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Production of α-amylase(s) by *Aspergillus flavus*, F7 attacking water hyacinth ground preparation (WHGP) under solid state fermentation

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ABSTRACT

Some environmental and nutritional parameters controlling the biosynthesis of α-amylase from *Aspergillus flavus,* F7 attacking the water hyacinth have been investigated under solid state fermentation conditions for maximum amylase production. The following optima's were recorded for the highest α-amylase yield; Incubation period 7 days; temperature, 30^{o}C; pH, 5; inoculum size, 3X10^{8} spores/ml; flask volume 100 ml capacity; hyacinth fresh weight 5 g; tap water, 25 ml. Under these conditions, starch showed remarkable stimulatory effect; nitrogen sources and amino acids have no stimulatory effect. Pyridoxal hydrochloride, B6 at a concentration of 200 ppm exhibited a stimulatory effect on biosynthesis of α-amylase.

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INTRODUCTION

Water hyacinth (*Eichhornia crassipes*) is an intruder floating water weed that has spread throughout freshwater bodies. Egypt has the oldest record of water hyacinth in Africa since the late 1800s. The weed prohibit electricity generation, clog irrigation canals, hindering fish production; increases water loss resulting from evaporation and block light from penetrating river water; and facilitates propagation of bilharzia and malaria diseases. Since 1991 Egypt has depended exclusively on mechanical harvesting method to control water hyacinth [1].

This plant contains a high proportion of protein (49.6%), total lipids (16.0%), total carbohydrates (26.9%), fibre (1.7%) and 5.8% ash. In addition, calcium, magnesium, copper, zinc, manganese and potassium were determined [2]. Recently, [3] reported elements of water hyacinth are C, O, N, Na, Mg, Al, Zr, Cl, K, Ca, Si, Ti, and Fe revealing dominant elements. Many trials were carried out to utilize water hyacinth as a sole carbon source and a fermentable substrate for fungal production of extracellular enzymes were reported [4–7] in addition production of biogas [8], bioethanol [9, 10] were reported.

One of the objectives of the biotechnology is the utilization of wastes from environment [11, 12], agriculture [13, 14], food industry [15–18] industrial [19, 20] for the production of enzymes, proteins, animal feed, and energy and at the same time combating the pollution of the environment. Waste disposal involves using appropriate microorganism to decompose organic wastes which are too hazardous to treat by other means [21].

Amylases are crucial enzymes which hydrolyze glycosidic linkages in starch and produce as primary products dextrins and oligosaccharides. They are

classified into α-, β-, and glucoamylase on the basis of their 3-dimensional structures, amino acid sequences and reaction mechanisms. Amylases have endless number of applications in analytical chemistry, clinical, medical, and as well as in food, textile, detergents, brewing, and distilling industries [22].

Amylase is the utmost and pivotal important enzyme that plays a crucial role in the field of biotechnology. It is induced mainly from microbial sources and is used in many industries. Amylases are spread widely in living systems and have specific substrates which are widely available from low-cost plant sources, rendering the potential applications of the enzyme more abundant in terms of costs, so it meets commercial needs [23–25].

However, microbial sources are the most preferred one for large scale production meeting industrial demands [26]. The major advantage of using microorganisms for the enzyme production is that the process is economically applicable and microbes are easy to manipulate to obtain enzymes of desired characteristics [27]. Fungal enzymes have the advantage of being secreted extracellularly. In addition, the ability of fungi to penetrate harsh and robust substrates enable the hydrolysis process. In addition, fungal species are highly appropriate for solid state fermentation. The most efficient producing species include those of genus *Aspergillus* (*A. flavus*, *A. niger*, *A. awamori*, *A. fumigatus*, and *A. oryzae* [5, 24, 27, 28].

This investigation has been undertaken for the purpose of making use of water hyacinth as a sole carbon source and as a fermentable substrate from one hand and production of commercially α-amylase enzyme from the other hand.

MATERIALS AND M ETHODS

Preparation of water hyacinth ground preparation (WHGP)

One Kg of water hyacinth (WH) (whole plant except the root) was washed and grinded in a blender using 100 ml tap water and stored frozen in a container in the refrigerator until use [11].

