Detection of the Optimal Conditions for Tannase Productivity and Activity by Erwinia Carotovora

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Abstract*—Tannase has great commercial significance in the feed, drink and medical industries, tannase is an enzyme catalyzing the hydrolysis ofthe ester bonds of tannic acid, a water soluble polyphenol secondary metabolites that present in many different parts of plants, to produce gallic acid and glucose and galloyl esters. Although several substrates for the production of tannase have been reported as being economically effective such as utilization of tannin-rich substrate rather than pure tannic acid, however, there is still need to develop the substrate to make the entire process much cheaper and more effective. Here we reported tannase production from *Erwinia* as there is not found any report on tannase production from *Erwinia*. The present work deals with physiological studies on bacterial tannase. Out of 50 spoilt cucumber and tomato samples from local markets in Baghdad city. Eleven isolates from *Erwiniacarotovora* were obtained (22%). Tannase activity was found in 7 isolates in primary and secondary screening and the isolate *Erwiniacarotovora*Et_3yielded the highest tannase production. The enzyme activity was increased to 62.6 U/ml when this isolate was cultivated under the optimal conditions which consisted of using basal medium containing(5% w/v) pomegranate peels extract and (2% w/v)ammonium nitrate with pH 5.0 at 30℃ for 24 hour. The metal ions K^+, Ca^{++}, Na^+, Mg^{++} and Mn^{++} did not affect enzyme production. However, metal ions like Zn^{++}, Cu^{++}, Co^{++}, Fe^{+++} and detergents inhibited the production of tannase. The enzyme revealed maximum activity (79.50U/ml) with pomegranate peels powder(1% w/v), followed by eucalyptus and pine barks powders with relative activities of 127,112 and 103%, respectively, as substrates. Consequently, pomegranate peel has a potential as an effective and much cheaper (economical) substrate for tannase production and tannase activity in comparison with traditionally used substrates like tannic acid and other saccharides.

***Index Terms*—Tannase, Erwinia**

I. INTRODUCTION

Erwiniacarotovora is a gram-negative, facultatively anaerobic, rod- shaped bacteria and belongs to family Enterobacteriaceae [1], [2]. This bacterium is a soft rot pathogen degrading succulent fleshy plant organs such as roots, tubers, stem cuttings and thick leaves. It is also a vascular wilt pathogen, colonizing xylem and becoming systemic within the plant [3], [4], also it is able to survive in the soil [3].

Erwiniacarotovora is a plant pathogen which can cause disease in a wide range of plants, including bananas, maize, onions, lettuce, garlic, potato, sweet-potato, eggplant, zucchini, onion, carrot, cauliflower, arracacha, melon, cucumber, sweet-pepper, okra, cabbage, tomato but is best known in temperate regions for causing blackleg (stem rot) and tuber soft rot in fruits and vegetables its success partly lies in its ability to produce many pectinases that are able to macerate and break down the plant cell wall material. This exposed part of the plant releases nutrients that can facilitate bacterial growth[1], [3].Fruit softening is associated with cell wall disassembly and modifications to the pectin fraction are some of the most apparent changes that take place in the cell wall during ripening [5]. Soft rot caused by several types of bacteria, such as *Erwinia*, *Pectobacterium*, *Bacillus*, *Enterobacter* and *Pseudomonas*, but primarily subspecies of *Erwiniacarotovora* and *E. chrysanthemi*, are a widespread and destructive disease of fleshy fruits, vegetables, and ornamentals throughout the world[6], [7].

Tannins are water soluble polyphenol secondary metabolites that present in many different parts of plants, such as pine(*Pinusdensiflora*), eucalyptus (Eucalyptus camaldulensis), cinnamon (*Cinnamomum cassia*) and Oak(*Quercusrubra*)tree galls, Peels of banana and pomegranate *(Punicagranatum*) fruits, fruits of date tree and apple, seeds of coffee, Black Cumin *(Nigella sativa*) and grape *(Vitisvinifera*) as well as in many tropical plant[8].Tannin rich parts of the plants such as fruits, leaves, branches and barks posses considerable amount of tannase [9]. Vegetable tannin extracts contain a variety of amorphous materials including polyphenolic tannins of large relative molecular mass, such as hydrolysable

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gallotannin and tannic acid, as well as a less-complex of non-tannins, such as flavones and gums [10]

Tannin is used mainly for the preparation of gallic acid, instant tea, acron wine, coffee flavoured soft drinks, high-grade leather tannin, clarification of beer and fruit juice, detannification of food and to clean-up highly polluting tannin from the effluent of leather industry. Gallic acid, a hydrolytic product of tannin, has different uses like preparation of trimethoprim, pyrogallol, propyl gallate, dyes, etc [11].

