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AMYLASE PRODUCTION ON SOLID STATE FERMENTATION BY WILD TYPE AND MUTANT BACILLUS LICHENIFORMIS & ASPERGILLUS NIGER FROM AGRO-WASTES

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ABSTRACT: Cultures of *Aspergillus niger* and *Bacillus licheniformis* were procured from NCL, Pune. Obtained cultures were exposed to UV irradiation. Mutants of *Aspergillus niger* exposed to 30 and 50 min UV and *Bacillus licheniformis* exposed to 20 and 30 min UV were selected. Growth of wild type and mutated *Aspergillus niger* and *Bacillus licheniformis* was found to be optimum at 48 hrs of incubation time, 30°C of incubation temperature and pH 6. SSF was carried out using two substrates namely banana peel and potato peel. Alpha amylase produced using the two substrates showed good activity but banana peel as a substrate exhibited highest activity of 113.6 (U/ml) in *Aspergillus niger* mutant exposed to 30 min UV treatment followed by banana peel exhibiting activity of 11.2 (U/ml) in *Aspergillus niger* mutant exposed to 50 min UV treatment. In this regard, production of amylase which can be used in food & pharmaceutical industry can be increased by using mutants & waste banana peel.

INTRODUCTION: Alpha-Amylases also named as 4-α-D-glucanglucanohydrolase, has found its application in a range of industries including food, brewing, distilling industry, textile, paper, pharmaceutical and bioconversion of solid waste etc. Large range of applications is the triggering factor for the industrialization of alpha amylase production. Amylases have been reported to be produced by plant, animal and microbial sources, although the microbial amylase production has been reported to be most effective.

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The synthetic media used for the production of amylases have been a bit costlier and that's why a matter of concern for the researchers is. Researchers are now busy in search of procedures to cut short the cost of production. Solid state fermentation which has been reported to be a bit cheaper because of the enzyme extraction procedures is a ray of hope. In case of SSF the cost of the substrate also plays a key role in deciding the cost of production. Agro industrial wastes have been reported to be good substrate for the cost effective production of alpha amylases and are thus attracting researchers for using agro industrial waste as a substrate for alpha amylase production. Studies on fungal amylases especially in developing countries have concentrated mainly on *Aspergillus niger*, probably because of their ubiquitous nature and non-fastidious nutritional requirements of these organisms [1].

Fungal species have been studied a lot for the production of alpha amylase because of the low cost of substrates used for the production of alpha amylases. The mutant strain of *Bacillus licheniformis* and *Aspergillus niger* have better ability to produce alpha amylase which can be derived by mutagenesis and extensive screening [2, 3].

In this study, wild type & mutant strains of microorganisms were used for enzyme production. Thus the present study was designed in the search of cheaper carbon sources for the production of alpha amylase enzyme by fungal strains [4].

MATERIALS AND METHODS:

Collection of substrates & cultures: Substrates like potato & banana peel were collected from Yash food processing industry Satpur MIDC, Nashik. Cultures of *Bacillus licheniformis* (NCIM 2051) and *Aspergillus niger* (NCIM 616) were collected from NCL, Pune.

Screening of wild type & mutant cultures for amylase production: Pure cultures each of *Aspergillus niger & Bacillus licheniformis* were screened for amylase production using starch agar media [4]. Plates were incubated at 28°C for 48 hours. All the plates along with blank were flooded with iodine and were observed for zone of hydrolysis.

Induction of mutation by Ultraviolet (UV) radiation: Mutation was induced in wild type *Bacillus licheniformis* & *Aspergillus niger* by UV treatment [5]. The Petri plates were exposed to UV for 10-60 min time interval. After every 10 min interval, respective petri plate was removed and placed in the incubator at 28^{o}C for 48 hrs.

Solid State Fermentation (SSF): All the treatments were run in duplicate. Potato & banana peel wastes were pretreated before undergoing fermentation. Potato & banana peels were washed to remove impurities present on their surface. After washing they were oven dried for 4 hours at 150^{o}C to remove the moisture content present in the peels. After oven drying the peels was ground into fine powder by using food processor. For SSF 20 gm of each of the powdered potato and banana peels were taken in 250 ml flasks and moistened with 50 ml of MSM medium. Flasks were autoclaved at 121^{o}C and then cooled to room temperature.1ml of 48 hours old grown broth culture of wild type & mutated *Aspergillus niger* & *Bacillus licheniformis* showing hydrolysis during screening were inoculated into MSM media and the flasks were incubated at 28°C for 5 days [4].

Effect of various parameters on alpha amylase production by SSF: One unit of amylase activity is defined as the amount of enzyme, which released 1 µM of glucose per minute per milligram protein (U/mg). All the factors were studied by enzyme assay in crude enzyme by DNS method [6].

Effect of Incubation Time: The effect of incubation period on enzyme production was investigated by checking the enzyme activity on 4th, 5th, 6th, 7th and 8th day of incubation in different solid substrates and at 28°C in incubator [7].

Effect of Temperature: The effect of temperature on enzyme production was investigated by SSF in different substrates and incubated at 25°C, 30°C, 35°C, 40°C, for 4 days [7].

Effect of pH: The effect of pH on amylase activity was determined by incubating the reaction mixture with different buffers of 0.1 morality (pH 3 Citrate buffer; pH 5, 6, and 7- phosphate buffer, pH 9- Tris-HCL buffer) [7].

