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Effect of Caffeine and Chlorogenic acid on α–galactosidase Enzyme

Al-zamely Oda Mizil Yasser[a], Qasim N. Thewaini[b] , Ameera M.Z. Fadli[c]

a: Department of Chemistry, College of Science, University of Babylon, Iraq.

b: Department of Biotechnology, College of Biotechnology, Al-Qasim Green University, Iraq.

c: Department of Community Health, Kut Technology Institute, Medill Technical University, Iraq.,

Corresponding Author, E.mail: **oda.alzamely@gmail.com,**

Abstract

A glycoside hydrolase enzyme known as α-galactosidase (EC 3.2.1.22) is responsible for hydrolyzing the terminal alpha-galactosyl moieties found in glycolipids and glycoproteins. Bacteria that produce this enzyme are isolated from *Erysipelothrix rhusiopathia.* Following the release of α-galactosidase from the cells, the purification process involves precipitating the protein with 40% ammonium-sulfate, resuspending it, and dialyzing it using a dialyze tube cut-off number (10–14 KD) overnight against phosphate buffer (pH=7). Finally, the protein is further purified using DEAE-cellulose column chromatography with (33.4) folds. At 45 °C, the enzyme had maximal activity, while 6.5 °C was its optimum pH. Through the use of SDS-PAGE technique, the molecular weight of the enzyme was estimated to be (55 KD). The enzyme's Km and Vmax were found to be (6.6 mM) and (833.3µmol/min) of protein, respectively, using p-nitrophenyl-α-D-galactopyranoside as the substrate. Following treatments of the purified enzyme with chlorogenic acid and caffeine, studies involving caffeine indicate that the inhibitor has the same affinity for the enzyme as the substrate pNPG. Measurements of the reaction rates at different concentrations of substrate and inhibitor observed a non-competitive inhibition and showed that the Km value was ineffective (6.66) mM and a decrease in Vmax values (555.5) µmol/min. While when we treated chlorogenic acid with α -galactosidase that is purified from *Erysipelothrix rhusiopathiae* this substrate acted as uncompetitive inhibition with substrate pNPG as the catalytic site that reflected the enzyme has single active that results from the change of Km and Vmax (3.22 mM) and (400 µmol/min) respectively.

Keywords*:* coffee, caffeine, chlorogenic acid, α–galactosidase, *Erysipelothrix rhusiopathia*e

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Introduction

The enzyme known as α-Galactosidases (EC 3.2.1.22) is an exo-type glycoside hydrolase that is responsible for catalyzing the breakdown of α-1,6-galactosidic linkages in galactose-containing oligosaccharides, including melibiose (galactose α-1,6-glucose), stachyose (galactose α-1,6-raffinose), and raffinose (galactose α-1,6-sucrose), which are frequently found in legumes and seeds [1; 2; 3]. Humans, animals, plants, and microbes all have α-galactosidases [4]. Different degrees of enzyme activity have been observed in the α-galactosidases isolated from different bacterial species, such as *Bacillus Stearothermophilus* [6], *Streptomyces griseoloalbus* [7], *Bifidobacterium bifidum* [8], and *Rmusbrockianus* [5]. This enzyme can be utilized to break down α-galactooligosaccharides, improving the nutritional value of foods made with legumes [9]. The cost of manufacturing determines whether industrial enzymes are commercially viable. The cost of the enzyme is mostly determined by the medium and fermentation techniques that are used to isolate stable forms with high yields. As a result, it is best to separate high-yielding and stable forms utilizing a medium devoid of repressors and containing product inducers [10].

Coffee beans, tea leaves, cocoa beans, cola nuts, and other plants are natural sources of caffeine (1,3,7-trimethyl xanthine), a natural alkaloid that is commonly found in coffee, tea, and soft drinks [11, 12, 13, 14]. In the adult diet, the main sources of caffeine are tea and coffee, whereas in the diet of children, the main sources are chocolate and caffeinated soft drinks [15; 16]. Arabica coffee beans [17] have a caffeine value of 10–12 mg/g, whereas Robusta coffee beans [18] have a caffeine content of 19–21 mg/g. Caffeine's ability to scavenge hydroxyl radicals [19] indicates that it has an antioxidant activity in addition to its well-known bioactive effects, which include improving alertness and reducing tiredness. A number of epidemiologic and clinical investigations conducted in the past several years have revealed that coffee drinking is linked to health advantages, including a decreased risk of type 2 diabetes [20; 21], Parkinson's disease, and Alzheimer's disease [22]; these findings are independent of caffeine intake.

