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TANNIN ACYL HYDROLASE AND GALLIC ACID PRODUCTION BY MUTATED STRAINS

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ABSTRACT

Tannase and gallic acid was produced from the mutated fungus Aspergillus niger 620 by submerged fermentation was studied. Mutant strains gives 32.81mg/ml and 27.84 mg/ml using UV treated L6 and after NTG treated LK8 at 48hrs of incubation time but at 36hrs. incubation of LK8 strain shows 28.12 mg/ml and 29.44 U/ml results of gallic acid and tannase activity respectively. The process parameter was optimized and higher production of tannase was found at 35°C and 96 hours of incubation with 2% tannic acid.

Key words: Tannase, Submerged fermentation, Purification, Screening.

Introduction

Current development in biotechnology is yielding new application for microbial enzymes. In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformation involving organic solvent media especially bioactive compounds. Enzymes have now become very much a part of a number of new industrial processes and appear to offer great potential in a wide variety of applications yet to be developed. Many microorganisms produce extra-cellular enzymes. They are chiefly hydrolysis and are involved primarily in the degradation of macromolecules to units capable of being taken into the living cell.

The development of recombinant DNA technologies has given the possibility of introducing targeted mutations (i.e. over-expressing genes or deletion of undesired ones)

instead of the random generation and further screening for the desired phenotypes which is very costly and time consuming. One of the most used transformation methods for filamentous fungi is the protoplast mediated transformation (PMT) method, developed earlier for *S. cerevisiae* and adapted for filamentous fungi. Nevertheless, this method preferentially produces multi copy integration events.

According to Michielse *et.al.* 2005, Ruiz-Diez. 2002 alternative methods for fungal transformation such as electroporation, biolistic transformation and *Agrobacterium* mediated transformation (AMT) have been developed.

Modification and improvement of the strain through mutation are typically achieved by subjecting the genetic material (*in vivo* or *in vitro*) to a variety of physical or chemical agents are called mutagens. N-methyl-N-nitro-N-nitrosoguanidine (MNNG; 0.15 mg mL-1) and ethidium bromide (0.5 mg mL-1) were used to induce mutagenesis in *A. niger* for enhance production of glucose oxidase.

The objective of mutagenesis for developing improved strain is to maximize the frequency of desired mutations in a population or culture. Random mutagenesis approach for industrial strain improvement is simple, easy and effective when compared with recombination technologies.

METHODS:

MICRO ORGANISM:

Tannase producing strain *Aspergillus niger* 620 used in the experimentation was obtained from NCIM (National collection of industrial microorganisms), Pune, Maharastra, India.

1.1. Cultural characteristics:

Aspergillus niger was grown of Potato Dextrose Agar medium for one week at thirty degree centigrade.

1.2. Culture maintenance:

For maintenance of the strain, was grown on Potato Dextose Agar (PDA) slants at 30°C and the slants were preserved at 4°C (Meera Gupta *et.al.* 2012).

After dissolving the agar in 100ml of above growth medium, it was transferred into test tubes. After sterilization the slants were allowed to cool in slanting position. After solidification, a slant was inoculated with pure culture of *Aspergillus niger* 620. After that the slants were incubated at 30°C for one week for good sporulation. The slants were stored in the refrigerator at 4°C for further use.

1.3. Spore suspension preparation:

8ml of sterile distilled water was taken in 50ml conical flask. The spores were dislodged using a sterile inoculation loop under aseptic condition in 2ml of distilled water containing TWEEN 80. The resulting spore suspension was vortexes to obtain a uniform suspension. The suspension was then added to the 8ml of distilled water to give 10ml of spore suspension. The spore count in the suspension was 3×10^7 spores/ml.

1.4. **Inoculum preparation:**

Inoculum was prepared by growing a loop full amount of stock culture of the fungi in 50ml sterile modified Czapek's dox medium. The medium used for growing fungi in modified czapek's dox broth containing gallotannin 0.5%w/v was taken into 100ml conical flask. It was then sterilized and inoculated with 2ml of spore suspension prepared from the culture slants. They were then kept into a rotator shaker (160rpm) at 30ºC for 48hrs. This induced inoculum was used for subsequent studies of SmF.

