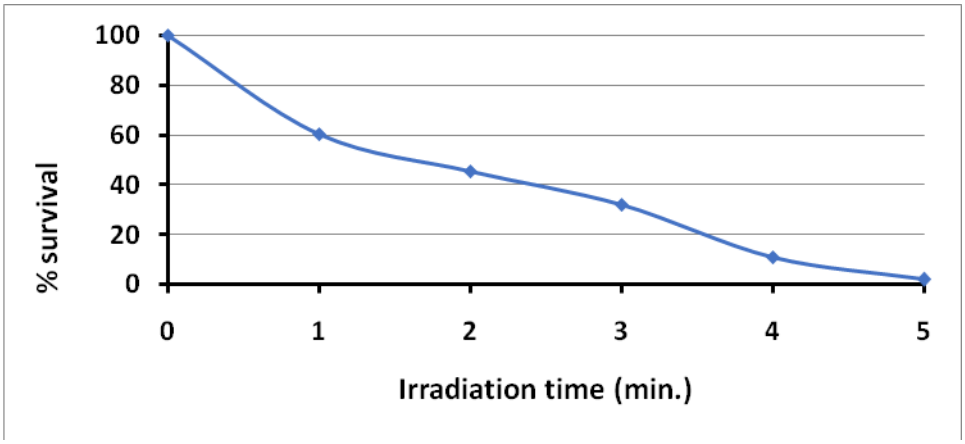

Figure 1: Survival curve of *Geobacillus thermoleovorans* after UV irradiation

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Table 1: Effect of UV on L-asparginase specific activity

UV Mutants	Specific activity U/mg protein
UVG1	65
UVG2	63
UVG 3	72
UVG 4	70
UVG 5	84
UVG 6	62
UVG 7	76
UVG 8	73
UVG 9	69
UVG 10	62
UVG 11	73
UVG 11	73
UVG 12	68
G. thermoleovorans (Parent Strain)	72

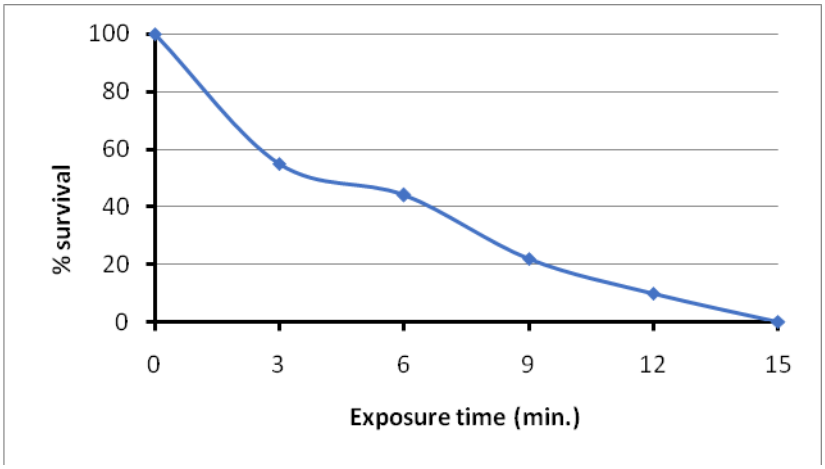

Figure 2: Survival curve *Geobacillus thermoleovorans* of after NTG treatemnt

Table 2: Effect of NTG on L-asparginase specific activity

NTG Mutants	Specific activity U/mg protein
UVG7N1	90
UVG7N 2	74
UVG7N 3	88
UVG7N 4	90
UVG7N 5	92
UVG7N 6	78
UVG7N 7	80
UVG7N 8	88
UVG7N 9	120.1
UVG7N 10	72
UVG7N 11	98
UVG7N 12	99
G. thermoleovorans (Parent Strain)	72

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Optimization of culture conditions

Effect of carbon source on L-asparaginase production

The data for the effect of carbon source on L-asparaginase production by UVG7-N12 strain was given in figure (3). It revealed that maximum L-asparaginase production was 128 U/mg by using lactose as sole carbon source. Reports have shown that lactose was the best carbon source for L-asparaginase production and the best concentration for maximum production by most bacteria was 0.5% (11,12).

