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UTILIZATION OF SOME AGRICULTURAL BY-PRODUCTS FOR *Aspergillus niger* M_2 AMYLASE PRODUCTION WITH SOILD-STAT FEMENTATION AND TO BE USED IN BREAD STALING EVALUATION.

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ABSTRACT

Because of the increasing demand on starch-hydrolyzing enzymes, this study was focused on studying factors controlling the production of α-amylase, β-amylase and glucoamylase from *Aspergillus niger* M_2 and also to study the effect of these enzyme on quality of balady bread and its staling properties. Spent brewing grain (SBG), was used as a solid substrate for the production of these enzymes. Different agro–industrial by-products were tested for the production of α, β and glucoamylase. Maximum amylases yields, α, β and glucoamylase synthesis by *A. niger* M_2 strain were 770, 390 and 950 IU/g dry substrate (g/ds), respectively. These values were obtained on the optimized media after 96 hrs and suitable conditions of initial moisture, inoculum size, initial pH and incubation temperature of 65%, 10^6 spores/gds, 5.0 and 30 °C, respectively. Maltose induced the enzymes biosynthesis and glucose syrup supported the enzymes formation. Amylases production were stimulated at 3.0% of glucose syrup. Corn steep liquor (CSL) at 0.21% as N gave highest amount of these enzymes. Regarding the durum wheat starches susceptibility to *A. niger* M_2 glucoamylase enzyme, it was lower than that of soft wheat starches. This may be due to the highest amylose lipid complexes in durum wheat starch. Organoliptic evaluation indicated that, durum bread has higher total scores being 87-97% as compared with soft wheat bread which was 85-86%.Also durum bread has 95-100% of freshness scores, while soft wheat bread has 85-90% of freshness scores, after 1 hr of baking. Staling properties expressed as the result of hydrolysis of crumb bread with *A. niger* M_2 glucoamylase indicated that, after 72 hrs of storage at room temperature, hydrolysis was in the range of (126.6-137.7 mg glucose/g crumb), and (94 -107.6 mg glucose/g crumb) in durum and soft bread crumb, respectively. This means that durum bread has longer shelf life and lower ability to staling mechanism due to its high content of amylopectin and amylose- lipid-complex.

Keywords: Solid substrate fermentation, *Aspergillus niger*, spent brewing grain, α, β and glucoamylase biosynthesis, staling evaluation.

INTRODUCTION

α-Amylase (α-1-4-glucanohydrolase [EC3.2.1.1] endo-amylase and dextrogenic) is of widespread occurrence in nature. The enzyme hydrolyzes α-1,4-glucosidic linkages in amylose, amylopectin, and glycogen in an endo-acting mechanism. Amylases in general have a wide spectrum of industrial applications. Industrial starch conversion involves liquefaction by α-amylase at temperature up to 110 °C followed debranching and saccharification by pulluianase and glucoamylase at 65 °C. The production of sweetness by conversion of starch involves two stages. In the first, or liqe-fying stage, α-

amylase is used to debranch the starch. In the second, or saccharifying stage, one interesting alternative in the use of β-amylase for the production of high fructose syrup (Shady *et al.* 2000)

Starch – hydrolyzing enzymes are produced by different microorganisms. Plant and microbial β-amylase (EC 3.2.1.2, α-1, 4-D-glucanomalto hydrolase, saccharogenic amylase are used in the food processing, brewing and distilling industries, and in the production of maltose–containing syrups. Extracellular microbial β-amyalses, which occur in various *Bacillus* species, are important in the syrup production (Fogarty & Kelly 1983; Priest, 1984; Saha & Zeikus 1987; Castro *et al.*, 1993 and shady *et al.* 2002). Several types of enzymes are involved in the industrial degradation of starch including glucoamylase (1, 4-α-D-glucanglucohydrolase, EC 3.2.1.3), which is an industrial enzyme that hydrolyzes 1,4 linked α-D-glucosyl residues successively from the nonreducing end of oligo– and poly–saccharide chains with the release of D-glucose (Saha and Zeikus 1989). The enzyme is secreted by numerous Fungi, including *Aspergillus* spp., *Penicillium oxalicum*, *Saccharomyces* spp., *Rhizopus oryzae*, and *Neurospora crassa* (Yamasaki *et al.*, 1977a&b; Tsuchiya *et al.*, 1994 and Hintz *et al.*, 1995). Production of glucoamylase has been especially well characterized in *Aspergillus* species and glucoamylase is used commercially for the production of food and beverage syrups (Saha and Zeikus,1989).

Solid substrate fermentation offers numerous advantages over submerged fermentation systems, including high volumetric productivity, high content of the products, less effluent generation and simple fermentation equipment. Fungal amylolytic enzymes are frequently produced by solid-substrate fermentation. Crude (*in situ*) hydrolytic enzymes prepared by solid-substrate fermentation (the whole solid substrate fermentation culture) can be used in biotechnologic processes, such as ensiling, feed supplementation, and bioprocessing of crops and crop residues. Among several such agro-industrial residues, which could be used as substrates, spent brewing grain (SBG) constitutes an interesting opportunity (Konik *et al.*, 1994; Sasaki *et al.*, 2000 and Bogar *et al.*, 2002).

