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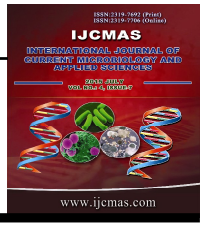

Original Research Article

Isolation and Screening of Tannase producing fungi

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Keywords

Tannin acyl hydrolase, Tannic Acid agar plate method, Zone of hydrolysis, Quantitative secondary screening, potent tannase producer

A B S T R A C T

Tannin acyl hydrolase (EC 3.1.1.20) which is commonly referred as tannase is one of the hydrolytic microbial enzymes. Tannase is an industrially important enzyme and has several applications in various industries such as foods, animal feeds, cosmetics, pharmaceutical, chemical, leather industries etc. Realizing the importance of the enzyme tannase, the present study was aimed to isolate and screen high tannase-producing fungi from different environmental sources such as from various tea Waste dump sites, agro-residue waste sites and, site nearby tannery industries. Forty one isolates of tannase-producing fungi were isolated from various sources; isolates were screened using tannic acid agar plate method. Zone of hydrolysis confirmed their Tannin degrading ability. Twenty nine isolates were screened as tannase producers. These isolates were subjected to quantitative secondary screening for tannase production under stationary condition. Accordingly, four Isolates, which exhibited high tannase activity, were chosen to undergo final screening. Thus the present study helped in screening the potent tannase producers from various sources.

Introduction

Tannins are naturally occurring polyphenolic compounds with varying molecular weights that occur naturally in the plant kingdom. These phenolic compounds differ from others by having the ability to precipitate proteins from solutions. Tannins can be divided into two major groups on the basis of their structure and properties; they are hydrolysable tannins and condensed tannins, an intermediate group also exist that combines both characteristics of hydrolysable tannins and condensed tannins and are called Catechin tannins. Tannins are abundantly present in natural plants like monocots, tea, coffee, sorghum, berries, nuts, pomegranates, legumes, some herbs and spices like cloves and cinnamon, palm kernel, Phyllanthus emblica (amla) and other different species of plants or plant products which are used for human consumption (Bhat et.al., 1998).Tannin in form of Catechin (Flavan-3-ols) is present in tea, cocoa, acacia and catechu plants. Catechins are present in all types of tea. Tannins are widespread in the plant kingdom, occurring mostly in leaves,fruits,bark and wood and are often considered nutritionally undesirable (Chung et.al.,1998;Murugan and

saleh,2010).Tannins inhibit growth of various microorganisms, by precipitating many enzymes (Field and Lettinga,1992)

Tannins are therefore known for their antimicrobial property and are resistant against microbes to protect plant bodies; they are toxic and release bacteriostatic compounds making non-reversible action with proteins (Bhat et.al., 1998). Although having antimicrobial activity, tannins serve as a nutrient compound or substrate for some microorganisms that utilize it with the help of the hydrolytic enzyme named Tannin acyl hydrolase. Tannic acid is a heteropolymer composed of glucose and gallic acid in 1:9 ratios and has various commercial applications. Industrial bioconversion of tannic acid is achieved with Tannase (Mondal et.al, 2001).

Tannase (Tannin acyl hydrolase, E.C.3.1.1.20) catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins, as tannic acid, releasing glucose and gallic acid. Tannase is an extracellular inducible enzyme. Bacteria, Yeast and filamentous fungi (Bertolin, T et.al., 2001, Cavalitto, S. et.al., 1996, Aoki, K et.al., 1976) are known tannase producers. Most of the commercial applications of tannase are in the manufacturing of instant tea, tannase is used to eliminate water-soluble precipitates called “tea cream” (Sanderson et.al., 1974).

Tannase is also used as a clarifying agent in some fruit juices and in cold drinks with coffee flavour where its use applies to the removal of the phenolic compounds present in plant materials. It has application in detanification of food and increasing the nutritive property of feed provided to cattle and also in bioremediation by cleaning up the tannins from effluents of industries specially from leather industries. In the production of beer, tannase could be used to remove tannins, since they are present in low quantities, specially as anthocyanidins.

