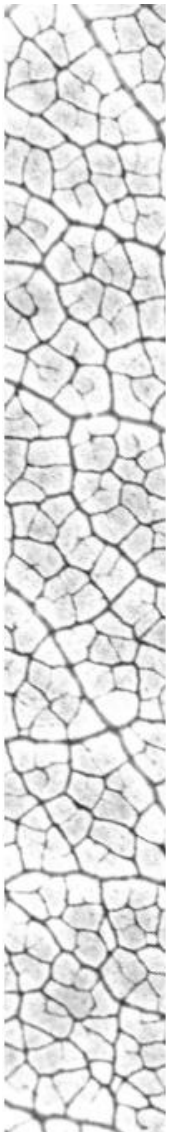

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Screening, extraction and purification for tannase produced from Iraqi *Klebsiella pneumonia* isolates and molecular detection of *tanA* gene

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Abstract
In this study, tannase enzyme was produced from an Iraq *Klebsiella pneumoniae* isolates. *K. pneumoniae* were identified by Vitek system and confirmed by housekeeping 16s rRNA gene (amplified size 155 bp). Tannase was genotpically detected by amplification *tanA* gene (amplified size 210 bp) followed by sequencing. The tannase activity reached its maximum level when this isolate was cultivated under the optimal conditions, which is consisted of using 2.8 g of nutrient agar containing 2% (w/v) tannic acid as a carbon source at pH 5.5 and temperature of 37°C for 24 h. The Tannase had been purified by using three methods: ammonium sulphate, ion exchange and gel filtration. The first method leads to gain a tannase precipitation at 70% ammonium sulphate which is considered as a partial purification where tannase activity was 80U/ml. In comparison, 300 U/mg tannase activity had been gained by using ion exchange with 4.31 fold of purification and a yield of 21.4%. Finally, a tannase activity of 500 U/mg is gained by using gel filtration with 5.75 fold of purification and a yield of 21.4%. The purified tannase is a single peptide with approximate molecular mass of 46.5 kDa as assessed by SDS-PAGE.

Keywords: *klebsiella pneumoniae*, tannase, tannase purification, 16srrna gene, tannase gene

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INTRODUCTION

K. pneumoniae is a Gram-negative rod-shaped microorganisms belongs to Enterobacteriaceae family (Ryan and Ray 2004). It is normally habitat the lumen of intestine in human and animal. For human being they are also found in mouth, nose and close to the gastrointestinal tract as normal flora. Thus, they behave as opportunistic human pathogens increasingly responsible for nosocomial infection. *K. pneumonia* has been identified as important common pathogens that can cause urinary tract infection, septicemia, wound infections hepatic infections, neonatal and bacteremia (Paterson et al. 2004). *Klebsiella* can produce many type of enzymes such as pullulanase, phytase and tannase which has several industrial applications. Tannase is adaptive, extracellular enzyme that belongs to esterase superfamily (Chen et al. 2015). Tannase has extensive applications in food, beverage, brewing, pharmaceutical and chemical industries such as production of gallic acid, instant tea, coffee flavor refreshing drinks and acorn wine (Chen et al. 2015). Tannase is an inducible enzyme that catalyzes the hydrolysis of ester bond (galloyl ester of an alcohol moiety) and the depside bond (galloyl ester of gallic acid) of hydrolysable tannins (Chen et al. 2015), releasing glucose, gallic acid and various galloyl esters of glucose. Tannase is produced by different microbial sources like bacteria, yeast and fungi. Amongst these, most of the researches have focused on fungal organisms (Rodríguez-Durán et al. 2013). Among bacteria, *Bacillus* and *Lactobacillus* genus have been widely investigated for the tannase production (Goel et al. 2011).

Microbial tannase is usually produced under submerged and solid state fermentation, each type has certain advantages and disadvantages (Ramírez-Coronel et al. 2003). In both of these fermentation techniques, high tannin containing materials are used as substrates (Belmares et al. 2004). In spite of great industrial significance, a few of these applications have been commercially exploited due to the constraints imposed by the high cost of tannic acid, which acts as substrate for tannase production. In this regard, crude tannin obtained from a number of agro-residues could

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be used as a tannin rich natural substrate for cost effective tannase production. Several strategies can be used for tannase purification after extraction from the biomass (solid state fermentation) or from the culture medium (submerged fermentation). For Tannase, most of the published purification protocols consist from multistep procedures able to obtain a highly purified enzyme but with a low-recovery yield. The common strategy used for the purification of tannase based on ammonium sulfate precipitation followed by ion exchange and/or gel filtration chromatography (Belmares et al. 2004).