Blazenka (1989) concluded that environmental condition and real differences in starch properties influenced the α-amylase susceptibility of starches and consequently the value of amylographic maximum viscosity. An increased amylose content in wheat possessed the best susceptibility to enzymatic attack (Fuwa *et al.*, 1978). Eerlingen *et al.*, (1993 and 1994) reported that increased retrogradation extents caused reduced enzyme susceptibilities to pancreatic α-amylose and amyloglucosidase (glucoamylase) at 37 °C. During the retrogradation of starch, a fraction may become resistant to amylolytic enzymes. It is believed that this resistant fraction consists mainly of retrograded amylase (Sievert 1989; Pomeranz, 1990 and Sievert *et al.*, 1991). The role of the starch component in bread quality including bread staling is being studied extensively. However, these studies provide insufficient information about the role of starch in bread

staling, especially the influence of amylose-to-amylopectin ratio on the starch retrogradation and bread staling mechanism.

The objectives of the present study were aimed to use solid substrate fermentation for α, β and glucoamylase on SBG. Also the enzymes were used for hydrolyzing some starchy substances and as well as to determine the influence of amylose to amylopectin ratio in soft durum wheat starches, on bread quality and bread staling during storage period depending on amylases enzyme hydrolysis.

MATERIALS AND METHODS

Microorganism and Culture Conditions:

Aspergillus niger M_2, was isolated from a screening programme for amylolytic fungi (Mansour and Saber, 2001). It was maintained on potato dextrose agar (PDA) medium at 4 °C and subcultured monthly. Inocula was prepared as follows: slant of 5 days culture grown on (PDA) medium with a heavy spore formation, were suspended in distilled water containing 0.1% Tween 80. Appropriate dilutions of this suspension were made in order to achieve a final concentration of $2x10^7$ spores ml^{-1}, approximately.

Substrate:

Raw materials used in this study were obtained locally. Spent Brewing Grain (SBG) was obtained from Al-Ahram for Manufacturing and Filling Company at Giza. The freshly collected (wet) sample was dried overnight at 80 °C. Corn steep liquor (CSL) and glucose syrup by–products were obtained from Egyptian Starch and Glucose Company at Torah. Sugar cane molasses and vinase by-products of sugar industry were obtained from Sugar Cane Integrated Industries Company at Giza. Four durum wheat kernels varieties (Beny Sweef-1, (B_1), Beny Sweef-3 (B_3), Sohag-2 (S_2), Sohag-3 (S_3) and two soft wheat kernels varieties Giza-168 (G-168) and Sakha-93 (SK-93) were supplied by wheat Research Section, Agricultural Research Center, Giza, Egypt.

Preparation of Samples:

Grain samples were cleaned and tempered to 16.5% moisture content for durum wheats and 14.5% moisture for soft wheat. A 5.0 K gm of each variety was milled a Quadramate Senior Laboratory mill. By using a remilled bran (refined in laboratory model 3100 which is a hammer type mill) the extraction rate of many flour sample was adjusted to the required extraction rate (82%). All flour samples were stored separately in air tight containers at 2 - 3 °C.

Solid-state fermentation:

Nonoptimized solid substrate fermentation was carried out in 250 ml Cotton-pluged Erlenmeyer flasks. Five grams of dry substrate was supplemented with salt solution to adjust the moisture level to between 45 and 80%. The composition of the salt solution was as follows: 5.0 g/l of NH_4NO_3, 5.0 g/l of KH_2PO_4, 1.0 g/l of NaCl, 1.0 g/l of $MgSO_4.7H_2HO$ and 2.0 mg/L of $CaCl_2.H_2O$, 1.6 mg/L of $MnSO_4$, 3.4 mg/L of $ZnSO_4.7H_2O$. and 5.0 mg/L of $FeSO_4.7H_2O$ (Selvakumar *et al.*,1996). in the time-course

experiment, the following composition of optimized medium was used: 5.0 g of SBG supplemented to 65% moisture content with the following solution: 30.0 g/L of glucose syrup, 6.6 ml/L (0.210% nitrogen content) of corn steep liquor. The prepared substrate was sterilized at 121 °C for 30 min. After cooling the medium was inoculated with a spore suspension of the tested fungus to a final concentration of 2 x 10^7 spores/g of dry matter (D.M). The inoculated flasks were incubated at 25 °C for 5d. All solid substrate fermentation experiments were carried out in duplicate in single experiments, and the results shown are average values (Selvakumar *et al.*,1998).

Analytical methods

Enzyme extraction:

Enzyme activity was determined using the culture extract of solid substrate fermentation samples: 5.0 g dry wt of fermented substrate was extracted with 100 ml of water containing 0.1% Tween-80, by shaking for 1 hr. at room temperature (25 °C). At the end of extraction, the suspension was centrifuged (3000g, lomin). Supernatant were stored at 4 °C until the assays were performed (Selvakumar *et al.*,1996).

Enzymes assay:

α-Amylase activity was determined as described by Okolo *et al.*, (1995). The reaction mixture consisted of 1.25 ml of 1% soluble starch (E. Merck) solution, 0.25 ml of 0.1 M sodium acetate buffer (pH 5.0), 0.25 ml distilled water, and 0.25 ml of properly diluted crude enzyme extract. After 10 min of incubation at 50 °C, the liberated reducing sugars (glucose equivalent) were estimated by the method of Somogyi (1952). One unit of α-amylase is defined as the amount of enzyme releasing 1 μ mol of glucose equivalent/ min under the assay condition.

