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Original Research

Optimization of tannase production by *Lactobacillus plantarum*

Authors:
Noor M and
Aswan H. Al-Bayyar

Institution:
Department of Food Science,
College of Agriculture,
Baghdad University, Iraq.

Corresponding author:
Noor M

ABSTRACT:

Three bacterial isolates were tested for tannase production; *Lactobacillus plantarum* was the best in enzyme production which was 6.842 U/mL. To stand on the best conditions for enzyme production, some conditions were studied such as inoculum size, incubation time, carbon source and its concentration, nitrogen source and its concentration beside the concentration of casamino acid, incubation temperature and pH. Results showed that the best production of tannase was by using: 1×10^7 spore/ml, 48 h, pomegranate peels 0.2%, sodium nitrate 0.3%, casamino acid 0.3% , at 35°C, pH 7 with using $FeSO_4$ 0.002% and $MgSO_4$ 0.2%.

Keywords:

Lactobacillus plantarum, Casamino acid, Tannase, Enzyme production.

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INTRODUCTION

Tannase or tannin acyl hydrolase is an extracellular or intracellular enzyme, depending on the environment of the microorganism and can be obtained from several sources such as fungi, bacteria, yeast, plants and animal stomach. Tannase (E.C.3.1.1.20) is placed within the hydrolysis family, acting specifically on the carboxyl ester bonds. The systematic name of this enzyme class is tannin acyl hydrolase. Tannase is a stimulated microbial enzyme that catalyzes the hydrolysis of ester and de-side bonds between varied substrate like gallotannin, epigallocatechin-3-gallate, gallic acid esters and hydrolysable tannins to release gallic acid and glucose (Belur and Mugeraya, 2011 and Gonzalez *et al*., 2012). Tannins are polyphenolic compounds with different molecular weights and in plants they play an important role in defense against viruses and bacteria. Tannins have the ability to precipitate protein by forming a complex compound with protein, making them undesirable in terms of nutrition. Tannins are a secondary metabolite of plants because they have no significant role in metabolism (Smith *et al.*, 2005), also Tannins cause tea cream in processed tea, turbidity in drinks and coffee, inhibiting the growth of many microorganisms, and they are complex compounds that do not dissolve easily (Rodriguez-Duran *et al*., 2011).

Lactobacillus plantarum is Gram positive and it is non-pathogenic to humans, always found naturally in human saliva and digestive system. *L. plantarum* is widely used in therapeutic food applications and plays an important role in fermenting the food. Major commercial applications, biotechnology, environmental applications, medicine and pharmacy, food, feed, beverages, medicines, production of gallic acid, instant tea through the production of many enzymes, and the most important of which is tannase (Gonzalez *et al*., 2012).

MATERIALS AND METHODS

Preparation of inoculum

MRS broth medium was prepared according to the manufacturer instructions and sterilized by autoclave at 121˚C for 20 min and poured into sterilized tubes (10 mL), after cooling to 45^{o}C. Then it was inoculated by taking a swab from the bacterial culture by the Loop incubated for 24 h at 30˚C. Calculation of the number of cells/ml by measuring the optical absorption at wavelength 600 nm was carried out using MacFarland equation, after that a series of dilutions was done to obtain the number of cells to be added to the center of production.

Estimation of protein concentration

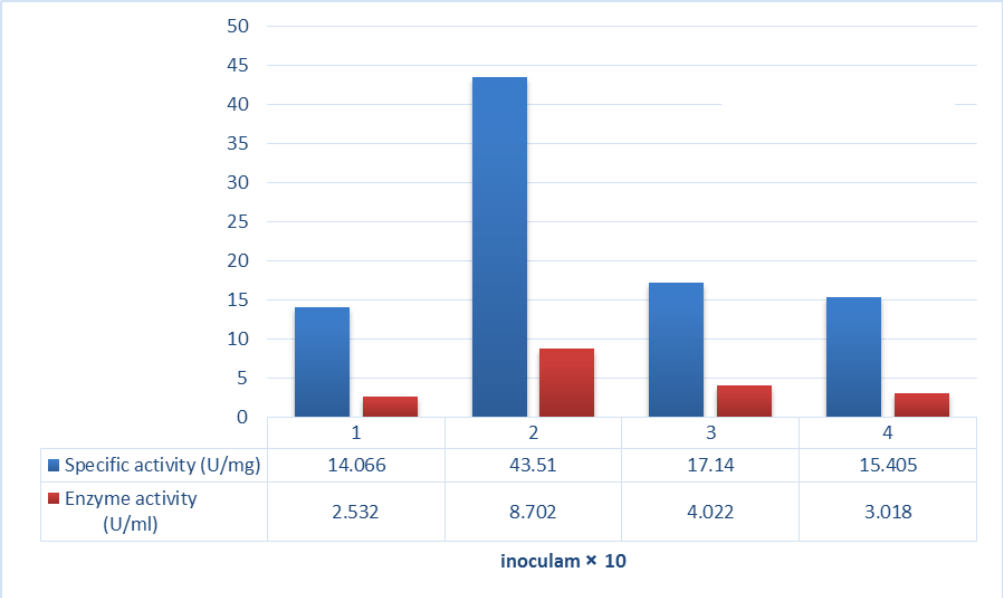
To estimate protein concentration, Bradford (1976) was followed by adding 0.1 mL of the crude enzyme extract to 1 mL of the Bradford Reagent, then mixed well and incubated for 5 min at room temperature. Absorbance at 595 nm was measured in the spectrophotometer. The protein concentration was then estimated based on the standard curve of the bovine serum albumin.

