Purification and Characterization of Tannase: A review

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Abstract— Tannin acyl hydrolase (E.C.3.1.1.20) which is also generally referred to as tannase can be described as a hydrolytic enzyme that hydrolyses the ester and depside bonds of hydrolyzable tannins like ellagitannins and gallotannins to further yield glucose, gallic acid, and galloyl esters. The obtained gallic acid from this process hasvarious therapeutic as well industrial applications leading to its high demand and requirement. The tannase enzyme is a very essential group of biotechnologically significant enzyme that are dispersed all through the plant, animal as well as the microbial kingdom. These enzymes have extensive applications in various fields and industries including pharmaceutical, chemical, food, feed, brewing, and beverage industries. Moreover, various tanneries produce toxic tannery effluents that contain tannins in the form of tannic acid. The tannase enzyme can be primarily used for the biodegradation of these tannins that are present in toxic effluents.

Even though extensive research has been done mainly on the production methodologies of tannase, it is still acknowledged as one of the costliest enzymes used in the industry, mainly owing to its lengthy fermentation time and less yield. Taking into account the growing demand for the enzyme, isolation of highly productive strainsand the development of economically feasible processes becomes very essential.This study reviews the purification and characterization of tannase enzyme, the microbial sources, the modes of production, temperature, and pH of the tannase enzyme.

Index Terms— Calcium Alginate, Gallic Acid, Immobilization, Tannase

1 Introduction

Tannase or tannin acyl hydrolase (E.C.3.1.1.20)can be defined as an intracellular or extracellular inducible enzyme that is produced by various microorganisms including bacteria, fungi, molds, and yeast [1]. This enzyme is known to catalyze thehydrolysis of the ester and depside bonds of hydrolyzable tannins, further releasing glucose and gallic acid [2]. Tannins, which are substrates for the production of tannase, are defined as polyphenolic compounds with a high molecular weight ranging from 500 kDa to about 3000 kDa [3]. These tannins are predominant constituents of plants and they are also seen to majorly occur as one of the dominant constituents of effluents that are emitted from different tanning industries which are lethal to animals, plants aswell as microorganisms. Hence, they pose potential threats to human health as well as the environment which can be avoided with the help of tannases [4]. Extracellular enzymes do not require expensive extraction methods and can be easily extracted [5].

The tannase enzyme can be retrieved from various sources including plant, animal, and microbial sources. However, microorganisms have been employed as the better approach for enzyme production due to several advantages like better control over the process parameters, biochemical heterogeneity, and better yield on a shorter period, reusability, and the ability to genetically alter microorganisms [6].

The gallic acid that is obtained from the bioconversion of tannins has various applications including being a very advantageous therapeutic agent because of its antiviral, anti-tumor, and anti-microbial properties. It also acts as a potential drug and aradioprotective agent. Further, it has applications in the agriculture field wherein it serves to protect crops as well as help in the storage of food which renders the gallic acid molecule very profitable, economically as well as commercially.

Besides gallic acid production, this enzyme is also considerably used for the clarification of beer and fruit juices, production of instant tea and acorn wine [7], improvement of flavor in grape wines, production of coffee-flavored soft drinks [8], and as a systematic probe for identifying the structure of naturally occurring gallic acid esters[9]. It can also be applied for plant cell wall digestibility by the cleaving of polyphenolics such as dehydrodimer cross-links that are present in the cell walls of plants [10].

Furthermore, it is used for synthesizing high-quality leather [11] and for the biodegradation of tannins that are present in toxic effluents of industries [12]. The current technology wherein tannins are hydrolyzed to yield gallic acid puts forth several disadvantages including less purity of gallic acid, low yield, generation of a high amount of toxic effluents that pose as environmental threats, and high production cost [13].Tannase which is the biocatalyst used for the biotransformation process to yield gallic acid consists of a significant industrial level of importance because of its extensive catalytic potential. Since there is a bioremediation need for tannin-containing agro-waste and industrial waste and also because of the variety of applications of gallic acid commercially, a low-cost and eco-friendly green technology method for the production of gallic acid can be devised to meet the high demand and requirement.

