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Optimum conditions for α- amylase production by *Aspergillus niger* mutant isolate using solid state fermentation

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ABSTRACT

Twenty one local isolates of *Aspergillus niger* were screened for their ability to produce α-amylase using solid-state fermentation (SSF), *A. niger* N11 local isolate exhibited maximum efficiency for amylase production, this isolate had been chosen for mutation by CO^{60} Gamma ray. Several food wastes were used, such as wheat bran, potato peel, banana peel and corn cob. The highest yield of amylase produced from *A. niger* N11 mutant isolate was obtained with wheat bran when used as a substrate. Soluble starch and Ammonium nitrate (1%w/w) used as the best additional carbon and nitrogen supplements respectively. The optimum pH, temperature and incubation period for α-amylase production by mutant isolate were found to be 5.5, 30°C and 6 days, respectively.

Keywords: α- Amylase, *Aspergillus niger*, Gamma ray, Solid-state fermentation

INTRODUCTION

Amylases are a class of enzymes that are capable of digesting glycosidic linkages in starch components and glycogen molecules, these enzymes refer to glycoside hydrolase enzymes group which have enzyme commission number (EC:3.2.1). They are widely distributed in plant, microbial and animal kingdoms, which show varying in action patterns depending on the source. Amylases have significant applications in food, fermentation, textile, paper and pharmaceutical industries. Currently, they comprise about 30% of the world enzyme production [1].

Amylases are widely distributed in microorganisms (bacteria and fungi). Molds are capable of producing high amounts of amylase; *Aspergillus niger* is used for commercial production of α -amylase. 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. It is possible to enlist the use of amylases under extreme condition of pH and temperature using thermo-acidophilic and alkaline amylases. [2].

For the microbial α-amylases production, two types of fermentation methods are mainly used : submerged and solid state fermentation .solid state fermentation system is reported to be the most appropriate process for developing countries ,because of the simplicity of the technique, superior volumetric productivity , low energy requirements , less water output, better product recovery, lack of foam build up. The low availability of water reduces the possibilities of contamination by bacteria and yeast, higher levels of aeration, low cost and availability of media employed in SSF [3]. The principle of solid state fermentation system derived from the nature or it is mimesis of the nature by which microorganism especially fungi preferred to grow in media nearly void of free water such as agricultural wastes, organic soil particles, cereals and fruit .The selection of a suitable microorganism is an important aspect of SSF for production of enzymes. The microorganism should be able to grow at low water activity (aw=0.6-0.7). Fungi and yeast were termed as suitable microorganisms for SSF (Solid state fermentation) according to the theoretical concept of water activity, whereas bacteria have been considered less suitable. However, experiences have shown that

bacterial cultures can be well managed and manipulated for SSF processes [4]. The selection of wastes as substrate for enzyme production in a SSF depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates [5]. In order to improve amylases production, number of researchers had been reached to mutation production in living microorganism which are the most important for enzyme production in commercial domain, these mutation may be duplicate enzyme production in contrast to mother culture or they may be cause to production low amount of enzyme and some undesirable compound. Mutations occurred by exposing microbial cultures into ionized radiation such as γ-Ray or by using chemical compounds, these treatments lead to killing some microorganism in culture and the residual microorganism recultured in suitable media in order to reach into distinct mutant for enzyme production in contrast to mother culture [6].

MATERIALS AND METHODS

2.1: Maintenance of *Aspergillus niger* isolates:

Twenty one local isolate of *Aspergillus niger* had been collected from different scientific departments of Baghdad university:

- Biotechnology department / Science college- Baghdad University.
- Biotechnology and Genetic engineering Institute - Baghdad University.
- Biotechnology branch /Applied Science department - University of Technology.

These isolates were cultured on potato dextrose agar in slant tubes for 5 days at 28˚C. Afterwards they were preserved in refrigerator at 4˚C until use.