Estimation of enzyme activity

The UV spectrophotometric method was used to measure the tannase enzyme activity, which involves taking 0.5 mL of the enzyme solution and adding it to 2 mL of the substrate, after that, 20 µl of the reaction mixture were taken and placed with 2 mL of the 90% ethanol solution to stop the enzyme reaction and the absorb-

Table 1. The efficiency of local bacteria isolates in the production of tannase by measuring the specific and enzyme activity

S. No	Specific activity (U/mg)	Enzyme activity (U/mL)	Bacterial isolation
1	285.08	6.842	*L. plantarum*
2	152.17	3.50	*Weissella paramesentrodes.*
3	168.8	4.220	*Weissella* sp



1. 1x 10^5 cell / mL; 2. 1x 10^6 cell / mL; 3. 1x 10^7 cell / mL; 4. 1x 10^8 cell / mL

Figure 1. Effect of inoculum volume in the production of tannase from *Lactobacillus plantarum* isolate

ance was immediately measured at 310 nm after the addition of ethanol solution. The contents were incubated at 37°C for 10 min and the absorbance was measured at 310 nm again. The following equation was applied to calculate the activity of the enzyme:

(U / mL) = 114 x Change in absorption / Difference in time (T_2-T_1) (Dhruvil and Modi, 2015).

Production of tannase from different bacterial isolates

Three bacterial isolates, *Lactobacillus plantarum, Weissella paramesenteroides* and *Weissella* sp were compared for their enzyme production. The enzyme production media were prepared based on Ayed and Hamdi (2002), where the media was distributed in 50 mL in flasks (250 mL) after which each flask was

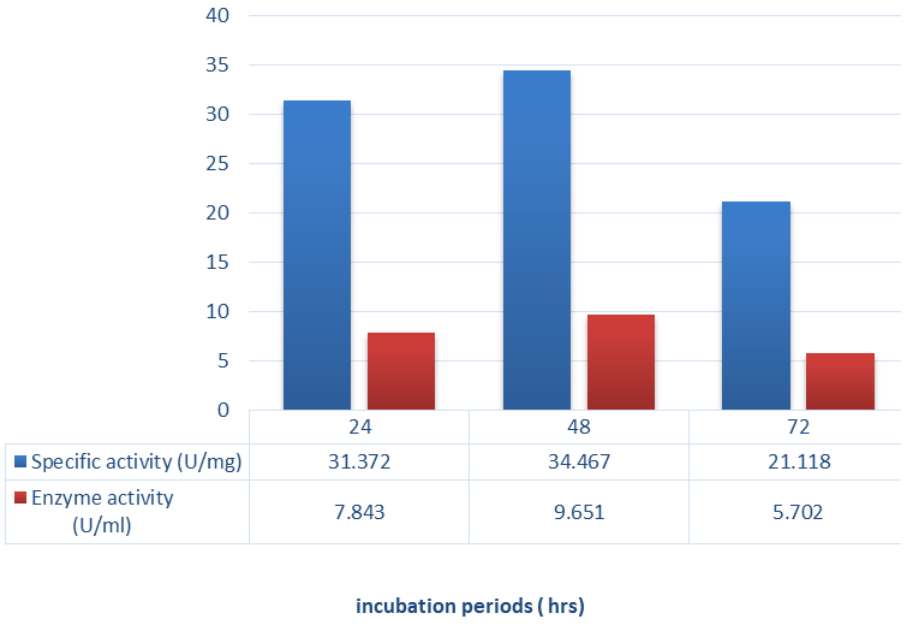


Figure 2. Effect of incubation period in the production of tannase from *Lactobacillus plantarum* isolate

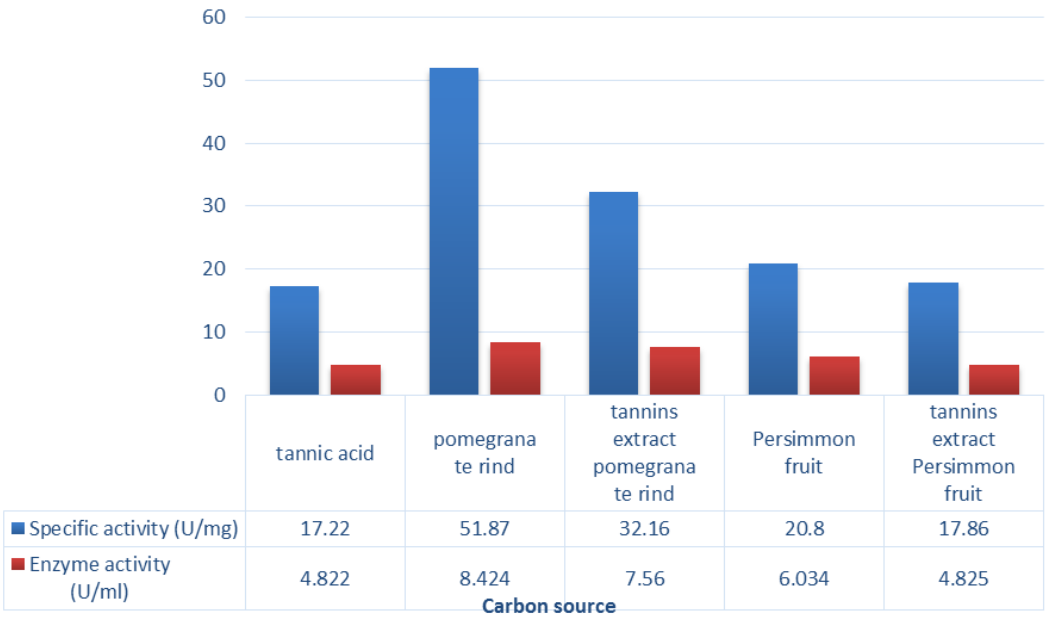


Figure 3. Effect of different carbon sources in the production of tannase by *Lactobacillus plantarum* isolate

