International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(4): 2907-2913
© 2019 IJCS
Received: 22-05-2019
Accepted: 24-06-2019

Tanvi
Ph.D. Scholar, Department of Biotechnology, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

C Duni
Professor, Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India

Correspondence
Tanvi
Ph.D. Scholar, Department of Biotechnology, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Enhanced production of tannase through response surface methodology and its partial purification from fungal isolate (TPF-13)

Tanvi and C Duni

Abstract
For various industrial purposes, an important enzyme Tannin acyl hydrolase (tannase) is used to catalyse the reaction by breaking up of the ester and depside bonds in tannin rich products. In present studies, screening of tannase yielding fungal strains was done and isolated strain was marked as tannase producing fungus (TPF-13). It was a hyper-producer strain that produced extracellular tannase under submerged fermentation using tannic acid powder. By using Response surface methodology, the culture conditions for best tannase production were optimized which included the effects of different parameters on TPF-13. Maximum tannase activity (346 U/ml) was obtained in medium supplemented with 0.05% each of K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, 0.3% NH_4NO_3, 0.03% $CaCl_2$ and 7% tannic acid at 30 ºC and pH 7 after 72 hours. The specific activity of 1.25 U/mg with a yield of 0.007 per cent was recorded from partially purified tannase with 0.25 fold.

Keywords: Tannin acyl hydrolase, tannic acid, RSM and partial purification

1. Introduction
The expediency of bioconversion process such as high turnout, process consistency and environmental safety using enzymes have been well accustomed over conventional chemical technologies. The dissociation of galloyl ester of gallic acid and an alcohol moiety in tannins is catalysed by tannase which is an inducible and hydrolytic enzyme which produces different products (Aguilar *et al*. 2007; Belur and Mugeraya, 2011) [2, 6]. The double activity of (breaking both ester and depside bonds) tannase have been attributed to the existence of bi-functionality of a single enzyme (Haslam and Stangroom 1966) [12]. Tannase is widespread in nature and is found to be notably similar in its overall size and composition of redox centres, as well as its ability to oxidize a wide variety of tannin-rich substrates. Various animals, plants, yeast, fungi and bacteria are known to be the potential sources of tannase (Lekha and Lonsane 1997) [16], but microbes are the preferential reservoir for generation of tannase enzyme because of diversity of their biotechnical, biochemical processes, cost effectiveness and are easy to be cultured in large amount in a short period of time by certified standard fermentation process. Different fungal strains (more than 60) have been recorded for tannase production. The economically feasible tannase production is also accomplished by *Aspergillus* strains (Lagemaat and Pyle 2006) [27]. A number of microorganisms which are potential sources have been reported and scientists are continuously exploring the organisms which are more prolific degraders and better sources of tannase.
Some bacterial species like *Enterobactor* spp., *Bacillus sphaericus*, *Bacillus pumilus, Serratia marcescens*, *Lactobacillus* species ASR-S1, *Lactobacillus hilgardii* (Deschamps *et al*. 1983; Belur *et al*. 2010; Sharma and John 2011) [10, 5, 25] and fungal sources like *Aspergillus* spp, *Penicillium verrucosum, Trichoderma harzianum* (Lal and gardener 2012; Bhoite *et al*. 2013; Lokeshwari and Lenin 2013) [15, 8, 17] are the potential sources of tannase production.
Molecular weight of tannases purified so far ranges from 50-300 kDa (Hatamoto *et al*. 1996; Lagemaat and Pyle 2006) [27] and contains 25-66 per cent w/w carbohydrate content. The optimum pH and temperature for tannase range between 5.0-6.0 and 30-50°C.
In plant kingdom, tannins are second most bounteous group of naturally occurring polyphenolic compounds with varying molecular weights, after lignin. Tannins are divided into four different major groups: gallotannins, condensed tannins, ellagitannins and complex tannins (Khanbabaee and Ree 2001) [14].

Enzyme reticence and substrate destitution reaction on membranes of microorganisms are the main cause of toxicity due to tannins (Reed 1995) [23].

Tannase also has a potential application in the tannery effluent, paper and pulp effluent and olive oil wastewater treatment (Ainnie *et al*. 2013) [3]. Recently, new fruit juices have been approved for their benefits related to health, specifically for its disease-fighting antioxidant prospective (Shrivastva and Kar 2009) [26]. Gallic acid produced during hydrolysis finds application in many areas such as manufacturing of ordinary writing iron gallate ink and dyes as a photographic developer, and some biological activities, like antibacterial, antiviral and analgesic (Beniwal *et al*. 2013) [7]. Synthesis of trimethoprim, an antibiotic which requires gallic acid as an intermediate (Mahendran *et al*. 2006) [18] which when given along with sulphamethoxazole, have a broad spectrum of action.

