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RESEARCH ARTICLE

SCREENING OF AGRO-RESIDUES FOR THE PRODUCTION OF MICROBIAL TANNASE.

S. N. Girdhari and S. A. Peshwe.
Department of Microbiology, Govt.Institute of Science, Aurangabad. (MS).India

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Abstract

Tannin Acyl Hydrolase (E.C 3.1.1.20) commonly referred as Tannase is one of the important hydrolytic microbial enzymes. It hydrolyses hydrolysable tannin and produces glucose and gallic acid. It is an industrially important enzyme and has several applications in various industries such as food, pharmaceutical, leather, animal feed and cosmetics. Realizing the importance of enzyme tannase, the present study aims to investigate the total phenolic content and hydrolysable tannin content from different agro-residues and utilize the efficient agro-residue as a substrate for potent tannase producing microorganism for the production of tannase. The amount of total phenols, were analyzed using a spectrophotometric technique, using Folin-ciocalteau reagent. Gallic acid was used as standard compound and the total phenols were expressed as mg/g gallic acid equivalents. The hydrolysable tannin content was analyzed by Mondal's method. The maximum phenolic content and hydrolysable tannin content was found in *Punica granatum* peel (pomegranate peel) and *Prunus dulcis* leaves (almond leaves) respectively. Thus out of the thirty one agro-residues; three agro-residues were selected for final screening having higher concentration of hydrolysable tannin content viz. *Prunus dulcis* leaves (almond leaves), *Annona reticulate* leaves (Custard apple leaves); *Azadirachta indica* leaves (Neem leaves). The selected agro residues were used for production of microbial tannase. Accordingly the actinomycetal isolate, *Streptomyces sp.*SKA1 and the leaves of *Azadirachta indica* were selected as potent combination for tannase production.

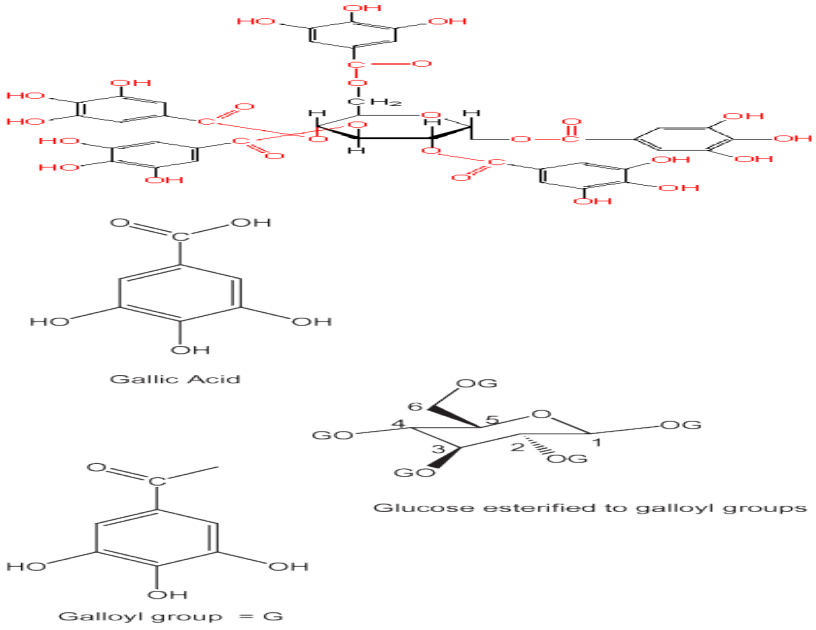
Introduction:-

Phenolics compounds are broadly distributed in the plant kingdom and they are the most abundant secondary metabolites in plant. These plant phenolic are involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators. They are abundant in all plant organs therefore they are integral part of human diet. They are important constituents of plant food (Fruits, vegetables, cereals, olive, legumes, etc.) and beverages (Tea, coffee, beer, wine etc.) and they are also responsible for bitterness and astringency of fruit and fruit juices. (Jin, D. and Russell, J. M 2010)

Corresponding Author:- S.N.Girdhari.
Address:- Department of Microbiology, Govt.Institute of Science, Aurangabad. (MS).India.