β- Amylase:

β-Amylase was assayed by the method of Bernfeld (1955).

Reagents: Dissolve 1.0 g of potato amylopectine, or 1.0g of Sochoch's B fraction from corn, or, if neither is available, 1.0g of soluble starch (E. Merck) in 100ml of 0.016 M acetate buffer, pH 4.8.

Procedure: 1.0 ml of properly diluted enzyme is incubated for 3.0 mins at 20 °C with 1.0 ml of the soluble starch solution. The enzyme reaction is interrupted and the reducing sugars determined according to the method of Somogyi (1952). A calibration curve established with maltose (is used to convert the colorimeter readings into μg of maltose). One unit of β-amylase activity is defined as the amount of enzyme required to release 1 μmol of maltose equivalent /min under assay condition.

Glucoamylase:

Glucoamylase (amyloglucosidase) was determined as described by Attia and Ali (1974).

Starch solution: Take 1.0g of Zulkowski starch (dry basis) for a slurry in a small amount of deionized water, add while stirring about 50 ml of boiling water and boil for additional 3 mins, cool to room temperature, add 5.0 ml of 1 M acetate buffer (pH 4.3), and make up too 100 ml with deionized water.

Procedure: To 1.0 ml of 1% starch solution, 1.0 ml of the enzyme solution was added; then the mixture was incubated at 60 °C for 5.0 mins. After the incubation period, 1.0 ml of Somogyi's reagent was added. The mixture was heated in a water bath for 10 min., then cooled, and 2.0 ml of Nelson's reagent were added and diluted to 25 ml of distilled water Optical density at 500nm was measured. One unit of glucoamylase (IU) is expressed as μmoles of glucose released/min by the total enzyme extracted from 1.0 g (dw) of substrate under assay conditions.

Enzymatic hydrolysis:

Different wheat flour and starchy substrates namely durum and soft wheat flour, durum and soft starches as well as crumb meal of bread stored at different periods (0, 24, 48 and 72 hrs) at room temperature (23-25 °C) in polyethylene tight bags were hydrolyzed by *Aspergillus niger* M_2 glucoamylase. The crude enzyme containing 5.0 units from *A. niger* M_2 glucoamylase was used. The hydrolysis was conducted in 50 ml capacity conical flasks containing 5% of substrate in acetate buffer (pH 4.3). the flasks were incubated in shaking waterbath at 60 °C for 8 hrs. Reducing sugars (as glucose) in hydrolyzed products were estimated according to Somogyi procedure (1952). The degree of hydrolysis (D.H) was expressed as the percentage of the reducing sugars against the weight of sample x 100 (Shady and Hassan, 1998).

Baking tests and sensory evaluation:

Balady bread was prepared according to Attia (1986) as follows: Bread loves were allowed to cool on rocks for one hr. before sensory evaluation. Sensory evaluation of bread was evaluated by ten staff members of Food Technology Inst. after one hr of baking for freshness (20), layer separation (10), crust color (10), crumb color (10), crumb distribution (20), odor (10) and taste (10) with total scores (100).

Staling test:

After storage time 0, 24, 48 and 72 hrs in tight polyethylene bags at room temperature (23-25 °C), the loves crumb of each bread sample was dried in an electric-oven at 40 °C overnight, then milled and sieved by sieve (60 mesh). The bread crumb meal sample prepared for staling tests depending on incubation with *Aspergillus niger* M_2 glucoamylase enzyme for 8 hrs in acetate buffer pH 4.3 at 60 °C in shaking waterbath, then the reducing sugars (as glucose) in hydrolyzed products were determined according to Somogyi (1952) procedure from every sample after different periods.

Isolation of starch:

Prime starch was isolated according to procedure described by Wolf (1964) and protein, ash, oil and prime starches were determined according to A.O.A.C. (1990).

Separation of amylose and amylopectin:

Separation of amylose and amylopectin from wheat starch (G-168) was done according to the method described by Pigman and Wolfform (1945). Also, the calibration curve of (Am) and (Ap) was prepared to determine (Am) and (Ap) in all wheat starch samples was investigated.

RESULTS AND DISCUSSION

I: Production of amylases:

I.1- Effect of initial moisture on amylolytic enzymes production:

The moisture content of the medium in solid substrate fermentation is very important for the growth of microorganisms and production of enzymes and enzyme activity. (Bogar *et al.,* 2002). Solid-state fermentations are distinguished from submerged cultures by the fact that microbial growth and product formation occur at or near surfaces of solid materials with low moisture content (Mudgett, 1986).

The water concentration in the solid substrate is critical factor in SSF (Ramadas *et al.*, 1996). To examine the effect of moisture content on α, β and glucoamylase production, moisture levels of 45, 50, 55, 60, 65, 70, 75 and 80% were prepared on SBG being set before autoclaving. *Aspergillus niger* M_2 was inoculated in the solid – state medium with an initial moisture described mentioned above and incubated at 25 °C for 120 hours. Data presented in Fig (I) show, the maximum enzymes production α, β and glucoamylase (140, 60 and 190 IU/gds) were obtained at the initial moisture content of 65% at 120 hours, respectively. The lowest α, β and glucoamylase yields (20, 5 and 40 IU /gds) were present at 45% as initial moisture content of the solid substrate. Panedy *et al.,* 1995 and Bogar *et al.,* 2002 found the maximum yield of α-amlylase production was obtained at 67% moisture content for all three SBGs.