The primary phenolic components in coffee are chlorogenic acids (CGA) (C16H18O9; mol. wt. = 354.31), an ester of caffeic acid and quinic acid. CGA is an ester of trans-cinnamic acids, including caffeic acid, ferulic and p-coumaric acids with (-) quinic acid [23; 24]. They are thought to possess antioxidant qualities, which may be crucial in preventing oxidative damage to food, cells, and any organ [25; 26].

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Materials and Methods

Isolation of α-Galactosidase Producing Bacteria from *Erysipelothrix rhusiopathia*

The strain was employed to produce α-galactosidase. It was cultured to prepare the inoculum in tryptic soy agar for 24 hours at 37℃. A 50 mL conical flask containing 5 mL of thioglaculate broth was filled with a single new colony and shaken at 220 rpm for 24 hours at 37℃. A 250 mL Erlenmeyer flask with 50 mL of tryptic soy broth was filled with 1% of the resultant 24-hour culture, which was then kept at 37℃ for 24 hours. The inoculum for solid-state fermentation (SSF) was this pre-culture.

Purification of α-galactosidase

Isolating the enzyme from cultivated cells is the initial stage in the purification process. Solid ammonium sulfate was added to the extract crude until it reached 40% saturation [27]. Proteins that had precipitated were recovered using chilled centrifugation (14000g for 20 min), *resuspended*, and dialyzed against phosphate buffer (pH=7.0) for a whole night. After the dialyzed extract (3mL) was equilibrated with 10 mM phosphate buffer (pH=7.0), it was put onto a DEAE-cellulose column (30 × 15mm). The column was cleaned using 2V phosphate buffer that was equilibrated with 10 mM (pH=7.0). Elution was performed at a flow rate of 0.5 ml/min using a gradient sodium chloride solution (0.1 M, 0.5 M, and 1.0 M), and fractions of 3 ml were collected [28]. Using a spectrophotometer UV (Nano-drop), the absorbance at 280 nm was measured after the proteins were eluted from the column. Each fraction's α-galactosidase activity was measured, and the fractions with the greatest enzyme activity were combined.

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Electrophoresis

Sodium dodecyl sulfate (SDS) was added to the separating gel at a concentration of 10% throughout the electrophoresis process. The sample was handled in accordance with Laemmli's procedure [29]. Bromophenol blue acts as a front run marker after being heated to 100°C for five minutes (Page Run–R WSE– 1100). In an electrophoresis apparatus, the gels were run for 80 minutes at 21mA and 150V. Following the run, Coomassie brilliant blue R-250 [30] was used to label them for protein.

Enzyme assay

The test for α-Galactosidase activity using the De et al. [31] technique with p-nitrophenyl-α-galactopyranoside (pNPG) at a concentration of 0.003M as the substrate. The addition of sodium carbonate (0.1M Na_2CO_3) terminated the reaction, which was conducted at 45°C. At 410 nm, the quantity of released p-nitrophenol (pNP) was measured. The quantity of α-galactosidase that releases 1 µmol of pNP (p-nitro phenyl) per minute under the specified test conditions [32] is known as an enzyme activity unit (U).

Enzyme characterization

Effect of temperature

By employing the enzyme assay at various incubation temperatures (30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, and 65°C), the optimal temperature of α-galactosidase was determined.

Effect of pH

An enzyme assay method was used to investigate the effects of pH on α-galactosidase activity at various pH levels of potassium phosphate buffer solutions (4.0 - 8.0).

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Kinetic parameter

Kinetics experiments were conducted at pH 6.5 and 45 °C. Using Lineweaver-Burk and Michaelis-Menten plots, the maximal response rates (Vmax) and Michaelis-Menten constant (Km) were determined [33]. By applying Michaelis-Menten kinetics, one may ascertain the kinetic parameters of enzymes in accordance with the (PHYWE SYSTEME GMBH) software. The number of particles transformed in a unit of time is known as the rate of a chemical reaction. It is not necessary to assess the reaction rate for the breakdown of pNP-α-D-galactopyranoside (substrate), although it is dependent on the initial substrate concentration. All that is measured is the conductivity's change over time. Thus, the increase in conductivity is directly related to the rate of reaction.