Production medium

Five grams of WHGP was added to only 25 ml tap water in each 100 ml capacity conical flask to obtain a selective medium for α-amylase production. The pH was adjusted at 6 and flasks were autoclaved at 1.5 atmospheric pressure (121°C) for 15 minutes.

Isolation and screening of fungi attacking WHGP and producing α -amylase enzyme

A piece of fermented WH plant was used as a source for isolation of microorganisms producing α-amylase. Using a sterile loop, streaking a piece of the fermented WH on starch agar plate was carried out as recorded by [29]. The produced isolates were evaluated according to their α-amylase productivity by measuring the clear zones in mm. Primary screening of all isolated microorganisms for alpha amylase production was done by the starch agar plate method [29]. Out of 20 bacterial and fungal isolates, the isolate that showed the biggest zone of clearance in starch hydrolysis were selected for production in Solid State Fermentation.

Characterization of the most potent isolate

The most potent isolate which shows the highest α-amylase productivity among all the tested isolates, was selected for characterization. Its characterization was carried out in the Faculty of Science, Al-Azhar Univ., Cairo, Egypt. Isolate preliminarily identified, based on macroscopic and microscopic morphology on Czapek-Dox agar (CDA). The identification was made based on gross colony morphology, color and on microscopic features (magnification of $100\times$ and $400\times$) in lactophenol cotton blue-stained wet mounts and characterized according to [30]. The most promising isolate was selected and identified as *Aspergillus flavus* F7 and used for further investigations.

α -Amylase Assay

Estimation of amylase activity was carried out according to the DNSA (3, 5 dinitro salicylic acid) method. Only 0.5 ml enzyme was added to a mixture of 0.5 ml of water and 0.5 ml of 1 % (w/v) soluble starch in phosphate buffer (0.02 M) at pH 6.9, then incubated at 37°C for 30 min. The reaction was stopped using 3,5-dinitrosalicylic acid, and reducing sugars were determined by the method of [31]. One unit of α-amylase activity was defined as the amount of enzyme that is produced by reducing sugar equivalent to 1 μmol of maltose per min in the reaction. All experiments were carried out in duplicate.

Optimization of culture conditions for amylase production

The optimization of various parameters by the most potent fungal isolate (*Aspergillus flavus*, F7) was carried out on the production medium (WHGP). Influencing α-amylase production was investigated, the effect of each parameter independently keeping others as constant. The optimized parameters were incorporated in subsequent experiments. All experiments were done in triplicate and the mean values of reducing sugars [31] are calculated.

Effect of environmental factors on α -amylase

productivity by the most potent isolate

Present investigation was carried out at different time incubations (2 up to 9 days), temperature (15, 20, 25, 30, 37 and 55°C) and pH (2 up to 9), inoculum size (0.25, 0.5, 1, 2, 3 and 4 ml of $1.8x10^7$ spores/ml), flask volume (50, 100, 250, 500, 1000, and 2000 ml).

Effect of nutritional factors on α -amylase productivity by the most potent isolate

Present investigation was carried out at different substrate concentrations of water hyacinth ground preparation (WHGP) (2, 4, 8, 20, 28 and 40 g/100 ml), carbon sources (starch, glucose and maltose, 1%), starch concentrations (0.25, 0.5, 1, 2, 4 and 6%), nitrogen sources ($NaNO_3$, $CaNO_3$, $(NH_4)_2SO_4$, NH_4NO_3,Urea, Peptone, Corn steep liquor, yeast extract and Malt extract, equivalent to nitrogen present in sodium nitrate), amino acids (15 DL-amino acids, a percentage of N_2 equivalents to that present in sodium nitrate), metallic ions ($FeSO_4.7H_2O$, $MgSO_4$, $CaSO_4$, $MnSO_4$, Na_2SO_4 and $ZnSO_4$ at 50, 100 & 200 ppm), vitamins (B_1, B_2, B_6, B_{12}, C, Biotin and Vitamin K, at 50, 100 & 200 ppm).

RESULTS AND DISCUSSION

Amylase is one of the most crucial industrial enzymes that have found practice in the baking, brewing, starch processing, pharmaceutical, detergents, and textile industries [32].