Tannase (tannin-acyl-hydrolase, E.C: 3.1.1.20) is an enzyme that hydrolyzes the ester bonds of tannic acid, to produce gallic acid and glucose and galloyl esters. Although tannase is present in the plants, animals and microorganisms, it is mainly produced by the microorganisms like fungi, bacteria and yeast. Nowadays, the enzyme has wide applications in food, beverage, brewing, cosmetics and chemical industries. Tannaseis used as clarifying agent in some wines, juices of fruits and refreshing drinks with coffee flavour. The use of tannase helps in overcoming the problem of undesirable turbidity in these drinks which poses the quality problem. Enzymatic treatment of fruit juices reduces bitterness, haze and sediment formation, hence are acclaimed for health benefits and industrial use [12]. Tannase is also being used for production of instant tea preparations. The enzyme has potential uses in treatment of tannery effluents and pretreatment of tannin containing animal feed [13]. One of the major application of tannase is the production of gallic acid. Gallic acid is used for the manufacture of an anti-malarial drug trimethoprim. Gallic acid is a substrate for the chemical and enzymatic synthesis of propyl gallate, used as anti-oxidants in fats and oils [14]. For these reasons, the aim of this study was to investigate tannase production by *Erwiniacarotovora* besides to optimize medium conditions for increasing the productivity of tannase and detection of substrate specificity.

II. Materials and Methods

A. Collection of Samples

The study included a total of 50 spoilt vegetables samples of which 25were from cucumber and 25 were from tomato collected local markets in Baghdad city. These samples were analyzed according to the method that described by [15]. Briefly, 25 g of sample (cucumber or tomato) was blended with 200 ml peptone water 0.1% by using a blender for 2 min and incubated at 30℃ for 18-24hour.

B. Isolation and Identification of Erwiniacarotovora

One loopfull of each samples was streaked on blood agar and MacConkeys agar, then incubated at 30℃ for 18-24 h. For isolation of *Erwinia* spp., cells were grown on selective medium that containing the followingper500ml: (1N NaOH,4.5ml;10% $CaCl_2.H_2O$, 3.0ml; $NaNO_3$,1.0g; agar,1.5g; sodium polypectate, 10g; 10% SDS,0.5ml and 0.075%crystal violet,1.0ml)[16]. Several biochemical tests were done to differentiate *Erwiniachrysanthemi* from the other species. These include the following tests: a negative indol test, a negative lipase test, unability to phosphatase and lecithinase production and ability to produce acid from trehalose and maltose [17]-[19]. Further, the *Erwiniacarotovora* isolate was confirmed by using API 20E biochemical kit and the Vitek 2 system by using Vitek GNI card (bio Mérieux, France) according to the manufacturer's instructions.

III. Screening for Tannase Production

A. Semi-Quantitative Analysis

All bacterial isolates were evaluated for their ability to produce tannase by culturing these isolates in the center of the TAA plate which contained per liter: tannic acid,10g;NaNO3,3g; KCl,0.5g; $MgSO_4.7H_2O$: 0.5g, $FeSO_4.7H_2O$: 0.01g, KH_2PO_4, 1g and agar-agar 30g [20] then incubated at 30℃ for 24 hour and measured the clear zones formed due to hydrolysis of tannic acid around the colony the ratio of clear diameter to colony diameter was obtained and this represented a semi-quantitative assay of tannase.

B. Quantitative Analysis

The selected bacterial isolates were grown in TAA medium without addition of agar and incubated on the rotary shaker at 150 rpm at 30℃ for 24 h. After removal of cells by cooling centrifugation at 8000 xg for 10min., the clear supernatant was used as the crude extracellular enzyme source and the amount of tannase produced was assayed.

