



## OPTIMIZATION OF PRODUCTION AND PARTIAL PURIFICATION OF TANNASE FROM LOCAL ISOLATE OF *BACILLUS LICHENIFORMIS* HJ2020 MT14171.1

Hameed Abbood Jebur

Department of Food Science, College of Agricultural Engineering Science, University of Baghdad, Iraq.

Email: dr\_hameedm59@yahoo.com

### Abstract

This study aimed to produce tannase with high activity from local isolate of *Bacillus licheniformis* HJ2020 MT14171.1 using submerged culture and partial purify this enzyme. The results were revealed that maximum production of tannase were found when using glucose as carbon source and tannic acid as inducer at concentration of 3 % (w/v) and 1.5 % (w/v) respectively, while yeast extract as nitrogen source at concentration of 2 % (w/v). Further inoculum ratio was 2% (v/v) contains  $1 \times 10^6$  CFU/ml. Optimum pH, temperature, Speed of shaker and fermentation period were 5.5, 35 °C, 200 rpm and 72 h, respectively. The enzyme specific activity of 688.32 U / mg was obtained at the optimum conditions mentioned previously. Crude enzyme was subjected to partial purification including precipitation with ammonium sulfate at 40 -80 % of saturation, then dialyzed and passed on ion exchange column using DEAE- cellulose, followed by Gel filtration using Sephadex G 150 column. The results were revealed that the enzyme specific activity was 3613.5 U / mg, while fold of purification was increased five times and attained to 5.24 with yield of 29.36 %.

**Keywords:** Tannase; *Bacillus licheniformis*; Partial purification; submerged culture.

### Introduction

Tannin acyl hydrolase (E.C.3.1.1.20) is commonly referred as Tannase, which can catalyze the hydrolysis of bonds present in the molecule of hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate, propylgallate and isoamyl gallate releasing glucose and gallic acid (Aguilar *et al.*, 2007; Aguilar– Gutirreze sanchez, 2001). Tannase can be obtained from plant, animal and microbial sources. As for plant sources, tannase is present in tannin-rich vegetables mainly in their fruits, leaves, branches and barks of trees. For animal resources, the enzyme can extract from bovine intestine and the ruminal mucous (Aguilar–Gutirreze sanchez, 2001). Tannase also produced from microbial origin, mainly bacterial and fungal, (Jian *et al.*, 2014). These sources represent the most enzymes used in biotechnological applications for several important reasons: (1) microorganisms grow rapidly and are ideal for intensive cultivation, (2) seasonal fluctuation of raw materials do not occur, (3) there is normally a high specific activity per mg protein (4) medium constituents are cheap and generally comprise agricultural products available in bulk, (5) can produce enzymes continuously and in large quantities, and (6) the enzymes thus obtained are more stable compared to those obtained by other means (Banerjee *et al.*, 2001; El-Shora *et al.*, 2015). Tannase is extensively used in the industrial processes such as clarification of beer and fruit juices, manufacture of coffee-flavored soft drink, manufacture of instant tea (Swaroop *et al.*, 2019) and as an analytical probe for determination of the structure of gallic acid ester (Seth and Chand, 2000; Sirvastava and Kar, 2010). Application of tannase for detannification of foods and feeds can improve their palatability, digestibility and nutritive value (Archambault *et al.*, 1996; Lekha and Lonsaneb, 1997). Gallic acid possesses a wide range of biological activities such as antioxidant, antibacterial, antiviral, analgesic etc. As antioxidant gallic acid acts as an antiapoptotic agent and helps to protect human cells from oxidative damage. Gallic acid is also found to show cytotoxic activity against cancer cells, without harming normal cells (Bainai and Patil, 2008). Enzyme biosynthesis through fermentation by microbes is

influenced by physical and chemical environment of cultural conditions, such as carbon and nitrogen source, presence of inducers, pH, temperature, aeration, etc (Beg and *et al.*, 2003).

The aim of the present investigation was to study the optimization of tannase production from local isolate of *Bacillus licheniformis* HJ2020 MT14171.1 by controlling some of the variables that affect enzyme production and partial purification of this enzyme.