In the present investigation, *Aspergillus flavus*, f7 was found to be the most potent amylolytic fungal isolate among all the isolated fungal micro flora investigated, they were selected purposely for studying some parameters controlling the biosynthesis of amylase(s) from water hyacinth under solid state fermentation conditions.

In the present study, Figure 1a showed the relation between amylase productivity and time of incubation. The level of amylase production increase gradually with increasing the incubation period up to a maximum of 7 days, beyond this maximum value decline in enzyme productivity could be observed [27] reported an optimum incubation period for amylases production of 6 days using *Aspergillus fumigatus* NTCC1222. The highest enzyme production by *A. niger* was obtained after 4 days [33, 34]. However, [5] investigated the production on water hyacinth by *Aspergillus* sp. S7 after 5-6 days. Also, other investigator reported that, maximum amylase production on wheat bran was obtained after 6 days of incubation by *Aspergillus flavus*, F2Mbb [21].

In the present study, the pH of the fermentation medium was adjusted by 6N NaOH or 6N HCl. The effect of different initial pH on amylase productivity for fungal isolates was represented graphically in Figure 1b. [35] reported that the majority members of Aspergilli producing amylases required acidic media i.e. 3.0 - 6.5. This in complete accordance with that recorded in the present study. Since a pH of 5 exhibited the highest amylase productivity for the most potent fungal isolate. Although the enzyme maintained the activity over a wide pH range 2-9. However, α-amylase maximum production by *A. niger* was exhibited at pH 6.2 and 6 [36, 37]. In addition, [38] found that pH 5.8 was the best for amylase production by *Aspergillus* sp. JGI 12.

In the present investigation, high level of amylase productivity was detected when the incubation temperature was adjusted at 30°C beyond this temperature led to decrease in enzyme productivity for *A. flavus*, F7 and considerable productivity was also noticed at 35°C with an ability to produce enzyme up to 55°C. On the other hand, the fact that the wide range of amylase(s) lies between 15- 55°C with a maximum at 30°C to indicate that the present of amylase tend to be heat stable. The results were represented graphically in Figure 1c. In view of the findings of other investigators, the optimum temperature for production of amylase by *A. niger* was 30°C [39–42].

Moreover, different concentrations of spore suspensions were used as an inoculum size. The inoculum size used were: 0.25, 0.5, 1, 2, 3 and 4 ml. Each 1 ml of *A.flavus*, F7 suspension contained $1.8x10^7$ spores/ml. The inoculum size was adjusted by means of haemocytometer. The optimum inoculum size was 1 ml for the most potent microbial isolate. Results are presented in Figure 1d. However, $3.4x10^7$ spore/ml was reported by [7] on amylase production by *Thermomyces lanuginosus* Tsiklinski Ferm-BAM. However, the best inoculum size was found to be 20% [43]. Moreover, it has been reported that α-amylase was increased by the use of increased amount of inoculum but β-amylase not influenced [44]. [5] found that a concentration of $3x10^8$ spores/ml exerted the highest amylolytic activity.

In the present investigation, the best volume for maximum amylase(s) productivity was 100 ml capacity, compared to other flask volumes used. The results were represented graphically in Figure 1e. An increase or decrease beyond this flask volume led to decrease in enzyme productivity. Similarly, [5] reported that, the best flask volume for amylase production by *Aspergillus* sp. S7 was 100 ml capacity.

It is well known that complex lignocellulosic wastes are supposed to be the best substrates for the

SSF processes [45, 46]. Fungi are used in the production of α-amylases under submerged fermentation (SmF), however, the production under solid state fermentation are preferred because of the higher yield, low capital investment, better product recovery and several characteristic advantages it offers [47, 48]. In the present investigation, the effect of different substrate concentrations (WHGP) used as a sole carbon source in fermentation medium on amylase(s) productivity by *A. flavus*, F7 was studied. WHGP contains sufficient nutrients that support good microbial growth and high yield of enzymes as well [5, 6, 11]. The results represented graphically in Figure 2a showed that, high level of amylase productivity was detected at 20% of (WHGP). An increase or decrease beyond this concentrations led to decrease in enzyme productivity.