2.2: screening of local isolate of *A. niger* for enzyme production:

2.2.1. Primary screening:

Aspergillus niger isolates had sifted primarily using starch agar media as described by Khan and Yadac [**7**] by dissolving the following materials in 1 liter of distilled water.(Yeast extract 1.5gm ,peptone 0.5gm ,sodium chloride 1.5gm ,starch 10gm and agar 15 gm), the pH of media regulated to 5.6 and sterilized. 75 µl of Spore suspensions (75000 spores) of all isolates were transferred into holes (radius 8 mm) in starch agar media and incubated at 28˚C for 48 hours. After incubation period, the radius of zone hydrolysis had been determined by using weak iodine solution which was prepared as described by Uguru *et al*., [**8**] by addition 0.3% (w/v) of iodine I2 to 3% (w/v) of potassium iodide KI. Later the isolates which had maximum hydrolysis zone had been selected for secondary screening.

2.2.2 Secondary screening (Quantitative screening):

The isolates which had distinct clear zone hydrolysis in primary screening were selected for secondary screening, which involved production of crude enzyme using solid state fermentation as in the following:

2.2.2.1. Wheat bran media:

Ten gram of wheat bran transferred into 250ml cotton plugged conical flask and moistened with 5ml of mineral salt solution, mixed and sterilized at 121˚C for 15 minute in an autoclave. Thereafter, the flasks materials cooled at room temperature.

2.2.2.2 Mineral salt solution:

According to [9], mineral salt solution prepared by dissolving the following materials in 1 Liter of distilled water:

Substance	Quantity (gram)
Potassium dihydro phosphate KH_2PO_4	10
Magnesium sulfate $MgSo_4$	2
Sodium Chloride NaCl	2
Manganese Sulfate $MnSo_4$	0.5

2.2.2.3 Method:

Solid state fermentation system had been used for α-amylase production. The highly productivity isolates which growth in high density on starch agar in primary screening was selected ,These isolates cultured in wheat bran media as prepared in (2.2.2.1) and added 1 ml of spore suspension (consist of 10^6 spores) of each isolate into media. The cultures incubated at 28˚C for 5 days then enzymes extracted by addition 50ml of distilled water, the extract shake and filtrated by No.1 Whitman filter paper .The filtrate considered as crude enzyme.

Determination of enzyme activity of crude enzyme was done by miller method [10] with DNSA reagent and determination of protein concentration by Bradford method [11] using Coomassie Brilliant Blue G-250. Afterword the specific activity of crude enzymes was determined and the highest efficiency isolate selected for Gamma ray mutagenesis. One enzyme activity unit was defined as the amount of enzyme releasing 1 µg of reducing sugar as glucose per gram of dry substrate per minute, under standard assay conditions [12].

2.3 Gamma ray mutagenesis:

The selected isolate of *A. niger* exposed to different doses of gamma ray ranged between (0.2-2) KGray using Co^{60}gamma cell-900 chamber which provide 200 Gray/h. The irradiated isolates had been screened using primary screening. Finally the selected mutant isolate which had more efficiency had been used for amylase production.

2.4 Optimum conditions for α- amylase production:

2.4.1. Type of Substrate:

Four types of culture media were tested for their efficiency in amylase production, these media consist of banana peel, potato peel, wheat bran and corn cob. 10 gm of each residual waste were humidified with 5 ml of mineral salt solution separately. Media was cooled and inoculated with 1 ml of spore suspension (10^6 spores/ml) from selected mutant isolate of *A. niger*. Then, flasks were incubated for 5days at 28°C.

2.4.2. Moistening solution:
Different types of moistening solutions were used to identify the optimum hydration solution, they consisted of:
• *Mineral salt media (MSM):*
It was prepared as described by khan and Yadav [**7**]. MSM containing the following material dissolved in 1 liter of distilled water (NaCl 0.8gm , KCl 0.8 gm , $CaCl_2$ 0.1gm , Na_2HPO_4 2.0 gm , $MgSO_4$ 0.2gm , $FeSO_4$ 0.1gm , Glucose 8.0 gm and NH_4Cl 2.0gm).