2 MICROBIAL SOURCES OF TANNASE:

Species such as Bacteria, Fungi, and Yeast are involved in the utilization of production of Tannase. Such microorganisms are very much efficient in tannase production and are used widely all around.

Bacteria: A large variety of tannase-producing bacteria have been isolated and identified. Extracellular tannase is produced from bacterial forms. The Molecular weight of the tannase from bacterial sources lies between 46.5-90 kDa. [14]. *Bacillus, Corynebacterium, Klebsiella, Streptococcus, and some Lactobacilli sp*. Can be utilized for tannase production. [15]. Fungi: Filamentous fungi can depolymerize tannin. Over 120 fungal species can produce tannase [14]. *Aspergillus, Myrothecium, Neurospora, Rhizopus, Fusarium, Trichoderma, and Penicillium* [15]. Yeast: Few forms of yeast can degrade Tannins.*Candida* species can depolymerize tannins [14].

Table 1: Microbial sources for tannase production

Bacteria	*Bacillus pumilus*
	Bacillus polymyxa
	Corynebacterium sp.
	Klebsiella pneumoniae
	Streptococcus bovis
	Selenomonas ruminantium
Yeast	*Candida* sp.
	Saccharomyces cerevisiae
	Mycotorula japonica
Fungi	*Aspergillus niger*
	Aspergillus oryzae
	Aspergillus japonicus
	Aspergillus gallonyces
	Aspergillus awamori
	Penicillium chrysogenum
	Rhizopus oryzae
	Trichoderma viride
	Fusarium solani
	Mucor sp.

Source: Belmares *et al*., 2004 [12].

3 STATISTICAL MODELING OF TANNASE PRODUCTION

An important tool to scale up the fermentation process for tannase production is the development of optimum parameters which is a crucial step in the overall process. The main aim of statistical modeling of tannase production is to interpret the relationship among different parameters and also for optimization. Several methodologies emphasize using statistical modeling for the optimization of tannase production, with Response surface methodology (RSM) and Taguchi methodology [16] being used widely across. Response surface methodology mainly examines the relationship between different variables and also helps to

determine the effect of various factors to reach maximum productivity.

4 TANNINS AS SUBSTRATES FOR TANNASE AND GALLIC ACIDPRODUCTION:

Tannase is known to catalyze the hydrolysis of ester bond and depside bond in hydrolyzable tannins to release gallic acid [17]. There are different classes of tannins namely, Hydrolysable tannins, Complex tannins, and Condensed polyflavonoid tannins [14]. Hydrolyzable Tannins include gallotannins, ellagitannin. They are present in plants in small amounts. They form gallic acid or ellagic acid by heatingit with hydrochloric or sulphuric acid. [18].Condensed tannins are polymers formed by thecondensation of flavonoids. They are calledproanthocyanidins [19]. Proanthocyanidins arechemical compounds, that are responsible for giving the fruit or flowers of many plants, colours [18].

Agricultural wastes like husk, barn, tea dust, nuts, shells, are used for gallic acid production. Sources of Hydrolysable tannins are Chinese Gall, Cashew Waste [14]. Tannins are also present in the leaves and legumes of certain plants [19].