[27] showed that, a concentration of 33% pomegranate peel and wheat bran induced maximum α-amylase production by *Aspergillus fumigatus* NTCC1222 under SSF, also recently the optimization and immobilization of amylase produced by *Aspergillus terreus* using pomegranate peel waste [49]. Also, it has been reported wheat bran could be used for economic production of amylase by SSF at a concentration 20% [21, 50]. In contrast, the concentration of 12% (g/l) of fresh hyacinth water homogenate exhibited the highest amylase productivity by the mesophilic fungus *Aspergillus flavus*, S-7 under laboratory scale fermentation conditions [5]. While [51] reported using orange waste powder in the α-amylase production by *Aspergillus niger* ATCC 16404. In addition, the production of thermostable amylase by *Thermomyces lanuginosus* Tsiklinski Ferm-BAM while utilizing water hyacinth as the sole carbon source at a concentration 20% under solid state fermentation [7]. Moreover, the biosynthesis of α-amylases from *Aspergillus flavus*, F2Mbb using bran as a sole carbon source was investigated [21].

In the present study, starch in combination with WHGP induces the maximum α-amylase production by *Aspergillus flavus* F7 (Figure 2b) while, glucose result in catabolic repression. The combination of different carbon sources may result in higher enzyme biosynthesis. Starch is known to induce α-amylase production in different bacterial and fungal strains [21, 52, 53]. However, [54] showed that, maltose exhibited maximum α-amylase production by *Aspergillus terreus* NCFT4269. It has been found that, starch resulted in maximum amylase production by *A. niger* and *R. stolonifer*, followed by maltose, while glucose and fructose were the least effective carbon sources [42].

In the present investigation, a concentration of starch up to 4% induces the highest biosynthesis of α-amylase production by *A. flavus* F7 as seen in Figure 2c. However, the optimum concentration of starch for α-amylase production was 0.5 % by *Aspergillus* niger [39].

The effect of different nitrogen sources on the enzyme productivity by the most potent microbial isolate was studied. Nine nitrogen sources were applied as shown in Figure 3a. In the present study, all the tested nitrogen sources failed to increase amylase productivity on growing *A. flavus*, F7 on WHGP under S.S.F condition. This means that, the nutritional value of WHGP contains the required nutrients included the nitrogen source as reported previously [4, 7]. So the addition of the WHGP to the production medium was a must and result in low capital investment. In view of the findings of other investigators, reported that water hyacinth extract has a potential effect as a new growth medium for culturing fungi and bacteria [55]. On the other hand, the highest yields of amylase by *A. niger* and *R. stolonifer* were achieved in cultures supplemented with ammonium sulphate, followed by peptone [42]. However, peptone was the optimum nitrogen source for α-amylase production [39]. However, various inorganic nitrogen sources salts have been reported to support better production in fungi [24].

Concerning the addition of different amino acids to the cultural medium, none of the added amino acids results in further increase in the α-amylase production by *A. flavus* F7 compared to the control and the tested amino acids were found to have inhibitory effect on amylase productivity (Figure 3b). However, [5, 6, 12] indicated that, the acidic amino acids group (glutamic and aspartic) was the best source for inducing a high α-amylase productivity. On the other hand, the sulfur containing amino acid methionine exhibited the highest enzyme productivity among the different 24 amino acids [21].

Certain microorganisms are sensitive to microelements in the medium, resulting either in suppression or stimulation of growth and enzyme yield [56, 57]. In the view of the present data, it could be concluded that all the added microelements exhibited no further α-amylase production by *A. flavus*, F7. Results were represented graphically in Figure 4a. concerning the present results, it appears that the addition of minerals exhibited α-amylase enzyme productivity lower than control by the most potent microbial isolate. However, Ca^{++} exhibited the same productivity of α-amylase as the control. This may be due to the WHGP contains high proportion of metallic ions as reported by [3]. More-