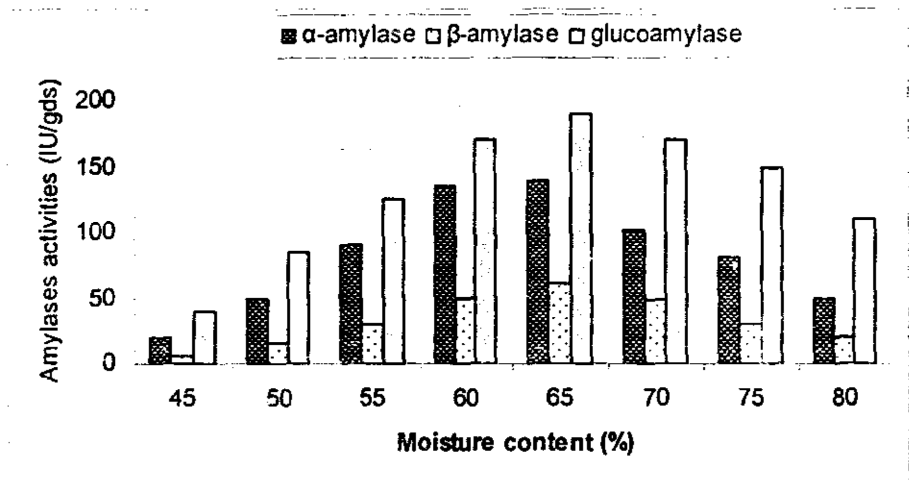


Fig. (1): Effect of moisture content on amylases production by *A. niger* M_2

I. 2- Effect of inoculum size:

It is well known that incoulum size has a marked effect on enzyme yields in fungal culture either in submerged or solid-state fermentation (Hours *et al.,* 1988). After 120 hrs, fermented matter was analyzed and the results were shown in Fig. (2) which revealed that a marked effect of inoculum size

on α, β and glucoamlase production. An optimum inoculum size 10^6 spores/g SBG was observed. Highest α, β and glucoamylase were 190, 80 and 266 IU/gds, respectively. There was no increase in all enzymes production when the inoculum size was further increased. At the amount of inoculum 10^8, enzyme production decreased which was related to the sugars metabolized for energy production.

This study indicated that an optimum level of inoculum was necessary for the best yield of enzyme. The importance of inoculum size in SSF has also been emphased by Pandey (1990).

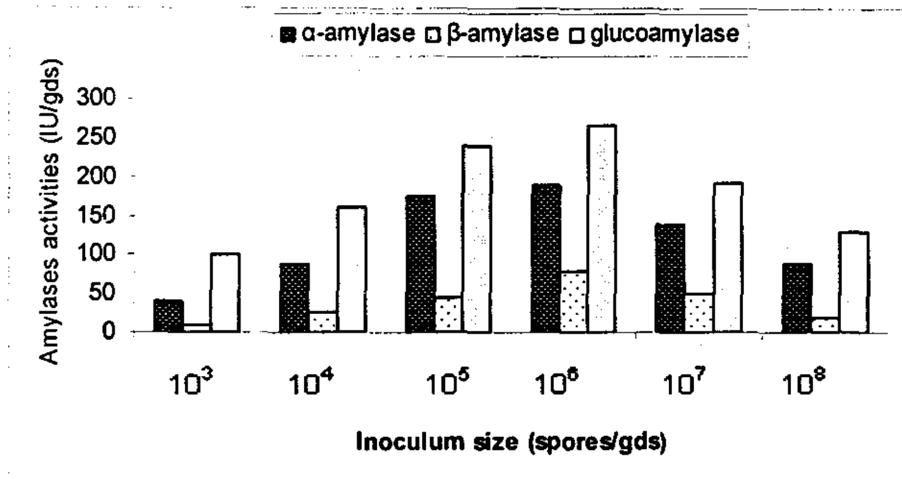


Fig. (2): Effect of inoculum sizet on amylases production by *A. niger* M_2.

I. 3- Effect of initial pH:

Biosynthesis of enzymes depends on the medium pH. Results illustrated in Fig. (3) show that the great variation on amylolytic enzymes biosynthesis with the different levels of pH of the cultivation medium. Maximum extracellular α, β amylase and glucoamylase took place at pH 5.0. The results also revealed that the optimum pH of this enzymes production were in acidic region. The activities of α, β and glucoamylase were decreased sharply, when the pH was found at natural side. This means that these enzymes bio-synthesis tend to be better in acidic region. Similar results were reported by Pandey (1990), Selvakumar *et al.,* (1998). Mansour and Saber (2001) and Shady *et al.,* (2002) found that maximum enzyme activity present at pH 5.0 for *Aspergillus awamori*, *A. niger* and *A. terreus*, respectively.

I. 4. Effect of incubation temperature:

To evaluate the effect of incubation temperature on α, β and glucoamylase production, solid substrate fermentation was carried out at five different fermentation temperatures: 20, 25, 30, 35, 40 and 45 °C. Results illustrated by Fig (4) show that the highest amylases production (α, β and glucoamylase) were observed at 30 °C. Above or below this temperatures,

the biosynthesis of these enzymes were decreased sharply. These results are similar to those obtained by Pandey (1990), Selvakumar *et al.*, (1998) and Ramadas *et al.*, (1996) while Bogar *et al.*, (2002) found that the highest yield of α-amylase production at 25 °C by *Aspergillus oryzae* after 3 days.

