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Taxonomical Characterization of Tannase Producing Fungi Collected from Local Tannery Effluent Water from Pedaravuru Village, Tenali, Guntur, Andhra Pradesh and India

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Abstract: ***The study was conducted to isolate and identify tannase producing fungi from tannery effluent water. Water or effluent samples were collected under the aseptic conditions screened with tannic acid agar media. The cultured plates had shown zone of clearance indicates that the microorganism had have ability to hydrolyse the tannic acid by releasing the tannin acyl hydrolase enzyme. According to the zone of clearance results, isolates were further sub-cultured to preserve in the form of pure culture. Chosen fungal isolates further screened for tannase enzyme activity, biomass weight, and production of gallic acid using shake flask level. Among the screened fungal isolates four was shown good tannin degradation, where the best isolate named as UK-2 further identified and characterized by using phylogenetic characterization and named the strain as Aspergillus niger KF514876. Further the strain which deposited in the NCBI was used to produce the gallic acid under submerged fermentation. It has shown good and enhanced tannase activity with highest production of gallic acid with tannase activity 68.5U/mL.***

Keywords: *Aspergillus sps., tannase, gallic acid*, NCBI, Tannin acyl hydrolase enzyme, phylogenetic tree, tannins, Biomass

1. Introduction

Tannin acyl hydrolase enzyme acts on polyphenolic compound named as tannins present in the plants as a secondary metabolite. Tannins are more toxic due to this reason; tannase enzyme converts the tannin into useful derivatives namely oligomeric tannins such as gallic acid (Milva Pepi et al., 2013). Tannase enzyme is tannin acyl hydrolase (EC 3.1.1.20) hydrolyses the galloyl residues of galloyl esters are having wide range of applications includes production of gallic acid used as an intermediate for trimethoprim synthesis in pharmaceutical industry. Pyrogallol or ester galates, used as the food preservatives in the food industry. Tannins also used to clarify the beer and beverages, manufacture of instant tea, manufacture of coffee flavored drinks, reduction of anti-nutritional effects of tannins in animal feed and high-grade leather tanning. Used as an antioxidant in fats, oils and beverages and in decontamination of tannery effluents. In the tea leaves polyphenols are not solubulized in the cold water, but the tannin acyl hydralases has shown promising role in the conversion tea into soluble form.

Tannin acyl hydrolase been produced by different organisms such as plants, microorganisms includes actinomycetes, fungi, bacteria, and yeast, among them fungi are the promising producer of the tannase. *Aspergillus sp.* is the prime source from the microbial world produce tannase at industrial level and its application among the other microbial organisms. Most of the tannase producing fungi are of terrestrial origin and present research findings on effluent source, also used for tannase production. In this context the present study led to the debt of *Aspergillus niger,* isolated from tannery effluent environment as a source for tannase.

2. Materials and Methods

Collection of Tannery Effluent Samples: The effluent samples were collected from Pre-Tanning (PrT1, PrT2, PrT3, PrT4) and Post-Tanning (PoT5, PoT6, PoT7, PoT8) stages of leather processing tannery industry, located at Pedaravuru village, Tenali town, Guntur district, Andhra Pradesh, India. The samples were collected from depth of the 15-30 cm from the surface level and transferred the sample into a sterile bottle without any foam formation.

Screening for Tannase Producing Microorganism: The collected effluent samples (Pr.T1, Pr. T2, Pr.T3, Pr.T4, Po.T5, Po.T6, Po.T7, Po.T8) were serially diluted with sterile distilled water and spread on agar plates containing enrichment medium and incubated at 30ºC to screen the tannase producing organisms. The colonies were streaked on modified nutrient agar and potato dextrose media to get pure cultures for further study. In order to get high yield tannase producing organism, all the pure cultures were streaked again on enrichment medium. The potent tannase producing microorganism was selected based on diameter of zone of clearance on tannic agar medium.

Tannase Activity: Tannase activity was assayed by the detection of liberated gallic acid from tannic acid. The released gallic acid was determined as one unit of tannase activity (U) was defined as the amount of enzyme that liberates 1.0 µmol gallic acid per min under assay conditions.

Storage of Culture Samples: The colonies which showed maximum tannase activity were identified and prepared in

50% glycerol stocks and stored in -20°C for long time preservation.

