Pharmacophore 2010, Vol. 1 (2), 112-122. ISSN 2229 – 5402

Pharmacophore

(An International Research Journal)

Available online at http://www.pharmacophorejournal.com/

Original Research Paper

MICROBIOLOGICAL PRODUCTION OF GALLIC ACID BY A MUTANT STRAIN OF *ASPERGILLUS ORYZAE* USING CASHEW HUSK

N. Lokeshwari*, D. Sri Rami Reddy

Centre for Biotechnology,

Department of Chemical Engineering,

AU College of Engineering, Andhra University,

Visakhapatnam – 530003, India

ABSTRACT

Gallic acid is a very useful chemical mostly utilized as an intermediate in the production of trimethoxy benzaldehyde, which is used in pharmaceutical industry for the production of Trimethoprim, a broad spectrum antibiotic. It has huge demand in India though it ia an imported item. It is mainly used for manufacture of propyl galllate, inks, and photographic developer, in testing free mineral acids, dihydroxy acetone and alkaloids. The worldwide annual demand of gallic acid is about 8,000 tons. Conventionally gallic acid is produced by acid hydrolysis of tannins, but this process releases a large amount of toxic effluent that causes environmental hazards. Production of gallic acid through fermentation of cashew tannins using suitable tannase producing microorganism is preferred today.

The Present study deals with the microbiological conversion of gallotannin to 3, 4, and 5 – tri hydroxy benzoic acid by a mutant strain of *Aspergillus oryzae*. Spores from *Aspergillus oryzae* were subjected to UV, heat and NTG (3-nitro, 5-methylguanidine) mutagenesis. A few colonies were screened from the selected media for tannase study. Amongst all, the best mutant isolated from the heat treatment (60 _C for 60 min) was ATTC9 3692. The maximum yield of gallic acid and tannase in case of mutant strain was 95.2% and 53.6 U/ml with an incubation period of 30 h as compared to wild strain where the incubation period was 48 h with an enzyme activity of 44.2 U/ml and gallic acid yield of 94%, respectively. The mutant was sensitive to tetracycline and was also an over-producer of protease and amylase

Keywords: Mutagenesis, Tannase, Gallic acid, *Aspergillus oryzae*, Gallotannins, Gallic acid.

INTRODUCTION

Tannase (tannin acyl hydrolase, EC 3.1.1.20), an inducible extra-cellular enzyme produced by a number of animals, plants and microbes, has wide application in tannery, alcohol industry, pharmaceuticals and beverage industries. It is also responsible for the bioconversion of hydrolysable tannins to gallic acid (Lekha and Lonsane, 1997; Mukherjee and Banerjee, 2003). Microbial tannase is most preferred as they are fast growing, more diverse and their environmental and genetic manipulation is easier, needed for generation of hyper producers in reduced time period. Microbial tannase is more stable than tannase from other sources like plants or animals. Tannase from fungal sources are reported to be active in a wide range of pH and temperature. Tannase also finds use in winemaking, beer chill proofing, and production of instant tea by solubilization of tea cream and in the pre-treatment of animal feed additives (Coggon *et al.*, 1975; Lekha and Lonsane, 1997). In addition it is also used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid ester (Haslam and Stangroom, 1996). Gallic acid, the product of tannin hydrolysis finds application in many fields including dye-making, pharmaceutical, leather, food industry and chemical industries (Hadi *et al.*, 1994; Lekha and Lonsane, 1997; Mukherjee and Banerjee, 2003). It can be used in the manufacture of ordinary writing inks and dyes, as photographic developer, in the enzymatic synthesis of propyl gallate, in tannery industry for homogenization of tannins, for the production of pyrogallol and gallic acid esters among other compounds.

Tannase production by solid-state fermentation (SSF) is more advantageous over submerged or liquid surface fermentation (Lekha and Lonsane, 1997; Aguilar ***et al***., 2001). A large quantity of heat is generated in fermenting solids due to the microbial metabolic activity in SSF process leading to rapid rise in temperature of the fermenting solid bed (Pandey, 1994). Removal of this heat is difficult in SSF due to lack of heat-exchange surface and poor heat transfer through the solid substrate bed causing large moisture loss and drying. To overcome the harmful effects on microbial growth and activity due to this heat, modified solidstate fermentation (MSSF) has been reported where the solid substrate placed on its float comes in continuous contact with the liquid medium in the vessel (Kar *et al*., 1999).