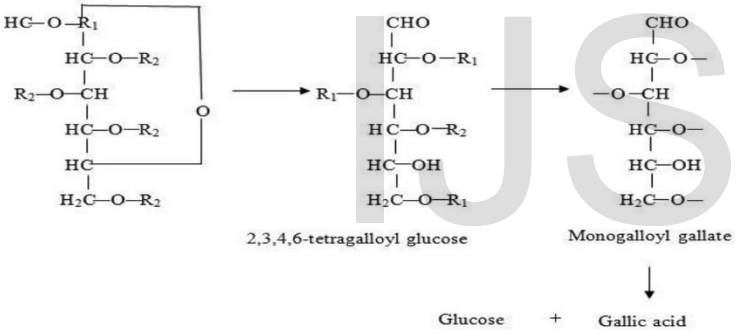

Fig.1. Hydrolytic Pathway of tannic acid by tannase

Source: Natarajan *et al*., 2008 [20]

5 STATISTICAL OPTIMIZATION:

The selection of fungus *Mucor circinelloides* isolates F6-3-12 was done using phenotypic analysis and 18S rRNA gene sequencing. Submerged fermentation (SmF) and solid-state fermentation (SSF) were performed to produceextracellular tannase from plants like Pomegranate, green tea, etc. Various strategies were carried out for optimization of several factors like substrate concentration, temperature, substrate: moisture ratio,incubation period, and inoculum age. It was recorded that *Mucor circinelloides* isolate F6-3- 12 was involved in producing a high level of Tannase (2.07 IU) at 72 hours fermentation with pomegranate rind concentration of about 1 g/flask in solid-state fermentation. Further, it was seen to decline after 96 hrs. Enhancement of Tannase production was possible by using Plackett-Burman design and increased from 2.07 IU to 5.83 IU and later on,increased up to 12.24 IU on applying the Box–Behnken design that had2.82 and 2.1-fold enhancement [21].

6 PURIFICATION OF TANNASE:

The purification of the tannase enzyme is mainly done to improve stability, prohibit unwanted reactions, and enhance catalyticactivity. The purification depends on various factors like the processing cost, the market, thefinal quality as well the available technology. Because of the need for cost-effective and large-scale protein purification, techniques thatprovided efficient, fast, and economical methods in much fewer steps were developed. A highly purified tannase enzyme is required for commercial use but the application part of the enzyme for purposes like waste management necessarily does not need high purity. The method of purification is generally not typical but is a combined method [22] which was shown to increase the yield from 2.9% tannase to 50% tannase in *A. niger* [23]. High-performance liquid chromatography (HPLC), electrophoresis, ultrafiltration, and various other methods have been used to achieve high levels of purification of the enzyme [24]. But these methods are costly and provides lower yields and in turn lower quantity. Therefore, the purified enzymes tend to become lesser in their activity and further unfit for biochemical studies. In many of the published purification methodologies, multiple steps are performed followed by ion exchange and/or gel filtration [25] [26]. The purification of the extracellular tannase enzyme can be done by following two chromatographic techniques. With the help of DEAE cellulose, ion exchange chromatography took place. And in the initial step, gel filtration chromatography using a G-150 Sephadex column was carried out effectively. The process of gel filtration chromatography that used Sephadex column 150 gel permits thedissociation of tannase with its two isoforms i.e., TAHI and TAH II. In comparison, TAH I was observed to be involved more in the total tannase activity. The activity of TAH I was calculated to be more than 70% of the totaltannase activity [27].

Deepanjali Lal *et al*., [28] purified the tannase at a temperature of about 4°C in a sterilized condition. At 48 hours of cultivation that had 1% tannic acid as a substrate, a supernatant culturebroth was obtained to purify the tannase. Filtration chromatography in a Sephadex G-150
the column was used to purify the extracellular tannase that allowed separation of the enzyme from minor proteins and fungal pigments. The purification of tannase was done at 7.17-fold atthe end of the process, and the specific activityof

101.428 U/mg protein had a yield of 18.35%.

Table 2: Purification results of tannase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Sephadex G-150	4.12	58.3	14.150	3.84	50.58
DEAE Sephadex	0.98	99.4	101.428	7.17	18.35

Source: Deepanjali Lal *et al*., 2012 [28]