Tannase also has a potential application in the olive oil wastewater treatment and partial decolourization by enrichment cultures because of its capability to hydrolyze tannic acid and gallic acid esters. Tannery effluents contain high quantities of tannins, mainly polyphones, which are dangerous pollutants; here the use of 3 tannase represents a cheap and effective treatment for the removal of these compounds. In addition this enzyme is also used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid ester. One of the most important applications of tannase is the production of gallic acid from plant by-products rich in tannins (Coggon et.al., 1975). Gallic acid is an important intermediary compound in the synthesis of the antibacterial drug, Trimethoprim, used in the pharmaceutical industry, in recent years it is find out that bacteria producing tannase have been associated with colon cancer allocating the possibility of bacterial tannase as biomarker for colon cancer (Lekha and Lonsane, 1997; Das Mohapatra et.al., 2012). Many reports regarding fungal and bacterial tannase are available and it is evident that fungal tannase are exploited and studied extensively as compared to the bacterial tannase, however reports on bacterial tannase are also available

Nevertheless there are also scientists working on isolation, identification and screening the novel species of actinomycetes from different samples or ecosystems. Thus by realizing the importance of the enzyme tannase, the present study aimed to isolate and screen high tannase-producing fungi from various environmental sources. The characteristics of the organisms and the

activity of tannase produced by them were studied.

Materials and Methods

Enrichment and isolation of tannase producing fungi

Soil samples of various tea waste dump sites, agro-residue waste site and site nearby local tannery industries were collected and enriched in Tannin containing medium. The enriched soil samples were diluted and plated on Potato Dextrose agar media (Hi Media, India) plates, and incubated for 72 hours at 30ºC. Fungal colonies developed were isolated and purified by repeated sub culturing (Fig, 1).

Total of 41 fungal isolates were obtained and they were screened for the production of tannase by spot inoculating the culture to a tannin agar medium, containing 1gm yeast extract, 0.5 gm Nacl, 0.5 gm tannin, 1 gm sucrose, 3gm Agar- agar, D/W 100 ml. The inoculated plates were incubated for 72 hours at 30°C.

The plates were flooded with 0.01 M $FeCl_3$ which reacts with tannic acid and forms a brown colour (R. Kumar et. al., 2010). The tannase producing fungal isolates were subjected to quantitative secondary screening by performing tannase assay by Mondal's colorimetric method. Fungi were preserved on Potato Dextrose agar slants, regular sub culturing were done after every 30 days.

Characteristic studies of isolates

Morphological features were observed from the plates according to their growth pattern and zone formations by the fungal isolates. Microscopic examination of fungi was done with lacto phenol cotton blue

Production of tannase

Enzyme production was carried out in 100 mL Erlenmeyer flask containing 50 mL tannic acid broth medium (yeast extract 1 gm, Nacl 0.5 gm, tannic acid 0.5 gm, sucrose 1 gm, D/W 50ml). The medium was sterilized at 121°C at 15 lbs for 15 min, Tannic acid was filter sterilized and added after sterilization. The pH of the medium was adjusted to 4

Preparation of Spore suspension

The fungal growth from the Potato Dextrose slants was scrapped off and suspended into 10 mL sterile 0.1 % Tween 80 solution. Suspension was mixed using cyclomixer to break cell aggregates. The spore suspension was serially diluted and plated on PDA for determination of colony forming unit.

Inoculum Preparation

5% v/v spore suspension of the isolates was used as inoculum in 50 mL tannic acid medium. Cells were removed by filtration through Whatman filter paper No. 1. Cell-free broth containing crude enzyme i.e. filtrate was used for estimating the tannase activity.

Tannase Assay

Standard graph of tannic acid was prepared by making the various concentrations of 1% tannic acid ranging from 1mg/ml to 10mg/ml, from every concentrations of tannic acid 0.5 ml of tannic acid, in 0.2 M acetate buffer (pH 5.0) mix with 2 ml of Bovine Serum Albumin (1 mg/ml) which precipitate the tannic acid. A control reaction was also carried out with heat denatured enzyme. The tubes were then centrifuged (5,000 x g, 10 min) and the precipitate was dissolved in 2 ml of SDS –

triethanolamine (1% w/v, triethanolamine) solution and the absorbance was measured at 530 nm after addition of 1 ml of $FeCl_3$ (0.13 M). (Mondal et.al., 2001)

One unit of the tannase was defined as the amount of enzyme, which is able to hydrolyse 1µ mole of ester linkage of tannic acid per min at specific condition.

Screening of potential tannase hydrolysing fungal isolates

For isolated fungal cultures tannase assay 5% of the spore suspension of each culture was inoculated in tannin broth and incubated it at 30°c for 72 hours. After incubation the broth was filtered and filtrate was used as source of crude enzyme. For the tannase assay 0.5mL of crude enzyme solution was incubated with 0.5 ml of 1.0% (w/v) tannic acid, in 0.2 M acetate buffer (pH 5.0). The control tube was run without the enzyme.