Plant phenolics include phenolic acids, flavonides, tannins, the stilbenes and lignans. Tannins constitute a large group of complex organic, non-nitrogenous phenolic compounds of high molecular weight. Tannins are natural polyphenolic compounds that are widely distributed in several parts of vascular plants. They are the second most abundant group of phenolics in nature and are considered as secondary metabolic compounds of plants because they play no direct role in plant metabolisms (Pepi M, Lampariello L R, Altieri R *et, al.2009*). These secondary metabolites are present in solution form in the cell sap and also in distinct vacuoles. Chemically tannins contain the mixture of complex organic substrate in which polyphenol are present, generally with O-dihydroxy or O-trihydroxy group on a phenyl ring. (Fig. I) Tannins are soluble in water and alcohol; they form colloidal solution with water and are non crystalline. They have the capacity to combine with tissue proteins and precipitate them. Tannins are characterized by their ability to form strong complexes with different minerals and macromolecules, such as proteins, cellulose, and starch among others (Bele, A.A., Jadhav, V. M., Kadam, V. J.2010). Tannins have several interesting biological activities. Active principles of medicinal plants are often polyphenolic compounds, and, in recent years, there has been a great scientific interest for this group of compounds due to their antioxidant, antiviral, and anticancer properties (Bors, W. Michel, C. 1999, Carretero-Accame M E.2000). High concentrations of tannins in beverage such as ice tea, beer, wine, fruit juices, and coffee-flavored beverages can result in the formation of precipitates due to their interaction with other molecules present in these beverages. These undesirable effects of tannins can be reduced or eliminated by a chemical or enzymatic treatment. (Belmares, R., Contreras-Esquivel, J. C., Rodríguez-Herrera, R. Coronel, A. R., Aguilar, C. N.2004). Tannins are toxic to fungi, bacteria, and viruses. However, many microorganisms have developed the mechanisms to overcome the effects of tannins. These mechanisms include tannin modification, degradation, dissociation of tannin-substrate complexes, tannin inactivation by high-affinity binders, membrane modification, and metal ion sequestration (Belur, P. D., Gopal, M., Nirmala, K. R., Basavaraj, N.2010) Tannins are subdivided into two groups: 1) Hydrolysable tannins and2) Condensed tannins.

Basic Structure of Tannin



Hydrolysable tannins undergo hydrolysis and produce gallic acid. According to acid produced, they are known as gallitannins or ellagitannins. Examples of hydrolysable tannins are gallotannin in nutgall, rhubarb, amla, clove and chestnut; ellagitannin from oak, myrobalans and pomegranate bark. Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond.

Interest in tannins has considerably increased in recent year because of their broad spectrum of chemical and diverse biological properties. One of the important properties of tannin is that it is used as substrate for the production of enzyme tannase. Though these polyphenolic compounds are widely distributed, the health effects of dietary Polyphenols have come to the attention of nutritionists in recent year (Jin, D. and Russell, J. M. 2010) however using the enzyme tannase the nutritional quality of dietary products can be improved. Tannins are 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)
Tannase catalyzes the hydrolysis of bonds present in the molecule of hydrolysable tannins and gallic acid esters (Lekha and Lonsane, 1997). The systematic name of tannase is Tannin acyl hydrolase (3.1.1.20). It is an extracellular, inducible enzyme that catalyses the hydrolysis of ester and depside linkages in hydrolysable tannins yielding glucose and gallic acid as products. It can be obtained from fungi, bacteria, some yeasts, higher plants and animal sources (Belur and Mugeraya 2010).It has wide application in preparation of instant tea, acron wine, coffee flavored soft drinks, clarification of beer and fruit juices, detanification of food and increasing the nutritive property of feed and also in bioremediation of effluent from leather industries. Tannase has wide application in cosmetics, chemicals and brewing industries; in preparation of gallic acid (Brahmbhatt D. *et.al*., 2014). Recently bacteria showing tannase activity is considered as a biomarker for colon cancer (*Lekha* and. *Lonsane*, 1997; *Das Mohapatra et.al.*, 2012). Due to Production costs and insufficient knowledge of the enzyme the use of tannase on a large scale is severely limited. (C.N. Aguilar and G. Gutie 2001)

Tannase is known to produce by bacteria, yeast and fungi, no reports available with actinomycetes. Filamentous fungi of the *Aspergillus,Penicillium* genus and bacteria of the *Bacillus* and *Lactobacillus* genus have been investigated for tannase production (Banerjee *et al.*2001; Mondal *et al*.2001).Techniques for production of tannase have been extensively studied and commercial production of tannase is achieved using synthetic tannic acid. The industrial process makes use of chemical tannic acid for tannase production but this process involving synthetic substrates has adverse environmental consequences. Conventionally gallic acid is also produced chemically by acid hydrolysis of synthetic tannic acid and suffer from disadvantages like high cost to yield ratio and low purity. (Swaran Nandini *et al.*2014).