• *Mineral salt solution (MSS):*
It was prepared as described by Irfan *et a*l., [**9**] by dissolving the following materials in 1 liter of distilled water (KH_2PO_4 10gm, $MgSO_4$ 2gm, NaCl 2gm and $MnSO_4$ 0.5gm).

• *Distilled water*
• *Tap water*

5 ml of each solution was added to 10 gm of wheat bran media separately. The humidified media were placed in 250ml Erlenmeyer flasks and autoclaved. The autoclaved media was cooled at room temperature and inoculated with 1 ml of spore suspension consist of 10^6 spores/ml of selected isolate and incubated for 5days at 28°C.

2.4.3. Moisture ratio:
10 gm of wheat bran were humidified with different volumes of mineral salt solution separately. Different moisture ratios were tested (0.5:1; 1:1; 1.5:1 and 2:1 w/v) in order to select the optimum moisture ratio for amylase enzyme production. The humidified media was autoclaved, inoculated and incubated for 5days at 28°C.

2.4.4. Optimum pH:
Production media were distributed into flasks after adjusting pH into 3.5; 4.0; 4.5; 5.0 ;5.5; 6.0; 6.5;7.0 and 8.0 separately; inoculated and incubated at 28°C for 5 days. The enzyme activity of amylase was determined after incubation to determine the optimal pH for α-amylase production.

2.4.5 Optimum temperature:
In order to determine the effect of incubation temperature in amylases production, 10 gm of wheat bran moistened with 10 ml of mineral salt solution was sterilized ,inoculated and incubated for 5 days at 25, 28, 30, 35,40,45 and 50 °C respectively to estimate the optimal temperature for amylase production.

2.4.6. Incubation period:
Mineral salt solution had been added to 10 gm of wheat bran .The humidified media was placed in 250 ml Erlenmeyer flasks and autoclaved. Sterilized media were inoculated and incubated at 30°C for 1, 2, 3, 4, 5, 6, 7 and 8 day in order to estimate the optimum incubation time for amylase enzyme production.

2.4.7. Effect of additional Carbon source:
Number of additional carbon sources had been tested for their ability to enhancing enzyme production ,they are (glucose, fructose ,maltose ,sucrose and soluble starch) which were added to humidified media in a ratio 1%w/w and incubated for 6 days at 30°C , and enzyme activity was determined, compared with control.

2.4.8. Effect of additional Nitrogen source:
Different types of nitrogen sources (organic and inorganic nitrogen sources) had been tested for their ability to increasing amylase production, they are yeast extract, peptone, urea, Ammonium sulfate, Ammonium chloride and Ammonium nitrate which were added to culture media in a ratio 1% w/w and incubated for 6 days at pH 5.5 and 30°C. Finally enzyme activity was determined and compared with control.

RESULTS AND DISCUSSION

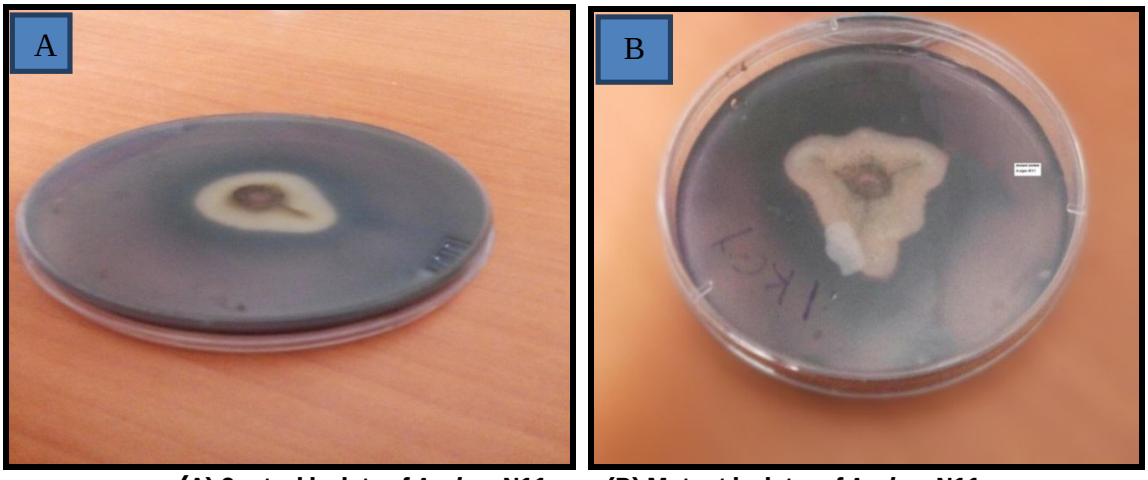
Primary and secondary screening of *A. niger* isolates:
Twenty one local isolate of *Aspergillus niger* had been tested primary for α-amylase production using starch agar media, the efficiency of isolates estimated primarily according to clear zone appearance around growth culture on agar media. The isolates efficiency was compared by determining the ratio between clear zone diameter around fungal cultures and the diameter of growth culture. Five isolates which had the most efficiency in primary screening had been selected for secondary screening to determine the specific activity of extracted crude enzymes of each isolate as in (table - 1) .The isolate *A. niger* N11 which had the higher specific activity of enzyme had been selected for the following mutation step.

