Detection and purification of tannase produced by clinical isolates of *Lactobacillus* spp.

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Abstract

The current study included thirty *Lactobacillus* spp. isolates obtained from breastfeeding infant stool and women vagina. They were seventeen *Lactobacillus plantarum* and thirteen *Lactobacillus acidophilus.* The screening for tannase producing isolates was done using semi-quantitative and quantitative methods, and the findings showed that 17/30 (56.66%) of the acquired isolates were tannase producers via the semi-quantitative method and all the producers were *Lactobacillus plantarum.* However, quantitative screening found that only 12 out of 30 isolates established specific activity ranging from (7.9 – 12.26 U/mg), the highest specific activity was for *Lactobacillus plantarum* S8, which was chosen as the best producing isolates. Tannase was purified using precipitation by ammonium sulfate and organic solvents followed by gel filtration chromatography. The results revealed that ammonium sulfate was superior to the organic solvents, with optimum saturation ratio for tannase precipitation was at 60% ammonium sulfate, which is considered a partial purification and yielded 122.77U/mg of tannase specific activity. Finally, the specific activity of a pure tannase reached 228.04 U/mg with 4.79 fold of purification and 49.57% yield was obtained by application to gel filteration chromatography.

Keywords: *Lactobacillus plantarum, Lactobacillus acidophilus*, purification of tannase

Introduction

The majority of *Lactobacillus* species are members of the human and animals commensal intestinal microbial flora and the Lactobacillus genus entails of a physiologically and genetically various group of rod-shaped, Gram-positive, non-pigmented, non-spore forming catalase negative, microaerophilic to anaerobic Lactic acid bacteria. *Lactobacillus* often known as friendly bacteria, are classified as generally recognized as (GRAS) or safe microorganisms (Britannica 2018).

Lactobacilli, which are naturally present in the newborn gut they are thought to be helpful to human hosts and are being researched as possible probiotics. *L. plantarum* strains given by breast-fed milk populate the gut microbiota of healthy newborns, which is a crucial source of LAB susceptible to colonization the gut mucosa and interacting with the host immune

system. The use of LAB from feces as prospective probiotic starter cultures for a variety of biomedical and technological applications is seen to be a viable strategy, because this bacterial group has a long history of human interaction and adaptability. Currently, *L. plantarum* is a fascinating biological agent with enormous biotechnological potential (Gheziel *et al* 2019 : Seddik *et al* 2017).

The tannin acyl-hydrolase (EC 3.1.1.20) belonging to the superfamily of esterase catalyzes hydrolysis of ester and depside bonds in hydrolysable tannin by releasing as gallic acid and glucose (Aharwar and Parihar, 2021). It is known as membrane bound enzyme and it also secrete extracellular and it can be obtained from various sources such as animals, plants and microorganisms but it is produced in large amount by microorganisms bacteria, fungi and yeast (Abdal *etal*, 2020a and Thiyonila *et al*., 2020). The most common strategy utilized to purify the tannase is based on ammonium sulfate deposition followed by ion exchange and/or gel filtration chromatography (Kim *et al*., 2019). The aim of this research was to detect the tanninolytic activity in *Lactobacillus* spp., besides to purification of tannase

Materials and methods

Collection of bacterial isolates

Thirty clinical isolates of *Lactobacillus plantarum* were collected from multiple gynecologist and paediatrician in Baghdad city. These bacterial isolates obtained from different clinical specimens included: premature birthed breastfeeding infant stool and women vagina. After collection, all bacterial isolates were re identified by using VITEK 2 compact system.

Detection of tannase production

All bacterial isolates were inoculated to nutrient agar supplemented with 0.2% tannic acid. Plates incubated at 37°C for 48 hrs., the formation of a greenish brown zone surrounding the colonies indicates the production of tannase, and then the diameters of clear zones around the colonies were measured (Muslim et al., 2015). Whereas in Quantitative analysis the chosen bacterial isolates were inoculated to nutrient broth medium supplemented with 0.2% tannic acid broth and incubated at 37°C for 48hrs., after centrifugation at 8000 rpm for 20 min. The resulting supernatant was used as the crude extract to establish the tannase activity(Muslim *et al*., 2015).