### Materials and Methods

#### Microorganism

This study used an isolate of *Bacillus licheniformis* HJ2020 MT192715.1 from Department of Food Science, College of Agricultural Engineering Sciences, University of Baghdad. This strain was isolated from Iraqi soil and identified by 16S RNA and designated in gene bank with accession number MT192715.1.

#### Enzyme production

Tannase was produced using an inoculum of *Bacillus licheniformis* HJ2020 MT192715.1 and submerged fermentation was carried out in triplicate. Experiments were achieved in Erlenmeyer flasks 250 ml in incubator shaker containing 50 ml of selective tannic acid-enriched medium (TA medium) consisting of (g/l): tannic acid 10;  $\text{NH}_4\text{NO}_3$  2;  $\text{KH}_2\text{PO}_4$  0.5;  $\text{K}_2\text{HPO}_4$  0.5;  $\text{CaCl}_2$  1 and  $\text{MgSO}_4$  0.5, pH 5, (Muhammad *et al.*, 2016). The flasks were cotton plugged and autoclaved at 15 lb/in<sup>2</sup> for 15 min. The medium was cooled and inoculated with 1 ml of the inoculum containing  $1 \times 10^6$  cfu/ml of above culture. The fermentation experiments were carried out at 35 °C in a rotary shaker at a stirring speed of 150 rpm for 48 h. The extract was subjected to centrifugation at 10000 Xg for 15 min at 4 °C. The supernatant is considered as a crude enzyme.

#### Enzyme assay

Tannase activity was determined by the colorimetric method of Mondal *et al.* (2001a). Briefly, 0.1 ml of enzyme was incubated with 0.3 ml of substrate tannic acid (1.0% w/v

in 0.2 M citrate buffer, pH 5.0) at 50 °C for 30 min. The enzymatic reaction was terminated by addition 3 ml of bovine serum albumin (BSA) solution (1mg/ml), which also precipitated the residual tannic acid. A control reaction was also done simultaneously with heat denatured enzyme. The tubes were then centrifuged at 5000 Xg for 10 min and the precipitate was dissolved in 2 ml of SDS–triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution. The absorbency was measured at 530 nm after addition of 1 ml of FeCl<sub>3</sub> (0.13 M). The specific extinction co-efficient of tannic acid at 530 nm was 0.577 . Using this co-efficient , one unite of tannase activity (Unit / ml) was defined as the amount of enzyme able to hydrolyze 1mM of substrate (tannic acid) in 1 min at 50 °C and pH 5.0 . Specific activity of an enzyme is calculated by dividing the enzyme activity on protein concentration.

### Protein estimation

The protein content in the culture filtrate was estimated according to Bradford *et al.* (1976) using bovine serum albumin (BSA) as standard and the values were expressed in (mg/ml).

### Optimization of tannase production

Factors influencing the production of tannase were optimized by a single factor of varying the parameters one at a time in submerged culture at the same conditions previously mentioned (Belur and Mugeraya, 2011; Muhammad *et al.*, 2016).

### Effect of carbon sources

Enzyme production was studied in presence of various carbon sources including sucrose, maltose, glucose, fructose and xylose. These compounds were tested at 2% (w/v) regarding their effects on tannase production, in presence of 1 % tannic acid as inducer.

### Effect of glucose concentration

Different concentrations of glucose including (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5%) were used as the best carbon source for tannase production from *Bacillus licheniformis* HJ2020 MT192715.1 by submerged culture.

### Effect of tannic acid concentration

Various concentration of tannic acid was added to the production medium (0.5, 1, 1.5, 2, 2.5 and 3%) and incubated at 35°C for 48 h in presences of optimum concentration of carbon source.

### Effect of nitrogen sources:

The effect of different nitrogen sources on tannase production was carried out using organic and inorganic nitrogen sources including yeast extract, casein, peptone, ammonium sulphate, and ammonium nitrate. These compounds were tested at 2% (w/v).

### Effect of nitrogen source concentration

Many concentrations of yeast extract including (0.5, 1, 1.5, 2, 2.5 and 3.0%) were used as the optimum nitrogen source used for tannase production *Bacillus licheniformis* HJ2020 MT192715.1 by submerged culture .

### Effect of temperature

The effect of temperature on the production of tannase was studied by assaying the enzyme production after 48

hours of incubation period in the culture medium at varying temperatures (25, 30, 35, 40 and 45 °C) .

