OPTIMIZATION OF TANNASE PRODUCTION FROM *ASPERGILLUS SP.*

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ABSTRACT

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 establish a solid state fermentation in order to obtain the enzyme and further the isolated enzymes's physicochemical properties are observed through RSM (Response Surface Methodology) and Tannase assay. Based on the RSM approach, the maximum tannase activity was observed i.e 4.86 U/ml thereby depicting that the most ambient condition of production are pH 4.0, temperature 30℃, incubation period of 6 days and moisture level of 10ml in 3g of substrate.

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DECLARATION

We hereby declare that the work reported in the B-Tech thesis entitled "**Optimization of tannase production from *Aspergillus sp.***" submitted at Jaypee University of Information Technology, Waknaghat India, is an authentic record of our work carried out under the supervision of Dr. Gunjan Goel. We have not submitted this work elsewhere for any other degree or diploma.

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Date: 25th May, 2016.

CERTIFICATE

This is to certify that the work titled "**Optimization of tannase production from *Aspergillus sp.***", submitted by Akanksha Walia(123801 and Yashika Sama(123804) in partial fulfillment for the award of 4 year degree program, Bachelors of Technology at Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or fully to any other university or institute for the award of this or any other degree or diploma.

Supervisor:

Dr. Gunjan Goel

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Date-

LIST OF ACRONYMS

SSF	SOLID STATE FERMENTATION
RSM	RESPONSE SURFACE METHODOLOGY
PPM	PARTS PER MILLION
CFU	COLONY FORMING UNITS
CZ	CZAPEK DOX
SMF	SUBMERGED FERMENTATION
PKC	PALM KERNEL CAKE
TPC	TOTAL PHENOLIC CONTENT
RPM	ROTATION PER MINUTE

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1. INTRODUCTION

Tannase is one of the hydrolases and is known to catalyze the breakdown of ester and depside bonds from hydrolysable tannins and gallic acid esters. This enzyme is known to display two different activities. The first one is an esterase activity; by which it can hydrolyze ester bonds of gallic acid esters with glucose (galloyl-glucose) or alcohols (e.g. methyl gallate). The second activity is called depsidase activity; by which it can hydrolyze depside bonds of digallic acid (Haslam and Stangroom, 1996; Saxena and Saxena, 2004; Sharma et al., 2000). Tannase is an industrially important enzyme and has several applications in various industries such as foods, animal feeds, cosmetic, pharmaceutical, chemical, leather industries and so on. (Aguilar and Gutierrez-Sanchez, 2001; Aguilar et al., 2007; Dũenas et al., 2007; Jun et al., 2007).

Tannase can be obtained from plants, animals, and microbial sources. Microorganisms are considered as the most important and commercial sources of tannase, that is because the produced tannases are more stable than similar ones obtained from the other sources. Moreover, microorganisms can produce tannase in high quantities in a constant way.

Microbial tannase is favoured also because the microbes can be subjected to genetic manipulation more readily than plants and animals, resulting in an increase in tannase production (Aguilar and Gutierrez-Sanchez, 2001; Purohit etal, 2006).

Fungi are the most studied microorganisms for tannase production. Fungi have the ability to degrade tannins as a sole carbon source (Aguilar and Gutierrez-Sanchez, 2001). The common genus used for tannase production either for research purposes or industrial production was *Aspergillus* and the common *Aspergillus* species used for tannase production was *Aspergillus niger*. Tannase-producing fungi were isolated from soils and tannery effluent (Enemuor and Odibo, 2009; Manjit et al., 2008). The present investigation aims to isolate and screen high tannase -producing fungi from tannin rich sources.

Tannase production by solid-state fermentation (SSF) is more advantageous over submerged or liquid surface fermentation (Lekha and Lonsane, 1994; Anguilar et al., 2001). Viniegra-González et al. (2003) demonstrated, using offer and Luedekind-Piret equations, that the higher productivity of invertase, pectinase and tannase in SSF was due to better growth of A. niger in SSF, resulting in

higher biomass production, and more economical synthesis of enzymes beneath conditions whereas not catabolite repression. Moreover, the breakdown of enzymes by contaminating proteases was eight times higher in submerged fermentation (SmF) than in SSF. Filamentous fungi grow in nature on solid substrates like wood, stem, root and leaf of plants in the absence of free water and even have the intrusion power for penetrating deep in to the intercellular house for higher utilization of the substrate. Hence filamentous fungi space unit ideally suited to a winning SSF. However, substrates used for tannase production under SSF had been like palm kernel cake (PKC), tamarind seed powder (TSP) (Sabu et al., 2005b), wheat bran (Gustavo et al., 2001; Sabu et al., 2005a), etc. Here for the first time, we space unit news use of jamun leaves (agro-waste) for tannase production, which space unit really low value and promptly on the market substrate throughout this a neighborhood of the world.

The utilization of measurable exploratory outline methods in maturation process advancement can bring about change of item yield, decrease process variability, give nearer affirmation of the yield reaction to ostensible and lessen general expenses. Ordinary routine of single element decrease enhancement by keeping up different variables required at an unspecified consistent level does not delineate the consolidated impact of all the elements included. This strategy is likewise tedious and requires various tests to decide ideal levels, which are inconsistent. These impediments can be wiped out by streamlining all the influencing parameters by and large by RSM.

RSM can be utilized to assess the relative noteworthiness of a few calculates even the nearness of complex associations. In order to to evaluate the optimum fermentation process conditions for tannase RSM act as a great tool for statistical and hassel free analysis and thereby help in determining ideal paramaters for production of tannase through solid state fermentation using tea residues.

2. REVIEW OF LITERATURE

The protein tannin acyl hydarolase (TAH), normally alluded as tannase is included in biodegradation of tannins and has imperative applications in different commercial ventures, especially in nourishment and pharmaceutical parts. TAH catalyzes the hydrolysis of ester bonds present in gallotannins, ellagitannin complex tannins, and gallic corrosive esters. Tannins are impervious to biodegradation, and the gathering because of release of tanneries and espresso handling commercial enterprises effluents can bring about natural contamination (Field and Lettinga 1992; Bhat et al. 1998).Tannins are characteristic polyphenolic mixes present in vascular plants. They are portrayed by their capacity to shape solid buildings with various minerals and macromolecules, for example, proteins, cellulose, starch, among others. Because of their solid capacity to tie with proteins, they have been utilized for tanning for a huge number of years (Frutos et al. 2004; Aguilar et al. 2007).Similarly for their capacity to accelerate substantial metals andsome alkaloids, tannins can be utilized as a part of the treatment of harming brought about by these substances. In conventional pharmaceutical of China and Japan, the plant separates rich in tannins have been utilized as astringent specialists, against diarrheal, diuretics, mitigating, disinfectant, and hostile to hemorrhagic. The nearness of tannins in the eating routine of ruminants contrarily influences their development and milk generation (Reed 1995).