Agro-residues and forest products serve well for being used as the substrate for production of microbial enzymes by SSF. In the present study tannin-rich agro-residues comprising of powdered seed cover of Anacardium occidentale were used for carrying out MSSF. In the present study attempts were made to enhance tannase production by three different mutagenic methods from the culture of *Aspergillus oryzae* by MSSF.

METHODS

Microorganism and growth medium

One of a potent gallic acid producing fungal *strain Aspergillus oryzae* 643ATTC9 3692, has been used in the present study. The strains were grown on 2% malt extract agar (MEA) slants at 32 _C and preserved at 4 _C. The slants were sub-cultured routinely at an interval of 4–5 weeks

Induced inoculum preparation

Induced inoculum was prepared by sub-culturing the strain in modified Czapek-dox media with 2% tannic acid (Chen, 1969). This induced inoculum was used for subsequent fermentations.

Preparation of spore suspension

Induced mycelium of *A. oryzae* were mixed with distilled water (15 ml) and resulting spore suspension mixture having 50% of spores was agitated in a vortex-cyclomixer for 5 min so as to get evenly dispersed spore suspension. The spore count in the suspension was 1 · 106 spores/ml.

Mutagenesis by heat treatment

From the spore suspension, 1 ml was taken in a sterilized test tube and was heat-treated to 60 _C for variable time periods (35–65 min). After this, 100 ll of treated spore suspension was taken and spread over 2% tannic acid agar plate under sterile condition. This process was done for all the plates (for 35, 40, 45, 50, 55, 60 and 65 min). All the plates were kept in dark for half an hour and were then incubated at 32 _C for 48 h.

Mutagenesis by UV treatment

The spore suspension was taken in six Petri plates (1000 ll in each plate) and was exposed to UV radiation (40 W UV bulb) for variable time periods (10– 60 min) keeping the distance of the UV source fixed to 10 cm (Defais et al., 1971). After exposure, they were kept in dark for half an hour, then plated on 2% tannic acid agar plates and were incubated at 32 _C for 48 h.

Mutagenesis by NTG (N-methyl N-nitro N-nitroso guanidine)

The spore suspension was treated with 10% NTG solution and then plated to tannic acid agar plates following exposure to variable time periods of 10– 60 min (Nakamura, 1994). All the six plates were kept in dark for half an hour and then incubated at 32 _C for 48 h.

Isolation of putative mutants

The colonies, which were hyper producers of tannase (grew faster than others), were isolated and transferred to Czapek-dox medium for growth. They were also sub-cultured on malt agar slants for their preservation and maintenance.

Tannin estimation

The estimation of tannin content was done following the protein precipitation method of Haggerman and Butler (1978). Bovine serum albumin (BSA) was taken as the standard protein.

Fermentation

Batch fermentation was carried out by MSSF in GROWTEK bioreactor under static state (Kar and Banerjee, 2000). The raw material consisted of cashew husk on the float and Czapek-dox below (solid: liquid—0.4:1). The reactor was a cylindrical vessel having a height of 16.0 cm and diameter of 11.3 cm with a spout (inclined 15_ to the vertical axis) near its base with length 8.5 and 2.6 cm diameter. One of the advantages of the spout is that the liquid medium can be removed or added through it. A float was inside the vessel on which the solid substrate was placed and it came in continuous contact with the liquid medium in the vessel. Fermentation of solid substrate was carried out on the float by the induced inoculum. The biotransformed product formed by the microorganism leached into the liquid medium. For fermentation, weighed amount of the tannin-rich material was taken on the mesh of the bioreactor, below which measured volume of medium was taken. The bioreactor was then autoclaved (121 _C, 15 min) and the required volume of induced inoculum of the fungal culture was added to the substrate. After incubation for the appropriate time, the entire material of the bioreactor was taken out in a beaker and mixed thoroughly followed by centrifugation at 10,000 rpm for 10 min. The supernatant was used as crude enzyme for assay.

Tannase assay

Tannase activity was determined spectrophotometrically according to the method of Iibuchi *et al.* (1967). One unit of enzyme activity is defined as the amount of enzyme able to release 1 lmol of gallic acid per min.

Gallic acid extraction

The bioconversion ability of the putative mutants was determined by estimating the gallic acid yield of the putative mutants, following the methodology described by Kar et al. (1999). The percentage yield of recovered gallic acid was determined on the basis of the available tannin content of the substrate. The protocol was followed for calculation of the data generated through different experimentation and the standard error was calculated. All the data were analyzed by using the standard software Microsoft office-excel. All experiments were conducted in triplicate.