Extraction of Crude Enzyme & its assay: After 5 days of fermentation of banana & potato peel wastes, the MSM medium was assayed for enzyme production. Crude enzyme was extracted from fermented medium by adding 100 ml of 100 mM Tris buffer pH 6.2 the flasks were agitated by continuous agitation on shaker at 180 rpm for 1 hour. After agitation, the mixture was filtered through muslin cloth and centrifuged at 8000 rpm at 4ºC for 5 min. After centrifugation, the supernatant was collected and treated as crude enzyme [4]. Protein estimation was done by Lowry method [8]. Enzyme assay was carried out by DNS method [6].

RESULTS & DISCUSSION:

Collection of substrate procurement of Culture from NCL, Pune:

FIG. 1: PRETREATED BANANA PEEL POWDER

FIG. 2: PRETREATED POTATO PEEL

FIG. 3: CULTURES OF BACILLUS & ASPERGILLUS

Substrates like potato & banana peel were collected from Yash food processing industry Satpur MIDC, Nashik. Following substrates shown below were used for the production of amylase. Fig. 1 shows pretreated banana peel powder and fig. 2 shows pretreated potato peel powder. Cultures of *Aspergillus* & *Bacillus* are shown in fig 3. A number of microbial sources exist for the efficient production of this enzyme, but only a few selected strains of fungi and bacteria meet the criteria for commercial production [9].

In this study one fungal strain *Aspergillus* (coded *as Aspergillus niger* NCIM 616), and one bacteria (coded as *Bacillus licheniformis* NCIM 2051) procured from National Chemical Laboratory, Pune was used.

Screening of obtained cultures for amylase production: The results of screening of wild type cultures are shown in **fig. 4 & 5**. Maximum zone of starch hydrolysis was seen on the plates of *Bacillus* & *Aspergillus* which indicated that *A. niger and B. licheniformis* are amylase producers.

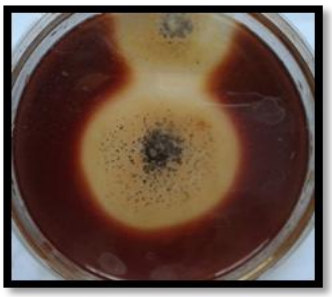


FIG. 4: ZONE OF INHIBITION SHOWN BY *ASPERGILLUS NIGER*

FIG. 5: ZONE OF INHIBITION SHOWN BY *BACILLUS LICHENIFORMIS*

Induction of mutation by Ultraviolet (UV) radiation: Growth was seen in mutated *A. niger* & mutated *B. licheniformis* as shown in **fig. 6 & 7.** Maximum growth was seen in plate exposed to 10 min UV treatment. Plate exposed to 60 min UV treatment showed negligible growth which indicated that death rate was 99%. Mutated *Bacillus licheniformis* showed maximum growth in plates exposed to 10, 20, 30 min UV treatment. No growth was seen in plates exposed to 50, 60 min UV treatment. The bacterial cells (*Bacillus amyloliquefaciens* UNG-16) were exposed to UV irradiation for 15-60 min. The mutants were picked up from the nutrient broth agar plates having at least 90 % death rate. In the present study the bacterial and fungal cultures were exposed to UV irradiation for 10-60 min and place it for 48 hrs incubation after incubation growth seen it was seen that as per increasing in UV treatment death rate of microorganism was increased. The mutants were picked up from starch agar plates having 90 % death rate [5].

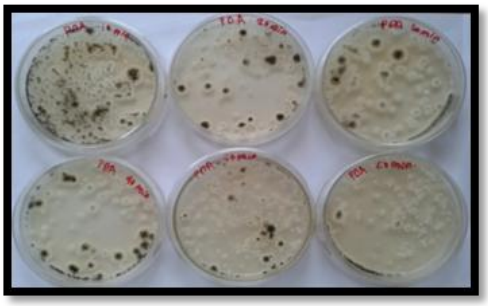


FIG. 6: GROWTH OF MUTATED *ASPERGILLUS NIGER* PDA

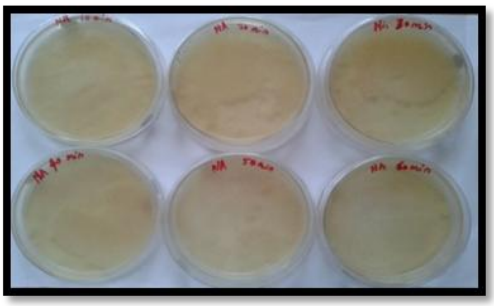


FIG. 7: GROWTH OF MUTATED *BACILLUS LICHENIFORMIS*

Screening of mutant strains for amylase production: Screening of the fungal isolates for amylase production was carried out in starch agar plates followed by iodine test as done earlier [10]. It can be found from **fig. 8** that *Aspergillus niger* exposed to 30 min and 50 min UV treatment showed maximum starch hydrolysis. This maximum starch hydrolyser's were selected for amylase production by SSF. It can be found from **fig. 9** that *Bacillus licheniformis* exposed to 20 min, 30 min & 40 min UV treatment showed maximum starch hydrolysis due to this, these plates showing maximum zone of starch hydrolysis were selected for amylase production by SSF.