pNP-α-D-Galactopyranoside + H_2O ⟶ p-Nitrophenol + D-Galactose

Preparing the solutions

To calculate the values of the Michaelis-Menten equation a series of dilutions of the substrate (pNP-α-D-Galactopyranoside) was used. First preparer a 0.04 molar (pNP-α-D-Galactopyranoside) stock solution. To make a series of dilution solutions of (pNP-α-D-Galactopyranoside) (0.02; 0.01; 0.005; 0.0025 and 0.00125 mol/L). It is best to have the solution ready as soon as possible before beginning the experiment. In the reaction, the (pNP-α-D-Galactopyranoside) is converted into the electrically conductive products (p-Nitrophenol), which enhance the flow of current. Plotting the specific conductivity values versus time is shown in the "Specific conductivity" graphic. There is a short initial phase followed by an almost linear rise in specific conductivity. As per the program (PHYWE SYSTEME GMBH), the higher the initial concentration of substrate, the faster it increases conductivity (an increase in concentration leads to an increase in conductivity) and converts into Vmax against [S].

In order to investigate the impact of caffeine and chlorogenic acid, first make a stock solution (0.04M) from (substrate pNPG plus caffeine) and (substrate pNPG plus chlorogenic acid) then prepare a dilution series of solution (0.02; 0.01; 0.005; 0.0025 and 0.00125 mol/L),using the (PHYWE SYSTEME GMBH) application, the conductivity was measured from the two stock solutions and converted into Vmax versus [S]

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Table 1. A description of the intracellular α-galactosidase purification process from *Erysipelothrix rhusiopathiae*

Purification steps	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg	Total Volume (ml)	Yield Units in total	Yield (%)	Purification (fold)
Crude extract	39.56	730.3	18.46	5	3651.5	100	1
Ammonium Sulfate (precipitation)	3.74	593.6	158.7	4	2374.4	65	8.6
DEAE-cellulose chromatography	0.537	331.15	616.6	3	993.45	27.2	33.4

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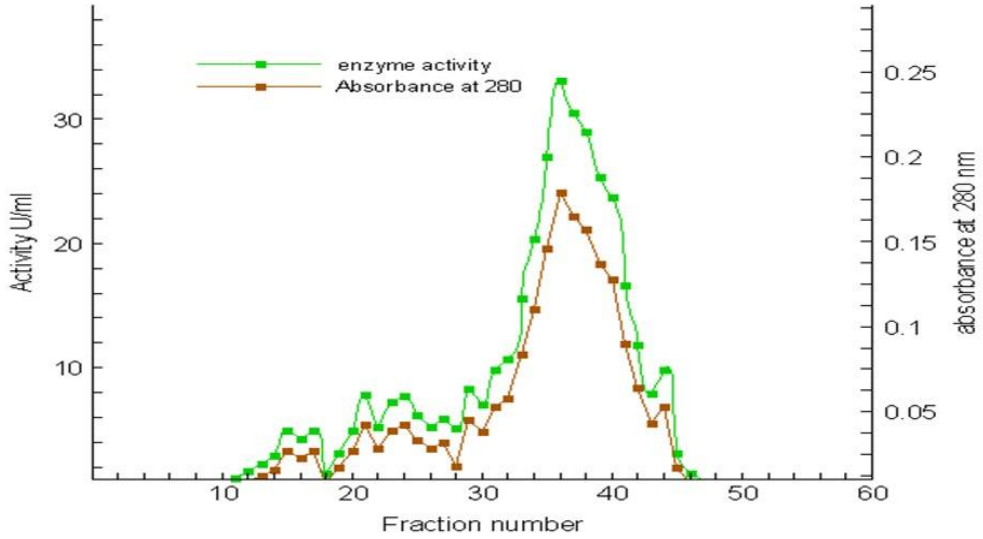
Steps in purification: Activity (U/ml), Purification (fold), Yield (%), Protein (mg), Specific activity (U/mg).