Morphological Identification of Isolated Strain: The identification of the selected fungal strain was done with macroscopic and microscopic observations in accordance with the colony diameter, colony texture, and pigmentation, structure of mycelia, branching, and arrangement of conidia, shape and size of conidia.

Identification of isolated Strain using FAME (Fatty acid methylester) Analysis: The FAME analysis was carried out by MIDI, Sherlock Microbial Identification System.

Molecular Identification by 18S rRNA Analysis: The most reliable tool to identify the unknown fungal species is by sequence the gene coding for 18S rRNA. The gene coding for the 18S rRNA was amplified using Polymerase Chain Reaction and the amplified product has been subjected to sequencing and the sequence obtained was compared with the sequence obtained from the Nucleotide Database of NCBI.

Construction and Phylogenic Tree Analysis: Construction of phylogenetic tree for the isolate UK-2 by determining the 5.8S ribosomal RNA gene and internal transcribed space 2 of the isolate *Aspergillus niger*UK-2 and compared with the related strains have been derived during the evolution. The evolutionary relationships among the sequences were depicted by placing them as outer branches on a phylogenetic tree.

Criteria for Species Identification: Identification of species through sequence similarity basis was performed according to the criteria used by (Bosshard et al., 2006) which states the following selection parameters: (a) when the percentage similarity of the query sequence and the reference sequence is 99% or above the unknown isolate would be assigned to reference species; (b) when percentage similarity is between 95–99%, the unknown isolate would be assigned to the corresponding genus; (c) when percentage similarity is less than 95 %, the unknown isolate would be assigned to a family.

3. Results and Discussion

Isolation and screening of tannase producing organisms: The present study is aimed at screening the organism with the ability for the maximum tannase production; hence both the Pre- Tanning (PrT1, PrT2, PrT3, and PrT4) and Post-Tanning (PoT5, PoT6, PoT7, PoT8) samples have been collected and inoculated in enriched media (Mutiat Adetayo Omotayo et al., 2023). Out of eight enriched samples, four samples (PoT5, PoT6, PoT7, and PoT8) were found to have distinct cell growth. These enriched samples were serially diluted and spread on agar plates containing 0.5% tannic acid as substrate (Slany et al., 2020). As a result, 60 pure colonies have been isolated according to their morphology. Among the isolated samples, 55 have been found to be fungi and the remaining 5 are bacterial species. They have been further examined for their activity on agar plates with tannic acid (1%) containing medium. Among the isolated 55 types of fungi, only 10 has shown good zone of inhibition and labeled as UK-1, UK-2, UK-3, UK-4, UK-5, UK-6, UK-7, UK-8, UK-9 and UK-10. Out ten fungi, UK-2 (plate-2) has shown maximum zone of inhibition of 2.35 cm (Fig. 4.1.1) and were selected for further study (Girdhari and Peshwe (2015)). The high tannase activity of UK-2 was further confirmed by tannase assay (Bardi et al., 2019). On the other hand, bacterial samples showed no significant growth on the tannic agar plate. Similar results were also reported in previous studies. The macroscopic and microscopic examinations have revealed that most of the isolated strains belong to *Aspergillus spp* (Saad et al., 2023). The reports of Mahadevan and Muthukumar (1980) clearly reveal that the fungal species like *Aspergillus, Cylindrocarpon, Penicillium, Chaetomium, Fusarium, Rhizoctonia* and Trichoderma were capable of degrading tannery waste constituents. Bradoo et al. (1996), has also revealed in his studies that the zone of clearance had been formed due to hydrolysis of tannic acid to gallic acid and glucose, thus leading to decrease in opacity of the media.

Figure 4.1.1: Zone of clearance of UK-2 plated on tannic agar medium: Plate-1 shows that the zone of clearance of tested UK-2 stain. Plate -2 kept as control growth culture. Among the isolated 55 types of fungi, only 10 had shown good zone of inhibition and labeled as UK-1, UK-2, UK-3, UK-4, UK-5, UK-6, UK-7, UK-8, UK-9, and UK-10 whereas in the case of bacteria no significant growth on tannic agar plates was observed.

#Experimental values are mean of triplicates test.