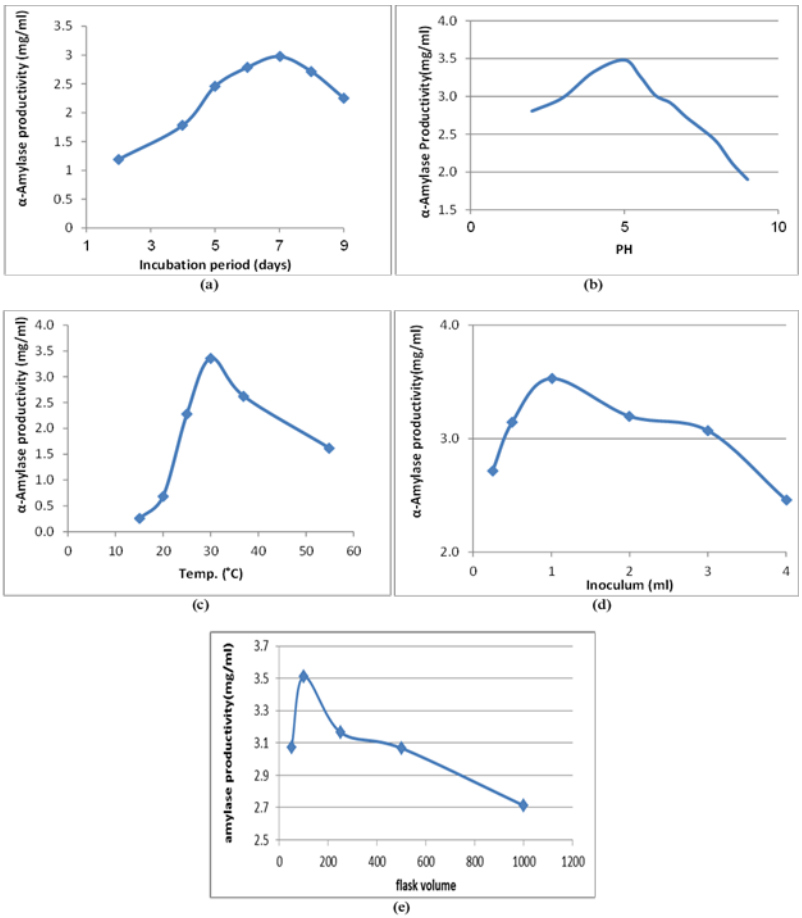

Figure 1: Relation of different incubation periods (a) pH (b) Temperature (c) Inoculum size (d) toα-amylase productivity by *A. flavus*, F7 growing on WHGP under SSF conditions

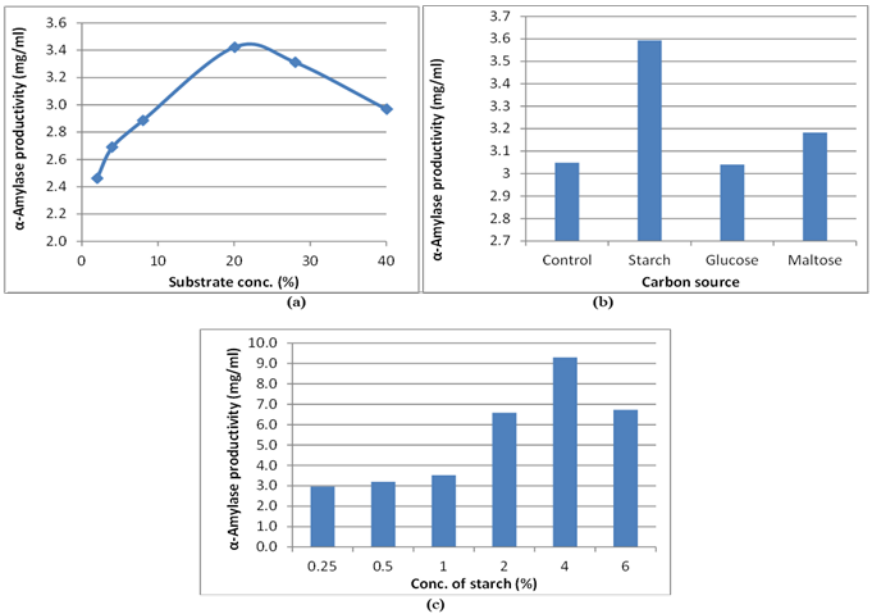

Figure 2: Relation of different substrate concentration (a) carbon source (b) starch concentration(c) to α-amylase productivity by *A.flavus*, F7 growing on WHGP under SSF conditions