### Effect of Incubation Period

Effect of incubation period for tannase production in submerged culture was studied for different period of incubation (24, 48,72, 96, 120 h.).

### Effect of pH

The effect of pH on the enzyme production was carried out using different pH values of the growth medium 3, 4, 5, 6, 7.0 and 8. The flasks were incubated at 35°C for 48 h and the enzyme production was measured as described earlier.

### Effect of Inoculum Size

Effect of different size of inoculum (1, 2, 3, 4 and 5ml) contains 1x 10<sup>6</sup> cfu/ ml of *Bacillus licheniformis* HJ2020 MT192715.1 calculated according to MacCFarland standard curve (Collee *et al.*, 1996) on tannase production was determined in submerged culture at 35 °C for 48 h .

### Effect of agitation

Effect of agitation was also studied with varying speed of 50, 100, 150, 200 and 250 rpm at pH 5.0 and 35°C.

### Enzyme purification

#### Precipitation with ammonium sulphate

The cell- free fermented broth was used as source of crude enzyme. The enzyme was precipitate with solid ammonium sulfate (40-80%) at 4°C for overnight with constant stirring. The precipitate was collected by centrifugation (10000 X g, 15 min), dissolved in citrate buffer (0.05 M, pH 5.0) and dialyzed against the same buffer for overnight, then concentrated against poly-ethylene glycol-6000 to about one-tenth of the original volume. The concentrated sample was taken out and the bag was rinsed with 0.05 M citrate buffer (pH 5.0) to ensure maximum recovery (Shweta *et al.*, 1999; Paranthaman *et al.*, 2009).

#### DEAE Cellulose chromatography

A Pharmacia column was packed to a bed size of (2.5 x 10 cm) and was equilibrated with 0.05 M citrate buffer (pH 5.0). The concentrated sample was applied on the column. Elution by Salt linear gradient of NaCl (0.05 - 0.5 M), flow rate 30 ml / h, and 3ml / tube. The fractions were monitored for the elution of proteins and tannase activity. The fractions corresponding to tannase activity peak were pooled, concentrated using PEG-6000 and were used for the next purification step. (Shweta *et al.*, 1999; Paranthaman *et al.*, 2009).

#### Sephadex G-150 chromatography

The swollen gel was degassed and was packed in a Pharmacia column to a bed size of (1.5 x 48 cm) bed volume 84.78 ml. The column was equilibrated with 0.05 M citrate buffer (pH 5.0). The concentrated sample from the preceding step was passed, then elution with 0.05 M citrate buffer (pH 5.0). About ten fractions of 2 ml each were collected after the void volume (30 ml) and monitored for elution of protein and tannase activity. Fractions containing high tannase activity were pooled and used to further investigations. (Shweta *et al.*, 1999).

## Results and Discussion

### Optimization of tannase production

Carbon acts as an important factor for the enzyme production. Among the various carbon sources included, glucose, fructose, sucrose, maltose and xylose, the glucose gave the highest enzyme activity of 35.16 Unit/ml. The effect of different concentration of glucose was studied and the best concentration of glucose was 3 %, so the enzyme activity was 41. 58 U/ ml. Tannic acid was used as inducer besides presence of glucose as carbon source and the best concentration of tannic acid was 1.5 % and the enzyme activity was attained to 48. 12 U/ml. These results were agree with Shweta *et al.*, (1999) which observed that the best concentration of tannic acid was 1.5 % when used the enzyme as immobilized form and must be 2% when used the enzyme as free cell form. Nitrogen can be use an important limiting factor for the microbial production of enzymes. The result revealed that yeast extract was the best one in comparison with organic and inorganic nitrogen sources such as casein, peptone, ammonium sulphate, and ammonium nitrate. The enzyme activity of tannase was attained to 55. 12 U / ml with yeast extract. These results were agree with Pallavi *et al.*, (2015) which appointed that yeast extract give high enzyme activity (39. 6 unit/ ml) because it was considered as rich source of many nutrients such as protein, carbon, vitamins etc. The effect of different concentrations of yeast extract also studied and included 1%, 1.5%, 2%, 2.5% and 3%. The highest productivity at the concentration of 2% and the enzymatic activity was (59.9 U/ml). The results of the present investigation show that optimum temperature of tannase production was recorded at 35 °C and attained to 64.14 U / ml (Table 1). The reduction of enzyme activity was observed at lower temperatures more than in higher temperature. This was in good agreement with the results obtained earlier for tannase from *Bacillus cereus* (Mondal *et al.*, 2001b). While Pallavi *et al.*, ( 2015 ) found the maximum tannase activity ( 29. 68 unit / ml ) was noticed at 32 °C although the organism had a capacity to grow over a wide temperature range of temperatures (25°C to 35°C) and with a rise in temperature the tannase production was decreased.