Therefore it is very necessary to design a process for the production of tannase which is more economical and environmental friendly. This has led to generating interest in searching natural source for tannic acid that can be effectively utilized by microbes. This effort may reduce the dependency on synthetic tannic acid.

Realizing the importance of enzyme tannase, the aim of the present study was to estimate the total phenolic content and hydrolysable tannin content in the methanolic extract of various agro-residues, to select the efficient agro-residue for production of microbial tannase.

Materials and Methods:-

Plant material:-
Different agro-residues like fruits, leaves, fruit peels, seeds were collected from local area and botanical garden in January 2014 and shade dried.

Extraction:-
Dried ground agro-residues were extracted with 99% methanol. The extract was concentrated by evaporation to yield gummy concentrate of greenish color.

Chemicals:-
Gallic acid, Methanol, Folin-Ciocalteau (FC) reagent, Bovine Serum Albumin, Sodium acetate, Ferric chloride, Acetic acid, SDS, Triethanolamine was obtained from Hi media chemicals. All the chemicals used were of analytical grade.

Microorganisms for the production of Tannase:-
The potent tannase producing fungal cultures SKF7, SKF11**,** Actinomycetal cultures SKA1, SKA2, and bacterial cultures SKB2, SKB3 were used for tannase production.

Screening of agro-residues:-
The methanolic extracts of thirty one agro-residues were screened for the total phenolic content by the Folin Denis Method and hydrolysable tannin content by the Mondal's method.

Estimation of total Phenolic content:-
A standard graph was prepared using different concentration of gallic acid ranging from 10 µg/ml-100 µg/ml was prepared. A volume of 1.5 ml FC reagent was added in each tube. After five minutes, 4 ml of 20% sodium carbonate

solution was added and the volume was made up to 10 ml with distilled water. The mixture was kept for 30 mins and absorbance was read at 738nm. (Premakumari K.B.*et.al.*2010). Tannic acid was used as standard compound and the total phenols were expressed as mg/g gallic acid equivalents

Sample Preparation and estimation:-
Methanolic extract of all agro-residues in 100 ml volumetric flask were made up to 50 ml with distilled water. (As shown in figure 3). Further 0.1 ml of methanolic extract, 1.5 ml of FC reagent, and 4ml of 20% sodium carbonate solution were mixed and made up to 10 ml with distilled water. After 30 mins, absorbance was read at 738nm. Premakumari K.B. *et.al.,* 2010 detected antioxidant activity and estimated total phenolic content of *Muntingia calabura* by employing this method.

Estimation of hydrolysable Tannin content by Mondal's method:-
Tannase Assay:-
Standard graph of tannic acid was prepared by making the various concentration of tannic acid ranging from 1 mg/ml to 10 mg/ml in 0.2M acetate buffer (pH 5). Then 0.5 ml of tannic acid from each tube, mixed with 2ml of Bovine Serum Albumin (1mg/ml) which precipitates 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 (As shown in Figure 4) and the absorbance was measured at 530 nm after addition of 1 ml of $FeCl_3$ (0.13 M) (Mondal *et.al.*, 2001)

Sample Preparation and estimation:-
Methanolic extract of all agro-residues taken in 100 ml volumetric flask were made up to 50 ml with 0.2 M acetate buffer (pH 5). From this solution 0.5 ml was taken out in a test tube and mixed with 2ml of Bovine Serum Albumin (1mg/ml). 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)

Results and Discussion:-

Phytochemical Screening:-
The observation revealed that methanolic extract of various agro-residues showed the presence of total phenols by Folin-Ciocateau method. Mariela Gonzalez, Bernardo Guzman *et.,al 2003* reported that, due to its reproducibility, the Folin-Ciocalteau method is recommended for *Propolis* with high phenolic compound concentrations.

