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Prof. Dr. Özer Çınar

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On behalf of the organizing committee, we are pleased to announce that the 3th International Conference on Environmental Science and Technology (ICOEST-2018) is held from September 19 to 23, 2018 in Kiev. ICOEST 2018 provides an ideal academic platform for researchers to present the latest research findings and describe emerging technologies, and directions in Environmental Science and Technology. The conference seeks to contribute to presenting novel research results in all aspects of Environmental Science and Technology. The conference aims to bring together leading academic scientists, researchers and research scholars to exchange and share their experiences and research results about all aspects of Environmental Science and Technology. It also provides the premier interdisciplinary forum for scientists, engineers, and practitioners to present their latest research results, ideas, developments, and applications in al lareas of Environmental Science and Technology. The conference will bring together leading academic scientists, researchers and scholars in the domain of interest from around the world. ICOEST 2018 is the oncoming event of the successful conference series focusing on Environmental Science and Technology. The scientific program focuses on current advances in th eresearch, production and use of Environmental Engineering and Sciences with particular focus on their role in maintaining academic level in Science and Technology and elevating the science level such as: Water and waste water treatment, sludge handling and management, Solid waste and management, Surface water quality monitoring, Noise pollution and control, Air pollution and control, Ecology and ecosystem management, Environmental data analysis and modeling, Environmental education, Environmental planning, management and policies for cities and regions, Green energy and sustainability, Water resources and river basin management. The conference's goals are to provide a scientific forum for all international prestige scholars around the world and enable the interactive exchange of state-of-the-art knowledge. The conference will focus on evidence-based benefits proven in environmental science and engineering experiments.

Best regards,

Prof. Dr.Özer ÇINAR

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Tannase Production and Enzyme Characterization from *Bacillus coagulans*

Esra Sunduz Yigittekin[1], Sadik Dincer[1*]

Abstract

In this study, the tannase from Bacillus sp. strains isolated from soil samples were produced and characterized. Tannase production of these strains were determined by production of clear zones around the colonies on the tannic acid containing medium, after 96 hours incubation at 37°C. The enzymes isolated from Bacillus sp. 2.11 strain used in this study because it showed the best activity. Bacillus sp. 2.11 was determined to be Bacillus coagulans with the VITEK-2 Compact System. Three bands with molecular weights of 17 kDa, 14 kDa and 5 kDa were detected by SDS-PAGE analysis of the tannase produced from Bacillus coagulans strain. Bacillus coagulans enzyme activity was determined to be 0.313 µmol/mL, enzyme showed optimum activity at 30°C and pH 4.2. The Bacillus coagulans enzyme was able to maintain its activity for 64% at 80 °C for 15 minutes. The enzyme activity of Bacillus coagulans was able to maintain its activity with 1mM $HgCl_2$ (74.7%) and was significantly inhibited with 1mM and 5mM $MnCl_2$ and 5mM $FeCl_2$ (0%). According to these results, due to the characteristics property of tannase produced from Bacillus coagulans, it can be suggested that the enzyme is appropriate for industrial applications. This study was supported project by Cukurova University Scientific Research Project Coordinator with coded FYL-2015-3782.

Keywords: Bacillus coagulans, tannase, tannic acid, characterization

1. INTRODUCTION

Tannins are naturally occurring and water-soluble polyphenols in plants. It is usually found in the fruits, leaves, roots, trunk and seeds of plants. Tannins are gallic acid and glucose esters and have the chemical formula $C_{14}H_{10}O_9$ ([1]-[3]). They bind to proteins and precipitate. It has a great influence on the food and feed quality of many food consumed by humans and animals. Tannins inhibits many microorganisms thanks to inhibition of enzyme activity and their development resistance to microbiological attack ([4]-[6]). Tannins are classified as Condensed Tannins, Hydrolysable Tannins (Gallotannins, Ellagitannin), and Complex Tannins.