individually inoculated with $1x10^6$ cell/mL from each bacterial isolates (bacterial colonies at age 24-48 h) and incubated for 24 h at 37°C. The enzyme was extracted from the production media by filtration through What-man filter paper (No.1) with vacuum, then the filtrate was taken and put in centrifuge at 3000xg at 4°C for 10 min, the supernatant was taken as a crude enzyme.

Tannin extraction

The tannin was extracted from the persimmon fruits and pomegranate peel. A quantity of 40 g dried material of both was homogenized in electrical shaker with 200 mL of 70% acetone, then through a filter paper in Buchner funnel with vacuum and the filtrate was washed with solvent solution twice. Aqueous layer was

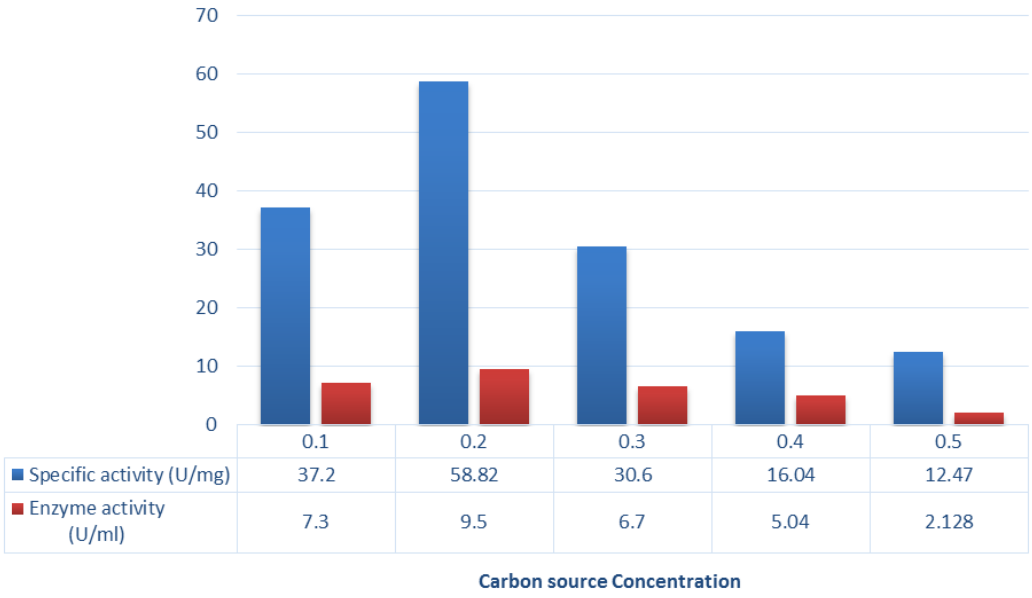


Figure 4. Effect of carbon source concentrations in the production of tannase by *Lactobacillus plantarum* isolate

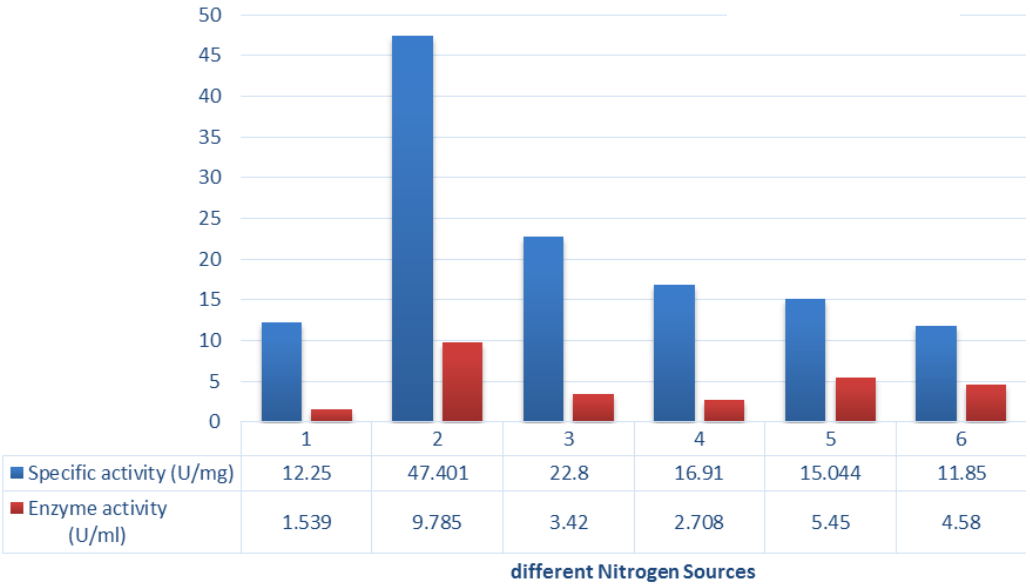


Figure 5. Effect of different nitrogen sources in the production of tannase from *Lactobacillus plantarum* isolate

taken and concentrated by Rotary evaporator, then it has been dried by using electrical oven at 60°C for 24 h and kept in the refrigerator until use (Yuliana *et al*., 2014).