(Fig 1). *Erysipelothrix rhusiopathiae's* α-galactosidase elution profile after DEAE-

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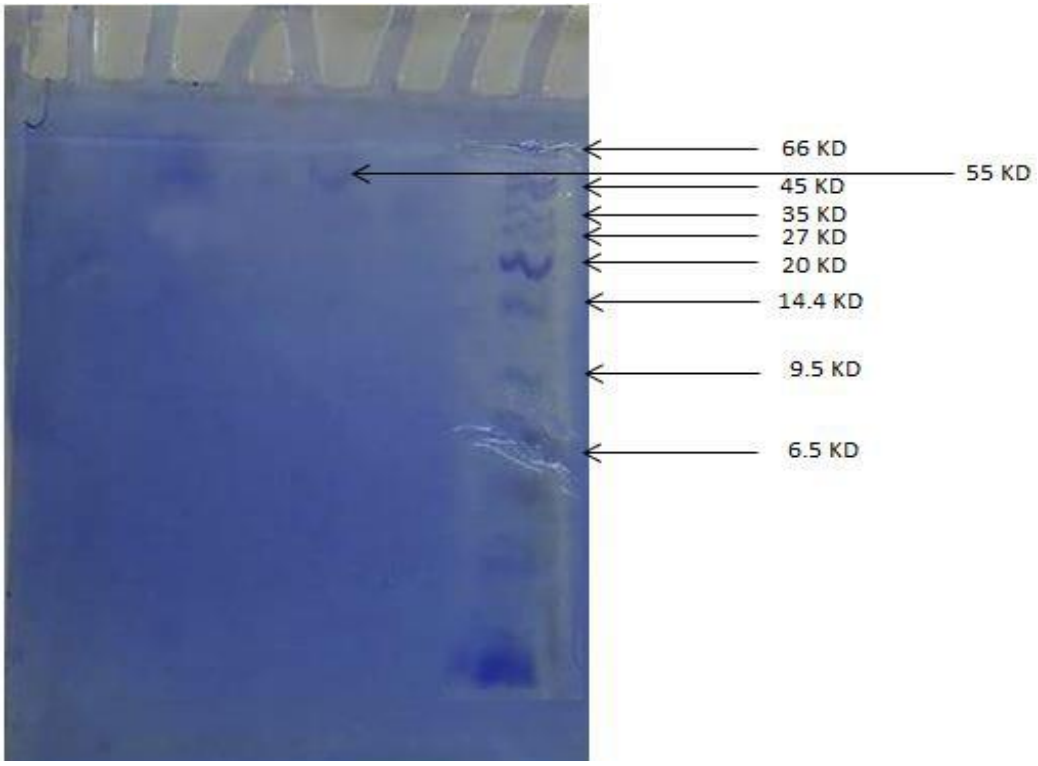

(Fig.2): Partially purified α-galactosidase produced from *Erysipelothrix rhusiopathiae* on 10% SDS-PAGE.

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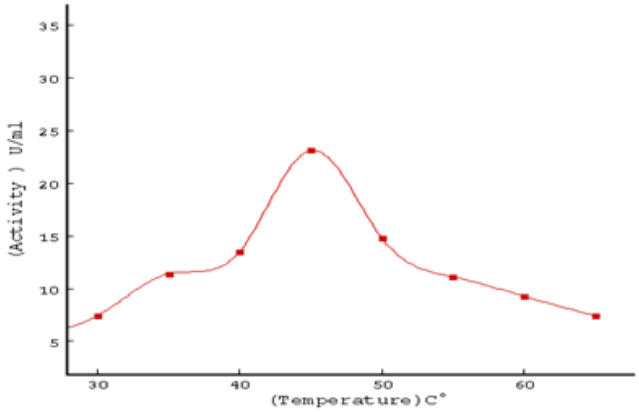
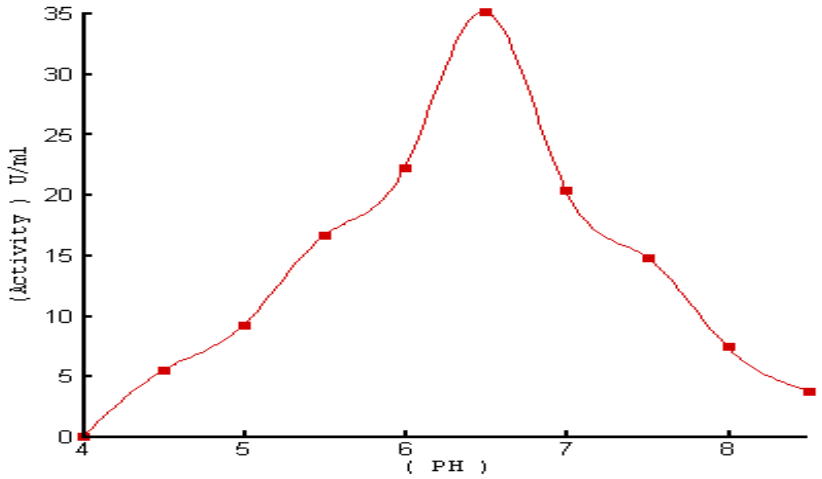