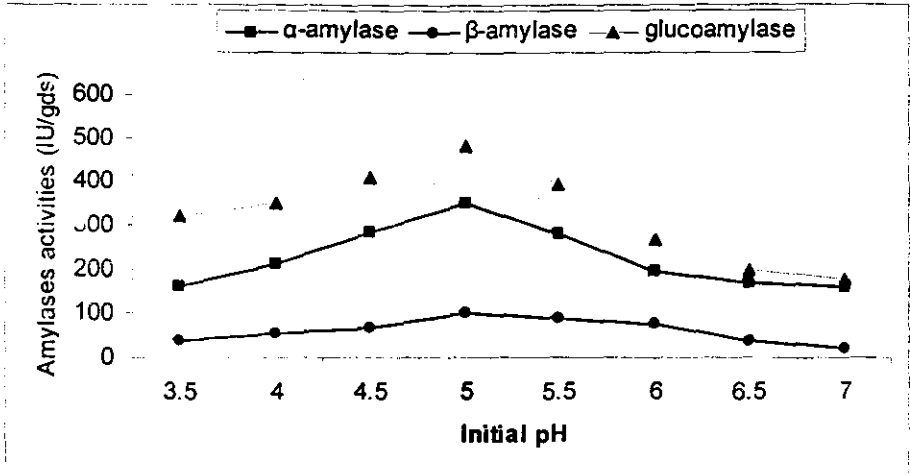


Fig. (3): Effect of initial pH on amylases production by *A. niger* M_2.

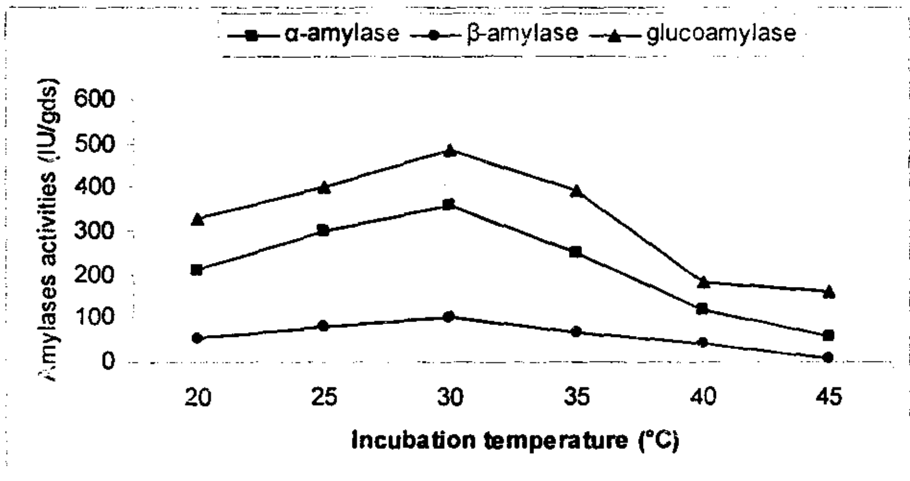


Fig. (4): Effect of incubation temperature on amylases production by *A. niger* M_2.

I. 5- Effect of carbon sources on amylases production:

Various carbon sources were added separately to the solid substrate medium at 1.0% (w/w, dry wt basis) concentration to assess their impact on enzymes production. Data presented in Fig. (5) showed the effect of different carbon and energy sources on enzymes production. Maltose, soluble starch, fructose and yellow dextrin were found as the best inducers for amylases

biosynthesis. Maximum enzymes productivity were found with maltose which induced the enzyme formation being 388, 114 and 545 (IU/gds) for α, β and glucoamylase, respectively. While glucose has no impact on enzymes yield. Lactose and galactose harmfully affected on the mould's enzyme productivity. These results clearly show that these amylases are constitutive in their nature and induced greatly with its substrates or carbohydrates contained materials. Shady & Hassan, (1998); Shady *et al.*, (2000), Mansour and Saber (2001), Shady *et al.*, (2002) and Bogar *et al.*, (2002) reported similar results. Selim *et al.*, (1998) found that 1% maltose induced the biosynthesis of α-amylase.

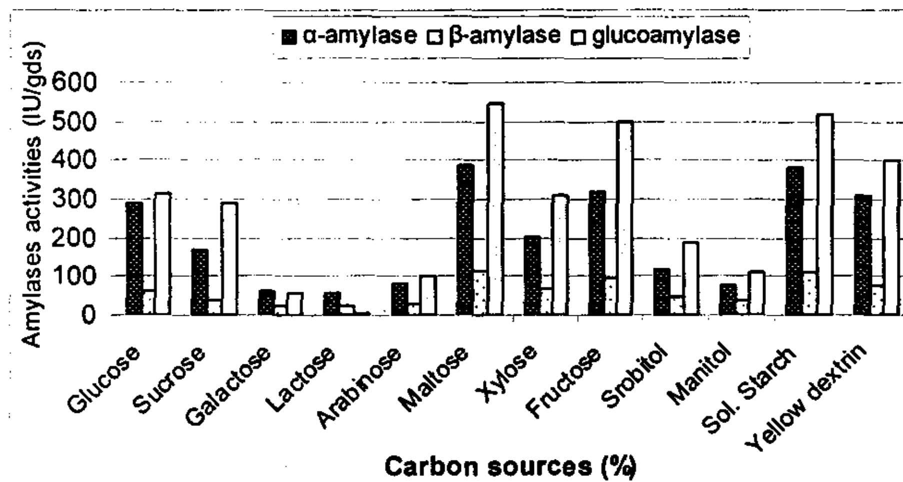


Fig. (5): Effect of different carbon sources on amylases production by *A. niger* M2.