Table 1. Screening of selected local isolates of *A. niger* for enzyme production.

Isolates symbols	Primary screening	Secondary screening		
	Ratio of zone Hydrolysis	Activity U/ml	Protein concentration mg/ml	Specific activity U/mg
***A. niger* N3**	**4.0**	**0.124**	**2.1**	**0.06**
***A. niger* N9**	**4.2**	**0.06**	**2.0**	**0.03**
***A. niger* N11**	**4.1**	**0.37**	**1.0**	**0.37**
***A. niger* N12**	**4.2**	**0.06**	**0.15**	**0.04**
***A. niger* N14**	**4.4**	**0.13**	**1.0**	**0.13**

Gamma ray mutation:

Mutation process appeared that the isolate *A. niger* N11 which was treated by 1 kGray of ionized gamma ray had distinct zone hydrolysis when experimented on starch agar as shown in figure (1) and the specific activity of extracted enzyme reached to 0.56 U/mg in contrast to control isolate activity which was 0.37U/mg as in(table-2).



(A) Control isolate of *A. niger* N11. (B) Mutant isolates of *A. niger* N11.

Figure 1. Clearance zone of *A. niger* N11 isolates (a) Control (untreated) isolate (b) mutant isolate treated by 1 KGray of gamma ray.

Table 2. Comparison study between control and mutant isolate.

Isolate	Ratio of zone Hydrolysis	Specific activity U/mg
Control	4.1	0.37
Mutant isolate	6.2	0.56

Fadel and El-Batal, [**13**] found that *A. niger* isolate when treated with 0.8 K Gray of gamma ray gave maximum alpha amylase specific activity reached to (6.1 U/mg) when they had experimented different doses of gamma ray.

Optimum conditions for α-amylase production:

Type of substrate

Four different types of substrates had been tested for their efficiency in amylase enzyme production, they are wheat bran, potato peel, banana peel and corn cob. Results in figure (2) indicated that the highest amylase production observed by wheat bran, amylase specific activity was (0.56U/mg), while the lower enzyme activity was by corn cob (0.22 U/mg).

The differences in enzyme productivity when using different substrates in solid state fermentation refer to many factors consist of the nature of internal structure of substrate, porosity degree and that effect on the microbial cell penetration and its growth. As a result it preferred to select highly porosity substrate or brittle materials as nutritious media because of easy of growth inside it. As well as enzyme productivity is highly affected by the size of media granules therefore the substrate which has small granules size increases the enzyme production by increasing the surface area of exposed material for microorganism [14]. Bhattacharya *et al*. [12] found that rice husk was the best residue for amylase production among five different wastes using *A. niger* isolate in solid state fermentation which gave enzyme specific activity 0.009 U/mg.