Assay of tannase activity

Tannase activity was detected using an ultra violet spectrophotometric technique. This strategy was based on modifying the absorption of ultra violet light. The hydrolysis of tannic acid's ester linkages was used to assess the enzyme's activity(Brahmbhatt and Modi, 2015).

Determination of protein concentration

Protein concentration was measured by the method of Bradfored, (1976) with Bovine serum albumin (BSA).

Tannase purification

Tannase purification steps were done according to the procedure projected by Miller (1959). The chosen isolate was grown in the nutrient broth supplemented with 2% tannic acid at 37°C for 48 hrs. The culture was centrifuged at 8000 rpm for 20 min (under cooling). The obtained supernatant (crude extract) was fractionated by two methods: the first involved precipitation with ammonium sulfate at saturation concentrations (20-90%), and the second involved adding tannase fractionated by organic solvents (ethanol, acetone, and isopropyl at a concentration of 95%) to the crude extract to obtain concentrations of 20, 30, 40, 50, 60, 70, and 80 percent in an ice bath. All samples were kept at 4°C overnight before the precipitates were collected by centrifugation at 10000 rpm for 15 minutes, dissolved in 0.1M acetate buffer pH 5, and dialyzed overnight against the same buffer.

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

Results and discussion

Collection of bacterial isolates

The findings revealed that the clinical isolates obtained were 17(56.66%) *Lactobacillus plantarum*, which included 9 isolates from newborn stool and 8 isolates from women vagina, and 13(43.33%). *Lactobacillus acidophilus*, which included 4 isolates from infant stool and 9 isolates from women vagina, as shown in figure(1).

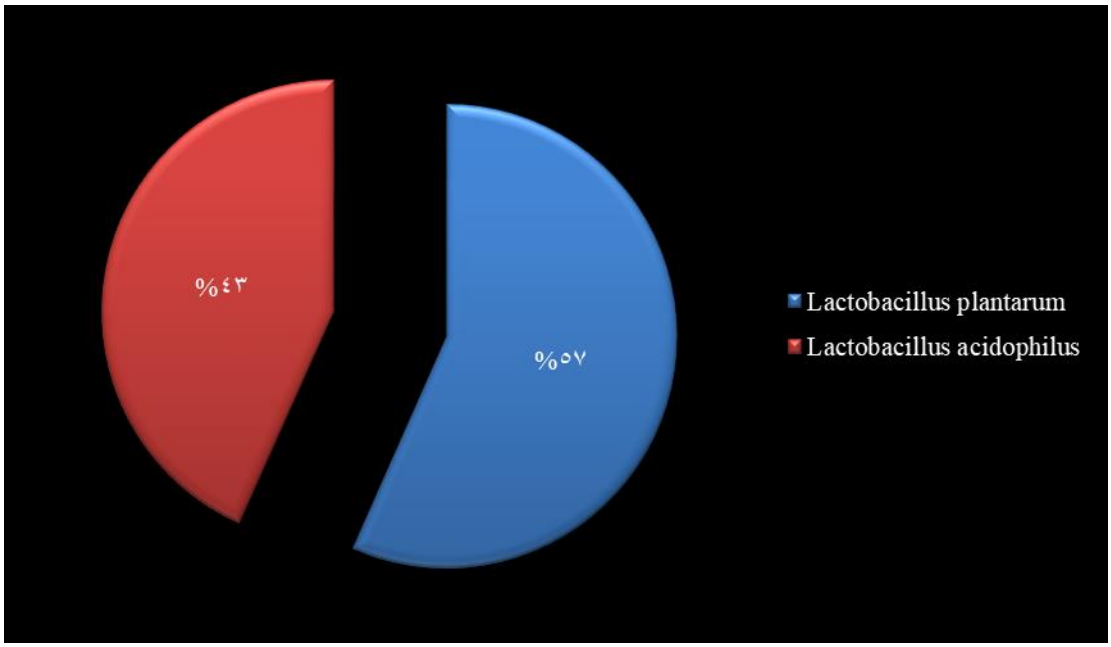

Figure (1): the percentage of *Lactobacillus* spp. From different sources.