Isolation of the best mutant

The best mutant was isolated from the screened strains, which showed minimum incubation period and maximum enzyme activity after MSSF for 13 generations.

Biochemical characterization of the mutant

RESULTS

Cashew husk contained 45% tannins.

Selection of mutant strain

The survival percentage of the cultures after heat, UV and NTG treatments is shown in Figs. 1 and 2. In case of UV mutagenesis, initial 10 min exposure produced countless colonies, which decreased as the exposure time increased and became nil when exposure time reached 60 min. Similar results were also obtained from heat and NTG treatments. From these plates nine different colonies were isolated at 5% survival rate from the tannic

Comparison of macromolecules utilization and metabolic activity of wild and mutants

Growth of wild and mutant strains was analyzed in Czapek-dox media containing 1% of different carbon sources such as mannose, mellibiose, mannitol, sorbitol, raffinose, galactose, glucose, fructose, maltose, sucrose and lactose individually. For nitrogen sources 1% sodium nitrate, potassium nitrate, sodium nitrite, ammonium nitrate, ammonium sulfate, liver extract and peptones were studied individually. Effect of amino acids on growth was studied by adding 10 mg/ml of different amino acids to tannic acid agar plates. All experiments were conducted in triplicate.

Test for antibiotic resistant strains

The test for antibiotic resistance was done by taking three concentrations (100, 200 and 400 lg/ml) of different antibiotics such as ampicillin, amoxycillin, penicillin and tetracycline on tannic acid agar plates.

Profile of other enzymes

The wild and mutant strains were inoculated in 2% tannic acid agar, 2% gelatin agar, 2% casein agar and 2% starch agar plates and the growth pattern was analyzed after 48 h of incubation.

acid agar plates (based on their efficiency of growth).

These were selected from the 50-min exposure plates in case of UV and NTG and 60 min exposure plate for heat mutagenesis. Enzyme production efficacy as well as the incubation period for maximum enzyme production of the nine selected putative mutants was compared with the wild culture in MSSF. It was found that the mutant *A.oryzae*, generated by 60-min heat treatment, the incubation period decreased to 30 h from 48 h with enhanced tannase activity of 53.6 U/ml from 44.2 U/ml when compared to the wild culture (Fig. 3).

The bioconversion efficiency of these putative mutants was also compared with the wild culture in MSSF and was found (Fig. 4) that the gallic acid yield of *A.oryzae* reached 95.2% compared to 94% in case of wild culture. The conversion of tannin rich agroresidues to gallic acid occurred within 30 h as compared to 48 h in case of wild culture. The enzyme producing capability of the mutant *A.oryzae* followed the same trend for 13 generations (Fig. 5) confirming the occurrence of stable mutant.

Biochemical characterization of mutant strain

From growth and biomass analysis it was found that except for mannose, mannitol and lactose, there was reduction in growth of the mutant when grown in presence of additional carbon sources. But the growth characteristic showed that during the early incubation period, the mutant showed maximum growth after which the wild strains compensated (Fig. 6). Different additional nitrogen sources also showed maximum growth in case of mutant strain in the initial hours of incubation but later on compensated by the wild strain (Fig. 7). Peptone was one deviation among all the nitrogen sources where the mutant continued to show maximum growth till the end i.e., 48 h of incubation. From the amino acid study (Table 1), it was evident that glycine, phenylalanine, arginine, histidine and aspartic acid were inhibitory for the growth of the mutant. Valine was inhibitory for *A. oryzae.*

Secretion of other enzymes by mutant strain

Analysis of the clear zones on the tannic acid agar plate showed that the radius was maximum for the mutant followed by the *Aspergillus oryzae* during the first 18 h of incubation. This confirmed the tannase hyper production efficacy of the mutant during the early phases. Similarly, analysis on gelatin agar plate confirmed the better ability of the mutant to produce protease followed by culture. In case of starch agar plate, after iodine reaction, the pale yellow zone was maximum for the mutant followed by the culture, showing the amylase hyper production efficiency of the mutant. But in the casein agar plate, the radius of the clear zone was maximum for *Aspergillus oryzae*.

DISCUSSION

Mutation was tried on the fungal spores by direct heat treatment, UV radiation and NTG treatment to find large quantity of putative mutants. The problem with the three 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. Heat works by producing an oxidative burst inside the cells. Heat stress acted as an oxidant and a mutagen in Saccharomyces cerevisiae, which was lethal at 50 _C (Davidson and Schiestl, 2000). It caused elevated frequencies of interchromosomal DNA recombination, DNA mutation, and mitochondrial membrane disruption and resulted in decrease of total sulfhydryl content.

