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EXPERIMENTAL ARTICLES

Condition Stabilization for *Aspergillus niger* FCBP-198 and Its Hyperactive Mutants to Yield High Titres of α-Amylase[1]

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Abstract—A number of substrates were tested for the cultivation of microorganisms to produce a host of enzymes. The effect of different substrates (wheat and rice straw, sugar cane waste, wood waste), incubation temperatures (20–40°C), initial pH levels (3.5–9.0), incubation periods (0–72 hours) and nitrogen sources (ammonium sulfate, urea, peptone, yeast extract, sodium nitrate) on growth and α-amylase activity was studied for the native and mutant strains. Maximum enzyme activity was observed at 1.5% wheat straw for *Aspergillus niger* FCBP-198 and An-Ch-4.7 and at 2% wheat straw for An-UV-5.6, with sodium nitrate as a principle nitrogen source. The optimum temperature for maximum enzyme activity was 30°C for the parental strain, while An-UV-5.6 and An-Ch-4.7 thrived well at 32.5°C. The best conditions of pH and incubation duration were 4.5 and 48 hours, respectively, for all the strains. Mass production under preoptimized growth conditions demonstrated the suitability of wheat straw for swift mycelial colonization and viability.

Key words: *A. niger* FCBP-198, α-amylase activity, optimization, mass production.

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Pakistan being an agricultural country has many agricultural byproducts. These byproducts are cheaply available for utilization in the fermentation processes [1], including solid state fermentation (SSF) for the production of enzymes. Some of the substrates that have been used are sugar cane bagasse, wheat straw, rice straw, rice husk, corncobs, banana waste, coconut oil cake, mustard oil cake, wheat flour, corn flour, starch, etc. [2–5]. These substrates offer potential advantages for the filamentous fungi, which are generally capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium. Application of this agro-industrial waste in bioprocesses also solves pollution problems, which their disposal may otherwise cause [6–8]. The hyphal mode of fungal growth and their good tolerance to low water activity (a_w) and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates [9].

The goal of the present study was to optimize the conditions for mass cultivation of indigenous and improved test species on economically feasible substrates to attain large amount of the active α-amylase.

MATERIALS AND METHODS

Growth conditions. *Aspergillus niger* strain FCBP-198 was mutagenized through UV and chemical (ethyl methane sulphonate) treatment mutation for hyper active α-amylase enzyme and two improved mutants, An-UV-5.6 and An-Ch-4.7 were obtained in previous study [10]. In order to use fungi for large scale enzyme production, these most efficient strains were cultivated on the cheapest possible sources. To make it economically feasible and cost-effective, cheap, and easily available substrates like wheat straw, rice straw, sugar cane waste, and wood wastes were tested. Wheat straw var. Maxi Pak and IRRI Pak-6 rice straw were obtained from the Government Agriculture Farm, Shiekhupura, sugar cane waste was obtained from the Juice corner of the Campus area of the University of the Punjab, Lahore; and wood waste of *Delbergia sissoo* was purchased from the Campus area of the University of the Punjab, Lahore. Prior to use, all the substrates were dried and chopped. Measured amounts of each substrate (2 g) were soaked in 100 ml distilled water at pH 4.5 and autoclaved. Afterwards, 1 ml of the spore inoculum (5×10^5 conidia ml^{-1}) of *A. niger* strain FCBP-198, An-UV-5.6, and An-Ch-4.7 was inoculated into the flasks and incubated at 30 ± 2°C for 72 hours on an orbital shaker incubator at 200 rpm with samples taken every 12 hours to determine the α-amylase activity.

The cultivation conditions (the best substrate concentration, temperature, initial pH, incubation period, and nitrogen source) were optimized for the active fungal strains on the best substrate and its optimal concentration.