In our present study the maximum tannase production was found in 72 h, and the maximum enzyme production was (76.8 U/ml). This might be due to the fact that the bacteria

would have entered in to its exponential phase, thereafter, the enzyme production started decreasing. Decreasing of tannase activity on prolonged incubation due to inhibition and denaturation of enzyme, Moreover the reduction of nutrients level of medium affecting the metabolic activity (Gautam *et al.* 2002). There are various reports of different incubation periods for maximal production of tannase, Lokeswari and Jaya Raju (2007), reported maximum extra cellular tannase production by *A. niger* at 36 h of incubation period. Banerjee *et al.* (2007) found maximum production of extracellular tannase by *A. aculaetus* after 72 h. While Pallavi *et al.* (2015) observed that the highest enzyme activity (35.6 U/ml) was recorded at 168 h and the productivity were decreased at prolonged period of incubation .These differences between reports in determination of incubation period may be due to differences in source of enzyme , Moreover differences of culturing conditions . Biological process was pH dependent, a small variation in the pH lead to change the rate of production. Hence, the optimal pH was very important for maximizing the yield of tannase production. The maximum enzyme activity (82.02 U/ ml) was observed at pH = 5.5. Tannase activity was increased as the pH increased from 3.5 to 5.5 then declined in alkaline conditions, this may be due to salt formation of tannic acid at higher pH, or possibility of low activity of tannase at higher pH which might have led to unavailability of tannic acid for the utilization and hence the tannase activity was observed to be less (El -Shora *et al.*, 2015). This result was similarly with Sabu *et al.*, (2005) which reported optimum pH of 5.5 for tannase production by *Aspergillus niger* ATCC 16620. Size of inoculum was also be studied and the suitable size of inoculum of *Bacillus licheniformis* HJ2020 MT192715.1 for tannase production by submerged culture was 2 ml containing 10<sup>6</sup>cfu / ml and the enzyme activity was attained to (91.12 U / ml) As shown in Table (1). The reduction of enzyme activity with increasing of inoculum size may be due to competition with high concentration of organism and run out of nutrients from medium early. Moreover loss of oxygen due to quickly growth of culture (Haritha *et al.*, 2011). The results revealed that the optimum agitation of shaker was 200 rpm and the enzyme activity was (98.43 U/ ml). Increasing of aeration velocity may affect cell destruction or lead to cell degradation and increased permeability, and thus affect enzyme production, (Sepahy and Jabalameli, 2011).

**Table 1** : Effect of different parameters on production of tannase from *Bacillus licheniformis* HJ2020 MT1927.1 by submerged culture .

Item No.	Parameters	Optimum parameter	Maximum enzyme activity (U/ml)
1	Carbon source	Glucose	35.16
2	Concentration of glucose	3% glucose	41. 58
3	Tannic acid	1.5	48. 12
4	Nitrogen source	Yeast extract	55. 12
5	Concentration of yeast extract	2 %	59.9
6	Temperature	35 °C	64.14
7	Incubation period	72h	76.8
7	pH	5.5	82.02
8	Size of inoculum	2 x 10 <sup>6</sup> cfu / ml	91.12
9	Speed of shaker	200 rpm	98.43

### Enzyme purification

Tannase was produced from locally isolate of *Bacillus licheniformis* HJ2020 MT1927.1 using submerged culture under optimum conditions and purified by following steps:

### Concentration by ammonium sulfite

This is an important step in purification and concentrated of enzyme. The results revealed that, the enzyme activity was 108.86 U / ml when using ammonium

sulfite at 40-80 % saturation while the enzyme specific activity was 863.96 U / mg and the fold of purification raised to 1.25 (Table 2). The previous studies were different in determination the best percentage of saturation of ammonium sulfite used for enzyme precipitation. Sabu *et al.* (2005) used ammonium sulfate with 40-60 % of saturation to concentrate tannase from *Aspergillus niger* ATCC 16620 and enzyme activity attained to 0.916 U / mg. While Mahendran *et al.*, (2006) used 70 % of ammonium sulfite to precipitate tannase from *Paecilomyces variotii* yielded 78.7% with 13.6-folds purification. Paranthaman *et al.*, (2009) indicated that 60-80 % saturation of ammonium sulfite was used to precipitation tannase produced from *Aspergillus flavus*. Sivashanmugam and Jayaraman, (2011) were subjected crud extract of tannase from *Klebsiella pneumoniae* MTCC 7162 to fractional precipitation with 80% ammonium sulphate and found that fold of purification was 2.85 and yield was 23.7%. Mukesh *et*

*al.* (2015) observed that the enzyme extract was subjected to 85%(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> of saturation and during this step enzyme was purified to 2.07 fold with 33.3% recovery.

### Dialysis

Dialysis was consider one of important steps in purification because non-enzymatic proteins were shown to be removed in addition to residual salts of ammonium sulfite which may be effect on enzyme activity (Paranthaman *et al.*, 2009). After dialysis the enzyme activity and enzyme specificity were 97.25 U / ml and 1080.55 U/mg respectively, while fold of purification raised to 1.56 and yield was decreased to 66.19%, (Table 2). Many research appointed that daiylsis led to increasing in enzyme specific activity and fold of purification, Where Paranthaman *et al.* (2009) noticed that specific activity of tannase from *Aspergillus flavus* increased to 55.92 U / g / min.

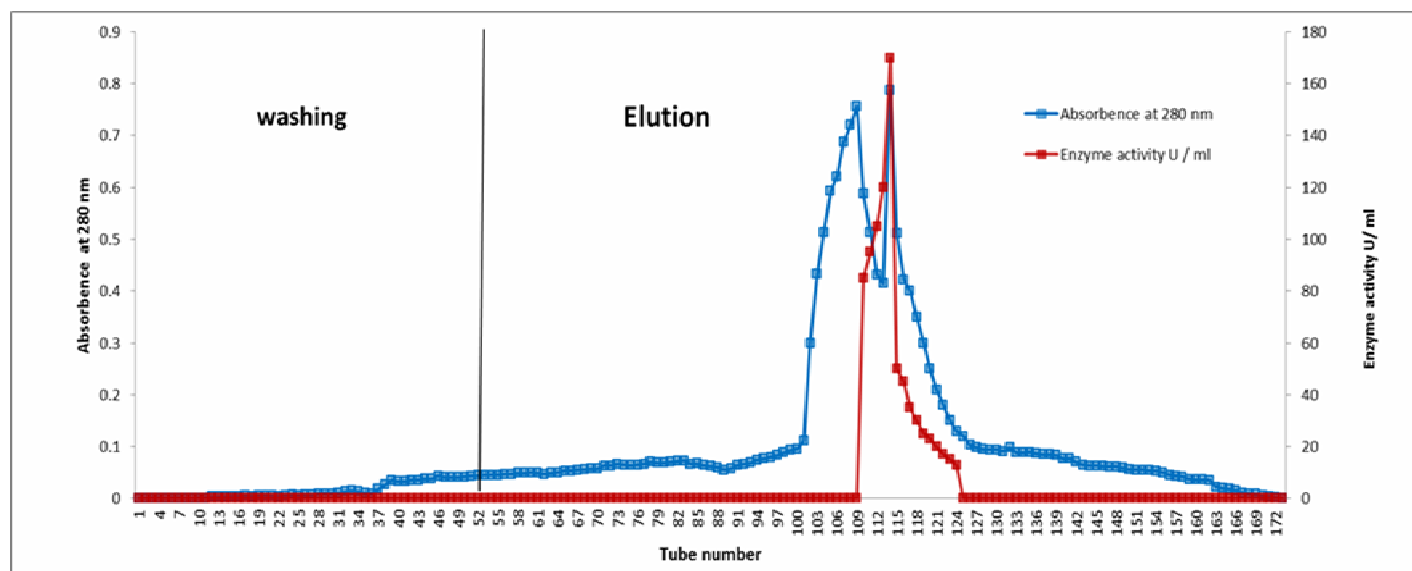
**Table 2 :** Step of tannase purification produced from local isolate of *Bacillus licheniformis* HJ2020 MT1927.1