Exposure at 60 _C for 35 and 40 min produced countless colonies and the number decreased as the radiation time increased from 40 to 60 min (Peak et al., 1984). The fastest growing colony was selected from heat mutagenesis exposed for 60 min, which was later found to be the best mutant amongst all. In case of the best performing mutant *A.oryzae*, the incubation time was reduced to 30 h from 48 h in wild.

The enzyme activity increased by 20% and the bioconversion efficiency also increased

showing gallic acid yield from 94% in case of wild to 95.2% in mutant strain. According to Zhong et al. (2004), the tannase from *Aspergillus oryzae* produced from recombinant *Pichia pastoris* showed 7000 IU/L activity after 96 h of fed batch culture. From the present studies it can be concluded that tannase activity though cannot be compared with MSSF and fed-batch cultivation system but the titer value in case of MSSF is 7.7 times than the reported literature. Tannase being an adaptive enzyme is used for degrading tannins to gallic acid and glucose where higher titer value helps efficient conversion (Haworth *et al.*, 1985).

The mutagens used, especially heat gave the cell an oxidative stress that had an inductive effect, which ultimately led to the increased enzyme production. In the present study the selected mutant strain maintained the production efficiency for 13 generations showing that the mutation was stable and can be inferred to be heritable too. Effect of additional carbon and nitrogen sources on the growth of wild and mutant strains revealed that all carbon and nitrogen sources had inhibitory effect on the growth of both the organisms except mannose, mannitol, lactose and peptone, respectively.

From the antibiotic study it was found that the mutant was sensitive to tetracycline whereas the wild was resistant to it. The antibiotic is selective in action, which depends on the presence of target for action, affinity for the target and permeability across the cell membrane. Tetracycline inhibits binding of the aminoacyl tRNA to the A site of 30 S sub-unit of ribosome and inhibits elongation of protein synthesis in prokaryotes. At higher concentrations it has metal sequestering activity.

CONCLUSIONS

There was no report till date on strain improvement of fungi for tannase overproduction. For bacteria, enhanced tannase production was reported from Bacillus licheniformis (Mondal and Pati, 2000). This report for the first time described mutagenesis by heat treatment in filamentous fungi. The improved strain obtained after mutation was not only able to produce enhanced tannase in reduced incubation time but also had enhanced bioconversion efficiency. The mutant was also a hyper producer of protease and amylase. Therefore, utilization and exploitation of the mutant *A.oryzae* would be sensible for large-scale production of tannase and other industrial enzymes.

REFERENCES

1. Aguilar, CN; Augur, C; Favela-Torres, E andViniegra-Gonzalez, G (2001), “Induction and repression patterns of fungal tannase in solidstate and submerged cultures”, ***Process Biochem***, 36, 565–570.
2. Chen, T (1969), ***Ph.D. Thesis***, University of California, Berkley, USA. Coggon, P., Graham, HN and Sanderson, GW (1975), ***Cold water soluble tea***, British Patent No. 1,380,135.
3. Davidson, J and Schiestl, R (2000), ***Cancer Cell Biology***, Harvard School Public Health, 665 Huntington Avenue, Boston, MA 02115, USA.
4. Defais, M; Fauquet, P; Rodman, M and Errera, M (1971), “Ultraviolet reactivation and ultraviolet mutagenesis of phages in different

genetic systems", ***Virology***, 43, 495–503.