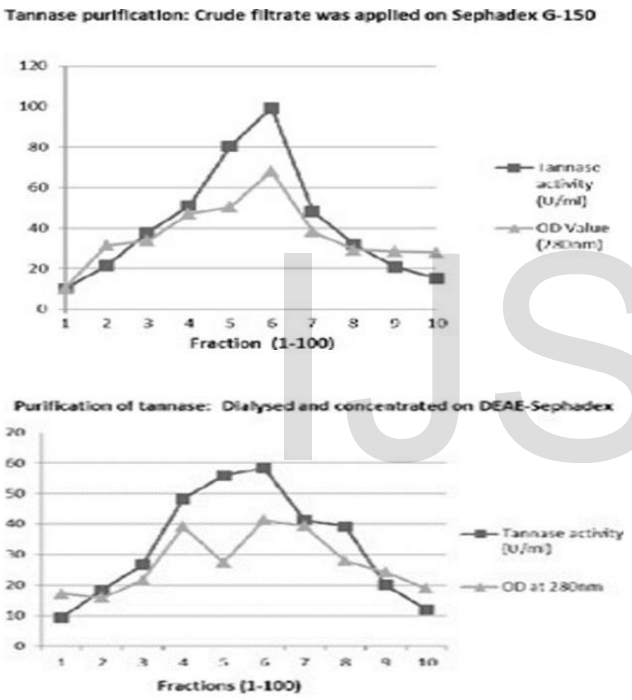

Fig. 2. Graphs depicting the results of purification of Tannase

Source: Deepanjali Lal *et al*., 2012 [28]

7 PURIFICATION METHODOLOGIES

7.1 Concentration by Ammonium Sulfate-

Al-Mraai, *et al*., [29] moderately extracted the crude enzyme by adding crystals of ammonium sulfate to a specific concentration of the enzyme. Accordingly, repeated mixing was done for about 4 hours until solubilization having a saturation ratio between 20-80% was obtained.Centrifugation at an rpm of 10000 was carried out for 15 min. at 4°C. The filtrate and precipitate wereanalyzed in each step to estimate enzyme activity and protein concentration.

7.2 DEAE-Cellulose Chromatography-

Saswati Gayen *et al*., [30] in a previously equilibrated DEAE-cellulose column 10 mL ofthe dialysate was applied. 0.1 M citrate phosphate buffer (pH 5) was eluted with the enzyme. And for the protein content and tannase activity, the eluted fractions of about 5mL were analyzed. The active fractions underwent freeze-drying and were stored for further studies at 4° C. Determination of enzyme yield and purification fold were studied.

7.3 Gel filtration-

Al-Mraai, *et al*., [29] carried out gel filtration methodology by utilizing ÄKTA Pure 25 apparatuswith Superdex 200 10/300 GL column that was filled with agarose and dextran. Calibration of the column was done with an acetate buffer of pH 5. Injection of the concentrated sample of about 0.5 ml obtained from the column of ammonium sulfate precipitate was done, followed by filtrate of 0.22 µm by micro filtrate with separated peaks on a wavelength of 280 nm. The enzymatic activity (unit/ml) was then measured.

7.4 Electrophoresis-

Polyacrylamide gel electrophoresis was used for the purification and estimation of the enzyme with an absence of denaturation substances like sodium dodecyl sulfate (SDS) [29].

8 CHARACTERIZATION OF TANNASE ENZYME

Polyphenol compounds that are present on the tannin substrates can form certain complexes with intracellular and extracellular enzymes that are formed from the microbes. This in turnleads to the inhibition of the biodegradation enzymes which further results in the decline ofmicrobial growth as well as a reduction in the decomposition of organic matter [31]. Other than tannic acid, the tannase enzyme facilitates the breakdown of n-propyl gallate, ethyl gallate, methyl gallate, and isoamyl gallate. Both yeast tannase, as well as fungal tannase, was seen to show higher activity in degrading hydrolyzable tannins. However, both the tannase enzymes were seen to show a reduction in the activity ofbreakdown of naturally occurring tannins [32].