Screening of tannase producing isolates

The capacity of bacterial isolates to produce Tannase was investigated using semi-quantitative and quantitative methods: nutrient agar with 0.2% tannic acid was utilized. There were strong, moderate, and weak producers of Tannase that were surrounded by a discernible dark green at different levels, While the negative isolates did not exhibit a hydrolysis dark zone in different levels as shown in figure (2). While the negative isolates had not shown hydrolysis dark zone.

Depending on the diameter of dark green zones, there were only 17(56.66%) isolates had the ability to produce tannase. All of tannase producers were *Lactobacillus plantarum* isolates with the diameter of dark zoon ranged between (6- 15.5mm) as shown in figure(2). In contrast, *L. acidophilus* isolates lack the ability to produce of tannase. According to the obtained results, *L. plantarum* S8 was the strongest producer for tannase with highest diameter (15.5 mm) and intensity dark green zone.

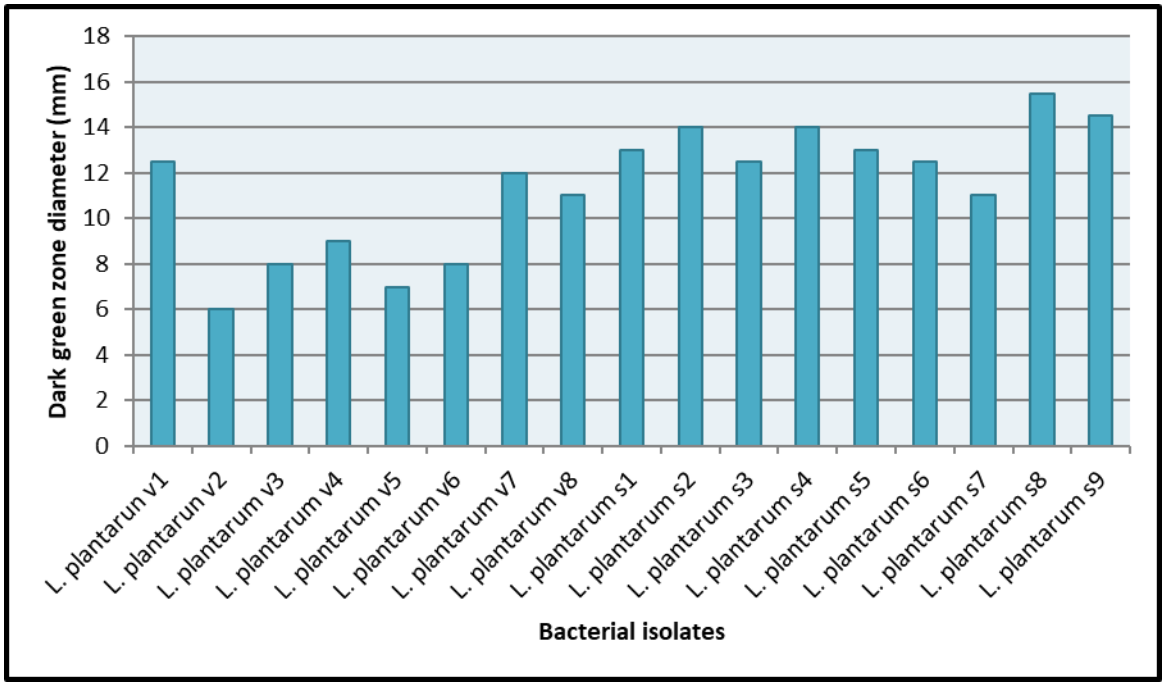

Figure (2): The diameters of dark green zone for each of tannase producing isolates

According to the semi-quantitative methodology, there were 17 bacterial isolates capable of producing tannase enzyme, which were *L. plantarum* isolates, and in the quantitative method, we chose only 12 isolates out of these 30 isolates that produced a larger hydrolysis zone of dark green.