5. Haggerman, AE and Butler, LG (1978), "Protein precipitation method for determination of tannins", ***J. Agric. Food. Chem.***, 26, 809–812.
6. Haslam, E and Stangroom, JE (1996), "The esterase and depsidase activities of tannase", ***Biochem. J.***, 99 (1), 28–31.
7. Haworth, RD; Jones, K and Rogers, HJ (1985), "Ion-exchange chromatography of Aspergillus niger extract", ***Proc. Chem. Soc.***, 8–9.
8. Iibuchi, S; Minoda, Y and Yamada, K (1967), "Studies on tannin acyl hydrolase of microorganisms part III. Purification of the enzyme and some properties of it", ***Agr. Biol. Chem.***, 32 (7), 803–809.
9. Kar, B and Banerjee, R (2000), "Biosynthesis of tannin acyl hydrolase from tannin rich forest residue under different fermentation conditions", ***J. Ind. Microbiol. Biotechnol***, 25, 29–38.
10. Kar, B; Banerjee, R and Bhattacharyya, BC (19990, "Microbial production of gallic acid by modified solid state fermentation" ***J. Ind. Microbiol. Biotechnol***, 23, 173–177.
11. Lekha, PK and Lonsane, BK (1997), "Production and application of tannin acyl hydrolase: state of the art", ***Adv. Appl. Microbiol***,44, 215–260.
12. Mondal, KC and Pati, BR (2000), "Studies on the extracellular tannase production from newly isolated Bacillus licheniformis KBR 6", ***J. Basic Microbiol***, 40 (4), 223–232.
13. Mukherjee, G and Banerjee, R (2003), "Production of gallic acid: biotechnological routes (Part 1)", ***Chim. Oggi. Chem. Today***, 21 (1/2), 59– 62.
14. Nakamura, T (1994), "Occurrence of two forms of extra-cellular endoinulinmase from Aspergillus niger mutant 817", ***J. Ferment. Bioengng***, 78, 134–139.
15. Pandey, A (1994), ***Solid state fermentation an overview***, In: Pandey, A. (Ed.), Solid State Fermentation. Wiley Eastern, New Delhi, 3– 10.
16. Peak, MJ; Peak, JG; Moehring, MP and Webb, RB (1984), "Ultraviolet action spectra for DNA dimer induction, lethality and mutagenesis on the UVB region", ***Photochem. Photobiol***,40, 613– 620.
17. Zhong, XF; Peng, LS; Zheng, SL; Sun, ZZ (2004), "Secretion, purification and characterization of a recombinant *Aspergillus oryzae* tannase in Pichia pastoris", ***Protein Expr. Pur.***, 36, 165–169.

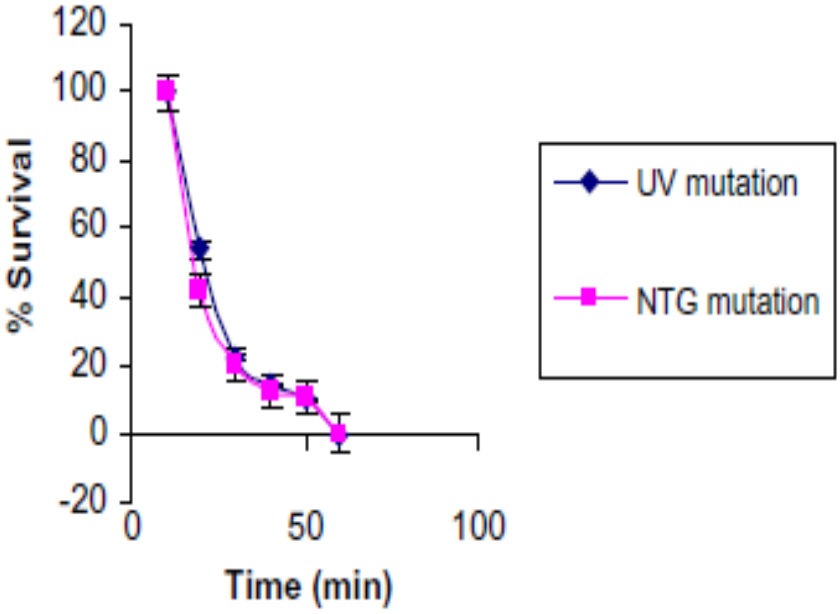

Fig. 1: Effect of UV and NTG treatment on percent survival of culture.