Steps of purification	Volume (ml)	Enzyme activity (U/ml)	Protein (mg/ml)	Enzyme specific activity (U/ mg)	Total activity (Unit)	Fold of purification	Yield %
Crud enzyme	100	98.43	0.143	688.32	9843	1	100
Precipitation by ammonium sulfite 40- 80 %	65	108.86	0.126	863.96	7075.9	1.25	71.88
Dialysis	67	97.25	0.09	1080.55	6515.75	1.56	66.19
Ion exchange on DEAE- cellulose	18	170.70	0.08	2133.75	3072.6	3.09	31.21
Gel filtration Sephadex G-100	20	144.54	0.04	3613.5	2890.8	5.24	29.36

### Ion exchange chromatography

The next step was passed the enzyme solution on ion exchange column chromatography contain DEAE –Cellulose. The absorbance of washing fractions (unbounded proteins

with positive charge) were measured at 280 nm. There is no peaks at washing step and the enzyme activity were close to base line because there is no positive charge of protein in enzyme extract (Fig. 1).



**Fig. 1 :** Ion exchange chromatography for purification of tannase produced from local isolate of *Bacillus licheniformis* HJ2020 MT1927.1 using DEAE – cellulose (2.5x 10) cm, Washing by 0.05 M citrate buffer (pH 5.0). Elution by Salt linear gradient of NaCl (0.05-0.5 M), flow rate 30ml/h, and 3ml/tube.

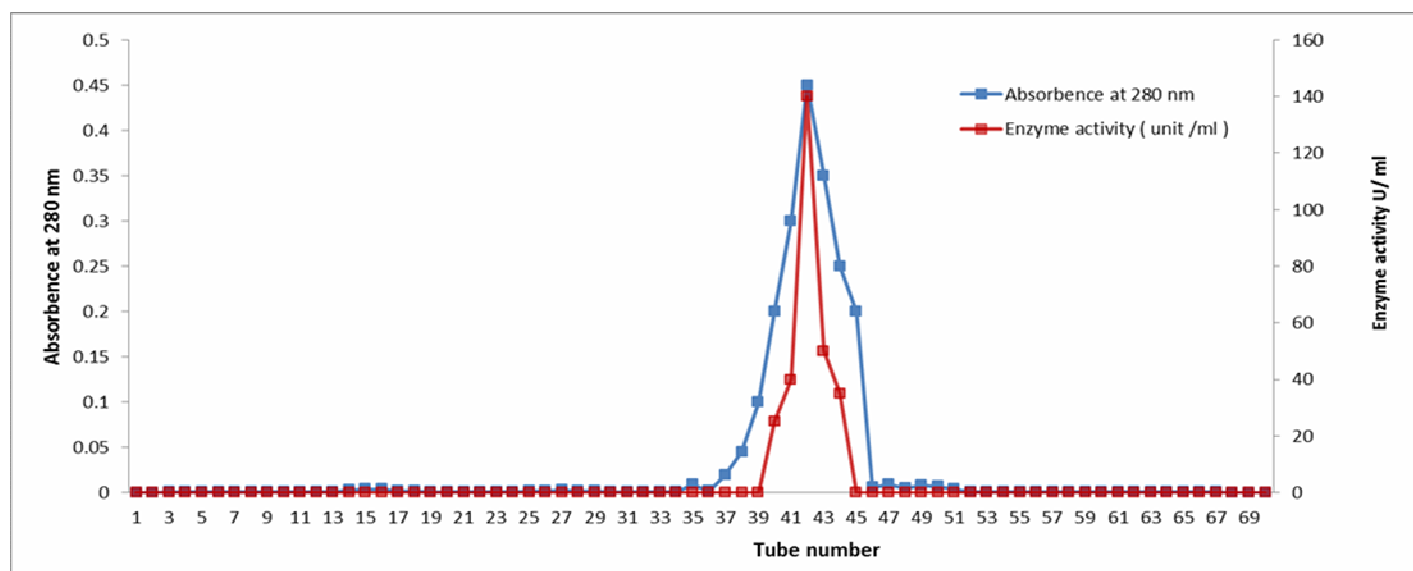
Elution of bounded protein with negative charge was performed by linear salt gradient using 0.05 M citrate buffer (pH 5.0) contain 0.05 - 0.5 M of NaCl. Two protein peak were appeared in elution fraction one of them have no enzyme activity, While the enzyme activity and enzyme specificity of the second peak were 170.70U / ml and 2133.75 U / mg respectively (Table 2). Fold of purification