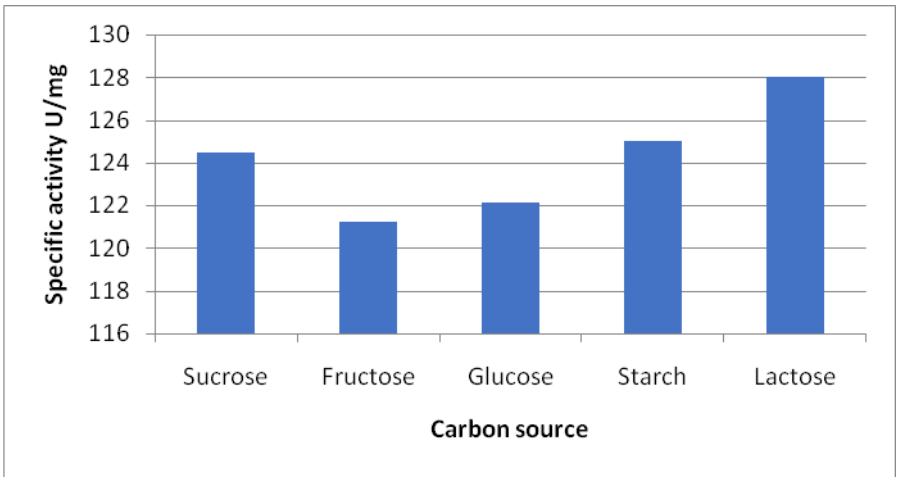

Figure 3: Effect of carbonsource on L-asparaginase production

Effect of nitrogen source on L-asparaginase production

Figure (4) shows the effect of nitrogen source on L-asparaginase production. It revealed that the highest amount L-asparaginase production was 131.1 U/mg. These results would agree with work of (1), who reported that yeast extract important for cell growth and L-asparginase synthesis.

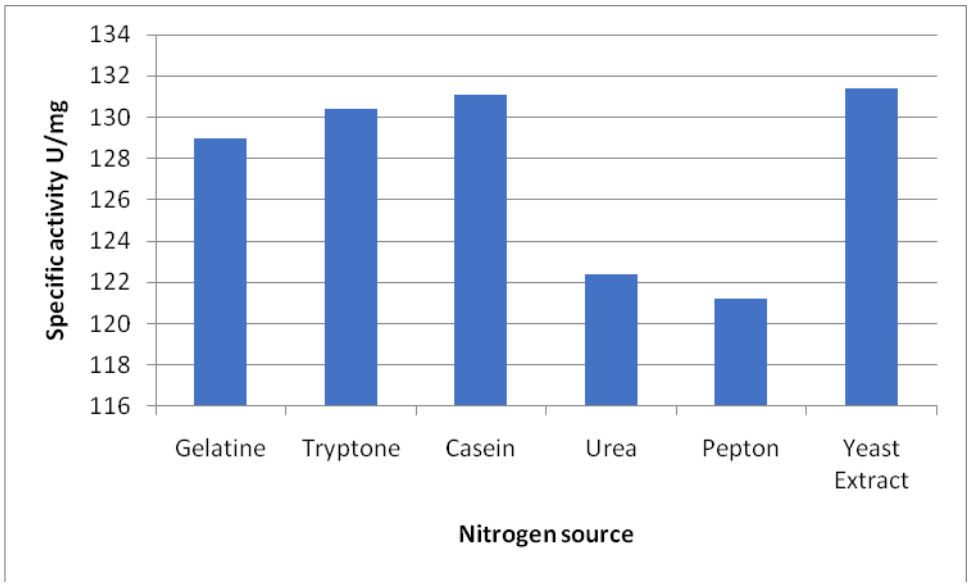

Figure 4: Effect of nitrogen source on L-asparaginase production

Effect of temperature on L-asparaginase production

Data on the effect of temperature on L-asparaginase enzyme production by *G. thermoleovorans* mutant strain UVG7-N12 was given in figure (5). The maximum L-asparaginase activity (132.2 U/mg) was

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recorded at 40°C. These results were agreed to Qeshmi *et al*., 2016 in which they found that the maximum L-asparaginase enzyme activity for *Bacillus* sp. was at 40°C. The optimum temperature of *Corynbacterium glutamicum* and *Bacillus subtilis* B11-06 was 40°C (13,14).