(Fig. 3). optimum temperature of α-galactosidase purified from *Erysipelothrix*



(Fig. 4). optimum pH of α-galactosidase purified from *Erysipelothrix rhusiopathiae*

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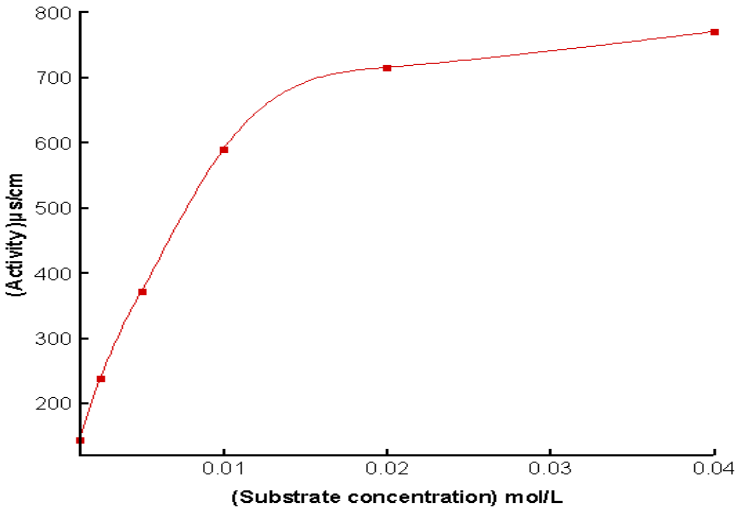

Fig. (5): Michalis-Minton for *Erysipelothrix rhusiopathiae's* purified α-galactosidase (substrate=pNPG)

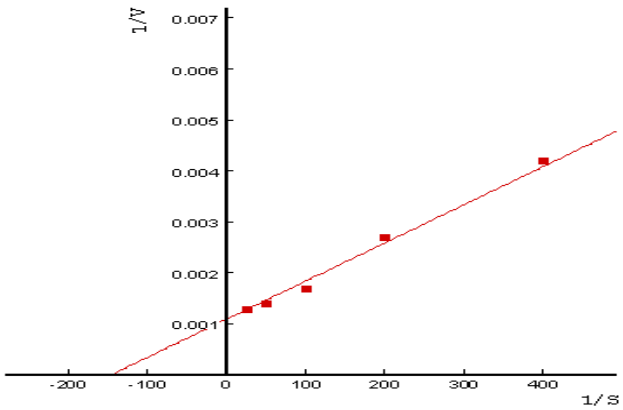

Fig. (6): Lineweaver-Burk plot for the α-galactosidase from *Erysipelothrix rhusiopathiae* that has been purified

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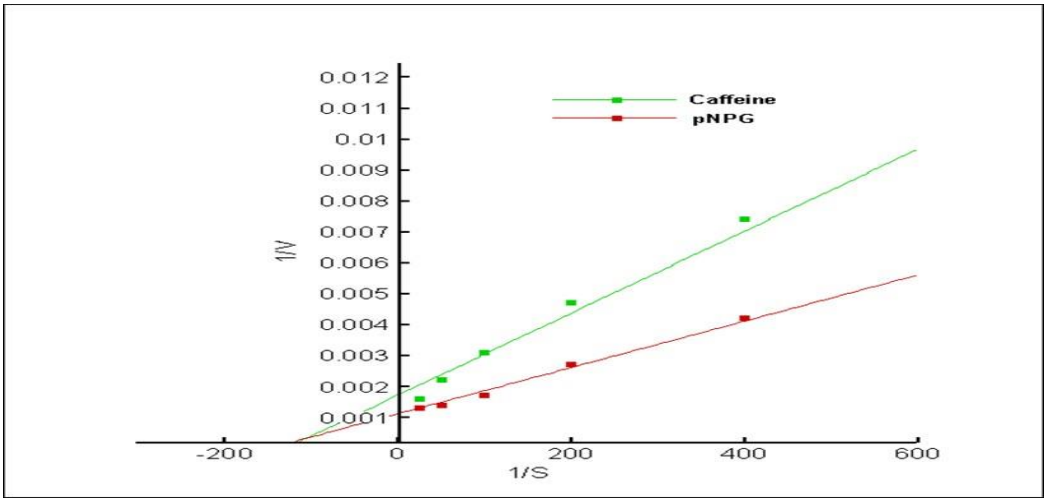