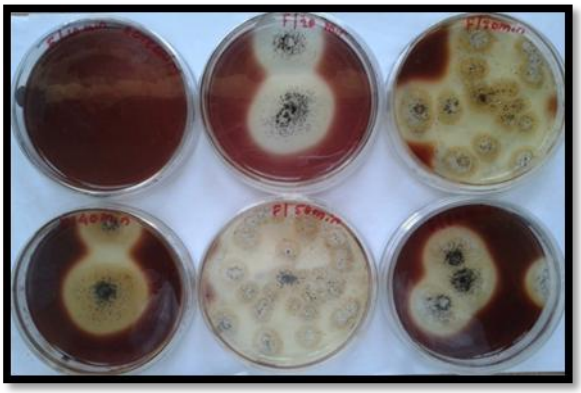


FIG. 8 PLATES SHOWING ZONE OF HYDROLYSIS BY *A. NIGER*

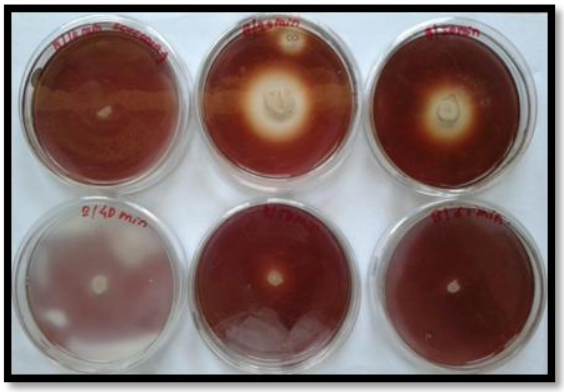


FIG. 9: PLATE SHOWING ZONE OF HYDROLYSIS BY *B. LICHENIFORMIS*

Amylase production by wild type *Bacillus licheniformis* and *Aspergillus niger*: Flasks in **fig. 10** shows wild type *Aspergillus niger* and *Bacillus licheniformis* inoculated in 50 ml MSM medium containing banana peel powder as substrate and kept for incubation at 28°C for 5 days in incubator. Flasks in **fig. 11** shows wild type *Aspergillus niger* and *Bacillus licheniformis* inoculated in 50 ml MSM medium containing potato peel powder as substrate and kept for incubation at 28°C for 5 days in incubator.

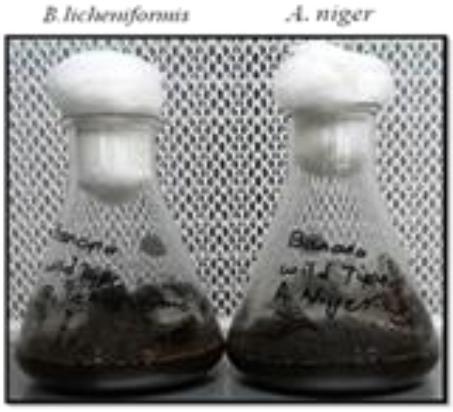


FIG. 10: SOLID STATE FERMENTED BANANA PEEL WASTES

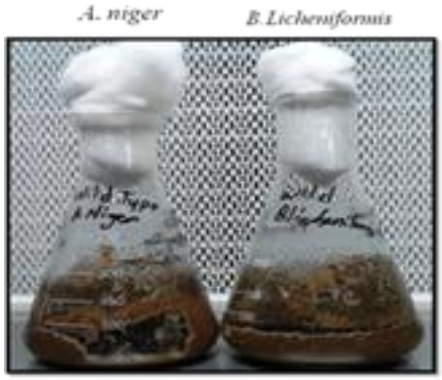


FIG. 11: SOLID STATE FERMENTED POTATO PEEL WASTES

Amylase production by mutated *Bacillus licheniformis* and *Aspergillus niger*: Flasks in fig.12 shows mutated *Aspergillus niger* exposed to 30 & 50 min UV exposure and mutated *Bacillus licheniformis* exposed to 20 & 30 min UV treatment were inoculated in 50 ml MSM medium containing banana peels. Flasks in fig.13 shows mutated *Aspergillus niger* exposed to 30 & 50 min UV exposure and mutated *Bacillus licheniformis* exposed to 20 & 30 min UV treatment were inoculated in 50 ml MSM medium containing potato peel powder as substrate and kept for incubation at 28°C for 5 days in incubator.

For SSF various agro industrial wastes including wheat bran, rice husk, vegetable waste (potato, tomato, brinjal) and banana peel were used as substrates [4]. All the four were found to be good substrates as the alpha amylase activity was seen in all the four flasks.

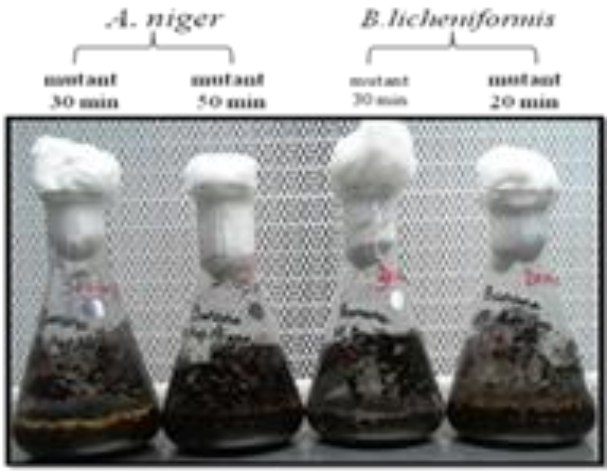


FIG. 12: SOLID STATE FERMENTED BANANA PEEL

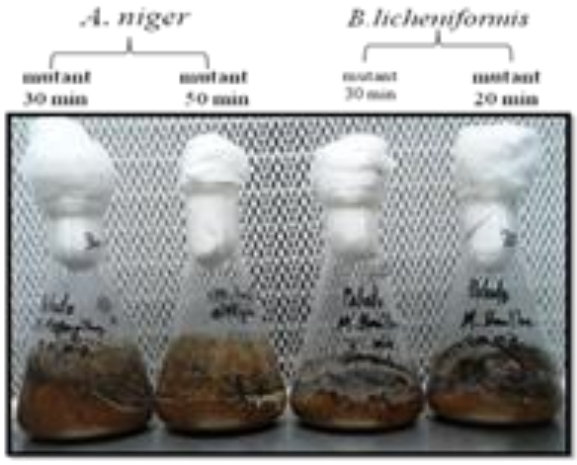


FIG. 13: SOLID STATE FERMENTED POTATO PEEL

Enzyme activity was maximum in the flask containing wheat bran as substrate and it was found to be 0.08U/ml/min followed by vegetable waste (0.06U/ml/min), banana peel (0.05U/ml/min) and rice husk (0.047U/ml/min). *Bacillus amylo-liquefaciens* strain UNG-16 was improved for alpha amylase production by UV irradiation[11, 12].