Selection of high tannase enzyme producing isolate by using enzyme assay: One unit of tannase activity is defined as the amount of enzyme required to liberate 1mM of gallic acid/min under defined conditions (Majewska et al., 2022; Alshaymaa et al., 2023) and expressd as U/ml. Out of ten

fungal isolates, the UK-2 strain showed highest enzyme activity of 5.12 ± 0.05U/ml (Fig.4.1.2) (Saad et al., 2023). This result is consistent with the results of the zone of diameter experiment, explained in the previous section. Murugan et al. (2007) also reported in their study that among their isolates the best tannase producer was *A. Niger* MS101 (16.77 U/ml) followed by *A. Niger* MTCC2425 (9.7 U/ml) and the poorest tannase producer was found to be *Penicillium,* which produced only 3 U/ml (Azzaz et al., 2021). The present reports are also consistnet with reports of Lekha and Lonsane, (1994) which states only about the extracellular production of tannase during the entire fermentation period.

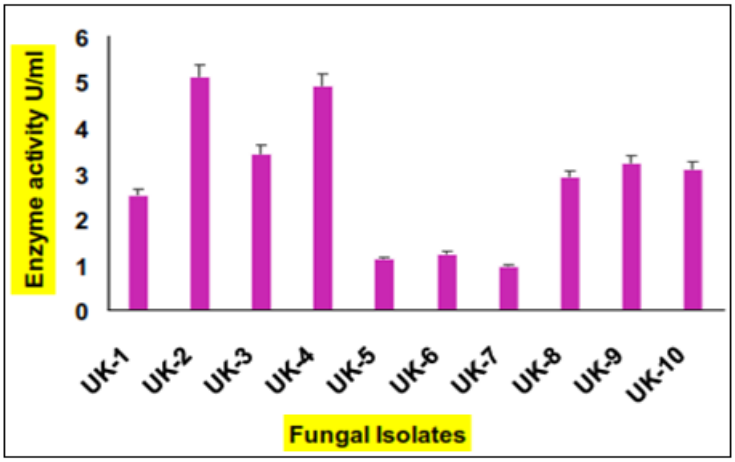

Figure 4.1.2: Tannase activity of 10 isolated fungal strains: The diameters of zone of clearance of these 10 fungal isolates were shown in the Fig. 4.1.2. Among all of them, the strain UK-2 showed a maximum diameter of 2.35 cm. Further, all the 10 screened strains are cultivated in broth cultures to compare the tannase activity.
#Experimental values are mean of triplicates test.

Taxonomic identification: ***Morphological identification of Aspergillus niger:*** Colony morphology of UK-2 on a petri plate initially showed a white colony within 24 hours of incubation and then it turned into blackish brown color (Avneet Kaur and Priya Katyal, (2018)). When the petri plate was inverted, the colony appeared to be pale yellowish in color with a conidial head, which is similar to the reports of Verweij et al., (2007) and Juliana Silva de Lima et al., (2014). Therefore, the isolated strain UK-2 is assumed to be species that belong to the genus *Aspergillus.* The identification of *Aspergillus* spp. Was done based on the morphological taxonomy, in accordance with the colony diameter, conidial color, mycelium texture, soluble pigment, shape and size of conidiophores, vesicles, metulae, phialides, conidia, their texture and color, previously used for the identification of genus *Aspergillus* as reported by Girmay Aragaw et al., (2023) and Roaf Ahmad Rather et al., (2022).

Identification of isolated strain using FAME (Fatty acid methyl ester) analysis: FAME analysis provides the fatty acid profile using a standardized procedure for identification the methyl esters of fungal fatty acids on gas chromatography. The resulting chromatogram provides a quantitative profile of an organism's fatty acids that can be used for identification of the genus of the fungal cultures using FAME by MIDI (Newark, Delaware). The FAs profile obtained in the present study, was higher than the one found for *Aspergillus* species by Blomquist et al. (1992); Stahl and Klug, (1996) and Nemec et al. (1997). The most common and abundant FA extracted were Palmitic acid (C16:0), Estearic acid (C18:0), Oleic acid (C18:1) and Linoleic acid (C18:2), which often correspond to 95% of the *Aspergillus spp*. total FA content (Fig.4.2.2). The studied isolated sample UK-2 also contained FA of carbon chain lengths ranging from 12-20 similar to the study of Blomquist et al. (1992); Stahl and Klug, (1996) and Nemec et al., (1997).