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Mass production of potential producers. For pilot scale production of the enzyme producers were cultivated on selected substrates under optimized conditions. For this purpose, wheat straw was soaked in water overnight. After that, the substrate was drained; the residual moisture content was 70%. The percentage of moisture content was calculated according to the following formula:

$$\text{Moisture (\%)} = \frac{\text{Difference in wt. of substrate before and after soaking (g)}}{\text{Initial wt. of substrate (g)}} \times 100.$$

The plastic bags of 20 × 30 cm were filled with 200 g of the substrate and sterilized at 121°C for 15 min. After cooling, each bag was supplemented with 0.5 g of sodium nitrate (optimized nitrogen source) under aseptic conditions. Two inoculum discs of 5 mm diameter from 7-day cultures of the parental strain, as well as of the UV and chemical mutants of the selected strains were used to inoculate the bags. Each treatment was replicated thrice. The prepared bags were incubated in a growth room for 15 days at 30 ± 2°C until the mycelium ramified/penetrated the whole substrate.

Assessment of fungal growth/sporulation on agro-waste. After 15 days, 2 g of material was taken from each bag and suspended in 20 ml of sterilized water. After thorough shaking, the suspension was permitted to settle for 15 min to ensure the loosening of conidia. The suspension was then filtered through muslin cloth to obtain the conidial suspension free from the mycelial biomass. The conidia concentration was determined in a haemacytometer in terms of number of conidia per 10 μl of suspension [11].

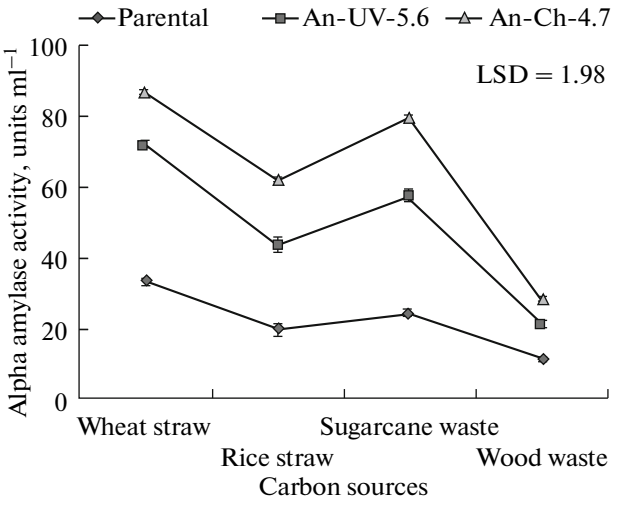

Fig. 1. Effect of carbon sources on α-amylase activity in *A. niger* FCBP-198 and its mutants An-UV-5.6 and An-Ch-4.7. Vertical bars indicated the standart error of means for three replicates. LSD at $P \leq 0.05$. Incubation temperature: 30 ± 2°C. Inoculum size: 1 ml of suspension containing 5×10^5 conidia ml^{-1}. pH: 4.5. Incubation period: 48 hours.

RESULTS

Effect of carbon source. Figure 1 presents the data on the activity of α-amylase from *A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7 grown on the following substrates: wheat straw, rice straw, sugar cane waste and wood waste. These agricultural by-products were added at a concentration of 2% (w/v). Each fungal strain (*A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7) grew well in the presence of the tested substrates. The enzymatic activity on these substrates differed from each other at $P \leq 0.05$ level of significance with 1.98 least significant difference (LSD) for *A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7. Although the subsistence of low level constitutive activity of α-amylase was detected in wood waste, the most active synthesis occurred on wheat straw, at the rates of 33.43, 71.42 and 86.38 U ml^{-1} for *A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7, respectively. Since wheat straw was evidently the best substrate for enzyme production, it was chosen for further investigations.

Effect of substrate concentration. The results revealed that both mutants exhibited significantly higher α-amylase activity than the wild type strain under the same conditions (Fig. 2). The enzyme activity was significantly higher ($P \leq 0.05$) in the fungi grown on 1.5% (*A. niger* FCBP-198 and An-Ch-4.7) or 2% (An-UV-5.6) wheat straw than at other concentrations of this substrate. Further increase in the substrate concentration resulted in decreased α-amylase activity.