Tannins structure edifices with dietary protein and different supplements that could meddle with their assimilation; tannins are likewise proficient to restrain the digestive compounds in salivation and rumen and subsequently lessen the supplements retention (Frutos et al. 2004). At last, tannins grant a biting taste, and this could essentially lessen the admission by domesticated animals (Belmares et al. 2004; Mingshu et al. 2006). The dynamic standards of restorative plants are frequently polyphenolic mixes, and as of late, ther been an awesome investigative enthusiasm for this gathering of mixes because of their cell reinforcement and anticancer property (Carretero-Accame 2000; Khanbabaee and Van Ree 2001).

The higher convergences of tannins in drink, suchas frosted tea, lager, wine, organic product juices, and espresso enhanced refreshments can bring about the arrangement of hastens because of their association with different particles present in these drinks. These undesirable impacts of tannins can be decreased or dispensed with by enzymatic treatment (Lekha and Lonsane 1997). The utilization of tannase could discharge the tannin monomers upgrading the nutritious and cancer prevention agent properties of these drinks.

Tannase has wide applications in sustenance industry, beverages, pharmaceuticals, skin treatment, and even in bioremediation. Tannases are increasing more consideration on account of their hydrolytic and in addition manufactured capacity in appropriate dissolvable frameworks. The complex reactant property of the tannases has upgraded their business significance.

It is for the most part utilized as a part of the procedure of frosted tea, oak seed alcohol, and generation of gallic corrosive from plant sources with high tannin substance. Gallic corrosive is, thus, utilized as a part of the generation of cell reinforcements and is a middle atom in the creation of the anti-infection trimethoprim (Aguilar et al. 2007).

In any case, its huge scale application has been seriously constrained by various elements, including high creation costs and inadequate learning on a portion of the chemical qualities (Aguilar and Gutiérrez-Sánchez 2001). This audit presents definite data on different parts of tannases, investigating logical and innovative aspects, with accentuation on substrates, physicochemical properties, creation, metabolic control components, recuperation and cleaning methodologies, applications, licensed innovation rights, and worldwide business sector situation.

2.1. **Tannins**

Tannin 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 2 major teams on the basis of their structure and properties; they are hydrolysable tannins and condensed tannins, an intermediate cluster conjointly exist that combines both characteristics of hydrolysable tannins and condensed tannins and are known as Catechin tannins. Tannins are abundant gift 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 merchandise which area unit used for human consumption (Bhat et.al., 1998).

Tannin in kind of Catechin (Flavan-3-ols) is present in tea, cocoa, acacia and catechu plants. Catechins are gift in all styles of tea. Tannins are widespread in the Plantae, occurring mostly in leaves,fruits,bark and wood and are typically thought of nutritionally undesirable

(Chung et.al.,1998;Murugan and saleh,2010).Tannins inhibit growth of various microorganisms, by precipitating many enzymes (Field and Lettinga,1992) .Tannic acid could be 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 aldohexose 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 celebrated tannase producers. Most of the commercial applications of tannase area unit in the manufacturing of instant tea, tannase is used to eliminate water-soluble precipitates called tea cream (Sanderson et.al., 1974).

2.2. **Biodegradation of Tannins**

Biodegradation by certain microorganism and proteins is a standout amongst the most proficient approaches to debase substantial tannin atoms into little particles with bio-exercises of high esteem. The capacity of microorganism to acclimatize tannin contrasts among yeast, microscopic organisms and parasites. Yeast, while acting adequately against gallotannins, loses it debasement capacity against high atomic mixes. Microorganisms can corrupt gallotannins and in addition ellagitannins. Growths can productively debase distinctive sorts of tannins (Bhat et al. 1998). A portion of the catalysts required in corruption of gallotannins are tannase and gallic corrosive decarboxilase. Tannase is maybe the most considered chemical so far in the biodegradation of tannins. It has activity on ester and depside obligations of gallotannin and might be microbial, plant, or creature in starting point, of which microorganisms are the most critical source (Aguilar et al.2007).

Tannase follows up on gallotannins, ellagitannins, and complex tannins by breaking just ester bonds without influencing the carbon–carbon bonds and henceforth does not influence the dense tannins (Haslam and Stangroom 1966). Gallic corrosive decarboxylase can catalyze the decarboxylation of gallic corrosive to pyrogallol. This chemical is extremely insecure as a result of its high affectability to oxygen, and along these lines, it is hard to confine and clean (Zeida et al. 1998). A few microscopic organisms, for example, Selenomonas gallolyticusand Escherichia coli, decarboxylate gallic corrosive to pyrogallol, yet this compound is not further changed.

The reason is hazy yet is liable to be less poisonous than gallic corrosive or that its creation is thermodynamically more positive and is perhaps connected to the era of vitality by pumping protons (Minghu et al. 2006).In the instance of ellagitannin biodegradation, the discharge ofellagic corrosive has been ascribed to another catalyst (ellagitannin acyl hydrolase). In any case, it is important to performa study to exhibit the reactant contrast between tannin acyl hydrolase and ellagitannin acyl hydrolase and to comprehend the biodegradation procedures of gallotannins and ellagitannins (Aguilera-Carbó et al. 2008).

Then again, the investigation of the debasement of consolidated tannins and complex is considerably more troublesome because of their entangled structures. In this manner, there is little advance in comprehension the systems of corruption of these mixes.

2.3. **Physicochemical Properties of Tannase**

Biodegradation by certain microorganism and proteins is a standout amongst the most proficient approaches to debase substantial tannin atoms into little particles with bio-exercises of high esteem. The capacity of microorganism to acclimatize tannin contrasts among yeast, microscopic organisms and parasites. Yeast, while acting adequately against gallotannins, loses it debasement capacity against high atomic mixes. Microorganisms can corrupt gallotannins and in addition ellagitannins. Growths can productively debase distinctive sorts of tannins (Bhat et al. 1998). A portion of the catalysts required in corruption of gallotannins are tannase and gallic corrosive decarboxilase. Tannase is maybe the most considered chemical so far in the biodegradation of tannins. It has activity on ester and depside obligations of gallotannin and might be microbial, plant, or creature in starting point, of which microorganisms are the most critical source (Aguilar et al.2007).