FAs include Palmitoleic acid (C16:0), Margaric acid (C17:0), Linoleic acid (C18:3), which represented less than 5% of the total FA content. Some *Aspergillus* species offered different quantities and profiles of FA and also differed in the relative concentrations of each type. The graphic dispersion, based on relative scores of the first two Analysis principal components (APC) shows the differences between the most similar *Aspergillus* species and Fig.4.2.2 shows the distribution of eight FAs and demonstrates the presence of four distinct groups (C10.0, C13.0, C15.0, C16:1, C16:0, C17:1, C16:975, C18:2, C18:1, C18:0, C18:0, C18:1).

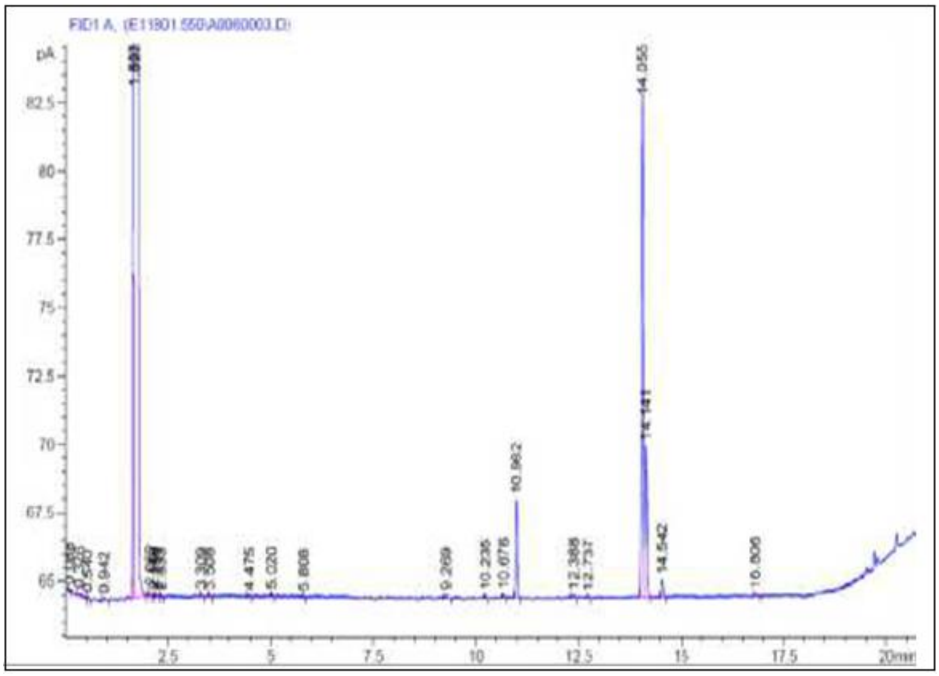

Figure 4.2.2: FAME analysis of tannase producing fungal strain: The analyzed fungi contained FA of carbon chain lengths ranging from 1220. The most common and abundant FA extracted were Palmitic acid (C16:0), Estearic acid (C18:0), Oleic acid (C18:1) and Linoleic acid (C18:2), which often corresponded to 95% of the *Aspergillus spp*. Total FA content (Fig.4.2.2).

Table 4.2.2 FAME analysis of isolated UK-2 strain: The graphic dispersion, based on relative scores of the first two Analysis principal components (APC) shows the differences between the most similar Table 4.2.2. and Fig.4.2.2 shows the distribution of eight FAs and demonstrates the presence of four distinct groups (C10.0, C13.0, C15.0, C16:1, C16:0, C17:1, C16:975, C18:2, C18:1, C18:0, C18:0, C18:1).