These 12 isolates indicated varying amounts of tannase production by tannase activity ranging from (1.24– 6.37 U/ml) and specific activities ranging from (7.9– 12.26U/mg), with *L. plantarum* S8 exhibiting the highest tannase activity of 6.37 U/ml and specific activity of 12.26 U/mg as shown in figure (3).

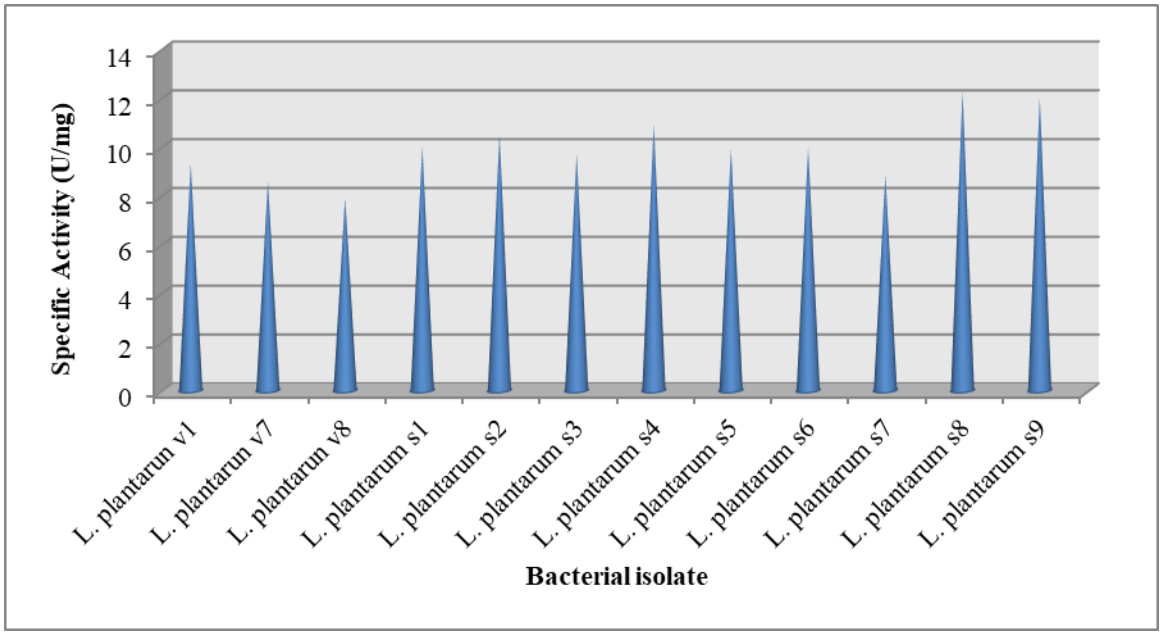

Figure 3: Specific activities for tannase production by the selected bacterial isolates in quantitative method

The results showed that *L. plantarum* isolated from the infant stool gave the heights enzyme activity and specific activity as opposed to *L. plantarum* isolated from women vagina and these variation could be attributed to the bacterial adaptation to the surrounding.

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 (Sheela *et al*., 2016). Muslim *et al*.(2017) investigated that tannase produced from *Serratia marcescens* which isolated from UTI clinical samples, that gave a higher yield of tannase and it chosen based on hydrolytic zone formation around the colonies and highest activity level of tannase.