I. 6- Effect of some agricultural by-products on amylases production:

Data recorded in Fig. (6) show that, agricultural by-products presented in Fig (6) induced α, β and glucoamylase production. The results also show that the three enzymes were highly produced with the use of all agricultural by-products, this means that all these materials were induced the biosynthesis of all enzymes formation. Glucose syrup was found as highly induced all enzymes formation, which enzymes productivity reached 401, 120 and 615 IU/gds for α, β and glucomylase respectively. These results may be due to their contents of these substances of adequate amounts of essential nutrients as well as its content of minerals which induced the biosynthesis of *A. niger* M_2 amylolytic enzymes. Thus, these enzymes were constitutive ones and induced with its substrates. These results are in agreement with those obtained by Fadel (2000), Mansour and Saber (2001) ans Shady *et al.*, (2002).

I. 7- Effect of different concentration of glucose syrup:

From the results recorded in Fig. (7), it could be observed that all enzymes (α, β and glucoamylase) production by tested strain were highly affected with differnt glucose syrup concentration. Maximum enzymes

productivity were found at 3.0% concentration, which, the enzyme activities were increased steadily with the increasing of glucose syrup concentration up to 3.0%, then start to decrease. On the other hand, the higher concentrations resulted sharp reduction in all enzymes biosynthesis. Malhotra *et al.*, (2000), and Bogar *et al.*, (2002) found that 2.0 and 5.0% of soluble starch enhanced the enzymes synthesis, respectively. Similar results was obtained by Shady *et al.*, (2002).

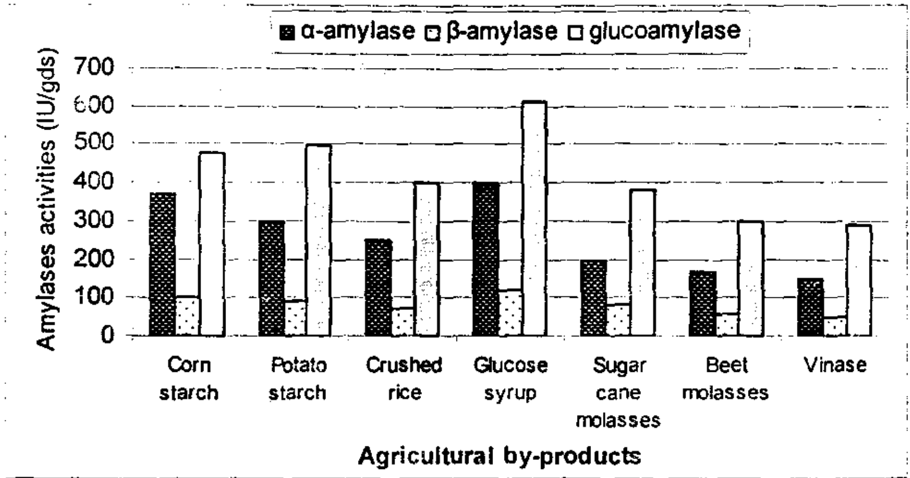


Fig. (6): Effect of some agricultural by-products on amylases production by *A. niger* M_2.

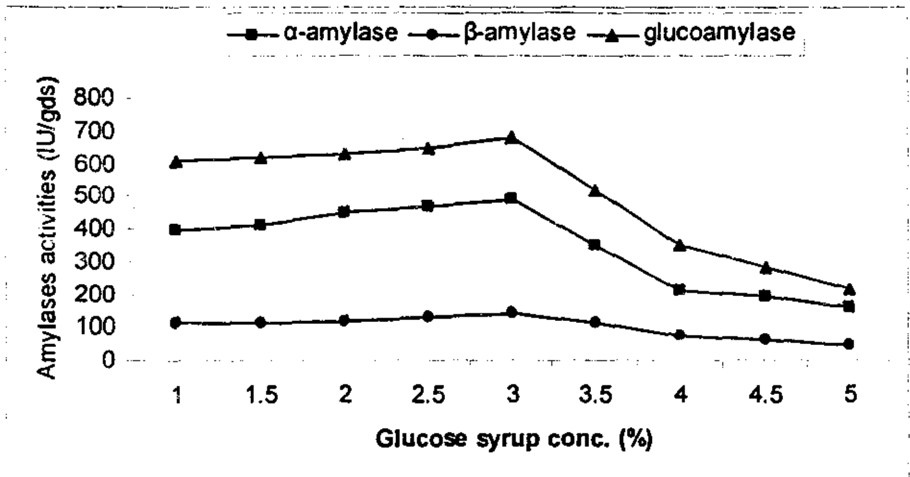


Fig. (7): Effect of glucose syrup concentration on amylases production by *A. niger* M_2.