Effect of incubation temperature. The influence of different incubation temperatures from 20 to 40°C on the amylolytic activity of the tested strains was assayed on wheat straw up to 3 days with 12 hours interval at 200 rpm. The results of activity assays are presented in Fig. 3 and demonstrate significantly different (at $P \leq 0.05$) amylolytic activity at different cultivation temperatures. The data obtained indicated that 30°C was the optimum temperature for the maximum α-amylase activity (37.94 U ml^{-1}) of *A. niger* FCBP-198, while for An-UV-5.6 and An-Ch-4.7 the optimum was at 32.5°C. However, substantial enzyme activity was detected within the temperature range of 22–37°C.

Effect of pH. The initial pH of the medium plays an important role in the synthesis of microbial enzymes. In the present assessment of the effect of initial pH of the medium, the enzyme activity was observed within a broad pH range of 3.5–8.5. However, the peak activity was observed at pH 4.5 for all the test strains

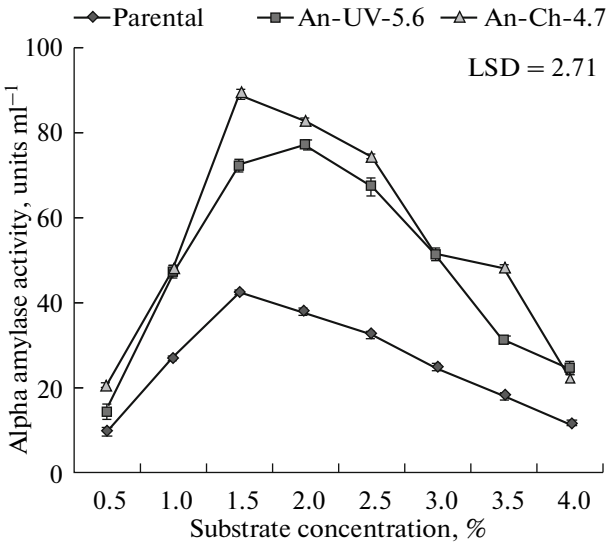

Fig. 2. Effect of different concentrations of substrate—water straw—on α-amylase activity in *A. niger* FCBP-198 and its mutants An-UV-5.6 and An-Ch-4.7. Vertical bars indicate the standard error of means for three replicates. LSD at $P \leq 0.05$. Substrate: wheat straw, pH: 4.5. Incubation temperature: 30 ± 2°C. Inoculum size: 1 ml of suspension containing 5×10^5 conidia ml^{-1}. Incubation period: 48 hours.

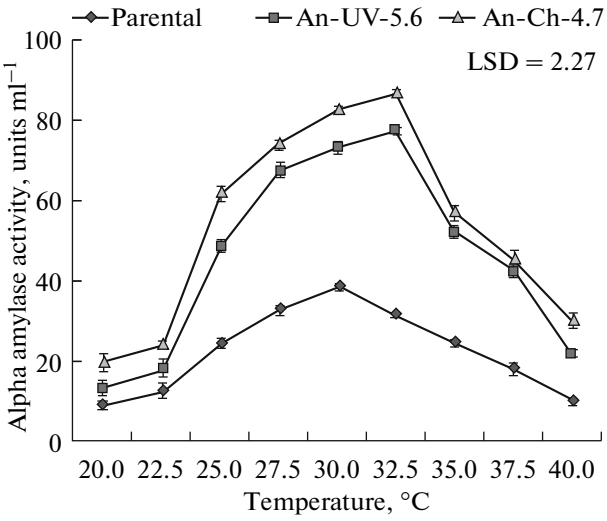

Fig. 3. Effect of incubation temperature on α-amylase activity in *A. niger* FC8P-198 and its mutants An-UV-5.6 and An-Ch-4.7. Vertical bars indicate the standard error of means for three replicates. LSD at $P \leq 0.05$. Inoculum size: 1 ml of suspension containing 5×10^5 conidia ml^{-1}, pH: 4.5. Incubation period: 48 hours. Substrate: 1.5% wheat straw for *A. niger* FCBP-198 and An-Ch-4.7, 2% wheat straw for An-UV-5.6.