Tannase is commercialized by different companies such as Biocon (India), Kikkoman (Japan), ASA special enzyme GmbH and JFC GmbH (Germany) with different catalytic units depending on the product presentation. Tannase is produced by Solid state culture by Kikkoman and Biocon.

Given the tremendous potential of tannase enzyme, efforts made to optimize the culture conditions for enhanced production and yield is a worthwhile endeavour. In achieving media optimization and to understand interactions among different parameters by using less number of experiments, use of statistical approaches has got substantial engrossment, recently. Statistical approaches saves much time and cost as Compared to conventional methods. The Plackett-Burman design (PBD) is most widely used to incline the expressive effect on response of independent variables (Xiao *et al*. 2015) [28]. As the extensity of applications of this enzyme is very immense, there is always a purview for peculiar tannase with better characteristics, which may be felicitous in the diverse application domains (Girdhari and Peshwe 2015) [11]. Despite that, many scientists are working on isolation, optimization and purification of the novel species of tannase from diverse areas or ecosystems. Thus by perceiving the significance of the enzyme tannase, the present study aimed to find hyper-producer isolate and further enhanced production of tannase by optimizing media components through RSM and its partial purification.

2. Material and Methods

2.1 Chemicals

Tannic acid, rhodanine and gallic acid were procured from Sigma-Aldrich (St. Louis, Mo). Media components were purchased from Hi-Media (India). All the other chemicals were of analytical grade and were obtained from Sigma-Aldrich and Hi-Media, unless otherwise specified.

2.2 Sample collection

Soil samples were collected from various sites of Himachal Pradesh and enriched in tannin.

2.3 Isolation and screening of tannase producing fungi

Isolation of different fungal and bacterial strains was done from one ml of enriched samples and was serially diluted to 10^{-4} to 10^{-6} times with 0.89 per cent saline and 100 µl of diluted sample and was spread over tannic acid agar plates. Plates containing 0.05 per cent each of K_2HPO_4, KH_2PO_4, $MgSO_4$, 0.3 per cent NH_4NO_3, 0.01 per cent $CaCl_2$ and 0.1 per cent tannic acid were incubated at 30ºC for 72 hours. Fungal colonies developed were isolated and purified by repeated sub-culturing. Fifteen isolates were isolated from different soil samples and each isolate was assigned separate code number. Out of these isolates, only 4 showed good tannase activity as stated in method described by Sharma *et al*. (2000) [24]. On the basis of which, one fungal isolate designated as TPF-13 was selected for further experimental studies and fresh plate of this isolate was prepared on tannic acid agar plate.

2.4 Tannase production

Production of enzyme was carried out in a flask containing 100 ml tannic acid broth medium having components (g/l): K_2HPO_4; 0.5, KH_2PO_4; 0.5, $MgSO_4$; 0.5, $CaCl_2$; 0.1, NH_4NO_3; 3.0. The medium was steam sterilized at 121°C for 15 minutes. One percent of filter sterilized tannic acid was added to the medium. The pH of the medium was adjusted to 5.5 and inoculated with fungal spore suspension and incubated at 30 °C for 72 hrs. in an incubator shaker (150 rpm). Then incubated media was filtered with Whatman Filter Paper and the filtrate was used as crude enzyme and was further assayed for enzyme activity.

2.5 Assay of tannase activity

Tannase hydrolysis was determined spectrophotometrically using rhodanine with some modifications (Sharma *et al*. 2000) [24]. The buffer substrate of 0.01M methyl gallate in 0.05M citrate buffer (pH 5.5) was used for tannase assay. The blank test tube contained only 1500 µl of buffer. Test and control tubes contained 500µl of substrate to which 995µl of buffer and then tannase (5 µl enzyme) was added to the test sample and incubated at 40ºC for 5 minutes and 600 µl of methanolic rhodanine (0.667per cent, w/v) was added and further incubated at 40ºC for 5 minutes. Thereafter, reaction was stopped by adding 400 µl of 0.5 M potassium hydroxide followed by further incubation at 40ºC for 5 minutes. The observations were recorded at 520 nm using spectrophotometer. One unit of tannase activity is defined as the amount of enzyme required to release 1 µmol of gallic acid per unit time under standard assay conditions.

2.6 Protein estimation

The protein estimation was done by a method using BSA as standard depicted by Bradford in 1976.