2. EXTRACTION OF TANNINS:

Testa of the *Anacardium occidentalis* was used for the extraction of tannins. Testa were dried and milled using ball mill to get the particle size below 5.0 mm. Water is preferred as solvent in view of its high saturation limit of the dissolved solids, inherent safety and ease of separation. Water should be soft and should not be contain iron. Tannins are extracted by using pressure autoclaving method. Autoclaving process at 10PSI for 30 min and obtained extract was evaporated with vacuum filter and obtained extract was used for the entire experimentation.

2.1. Braemer's test:

For the identification of tannins present, to 2ml of aqueous extract 2ml of 5% $FeCl_3$ was added. Formation of yellow brown or greenish black precipitate indicates that tannins are present.

3. STRAIN IMPROVEMENT:

For the strain improvement process for the production of tannase and gallic acid by Ultraviolet and NTG treatments were followed as follows:

4. UV treatment

UV light carried out to dispensing cabinet fitted with TUP 40W germicidal lamp 90% of its radiation at 2540 -2550 A° exposure carried out at 20.0 cm away from light. UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, the serially diluted

in Phospate Buffer and plated on PDA plates incubated at 28°C for 7 day. Then no. of colonies in each plate was counted. Each colony was assumed to be formed from a single spore. A total of 10 colonies were selected from the plates, naming as L1, L2, L3, L4, L5, L6, L7, L8, L9 and L10 showing less than 1% survival rate and tested for tannase and gallic acid production. Among all selected, the best UV mutant strain was used for further process of the experiment.

4.1. NTG treatment (N-methyl N-nitro N-nitroso guanidine):

9 ml spore suspension, 1ml of sterile NTG sol. (3 mg/ml in 0.02M phosphate buffer pH 7.0) control was treated in the way except that no NTG was added to the buffer. Samples were withdrawn from the reaction mixture at intervals of 30, 60, 90, 120, 150 and 180 min. and immediately centrifuged for 10 min at 5000 rpm and the supernatant sol. was decanted. Cells were washed 3 times with sterile water and resuspended in 10 ml of sterile phosphate buffer. The samples were serially diluted in the same buffer and plated over PDA as mentioned earlier. A total of 10 colonies were selected from the plates, naming as LK1, LK2, LK3, LK4, LK5, LK6, LK7, LK8, LK9 and LK10 showing less that 1% survival rate and tested for tannase and gallic acid production.

Mutation was tried on the fungal spores by UV radiation and NTG treatment to find large quantity of putative mutants. The problem with these methods of mutation was that they did not just mutate cells in the desired genes but could also mutate essential functions of the cells beyond repair and in doing so, kill them. The fast growing colonies that might have any possibility of being mutated were streaked to fresh plates to confirm presence of putative mutants.

Results and Discussion:

1. SELECTION OF MUTANT STRAIN UNDER UV TREATMENT:

The survival percentage of the cultures after UV treatment was shown in **Table 1.1** and **Graph 1.1.** Initial 15 min exposure produced 66% colonies, which decreased as the exposure time increased and became nil when exposure time reached 90 min. Showing maximum less survival rate colonies **(Table 1.1)** were selected for tannase and gallic acid production. Among all ten selected strains, L6 shows highest production rate of tannase and gallic acid respectively 34.95U/ml and 32.81mg/ml **(Table 1.1)**. L6 strain was selected mutation further experiments. Results were tabulated in **Table 1.1**.

2. SELECTION OF MUTANT STRAIN UNDER NTG TREATMENT:

Selected L6 strain was treated with N-methyl N-nitro N-nitroso guanidine as mentioned in previous chapter. In case of NTG treatment initial 30min treatment

produced 44% colonies, after increasing of the time colonies were decreased. Showing least survival rate colonies were selected and the results of effect on NTG treatment were shown in **Table 1.2** and **Graph 1.2.**

Among all ten selected strains, LK8 shows highest gallic acid concentration 19.97 mg/ml and tannase activity 22.14 U/ml (**Table 1.2**). Compare with UV treatment, the growth rate of selected strain by NTG treatment was high but the production rate of tannase and gallic acid was decreased. Enzyme production efficiency as well as gallic acid production of ten selected putative mutants was compared with wild co-culture in submerged fermentation at optimized medium was very less. Respective production rate of gallic acid as follows with wild co-culture was 34.87mg/ml was slightly decreased with UV treatment of strain to 32.81mg/ml and after that it was totally decreased, after treatment with NTG to 27.84mg/ml. Results of NTG treated strain were present in **Table 1.2.**

Based on efficiency of growth (least percentage of survival culture (60min UV, 120 min NTG)) ten different colonies were isolated. Among all LK8 strain was selected for production of enzyme and gallic acid. Experiment was conducted at 35ºC with the parameters of inoculum size 4ml, agitation 120rpm, gallotannin concentration 1.5% w/v, Glucose concentration 1.5% w/v, Urea concentration 0.002% w/v, Dipotasium phosphate concentration 0.3% w/v, Zinc sulphate concentration 0.02% w/v for 12 hrs to 72 hrs. Every 12hrs interval obtained results were tabulated in **Table 1.3** and **Graph 1.3.** Enzyme production efficiency of the mutant *A.niger* was decreased compare with wild culture.