C. Tannase Assay

Tannase was assayed following the method of Mondal *et al*. [21] using tannic acid as substrate at a concentration of 1% in 0.2M acetate buffer (pH 5.5). The reaction mixture was prepared by the addition of 0.5 ml substrate with 0.1 ml of the crude enzyme and incubated at 35° C for 20 minutes. The enzymatic reaction was stopped by adding 3ml bovine serum albumin (BSA) (1mg/ml). The tubes were centrifuged at 5000g for 10 min. The precipitate was dissolved in 2ml SDS-triethanolamine solution followed by the addition of 1ml of FeCl3 reagent. The contents were kept for 15 min for stabilizing the color formed and the absorbance was measured at 530nm against the blank. One unit of tannase activity can be defined as the amount of enzyme which is able to hydrolyze 1mM of substrate tannic acid in 1min under assay conditions.

D. Preparation of Substrate and Crude Tannin Extract from Different Plants

Pomegranate, eucalyptus, grape, black cumin, pine, oak and cinnamon were obtained from local market. Pomegranate peels were obtained by removing seeds handly and cleaned by water. The grape seeds were extracted by electrical rotary juicer machine, washed by water, scoured by hand to separate tissue and peel of fruits. Peels of pomegranate, seeds of grape and black cumin and bark of pine, oak, eucalyptus and cinnamon were dried separately in drying oven at 55℃ until their

moisture content reached 5% , then milled twice separately to obtain homogenized powders and used as substrates. Extraction of tannic acid from peels of pomegranate, seeds of grape and black cumin and bark of pine, oak, eucalyptus and cinnamon was done with modification the method that described by[22-25] as follow: 10 g of their powders was added to 100 ml distilled water then boiled for 10 min. After that it was filtered through muslin cloth, centrifuged at 8000 rpm for 10 min and used as a sole carbon sources throughout the study.

IV. Optimization of Culture Conditions for Tannase Production

A. Effect of Carbon Sources on Tannase Production

Twenty-five ml of 5% pomegranate peels, grape and black cumin seeds and pine, oak, eucalyptus and cinnamon barks extracts(pH7.0) besides to 5% tannic acid, glucose, sucrose, lactose, xylose, dextrose, starch, mannitol and milled wheat bran and rice bran were used as carbon sources and added instead of 1%tannic acid that found in TAA medium for tannase production. The selected isolate was inoculated to these media and incubated at 30 ℃for 24 hour. The fermented broth was centrifuged at 3500 rpm for 10 minutes and the cell free supernatant obtained was collected and used as crude tannase enzyme, then tannase activity was determined.

B. Effect of Nitrogen Sources on Tannase Production

Effect of different nitrogen sources including peptone, treptone, beef extract, yeast extract, casein and urea (organic nitrogen sources) and ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate and ammonium sulfate a(inorganic nitrogen sources) was studied by incorporating 2% (w/v) of each nitrogen source in pomegranate peels extract medium. The selected isolate was inoculated to these media and incubated at 30 ℃ for 24 hour, then tannase activity was determined.

C. Effect of Different Concentrations of Pomegranate Peels Extract on Tannase Production

Different concentrations of pomegranate peels extract of (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%) were prepared and added instead of 1%tannic acid that found in TAA medium. The pH was adjusted at 7.0, inoculated with selected isolate and incubated at 30 ℃for 24 hour, then the tannase activity was determined.

D. Effect of pH on Tannase Production

Pomegranate peels extract medium was prepared at different pH values (2-8).This medium was inoculated and incubated at 30 ℃ for 24hours. The pectate lyase activity was determined.

E. Effect of Incubation Temperature on Tannase Production

Pomegranate peels extract medium was inoculated and incubated with selected isolate at different temperatures (10, 15, 20, 25, 30, 35, 37 and40 ℃) for 24 hours. The tannase activity was measured.

F. Effect of Different Incubation Periods on Tannase Production

Pomegranate peels extract medium was incubated with selected isolate at 30 ℃ for different periods (12, 18, 24, 36, 48 and 60 hours). The tannase activity was determined.