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
[illegible]	221	[illegible]		4.045			< min rt	
[illegible]	1600	0.030		4.465			< min rt	
[illegible]	661	0.045		4.875			< min rt	
[illegible]	[illegible]	0.050		5.626			< min rt	
1.663	594915	0.018		6.942			< min rt	
1.697	3.981E+8	0.032		7.001	SOLVENT PEAK		< min rt	
2.062	912	0.030		7.67[illegible]			< min rt	
[illegible]	368	0.029	---	7.834		---	< min rt	
[illegible]	321	0.026		8.001			< min rt	
[illegible]	411	0.031		[illegible]			< min rt	
3.309	383	0.030	---	9.968		---		
[illegible]	411	0.041		10.245				
4.475	455	0.034	1.063	11.413	10:0 3OH	0.30	ECL deviates -0.005	
5.020	377	0.027	---	11.952		---		
5.808	354	0.032	1.006	12.612	13:0 ISO	0.21	ECL deviates 0.000	Reference 0.011
9.269	914	0.040	0.950	14.992	15:0	0.54	ECL deviates -0.005	
10.235	800	0.042		15.561				
10.676	764	0.038	0.943	15.820	16:1 CIS 9 (w7)	0.45	ECL deviates 0.005	
10.962	2000[illegible]	0.043	0.942	16.000	16:0	11.71	ECL deviates 0.000	Reference 0.015
12.388	851	0.046	0.941	16.801	17:1 CIS 8 (w8)	0.48	ECL deviates 0.009	
12.737	400	0.030	0.941	16.979	Unknown 16.975	0.23	ECL deviates 0.004	
14.055	[illegible]	0.046	0.941	17.725	18:2 CIS 9,12/18:0a	64.02	ECL deviates 0.005	
14.141	33117	0.046	0.941	17.762	Sum In Feature 5	19.41	ECL deviates -0.011	18:1 CIS 9 (w9)
14.542	3710	0.046	0.942	17.987	18:0	2.17	ECL deviates 0.013	
16.806	825	0.052	0.944	19.261	18:0 2OH	0.48	ECL deviates 0.000	
---	33117	---	---	---	Summed Feature 5	19.41	18:1 CIS 9 (w9)	18:1 (w5)

ECL Deviation: 0.007 Reference ECL Shift: 0.013 Number Reference Peaks: 2

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Molecular identification of UK-2 by 18S rRNA analysis: The molecular techniques are being very much employed for classification and characterization of various fungal species. One of the molecular techniques the polymerase chain reaction (PCR) has been significantly used to study the complexities of fungi. The simplicity and speed of PCR techniques, coupled with the genomic studies, increased the understanding of fungal taxonomic groupings, evolutionary relationships and functional properties (Mutiat Adetayo Omotayo et al., 2023). This PCR-based technique is also used to develop the characteristics of DNA which further encodes the ribosomal RNA (rDNA) and in discriminating fungi at inter and intraspecific levels. The above statement also been reported by Ueda et al., (2014).

GCTCTATCCCCAGCACGACAGGTTTAACAAGATTACCCGGGCCTCTCGGCCAAGGTG

ATGTACTCGCTGGCCCTGTCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGC

ATCACAGACCTGTTATTGCCGCGCACTTCCATCGGCTTGAGCCGATAGTCCCCCTAAG

AAGCCAGCGGCCCGCGGACGCGGACCGGGCTATTTAAGGGCCGAGGTCTCGTTCGT

TATCGCAATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCAGCATC

CAAAAGATCAAGAAAGAGCTCTCAATCTGTCAATCCTTATTTTGTCTGGACCTGGTGA

GTTTCCCCGTGTG

Figure 4.2.3.18S rRNA gene sequence of UK-2 isolate: Genomic DNA was extracted by a standard protocol and analyzed on 0.8% agarose electrophoresis (Fig.4.2.3.) PCR was carried out to amplify the 18s rRNA gene of the extracted genomic DNA of UK-2 fungi using Gene Amp PCR with the forward and reverse primers. Forward Primer 5'GAGTTTGATCCTGGCTCAG-3' and Reverse primer 5'-GAAAGGA GGTGATCCAGCC-3'.

In the present study, genomic DNA was extracted by a standard protocol and analyzed on 0.8% agarose electrophoresis. Further, PCR was carried out to amplify the 18S rRNA gene of the extracted genomic DNA of UK-2 fungi using Gene Amp PCR with the forward and reversen primers (Forward Primer 5'-GAGTTTGATCCTGGCTCAG-3' and Reverse primer 5'-GAAAGGA GGTGATCCAGCC-3'). The genus of the strain was determined based on the sequence of 356 bp of 18S rRNA gene. The obtained 18S rRNA gene sequences were assembled (Fig.4.2.3) and exploited for phylogenetic analysis. The 18S rRNA gene sequence related taxa were acquired from the GenBank database at the National Center for Biotechnology Information. Multiple sequence alignment was conducted using the ClustalW program and the phylogenetic tree for the data set was constructed (Fig.4.2.4) by using phylip software. Phylogenetic analysis of UK-2 is similar to the compared sequence of *Aspergillus* species which is closely related to *Aspergillus niger* (Fig.4.2.4). Although the present fungal isolate has shown similarity (96–98%) with other *Aspergillus niger in* multiple sequence alignment, there are certain differences in their growth parameters compared with the reported *Aspergillus niger* (Goncalves et al., 2012). Hence, it is proposed to classify the UK-2 isolate as a novel species of genus *Aspergillus* and named as *Aspergillus nigerUK-2.*