Tannin acyl hydrolase (EC 3.1.1.20), known as tannase, catalyzes the hydrolysis of debacle bonds in gallic acid esters and hydrolysable tannins such as tannic acid. As a result of hydrolysis of tannic acid with tannase occurs gallic acid and glucose ([7]- [9]). Resources of tannase are plants, animals and microorganisms (bacteria, fungi, yeast). Tannase is used for various industrial application such as, ready tea and cold tea production, wine and beer production, gallic acid production, animal feed additive and industrial wastewater treatment. In this study, it was aimed that production and characterization of tannase from *Bacillus* strain showing the best tannase activity. In this study, it was aimed that production of tannase which appropriate for using removing and clarifying of foam occurring during the production of cold tea, beer and wine, increasing the digestibility of tannins in animal feed industry, with gain of Gallic acid ensure that its using at pharmacology, remove organic pollutant tannins from industrial wastewater.

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2. MATERIALS AND METHODS

2.1. Isolation of Bacillus sp.

Soil samples were taken from *Platanus orientalis*, *Thuja orientalis* and *Cupressus arizonica* trees and 2 gr from each samples were weighed and homogenized in 10 ml sterilized saline with vortex. For the isolation of bacteria two different Isolation process is carried out .in the one of them before the inoculation soil samples incubated for 15 min at 85°C to eliminate non spore forming bacteria. 1ml of each soil suspension samples were inoculated to 10 mL bullion containing tannic acid (Tannic acid, 10 g/L; K2HPO4, 0,5 g/L; KH2PO4, 0,5 g/L; MgSO4, 0,5; NH4Cl, 1 g/L; CaCl2, 0.01 g/L; D-Glucose, 0,5 g/L) ([10]) and allowed to grow at 37°C in orbital shaker at 150 rpm for 72 hours. At the end of the incubation, these mediums were seeded onto PCA plate by reduction method and incubated for 24 hours at 37 °C. The single colony was seeded onto PCA again and incubated at 37°C for 24 hours. After incubation, plates were stored in t refrigerator at 4°C.

2.2. Determination of Tannase Producer Microorganism

The isolated bacteria were spotted on tannin agar plate and incubated for at 37 °C. for 72 hours. After incubation, to determine the biodegradation zone, plates were respectively staining with 0.01 M $FeCl_3$ and washing with 1M NaCl ([11]).

2.3. Identification of Bacteria

Gram-stained bacteria were identified by VITEK-II Compact System.

2.4. Production of Tannase Enzyme in Liquid Media

Tannase positive bacteria strains on solid medium were transferred liquid enzyme production medium and incubated at 37°C, 150 rmp for 48 hours. After incubation, the bacterial culture was centrifuged at 10,000 rpm for 15 minutes at + 4 °C (Sigma 2-16 K) the supernatant which containing tannase enzyme was used for enzyme activity assays.

2.5. Production of Tannase in Liquid Medium and Determination of Tannase Activity

To determine tannase activity from the isolated enzyme, first of all the enzyme, substrate and 0.05 M citrate buffer were incubated separately at 30°C for 5 minutes. Then Enzyme samples (250 µl enzyme + 250 µl substrate), blank (250 µl 0.05 M citrate buffer + 250 µl substrate) and control group solutions (250 µl substrate) were prepared and incubated at 30°C for 5 minutes. End of the incubation 300 µl of Rhodanin was added to each solution and incubated at 30°C for 5 minutes. After that ,200 µl 0.5 N KOH was added to each solution and incubated for 5 minutes at 30°C. in the next step 250 µl of enzyme (previously incubated at 30 °C for 5 minutes.) was added to only the control group. Finally, 4 mL of pure water was added to each tube and incubated at 30 °C for 5 minutes. End of the reactions. Tannase activity was measured at a wavelength of 520 nm with 96-well 'Multiskan ™ FC Microplate Photometer' (Thermo Scientific ™). Incubation experiment were performed in a Bioer ThermoCell cooling & heating block and water bath. At the end of the measurement, tannase activity was calculated according to the following equation ([8]).

Activity= (Enzyme Sample-Blank)-(Control-Blank) µmol/mL

2.6. Characterization of Enzyme

2.6.1. Determination of the optimum pH Value for the Enzyme Activity

To determine the optimum pH of the enzyme activity, experiments were carried out at various pH values using citrate (pH 3.0-5.8), Tris-Maleat (pH 6.2-7.4), Tris (pH 7.6-9.0) and Carbonate-Bicarbonate (pH 9.2-10.7) buffers containing methyl gallate substrate (0.01 M concentration) was prepared and standard enzyme activity was determined.