Optimal conditions for enzyme production

To determine the optimal conditions for the production of the enzyme from the isolation of the most efficient bacteria (*Lactobacillus plantarum*), many factors affecting enzyme production were studied, which included: Inoculum size, incubation time, carbon source, concentration of optimum carbon source, nitrogen source, concentration of optimum nitrogen source, pH effect, temperature and the concentration of magnesium sulphate, concentration of iron sulphate in the production media (Beniwal *et al*., 2010).

Influence of inoculum size

Enzyme production media were inoculated with

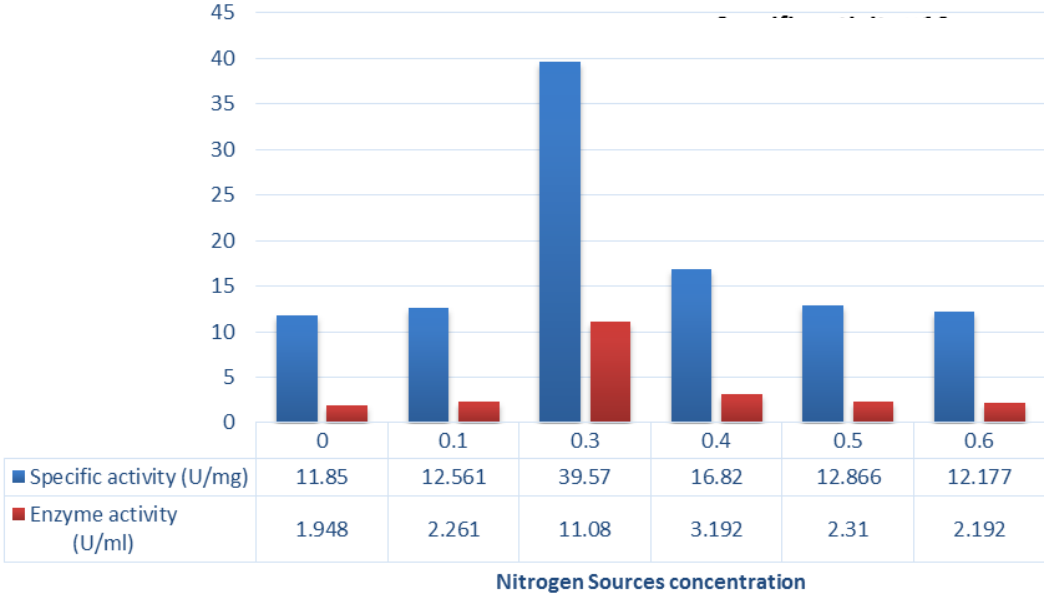


Figure 6. Effect of nitrogen source concentrations in the production of tannase by *Lactobacillus plantarum* isolate

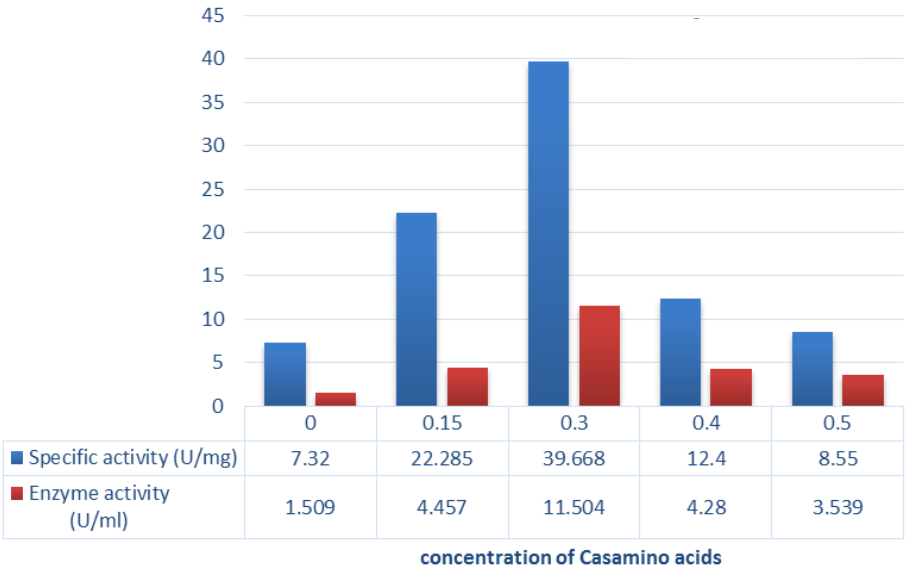


Figure 7. Effect of casamino acids concentration in the production of tannase from *Lactobacillus plantarum* isolate

different concentrations from bacteria cells as following (1x 10^5, 10^6, 10^7 and 10^8 cell / mL).

Influence of incubation period

Enzyme production media containing bacteria cells were incubated for different periods of time (24, 48 and 72 h) at 35°C, to determine the optimal periods of time for enzyme production.