Fig.4.2.4. Phylogenetic tree analysis of isolate UK-2

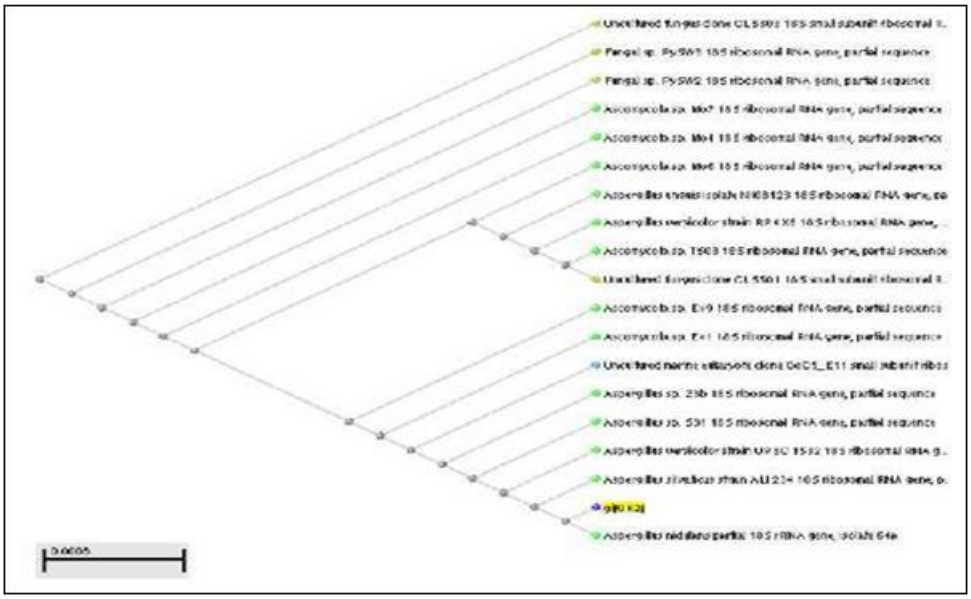

Figure 4.2.4. Phylogenetic tree analysis of isolate UK-2: The obtained 18S rRNA gene sequences were assembled (Fig.4.2.3) and were utilized for phylogenetic analysis. Phylogenetic analysis of UK-2 based on 18S rRNA gene sequence has shown that the isolate was affiliated to the genus of *Aspergillus* and closely related to *Aspergillus niger* (Fig.4.2.4). Although the present fungal isolate has shown similarity (96–98%) with other *Aspergillus niger* in multiple sequence alignment, there are certain differences in their growth parameters compared with the reported *Aspergillus niger*.

Homology modeling of tannase enzyme from Aspergillus niger: As per the literature there is no 3D structure reported in the protein data bank (PDB) for the tannase enzyme from *Aspergillus niger*. So herein, it is proposed to develop a predicted 3D structure for the sequence of Accession No.BN001306.1 (Fig.4.3.1) using Modeler 9v8 software. Template structure for modeling the protein derived through BLAST and among all the hits obtained, PDB ID 1IVYA are selected which is having 0.0 E-value and by using this, a valid 3D structure was generated by the help of Modeler 9v8. The score values for DOPE (Discrete optimized protein Energy) of all the four generated models was -113439.30469, -112441.01563, -113630.60938 and -112431.86719 (Fig.4.3.2). Among all, the model having the lowest DOPE score (-112431.86719) was considered as the final model (Fig.4.3.3) and used for further studies. The Pro SA Z-score value of -9.6 and 88% of favoured region were obtained by Ramachandran plot (Fig.4.3.4). Ren et al., (2013) has given first report about three-dimensional structures of a tannase from *Lactobacillus plantarum*. The enzyme displayed a α/β structure, featured by a large cap domain which was inserted into the classical serine hydrolase fold. A catalytic triad was identified in the structure, which was composed of Ser163, His451 and Asp419. During the binding of gallic acid, the carboxyl group of the molecule forged hydrogen-bonding interactions with the catalytic triad of the enzyme. The three hydroxyl groups made contacts with Asp421, Lys343 and Glu357 in order to form another hydrogen-bonding network. The tannase 3D model which was obtained from molecular modelling method would be helpful in investigation of the structure dynamics, surface properties of enzymes, biological activity, folding, enzyme catalysis, enzyme stability and conformational changes associated with biomolecular functions of tannase in further studies.