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Microorganism	**Optimum temperature (°C)**	**Optimum pH**	**MW (kDa)**	**KM (mM)**	**Reference**
Aspergillus fFlavus	50–60	5.0–5.5	192	0.05	Adachi et al. 1968; Yamada et al.1968a
A. niger LCF 8	35	6.0	186		Barthomeuf et al. 1994
A. niger van Tieghem	30	5.0–6.0	185		Rana and Bhat 2005
Candida sp.	50	6.0	250		Aoki et al. 1976a, b
Cryphonectria par asitica	30	5.5	240	0.95	Farías et al. 1994
Paecilomyces variotii	55	5.5	87.3, 71.5	0.61× 10−3	Battestin and Macedo 2007
Penicillium chrysogenum	30–40	5.0–6.0		0.048	Rajakumar and Nandy 1983

Table 1: Microorganisms and the ambient conditions at which they produce tannase

2.4. **Biotechnological Production of Tannase**

TAH can be extricated from microbial, creature, and vegetal sources, yet microorganisms are normally utilized for business generation since they deliver tannases more steady than the vegetal or creature chemicals. What's more a steady extensive scale creation of tannase can be accomplished by microbial maturation process (Lekha and Lonsane 1997). Despite the fact that it is surely understood that tannins hinder microbial development, there are various microorganisms ready to debase these mixes and even develop with tannic corrosive as sole carbon source (Banerjee and Pati 2007). These microorganisms have built up the essential instruments to defeat the inhibitory impact of tannin, for example, the creation of tannase. Table 2 outlines the microorganism reported as tannase makers.

For a long time, the mechanical creation of tannase was done only in submerged fluid maturation frameworks (SLF). However the advantages of strong state maturation (SSF) for generation of tannase and different chemicals are being accounted for broadly. A portion of the points of interest are the accompanying: extracellular nature of protein, expanded profitability, higher action titers, and expanded steadiness to pH and temperature changes. Furthermore, the SSF permits the development of more conservative reactors with less vitality utilization, less water utilization, and profluent dispose of and consequently lessening the vitality effect to nature (Lekha and Lonsane 1994; Viniegra-González et al. 2003).

Cruz-Hernández et al. (2006) thought about the effectiveness of SSF and SLF on tannase generation from A. niger GH1 and reported that the compound creation was more than four times higher in SSF than SLF. Beforehand, Aguilar et al. (2001a,b) reported comparative results for A. niger; they recorded no less than 2.5 times higher action and efficiency in SSF. They recommended that the low efficiency of the SLF is because of a conceivable corruption of the compound when it is created in SLF, and such debasement does not happen in SSF.

Aguilar et al. (2002) reported that protease generation by A. niger relies on upon the way of life framework utilized and on the underlying tannic corrosive fixation. Articulation of protease action in SLF was higher (up to 10 times) than that got in SSF utilizing the same society medium. Lekha and Lonsane (1994) analyzed the tannase creation in SLF, SSF, and maturation in fluid surface (FLS) for A. niger PKL 104 and found that chemical generation in SSF was around 2.5 and 4.8 times more than that acquired for SLF and FLS individually.

Aside from higher profitability and action, the crest action came to in SSF was acquired in about portion of the time required by the other two frameworks. Rana and Bhat (2005) likewise demonstrated that the SSF framework is best for the tannase creation by an alternate strain of A. niger. They reported a yield 1.6 times higher in SSF than that in SLF.

2.5. **Tannase Research: Historical Developments**

A superb survey on chronicled improvements of generation and utilizations of tannase was accounted for by Lekha and Lonsane (1997). Scheele watched the nearness of gallic corrosive in the fluid concentrate of gallnut in 1786 (Knudson, 1913). Robiquet associated the movement with a life form which causes aging and ensuing arrival of gallic corrosive in nerve nuts. Loraque considered the development of gallic corrosive from tannic corrosive to be because of a life form or oxidation. To bolster this hypothesis he promote found that few poisonous substances repress the development of gallic corrosive from tannic corrosive in nerve nut (Knudson, 1913).

Van Teighem was the first to show that the development of gallic corrosive is because of the activity of growth living being and neither to compounds prior in the nerves, nor to oxidation by the air in 1867 (Knudson, 1913). He expressed further that the living beings were Penicillium glaucum and the new living being which he named as Aspergillus niger. He assist examined the submerged and surface development of this life form and the degree of tannic corrosive corruption.

Fernbach developed Aspergillus niger in Raulin's answer with the sugar supplanted by tannic corrosive and afterward removed from the creature the chemical tannase in 1901 (Knudson, 1913).

The primary broad studies on tannase properties, sources, applications, response instruments and specificity were directed by laborers, for example, Fernbach, Pottevin, Dykerhoff and Ambruster and Thom and Raper toward the begin of the twentieth century. These concentrates in addition demonstrated that tannase was an inducible catalyst and could be blended in strong state maturation by filamentous organisms, for example, Aspergillus and Penicillium.

The utilization of tannase for the production of gallic corrosive from tannin-containing materials was soon perceived. In 1943, Toth and Barsony showed that tannase comprises of an esterase and depsidase movement (Lekha and Lonsane, 1997). In 1960's tannase was portrayed and decontaminated from plant and contagious source by Madhavakrishna-Bose and Dhar-Bose, individually (Lekha and Lonsane, 1997).

Japanese laborers Iibuchi et al. (1967) and Yamada et al. (1968) did cleansing and portrayal concentrates on Aspergillus tannase. Iibuchi et al. (1967) additionally built up a spectrophotometric measure for the determination of tannase movement, which was in the past taking into account titration. In the mid seventies, a few licenses were petitioned for potential use of tannase in sustenance and drink industry (Van de Lagemaat and Pyle, 2006).

Around 1980's numerous studies on the sources, test, applications, immobilization, sanitization and portrayal of tannase were distributed. It was found that separated from filamentous growths, tannase was additionally observed to be created by creatures (Lekha and Lonsane, 1997) and microscopic organisms (Deschamps et al., 1983).

Later on strategies were created to decide/distinguish tannase movement utilizing gas chromatography (Jean et al., 1981).

There was a solid spotlight on the generation of tannase by bacterial strains from 1990 onwards (Mondal et al., 2000, 2001; Mondal and Pati, 2000; Osawa et al., 2000; Ayed and Hamdi, 2002; Das Mohapatra et al., 2006; Selwal et al., 2010; Belur et al., 2010). A few studies showed the potential preferences of strong state maturation over submerged state aging utilizing contagious societies (Lekha and Lonsane, 1994; Kar and Banerjee, 2000; Aguilar et al., 2001b, 2002). Van de Lagemaat and Pyle (2001) built up a persistent strong state maturation process for the generation of contagious tannase.