Estimation of total phenolic content:-
The amount of total phenols was determined with Folin-Ciocalteu reagent. Gallic acid was used as standard compound. The absorbance for various dilutions of gallic acid with Folin-Ciocalteu reagent and sodium carbonate were noted and are shown in table I. The total phenolic content (gallic acid equivalent) in methanolic extract was found to high in *Punica granatum* peel (pomegranate peel) which was shade dried (7.04 mg/ml). Sharma G.N.*et.al*., 2011 reported the total phenol content of methanolic extract of *Aegle marmelos* as 65.20 mg/g

Screening of agro- residues for microbial tannase production:-
Out of thirty one agro-residues finally three agro-residues *Prunus Dulcis* leaves (Almond leaves) *Azadirachta indica* leaves (Neem leaves) and *Annona reticulate* leaves (Custard apple leaves) showed high hydrolysable tannin content, among these three agro-residues combination of *Azadirachta indica* leaves as a substrate and actinomycetal isolate SKA1 , show promising tannase activity (As shown in figure 1).Paranthaman R.(2008) reported that Rice straw and Sugarcane can be used as substrate for the production of tannase. Kulkarni, A. patil, P. and Kininge, P. (2012) reported tannase production from *Aspergillus oryzae* NCIM 1032 using mixture of Jamun (*Syzigium cumini*) and Babul (*Acacia nilotica*) stem barks under solid state fermentation.

Table 1:- Concentration of total phenolic content and hydrolysable tannic acid of different Agro-residues

Sr.no.	Botanical Name of Agro-residues	Common name of Agro - residues	Conc. of total phenol mg/ml	Conc. Of hydrolysable tannin mg/ml
1	*Terminalia belirica*	Behda	4.75	2.52
2	*Phyllanthus emblica fruit*	Amla	5.34	3.33
3	*Terminallia chebula fruit*	Hirda	4.87	3.21

4	*Syzygium cumini leaves*	Jamun	3.96	3.27
5	*Citrus limun leaves*	Nimu	2.27	1.89
6	*Santalum alba leaves*	Chandan	3.24	2.97
7	*Sorghum leaves*	Jawar	3.85	2.55
8	*Manikara zapota leaves*	Chiku	3.71	2.88
9	*Nilgiritragus hylocrius leaves*	Nilgiri	4.67	0.6
10	*Tamarindus indica leaves*	Imli	5.09	0.87
11	*Pithecellobium dulce leaves*	Firangi Imli	2.34	0.03
12	*Saraca asoca leaves*	Asoca	3.12	2.01
13	*Ziziphus maritiana leaves*	Ber	2.76	1.2
14	*Barleria cristata leaves*	Vajra danti	1.80	0.45
15	*Sapindusmukorossi leaves*	Ritha	4.07	1.11
16	*Phyllanthus emblica leaves*	Amla	5.38	2.67
17	*Azadirachta indica leaves*	Neem	4.95	3.51
18	*Annona reticulate leaves*	Custard apple	4.44	3.48
19	*Psidium guajava leaves*	Guava	5.34	3.39
20	*Mangifera indica leaves*	Mango	3.49	3.42
21	*Prunus dulcis leaves*	Almond	5.51	4.32
22	*Morus nigra leaves*	Mulberry	1.18	0.9
23	*Calotropis gigantean leaves*	Madar	2.98	1.74
24	*Ficus religiosa leaves*	Pipal	2.78	0.93
25	*Vachellia nilotica leaves*	Babul	6.48	1.5
26	*Citrus aurantium peel*	Orange	3.86	1.5
27	*Punica granatum peel*	Pomegranate	7.04	0.6
28	*Tamarindus indica seeds*	Imli	0.99	0.51
29	*Vistis vinitera*	Grape	0.41	0.006
30	*Saccharum officinarum stalks*	Sugarcane	0.12	0.001
31	*Cieer arientinum leaves*	Chana	0.23	0.003