I. 8- Effect of different nitrogen sources on amylases production:

Various inorganic and organic nitrogen sources were added separately to the solid substrate medium at the concentration of 0.175% as nitrogen to study the effects of these materials on enzymes production (Selvakumar et al., 1998). Data illustrated in Fig. (8) show that the effect of different nitrogen sources in amylases production by *A. niger* M_2. Among different nitrogen sources tested, corn steep liquor (CSL) supported α, β and glucoamylase production by the tested strain, followed by peptone and casein hydrolysate. The maximum enzyme yields of α, β and glucoamylase were 530, 195 and 730 IU/gds, respectively in the medium containing CSL at 120 hrs, in comparison to the control (481, 142 and 667 IU/gds), respectively. Another nitrogen sources reduced or repressed the enzyme formation. These results may be due to that nitrogen sources which contained vitamins, minerals and other growth factors, *e.g.*, corn steep liquor, stimulated the enzyme synthesis. These results are similar to those obtained by Gogoi *et al.*, (1987), Selim *et al.*, (1998), Shady *et al.*, (2000), Mansour & Saber (2001), Bogar *et al.*, (2002) and Shady *et al.*, (2002).

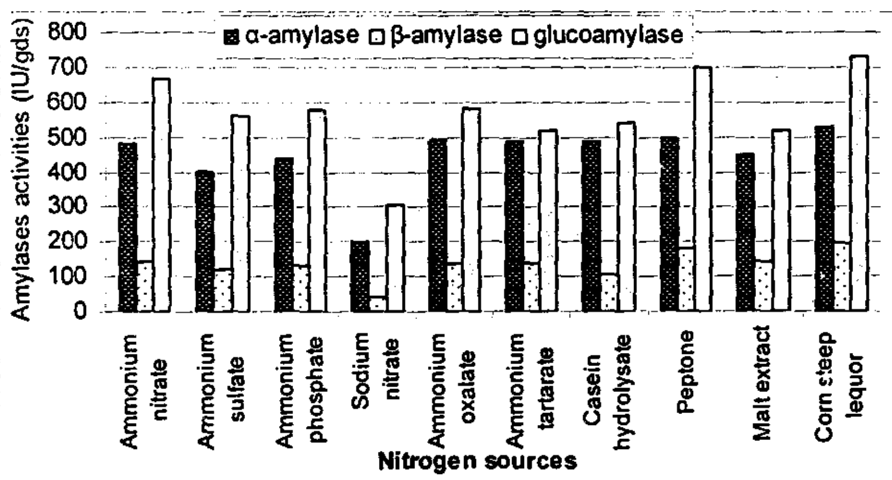


Fig. (8): Effect of different nitrogen sources on amylases production by *A. niger* M_2.

I. 9- Effect of concentration of corn steep liquor:

The medium was supplemented with corn steep liquor (CSL) to various concentration ranged from 0.105 to 0.315 as nitrogen content. Data illustrated in Fig. (9) shows that increasing of CSL up to 0.210% as nitrogen content increased the secretion of all amylases tested with much more amount. In other words, CSL up to 0.210% nitrogen stimulated and induced greatly the biosynthesis of these enzymes. These results may be due to its contains of growth factors such as minerals and vitamins and other ingredients at such concentration induced the biosynthesis of these enzymes. Maximum enzymes productivity were found at 0.210% nitrogen concentration being 589, 196 and 798 IU/gds for α, β and glucoamylase, respectively. This

means that the present of CSL in the production medium was very necessary for amylases production. Similar results were obtained by Mansour, (2001), Mansour and Saber (2001) and Bogar *et al.*, (2002).

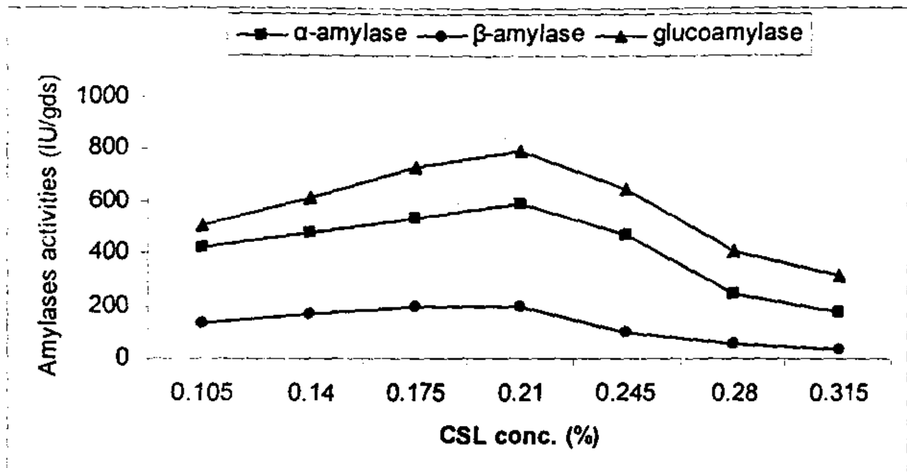


Fig. (9): Effect of different concentrations of CSL on amylases production by *A. niger* M_2.