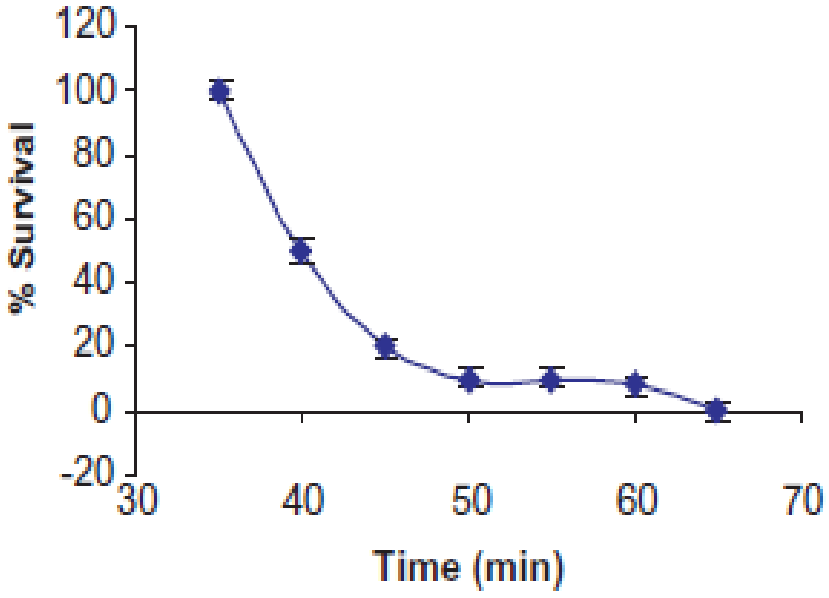

Fig. 2: Effect of heat treatment on percent survival of culture.

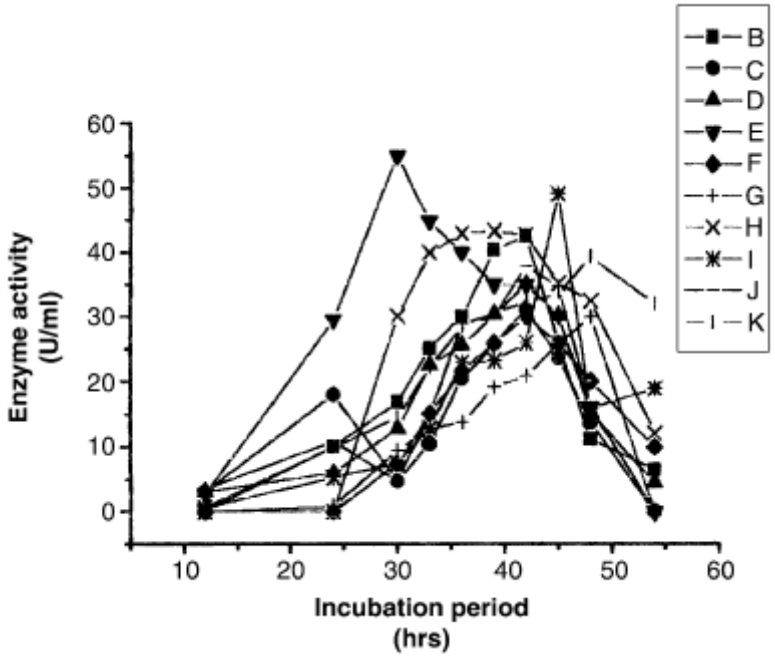

Fig. 3: Effect of incubation period on enzyme activity of the mutant and wild culture.

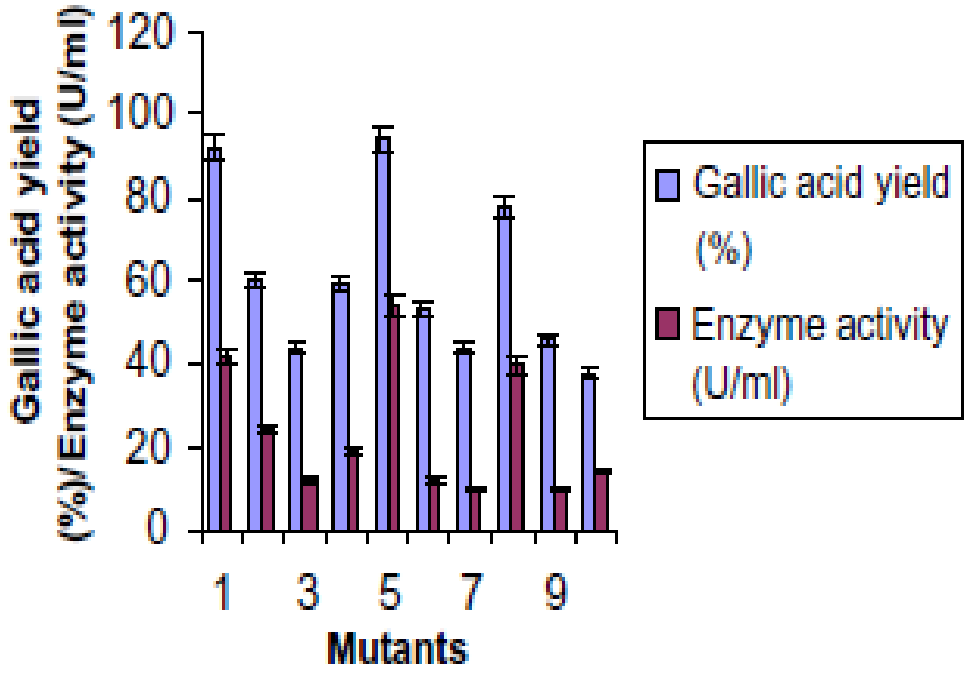

Fig. 4: Bioconversion efficiency test (i.e., % gallic acid yield) of the mutant and wild culture**.**