Influence of carbon source and its concentration

Various carbon sources were used in this study: tannic acid, pomegranate peel, tannin extract from pomegranate husks, dried persimmons, tannin extract of dried persimmons and to determine the best carbon source for the enzyme production, 0.1% concentration of each carbon source was used. In addition, the effects of different concentrations of the optimal carbon source to the liquid production medium were: 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%.

Influence of nitrogen source and concentration

Two types of nitrogen sources (organic and inorganic) were selected and added to the enzyme produc-

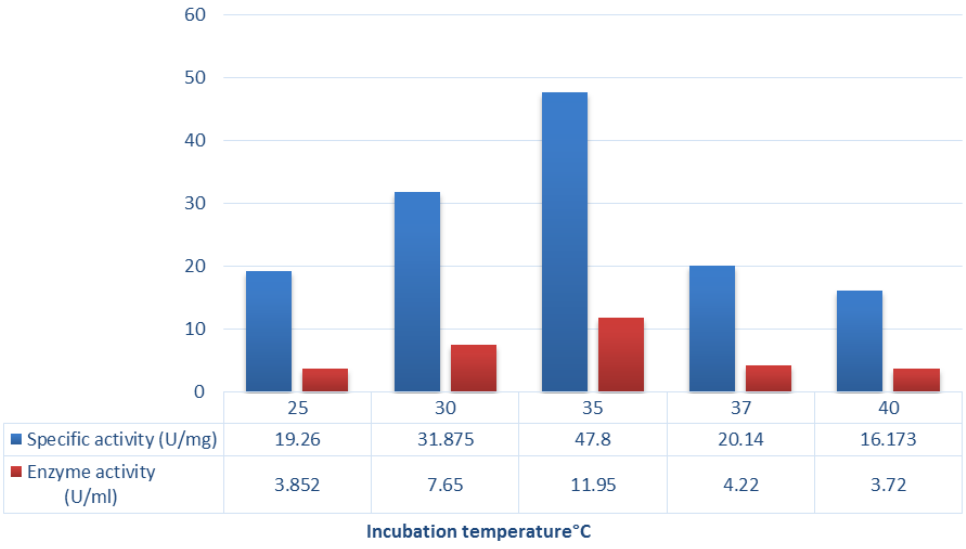


Figure 8. Effect of incubation temperatures in the production of tannase from *Lactobacillus plantarum* isolate

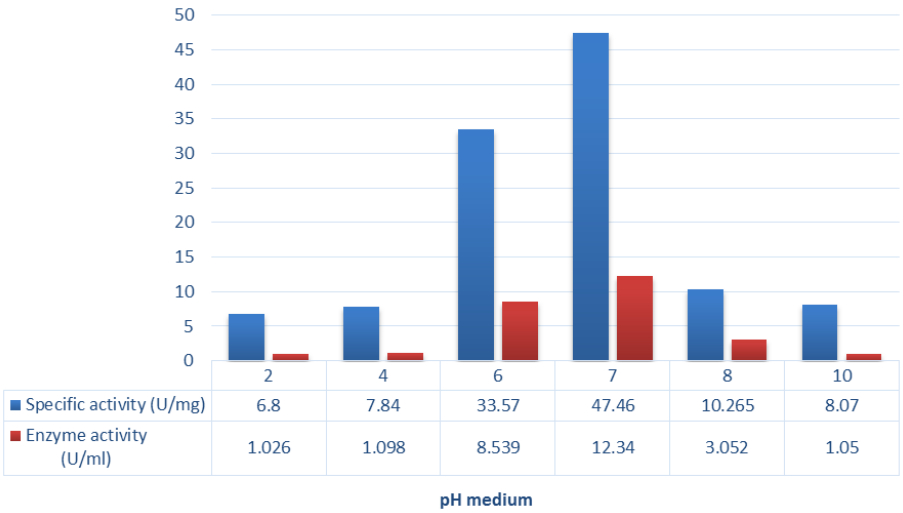


Figure 9. Effect of pH medium in the production of tannase from *Lactobacillus plantarum* isolate

tion media at a concentration of 0.3

1. Casamino acids + ammonium sulphate ($(NH_4)_2SO_4$)
2. Casamino acids + sodium nitrate ($NaNO_3$)
3. Casamino acids + peptone
4. Casamino acids + yeast extract
5. Casamino acids + potassium nitrate (KNO_3)
6. Ammonium sulphate ($(NH_4)_2SO_4$)

The effect of different concentrations of the optimal nitrogen source for production (0.1, 0.2, 0.3, 0.4 0.5, 0.6%) were also studied.

Influence of different concentrations of casamino acids

To determine the optimal concentration of nitrogen source for the production of tannase enzyme, different concentrations of casamino acids (0.15, 0.3, 0.4 and 0.5) were used as a nitrogen source in the preparation of enzyme production media.

Influence of fermentation temperature

Enzyme production media containing bacteria cells were incubated at different temperatures (25, 30, 35, 37 and 40 °C) for 24 h, to determine the optimal temperature for enzyme production.