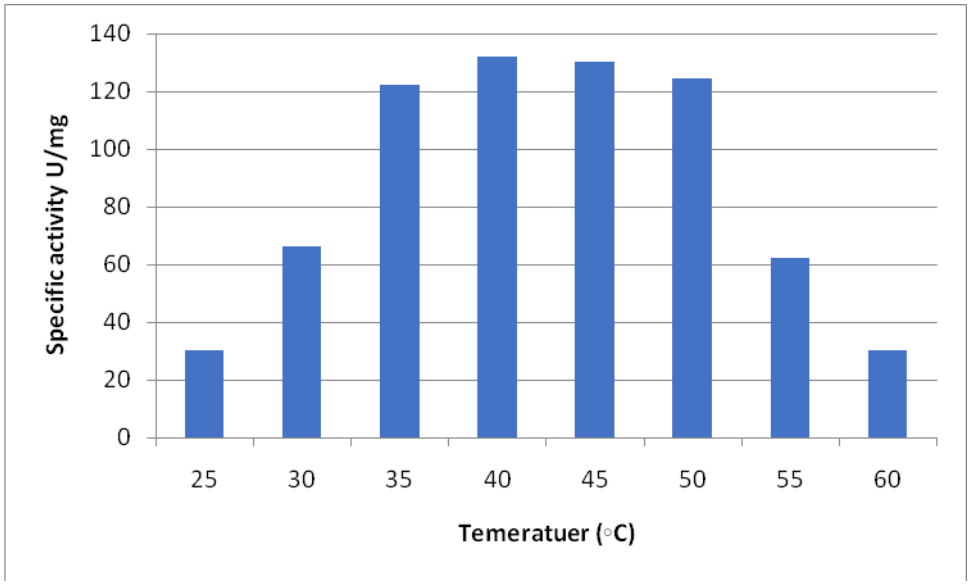

Figure 5: Effect of temerature on L-asparaginase production

Effect of pH on L-asparaginase production

The effect of pH on L-asparaginase production was shown in figure (6). It revealed that maximum L-asparaginase production was 133 U/mg at pH 7.0. These results were the same as those in (13,15). Since the physiological optimum pH 7.0 is one of the main requirements for antitumor activity and this is a desirable marker to make this enzyme suitable for both research studies and industrial and pharmacological processes.