2.6.2. Determination of Optimum Temperature Value of Enzyme

To determine the optimum temperature value of the enzyme activity, experiments were carried out at various temperatures (20, 30, 40, 50, 60, 70, 80, 90 and 100 ºC). the experiments that temperature range 20ºC from to 80ºC were conducted in the Bioer ThermoCell Cooling&Heating Block and temperature range from 90-100ºC

experiments were conducted in oil bath. Standard activity assay was carried out using substrate prepared at optimum pH.

2.6.3. Determination of Thermal (Temperature) Stability of Enzyme

For the detection of thermal (temperature) stability, the enzyme was pre-incubated at 80,90 and 100°C for various times (5,10,15,20 and 30 minute) After preincubation, activity experiment was carried out at optimum temperature and pH values.

2.6.4. The Effect of Inhibitor and Divalent Cations on Enzyme Activity

For the determination of the divalent cations effect on the tannase activity, stock metal solutions were prepared such as $MgCl_2$, $CuCl_2$, $CoCl_2$, $HgCl_2$, $NiCl_2$, $MnCl_2$, $CaCl_2$, $FeCl_2$ and $ZnCl_2$ also metal and enzyme solution mixtures were prepared for pre-incubation of enzyme. Final volume of these mixtures were 100µl and final cation concentrations were prepared as 1mM and 5 mM. Pre-incubation of enzyme was carried out at 37°C. As an enzyme inhibitor, EDTA's effect on enzyme activity was investigated with same method. The activity experiments were carried out at the temperature which the optimum activity was observed and the substrate prepared at the pH value which the optimum activity was achieved.

2.7. Determination of Enzyme Molecular Weight by SDS-PAGE Method

The molecular weight of the tannase was determined with SDS-PAGE and Zymogram analyzes ([12],[13]).

3. RESULTS AND DISCUSSION

3.1. Identification of Bacteria

As a result of gram staining, *Bacillus* sp. 2.11 strains were defined as gram-positive and rod-shaped bacteria with microscopic examination. According to VITEK-2 Compact System results, isolated microorganism was identified as *Bacillus coagulans* strain.

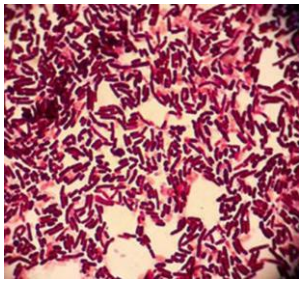


Figure 1. Microscopic appearance of gram stained Bacillus sp. 2.11



Figure 2. Result of VITEK-II Compact System type identification test

3.2. Determination of Tannase Producing Microorganism in Solid Media

Bacteria was spot-seeded onto Tannic Acid-Agar plates and incubated for 72 hours at 37°C. At the end of the incubation, the medium was stained with 0.01 M FeCl3 and photographed before and after staining. It was observed that there is positive tannase activity considering, occurring transparency zone around the bacteria.

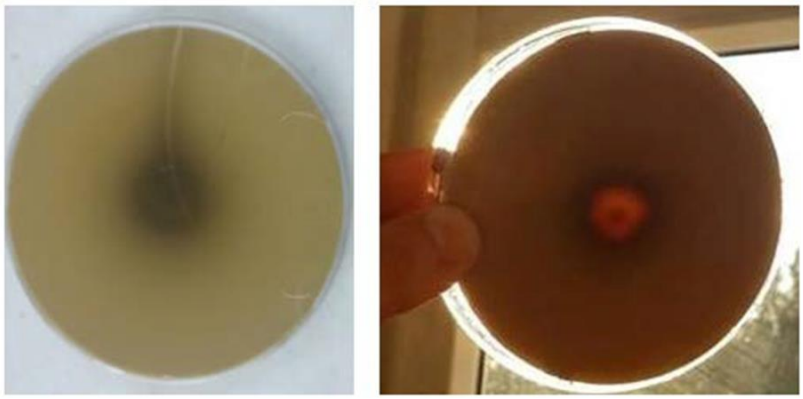


Figure 3. The appearance of the tannase positive microorganism before and after staining

3.3. Determination of The Strain Showing Tannase Activity

Bacillus coagulans enzyme activity was determined as spectrophotometric measurements at 0.313 µmol / mL.