2.6. **Microbial wellsprings of tannase**

Research over recent years brought about the revelation of extraordinary assortment of tannase makers. Microorganisms, parasites and yeasts are the unmistakable makers. Couple of creatures likewise have been observed to be the makers of tannase. Further research demonstrated that the colonizing microorganisms of these creatures are the real makers, not the creatures.

Parasites: Filamentous organisms of the Aspergillus and Penicillium class have been generally utilized for tannase creation. Lion's share of the examination work utilized these life forms. A brief rundown of these molds is given in Table 1.

Yeasts: There are not very many reports of tannase creation from yeasts, which are recorded in Table 2.

Microscopic organisms: Report on tannase from bacterial source is meager in writing before 1980's. In most recent 25 years or somewhere in the vicinity, around twelve reports were distributed on bacterial tannase and around 25 new tannase positive microbes have been segregated. Deschamps et al. (1983) confined number of bacterial strains that can use tannic corrosive as the sole carbon source.

Table 2. Fungal sources of tannase

Microorganism	References
Aspergillus flavus	Yamada *et al.* (1968)
Aspergillus awamori	Bradoo *et al.* (1996)
Aspergillus niger	Bradoo *et al.* (1996)
Aspergillus japonicus	Bradoo *et al.* (1997)
Aspergillus oryzae	Bradoo *et al.* (1996)
Aspergillus aureus	Bajpai and Patil (1997)
Aspergillus fischeri	Bajpai and Patil (1997)
Aspergillus gallonyces	Belmares *et al.* (2004)
Aspergillus fumigatus	Batra and Saxena (2005)
Aspergillus versicolor	Batra and Saxena (2005)
Aspergillus caespitosum	Batra and Saxena (2005)
Aspergillus aculeatus	Banerjee *et al.* (2001)
Aspergillus rugulosus	Bradoo *et al.* (1996)
Aspergillus terreus	Bajpai and Patil (1997)
Aspergillus foetidus	Banerjee *et al.* (2001)
Penicillium notatum	Ganga *et al.* (1977)
Penicillium islandicum	Ganga *et al.* (1977)
Penicillium chrysogenum	Bradoo *et al.* (1996)
Penicillium digitatum	Bradoo *et al.* (1996)
Penicillium acrellanum	Bradoo *et al.* (1996)
Penicillium carylophilum	Bradoo *et al.* (1996)
Penicillium citrinum	Bradoo *et al.* (1996)
Penicillium charlessi	Bradoo *et al.* (1996)

Table 3. Bacterial sources of tannase

Microorganism	References
Bacillus plumilus	Deschamps *et al.* (1983)
Bacillus polymyxa	Deschamps *et al.* (1983)
Corynebacterium sp.	Deschamps *et al.* (1983)
Klebisella pneumoniae	Deschamps *et al.* (1983)
Pseudomonas solanaceaum	Deschamps *et al.* (1983)
Citrobacter freundii	Kumar *et al.* (1999)
Lactobacillus plantarum	Osawa *et al.* (2000)
Lactobacillus paraplantarum	Osawa *et al.* (2000)
Lactobacillus pentosus	Osawa *et al.* (2000)
Bacillus lichiniformis	Mondal *et al.* (2000)
Bacillus cereus	Mondal *et al.* (2001)
Lactobacillus plantarum	Ayed and Hamdi (2002)
Lactobacillus paraplantarum	Nishitani *et al.* (2004)
Lactobacillus acidophiuis	Nishitani *et al.* (2004)
Lactobacillus pentosus	Nishitani *et al.* (2004)
Lactobacillus animalis	Nishitani *et al.* (2004)
Lactobacillus murinus	Nishitani *et al.* (2004)
Lactobacillus faecalis	Nishitani *et al.* (2004)
Lactobacillus acidilactici	Nishitani *et al.* (2004)
Lactobacillus pentosaceaus	Nishitani *et al.* (2004)

2.7. **Tannase Purification**

TAH delivered by SSF indicated higher temperature (40–60 °C) and pH (6.0–6.5) steadiness. A few creators have additionally reported that tannase delivered by SSF is more steady at an extensive variety of temperatures and pH (Lekha and Lonsane 1994; Rana and Bhat 2005). All in all, substrates with high tannin substance are utilized for the creation of tannase by SSF. The substrate is soaked with a mineral arrangement and is vaccinated with the chose life form. Among the regular backings that have been utilized for the generation of tannase are sugar stick bagasse, wheat grain, tamarind seed powder, creosote shrub leaves (Larrea tridentata), bark of chestnut tannins (Caesalpinia spinosa), oakgalls (Quercus infectoria), leaves of sumac (Rhus coriaria), myrobalan natural product (Terminalia chebula), leaves of (Sorghum vulgaris), and leaves of Indian gooseberry.

In any case, as of late, dormant backings, (for example, polyurethane froth) impregnated with supplement media are progressively being utilized (Cruz-Hernández et al. 2006; Mata-Gómez et al. 2009; Renovato et al. 2011). The utilization of dormant backings and characterized society media

encourages the checking and control of parameters amid the SSF procedure (Zhu et al. 1994). Kar et al. (1999) utilized an altered strong state aging (MSSF) for synchronous creation of gallic corrosive and tannase utilizing a strain of R. oryzae.

In correlation with conventional SSF frameworks, MSSF expanded the tannase generation and gallic corrosive yield just about 1.7 and 3 times separately (Kar and Banerjee 2000). Van de Lagemaat and Pyle (2001) built up a model of consistent strong state aging for the contagious tannase generation. The bioreactor could work consistently and without immunization of the food. Nonetheless, bring down biomass, tannase yield, and sporulation rate were accomplished when contrasting and a static bunch society, most likely because of the pernicious impact of shear.

Tannase is at present marketed by a couple organizations: Biocon (India), Kikkoman (Japan), ASA uncommon protein GmbH (Germany), Julich Chiral Solutions GmbH (Germany), Wako Pure Chemical Industries, Ltd. (Japan), Novo Nordisk (Denmark), and Sigma-Aldrich Co. (USA). They are the principle suppliers, and they offer tannase arrangements with various immaculateness and reactant units relying upon the presentation of the item (Aguilar et al. 2007).