Fig. (7): Lineweaver-Burk plot for *Erysipelothrix rhusiopathiae's* pure α-galactosidase (substitute with caffeine)

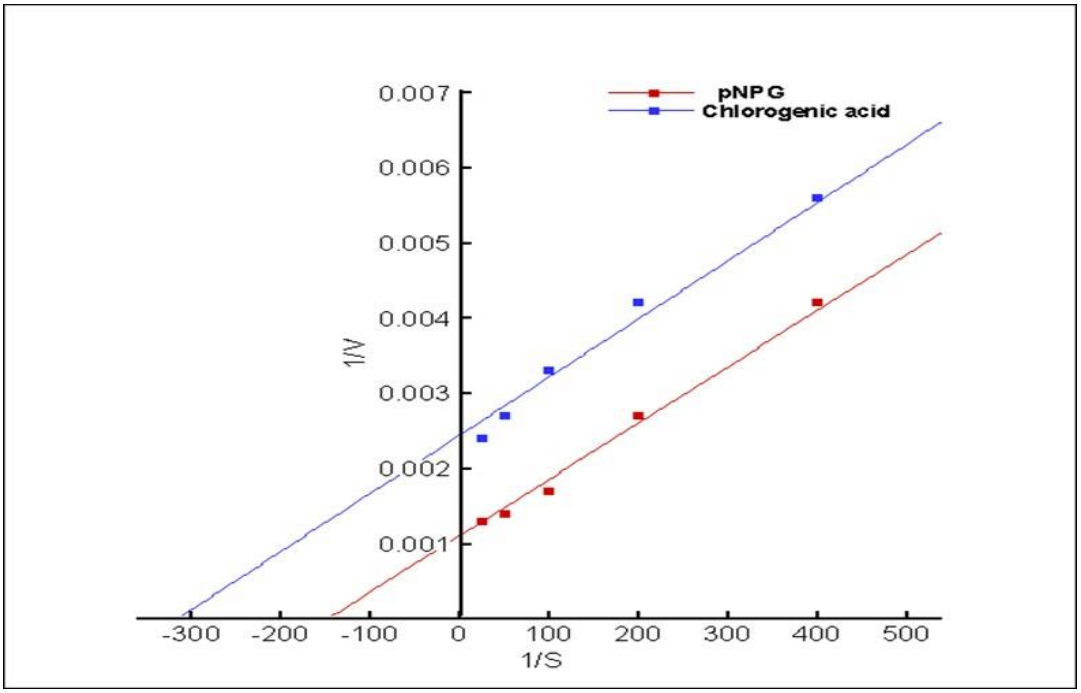

Fig.(8): Lineweaver-Burk plot for *Erysipelothrix rhusiopathiae's* pure α-galactosidase (substrate containing chlorogenic acid)

Discussion

Several investigations into the anti-infective qualities of coffee on various microorganisms have been conducted over the past ten years (34, 35), and the results have shown possibilities for improving the management of a variety of medical conditions. Caffeic acid,

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caffeine, and chlorogenic acids are the primary components in roasted coffee extracts that exhibit this kind of action (36, 37).