3.4. Effect of pH on Tannase Activity

In this study the highest enzyme activity was found at pH 4.2 (100%) in citrate buffer (Figure 4). While the enzyme showed an average of 92.5% activity in pH 4.2-4.6, activity average at pH 5.0-9.0 decreased to 49.42% and at pH 9.2-10.4 to 27.5%.

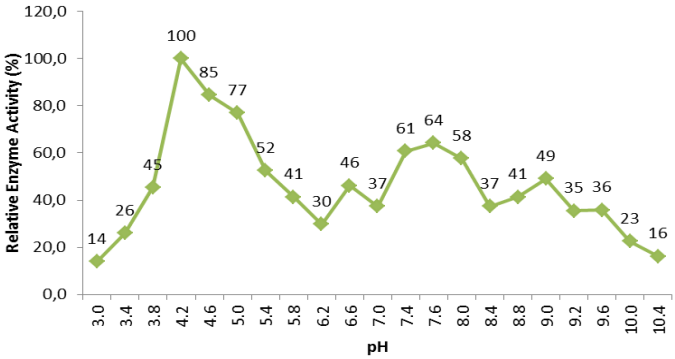


Figure 4. Optimum pH value of tannase enzyme activity produced from Bacillus coagulans

In other studies, optimum activity pH value of tannase produced from *Aspergillus niger* LCF 8 was determined to be 5 ([14]), optimum tannase activity of *Bacillus* sp. was obtained at pH 4.5 ([15]), the optimum tannase activity of *Bacillus subtilis* at pH 6 and the tannase which was cloned to *Lactobacillus plantarum* was observed at pH 5 ([16]).

Considering these results, it can be say that the enzyme is appropriate for using in acidic conditions.

3.5. Effect of Temperature on Tannase Activity

The optimum enzyme activity of *Bacillus coagulans* was showed at 30 °C (Figure 5). At the other temperatures it is observed that enzyme activity maintained 22% at 20 °C, 55% at 40 °C, 59% at 50 °C, %54 at 60 °C, 47% at 70 °C, 20% at 80 °C, 10% at 90 °C and 0% at 100 °C. While the average activity of the %range from 20 to 70 °C was % 56.28 decreased to 8.9% at range from 80 to 100°C. Considering these results, the *Bacillus coagulans* enzyme has a high activity in the range of 20-70°C and has a mesophilic property.

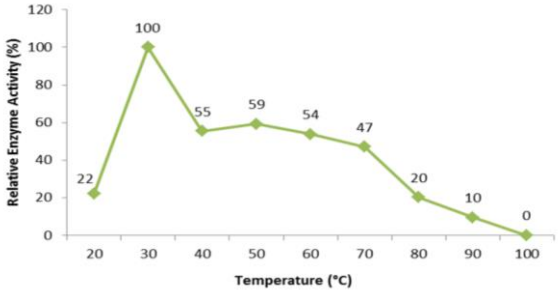


Figure 5. Optimum temperature value of tannase enzyme activity produced from Bacillus coagulans

In study of Yao et al. (2011), they obtained tannase from tannase-coded tan410 gene and purify, then optimum tannase activity temperature was determined to be 30°C ([17]). In another study tannase gene was cloned to *Lactobacillus plantarum* then the tannase produced from that microorganism showed optimum activity at 45°C ([16]) and in the study conducted with tannase produced from *Bacillus cereus* KBR9 strain, optimum activity temperature of enzyme was determined 40°C ([18]).

3.6. Findings on the Thermal Stability of Enzyme Bacillus coagulans

The *Bacillus coagulans* enzyme was able to maintain its activity 64.80% at 80 °C for 15 minutes (Figure 6). Considering the obtained data, it can be say that *Bacillus coagulans* enzyme maintained its activity an average of 39.75% after preincubation period of 0-30 minutes at all temperature values. This result revealed that the enzyme is mesophyll.