Amount of tannic acid hydrolyzed by enzyme was calculated by subtracting amount of residual tannic from the un-hydrolyzed tannic acid in the control tube.

The activity was calculated using following formula: Enzyme activity (U/mL) =

$$\frac{\text{microgram of tannic acid hydrolyzed by enzyme x 1000}}{1701(\text{Mol.wt of enzyme}) \times \text{ml of enzyme}}$$

The amount of tannic acid hydrolyzed by enzyme was obtained from standard graph.

Results and Discussion

Enrichment and isolation of tannase producing fungi

A total of 29 (SK1 to SK29) fungal isolates were obtained which exhibited zone of tannin hydrolysis (Fig.2), thus confirming their ability to degrade tannin. The fungal isolates were preserved on Potato Dextrose Agar slants. Brahmbhatt D., et.al., 2014 isolated seven tannin hydrolyzing fungi from tea waste dump sites and agro residue waste sites.

Characteristic studies of isolates

Morphological features (colony characteristics) based on size, shape, margin, texture, opacity; elevation and pigment (Table 1 and Table 2) were observed. Zone of tannin hydrolysis was measured after 72 hours incubation (Table 2).

Microscopic examination of fungal isolates revealed that the isolates Sk7 and Sk12 belonged to *Aspergillus* species, while Sk4 and Sk11 belonged to *Penicillium*, species. Gustavo et.al, 2001 report the isolation of tannin hydrolyzing *Aspergillus niger*.

Secondary Screening of potential tannase producing fungal isolates

Twenty nine fungal isolates were screened on the basis of their tannase producing efficiency under stationary condition. . The results are as shown in Fig. 3. The isolates Sk4, Sk7, Sk11 and Sk12 exhibited maximum tannase activity viz. -54.6 U/ml 76.4U/ml, 60.5 U/m 53.4U/ml respectively. On the basis of morphology and microscopic examination Sk7 as *Aspergillus fumigatus*, and Sk12 were identified as *Aspergillus carbonarius* while Sk11 as *Penicillium* lividum westling and Sk4 as *Penicillium citrinum* thom. The microscopic observation is as shown in Fig.4.

The fungal isolate Sk7 exhibiting maximum tannase activity was selected for further studies. Similarly Hamdy H.S.and Fawzy E.M. (2011) reported economic production of tannase by Aspergillus niger.

Table.1 Morphological characterization of fungal isolates

Sr.no.	Fungal isolate	Size	Form	Margin	Elevation	Texture	Opacity	Pigment
1	Sk1	Small	Circular	Entire	Raised	Rough	Opaque	Black
2	Sk2	Small	Circular	Entire	Raised	Rough and dry	Opaque	Off White
3	Sk3	Small	Circular	Entire	Raised	Rough and dry	Opaque	White
4	Sk4	Big	Rhizoidal	Undulate	Raised	Rough and dry	Opaque	Brown
5	Sk5	Small	Circular	Lobate	Umbonate	Rough	Opaque	Green
6	Sk6	Small	Irregular	Entire	Raised	Rough	Opaque	Green
7	Sk7	Big	Irregular	Entire	Raised	Rough	Opaque	Green
8	Sk8	Small	Rhizoidal	Entire	Raised	Rough	Opaque	Light green
9	Sk9	Small	Filamentous	Entire	Raised	Rough	Opaque	Green
10	Sk10	Small	Circular	Lobate	Raised	Smooth	Opaque	Dark Black
11	Sk11	Big	Irregular	Undulate	Umbonate	Rough	Opaque	Light Green
12	Sk12	Big	Rhizoidal	Undulate	Umbonate	Rough and dry	Opaque	Black
13	Sk13	Small	Circular	Entire	Raised	Rough and dry	Opaque	Green
14	Sk14	Small	Irregular	Entire	Umbonate	Smooth	Opaque	Yellow
15	Sk15	small	Circular	Entire	Raised	Dry	Opaque	Orange
16	Sk16	Small	Circular	Entire	Raised	Rough	Opaque	Green
17	Sk17	Big	Filamentous	Entire	Raised	Rough	Opaque	Black
18	Sk18	Small	Circular	Entire	Raised	Rough and dry	Opaque	Brown
19	Sk19	Small	Circular	Undulate	Raised	Rough and dry	Opaque	Black
20	Sk20	Big	Filamentous	Entire	Raised	Rough	Opaque	Orange
21	Sk21	Big	Irregular	Entire	Raised	Rough	Opaque	Light Orange
22	Sk22	Small	Irregular	Undulate	Umbonate	Rough	Opaque	Off white
23	Sk23	Small	Circular	Lobate	Umbonate	Rough and dry	Opaque	Yellow
24	Sk24	Small	Irregular	Undulate	Raised	Rough and dry	Opaque	Off white
25	Sk25	Small	Circular	Lobate	Raised	Rough and dry	Opaque	Yellow
26	Sk26	Small	Circular	Entire	Raised	Smooth	Opaque	White
27	Sk27	Big	Rhizoidal	Entire	Raised	Rough and dry	Opaque	Off white
28	Sk28	Small	Irregular	Entire	Raised	Rough	Opaque	White
29	Sk29	Small	Circular	Entire	Raised	Rough	Opaque	White