2.7 Optimization of tannase production using response surface methodology

Optimization of tannase production by response surface methodology was done using two designs:

2.7.1 Plackett-Burman design

Effect of nine independent factors i.e. K_2HPO_4 (0.03-0.07 per cent), KH_2PO_4 (0.03-0.07 per cent), $MgSO_4.7H_2O$ (0.03-0.07 per cent), ammonium nitrate (0.1-0.5 per cent), calcium chloride (0.01-0.05 per cent), tannic acid (0.5-5 per cent), pH (3-7), temperature (20-50°C), time (2-5 days) on tannase production were screened using Plackett-Burman design of Design expert software 9.0.5. Experiments were performed at different combinations of low and high levels of variables and scrutinized for their effect on enzyme production. Pareto chart was fabricated to find out the positive factors or negative factors.

2.7.2 Central composite design

Central composite design (CCD) experiment was drafted for the optimization of the values of ammonium nitrate, calcium

chloride, tannic acid, pH, which showed a positive effect on tannase production. Second order (quadratic) polynomial coefficient was calculated and analysed by using Design expert software 9.0.5 (Adinarayana and Ellaiah 2002) [1].

2.8 Partial purification of tannase from TPF-13

2.8.1 Ammonium sulphate precipitation

Tannase was extracted by filteration at the end of incubation and ammonium sulphate was added to the crude enzyme to achieve 10-90 per cent saturation. The contents were kept overnight at 4°C to achieve optimum precipitation. The sediment was centrifuged at 10,000 'g' for 30 minutes at 4°C to collect the precipitates containing enzyme and a minimum amount of buffer (0.05 M citrate buffer, pH 5.5) was added to this precipitate to dissolve it. The tannase activity and protein concentrations were determined as described earlier.

2.8.2 Dialysis and column chromatography

The enzyme was dialyzed against citrate buffer (0.05 M, pH 5.5) for 24 hours and then it was loaded onto a column packed with Sephadex G-200. The column was subsequently eluted with 0.05 M citrate buffer (pH 5.5) and fractions of 2.0 ml were collected. The protein and tannase activity were determined in each collected fraction. The purity of tannase was established by polyacrylamide gel electrophoresis.

2.8.3 SDS polyacrylamide gel electrophoresis and molecular weight determination

SDS-PAGE was carried out to separate protein on the basis of its molecular weight and to determine purity of the protein. Concentration of acrylamide was 4 per cent for stacking gel and 8 per cent for separation gel. The gel was run untill the bromophenol blue dye (tracking dye) reached the other end of the gel at 120V. Gel was stained by a solution of Coomassie blue in methanol, glacial acetic acid and distilled water for half an hour at room temperature. Gel was de-stained in a solution of glacial acetic acid and methanol to remove excess dye and process continues till the gel became transparent (Laemmli, U.K. 1970).

3. Results and Discussions

3.1 Isolation and screening of tannase producing microbes

The fungal isolate TPF-13 showed maximum activity and was selected for further experimental studies.

3.2 Optimization of production of tannase using Response Surface Methodology

3.2.1 Evaluation of significant variables

The effect of nine independent variables (K_2HPO_4, KH_2PO_4, $MgSO_4$, NH_4NO_3, $CaCl_2$, tannic acid, pH, temperature and time) was observed on the tannase production and study was carried out in 12 runs using Plackett-Burman design. Out of nine selected variables four variables viz. tannic acid, NH_4NO_3, $CaCl_2$ and pH have showed a positive effect on enzyme production through Pareto chart. The results were fitted onto equation:-

$$R_1 = -7.66037+10.19167*NH_4NO_3 + 59.75000*CaCl_2 + 3.37407*\text{Tannic acid} + 0.86000*pH$$

Where R_1 is the response of tannase activity and ammonium nitrate, calcium chloride, tannic acid and pH were the factors which showed a positive impact on tannase production.