MATERIAL AND METHODS

Collection of Samples

In the present study, 56 *K. Pneumoniae* isolates are collected from different source including 40 (71.5%) isolates from UTI and 16 (28.5%) isolates from wound infection, they were diagnosed using Vitek system. For molecular diagnosis depending housekeeping gene 16s rRNA was used, using a set of primers F **5'**CATCAGCGGGGTAAGGAGAT**3'** and R **5'** TTCATGCCTATCAACAGCGC**3'** with amplified size 221 bp. For the PCR procedure which is composed of the following conditions: primary denaturation step at 95°C for 5 min, 35 repeated cycles start with denaturation step at 94°C for 30 sec, annealing at 59°C and 58 °C for 30 sec to 16s rRNA and *tanA,* respectively, 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min. The products were kept in deep freeze till been subjected to electrophoresis. PCR product was sequenced and the data was analyzed using genious software as compared with NCBI.

Screening of Tannase Production Bacteria

Semi-quantitative analysis

All bacterial isolates were inoculated to nutrient agar plates supplemented with 2% (w/v) tannic acid. Plates incubated at 37°C for clinical isolates for 24 hour, an appearance of a greenish brown zone around the colonies refer to tannase production and then the diameters of clear zones around the colonies were measured (Muslim et al. 2015).

Quantitative analysis

All bacterial isolates were inoculated to nutrient broth supplemented with 2 % (w/v) tannic acid and incubated at 37°C for clinical isolates and 30°C for hospital isolates for 24 hour, after centrifugation at 8000 rpm for 20 min. The resulted supernatant was used as the crude extract to establish the tannase activity (Brahmbhatt and Modi 2015).

Media for tannase production [tannic acid agar (taa)]

Tannic acid agar prepared by dissolving 2.8 gm of nutrient agar in 95ml of distill water then sterilized by autoclaving. Tannic acid stock solution (2%) was prepared by dissolving 2 gm of tannic acid in 100 ml of distilled water then was added to the above medium after cooling it to 45°C. The media was poured in to sterile petri plates.

Tannase activity assay

Tannase activity was spectrophotometrically measured by using tannic acid as a substrate and formation of gallic acid and glucose by modification of the method that described by Miller (1959). One ml of crude extract was incubated for 30 min at 37°C with 1 ml of 0.1M acetate buffer at pH =5.5 supplemented with 0.2% tannic acid as substrate. Thereafter, the mixture was incubated in boiling water bath for 15 min seeking for stopping the enzyme reaction. A quantity of 1 ml is taken from the above mentioned mixture to be mixed with 3, 5- Dinitrosalicyclic acid reagent where the final volume was diluted by adding 10 ml of distilled water. As a result, the absorbance was measured at 540 nm. Tannase activity was calculated from the following formula:

Enzyme activity (U/ml) = µg of liberated glucose / V x T

Where: µg of liberated glucose can be taken from the standard curve; V is the volume of enzyme sample; T is the hydrolysis time.

One unit of tannase activity is defined as the amount of enzyme releasing 1µmol min^{-1} of glucose under assay conditions.

Tannase purification

The tannase purification steps were carried out according to the procedure proposed by Miller (1959). The selected isolate was grown in the nutrient broth supplemented with 2% (w/v) of tannic acid at 37°C for 24 hrs. The culture was centrifuged at 8000 rpm for 20 min (under cooling), the obtained supernatant (crude extract) was subjected to fractionate to 70% ammonium sulfate. All samples were left overnight at 4°C, and then the precipitates were collected by centrifugation at 10000 rpm for 15 min and dissolved in 0.1M acetate buffer pH 5.5 then dialyzed overnight against the same buffer. The sample loaded onto column of DEAE-Cellulose (2 x 15 cm) equilibrated with 0.1 M acetate buffer, pH 5.5 and eluted with gradient of 0.1-0.5 M NaCl prepared in the same buffer.

Fraction (5 ml) were collected and assayed tannase enzyme activity then pooled and applied to sepharose-6B column (1.5 x 85) cm. that was pre-equilibrated and washed with 0.2 phosphate buffer and the elution done by the same buffer. Protein concentration at 280 nm and tannase activity were estimated and the active fractions were pooled for further experiments.

RESULTS AND DISCUSSIONS

In this study, 56 *K. pneumoniae* isolates, collected from different source of Baghdad hospitals including 40 (71.5%) isolate from UTI and 16 (28.5%) isolate from wounds infections , and as shown in **Fig. 1**.