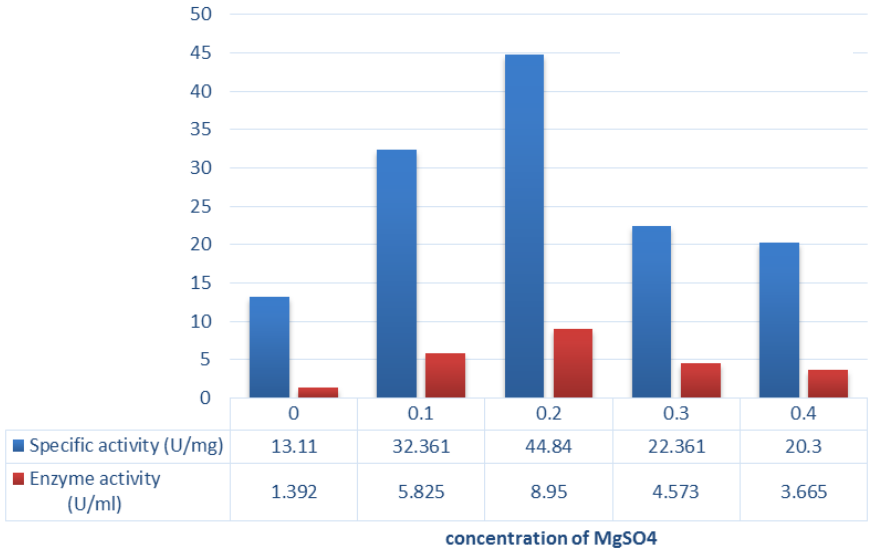


Figure 10. Effect of $MgSO_4$ concentration in the production of tannase from *Lactobacillus plantarum* isolate

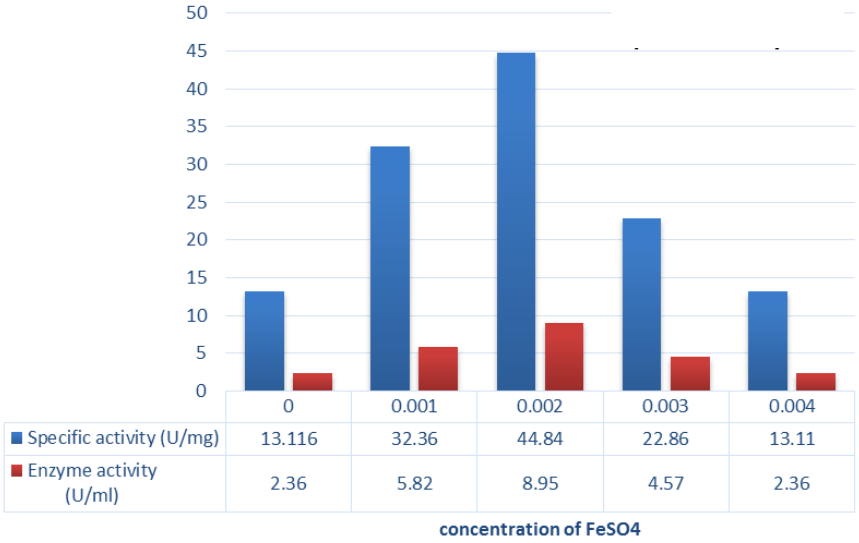


Figure 11. Effect of concentration $FeSO_4$ in the production of tannase from *Lactobacillus plantarum* isolate

Influence of pH

Enzyme production media containing bacteria cells were prepared with different pH (2, 4, 6, 6.5, 7, 8 and 10) and incubated at 35°C for 24 h to determine the optimal pH for the production of the enzyme.

Influence of different concentrations of magnesium sulphate

Different concentrations of magnesium sulphate (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%) were used in the liquid enzyme production medium to determine the optimum concentration of this salt to produce the enzyme.

Influence of different concentrations of $FeSO_4$

Different concentrations of $FeSO_4$ (0.01, 0.02, 0.03 and 0.04%) were used in the liquid enzyme production medium to determine the optimum concentration of this salt to produce the enzyme.

RESULTS AND DISCUSSION

The best isolate for the tannase enzyme production

The results showed that *L. plantarum* isolate had a higher efficacy for the tannase activity production than the other *Weissella paramesentrods* and *Weissella* sp isolates Table 1 and based on this result, *L. plantarum* isolate was selected to complete the research. Many studies have reported the efficacy of *Lactobacillus plantarum* in the production of tannase. Nishitani *et al*. (2004) noted that tannase is not only produced from *L. plantarum* but also produced from *L. paraplantarum* bacteria and *L. pentosus*. Also, Matsuda *et al*. (2016) noted that the tannase produced by *L. sakei* was less active than those produced by *L. plantarum* H78.

Effect of inoculum size

Results of the study of different levels of *L. plantarum* inoculum Figure 1 indicated that the highest yield of the tannase was at$1x10^7$ cell/ml in terms of enzyme and specific activity, recording 8.702 U/mL and 435.1 U/mg respectively. Thereafter, a significant decrease in production of tannase was observed in the enzyme and specific activity to 2.804 U/mL and 125.8 U/mg when using the size of inoculum of $1x10^8$ cell/mL, and this can be attributed to a competition between the cells on the nutrients and may lead to food consumption in the medium of production early in production. Jana *et al*. (2012a) mentioned that the maximum yield of the enzyme *Enterobacter cloacae* MTCC 9125 when using a 1% concentration of cells at the age of 24 h, while Banerjee *et al*. (2005) reported that the volume of 2% (v/v) was the best for enzyme production from *Aureobasidium pullulans* DBS66. But Sabu *et al.* (2006) found that the best inoculum was $1x10^8$ cell/mL for

tannase production from *Lactobacillus* sp other studies indicated that the inoculum size 1% (v/v) achieves the maximum yield of the enzyme (Mondal *et al*., 2000; Mondal and Pati 2000; Das *et al*. 2006).