Depending on the source of the tannase enzymes from particular organisms, the properties of the tannase enzyme may be very diverse. The molecular weight of the bacterial tannase enzyme ranges from 46.5 kDa to around 90 kDa. [33] [34] [26] [35].

8.1 Molecular mass and carbohydrate content:

Tannase has a varied molecular weight that ranges from 186,000 Da to 300,000. Various Tannase of microorganisms

consists of more than two pairs with two sub-units. Such as tannase from *candida sp*.K-1 consists of two subunits of 120,000 Da each and, these subunits could be separated by SDS treatment and 2- mercaptoethanol [36]. Another Tannase from *A. flavus* was found to be of 192000 Da molecular weight with a carbohydrate content of 25.40 % [37]. An organism *Chryphonectria parasitica* had a molecular weight of its Tannase of about 240000 Da and its carbohydrate content was found to be 64% [36]. The molecular weight of tannase produced from *A. niger* and *Paecilomyces variotii* was found to be 95.49 KDa. [29] and 149.8 kDa [38] respectively. Tannase from *P.variable* is a dimer, made up of two subunits having a molecular weight of 158 kDa **[39].**

8.2 Effect of Temperature on Tannase

8.2.1 Optimum Temperature

Optimum temperature affects enzyme activity. Optimum temperature is defined as the temperature at which the activity of the enzyme is high. The optimum temperature varies from enzyme to enzyme and it also varies from species to species. Nevertheless, for many enzymes, the optimum temperature usually liesbetween 25 °C to 37 °C [40].

The foremost active tannase-producing fungus is *Aspergillus niger*. Tannase production by *A. niger* can be assessed at room temperature. However, experimentally, the optimum temperature at which tannase production by *A. niger* is high at 30 °C. [28].

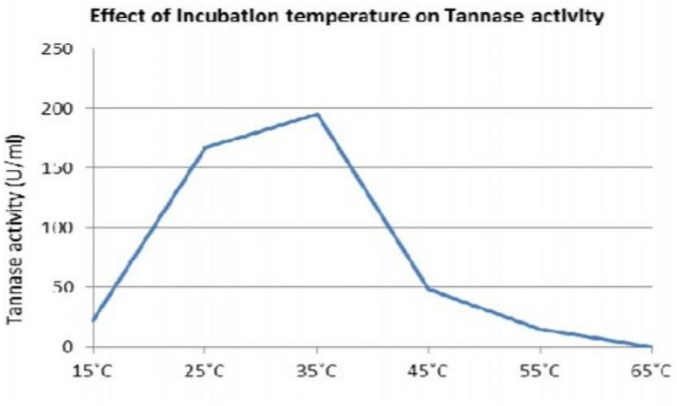

Fig. 3. Effect of Temperature on Tannase Activity in *A. niger*

Source: Lal and Gardner, 2012 [28]

In Bacteria, Tannase shows an optimal temperature between 30 °C and 40 °C [42]. Theactivity of immobilized tannase, crude tannase, and purified tannase was 90%, 85%, and 75 % respectively at 50 °C. Also, the activity of immobilized tannase, crude tannase, and purified tannase was 80 %, 60%, and 40% respectively at 4°C [43].

8.2.2 Temperature Stability

Temperature stability is a procedure where the enzyme is incubated with the buffer at differenttemperatures and then assayed. This procedure allows us to know at which temperature the enzyme is stable.

Tannase produced from *A. fumigatus* showed high stability at 60 °C. Tannase produced from *A. caespitosum* showed less stability at higher temperatures [44]. Tannase producedfrom *A. tamarii* showed utmost stability between 30-50 °C. Tannase produced from *A. niger* showed high stability between 40-60°C [29]. Tannase produced from *Bacillus cereus* showed maximum stability at 30 °C [45].