G. Effect of Additives on Tannase Production

Different salts such as NaCl, KCl, $CaCl_2$, $CuSO_4$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$ and $FeCl_3$ and different detergents like Tween-20, Tween-40, Tween-60, Tween-80, Triton X-100 and SDS were added to the production medium. After inoculation and incubation with selected isolate at 30 ℃ for 24hour, tannase activity was determined as described earlier.

H. Substrate Specificity

A study of substrate specificity for the tannase was made by using pomegranate peels, grape and black cumin seeds, pine, oak, eucalyptus and cinnamon barks, wheat bran and rice bran as powders. The tannase assay was done by using these plant powders at concentration 1% instead of tannic acid.

V. Results and Discussion

A. Isolation of Erwiniacarotovora

Out of 50 plant samples collected, 11(22%) *Erwiniacarotovora* was isolated. Among which 4/11 (36%) were from cucumber, 7/11(63%) were from tomato (Fig. 1a and b). This bacterium causes blackleg of potatoes of which it is the chief, if not the only cause, in cold climates. However, in warmer climates *Erwiniachrysanthemi* and *Erwiniacarotovora* can cause similar or identical symptoms [26]. *Erwiniacarotovora* strains were isolated from potatoes, cucumber, broccoli, radish, tomato and sweet pepper and survived for 5 months at temperatures of 10 ℃ and 20 ℃ and relative humidities of 81 and 93%, and temperatures of 30 ℃ and 35 ℃ [27]. *Erwiniacarotovora* and *Erwiniachrysanthemi* can cause symptoms similar to blackleg and contribute to tuber rotting, also *Erwiniacarotovora* can cause a slow wilt in the field with darkened vascular tissue and a brown discoloration in the stems [28], [29].The insoluble plant cell wall polymers are significant barriers to microorganisms, yet represent an abundant source of carbohydrates. The disease process in general requires pectin degradation and is often dependent on plant sensitivity and environmental conditions such as temperature, nitrogen starvation, osmolarity and oxygen limitations [30]. In cucumber plants fed unbalanced solutions, bacterial wilt was increased significantly in low N and low K solutions, while the disease indices of plants grown in low P, high N, high P, and high K solutions were either not significantly or not consistently different from those in the balanced solution [28].

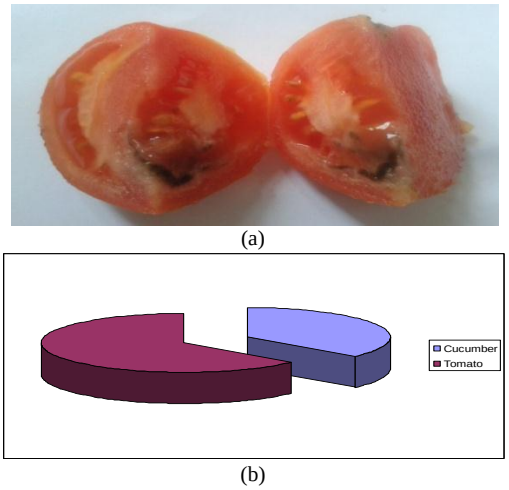

Figure 1 a)Tomato sample infected withErwiniacarotovora, b) Percentage of Erwiniacarotovora from different plant sources

VI. Screening for Tannase Production

A. Semi-quantitative Analysis

Among 11 *Erwiniacarotovora* isolates subjected to rapid screening for extracellular tannase production by using TAA medium plates, 7 *Erwiniacarotovora* isolates were found to be positive for tannase activity by growth on this medium and production of clear zones. *Erwiniacarotovora*Et_3 gave higher clear zone around the colony in comparison with other isolates (Table I). Zones formed due to hydrolysis of tannic acid to gallic acid and glucose, leading to a decrease in zone formation by *A.flavus* opacity of the media. Direct measurement of the colony diameter was a good indicator of the ability of tannic acid utilization as a carbon source due to the tannase activity in the medium[31].The tannase production was induced by tannic acid or some of its derivatives, but the exact mechanism is unclear[32]. The clear zone was observed around the growth of *Rhodococcus* NCIM 2891 after 48 h of incubation indicates that the microorganism producing tannase enzyme cleave tannic acid protein complex by hydrolyzing tannic acid[20].