In the present study, agro industrial wastes including banana peel and potato peel were used as substrates. Enzyme activity was maximum in the flask containing banana as substrate and it was found to be 103.4 (U/ml) in *B. licheniformis* mutant 30 min UV treatment followed by potato peel waste, 101.6 (U/ml) seen in *B. licheniformis* mutant 20 min UV treatment.

Extraction of Crude Enzyme from wild type microorganisms after SSF:

Extraction of Crude Enzyme from mutant microorganisms after SSF: After 5 days of incubation, crude enzyme was extracted by adding 100 ml of 1mM tris buffer in the flask and kept on rotatory shaker at 180 rpm for 1 hour which was subjected to centrifugation at 8000 rpm at 4ºC for 5 min. The results obtained after centrifugation of medium containing crude enzyme produced by wild type & mutated *Aspergillus niger* and *Bacillus licheniformis* by using banana peel & potato peel as substrate are shown in **fig. 14 & 15** respectively. Similarly the results obtained after centrifugation of crude enzyme from mutated microorganisms by using banana peel & potato peel as substrate are shown in **fig. 16 & 17** respectively.

FIG. 14: SUPERNATANT (CRUDE ENZYME) COLLECTED AFTER CENTRIFUGATION BY BANANA PEEL WASTES

FIG. 15: SUPERNATANT (CRUDE ENZYME) COLLECTED AFTER CENTRIFUGATION BY POTATO PEEL WASTES

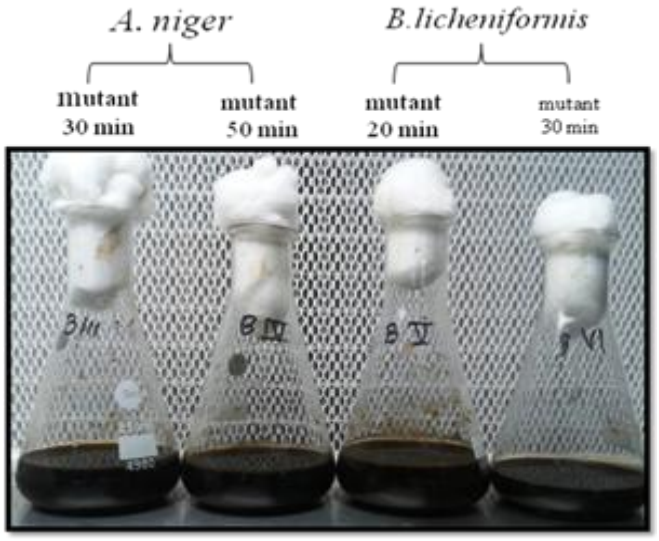


FIG. 16: SUPERNATANT (CRUDE ENZYME) COLLECTED AFTER CENTRIFUGATION BY BANANA PEEL WASTES

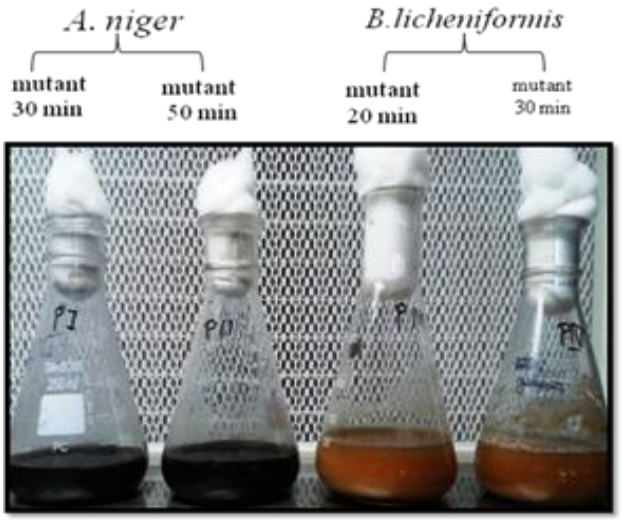
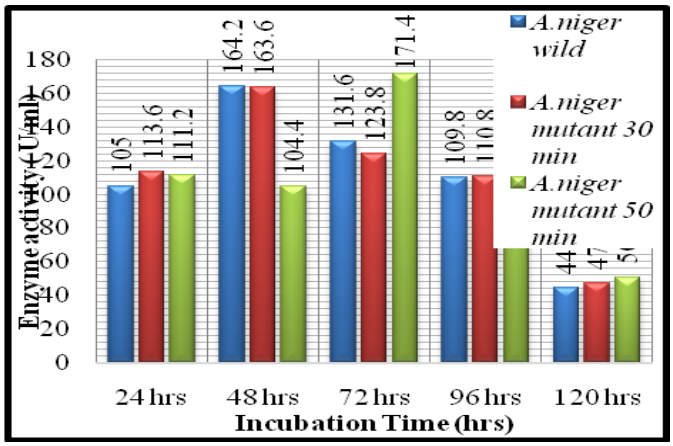