2.8. **Tannase Applications and Potential Uses**

Tannase is generally utilized as a part of production of moment tea and oak seed wine.Tannase has potential application in the elucidation of brew and organic product juices, assembling of espresso seasoned soda pops, and change in kind of grape wine, and as a diagnostic test for deciding the structure of normally happening gallic corrosive esters (Seth and Chand, 2000).

Tannase has likewise been connected for cleavage of poly phenolics, for example, dehydrodimer crosslinks present in the cell mass of plants, which is fundamental for plant cell divider absorbability (Garcia-Conesa et al., 2001). The modern uses of tannase have not been completely misused as a result of its high cost, in spite of the fact that there are a substantial number of reports on the creation of tannase by submerged maturation, the greater part of these don't include the distinguishing proof of basic parameters for catalyst biosynthesis and their streamlining.

Gallic corrosive is likewise utilized as an imperative substrate for amalgamation of propyl gallate in nourishment industry as an additive and as a hostile to oxidant (Lekha and Lonsane, 1997). Mechanical bioconversion of tannic corrosive is by and large achieved by the protein tannase for the creation of gallic corrosive.

2.8.1. **Modern Applications**

A. **Lager and wine creation**

Tannase could hydrolyze wort phenolics which complex with different chemicals in brew blend and result in cloudiness arrangement (Giovanelli, 1989). c (Chae and Yu,1983). On account of wines, express that the fundamental tannins present are catechins and epicatechins, which can get a complex with galacto-catechins and others galloyl derivated.

Fifty percent of the shade of the wines is because of the nearness of the tannins; in any case, if these mixes are oxidized to quinines by contact with the air it could frame an undesirable turbidity, showing extreme quality issues.

At the point when the proteins of the lager are in significantly high amounts an undesirable turbidity is displayed by fulfilling with these tannins. The utilization of tannase can be an answer for these issues.

B**. Cool tea items**

The cloudiness arrangement in tea is because of coacervation of tea flavanoids, comprising for the most part of epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate. Tea polyphenols structure hydrogen bonds with caffeine which prompts cream arrangement. Purchasers

would lean toward clear items, so the mixes shaping murkiness ought to be expelled to get an item free of turbidity and chemicals utilized as clarifiers.

Tannase has reactant action to expel gallic corrosive moieties from tannins and the polyphenols from tea separates which results in coldwater solvent items. The treatment of tea with tannase improves the common levels of epicatechin and gallic corrosive which thusly supports the development of epicatechin flavic corrosive which is in charge of splendid rosy shade of tea with great chilly water solvency and shading (Albertse, 2002).

C. **Creature nourish**

The dimness development in tea is because of coacervation of tea flavanoids, comprising primarily of epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate. Tea polyphenols structure hydrogen bonds with caffeine which prompts cream arrangement. Customers would lean toward clear items, so the mixes framing murkiness ought to be evacuated to get an item free of turbidity and chemicals utilized as clarifiers.

Tannase has synergist movement to expel gallic corrosive moieties from tannins and the polyphenols from tea extricates which results in coldwater solvent items. The treatment of tea with tannase upgrades the common levels of epicatechin and gallic corrosive which thus supports the development of epicatechin flavic corrosive which is in charge of splendid ruddy shade of tea with great chilly water solvency and shading (Albertse, 2002).

D. **Pharmaceutical industry**

Gallic corrosive (3, 4, 5-trihydroxy benzoic corrosive), which is integrated artificially is utilized as a part of pharmaceutical industry for generation of hostile to bacterial medication trimethoprim (Bajpai and Patil, 1996; Lekha and Lonsane, 1997) utilized as a part of blend with sulphonamide.

Gallic corrosive has been incorporated synthetically, however this concoction blend has been known not exceptionally costly and not generally extremely specific. Gallic corrosive is one of the items endless supply of tannic corrosive with tannase (Iibuchi et al., 1972). It is utilized as an engineered middle of the road for the creation of pyrogallols and gallic corrosive esters. Propyl gallate which is extremely costly is utilized as a hostile to oxidant as a part of fats and oils, in sustenances, makeup, hair items, cements and oil commercial enterprises (Gaathon et al., 1989; Haadi et al., 1994; Yamada and Tannaka, 1972). Tannase catalyst is gainful in encouraging the breakdown of extraneous stain and is helpful in hydrolysis of tannins which is known not the tooth surface (Laurence Du-Thumm, et al.,2005).

2.9 **Response Surface Methodology**

Optimizing alludes to enhancing the execution of a framework, aprocess, or an item with a specific end goal to get the most extreme benefit fromit. The term enhancement has been usually utilized as a part of analytical chemistry as a method for finding conditions at which to apply a procedure that creates the most ideal reaction .Traditionally, advancement in logical science has been auto ried out by observing the influence of one element at once on anexperimental reaction. While stand out parameter is changed, oth-ers are kept at a steady level. This streamlining strategy is calledone-variable-at once. Its real weakness is that it does notinclude the intuitive impacts among the variables considered. As aconsequence, this strategy does not delineate the complete effectsof the parameter on the reaction . Another detriment of theone-component streamlining is the expansion in the quantity of experi-ments important to direct the exploration, which prompts an increaseof time and costs and also an increment in the utilization ofreagents and materials.In request to defeat this issue, the advancement of analyti-cal techniques has been done by utilizing multivariate statistictechniques. Among the most important multivariate strategies used in expository improvement is reaction surface system (RSM).Response surface procedure is a gathering of numerical and statistical methods in view of the fit of a polynomial equation to the trial information, which must portray the conduct of a data set with the goal of making factual previsions. It can be very much connected when a reaction or an arrangement of reactions influenced by a few variables. The goal is to simultane-ously enhance the levels of these variables to accomplish the best system performance.

Before applying the RSM strategy, it is first fundamental to choose a trial outline that will define which experiments should be done in the trial district being studied.There are some trial networks for this reason. Experi-mental outlines for first-request models (e.g., factorial plans) canbe utilized when the information set does not present arch .How-ever, to rough a reaction capacity to test datathat can't be depicted by direct capacities, test designsfor quadratic reaction surfaces ought to be utilized, for example, three-level factorial, Box–Behnken, focal composite, and Doehlertdesigns.The present paper talks about the utilization of RSM for advancement inanalytical science. To begin with, its essential standards are exhibited. Then,the way to deal with the utilizations of its more frequentlyused second-arrange test plans is proposed, and the optimizationof methods that create numerous reactions.