Tannase purification

Tannase is an extracellular enzyme found in *L. plantarum* culture broth. Table (1) summarizes the three tannase purifying procedures. The tannase from *L. plantarum* S8 was precipitated using two different methods: one with ammonium sulfate and the other with organic solvents such ethanol, acetone, and isopropanol. It was discovered that 60% percent saturation of ammonium sulfate resulted in the precipitation of tannase with tannase specific activity of 122.74U/mg, making it more efficient than organic solvents. In contrast, 30% ethanol, acetone, and isopropanol precipitated tannase with specific activity ranging from (29.4 - 79.44 U/mg), with the maximum level of specific activity being 79.44 U/mg in the case of acetone, as shown in figure (4)

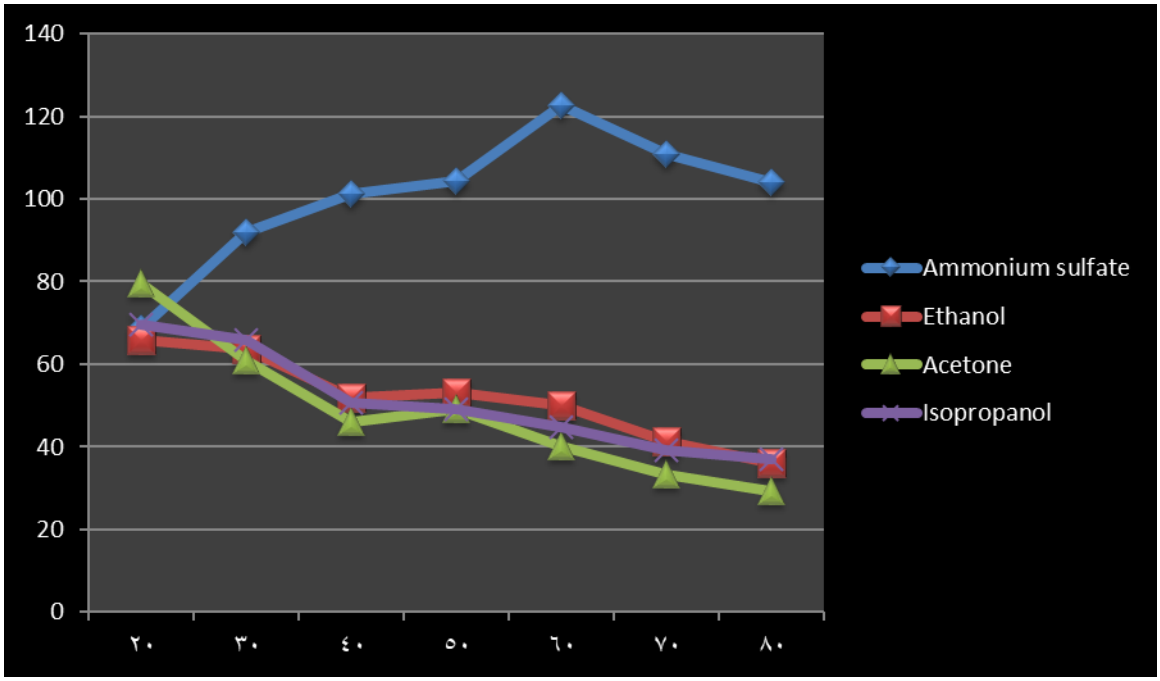

Figure 4: precipitation of tannase via organic solvents and ammonium sulphate

The protein was precipitated from the cell-free supernatant using ammonium sulphate at 60% saturation ratio with tannase specific activity of 122.74U/mg in the first phase of purification. The ammonium sulphate precipitation supernatant was applied to gel filtration Sepharose 6B column, and the findings showed that tannase was purified 4.79 folds with a yield of 49.57%, resulting in a final specific activity of 228.04U/mg, as shown in figure 5.