was raised to 3.09 and yield were 31.21%. These results were consider an important indicators of purity and it appointed that the native charge of enzyme was negative under purification conditions. The present results in agreement with those of Sivashanmugam and Jayaraman (2011) who obtained that DEAE-cellulose column chromatography led to an overall purification of 8.8 fold with a yield of 4.84% for

partial purification of extracellular tannase by *Klebsiella pneumoniae* MTCC 7162 isolated from tannery effluent. While Mukesh *et al.* (2015) indicated that the concentrated enzyme was purified to homogeneity by ion exchange chromatography using DEAE cellulose, which resulted in 9.5% enzyme yield with a purification fold of 8.55.

## Gel filtration

The fractions possessing tannase activity during ion exchange chromatography step were pooled and applied on gel filtration column using Sephadex G- 100. The results revealed there is one peak of enzyme activity which identical to one peak of absorbance at 280nm which indicate increasing of purity (Fig. 2).



**Fig. 2 :** Gel filtration chromatography of Tannase produced from *Bacillus licheniformis* HJ2020 MT1927 using sephadex G-100 (1.5 x 48 cm), Elution with 0.05 M citrate buffer (pH 5.0), flow rate 30ml/ h. and 2ml/tube.

The fractions of main peak were collected and determined their volume, protein content and enzyme specific activity. The enzyme activity were 144.54 U/ ml , While enzyme specific activity 3613.5 U/ mg. Fold of purification was raised to about five times and yield were 29.36 % (Table 2). These results were also considered an important indicators of purity (Segal, 1976). Many studies pointed out purification of tannase by gel filtration, Sharma and John (2011) can purified tannase from *Enterobacter* sp to 162 fold with a recovery of 7.1% using DEAE-cellulose chromatography followed by SephadexG-100 gel filtration.

Also Jana *et al.* (2013) purified tannase of *Bacillus subtilis* to 24.18 fold, using  $(\text{NH}_4)_2\text{SO}_4$  precipitation (80%) followed by SephadexG-75 gel filtration chromatography with 5.04 % recovery. The differences between these studies in purity evidences may be due one or more factor such as source of enzyme, type of purification method, purification conditions especially in respect of ammonium sulphite saturation, type of column used in ion exchange and gel filtration chromatography, moreover flow rate used in washing and elution in all steps of purification.

## Conclusion

The focus of present research was include optimizing of tannase production from local isolate of *Bacillus licheniformis* HJ2020 MT14171.1 by submerged culture. The results revealed that the optimum conditions by using glucose at 3 % as carbon source, tannic acid at 1.5 % as inducer, yeast extract at 2% as nitrogen source, temperature of incubator shaker was 35 °C at 72 h, pH was 5.5, size of inoculum was  $2 \times 10^6$  cfu /ml and shaker agitation speed was 200 rpm . After optimizing the enzyme specific activity of tannase were (688.32 U/mg) which can be increased to (3613.5 U/mg) due to subjected crud enzyme to partial

purification including concentration by ammonium sulfite at 40-80 % saturation followed by dialysis, ion-exchange chromatography on DEAE- cellulose column and gel filtration using Sephadex G-150 column. The fold of purification was increased about five times with yield of 29%. The emergence of a peak of enzymatic activity in the stage of elution using Ion exchange chromatography was indicate that the enzyme possesses a negative charge, While the appearance of one single peak identical to peak of absorbance at 280nm when using gel filtration refers to obtaining the enzyme with high purity.

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