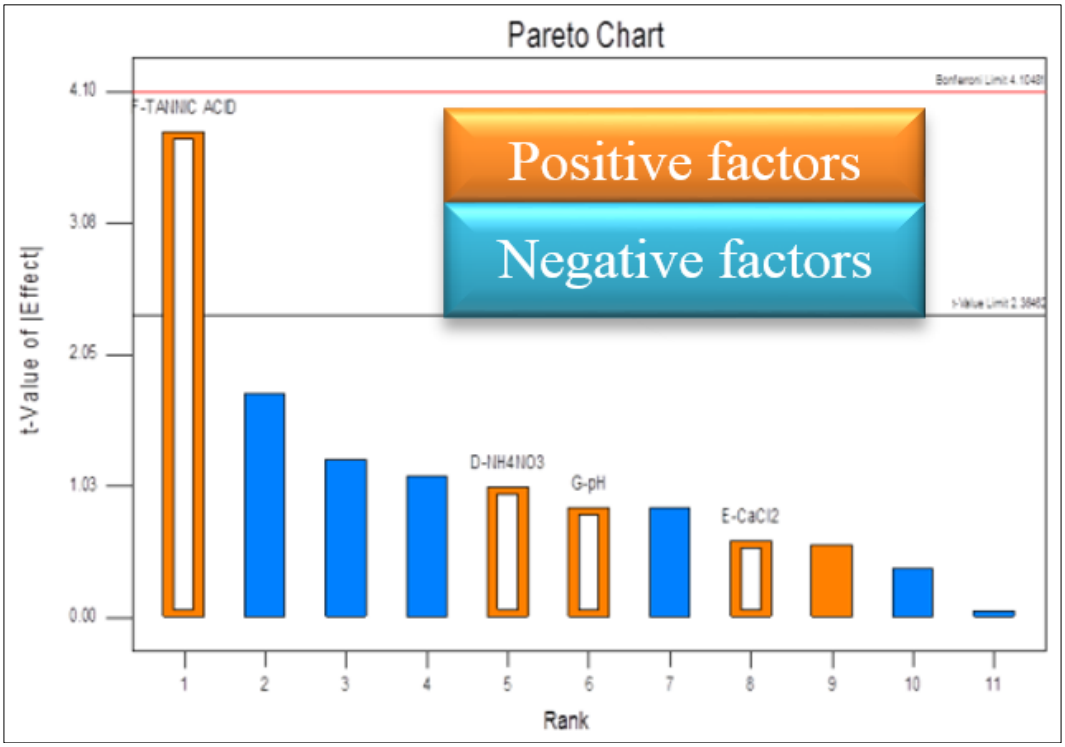


Fig 1: (a) A Pareto chart showing the positive and negative effects of selected variables (KH_2PO_4, K_2HPO_4, Magnesium sulphate, Ammonium nitrate, Calcium chloride, temperature, pH, tannic acid, time).

3.2.2 Optimization of significant factors by CCD

For the resolution of optimum level and the interactive effects of four variables showing positive effect (ammonium nitrate, $CaCl_2$, tannic acid, pH), central composite design was used. From experimental designs, the perturbation plot showed that change in response is mediated by change in that factor over its range, with all other factors constant at the reference value. The curve in the factor showed that the response is sensitive to that factor.

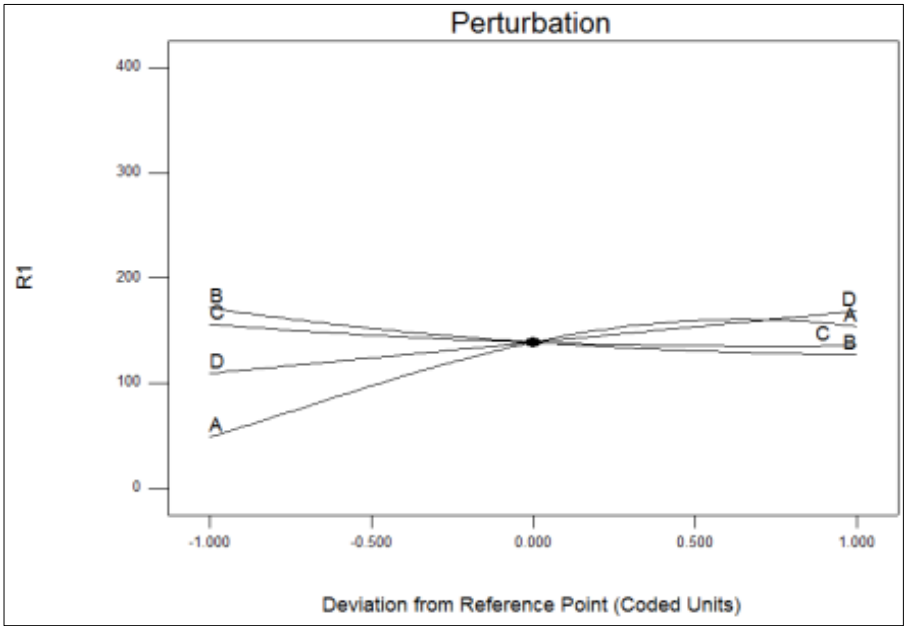


Fig 1: (b) Perturbation plot chart showing the deviation from reference point of selected variables.