Table1: Purification steps of tannase from *Lactobacillus plantarum* S8

Purification step	Volume (ml)	Tanaase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude extract	90	46.1	0.97	47.52	4149	1	100
$(NH_4)_2SO_4$ percipitate	35	76.1	0.62	122.74	2663.5	2.58	64.19
Sepharose 6B	22	93.5	0.41	228.04	2057	4.79	49.57

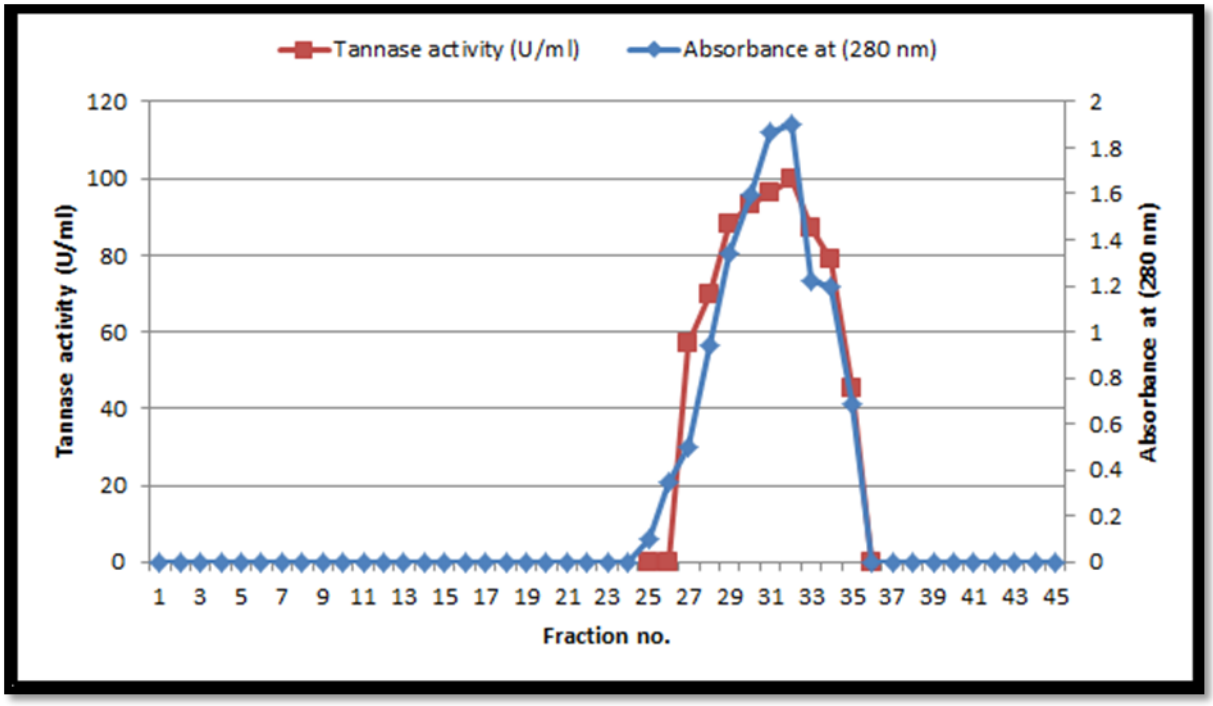

Figure5: Gel filtration chromatography for purified tannase by using Sepharose –6B column (1.5x80) cm. The column was calibrated with 0.1M acetate buffer pH 5; flow rate 30 ml/hrs and 5 ml/fraction.

Tork *et al*. (2019) purified tannase from *Lactobacillus plantarum* by the use of several column chromatography techniques. With 60% ammonium sulfate, high enzyme precipitation was achieved. Further purification ultrafiltration using Sephadex G-200 and DEAE-cellulose gel filtration chromatography resulted in a large amount of pure enzyme with tannase activity up to 0.55 U/ml and 19.89-fold with yield of 11.77%.

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