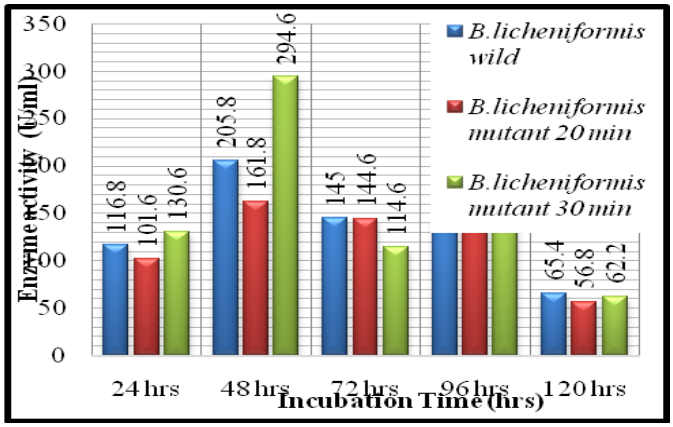
FIG. 17: SUPERNATANT (CRUDE ENZYME) COLLECTED AFTER CENTRIFUGATION BY POTATO PEEL WASTES

Effect of incubation time:

Effect of incubation time on enzyme activity using banana as substrate by *Aspergillus niger* & *B. licheisformis*: From Graph 1, it can be seen that maximum enzyme activity of 171.4 U/ml was observed in mutated *A. niger* exposed to 50 min UV treatment at 72 hrs incubation period. Minimum enzyme activity of 44.6 U/ml was observed in wild type *A. niger* at 120 hrs. incubation period as compared to wild type & mutated fungus exposed to other incubation period. From **Graph 2**, it can be seen that maximum enzyme activity of 294.6 U/ml was observed in Mutated *B. licheniformis* exposed to 30 min UV treatment at 72 hrs and minimum enzyme activity of 56.8 U/ml was observed in mutant *B. licheniformis* exposed to 20 min UV treatment at 120 hrs. incubation period as compared to wild type & mutated fungus exposed to other incubation periods.

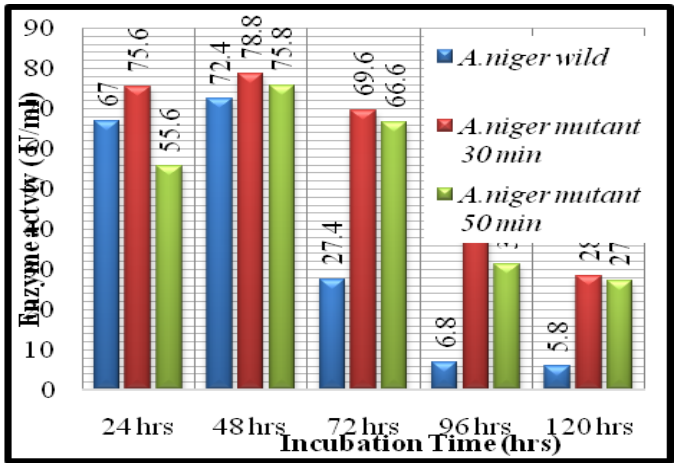


GRAPH 1: EFFECT OF INCUBATION TIME ON ENZYME USING BANANA AS SUBSTRATE BY *A. NIGER*

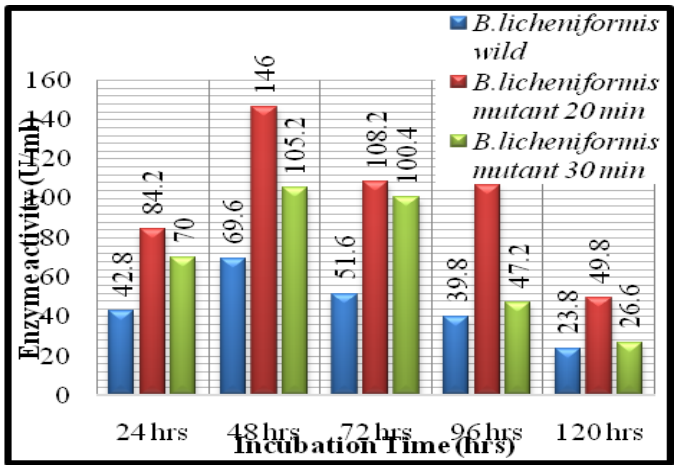


GRAPH 2: EFFECT OF INCUBATION TIME ON ENZYME ACTIVITY ACTIVITY USING BANANA AS SUBSTRATE BY *B. LICHENIFROMIS*

Effect of incubation time on enzyme activity using potato as substrate by *Aspergillus niger* & *B. licheisformis*: From **Graph 3**, it can be seen that maximum enzyme activity of 78.8U/ml was observed in mutated *A. niger* exposed to 30 min UV treatment at 48 hrs Minimum enzyme activity of 5.8U/ml was observed in wild type *A. niger* at 120 hrs. incubation period as compared to wild type & mutated fungus exposed to other incubation periods.



GRAPH 3: EFFECT OF INCUBATION TIME ON ENZYME ACTIVITY USING POTATO AS SUBSTRATE BY *A. NIGER*



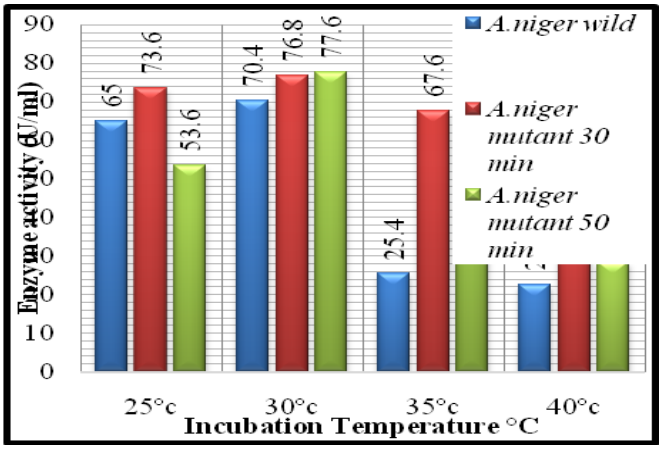
GRAPH 4: EFFECT OF INCUBATION TIME ON ENZYME ACTIVITY USING POTATO AS SUBSTRATE BY *B. LICHENIFORMIS*

From **Graph 6**, it can be seen that maximum enzyme activity of 146U/ml was observed in mutated *B. licheniformis* exposed to 20 min UV treatment at 48 hrs and Minimum enzyme activity of 23.8U/ml was observed in wild type *B.licheniformis* at 120 hrs. incubation period as compared to wild type & mutated fungus exposed to other incubation periods.