Table 1: ANOVA for response surface quadratic model Analysis of variance table [Partial sum of squares-Type III]

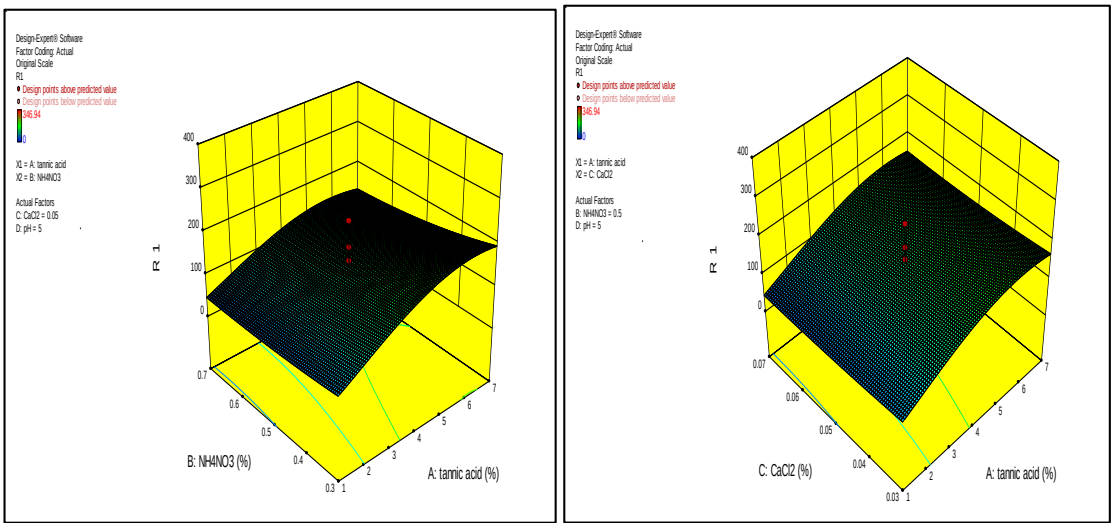
Source	Sum of squares	dF	Mean square	F-value	p-value Prob<F
Model	430.93	14	30.78	2.94	0.0233*
A-Tannic acid	198.83	1	198.83	19.00	0.0006
B-NH_4NO_3	20.96	1	20.96	2.00	0.1774
C-$CaCl_2$	4.58	1	4.58	0.44	0.5184
D-pH	41.12	1	41.12	3.93	0.0660
AB	1.25	1	1.25	0.12	0.7342
AC	0.50	1	0.50	0.048	0.8298
AD	0.49	1	0.49	0.046	0.8324
BC	0.23	1	0.23	0.022	0.8837
BD	2.31	1	2.31	0.22	0.6454
CD	0.14	1	0.14	0.013	0.9108
A^2	137.49	1	137.49	13.14	0.0025
B^2	4.69	1	4.69	0.45	0.5134
C^2	2.35	1	2.35	0.22	0.6421
D^2	0.16	1	0.16	0.015	0.9027
Residual	156.94	15	10.46		
Lack of Fit	117.78	10	11.78	1.50	0.3413**
Pure error	39.15	5	7.83		
Cor total	587.87	29			

* indicates model terms are significant.
** indicates lack of fit is non- significant.

R_1= +10.39896 + 2.89013*tannic acid - 19.39409*NH_4NO_3 - 110.38430*$CaCl_2$ + 0.37077*pH-0.46614*tannic acid*NH_4NO_3 + 2.94879*tannic acid*$CaCl_2$+ 0.029018*tannic acid*pH + 30.06862*NH_4NO_3*$CaCl_2$ + 0.94946*NH_4NO_3*pH –2.30287*$CaCl_2$*pH – 0.24877*tannic $acid^2$ + 10.33519*$NH_4NO_3^2$ + 732.34862*$CaCl_2^2$ – 0.019192*pH