(Fig. 4). Increase in pH beyond 4.5 resulted in a decrease in α-amylase activity. The activity was greatly inhibited at alkaline pH (8.5) and dropped to zero at pH 9.0 in case of parental (*A. niger* FCBP-198) and to negligibly low enzymatic activity for An-UV-5.6 and An-Ch-4.7. Thus, the acidic pH 4.5 was selected for further enzyme assays. Compatibility test revealed the LSD value of 2.19 between the strains.

Effect of the incubation period. The effect of time course was evaluated for up to 72 hours for α-amylase activity; the results are presented in Fig. 5. In the course if cultivation of *A. niger* FCBP-198 and its mutant derivatives, α-amylase activity increased with an increasing incubation time. The highest α-amylase activities (35.71, 76.04 and 89 U ml^{-1} for *A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7, respectively) were discerned after 48 hours of incubation. Further increase in the incubation time resulted in decreased α-amylase activity.

Effect of nitrogen source. The effect of nitrogen sources, namely urea, peptone, and yeast extract (organic nitrogen sources), ammonium sulfate and sodium nitrate (inorganic nitrogen sources) on α-amylase activity of the wild type strain *A. niger* FCBP-198 and its mutant derivatives, with wheat straw as a substrate was studied (Fig. 6). All organic and inorganic nitrogen sources tested, at a concentration of 0.5% in the growth medium, were found effective for obtaining the maximum activity of the enzyme. All the wild and mutant isolates exhibited significantly ($P \leq$ 0.05) high levels of α-amylase activity using ammonium sulfate, urea, peptone and yeast extract as nitrogen sources. However, urea displayed poor efficiency as inducer of enzyme activity for wild strain of *A. niger* FCBP-198 and An-UV-5.6. Similarly yeast extract was found weak inducer for An-Ch-4.7. Among all nitrogen sources, sodium nitrate was chosen as the best nitrogen source for α-amylase production by all isolates with the highest activity of 61.71, 99.59 and 110.94 U ml^{-1}, for *A. niger* FCBP-198, An-UV-5.6 and An-Ch-4.7, respectively.

Stability of derived mutants. To evaluate the stability of the mutant derivatives of *A. niger* FCBP-198 i.e., An-UV-5.6 and An-Ch-4.7, these isolates were analyzed for up to 10 generations after every two months for their ability to produce the enzyme under optimized assay conditions. For up to 10 generations, the efficiency of improved isolates was found to be stable, with the same yields with insignificant difference (Fig. 7).

Mass production of mutants on pilot scale under optimized conditions. Wheat straw was the most suitable substrate for the fungal growth and rapid colonization by *A. niger* FCBP-198 and its mutants. In case of *A. niger* FCBP-198 and An-Ch-4.7, the 1.5% wheat straw supplemented with 0.5 g sodium nitrate per 200 g of substrate supported maximum conidial production. In the case of An-UV-5.6 the same nitrogen source was found to be comparatively less effective. Thus, 2% wheat straw supplemented with 0.5 g sodium nitrate (0.5 g/200 g of substrate) was the best