Further increase in incubation period the production of gallic acid concentration and tannase activity was decreased compared with wild co-culture it was occurred may be treatment with UV, and NTG to wild culture. The mutagens used, which ultimately led to the increased enzyme production. In the present study the selected mutant strain was maintained production efficiency constant, showing that the mutation was stable and can be inferred to be heritable too.

The following test was carried out for the confirmation of the compound (gallic acid).

3. GALLIC ACID CONFIRMATION TEST BY THIN LAYER CHROMATOGRAPHY (TLC):

TLC of gallic acid in three samples (sample 2, sample 3, sample 4 in **Fig .1**) having Rf value 0.34 (**Fig .1**) when a solvent phase benzene, dioxin and acetic acid (90:25:4) was used. TLC clearly indicated the presence of gallic acid in the samples when compared with

standard and the Rf value was found to be 0.34 which correlated with the already reported value (0.34) (**Fig .1**).

Conclusions

The gallic acid yield of 31.55 mg/ml and 36.76mg/ml using free and immobilized enzyme at shake flask level resulted in an efficient conversion of substrate to gallic acid, whereas putative mutant strains gives 32.81mg/ml and 27.84 mg/ml using UV treated L6 and after NTG treated LK8 at 48hrs. of incubation time but at 36hrs. incubation of LK8 strain shows 28.12 mg/ml and 29.44 U/ml results of gallic acid and tannase activity respectively. By this comparative study, it is noticed that gallic acid yield was obtained by the immobilized enzyme of *Aspergillus niger* NCIM 620 is useful for repeated production of maximum gallic acid yield. Another advantage of this strain is that it can produce maximum enzyme within a short period of cultivation. These features make the strain an excellence for production of tannase and gallic acid on a commercial scale.

Table 1.1: Effect of UV treatment

Irradiation Time (min)	No. of Survivors / ml	% of survivors
0 (control)	32×10^6	100
15 min	21×10^6	65.625
30 min	42×10^5	13.125
45 min	11×10^4	0.343
60 min	3×10^4	0.093
75 min	2×10^3	0.006
90 min	0	0.000

Table1 1.2: Tannase and Gallic acid production by UV treated strains:

UV treated strain	Gallic acid con. (mg/ml)	Tannase activity (U/ml)
L1	20.2	20.21
L2	20.69	20.78
L3	24.52	26.41
L4	30.11	32.14
L5	20.78	19.87
L6	**32.81**	**34.95**
L7	31.21	30.16
L8	30.07	30.10
L9	28.64	29.48
L10	20.69	20.78

Table 1.3: Effect of NTG treatment:

Irradiation Time (min)	No. of Survivors / ml	% of survivors
0 (control)	36×10^5	100
30 min	16×10^5	44.444
60 min	23×10^4	6.388
90 min	7×10^3	0.194
120 min	19×10^2	0.052
150 min	2×10^2	0.005
180 min	0	0.000