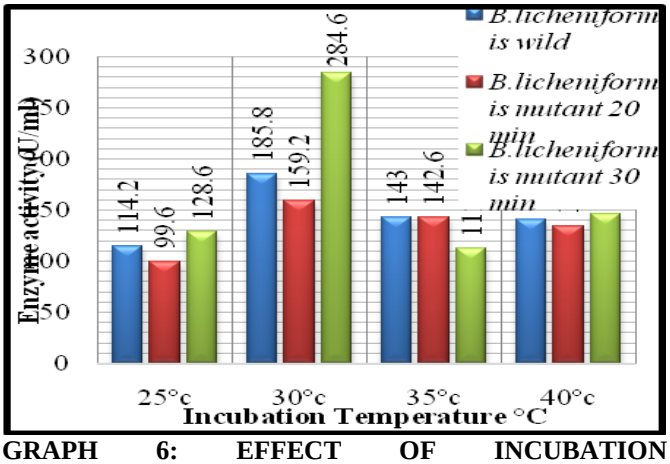
Short incubation period offers potential for in expensive production of enzyme [13]. Similar results were reported earlier that the mycelial growth on starch reached a maximum after five days and maximum amylase activity was produced after two days of cultivation [14]. The decreased activity in the later phase of growth was probably due to catabolite repression by glucose released from starch hydrolysis, in agreement with the results reported in *Humicolagrisea* and *H. Brevis*, but different from *Papulasporia thermofilia* in which the maximum amylase activity was recorded during the period of fungus autolysis and reported the maximum production of amylase enzyme at five days of incubation period at 30°C [15].

Incubation temperature:

Effect of incubation temperature on enzyme activity using banana as substrate by *Aspergillus niger* & *Bacillus licheniformis*:

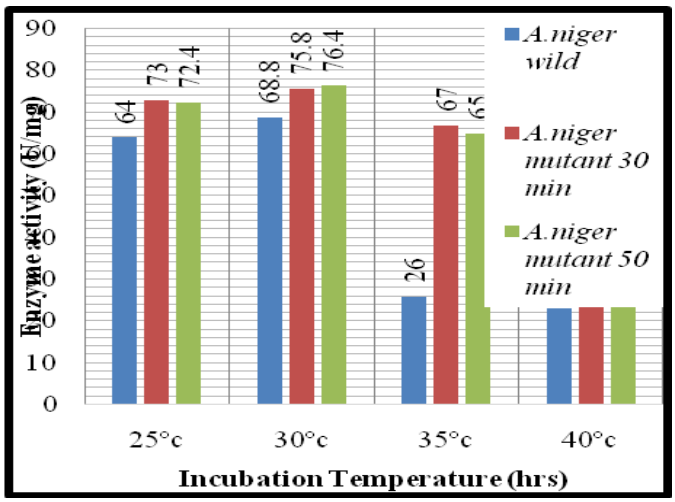


GRAPH 5: EFFECT OF INCUBATION TEMPERATURE ON ENZYME ACTIVITY USING BANANA AS SUBSTRATE BY *A. NIGER*

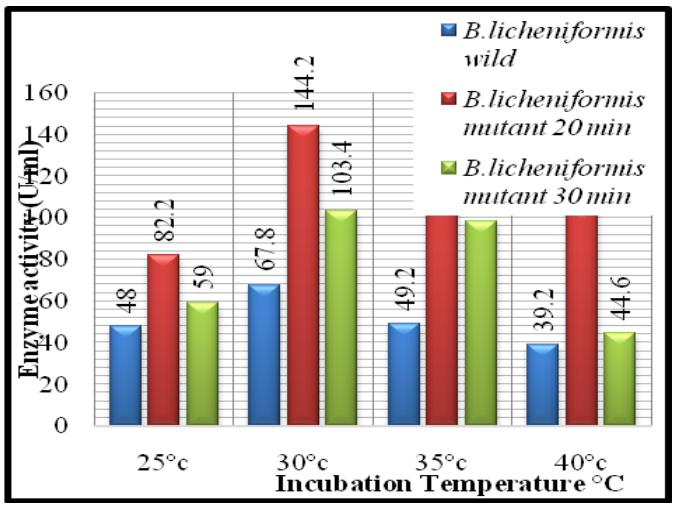


GRAPH 6: EFFECT OF INCUBATION TEMPERATURE ON ENZYME ACTIVITY USING BANANA AS SUBSTRATE BY *B. LICHENIFORMIS*

From **Graph 5**, it can be seen that maximum enzyme activity of 77.6 U/ml was observed in mutated *A. niger* exposed to 50 min UV treatment at 30ºC. Minimum enzyme activity of 22.6U/ml was observed in wild type *A. niger* at 40ºC incubation period as compared to wild type & mutated fungus exposed to other incubation temperatures. From **Graph 6**, it can be seen that maximum enzyme activity of 284.6 U/ml was observed in mutated *B. licheniformis* exposed to 30 min UV treatment at 48 hrs. Minimum enzyme activity of 134.6 U/ml was observed in mutated *B. licheniformis* exposed to 20 min UV treatment at 120 hrs incubation period as compared to wild type & mutated fungus exposed to other incubation temperatures.