MRIITRNTIFAMATVGAASAKSMGDVCTVSHVRSILPTNGTLEGIDLLPSLTTASVYYNASSASSS

SASAGGMAMGGSSSSYDYCNVTVAYTHTGKNDKVVVKYAFPSPSEFKNRFYVAGGGGYSLSS

DATGGLQYGAVSGAIDAGYDAFDYSFDEVVLYGNGSINWDATYMFSYQALGEMTKLGKALT

RGFYGKSSDAKVYTYYEGCSDGGREGMSQVQRYGDEYDGATGAPAFRHSQQQVNHLFPAE

VEYTLDYYPPPCELAKCVNATIEACDPLDGRTDGVISRTDLCMLNFDLSSVIGESYYCAEQNYT

SLGEGFSKRADGSTTSYQFAQNGTVSKEGVAVAQAIYNGLHSTSGERAYLSWQIGSELSDADTT

YNSDTGKWTLTQSTGGVTVAKMVELLQLDNTFSLFNTTYDTTIQWMETGMVRYLDSLQTTL

PDITTTQSSGGKLLIYIGESDPSVPAASSVIIYWQAVRSIMYSDVSYKKSLEEMQDWYQFYLIP

GAAHCGSNSLQPGPYPENNMEIMIDWVENGVKPSRLNATVSSGTYEGETQMLCQWPKRPLW

KDNSDDFECVSDAKSIETWTYSFPAFKVPVY

Figure 4.3.1: ***Sequence retrieved from NCBI to predict the 3D model:*** The 18S rRNA gene sequences related taxa were acquired from the Gen Bank database at the National Center for Biotechnology Information. So herein, we propose a predicted 3D structure for the sequence of Accession No.BN001306.1 (Fig.4.3.1) by using Modeller 9v8 software.