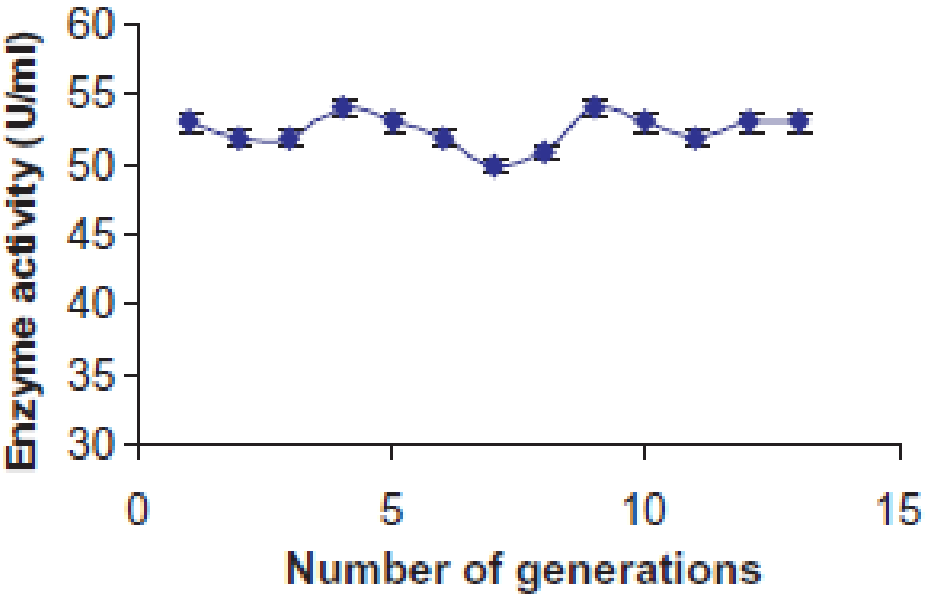

Fig. 5: Stability test for enzyme production by *A.oryzae*

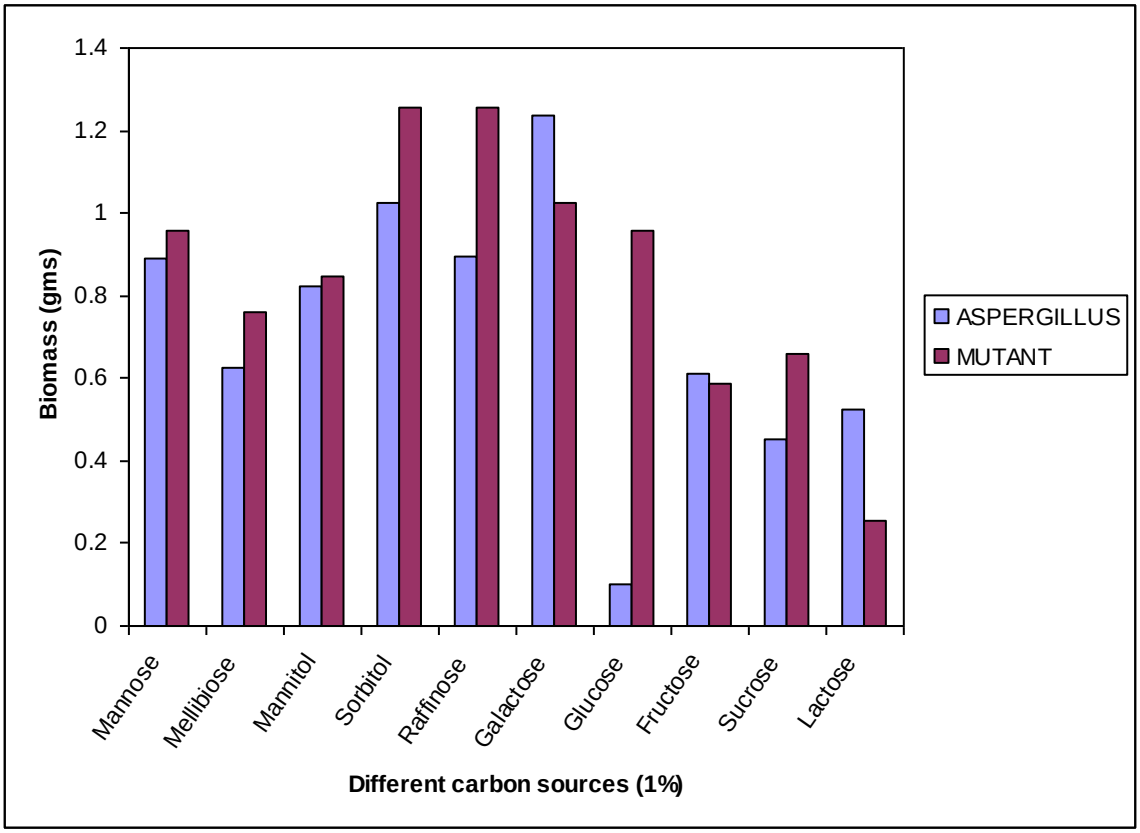

Fig. 6: Effect of different carbon sources on