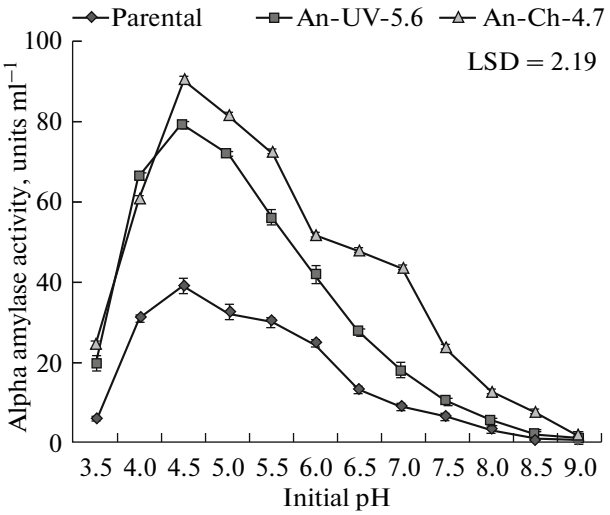

Fig. 4. Effect of initial pH of basal medium on α-amylase activity in *A. niger* FCBP-198 and its mutants An-UV-5.6 and An-Ch-4.7. Vertical bars indicate the standard error of means for three replicates. LSD at $P \leq 0.05$. Inoculum size: 1 ml of suspension containing 5×10^5 conidia ml^{-1}. Substrate: 1.5% wheat straw for *A. niger* FCBP-198 and An-Ch-4.7, 2% wheat straw for An-UV-5.6. Incubation period: 48 hours. Incubation temperature: 30°C for *A. niger* FCBP-198 and 32.5°C for An-UV-5.6 and An-Ch-4.7.

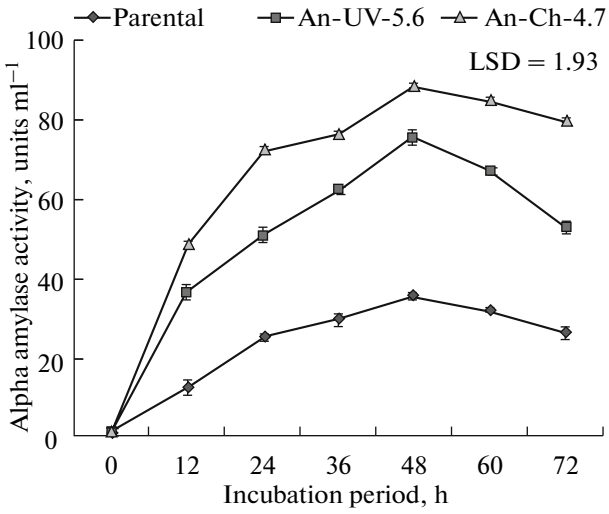

Fig. 5. Effect of incubation period on α-amylase activity in *A. niger* FCBP-198 and its mutants An-UV-5.6 and An-Ch-4.7. Vertical bars indicate the standard error of means for three replicates. LSD at $P \leq 0.05$. The same conditions as in the Fig. 4.

source for mycelial growth and conidial yield of An-UV-5.6. The results of mass production presented in Fig. 8 reveal that the conidial production was 3.63×10^6 conidia 10 μl^{-1} for the wild type strain *A. niger* FCBP-198; however, the highest conidial yield (3.92×10^6 conidia 10 μl^{-1}) was recorded in case of An-Ch-4.7 under similar conditions. Under the conditions for mass production, An-UV-5.6 formed 3.84×10^6 conidia 10 μl^{-1}. It was biologically the best substrate that supported healthy growth of the fungus which was evidenced in terms of conidial productivity (Fig. 8).

The viability tests of the wild type and mutant strains, mass produced on wheat straw + sodium nitrate revealed that α-amylase activity remained the same during mass culturing. The strains *A. niger* FCBP-198, An-UV-5.6, and An-Ch-4.7 displayed the enzymatic activity of about 34, 73.5, and 88 U ml^{-1}, respectively.