GRAPH 7: EFFECT OF INCUBATION TEMPERATURE ON ENZYME USING POTATO AS SUBSTRATE BY *A. NIGER*



GRAPH 8: EFFECT OF INCUBATION TEMPERATURE ON ACTIVITY ENZYME ACTIVITY USING POTATO AS SUBSTRATE BY *B. LICHENIFORMIS*

From **Graph 7**, it can be seen that maximum enzyme activity of 76.6 U/ml was observed in mutated *A. niger* exposed to 50 min UV treatment at 30ºC. Minimum enzyme activity of 23.0 U/ml was observed in wild type *A. niger* at 40 º C incubation periods. As compared to wild type & mutated fungus exposed to other incubation temperatures. From **Graph 8**, it can be seen that maximum enzyme activity of 144.2 U/ml was observed in Mutated *B. licheniformis* exposed to 20 min UV treatment at 48 hrs and Minimum enzyme activity of 39.2 U/ml was observed in wild type *B. licheniformis* at 120 hrs incubation period as compared to wild type & mutated fungus exposed to other incubation temperatures.

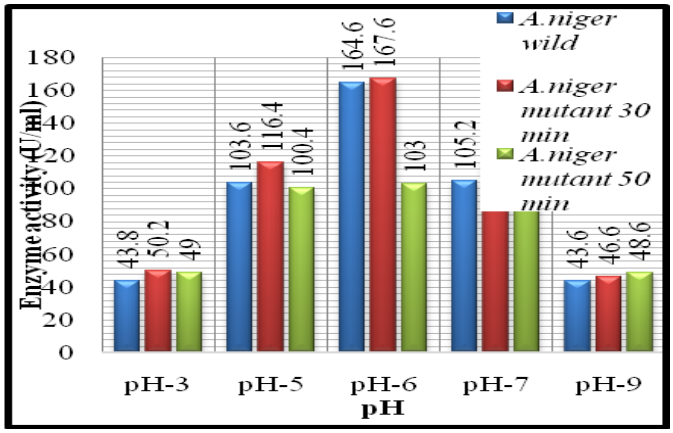
In the present study *Aspergillus niger* NCIM (616) and *Bacillus licheniformis* NCIM (2051) and its mutant was inoculated at different temperatures 25°C, 30°C, 35°C and 40°C showed maximum yield of amylase in banana peel and potato peel. Maximum enzyme activity was observed in temperature 30°C of wild type and mutated *Aspergillus niger* and *Bacillus licheniformis.* There was increase in yield in 40°C.Then a gradual decrease in yield was observed in banana peels (Graph 3 and 4) and in potato peel (Graph 5 and 6) as substrate was recorded. It is reported that best enzyme production in *A. niger* occurs at room temperature both in SmF and SSF and reported 30°C as the optimum temp. to be the best for enzyme production by *Penicillium fellutanum* [16, 17].

This shows clearly that the enzyme production in solid state is greatly affected by temperature. The great yield temperatures of amylase were between 30°C - 37°C [18, 19]. However, the optimum temperature for enzyme production was reported as 30°C in many literatures [15, 20]. Previously 30°C and 45°C were reported as optimum temperature for amylase production by *A. flavus* and *Myceliophthora thermophilia* [21, 22].

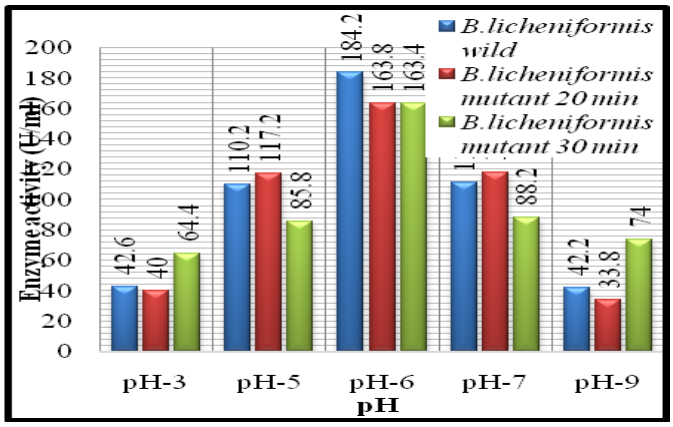
Effect of pH:

Effect of pH on enzyme activity using banana as substrate by *Aspergillus niger* & *Bacillus licheniformis*: From Graph 9, it can be seen that maximum enzyme activity of 167.6 U/ml was observed in mutated *A. niger* exposed to 30 min UV treatment at pH-6.

Minimum enzyme activity of 43.6 U/ml was observed in wild type *A. niger* at pH-9. From the above Graph No. 10, it can be seen that maximum enzyme activity of 184.2 U/ml was observed in wild type *B. licheniformis* at pH-6. Minimum enzyme activity of 33.8 U/ml was observed in mutated *B. licheniformis* exposed to 20 min UV treatment at pH 9.