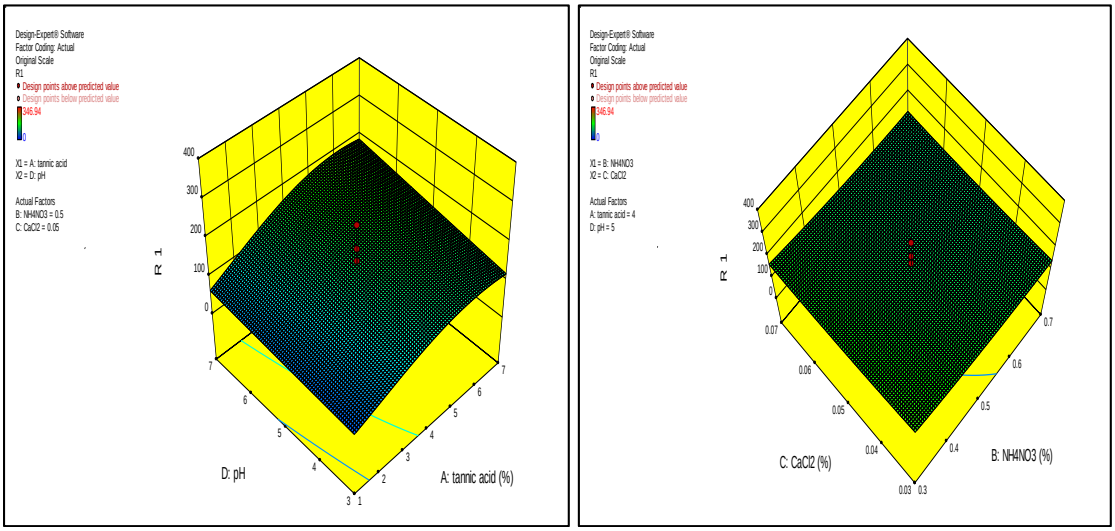
Where R_1 presents the tannase activity and the ANOVA of model indicated that it was highly significant. The model F-value of 2.94 and p-value of 0.0233 indicates that the model is significant. There is only a 2.33per cent chance that an F-value this large may occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant and greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 1.50 implies the Lack of Fit is not significant relative to the pure error. There is a 34.13per cent chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit.

The interactive effects of variables on production of tannase were considered by plotting 3D surface curves against two different independent variables while keeping the other variable constant. Figure A shows the dependence of tannase production on tannic acid concentration and ammonium nitrate. The tannase activity increased with an increase in tannic acid concentration to about 7 per cent (Fig. A, B, C). Tannic acid acts as inducer for the tannase production. These are in agreements with the results obtained by Mohan SK (2014b) [20]. Figure A, D, E shows the independence of an effect of ammonium nitrate on tannase activity after a concentration of 0.3per cent. Figures B, D, F shows the independence of an effect of calcium chloride on tannase activity after a concentration of 0.03 per cent and the effect of pH on tannase activity is described by figures C, E, F and it was inferred that increasing pH (3 to 7) resulted in an increase in tannase activity.



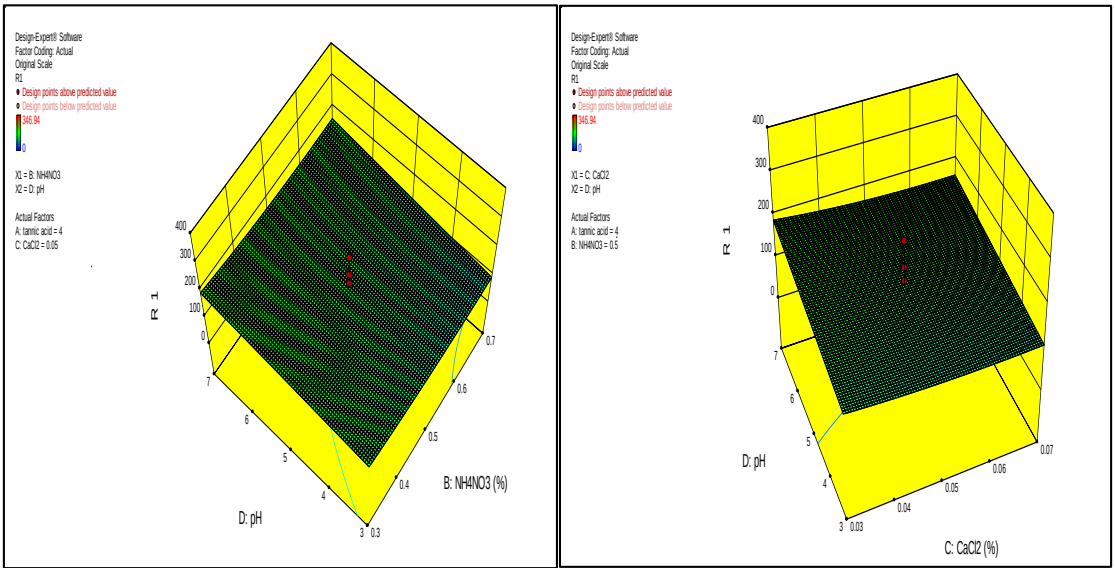
A B

Representation of interaction between tannic acid and ammonium nitrate (A) and tannic acid and $CaCl_2$ (B)