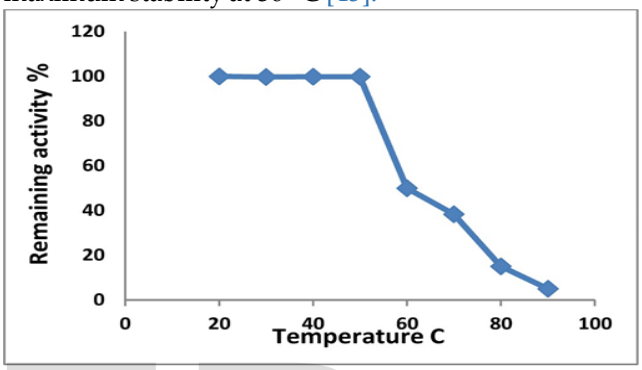

Fig. 4. Graph representing Temperature Stability in *A.niger*

Source: Fekaiki and Manhel, 2018 [29].

8.3 Effect of pH on Tannase

8.3.1 Optimum pH

Optimum pH is defined as the pH at which the activity of the enzyme is high. Optimum pH varies from enzyme to enzyme. The optimum pH for tannase is usually slightly acidic. Maximum tannase activity at a pH of 5.0 was seen in *A. niger*. [28]. In *Paecilomyces variotii*,crude tannase showed maximum activity at pH 6.5 and purified tannase exhibited maximum activity at pH 5.5 [46]. In *Bacillus* licheniformis, maximum activity was observed at pH 5.0 [47].

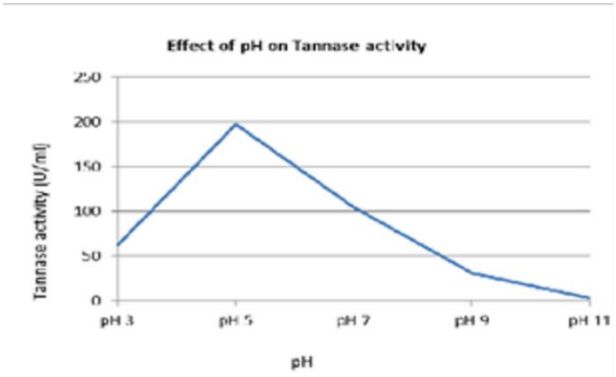

Fig. 5. Effect of pH on Tannase Activity in *A. niger*

Source: Lal and Gardner, 2012 [28].

8.3.2 pH Stability

pH stability is used to assess at which pH the enzyme is stable. Tannase produced from *Bacillus cereus* showed maximum stability between pH 4.5-5.0 [45]. Tannase producedfrom *P. restrictum* and *A. versicolor* was stablebetween pH range of 3.0-8.0. [44]. Tannase produced from *A. niger* showed maximum stability between pH 3.0-6.0 [29].

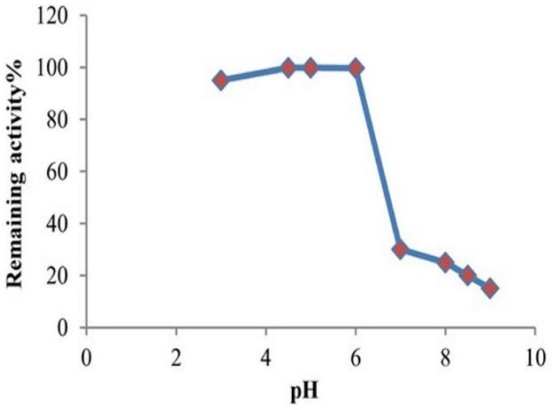

Fig. 6. Graph representing pH Stability in *A.niger*

Source: Fekaiki and Manhel, 2018 [29]

8.4 Kinetic parameters of the tannase enzyme

Vmax is characterized as the maximum rate of a chemical catalyzed response. Km is characterized as the substrate concentration at which the initial rate of response is half Vmax. The affinity of an enzyme towards a substrate can be determined using Km. When Km values are low, the affinity of an enzyme towards the substrate is high and it is the vice-versa when the Km values are high. Tannase produced by *Aspergillus niger* on Pomegranate Rind showed Km = 0.012 mM and Vmax =33.3 µmole/min [48]. Tannase produced by *Bacillus subtilis* PAB2 showed Km = 0.445 mM and Vmax =125.8 µmole/min [35]. Tannase produced by *Aspergillus tamarii* showed Km = 0.77 mM and Vmax = 682.8 U/mg [27].