growth and metabolism of *Aspergillus* and mutant.

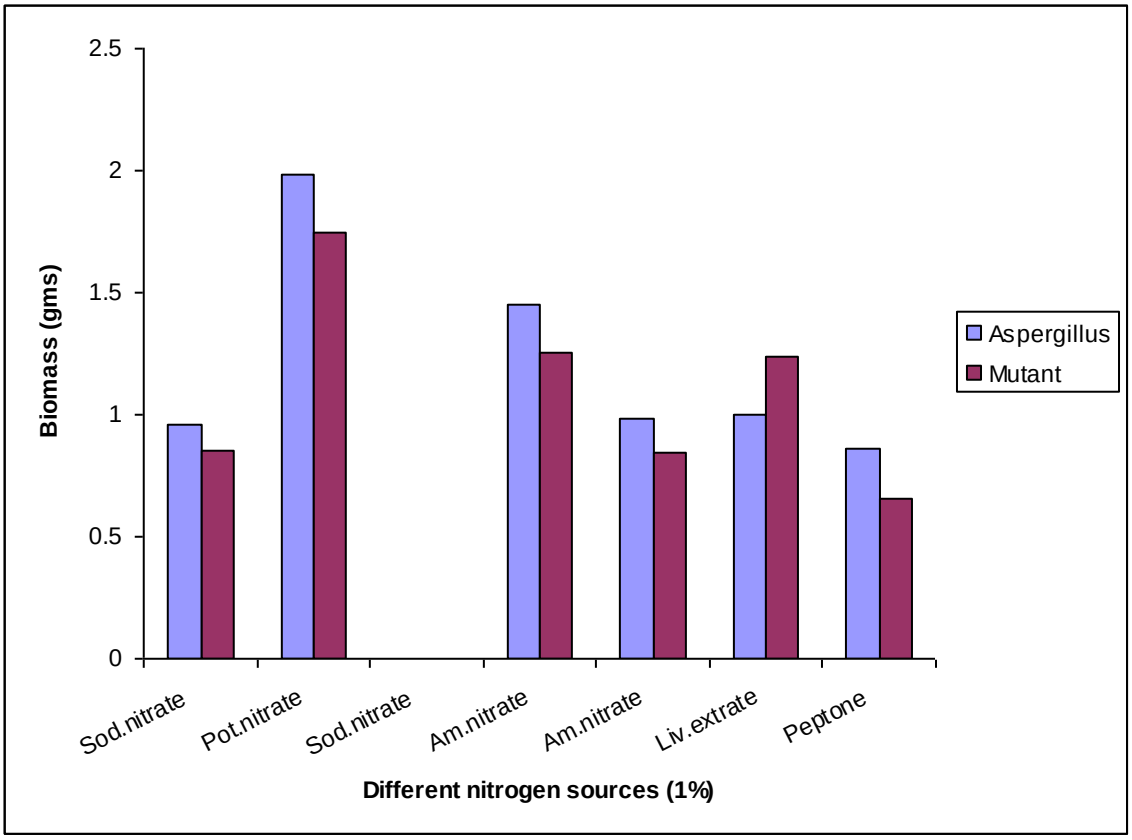

Fig. 7: Effect of different nitrogen sources on growth and metabolism of *Aspergillus* and mutant.