Effect of incubation period

The results of this study showed that the highest enzyme production was 48 h after incubation in terms of enzyme and specific activity 9.651 U/mL and 344.56 U/ mg, respectively Figure 2. This indicated that the best incubation period was 48 h for the production of tannase. A reduction in production after 72 h of incubation can be attributed to nutrient depletion in the medium during the incubation period, as this negatively affects the production of the enzyme or because of changes in the medium of production by metabolic products with the continued growth of bacteria and also possibly due to the production of bacteria for enzymes such as proteases, which can analyze some of the tannase molecules that found in the media (Suseela and Nandy, 1983). Many studies have focused on determining the best duration of incubation for the production of enzyme and this depends on the type of microorganisms and components of the medium and the different production conditions. These results were agreed with Lokeshwari *et al*. (2010), who found that 48 h were optimal for the production of *Trichoderma viride*. While Manjit *et al*. (2010) indicated that the best incubation period for the production of tannase was after 24 h of production, where the enzyme activity was 13.09 U/mL. Also, Natarajan and Rajendran (2012) noted that 30 h is optimal for the production of *Lactobacillus plantarum* where the enzyme activity was 9.13 U/mL.

Influence of different carbon sources

The results in Figure 3 showed that the production medium, which contained dried pomegranate peel as a carbon source, was the best in the production of the enzyme by *Lactobacillus plantarum* during 48 h of incubation at 30°C, in terms of enzyme and specific activity of 8.424 U/mL and 51.87 U/mg respectively, followed by tannins extract from pomegranate peel with enzyme and specific activity 7.56 U/mL and 32.16 U/mg respectively. Many studies have reported that the use of plant parts rich in tannin as a single carbon source stimulates the production of tannis. In a study conducted by Iqbal and Kapoor (2012), several tannin-rich hydrocarbons were used in the production of the tannase enzyme by fungus *Trichoderma harzianum*, where the highest efficacy was found when using Amla fruit as a carbonate source for enzyme production with an enzyme activity of 31.56 U/mL followed by pomegranate peel with enzyme activity 26.63 U/mL, while the lowest enzyme and specific activity was obtained when Eucalyptus was used as a carbonate source, with 4.37 U/mL. Manjit *et al*. (2010) reported the use of dried leaves from four different trees and the highest enzyme activity was 11.06 U/mL when using leaves of a tree *Phyllanthus emblica*. While Srivastava and Kar (2009) used pomegranate peel as a single source of carbon in the enzyme production media by the fungus *Aspergillus niger* ITCC where the highest enzyme activity was 28.72 U/mL. Jana *et al*. (2012 b) studied the effect of five carbonate sources: tamarind seed, tea residue, arjun fruit, pomegranate residue and haritaki fruit. The highest enzyme activity was 1.536 U/g when tamarind seeds were used as the source for tannase production by *Penicillium purpurogenum* PAF6.

Effect of carbon sources concentration

Dried pomegranate powder was selected as a carbon source for the production of tannase from *Lactobacillus plantarum* isolate in the later experiments of this study. The results Figure 4 showed that the concentration at 0.2 was superior to the other concentrations by the production of the enzyme. Therefore, it was considered the best concentration of the source of carbon can be added to the tannase production media, in terms of enzyme and specific activity 9.5 U/mL and 588.2 U/mg respectively. In another study Manjit *et al*. (2010), they used different concentrations of carbon

sources (0.5-5.0%). they found that the best concentration of carbon sources of Amla fruit was 2%, and in terms of enzyme activity 11.06 U/mL. Monika and Turkiewicz (2007) showed that maximum production of TNS in 0.5% concentration when using crude tannin extract from *Anacardium occidentale* bark. Pallavi *et al*. (2015) reported that the best concentration of tannic acid to produce the tannase enzyme by *Aspergillus* sp was 1%, in terms of enzyme activity 20 U/mL.

Effect of different nitrogen sources

The results showed in Figure 5 that the inorganic source of sodium nitrate exceeded the production of tannase compared to other nitrogen sources, which had a significant effect on the increase in enzyme and specific activity 9.78 U/mL and 47.401 U/mg respectively.

Casamino acids + ammonium sulphate $(NH_4)\ 2SO_4$

Casamino acids + sodium nitrate NaN_3

Casamino acids + peptone

Casamino acids + yeast extract

Casamino acids + potassium nitrate KNO_3

Ammonium sulphate $(NH_4)\ 2SO_4$

This result was agreed with Srivastava and Kar (2009) and George and Ong (2013) where they were reported that the best source of nitrogen for the tannase production by *Aspergillus niger* was sodium nitrate. While Natarajan and Rajendran (2012) indicated that the maximum production of tannase was when the use of ammonium chloride as a nitrogen source in terms of enzyme activity at 9.13 U/mL. Most microorganisms are capable of growth and metabolic production in both organic and inorganic sources for the production of nucleic acids, proteins, amino acids and cell wall components (Jana *et al*., 2013). Kasieczka-Burnecka *et al*. (2007) reported that the presence of sodium nitrate was in the optimal conditions for the tannase production media from *Verticillium* sp**.**

Effect of nitrogen sources concentrations

The results showed in Figure 6 that the best concentration of the optimum nitrogen source was sodium nitrate, which was 0.3%, because it recorded an enzyme activity higher than any other concentrations in terms of enzyme and specific activity, reaching 11.08 U/mL and 39.57 U/mg respectively. This concentration of sodium nitrate was based on the tannase production media in the later experiments of this study. This result was a confirmation of Beniwal *et al*. (2010), where they indicated that the highest enzyme activity was obtained at the concentration of 2% of sodium nitrate when used as a source of nitrogen, while (Sharma *et al*., 2015) which observed that the best concentration of ammonium chloride was 0.5% for the tannase production from *Bacillus sphaericus*, which achieved enzyme activity 7.29 U/mL.