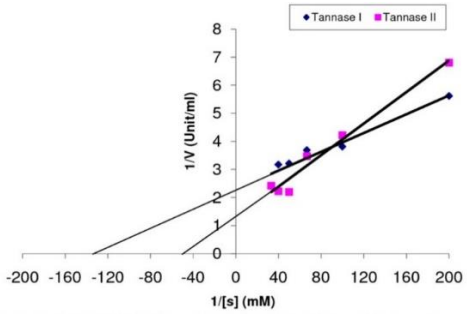

Fig. 7. Lineweaver Burk plot for the determination of Km Values in *Aspergillus tamarii.*

Source: Enemuor *et.al.*, 2010 [49].

9 THE SPECIFIC ACTIVITY OFTANNASE:

The amount of enzyme needed to release one micromole of gallic acid produced per minute is known as one unit (U) of the enzyme [28].

There are various methods used for the estimation of tannase activity. Each of the methods works on different principles and mechanisms. The most commonly used techniques are colorimetric or spectrophotometric [50]. A spectroscopic method is carried out using Rhodanine. Microbial tannase reacts with methyl gallate to produce gallic acid. The gallic acid released reacts with Rhodanine in the presence of an alkali to form a chromogen [51].

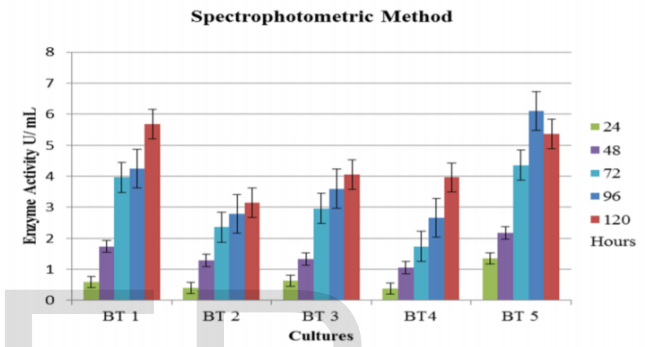

Fig. 8. Enzyme activity by spectroscopic method

Source: Brahmbhatt and Modi, 2015 [50]

A colorimetric method (Miller Method [52]), where tannic acid which acts as a substrate is broken down by tannase to form gallic acid and liberate glucose. The activity of tannase is estimated by estimating the amount of reduced glucose released using the 3, 5-dinitrosalicylic acid (DNS) reagent. [50].

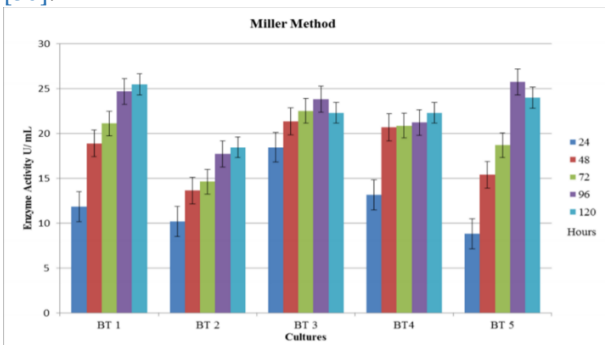

Fig.9. Enzyme activity by Miller method

Source: Brahmbhatt and Modi, 2015 [50]

The total protein content was determined using the Bovine Serum Albumin (BSA) standard curve. Specific Activity is calculated using the total activity and the total protein content. In *Aspergillus oryzae*, the specific activity of the immobilized

tannase was14.6 U/ mg protein, and the specific activity of the free enzyme was 72.2 U/mg protein [53]. In *Aspergillus niger*, the specific activity of tannase was 101.428 U/ml [28]. TanSg1 protein (Gene in the locus GALLO_1609 from *Streptococcus gallolyticus* UCN34 wascloned and expressed as an active proteinin *Escherichia coli* BL21[DE3], has a specific activity of 577 U/mg [54].