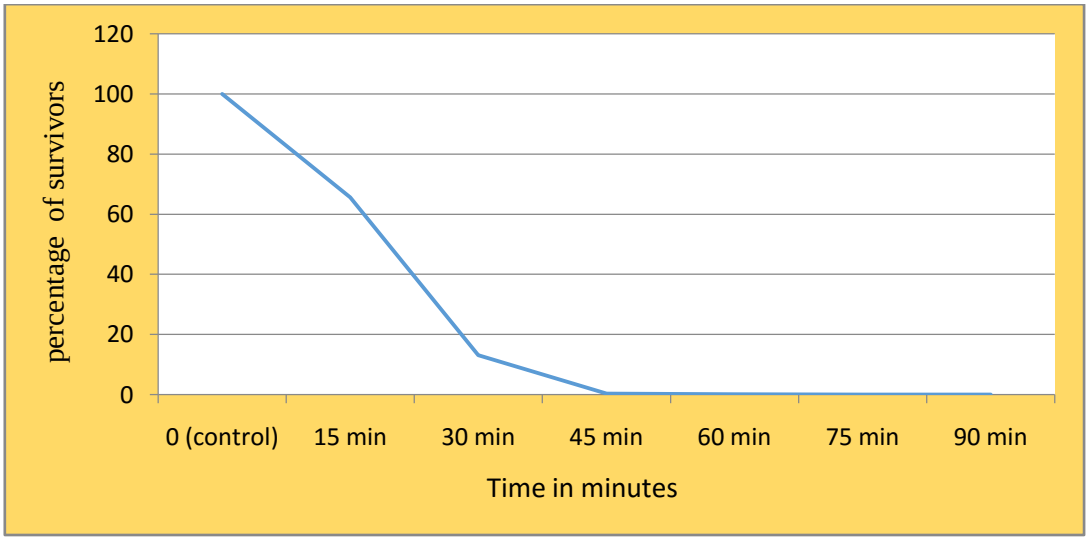
Table 1.4: Tannase and gallic acid production on NTG treated strain:

NTG Treated Strains	Gallic acid con. (mg/ml)	Tannase activity (U/ml)
LK1	20.12	21.32
LK2	22.46	23.14
LK3	24.32	24.72
LK4	22.97	23.14
LK5	25.01	26.64
LK6	20.41	20.82
LK7	26.55	27.01
LK8	**27.84**	**28.12**
LK9	25.99	26.04
LK10	22.45	22.91

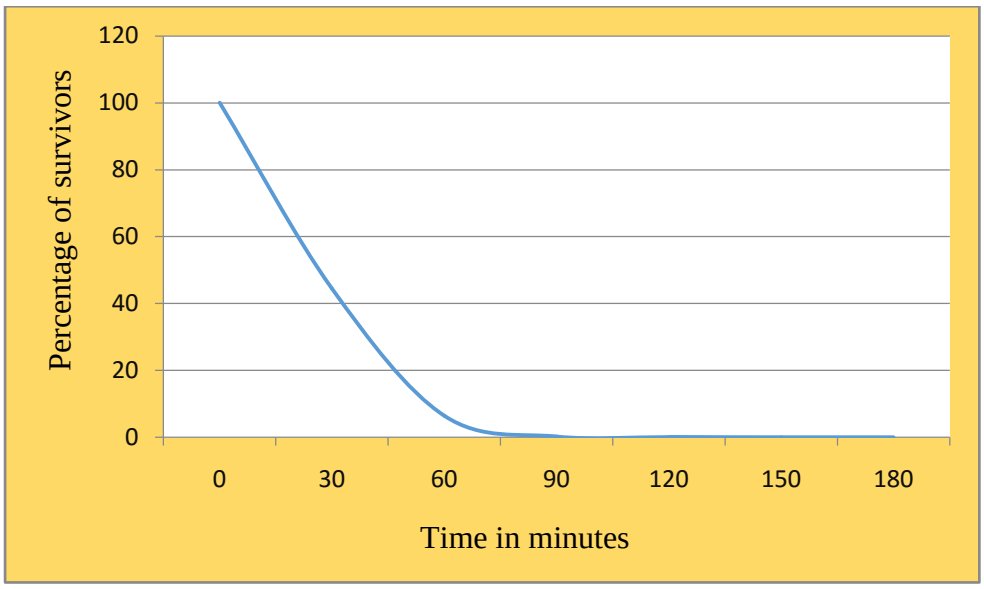
Table: 1.5: Production of gallic acid with mutant strain LK8:

Time (hrs)	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
Gallic acid con.(mg/ml)	26.72	27.24	**28.12**	27.94	27.11	26.72
Tannase activity (U/ml)	27.91	27.76	**29.44**	28.72	28.04	27.14