DISCUSSION

Optimization of growth conditions is essential to obtain high α-amylase activity of the selected fungal strains. Literature clearly demonstrates that the desired microbial activities can be tremendously enhanced by establishing appropriate cultivation protocols. Different factors affect the enzyme activity, including composition of the substrate, type and concentration of carbon and nitrogen sources, concentration of certain other ion/s, pH, and temperature. The environmental parameters affect microbial growth on solid as well as in liquid media and therefore the production of enzymes or other metabolites [12–14]. Agricultural by-products, are low cost substrates widely used for enzyme production [1, 2, 15, 16]. α-Amylase is an inducible enzyme and is generally induced in the presence of carbon sources such as starch, products of its hydrolysis, or maltose [17, 18]. In the present study, among various substrates employed, wheat straw proved to be the best substrate contributing all the nutrients required for growth and enzyme formation. Maximum activity of the enzyme was recorded at 1.5% wheat straw in *A. niger* FCBP-198 and An-Ch-4.7 while 2% wheat straw was optimal for An-UV-5.6. Further increase in substrate concentration resulted in decreased activity of the enzyme. Several previous studies have demonstrated that an increase in nutrient concentration with a consequent increase in the stationary phase duration inhibits α-amylase accumulation [19, 20]. Earlier Tsen et al. [21] tested 11 carbon sources as inducers of α-galactosidase activity in *Rhizopus thailandensis*. The substrates showing the best activities were sucrose, raffinose, and lactose whereas no activity was induced by glucose, maltose, starch, and xylose.

The production and stability of α-amylase is greatly affected by the addition of salts in the fermentation medium because their metal ions act as activators for the enzyme activity [22]. Enzyme activity depends upon pH, temperature, incubation time, and many other factors. It is necessary to optimize different limiting factors for maximum activity of amylase enzyme. The amount of nitrogen source has also been

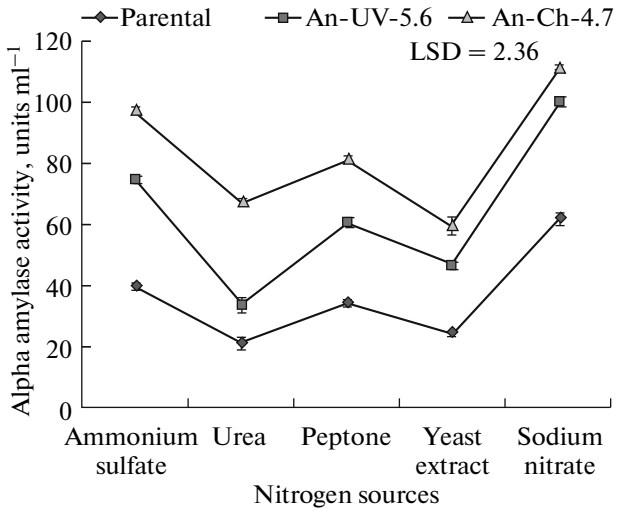

Fig. 6. Effect of different nitrogen sources on α-amylase activity in *A. niger* FCBP-198 and its mutants An-UV-5.6 and An-Ch-4.7. Vertical bars indicate the standard error of means for three replicates. LSD at $P \leq 0.05$; incubation conditions are the same as in the Figs. 4, 5.

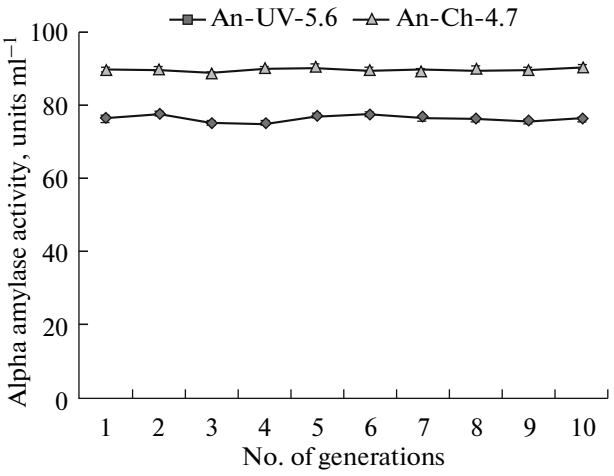

Fig. 7. Stability of *A. niger* FCBP-198 and its mutant derivatives An-UV-5.6 and An-Ch-4.7 for α-amylase activity. Vertical bars indicate the standard error of means for three replicates. Inoculum size: 1 ml of suspension containing 5×10^5 conidia ml^{-1}, pH: 4.5. Incubation period: 48 hours. Incubation temperature: 30 ± 2°C.