Table.2 Screening of fungal isolates for tannin hydrolysis

Sr.No	Fungal isolate	Zone of tannin hydrolysis (mm)
1	Sk1	15
2	Sk2	10
3	Sk3	12
4	Sk4	15
5	Sk5	20
6	Sk6	20
7	Sk7	25
8	Sk8	04
9	Sk9	25
10	Sk10	30
11	Sk11	20
12	Sk12	25
13	Sk13	30
14	Sk14	20
15	Sk15	10
16	Sk16	10
17	Sk17	15
18	Sk18	15
19	Sk19	15
20	Sk20	20
21	Sk21	20
22	Sk22	15
23	Sk23	25
24	Sk24	15
25	Sk25	20
26	Sk26	20
27	Sk27	20
28	Sk28	20
29	Sk29	20

Figure.1 Isolation of fungi from different tannin rich sites

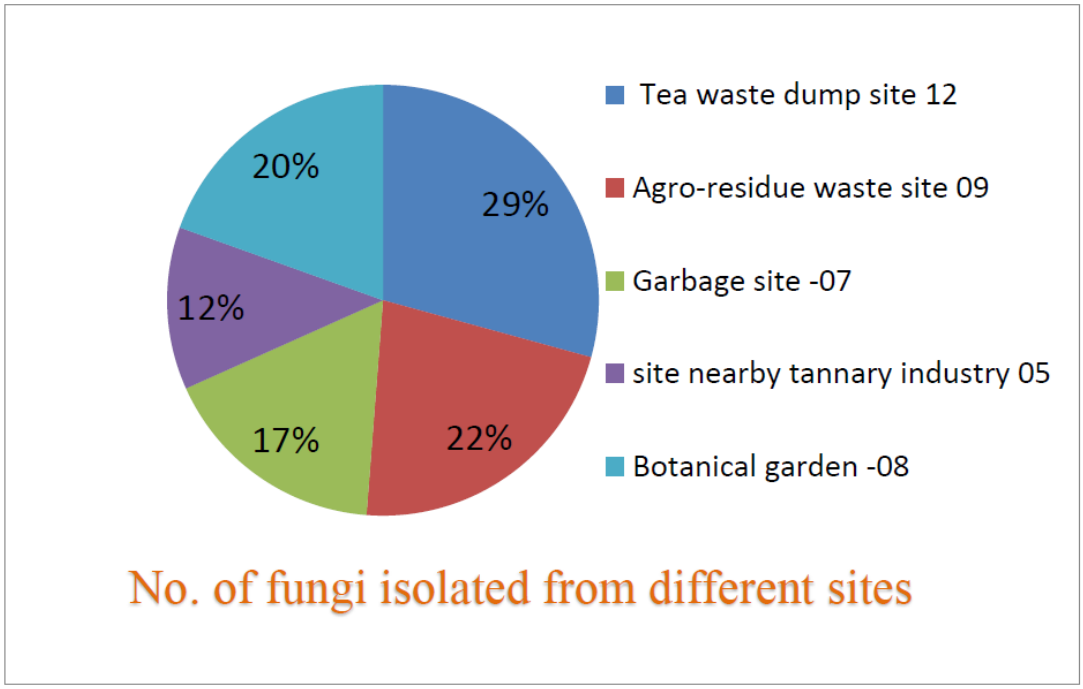


Figure.2 Fungal Isolates showing zone of tannin hydrolysis

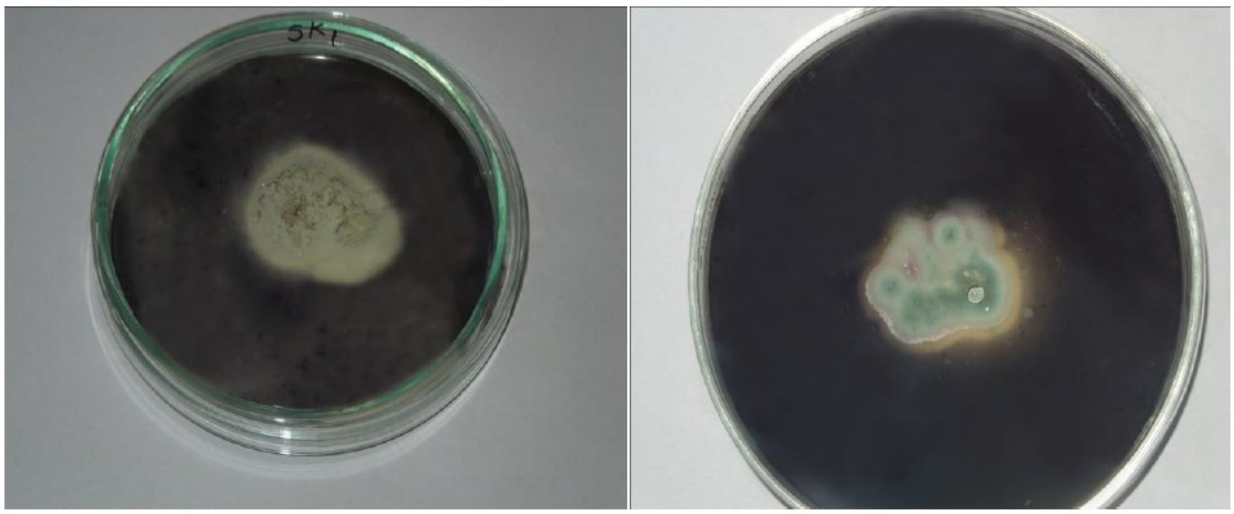
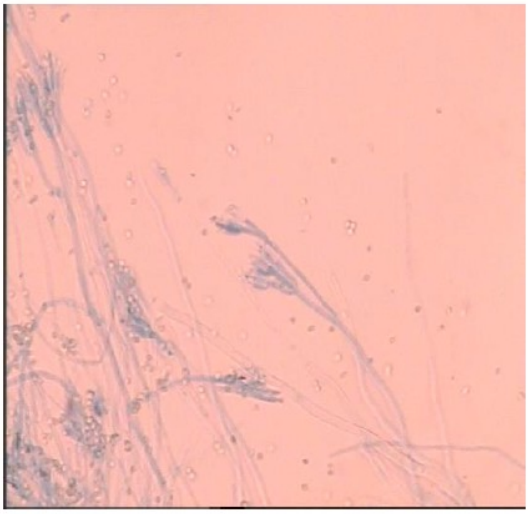
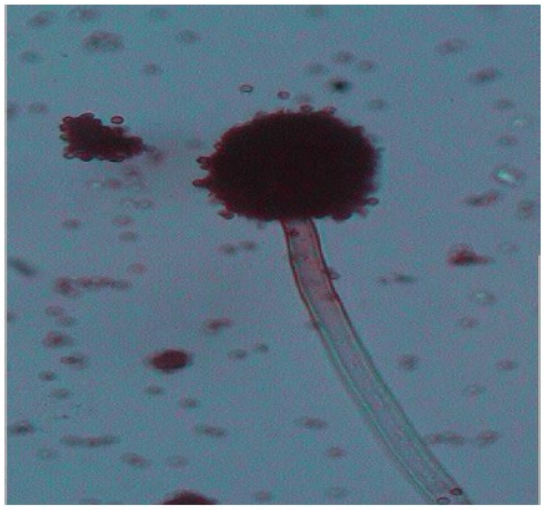