TABLE I. Diameter of Inhibition Zone and Pectatelyase Activities for Erwiniachrysanthemi Isolates

Isolate No.	Diameter of inhibition zone(mm)	Tannase activity(U/ ml)
Et_1	16	21.12
Et_2	14	18.75
Et_3	24	33.53
Et_4	19	27.15
Ec_5	12	16.17
Ec_6	17	23.04
Ec_7	12	14.33

B. Quantitative Analysis

All *Erwinia Erwiniacarotovora* isolates that exhibited clear zone around the colonies were tested for tannase production in liquid condition by growing in TAA medium without addition of agar. These isolates revealed tannase activity between 9.14-33.53 Unit/ml (Table I). *Erwiniacarotovora*Et_3demonstrated high tannase activity 33.53 Unit/ml, so that this isolate was selected as the besttannase producer. The tannases are inducible enzymes that require the presence of the inducer to be synthesized. Mondal*et al.* [21] eported that the tannase production was directly proportional to the growth of Bacillus licheniformis KBR6 and the extracellular enzyme accumulation increased with the number of cells [33]. In tannase fermentations using fungal cultures, tannase is mostly extracellular when produced by solid state fermentation and intracellular in submerged fermentations, while in case of bacterial fermentations, the tannase is mostly extracellular [33].

VII. Optimization of Culture Conditions for Maximum Tannase Production

A. Effect of Carbon Sources on Tannase Production

Among the various carbon sources tested tannase production (Fig. 2), tannin was the best carbon source tannase production yielding the maximum enzyme activities of about 51.62U/ml with (5%) pomegranate peels extract followed by(5%) pine and eucalyptus barks with about 44.35 and 41.09 U/ml, respectively. While the inhibition in enzyme production was observed when glucose, sucrose, lactose, xylose, dextrose, starch, mannitol and milled wheat bran and rice bran were added to TAA medium instead oftannic acid as carbon sources. pomegranate peel is an interesting alternative, since, it is a relatively cheap and easily available substrate, that can serve as a feedstock for large-scale fermentation, whereas pure tannic acidis only available in limited quantities at very high cost and it wasn't suitable for the commercial production of the enzyme.

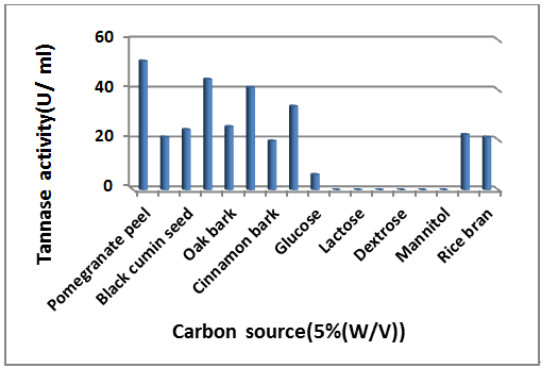

Figure 2 Effect of carbon source on tannase production byErwiniacarotovoraEt3

An extraction process is the most important operation to obtain tannin from vegetal tissue. Tannin extraction is a multiple-stage physical–chemical process in which hydrolysis and extraction of tannin macromolecules from plant tissue and their solubilisation take place under the influence of different factors, mainly temperature, pH and time [34]. Similar result was reported by [35], in accordance to this study where the pomegranate rind gave maximum production of tannase by *Trichodermaharzianum* MTCC 10841 compared to jamun bark and amaltashleaves. There was an inhibitory

effect on the synthesis of pectatelyase. This result is in agreement with the study of [36] where the production tannase was inhibited when mannose, galactose, glycerol and ribose were added to the medium compared to the presence of tannin as the sole carbon source in submerged fermentation. The low enzyme production with other carbon sources is maybe because of catabolite repression in presence of these materials.