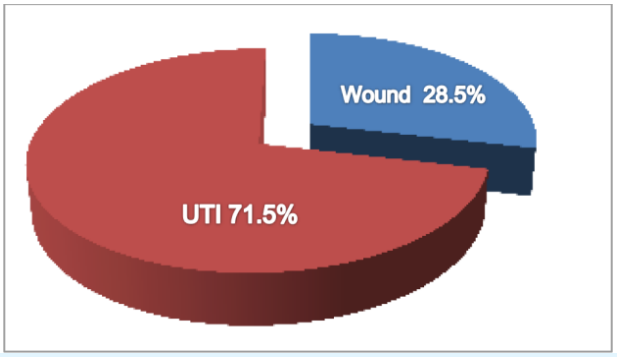

Fig. 1. Percentage of *Klebsiella pneumoniae* isolations from UTI & wounds

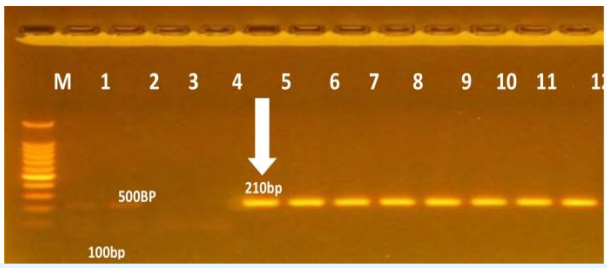

Fig. 2. Agarose gel electrophoresis (2% agarose, 7V/cm2 for 90 min) for tanA gene (size 210 bp) as compared with (100pb) DNA ladder lane (M)

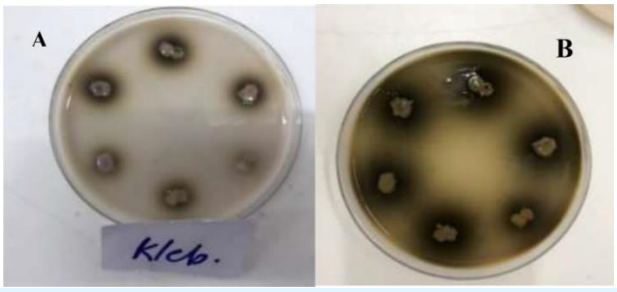

Fig. 3. Semi-quantitative assay for tannase detection (A) Heavily positive results for tannase production with dark green halos surrounding some colonies of *K. pneumoniae* isolates, (B) weakly positive result

Molecular Detection of *Tana* Gene for *K. pneumoniae*

All 40 isolates of *K. pneumoniae* that showed positive result in the morphology test on the tannic acid medium, amplificated in the PCR, and the PCR products have been confirmed by analysis of the bands on gel electrophoresis and by comparing their molecular weight with 100 bp DNA Ladder **Fig. 2**.

Screening of Tannase Production Bacteria

In the preliminary screening for isolation of tannase producing bacteria from different hospitals in Baghdad city, all isolates of *K. pneumoniae* were determined morphologically, 41 isolates of them give a positive results, this results detected by showing a visible dark green halo of gallic acid surrounding the bacterial colonies after incubation on tannase agar plate and concederd as tannase producers while 15 isolates showed a weakly positive results **Fig. 3-A** and **Fig. 3-B**. *K. pneumoniae* U40 (Klebsiella from wound source) was a better producer of gallic acid with a diameter of dark green zone equals to 23 mm and considered as a better producer of tannase in present study.

The mechanism in which the stimulation of tannase production in the microorganism still unclear but there was suggestion that intermediate compounds produced from the hydrolysis of tannic acid since tannic acid cannot penetrate the cell membrane and stimulates tannase production. The presence of glucose in the medium refers to tannase production and initial hydrolysis of tannic acid to intermediate compounds (Riul et al. 2013). Also in this paper observed that high tannic acid concentrations led to reduction in tannase production as a result to Gallic acid deposition on the cell surface. The liberated glucose and gallic acid in the culture medium refer to breakdown of tannic acid that is added as the sole carbon and energy source (Riul et al. 2013). The differences in tannase production by bacterial isolates may due to differences in the sources of these isolates or the variation in the gene expression for synthesis of tannase (Ahmed et al. 2016).