10 IMMOBILIZATION OF TANNASE:

Based on certain applications, the immobilization of the tannase enzyme can be done depending on one's need. Different methods such as physical adsorption, covalent coupling, encapsulation,entrapment, or crosslinking can be used to immobilize tannase. Different activities of tannase such as catalytic activity and its stability, etc. can be enhanced by immobilization. Encapsulation of Tannase is possible in alginate, carrageenan, or pectin gel matrices. For the production of gallic acid, immobilized tannase [38] [39] and its esters have been used in a non-aqueous medium; [55] [56]. Immobilization of *Aspergillus oryzae* tannase was seen to be possible because of the covalentbinding of its glycosidic part on chitosan, chitin, Dowex 50 W, DEAE- Sephadex A-25 [57]. Other non-covalently immobilized *A. niger* van tighem tannase was possible due to a bio affinity interaction. Chitin was found to have had the best support matrix with 88% operational stability after seven cycles of reactions. After six times reuse immobilized enzyme seemed to still retain its catalytic activity [58]. The immobilized tannase resulted in 86.9 % residual activity on alginate by the crosslinking-entrapment-crosslinking method after 30 times repeated use. It was seen that in covalently immobilized recombinant *L. plantarum* tannase when put onto glyoxyl agarose was seen to be retaining its catalytic activity even after a month. Calcium alginate and Amberlite [59] resins were also found to be the best acceptable support since the immobilized enzyme retained its activity efficiently. The tannase which was immobilized covalently onto magnetic nanoparticles composited with polyanilinecoated diatomaceous earth [60] was seen to be retaining its catalytic activity

11 STABILITY OF IMMOBILIZED TANNASE:

The optimum pH for immobilized enzyme ranges from pH 5.5 to 5.0, making it more acidic. The optimum temperature ranges from 50 °C for the free enzyme and 60 °C for the immobilized form. The immobilization process is seen to significantly improve thermal stability as well as stability over a varied rangeof pH. It is also seen that immobilized enzymes can retain their catalytic activity after many cycles of more than 50 [61].

Schons and co-workers recently described the immobilization of *Paecilomyces variotti* tannase by using the method of entrapment into definite polysaccharide matrixes [62] [63]. *Aspergillus awamori* can be used to produce tannase and the immobilization studies can be carried out using several carrierslike pectin, DEAE-Sephadex, Sephadex 25-40, Silica, and Chitin [64].

Immobilization of tannase from Phycomycetes, like *Rhizopus oryzae* was also reported using calcium alginate gel entrapment method mainly for the bioconversion of tannin-rich agro/forest-residues [65].

12 CONCLUSION:

The tannase enzyme is a resourceful enzyme that has a wide range of applications in several industries. However, due to higher production costs & lower yield, the current use of the enzyme remains very limited.

Several studies demonstrate the usage of the tannase enzyme for the treatment of tannin-richeffluents, such as wastewater that is emitted from industries. Nevertheless, attempts must be made to overcome certain complications during the procedure like the inhibition of the enzyme strains by various heavy metals that are present in the effluents.

Certain studies reveal that there is a probability for the development of tannase-based biosensors to determine the levels oftannic acid or gallic acid in food industries. This was put forth as controlled levels of tannic acid and gallic acid are known to be beneficial whereas higher levels can lead to the development of certain undesirable and unsatisfactory characteristics.

Generalization of the conditions to produce tannase enzyme cannot be described as it may differ from organism to organism. Considering the increasing demand for tannase for industrial applications, it is very important to develop high-yielding & cost-effective procedures to produce the enzyme.

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