reported as a very critical parameter in the production of α-amylases [5, 23]. Therefore, the substrate was supplemented with different nitrogen sources and the cultural conditions were evaluated further in optimization studies. The highest α-amylase activity was detected at pH 4.5 after 48 hours for all the test strains with sodium nitrate as a principle nitrogen source. The optimum temperature was 30°C for the parental strain while An-UV-5.6 and An-Ch-4.7 thrived well at 32.5°C. Earlier, in a study of *Aspergillus fumigatus* (Fres.), the highest amylase activity was observed in the presence of 4% starch as a carbon source and 0.25% $(NH_4)_2HPO_4$ as a nitrogen source at 35°C and pH 7.0 [24]. The optimization studies on *Fusarium solani* (Mart.) Sacc. were carried out for enhanced production of glucoamylase using different substrates. Wheat bran as a substrate yielded the highest enzyme activity of 61.35 U g^{-1} when supplemented with 1% fructose as a carbon and energy source with 1% urea as a nitrogen source at 35 ± 1°C and pH 5.0. It was further observed that the addition of surfactants caused a decrease in enzyme biosynthesis by *F. solani* in SSF of wheat bran [14].

In the present work, investigations were further extended to upscale production of mycelial biomass on wheat straw under pre-optimized conditions to enhance the production potential of α-amylase by the tested strains. Mass production of both wild and mutant strains was successfully achieved as wheat straw was very rapidly colonized under pre-set conditions. In several similar studies economically available agricultural by-products have been used individually or in various combinations for mass production of microbial biomass for α-amylase production [20, 25, 26]. In solid-state fermentation assays, carried out for α-amylase production using *Aspergillus oryzae*, Sumitra et al. [27] among a variety of substrates identified groundnut oil cake as the best substrate. Combination of wheat bran and groundnut oil cake (1 : 1) displayed higher enzyme titers than the individual substrates. Maximum amount of the enzyme (9196 U g^{-1}) was achieved when solid state fermentation was carried out using wheat bran + groundnut oil cake, with initial moisture of 64%, supplemented with lactose and

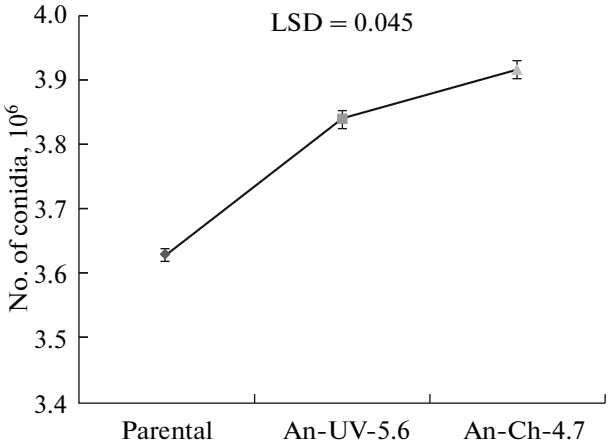

Fig. 8. Mass production of *A. niger* FCBP-198 and its mutant derivatives An-UV-5.6 and An-Ch-4.7.

ammonium nitrate (1% each) at 30°C for 72 hours using 2 ml spore suspension (6×10^7 spores ml^{-1}). The present observation implies that there is an excellent scope for utilizing these strains for commercial production of α-amylases.

In auxiliary studies, the mutant strains An-UV-5.6 and An-Ch-4.7 were evaluated for up to 10 generations for their potential to secrete highly active α-amylase under pre-optimized conditions. The mutants exhibited stable activity of α-amylase. These findings are in accordance with the work of Mohsin [28] who assessed the mutant stability with respect to enzyme secretion and reported markedly stable mutants under defined conditions.

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