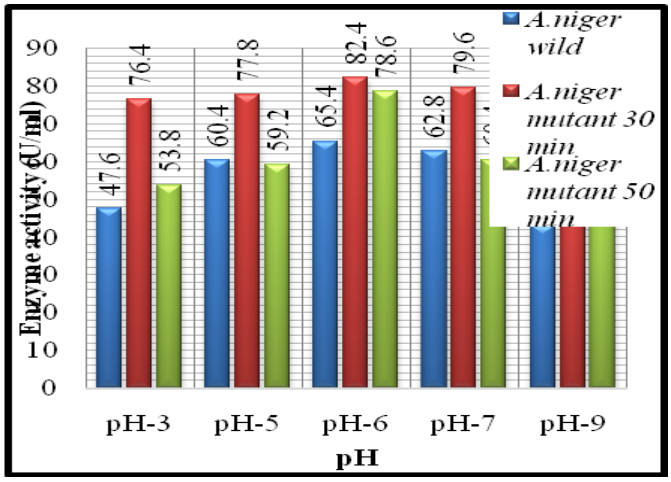


GRAPH 9 EFFECT OF pH ON ENZYME ACTIVITY USING BANANA AS SUBSTRATE BY *A. NIGER*

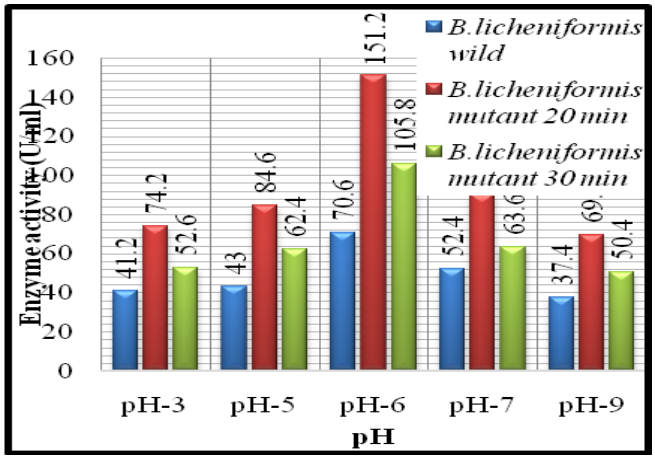


GRAPH 10: EFFECT OF pH ON ENZYME ACTIVITY USING BANANA AS SUBSTRATE *BY B. LICHENIFORMIS*

Effect of pH on enzyme activity using potato as substrate by *Aspergillus niger* & *Bacillus licheniformis*: From **Graph 11**, it can be seen that maximum enzyme activity of 82.4 U/ml was observed in Mutated *A. niger* exposed to 30 min UV treatment at pH-6. Minimum enzyme activity of 43.2U/ml was observed in wild type *A.niger* at pH-9. From **Graph 12**, it can be seen that maximum enzyme activity of 151.2 U/ml was observed in mutated *B. licheniformis* exposed to 20 min UV treatment at pH-6. Minimum enzyme activity of 37.4 U/ml was observed in wild type *B. licheniformis* at pH-9.



GRAPH 11: EFFECT OF pH ON ENZYME ACTIVITY USING POTATO AS SUBSTRATE BY *A. NIGER*



GRAPH 12: EFFECT OF pH ON ENZYME ACTIVITY USING POTATO AS SUBSTRATE BY *B.LICHENIFORMIS*

In the present study, *Aspergillus niger* NCIM (616) and *Bacillus licheniformis* NCIM (2051) and its mutants were inoculated into different substrates like banana peel powder and potato peel powder used as substrates for SSF for 5 days The enzyme was extracted and the activities of the amylase produced at different pH was recorded. The maximum yield of amylase was found at pH 6.0 and the activity was high in substrate banana peel in *A. niger* mutated at 30 min was 838 U/ml & high in wild *B. licheniformis* 921 µg/ml in 5 days of incubation.

The specific activity observed was as high as in the substrate potato peel *A.niger* mutated 30 min 421 µg/ml & high in *B.licheniformis* mutated 20 min 756 µg/ml potato peel. As compared to wild type and mutated *A. niger* and *B. licheniformis.* Therefore it is clear that, each substrate was

utilized better in pH-6.5. There was marked increase in the yield till pH 8, and then there was very minimum activity at pH 9 in all the substrates used in SSF. In contrary to present study, Varalakshmi[16] reported the maximum enzyme activity of 75 U/mg of protein at pH 6.5 others have reported acidic pH optima for amylases from *A. niger* [23, 24].

Bacterial amylases having alkaline pH optima were reported by different workers [25]. *A. niger* LPB 28 had a preference to grow and produce amylase in acidic conditions and related that fungi have a growth capacity, although limited in extreme acidic and alkaline conditions [26]. This characteristic is most important to fermentation processes because it shows that in these conditions most of the bacteria responsible for fermentation contamination processes are inhibited. It was found that an optimum pH of 4.6 for amyloglucosidase production in SSF by *A. niger* NRRL 3122 using rice bran [27]. It is reported that amylase production is high at pH 5 [15].

In the present study, the effect of pH on the enzyme activity indicates that the amylase is active in the pH range 5.5 – 6.5. Similarly multiple pH optima were observed for amylolytic activities in the crude amylase preparation in various literatures [28, 29, 30, 31].

CONCLUSION: In the present study, it is concluded that the *Aspergillus niger* and *Bacillus licheniformis* are potent producers of alpha amylase by Solid State Fermentation condition. This potential can be further improved by mutation of fungus and bacteria. In this regard parental strain was subjected to UV irradiation. The UV irradiation for 10- 60 minutes was found to be sufficient for the production of viable and stable mutants of fungus and *bacillus*.

The productivity of alpha amylase was significantly improved after screening and successive treatment of UV. The selected mutants of *Aspergillus niger* i.e. in 30 and 50 min UV exposure plates and *Bacillus licheniformis* in 20 and 30 UV exposure plates were selected to produce alpha amylase in solid state fermentation. For the production of alpha amylase optimization of temperature of 30ºC, pH 6.5 and time of incubation 48 hrs was found to be optimum for the biosynthesis of alpha amylase.

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