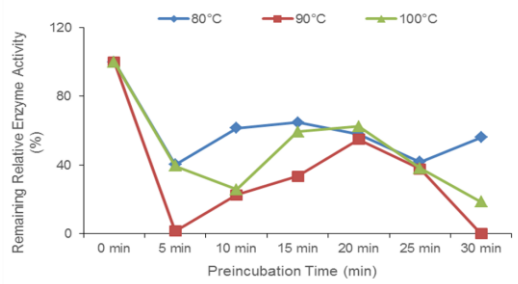


Figure 6. Thermal stability of enzyme Bacillus coagulans

Zakipour-Molkabadi et al. (2013) in their study partially purified and characterized the tannase which produced from *Penicillium* sp. EZ-ZH190 and they observed that the enzyme activity maintained 50% of the maximum activity at 50°C ([19]). The GALLO_1609 gene from *Streptococcus gallolyticus* UCN34 cloned to *Escherichia coli* BL21 (DE3) and expressed as an active protein then its determined that the enzyme maintained its activity of 25% at 45°C ([20]).

3.7. Effect of Inhibitor and Divalent Cations on Enzyme of Bacillus coagulans

The tannase enzyme maintained its activity 74.7% after preincubated in 1mM concentration of $CuCl_2$. Enzyme activity was totally inhibited with both 1mM and 5mM concentration of $MnCl_2$ and 5mM concentration of $FeCl_2$. Experiments results related with effects of inhibitors and divalent cations on enzyme activity were given Table 1.

Table 1. Effect of inhibitor and divalent cations on enzyme of Bacillus coagulans activity

Chemical	Relative Remaining Activity (%) (%) 1mM Concentration	Relative Remaining Activity (%) 5 mM Concentration
Control	100	100
EDTA	26,1	45,4
$CaCl_2$	18,3	13,3
$HgCl_2$	74,7	35,6
$MnCl_2$	0	0
$ZnCl_2$	37,6	30,4
$MgCl_2$	48,4	46,2
$NiCl_2$	10,3	52,2
$CuCl_2$	43	22,1
$CoCl_2$	41,4	28
$FeCl_2$	20,5	0

The study of Dincer et al. (2015), tannase cloned to *Lactobacillus plantarum* and tannase produced that microorganism pre incubated with $ZnCl_2$ then it is revealed that the enzyme was significantly stimulated with $ZnCl_2$ (105%) ([16]). In the study conducted with Klebsiella, tannase production, purification and characterization from Klebsiella pneumoniae KP715242 strain was conducted. in this study it is determined that He had the most inhibitory effect on tannase activity and Zn+2, Mg+2, Mn+2 had the enhancing effect on enzyme activity and any significant effect of EDTA no found ([21]).

3.8. Findings Related to SDS-PAGE Analysis

The protein bands obtained from the SDS-PAGE (10%) analysis of the *Bacillus coagulans* enzyme are shown in Figure 7. SDS-PAGE analysis revealed a three bands which molecular weights are 17 kDa, 14 kDa and 5 kDa. To determine molecular weights of protein bands, standard protein markers (Broad Range Markers: sc-2361- 500 UL 200 kDa 97 kDa 66 kDa 44 kDa 29 kDa 17 kDa 14 kDa 6 kDa) were used.

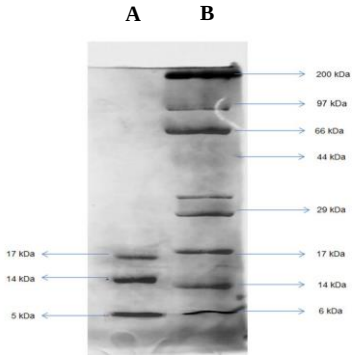


Figure 7. SDS-PAGE and zymogram result of Bacillus coagulans enzyme (A: Protein bands of Bacillus coagulans B: marker proteins)

In the other studies tannase produced from *Bacillus subtilis* PAB2 had the 52 kDa molecular weight ([22]), and tannase produced from *Enterococcus fecalis* had 45 kDa molecular weight ([23]).

4. CONCLUSIONS

According to the results of this study, it was revealed that *Bacillus coagulans* is a tannase producer microorganism. Thanks to the characteristics of this enzyme, it can be recommended that use it in many industrial fields. also its use supply economically advantage. As a result, microbial tannase of produced can be used that cold tea, beer and wine production of the occurring in the removal of foam and clarify, hydrolyzed of excess tannins present in plant leaves and feed that consumed by ruminant animals, gain of gallic acid which used in the pharmacology area, also in the elimination of tannins that are organic pollutants in industrial wastewater.

ACKNOWLEDGMENT

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