This term was begun from the graphical viewpoint created after fitness of the mathematical model, and its utilization has been broadly embraced in writings on chemo-measurements. RSM comprises of a gathering of numerical and statisticaltechniques that depend on the fit of exact models to the experimental information acquired in connection to trial design.Toward this target, straight or square polynomial capacities areemployed to depict the framework contemplated and, therefore, toexplore (displaying and dislodging) exploratory conditions untilits streamlining .Some stages in the utilization of RSM as an improvement tech-nique are as per the following:

(1) the choice of free variables of significant consequences for the framework through screening studies and thedelimitation of the trial district, as per the objec-tive of the study and the experience of the specialist;

(2) thechoice of the test outline and doing the exper-iments as per the chose test network;

(3) themathematic–statistical treatment of the got experimentaldata through the fit of a polynomial capacity;

(4) the evaluationof the model's fitness;

(5) the verification of the need andpossibility of performing a relocation in bearing to the opti-mal area;

(6) getting the ideal qualities for each studied variable.

3. MATERIALS AND METHODS

3.1 **Isolation of Tannase Producing Microorganisms**

The tannase producing strains were revived on a solid CZ (Czapek Dox) medium and also within a liquid CZ suspension containing 1% filter sterilized tannic acid (TA) as C-source. The initial screening of the isolates was done on Czapek Dox's minimal media supplemented with tannic acid (1%) as a sole carbon source. The tannase producers were identified by clear zone of tannic acid hydrolysis around their growth.

3.1.1 **Medium**

Czapek Dox medium with the creation given underneath was utilized all through the study for all the strains.

CZAPEK DOX MEDIUM

Chemical	Concentration
$(NH_4)_2SO_4$	0.87%
KH_2PO_4	0.44%
$MgSO_4.H_20$	0.088%
$CaCl_2.7H_2O$	0.009%
$NaMoO_4.2H_20$	0.0008%
$FeSO_4.7H_2O$	0.012%
$MnCl_2.6H_2O$	0.002%

Table 4 : Components of CZ media

3.1.2 **Plating strategy**

The plates and the liquid suspension was incubated at 40ºC for 6 days. The plates were watched for clear zone depicting the presence or absence of tannase . On the other hand liquid suspension is observed for turbidity .

3.2 **Quantitative measure of tannase activity**

For the quantitative measure of tannase the technique for methanolic rhodanine was utilized. The way of life was developed in negligible media containing TA (1%) as sole carbon source. The inoculum was included as agar glasses with 2.5x105 spores/agar container as tallied by hemocytometer. Following 6 days of brooding at 37°C, the way of life aliquots were centrifuged and the supernatant was examined for tannase test as point by point beneath:

3.2.1 **Gallic acid standard plot**

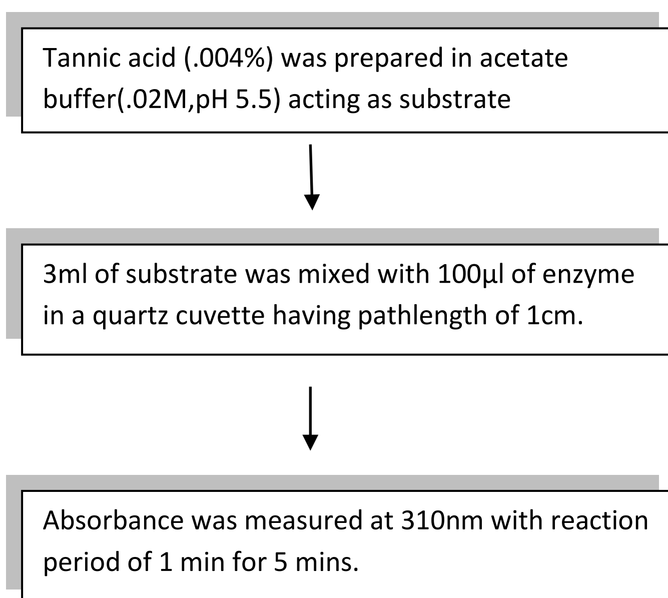
Gallic corrosive standard plot was produced from a technique. This was done to check the action of tannase.

i. The response blend was set up by utilizing convergences of 0.04gm gallic corrosive in 40ml refined water and 0.05M citrate support. This was trailed by expansion of 300µl of 0.667 % rhodanine and 200µl of 0.5 M Potassium hydroxide.

ii. After incubating at 30ºC for 5 min the blend was weakened 5 times with refined water.

iii. Absorbance was taken at 520nm.

3.2.2 **Tannase Assay**



3.4. **Production of tannase utilizing tea deposits**

For the generation of the tannase , the organism(Aspergillus sp.) having the potential to synthesize tannase was added into a flask containing 5gm of tea residue(agro waste) as solid substrate and 15ml of CZ broth as moistening medium in order to establish SSF . The tea buildups were gathered from cafetaria and mess of JUIT itself. The tea deposits were washed with water and after that dried for further utilize.

Before the innoculation of the organism the flask along with tea residue and moistening medium is autoclaved to avoid contamination. Later on in the LAF (laminar air flow hood) the flask is inoculated with three discs each from the Aspergillus sp. containing plate . At that point the flask was fixed legitimately and incubated in different set of pysico-chemical conditions taking four contraints into consideration that are:

i. Moisture Level

ii. pH

iii. Temperature

iv. Incubation Time:

SSF was established using above mentioned four constraints with their varying combinations :

pH	3.0	4.0	5.0
Temperature (˚C)	30	37	40
Incubation Time (days)	6	8	10
Moisture Content (ml)	10	15	20

Table 5: Combination of parameters taken into consideration for SSF

3.5 **Enzyme extraction**

After indicated time of incubation , 25 ml of 1% (w/v) NaCl was added to the fermented residue. The protein was extricated from strong substrate by shaking at 200 rpm for 1 h. The concentrate was gone through muslin fabric. The concentrate acquired was centrifuged at 10,000g for 5 min and the pellet was spread on a glass plate and kept for overnight drying in oven.

3.6 **Methanol extraction**

Methanol has a polarity index of 5.1. Mostly methanol is used for extraction various polar compounds but certain group of non polar compounds are fairly soluble in methanol if not readily soluble. Therefore methanol is commonly used for extraction of bioactive compounds. Moreover methanol among all the alcohols has low boiling point of just 65 degree Celsius. So extraction and concentration of bioactive compounds is easy .