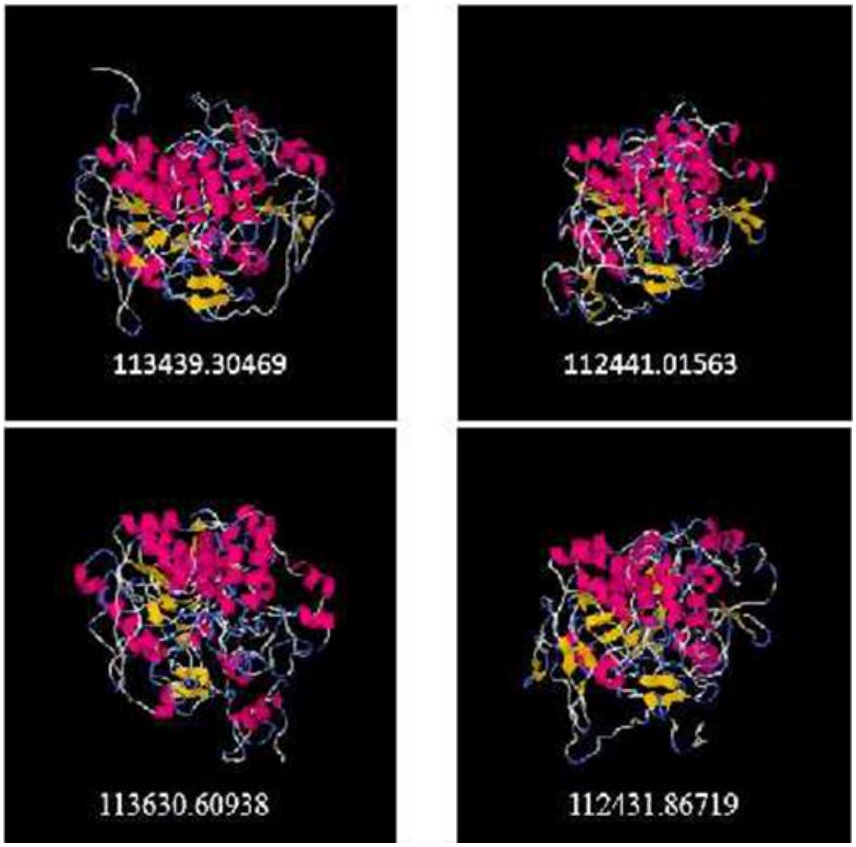

Figure 4.3.2: ***Generated models of enzyme tannase:*** Proposed predicted 3D structure for the sequence of Accession No.BN001306.1 (Fig.4.3.1) by using Modeler 9v8 software. Template structure for modeling the protein was searched through BLAST and among all the hits obtained, PDB ID 1IVYA was selected which is having 0.0 E-value and by using this, a valid 3D structure was generated by the help of Modeler 9v8. The DOPE (Discrete optimized protein Energy) score values for all the four models generated (Fig.4.3.2) were -113439.30469, 112441.01563, -113630.60938 and -112431.86719 and among all, the model having the lowest DOPE score (-112431.86719) is considered as the final model (Fig. 4.3.2).

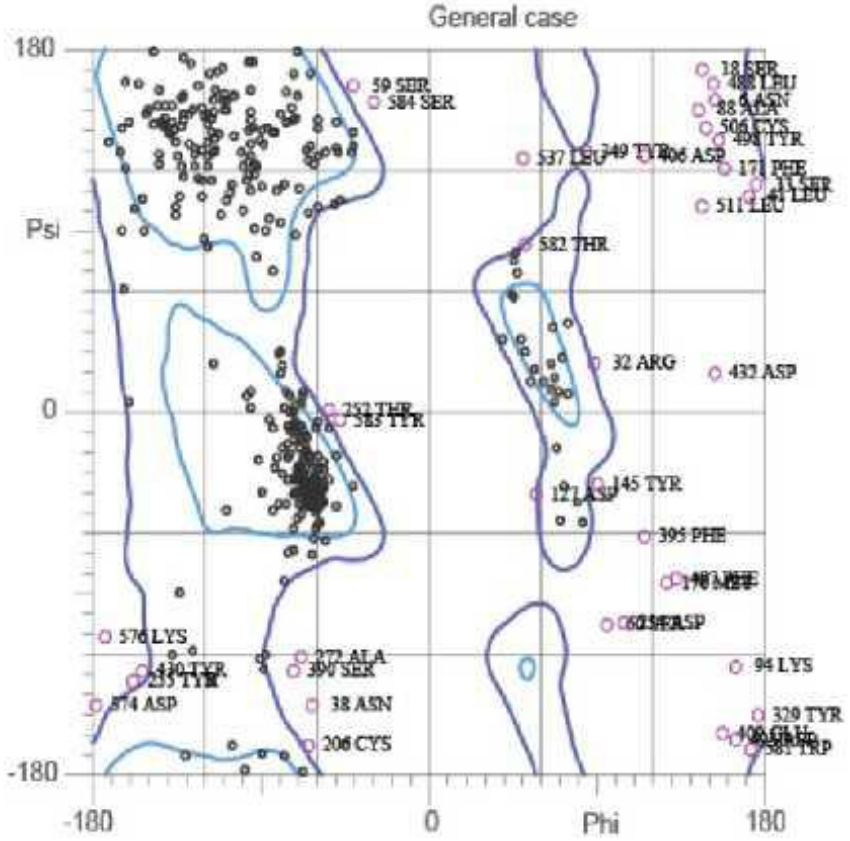

Figure 4.3.3: Ramachandran plot for enzyme tannase showing different regions: A final model was confirmed and used for further studies as the Pro SA Z-score value of -9.6 and 88% of favoured region was obtained through ramachandran plot (Fig 4.3.3).

4. Conclusion

Tannase producing microorganisms were isolated from local tannery effluent water collected from Pedaravuru, Tenali, Guntur and Andhra Pradesh. Further screened for high yield gallic acid producing microorganisms namely bacteria, fungi, yeast and actinomycetes. Among the total 150 isolates four fungi were shown good and UK-2 was the best producer. UK-2 was further identified and characterized by taxonomical FAME analysis and conformed by 18S rRNA gene sequence of UK-2 isolate and characterized that fungi belong to *Aspergillus niger* and deposited in the NCBI. Where the stain was named as ***Aspergillus niger KF514876.***

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