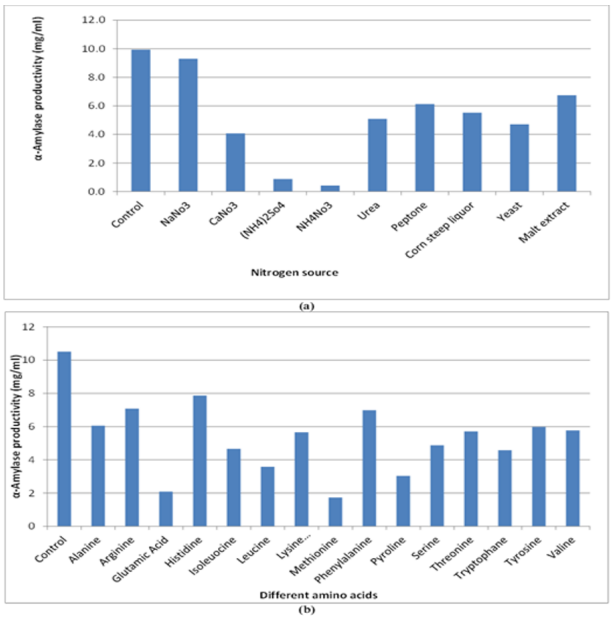

Figure 3: Relation of different nitrogen sources (a) and amino acids (b) to α-amylase productivity by *A.flavus*, F7, growing on WHGP under SSF conditions

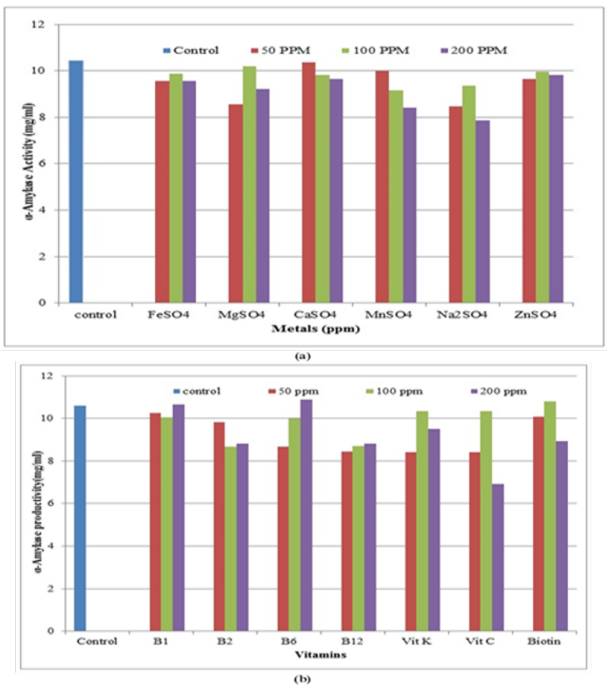

Figure 4: Relation of different metals (a) and vitamins (b) to α-amylase productivity by *A.flavus*, F7, growing on WHGP under SSF conditions

over, [43] reported that, certain metallic ions as Na^{+}, Ca^{++}, Mg^{++} and Co^{++} enhanced α-amylase productivity. In addition, Most of the amylases are metalloenzyme requiring Ca^{+2} for their activity, structural integrity, and stabilization [58, 59].

The effect of the addition of different vitamins on amylases productivity by the most potent microbial isolate *A. flavus*, F7 was studied. Results were represented graphically in Figure 4b. Data indicated that the highest enzyme productivity was recorded at concentration 200ppm of Pyridoxal hydrochloride, and 100ppm of Biotin [30]. However, 100ppm thiamine exhibited a remarkable stimulatory effect on α-amylase production by *Aspergillus* sp. S7 [5]. While, it has been reported that 400 ppm choline resulted in the increase biosynthesis of α-amylase by thermophilic *Thermomyces lanuginosus* Tsiklinski Ferm-BAM while utilizing water hyacinth as the sole carbon source [7].

CONCLUSIONS

The present investigation thorough a light on the possibility of use Water hyacinth which cause many environmental problems in Egypt as the sole carbon source in the production of commercially useful α-amylase enzyme.

Future Plan

The produced α-amylase must be purified and optimized in the next study. In addition, the production must be optimized in Batch fermentor.

Author contributions

All the authors have contributed equally in designing, drafting the manuscript as per the journal submission format. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that there are no competing conflicts of interest.

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