The pellets are processed for methanol extraction after overnight drying by following method:

Add dried pellet into the vial

Add 5ml 70% methanol and keep in waterbath for 70°C

Cool at room temperature

Centrifuge at 200g for ten minute

Collect supernatent

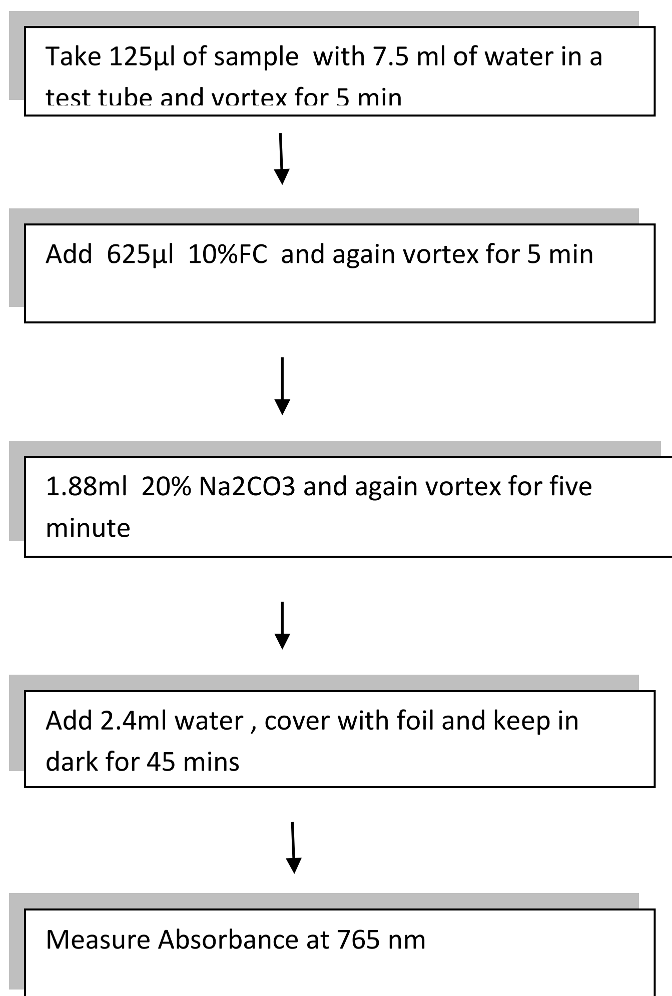
Pool extract and make final volume 10ml with 70%methanol

Dilute 1ml of extract in 100ml water.

3.7 **Total Phenolic Content (TPC) estimation**

Polyphenols in plant extracts react with specific redox reagents FC (Folin-Ciocalteu reagent) to form a blue complex that can be quantified by visible-light spectrophotometry . The reaction forms a blue chromophore constituted by a phosphotungsticphosphomolybdenum complex , where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds.

The following methodology was used in the TPC determination of the sample after methanol extraction :



3.8 **Response Surface Methedology (RSM)**

Response Surface Methodology (RSM) is an experimental technique invented to find the optimal response within specified ranges of the factors. These designs are capable of fitting a second-order prediction equation for the response. The quadratic terms in these equations model the curvature in the true response function. If a maximum or minimum exists inside the factor region, RSM can estimate it. In industrial applications, RSM designs usually involve a small number of factors.

This is because the required number of runs increases dramatically with the number of factors. Using the response surface designer, you choose to use well-known RSM designs for two to eight continuous factors. Some of these designs also allow blocking.

The software used was JMP for statistical analysis.

To start a response surface design, select **DOE > Response Surface Design**, or click the **Response Surface Design** button on the JMP Starter **DOE** page. Then, follow the steps described in the following sections.

•Enter Responses and Factors

•Choose a Design

•Specify Axial Value (Central Composite Designs Only)

•Specify Output Options

•View the Design Table

4. RESULTS AND DISCUSSION

1**. Morphological characteristics of tannase producing fungus:**

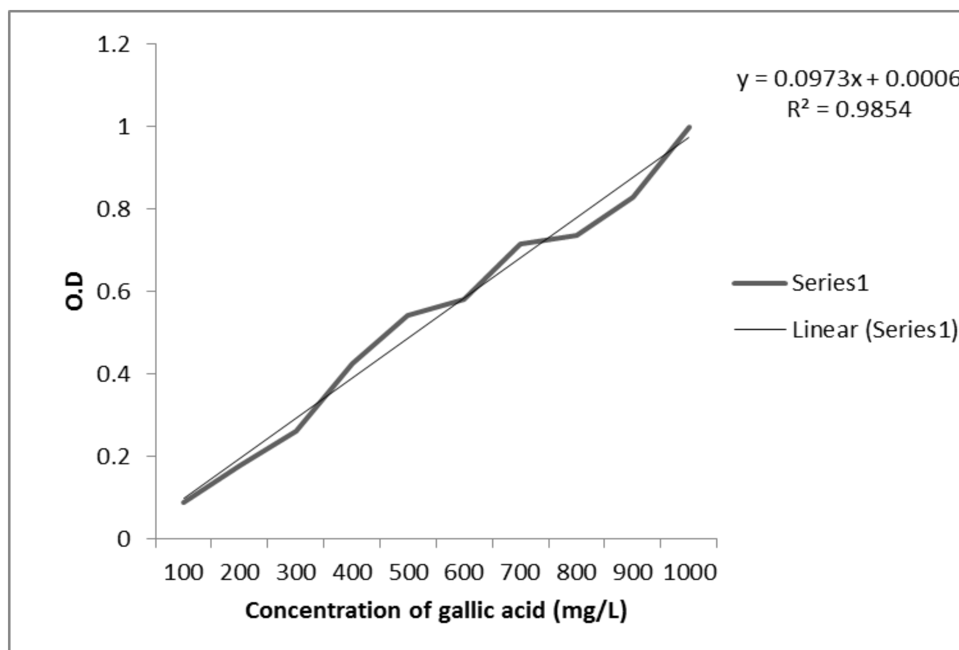
The fungal strain was kept at 37°C and 30̊ C respectively. The culture was a fast grower, the colony size increased within a week when grown on Czapek-Dox agar at 37°C. However, the growth and tannase activity was lower when the fungus was grown at 30°C

5.a. Zone of Clearance on CZ Media.

2. **Quantitative analysis of tannase:**

For qualitative assay of the enzyme tannase, gallic acid standard plot was prepared and tannase assay was done. The observation concluded from these experiments are given in the following tables

3. **Gallic acid standard plot**



4. **Tannase Assay**

The tannase assay was carried out from the czapek dox broth samples collected at different time intervals of 48, 120, 144 h. To neglect the effect of gallic acid present in fermentation, the enzyme sample was heat treated at 95° C for 10 min and was taken as control to calculate Tannase activity units/ ml.