C D

Representation of interaction between tannic acid and pH (C) and NH_4NO_3 and $CaCl_2$ (D)



E F

Representation of interaction between ammonium nitrate and pH (E) and $CaCl_2$ and pH (F)

In earlier studies, a tannase titre of 157.6 U/ml has been reported from *Aspergillus foetidus* (Mohan *et al*. 2014a) [19] and 139.0 U/ml has been reported from *A. flavus* (Mohan *et al*. 2014b) [20] and of 21.73 U/ml reported from *Penicillium* sp. (Molkabadi *et al*. 2015) [21]. Based on the results of the present study, it is concluded that the optimized conditions for tannase productivity by TPF-13 is increased by 8.23 folds from un-optimized conditions.

3.3 Partial purification of tannase from TPF-13

Tannase produced extracellularly by TPF-13 was subjected to fractional precipitation with 60 per cent ammonium sulphate. Dialysis was done against 0.05M citrate buffer and was loaded into a column of Sephadex G-200 which led to its partial purification of 0.25 fold and yield of 0.007 per cent. The molecular weight of partially purified enzyme driven by SDS-PAGE declared that tannase from TPF-13 is a monomeric protein of 30 kDa. Most of the purified tannase from fungal sources have a high molecular mass in the range of 168-310 kDa (Ramirez-Coronel *et al*. 2003) [22]. Previous studies showed a monomeric tannase with the molecular mass of 66 kDa from *Aspergillus niger* (Lekha and Lonsane 1997) [16] and also of 68 kDa from *Mucor* sp. (Anitha and Arunkumar 2013) [4].

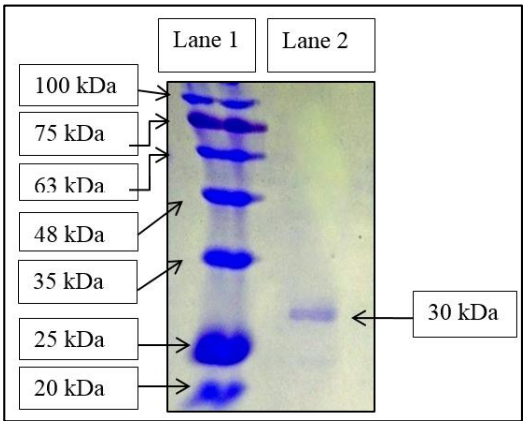


Fig 3: Lane 1: Molecular weight markers
Lane 2: A single band of purified tannase

4. Conclusion

The activity of tannase of different isolates helped in the screening of the hyper-producer microorganism and was marked as Tannase producing fungus (TPF-13) and further culture conditions for tannase production from TPF-13 strain were optimized and standardized. Due to vast range of applications of tannase enzyme, there is always a scope for peculiar tannase with better characteristics. Since, fungal activity is the main aspect in this area, there is an extensive opportunity for the profitable tannase production. The response surface methodology was effectively applied for the production of tannase from fungal strain-13, using tannic acid powder as a sole carbon source and Central composite design was used to analyse the effects of different concentrations of tannic acid, ammonium nitrate, calcium chloride and pH. After optimizing nutrient solution, maximum tannase activity of 346 U/ml was obtained at tannic acid concentration of 7 per cent, ammonium nitrate of 0.3 per cent, calcium chloride of 0.03 per cent, pH 7, 0.05 per cent each of potassium di-hydrogen phosphate, di-potassium phosphate, magnesium sulphate hepta-hydrate and the temperature of 30 °C for 72 hours. The statistical design of experiment provides an effective methodology to recognize the expressive variables and to optimize the factors with the minimum number of experimental runs. Tannase was partially purified to 0.25 fold having a specific activity of 1.25 U/ml and a yield of 0.007 per cent. It is of great interest to scale up the tannase production by using different genetic engineering techniques so that the insistence for tannase is taken care of on a large scale. The activity of this enzyme can also be enhanced by cloning and expression of the genes.