Figure.3 Morphology of the potential tannase producing fungi

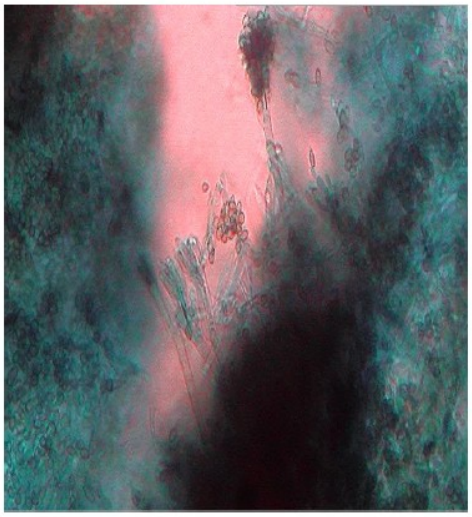


Sk4: *Penicillium citrinum Thom*

Sk7: *Aspergillus fumigatus*

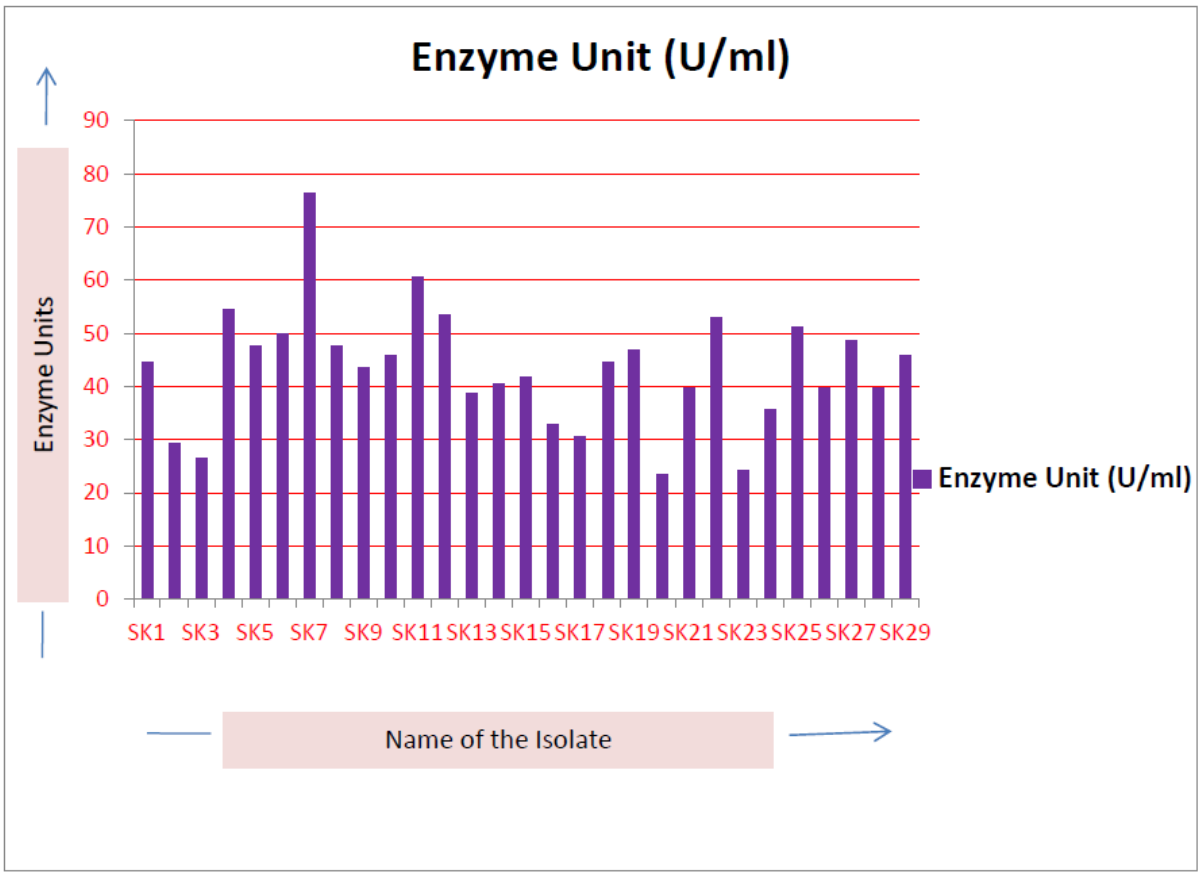


SK12:*Aspergillus carbonarius*



SK11: Penicillium lividum westling

Figure.4 Quantitative tannase profile (U/mL) of fungal isolates



K.Murugan and Saleh A (2010) report the application of Tannase produced by Aspergillus candidus for removal of tannin from the tannery effluent.

Thus in the present study, various fungal tannase producers were isolated from various tannin rich sites, screened on the basis of tannase producing efficiency and their morphological, and microscopic characters were studied. The potential isolate Sk 7, identified as Aspergillus fumigatus , will be used for tannase production and purification and its application will be exploited in food industry for debittering of fruit juices, clarification of wine etc. It will also be applied for treatment of tannery effluent.

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