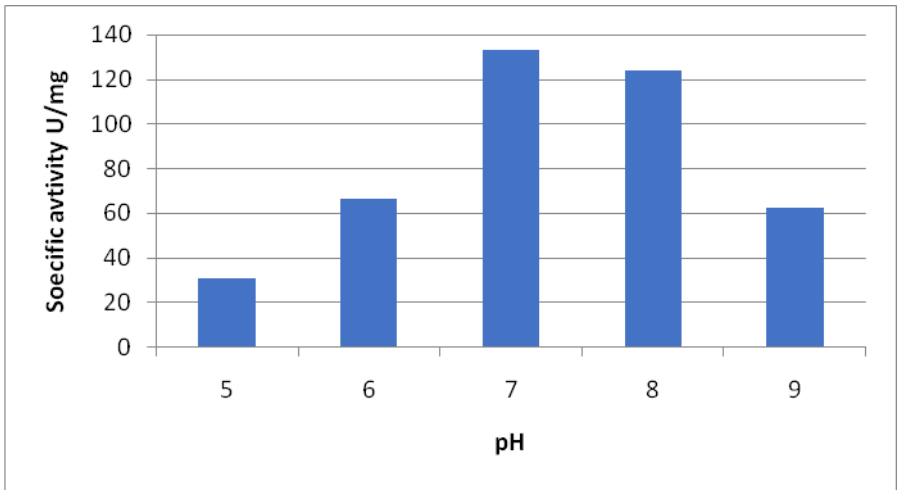

Figure 6: Effect of pH on L-asparaginase production

Effect of amino acids on L-asparginase production

Figure (7) show the effect of amino acids on L-asparaginase production. It revealed that the highest amount L-asparaginase production was 135 U/mg. These results was agreed with work of (1,15), who reported yeast extract important for cell growth and L-asparginase synthesis.

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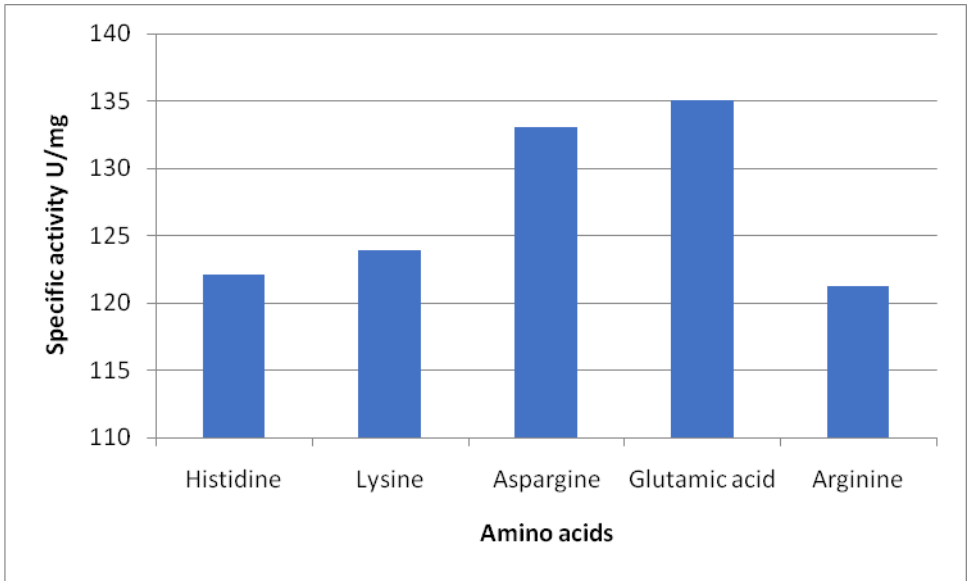

Figure 7: Effect of amino acids on L-asparaginase production

Purification profile of L-asparaginase

The L-asparaginase enzyme from mutant strain UVG7N12 are summarized in Table (3).

Table 3: Purification steps of L-asparginase produced by mutant strain UVG7N12

Step	Vol.	Activity	Protein Conc.	Specific activity	Total activity	Purification fold	Yield (%)
Crude extract	100	287.5	2.13	134.9	28,750	1	100
Ammonium sulphate (40-60%)	40	405	1.2	337.5	16,200	2.50	56.34
DEAE-Cellulose	20	600	0.3	2000	12000	14.82	71.73

In order to concentrate the crude extract of L-asparginse and remove as much water and some protein molecules as possible, the saturation ratio (40-60%) was used. It achieves specific activity 337.5 U/mg, 2.50 purification fold with 56.34% yield. From these results approximately there was a duplication in activity within 40-60% saturation ration comparing with crude extract, in addition to an increase in specific activity. So the ammonium sulphate precipitation is recommended to this stage of purification. Those results were similar to those of (16,17) they found that specific activity increase from 9.88 to19.92 by using ammonium sulphate.

DEAE-cellulose is a weak anion exchanger with excellent flow properties and high capacity for protein of most pI values with a high resolution. The ion exchange functional group is diethylaminoethyl that remain charged and maintain consistently high capacities over the entire working range. With DEAE-cellulose specific activity increase to 2000U/mg, 14.82 purification fold with 71.73 yield, which agreed with (18,19).

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