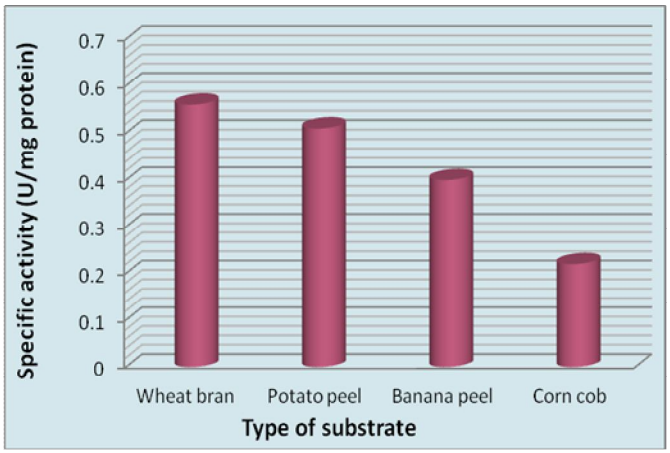


Figure 2. Effect of substrate type on α- amylase enzyme production by mutant isolate of *A .niger* N 11, incubation for 5 days at 28°C.

Moistening solution

In order to specify the best moistening solution for amylase production, four different solutions were examined which were (mineral salt media, mineral salt solution, distilled water and tap water). Mineral salt solution gave the highest specific activity (0.57U/mg) while tap water gave the lower enzyme activity (0.24 U/mg) as in (figure -3). Results showed that the highest specific activity for amylase production obtained from mineral salt solution, this solution provide appropriate quantity of ions which are essential for fungal growth and enzyme production [**6**]. Khan and Yadav[**7**], used mineral salt media as a moistening solution using wheat bran in solid state fermentation for amylases production and the enzyme specific activity reached to 0.43 U/mg .

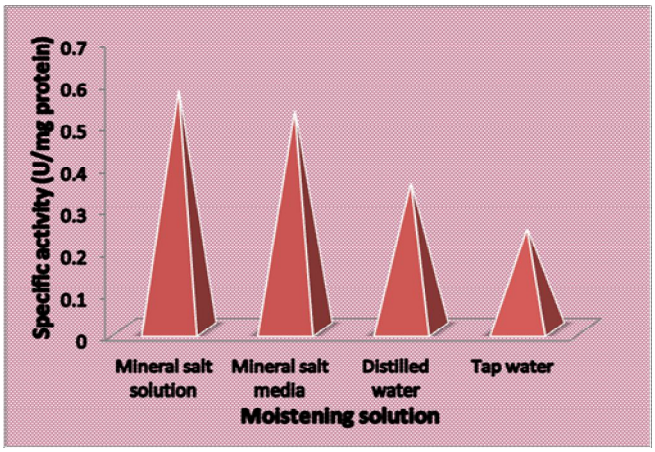


Figure 3. Effect of different moistening solutions on α-amylase production from *A. niger* N11 mutant isolate, using wheat bran, incubation for 5 days at 28°C.

Moistening ratio

Wheat bran was moistened with different volumes of mineral salt solution and were tested to select the optimum moisture ratio for amylase production. The best moisture ratio was 1:1(w/v) which gave specific activity (0.64U/mg) while 0.5:1, 1.5:1 and 2:1 ratios gave 0.57, 0.43 and 0.34 U/mg respectively (figure-4). Vu *et al*. [**15**], found the optimum moisture ratio for raw-starch digesting enzyme using mutant isolates was 50% which gave enzyme activity 62.52U/mg. The degree of hydration of the substrate plays an important role in the growth of the fungi and subsequently the enzyme production. Water causes the swelling of the substrate and increasing microorganism ability for substrates utilization. Increased moisture level is believed to have minimized the substrate porosity as a result it limits the oxygen transfer into the substrate. In the other side a lower moisture ratio causes solubility reduction of the nutrients of the solid substrate, lower degree of swelling and a higher water tension [16].