Methyl Gallate (µl)	**Buffer(µl)**	**Enzyme Sample(µl)**	**Rhodanine (µl)**	**KOH(µl)**		**Distilled water (ml)**	**Tannase activity (U/ml)**	**Control**	**Tannase activity (U/ml)**
250	250	100 (48 hours)	300	200	Incubation at 30°C for 5min.	4	203.6	50.0	281
250	250	100 (120 hours)	300	200		4	259.3	63.0	296
250	250	100 (144 hours)	300	200		4	290.3	74.6	216

Table 6: tannase assay of CZ broth samples collected at different time intervals

Here the enzyme sample which was incubated for 120 hours had the highest activity while the sample which was incubated for 144 hours had the lowest activity of enzyme . In the heat treated samples, residual amount of gallic acid was estimated. The enzyme activity was calculated from the change in absorbance at 520nm

5**. Fermentation of tea residues**

SSF offers a number of advantages over conventional submerged fermentation for enzyme production (Mudgett, 1986). The production medium is often simple, using agro-industrial by-products like wheat bran, rice bran or wheat straw as substrate (Mitchell and Lonsane, 1992). Because the moisture level is low, the volume of medium per unit weight of substrate is low. Hence, enzyme activity is usually very high. Thus to achieve a given enzyme productivity, fermentor volumes can be much smaller than in submerged fermentation systems (Mitchell and Lonsane, 1992).

In this study, tea residue was selected as a substrate for the production of tannase under SSF. It is reported that the tea residue contains 20–30% polyphenols, 4–5% caffeine, 2–4% amino acids, 2–4% sugars, 2.5% organic acids, 4–6% pectic substances, 4–8% lipids, 0.5% pigments, 16% proteins, 5% minerals, and less than 0.1% volatiles, besides cellulose, hemicellulose, and lignin.] The diverse chemical constituents of tea residue, mainly phenols, sugars, and amino acids, are favorable to fungal growth and tannase production. Further, tea residue shows higher water retation capacity that absorbs medium and makes it readily available to the fungal mycelia; in addition, tea particles also provide increased surface area to the fungal mycelia for attachment, leading to pseudo-immobilization of the mycelia.

SSF was performed using different combinations of four variables (temperature,pH,moisture content,incubation days) and the most ideal conditions were determined for production of tannase through tannase assay. As the incubation began, the mycelium started spreading in the flasks and it was observed that whole of the substrate was covered with mycelium.Tannase assay eas performed after enzyme extraction through NaCl followed by Methanol extraction.Activity of tannase was observed in each flask with the help of tannase assay.

pH	3.0 (P1)	4.0 (P2)	5.0 (P3)
Tannase Activity(units/ml)	1.22	1.99	0.98
Temperature (˚C)	30 (T1)	37 (T2)	40 (T3)
Tannase Activity(units/ml)	1.51	1.41	0.45
Incubation Time (days)	6 (D1)	8 (D2)	10 (D3)
Tannase Activity(units/ml)	1.01	1.69	1.67
Moisture Content (ml)	10 (V1)	15 (V2)	20 (V3)
Tannase activity(units/ml)	2.89	1.91	1.90

Table 6 : tannase activity in the jars with SSF established

The above data depicts that the most ambient conditions for production of tannase through SSF using tea residue are pH 4, temperature 37̊ C,incubation period of 6 days and 10ml of CZ broth as moistening medium.

5.b Before and After inoculation

5.c SSF jars after incubation period has been completed

5.d Overnight dried tea residue after SSF processed for Methanol Extraction

6. Response Surface Methodology

Among the most important multivariate strategies used in expository improvement is reaction surface system (RSM).Response surface procedure is a gathering of numerical and statistical methods in view of the fit of a polynomial equation to the trial information, which must portray the conduct of a data set with the goal of making factual previsions. It can be very much connected when a reaction or an arrangement of reactions influenced by a few variables. The goal is to simultane-ously enhance the levels of these variables to accomplish the best system performance.

RSM was performed using a JMP software that took into consideration all possible combinations of pH,temperature,moisture content and incubation period to determine best system for production of tannase enzyme.

After SSF has been established in each system of multivariable , the jars are then processed further for TPC and Tannase assay to determine most ideal condition for tannase production .

S.No	pH	Temperature (˚C)	Moisture content (ml)	Incubation time (days)	Loss in weight (%)	TPC content (µg/ml)	Tannase assay (enzyme units/ml)
1	3	35	7	6	14.6	26.18	3.15
2	5	30	10	6	26.3	30	1.49
3	4	35	10	9	23.3	24	1.42
4	4	35	10	3	14.6	17.9	2.67
5	4	40	13	6	36.6	22.54	1.62
6	4	40	10	3	22.3	25.36	10.1
7	4	40	7	6	31	35.36	1.32
8	4	35	7	9	29.3	22.54	1.28
9	4	30	7	9	21.6	28.81	1.82
10	3	35	10	9	13.6	35.81	0.93
11	4	30	13	9	24	26.81	2.57
12	4	40	10	3	21.6	29.90	1.60
13	5	35	10	3	29.3	31.27	1.99
14	3	35	13	3	40	27.09	0.52

Table 8: TPC and tannase assay of RSM jars

15	5	35	10	6	21.3	16.27	4.
16	4	35	7	6	11	21.09	1.
17	4	35	10	6	24	25.36	2.
18	5	35	13	9	22	25.09	3.
19	4	35	13	6	11.3	32.72	4.
20	5	40	10	9	17.6	28.27	2.
21	4	35	13	9	26	27.90	1.
22	4	30	10	6	32.6	22.90	4.
23	3	40	10	6	7	25.81	3.
24	4	30	10	9	13	20.72	1.
25	4	35	10	6	29	27.63	1.
26	5	35	7	6	20.3	29.45	3.
27	3	30	10	6	18.3	28.45	1.
28	4	35	10	6	27.6	32.45	2.
29	3	35	10	3	23.6	27.45	4.

NOTE: one unit of enzyme was defined as decrease of OD of 0.01 (for tannase assay)

In flask 22 highest tannase activity was observed i.e 4.86 enzyme units/ml thereby depicting that the most ambient conditions for tannase production are pH 4.0, temperature 30℃, incubation period of 6 days and moisture level of 10ml in 3gm of substrate.

5.d RSM Flasks after incubation for their respective time period

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