As shown in (Table 1) The enzyme was partially purified by many steps. The first ammonium sulfate precipitation and the total enzyme activity was found to be in 40% saturation, which was then dialyzed to remove undesirable protein. Before dialysis, this crude α-galactosidase was assayed to determine the range of its activity. At pH (6.5), as seen in (Fig. 4), maximum enzymatic activity was observed and optimum temperature at (45°C) shown in (Fig 3). The findings agree with those of other α-galactosidases found in *Bifidobacteria* [38] and *Lactobacilli* [39] however, α-galactosidase found in fungi [40; 41] demonstrated maximum activity at pH levels that are acidic. After concentrating and further purifying the enzyme preparation using DEAE-cellulose chromatography (Fig. 1), α-galactosidase was purified by a 33.4-fold using the aforementioned purification processes, resulting in an increase in specific activity (616.6 U/mg) and a yield of 27.2%.
The enzyme's purity was assessed using SDS-PAGE of the purified fractions. The figure (2) illustrates the band separation. Standard markers and purified α-galactosidase were electrophoresed in a 10% (w/v) polyacrylamide gel under denaturing conditions. This method was also beneficial for counting the number of tubes that were collected and contained the separated band. The isolated enzyme displayed a single band measuring 55 KDa. This method was employed to gather comparable tubes containing just pure α-galactosidase. The purification α-galactosidase from L. acidophilus had a molecular mass of (45 KDa), as determined by SDS-PAGE [43]. Our result is different from that obtained by [44], which was based on the zymographic analysis. The molecular weight of the purified enzyme from *PSEUDOMONAS* SP. MCCMB3 was approximately (80 KDa). The molecular mass of α-galactosidase was slightly different from that of earlier purified α-galactosidase from other bacteria, such as *Saccharopolyspora erythraea* (45 kDa) [42].

α-galactosidase which is purified from *Erysipelothrix rhusiopathiae* bacteria causing halitosis and detected the optimum temperature at (45 C^0) and optimum pH (6.5) and the activity with different concentration of substrate pNPG with constant concentration of enzyme ,the enzyme α-galactosidase for these physical properties was obey Mechalis- Menten equation by showing the Mechalis-Menten curve as in fig.(5,6). Using p-nitrophenyl-α-D-galactopyranoside (pNPG) as the substrate, the Km and Vmax values for the isolated enzyme were ascertained to be 6.66 mM and 833.3 µmol/min per mg of protein, respectively.

The enzyme's Km value is greater than the *Lactobacillus reuteri* Km value of 0.48 mM published by [45]. On the other hand, it was less than that of the a-

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galactosidase that was isolated from different microbes using the same substrate (pNPG). Based on the Km values from *Bifidobacterium adolescentis* (0.9 mM) [47] and *Citrobacter freundii* (5 mM) [46] that other researchers have published, which could be that the isolated enzyme in this study contain isoenzyme compared with another source which has another different value of Km and Vmax.

In order to examine how caffeine affected the activity of α-galactosidase, which was isolated from *Erysipelothrix rhusiopathiae*, it was found that caffeine inhibited the enzyme (pNPG is a substrate S1) when it was applied to other individuals. A reduction in the Vmax values of α-galactosidase was seen in (Figure 7) when α-galactosidase reactions with equal quantities of both substrates, pNPG (S1) and caffeine substrate (S2), were assessed kinetically in the presence of increasing doses of caffeine. Caffeine-related experiments indicate that the inhibitor and the enzyme's substrate, pNPG, have similar affinities. Similar affinities are shown by the fact that the molar concentration of caffeine at which it starts to impact enzyme activity is rather close to the concentration of the pNPG substrate. Measurements of the reaction rates at various substrate and inhibitor concentrations revealed a non-competitive inhibition, a decrease in Vmax values (555.5) µmol/min that is less than that of the enzyme without inhibitors (833.3) µmol/min, and an ineffective Km value (6.66 mM) as shown in Figure (7). By binding to the enzyme at the active site, which is distinct from the substrate binding site, caffeine functions as an inhibitor. As a result, it may bind to both the free enzyme and the enzyme-substrate complex, lowering (Vmax) while having no impact on (Km).

When we treated chlorogenic acid with α-galactosidase that is purified from *Erysipelothrix rhusiopathiae* this substrate acts as uncompetitive inhibition with substrate pNPG as the catalytic site that is reflected the enzyme have single active [48], that is become from the change of Km and Vmax
mM) and (400 µmol/min) respectively as shown in Figure (8). We found a decrease in both (Km) and (Vmax) because chlorogenic acid binds reversibly to the enzyme-substrate complex, resulting in the formation of an inactive (ESI) complex.
Coffee is an inexpensive, naturally occurring product that may be purchased in bulk. The fact that coffee is ingested in a concentrated form (6–10%) as opposed to numerous medical infusions that have demonstrated such an effect in vitro and are typically eaten at 1-2% [49] is another benefit of coffee as an antibacterial beverage. Within the parameters of the study, it can be concluded that using mouthwash on a regular basis to supplement mechanical halitosis management methods may help prevent or block the production of enzymes by bacteria that cause halitosis and lessen the likelihood that unpleasant odors would arise.

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