B. Effect of Nitrogen Sources on Tannase Production

The effect of nitrogen source on tannase production from *Erwiniacarotovora* was studied by supplementing the production medium with various organic and inorganic nitrogenous sources. The best nitrogen source was ammonium nitrate (55.24U/ml) followed by ammonium chloride (52.12 U/ml). On the other hand, peptone, treptone, beef extract, yeast extract, casein, potassium nitrate and ammonium sulfate seem to inhibit tannase production (Fig. 3). The organic nitrogen sources such as casein and peptone gave considerable enzyme production. Urea was a very poor nitrogen source for tannase production. Malt extract (2%) with NH4Cl (0.2%) was found to the best nitrogen source for tannase production by *Trichodermaharzianum* MTCC 10841 [11]. 1%(w/v) sodium nitrate as nitrogen source for maximum tannase activity in *Aspergillusniger* [36].

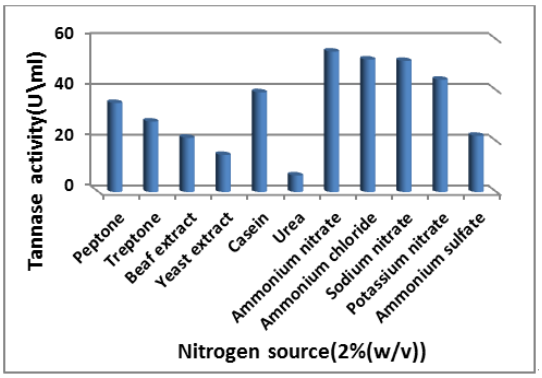

Figure 3 Effect of nitrogen source on tannase production by ErwiniacarotovoraEt3

C. Effect of Different Concentrations of Pomegranate Peels Extract on Tannase Production

The best concentration of pomegranate peels extract in media for tannase production was 6% with 56.73U/ml tannase activity (Fig. 4) and the enzyme production decreased with further increased in substrate concentration (7-10%). Probably, this is due to the presence of a high gallic acid concentration from tannin degradation. The concentration of tannin is a very important determining factor for tannase biosynthesis for most fungi and bacteria. The actual mode of tannase induction in a particular concentration of tannin has not been properly explained until now. the higher concentrations of tannin lead to non-reversible bonds with surface proteins and impair the metabolism as well as growth of the organism.(tannase plant) increased in tannase production by *Aspergillusflavus* was observed with Redgram Husk (RGH) and Tamarind seed powder (TSP) as a substrates at 3% and further increased in the concentration, resulted in slight decreased in enzyme activity[37].

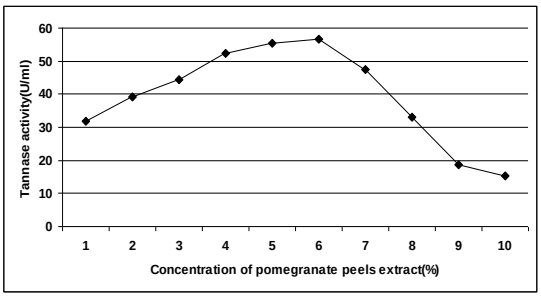

Figure 4 Effect of different concentrations of pomegranate peels extract on tannase production byErwiniacarotovora Et3

D. Effect of pH on Tannase Production

Experiments were executed to find out the optimum pH in order to maintain the favorable conditions for increasing tannase production. The fermentation medium pH was adjusted accordingly with 1N HCl /NaOH from 2-8. The significance of pH on the production of tannase was observed. The maximum tannase production of 61.03U/ml was obtained at pH 5.0(Fig. 5).