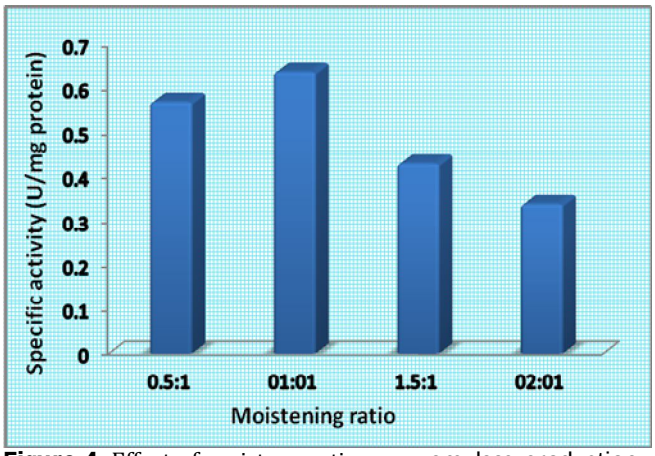


Figure 4. Effect of moisture ratio on α- amylase production from mutant isolate *A. niger* N11, using wheat bran, mineral salt solution and incubation for 5 days at 28°C.

Optimum pH of production media

In order to estimate the effect of pH in amylases production, nutritional media had been prepared in different pH values consist of (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0). Results in figure (5) illustrate the optimal pH was 5.5 which gave the highest enzyme productivity and the specific activity evaluated to (0.78U/mg),while the other pH values 3.5, 4.0, 4.5, 5.0, 6.0, 6.5, 7.0 and 8.0 gave specific activities 0.23; 0.46; 0.52; 0.67; 0.72; 0.595; 0.47 and 0.34U/mg respectively. The reason for the growth rate descending in either side of the optimum value is due to alterations in three-dimensional of protein structure [**16**]. Generally fungi prefer slightly acid conditions and the pH affects in enzyme productivity because of its influence in the solubility of medium substrates and its effect on the ionization process of the substrate in addition to its availability for the mold growth .It is unnecessary the congruity between the optimal pH for microorganism growth and the optimal pH for enzyme activity [**17**]. Vardhini *et al*. [**18**] found the optimal pH for α-amylase enzyme production by *A. niger* was 6.5 which gave enzyme specific activity 40 U/mg.

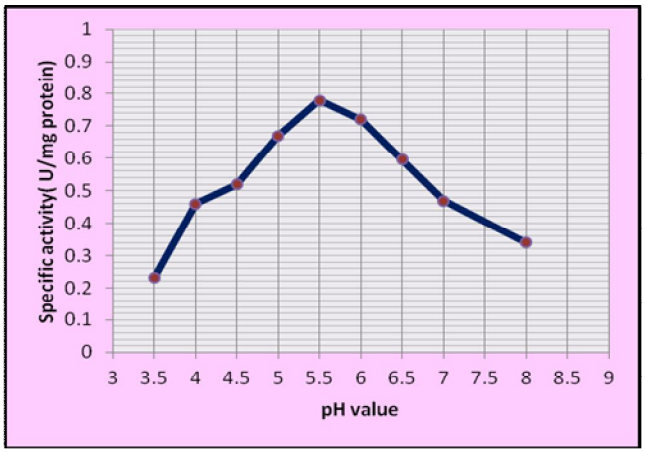


Figure 5. Effect of pH on α-amylase production from mutant isolate *A. niger* N11 in wheat bran, using mineral salt solution with 1:1 moisture ratio, incubation for 5 days at 28°C.

Optimum incubation temperature

In order to investigate the role of temperature on amylase enzyme production, different incubation temperature were used consist of 25, 28, 30, 35, 40, 45and 50°C. Results in Figure (6), show that the optimum incubation temperature was 30°C which gave the enzyme specific activity of 0.92 U/mg. While the other temperatures 25,28,35,40,45 and 50°C gave 0.53 ,0.75 ,0.86 ,0.63 ,0.55 and 0.26 U/mg respectively. Lower and higher temperatures appear falling in the specific activities values, Lower temperature decreases the specific activity because it is unsuitable for mold growth as a result it lowers enzyme production while higher temperature lead to minimize media water content by vaporization as a result it effects cells growth, in addition to higher temperature limit oxygen concentration. Previous studies found it is unnecessary the congruity between the optimal temperature for microorganism growth and the optimal temperature for enzyme activity [**17**]. Bhattacharya *et al*. [**12**] found that the optimal temperature for amylase enzyme production was 30°C which gave the maximum enzyme activity approximated to 0.002U/mg.