Effect of casamino acids concentration

This study used different concentrations of organic nitrogen, casamino acids, to determine the optimum concentration of tannase production. The results showed in Figure 7a decrease in enzyme and specific activity in the absence of casamino acids, which recorded 1.509 U/mL and 7.32 U/mg respectively. While the maximum production of tannase was at a concentration (0.3%) where the enzyme and specific activity was 11.504 U/mL and 39.668 U/mg respectively, and this means that it is the optimum concentration in the production of tannase from *Lactobacillus plantarum*. Ayed and Hamdi (2002) noted that the use of casamino acids in the production of tannase enzyme from *Lactobacillus plantarum* at a concentration of 0.3% was the best. Also, Aithal and Belur (2011) noted that the use of casein at the tannase production media was very important.

Effect of incubation temperature

Temperature plays an important role in the activity of microorganisms, and is the main means of controlling the biological activity of these organisms. The optimal temperature for the production of enzymes from microorganisms varied according to the type of microorganisms. In general, the optimal temperature for microbial production is 20-60°C. The tannase production

from *Lactobacillus plantarum* isolate increased with increasing incubation temperature until it reaches the highest enzyme production at 35°C where the enzyme and specific activity was 1.95 U/mL 478 U/mg respectively. A decrease in productivity was observed at a temperature higher than 35°C, indicating that the temperature of 35°C was the optimal thermal degree for the enzyme production Figure 8. This result was similar to that of Trevin˜o-Cueto *et al*. (2007), which found that 35°C was the optimal temperature for the production of the tannase enzyme from *Oryzae*. Also, Iibuchi *et al*. (1966) found that the maximum production of tannase was at 35°C from *B. licheniformis* KBR6. While the maximum yield of the tannase was obtained at 37°C when Amla and keekar leaves which used as a substrate for enzyme production, where maximum enzyme activity was 11.95 U/ml and 47.8 U/mL respectively. Other research reported that the maximum production of tannase from *Lactobacillus sp* was found at 30°C (Trevin˜o -Cueto *et al*., 2007; Sabu *et al*., 2006).

Effect of pH

Results in Figure 9 showed that the maximum production of tannase was at pH 7.0 where enzyme and specific activity recorded 12.34 U/mL and 47.46 U/mg respectively. These results are similar to Manjit *et al*. (2010), who found that the best production of tannase was obtained at pH 7.0, with the enzyme activity of 13.14 U/mL when using Amla as a carbon source. While other studies reported that pH 5.0 was the best for the maximum production of tannase (Bradford, 1976), who found that pH 5.0 was the best in the process of producing the enzyme from *A. oryzae* in terms of enzyme activity of 112.31 U/mL when using pomegranate peel as a carbonic source. Also Sharma *et al*. (2014) noted that the maximum production of tannase was at pH 5.0 with enzyme activity was 1.86 U/mL.

Effect of $MgSO_4$ concentration

Different concentrations of magnesium sulphate salts were tested in enzyme production media to determine the best concentration of tannase production. The results of Figure 10 showed that the absence of magnesium sulphate salts from the production media resulted in a decrease in the tannase production, where enzyme and specific activity recorded 1.392 U/mL and 13.11 U/mg respectively. While the maximum production of tannase was at a concentration (0.2) where the enzyme and specific activity was 8.95 U/mL and 44.84 U/mg respectively and this means that it is the optimum concentration in the production of tannase from *Lactobacillus plantarum*. Several studies have indicated the use of magnesium sulphate salts within the productive medium of tannase. Manjit *et al*. (2010) reported that salts were used at a concentration of 0.2 in the tannase production media, also both Sabu *et al*. (2006); Raghuwanshi *et al*. (2011) and Jana *et al*. (2013) showed that the $MgSO_4$ was used in the tannase production media at (0.1) concertation.

Effect of $FeSO_4$ concentration

The study used different concentrations of $FeSO_4$ salts to determine the optimal concentration for tannase production. The results of Figure 11 showed that the best tannase production at the concentration 0.02%, with the highest enzyme and specific activity, recording 8.95 U/mL and 44.84 U/mg respectively and this means that it is the optimum concentration in the production of tannase from *Lactobacillus plantarum*. Ayed and Hamdi, (2002) mentioned the use of $FeSO_4$ salts at 0.002% concentration in the tannase production media, while Aguilar and Gutierrez-Sanchez (2001) pointed to the use of $FeSO_4$ salts at a concentration 0.0001%.

CONCLUSION

The tannase enzyme production was influenced significantly by various parameters *viz:* inoculum size, incubation period, temperature, pH and different concentrations of carbon sources, nitrogen sources, casamino acids, $MgSO_4$ and $FeSO_4$.

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