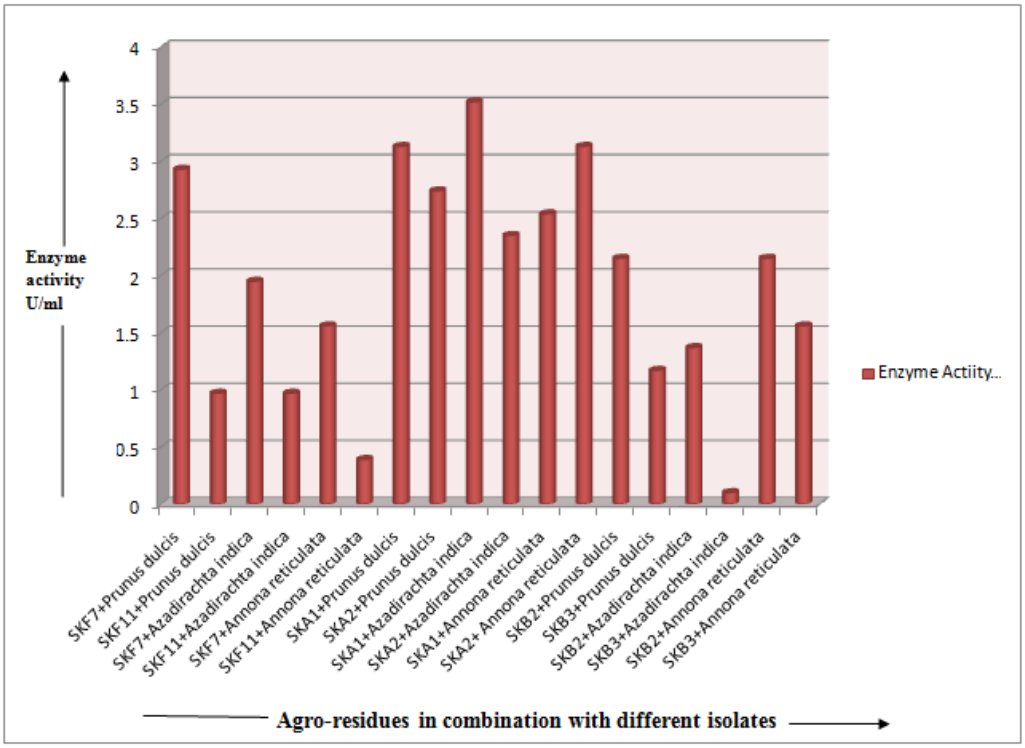


Figure 1:- Tannase activity of different isolates with combination of selected Agro-residues.

Figure 2:- Agro-residues Powder used for study

Figure 3:- Methanolic extraction of agro-residues.

Figure 4:- Mondal's Assay.

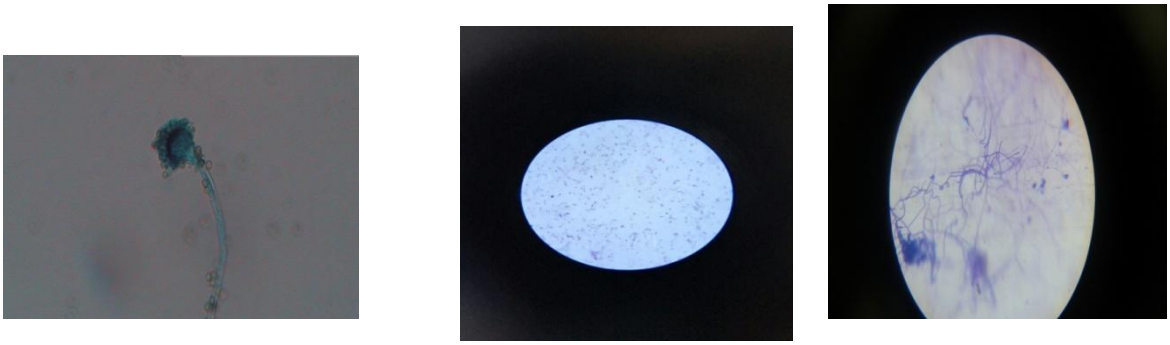


Figure 5:- Potent tannase producer fungal (SKF7), bacterial (SKB2) and actinomycetal (SKA1) isolates

Conclusion:-

The total phenolic content of thirty one agro-residues was estimated using Folin-Ciocalteau reagent. The results of Phytochemical screening reveals that the maximum phenolic content was found in methanolic extract of pomegranate peel, whereas the maximum hyolysable tannic acid concentration was found in methanolic extract of almond leaves. When different combinations of all the types of Isolates with three efficient agro-residues were carried out it was found that combination of actinomycetal culture *Streptomyces* sp. SKA1 and *Azadirachta indica* leaves (Neem leaves) as a substrate was found to be best combination for tannase production. Further study of process optimization will be carried out using this high tannin containing substrate.

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