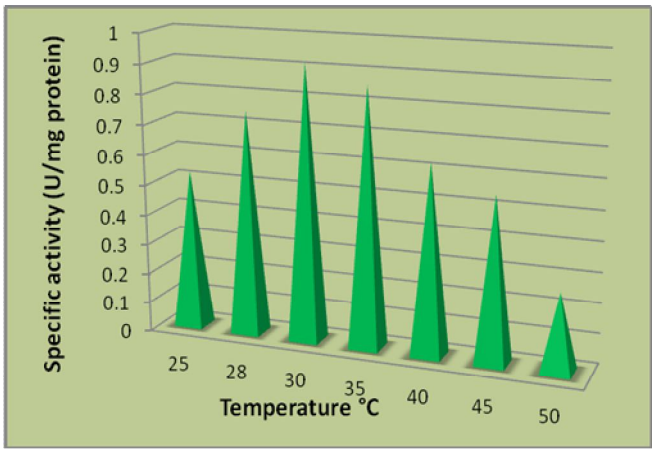


Figure 6. Effect of incubation temperature on amylase production from mutant isolates A. niger N11, using wheat bran, with 1:1 moisture ratio, incubation for 5 days at pH 5.5.

Incubation period

Results in (figure -7) show the effect of incubation period (1–8 days) on α-amylase production from *A. niger* N11 mutant isolate, the highest specific activity was at 6 days of incubation which was 0.93 U/mg. These results corresponds to the studies of Alva *et al.*[19], who found the best incubation time for amylase production by *Aspergillus* species in SSF was 6 days which gave specific activity 7 U/mg .The enzyme production decreases after 6 days of incubation period it may refer to the production of reducing sugar in culture media which lead to repression of amylase production because these sugars are more readily carbon source than starch, in addition to depletion of the nutrients in the medium [12].

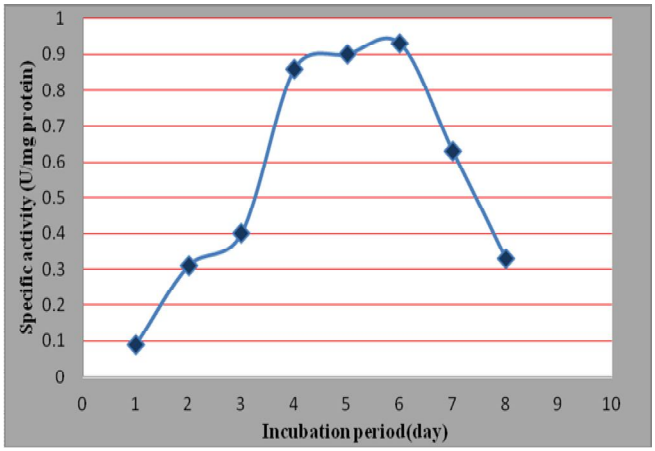


Figure 7. Effect of incubation period on α- amylase production from mutant isolate of *A. niger* N11, using wheat bran, with 1:1 moisture ratio, incubation for (1-8) day at 30 °C and pH 5.5.

Effect of additional carbon source

Different additional carbon sources were tested for their efficiency in amylases production. These sources consist of glucose, fructose, maltose, sucrose and soluble starch (Figure -8). The highest specific activity is shown in soluble starch with specific activity 0.97 U/mg, while glucose, fructose, maltose and sucrose showed specific activities 0.83 U/mg, 0.85U/mg, 0.92 and 0.73 U/mg respectively. This indicates that soluble starch is the most efficient source for amylases production from *A. niger* N11 mutant isolate .Alva *et al.* [19], found the maximum production of amylase was achieved when glucose was the carbon source using *Aspergillus* species in SSF and gave specific activity (12.2 U/mg).