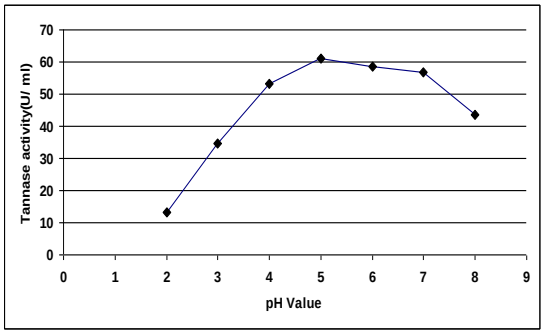

Figure 5 Effect of pH value on tannase production by Erwiniacarotovora Et3

This may be attributed to the balance of ionic strength of plasma membrane. The pH affects in enzyme production because of its role in the solubility of medium substrates and its effect on the ionization of the substrate and it's availability for the bacterial growth. Moreover, the pH affects the productivity and enzyme stability [38]. This could also be explained by the fact that *Erwiniacarotovora* was isolated from the spoilage vegetables such as tomato which was an acidic by nature. Moreover, the optimal initial medium pH for tannase production by *Lactobacillus plantarum* MTCC 1407 and *Klebsiella pneumoniae* MTCC 7162 in submerged fermentations have been reported in the acidic range of 6.0 [9], [39].Also found that the optimum tannase production in *Klebsiella pneumoniae*MTCC 7162 at alkaline pH(7.5)[40]. In contrast, the tannase from fungal isolates shows pH optima between 4 and 5.5 [36], [37], [41].

E. Effect of Incubation Temperature on Tannase Production

The effect of incubation temperature on tannase production from *Erwiniacarotovora* was studied in the

temperature range of 10-40℃ under submerged stationary fermentation conditions. The optimum temperature for tannase production was found to be 30℃ with 62.63U/ml (Fig. 6). Further rise in temperature, decreased the production of tannase and the minimum tannase activity was observed at temperature 40℃. The enzyme production reduced gradually with further increase in incubation temperature.

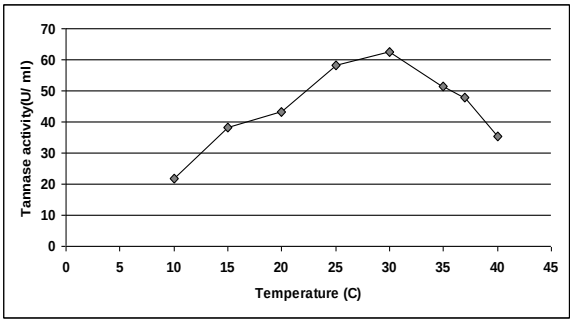

Figure 6 Effect of temperature on tannase production by Erwiniacarotovora Et3.

This may be due to the denaturation of microbial strain at higher temperatures. Lower and higher temperatures decreases the specific activities because of the thermal effects of these temperatures on the microorganism growth and on the enzymatic reaction rate inside the cells which reflects on the vital creation of the enzyme [38]. Therefore, the data obtained indicated the enzyme production was not growth dependent. The fermentation temperature for optimum production of tannase is mostly reported to be 30℃ [9]. Some reports also mentioned tannase production at temperature 35℃-37℃ [39], [42]. Normally the effect of pH on the enzyme activity is determined by the nature of the amino acid at the active site, which undergoes protonation and deprotonation and by the conformational changes induced by the ionization of the amino acids [9]. When the temperature increases, the kinetic energy of the substrate and enzyme molecules also increase which affects the reaction rate. The number of collisions per unit time of tannase activity and its substrate, tannic acid increases, resulting in a higher activity with the continuous increases in the temperature level. When the optimum level of temperature obtained, the energy of the molecules increased thorough out the process, but the chemical potential energy increases enough, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation of the tannase protein causing its inactivation. Thus, an increase in temperature beyond the optimum value caused reduces in the catalytic rate of tannase as either the enzyme or substrate became denatured and inactive [9].

F. Effect of Different Incubation Periods on Tannase Production

Fig. 7 shows a rapid increased in tannase production by *Erwiniacarotovora*Et_3until it achieved maximal production at 24 hours of cultivation (62.66U/ml). The enzyme production decreased rapidly after achieving its maximal production. This might be due to sugar utilization or enzyme denaturation as a result of interaction of other compounds in the medium or reducing the nutrient level of medium [43]. When compared to previous findings, the cultivation time by *Erwiniacarotovora* (24 hour) was longer compared to cultivation time showed by *Bacillus* licheniform is KBR6 and *Klebsiella pneumonia* MTCC 7162 which yield maximum pectinase activity at 15-18and 21h of cultivation, respectively [40], [44]. *Rhodococcus* NCIM 2891cultivated in medium containing 0.1% tannic acid, produced tannase which showed maximum activity after 24 h [20].