5. References

1. Adinarayana K, Ellaiah P. Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp. J Pharm Pharm Sci. 2002; 5:272-278.
2. Aguilar CN, Rodriguez R, Gutierrez-Sanchez G, Augur C, Contreras-Esquivel JC. Microbial tannase: Advances and perspectives. Appl Microbiol & Biotechnol. 2007; 76:47-59.
3. Ainnie RA, Nor AB, Hasnah O. Study on the Relationship of the Phenolic, Flavonoid and Tannin Content to the Antioxidant Activity of *Garcinia atroviridis*. Universal J Appl Sci. 2013; 1(3):95-100.
4. Anitha A, Arunkumar D. Extraction, Partial Purification and Characterization of Tannase Enzyme from *Mucor* sp. Neheu E- J. 2013; 1:25-29.
5. Belur PD, Gopal M, Nirmala KR, Basavaraj N. Production Of novel cell-associated tannase from newly isolated *Serratia ficaria* DTC. J Microbiol Biotechnol. 2010; 20:732-736.
6. Belur PD, Mugeraya G. Microbial Production of Tannase: The State of Art. Res J Microbiol. 2011; 6:25-40.
7. Beniwal V, Kumar A, Sharma J, Chhokar V. Recent advances in industrial application of tannases: A review. Recent Pat Biotechnol. 2013; 7:228-233.
8. Bhoite RN, Navya PN, Murthy PS. Purification and characterization of a coffee pulp tannase produced by *Penicillium verrucosum*. J Food Sci and Eng. 2013; 3:323-331.
9. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.
10. Deschamps AM, Otuk G, Lebeault JM. Production of tannase and degradation of chestnut tannins by bacteria. J Ferment Technol. 1983; 61:55-59.
11. Girdhari SN, Peshwe SA. Isolation and Screening of Tannase producing fungi. Int J Curr Microbiol App Sci. 2015; 4(7):765-774.
12. Haslam E, Stangroom JE. The esterase and depsidase activities of tannase. J Biochem. 1966; 99:28-31.
13. Hatamoto O, Watarai T, Kikuchi M, Mizusawa K, Sekine H. Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*. Gene. 1996; 175:215-221.
14. Khanbabaee K, Ree TV. Tannins: Classification and Definition. Nat Prod Rep. 2001; 18:641-649.
15. Lal D, Gardener JJ. Production, Characterization and Purification of Tannase from *Aspergillus niger*. European J of Exp Biol. 2012; 2:1430-1438.
16. Lekha PK, Lonsane BK. Production and application of tannin acyl hydrolase: state of the art. Adv appl microbial. 1997; 44:215-260.

17. Lokeshwari N, Lenin Kumar B. Tannase production from cashew husk by solid-state fermentation. Int J Adv Bio Res. 2013; 3(2):295-299.
18. Mahendran B, Raman N, Kim DJ. Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. Appl Microbiol Biotechnol. 2006; 70:444-450.
19. Mohan SK, Thangavelu V, Chockalingam AK. Application of response surface methodology for the maximum production of tannase using *Aspergillus foetidus* (MTCC 3557). Int J Pharm Develop Tech. 2014a; 4:151-156.
20. Mohan SK, Thangavelu V, Chockalingam AK. Statistical optimization of process parameters for the production of tannase by *Aspergillus flavus* under submerged fermentation. 3 Biotech. 2014b; 4:159-166.
21. Molkabadi EZ, Hamidi-Esfahani Z, Sahari MA, Azizi MH. Statistical Optimization of Tannase Production by *Penicillium sp.* EZ-ZH390 in Submerged Fermentation. Appl Food Biotechnol. 2015; 2(3):63-70.
22. Ramirez-Coronel MA, Viniegra-Gonzalez G, Darvill A, Augur C. A novel tannase from *Aspergillus niger* with β-glucosidase activity. Microbiol. 2003; 149:2941-2946.
23. Reed JD. Nutritional toxicology of tannins and related polyphenols in forage legumes. J Anim Sci. 1995; 73:1516-1528.
24. Sharma S, Bhat TK, Dawra RK. A spectrophotometric method for assay of tannase using rhodanine. Anal Biochem. 2000; 279:85-89.
25. Sharma KP, John PJ. Purification and characterization of tannase and tannase gene from *Enterobacter* sp. Process Biochem. 2011; 46:240-244.
26. Shrivastva A, Kar R. Characterization and application of tannase produced by *Aspergillus niger* ITCC 6514.07 on pomegranate rind. Braz J Microbiol. 2009, 782-789.
27. Van De Lagemaat J, Pyle DL. Tannase: In Enzyme Technology (eds. Pandey A, Webb C, Soccol CR and Larroche C), Springer, New York, 2006, 381-398.
28. Xiao A, Huang Yufeng, Ni H, Cai H, Yang Q. Statistical optimization for tannase production by *Aspergillus tubingensis* in solid state fermentation using tea stalks. Elect J Biotechnol. 2015, 143-147.