Tannase Purification

Tannase is an extracellular enzyme in the culture broth of *Klebsiella* **Table 1** summaries the three steps of tannase purification. The first step of purification was carried out by precipitation of protein from the cell-free supernatant by using ammonium sulphate at 70% saturation ratio with tannase activity 80U/ml **Fig. 4**. The supernatant obtained from ammonium sulphate precipitation was first loaded on ion exchange DEAE-cellulose and eluted by using sodium chloride gradient. In this step, tannase was purified 4.31 fold with yield about 21.4%. The tannase activity which obtained from this step was 300 U/mg **Fig. 5**. The purification procedure completed by gel filtration Sepharose 6B column. The results demonstrated that tannase was purified with 5.75 fold of purification and a yield of 21.4% obtaining a final specific activity of 500 U/mg protein (**Fig. 6**).

Table 1. Summary of the purification of *K. pneumoniae*

Purification steps	Volume (ml)	Tannase Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Enzyme Activity (U)	Purification fold	Yields (%)
Crude extract	35	40	0.46	86.9	1400	1	100
70% (NH2)SO4	10	80	0.28	285.7	800	3.28	57.2
DEAE Cellulose	5	60	0.16	375	300	4.31	21.4
Sepharose-6B	5	60	0.12	500	300	5.75	21.4

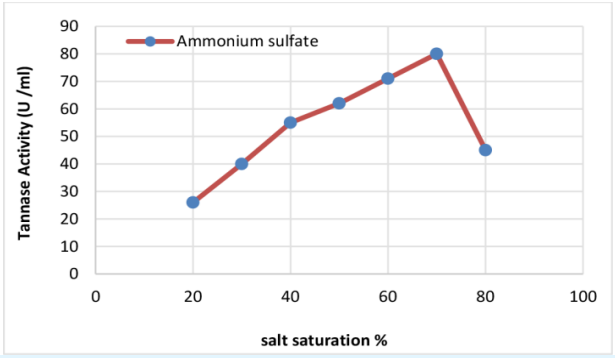

Fig. 4. Extraction of tannase by using ammonium Sulfate

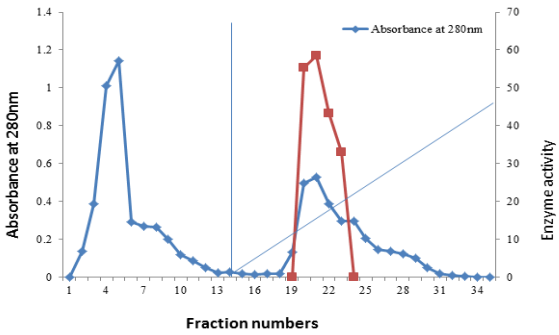

Fig. 5. Ion exchange chromatography for tannase enzyme through DEAE cellulose column (2 x 15) cm. The column was calibrated with acetate buffer pH5.5.flow rate 60 ml /hrs. And 5 ml /fraction

Earlier study on tannase demonstrated that use of ion exchange columns in the beginning and gel filtration technique in the last in purification of tannase gave highly purified phase (El-Toukhy et al. 2013). The initial fractionation with ammonium sulfate precipitation showed a very low recovery of tannase (Battestin and Macedo 2007). The precipitation with 70% ammonium sulphate saturation kept 34% of the total tannase and removed some of the non-enzymatic proteins [9]. On the other hand, the results of the current study are agreement with Muslim *et al*. (2015) who illustrated that the precipitation with Ammonium sulfate was more

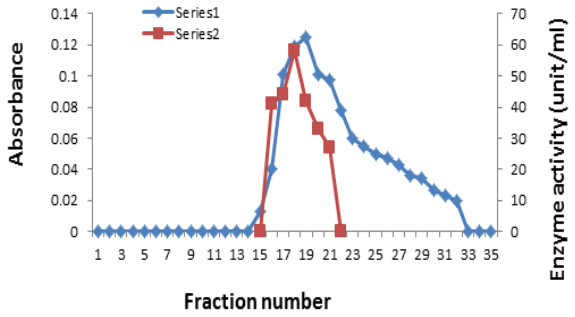

Fig. 6. Gel-filtration chromatography for purification tannase by using Sepharose 6B column (1.5 x 85) cm. The column was calibrated with acetate pH 5.5 flow rate 50 ml/hrs. And 5ml/fraction, series1 Absorbance at 280nm and series2 Enzyme activity U/ml

efficient than organic solvents, since who was found that 70% saturation of ammonium sulfate led to precipitation of tannase with tannase activity of 80U/ml and specific activity of 320U/mg (Farag et al. 2018).

CONCLUSIONS

K. pneumoniae is caused infections in higher rate. 41 *K. pneumoniae* isolates were able to produce tannase, and *K. pneumoniae* (U40) was a better producer for tannase, current study on tannase demonstrated that use of ion exchange columns in the beginning and gel filtration technique in the last in purification of tannase gave highly purified phase.

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