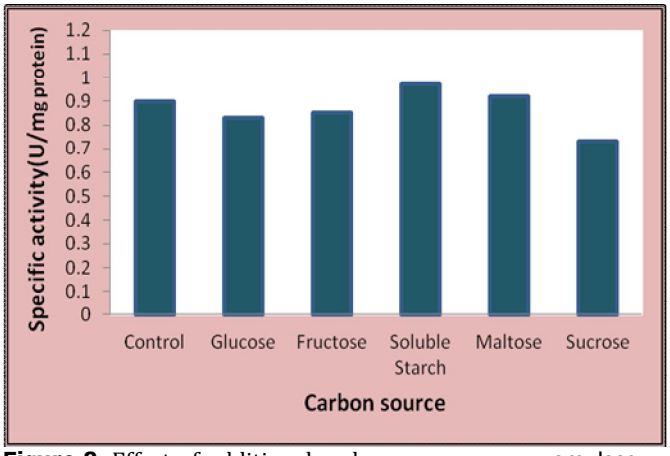


Figure 8. Effect of additional carbon sources on α- amylase production from mutant isolate of *A. niger* N11, using wheat bran with 1:1 moisture ratio, incubation for 6 days at 30 °C and pH 5.5.

Effect of additional nitrogen source

Different nitrogen sources (organic and inorganic nitrogen sources) were selected to study their efficiency to enhance amylases production. These sources consist of yeast extract, peptone, urea, ammonium nitrate, ammonium chloride and ammonium sulfate as in (figure-9). The highest specific activity is shown in ammonium nitrate as inorganic source which gave specific activity1.5U/mg and urea as organic source which gave 1.4U/mg, while yeast extract , peptone, ammonium sulfate and ammonium chloride showed specific activities as follows 1.26, 1.0, 0.82 and 0.83 U/mg respectively. This indicates that ammonium nitrate and urea are the most efficient nitrogen source for amylases production from *A. niger* N11 mutant isolate. Hashemi *et al.* [20], found that ammonium nitrate NH_4NO_3 was the best nitrogen source for α-amylase production in SSF and gave enzyme specific activity 0.14 U/mg.

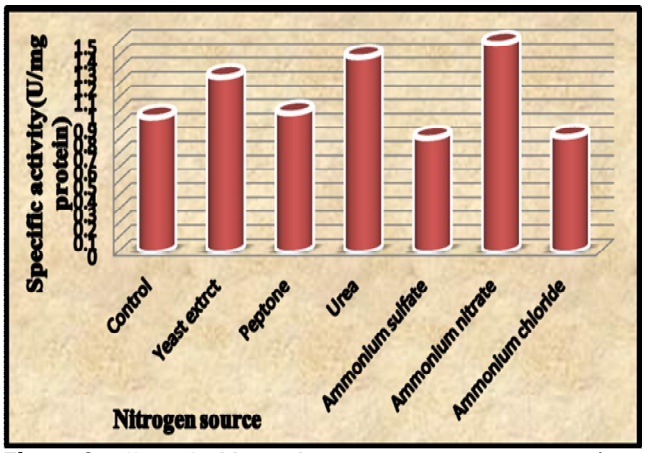


Figure 9. Effect of additional nitrogen sources on α- amylase production from mutant isolate of *A. niger* N11, using wheat bran, 1% carbon source, 1% nitrogen source with 1:1 moisture ratio, incubation for 6 days at 30 °C and pH 5.5.

CONCLUSION

The research indicated that amylolytic molds such as *Aspergillus niger* is very important for optimizing amylase enzyme production using different waste products which are rich in starch and can be help full in reducing the production cost. The obtained results show that wheat bran serve as a good substrate that enabling the growth of *Aspergillus niger* and production a considerable amount of the amylases. So, the composition of the medium is a major factor in regulating the synthesis of α-amylase. Under respective optimum conditions, fermentation period of 6 days , temperature of 30°C, pH(5.5), soluble starch (1%w/w) and ammonium nitrate (1%w/w).

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