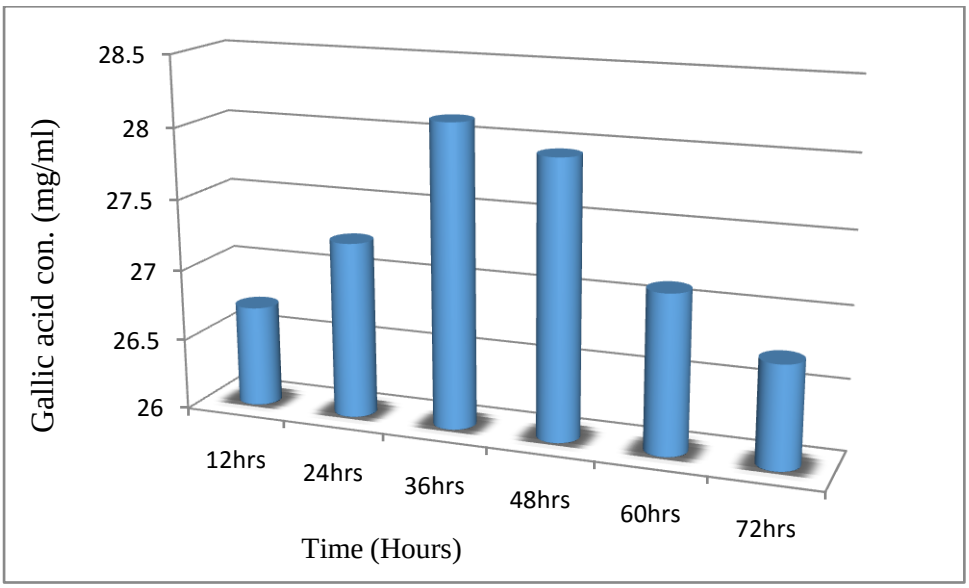
Graph 1.1: Effect of UV treatment on percentage survival of co-culture.



Graph 1.2: Effect of NTG treatment on percent survival of co-culture.



Graph 1.3: gallic acid production with mutant strain LK8:



Graph 1.4: Tannase production with LK8 strain

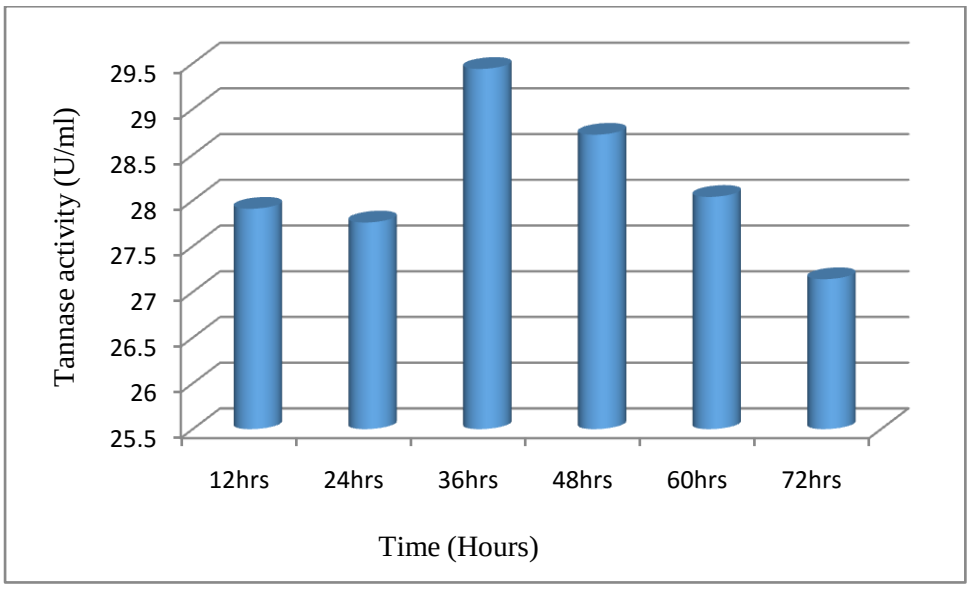
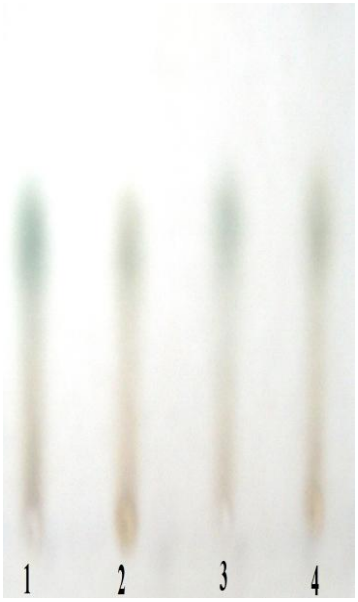


Fig. 1: TLC analysis of 3,4,5 tri hydroxyl benzoic acid.



Lane 1: Standard., **Lane 2:** Sample 1., **Lane 3:** Sample 2 and **Lane 4:** Sample 3.

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