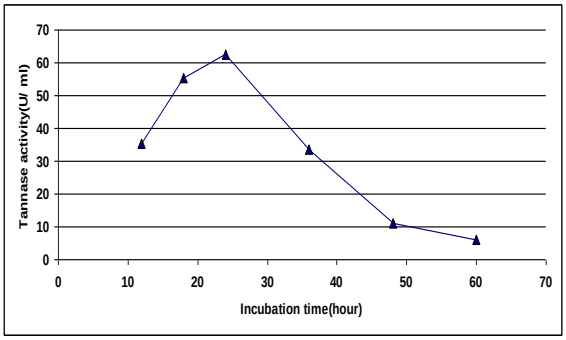

Figure 7 Effect of different incubation periods e on tannase production by Erwiniacarotovora Et3

G. Effect of Additives

The effect of additives on tannase production was studied by adding the different salts and detergents in the production medium. The metal ions K^{+}, Ca^{++}, Na^{+}, Mg^{++}, Mn^{++} and did not affect enzyme production. However, metal ions like Zn^{++}, Cu^{++}, Co^{++}, Fe^{+++} and detergents (Tween-20, Tween-40, Tween-60, Tween-80, Triton X-100 and SDS) inhibited the production of tannase (table-2). The addition of some metal ions such as Zn^{+2}, Mo^{+6}, Al^{+3}, Mn^{+2}, Cu^{+3} and Li^{+3} to the production medium was inhibited tannase production by *Aspergillusniger* [36].I n a study done by[11] reported that some detergents and metal ions like Cu^{++}, Co^{++} and Fe^{+++} inhibited the production of tannase.

TABLE II. EFFECT OF SALTS AND DETERGENTS ON TANNASE A

salt	Remaining activity(%)	Detergent	Remaining activity(%)
NaCl	100	Tween-20	17
KCl	100	Tween-20	13
$CaCl_2$	100	Tween-20	4
$CuSO_4$	15	Tween-20	9
$CoCl_2$	53	Triton X-100	6
$MgCl_2$	100	SDS	11
$MnCl_2$	100		
$ZnSO_4$	65		
$FeCl_3$	24		

H. Substrate Specificity

The crude tannase from *Erwiniacarotovora* Et_3 showed maximum activity (79.50U/ml) with pomegranate peels powder(1% w/v), followed by eucalyptusand pine barks powders, respectively(Table

III), with relative activities of 127,112 and 103%, respectively, but it decreased with all other substrates such as grape and black cumin seeds, oak and cinnamon barks, wheat bran and rice bran powders. This indicating that pomegranate peels powder might be the most suitable substrate for maximum tannase activity. It is interesting to note that although plants considered in this study all contain tannin but there is distinct variable differences in tannase activity. The possible differences in tannin synthesis in these species**.**

The content of tannic acid in grape seeds and pomegranate peels were 4.1 and 27.6 g/100g (dry weight), respectively. thus grape seeds is not considered as an important source of tannic acid [8]. The basis content of tannin inpomegranate of approximately 73%in peels and 27% in seeds [22]. Tannin has been confirmed to be present in EC bark and a yield of between 5 and 12% was obtained and the yield obtained was found to vary inversely with the particle size of the bark [10]. while cinnamon barks contain tannins levels ranging among (0.65-2.18%) [24]. Pure tannic acid is a very costly substrate and is not suitable for large-scale production of the enzyme. In this respect crude tannin could be cost effective and suitable for the commercial production of the enzyme. Agro-residues and forest products are generally considered the best source of tannin-rich substrate. The use of inexpensive substrates can economize the process of production. Therefore the agricultural wastes containing tannin could be considered as an alternative source of tannic acid for producing this enzyme.

TABLE III. EFFECT OF SUBSTRATES ON TANNASE ACTIVITY

Substrate	Tannase activity(U/ ml)	Remaining activity(%)
Pomegranate peel	79.50	127
Grape seed	40.06	64
Black cumin seed	38.81	92
Pine bark	64.47	103
Oak bark	45.69	73
Eucalyptus bark	70.11	112
Cinnamon bark	51.33	82
Wheat bran	9.39	15
Rice bran	11.26	18
Tannic acid(control)	62.66	100

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