I. 10- Effect of ingredients elimination of SBG medium on amylases production:

It was found interest to study the effect of the presence or absence of each ingredients of SBG medium on *Aspergillus niger* M_2 amylases production. Eleven trials were compared with complete SBG medium (control) as shown in data recorded in Table (1). It could be noticed from the result that amylases biosynthesis were affected greatly with the presence of glucose syrup and CSL. Data recorded in Table (1) clearly show that elimination from the SBG medium either KH_2PO_4, NaCl, $MgSO_4.7H_2O$ and $CaCl_2.6H_2O$ in decreasing the all enzyme activities. The omission of $MnSO_4$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ from the tested medium gave the same effect on the production of *A. niger* M_2 amylases. The elimination of G. syrup and CSL from the SBG medium gave harmful effect on amylase production which may be due to the presence of growth factors, vitamins and minerals in G-syrup and CSL. The medium which contains SBG in addition to glucose syrup and corn steep liquor gave the highest amylase activities being 600, 194 and 800 IU/gds for α, β and glucoamylase, respectively. From the above results it could be concluded that the highest *Aspergillus niger* M_2 amylases production were obtained on SBG medium supplemented with G syrup and CSL. Similar results were obtained by Mansour (2001), Mansour and Saber (2001), and Bogar *et al.*, (2002).

Table (1):Effect of ingredients elimination of medium on amylases production by *A. niger* M_2.

Trials	Ingredients									Amylase enzyme activities (IU/gds)**		
No.	G. syrup	CSL	KH_2PO_4	NaCl	$MgSO_4 \cdot 7H_2O$	$CaCl_2 \cdot 6H_2O$	$ZnSO_4 \cdot 7H_2O$	$FeSO_4 \cdot 7H_2O$	$MnSO_4$	α	β	Gluco-amylase
1C*	+	+	+	+	+	+	+	+	+	590	195	798
2	-	+	+	+	+	+	+	+	+	430	97	601
3	+	-	+	+	+	+	+	+	+	450	100	640
4	+	+	-	+	+	+	+	+	+	595	196	800
5	+	+	+	-	+	+	+	+	+	594	190	798
6	+	+	+	+	-	+	+	+	+	595	194	790
7	+	+	+	+	+	-	+	+	+	590	190	786
8	+	+	+	+	+	+	-	+	+	594	194	790
9	+	+	+	+	+	+	+	-	+	595	193	798
10	+	+	+	+	+	+	+	+	-	593	192	785
11	+	+	-	-	-	-	-	-	-	600	194	800

(-) The source absent medium .
(*) International unit/g dry substrate
(**) Control (complete tested medium)

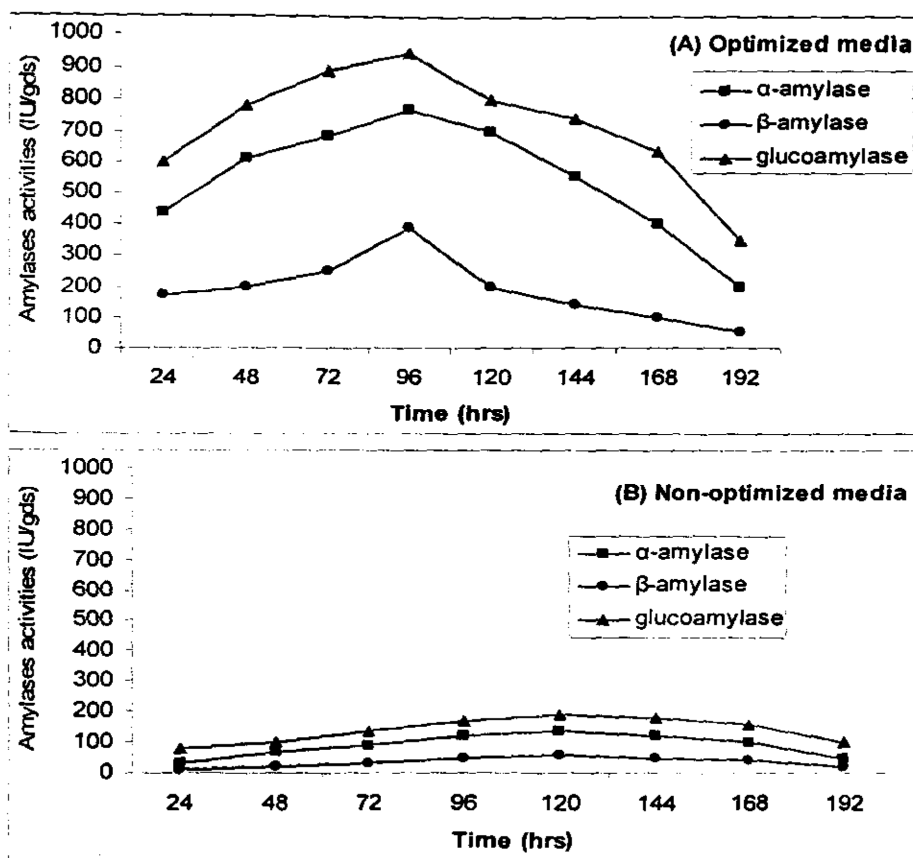
I. 11- Effect of incubation period on amylases production:

Fig. (10 a & b) shows the level of α, β and glucoamylase activities released in non-optimized and optimized medium of *Aspergillus niger* M_2. The highest levels of these enzymes were found after 96 hours. Then, the enzymes formation decreased sharply. The optimum of α, β and glucoamylase production on optimize medium were 770, 390 and 950 IU/gds, respectively. Also, the results indicate that various amyloytic enzymes are present in the culture extract of *A. niger* M_2 which those enzymes were constitutive in their nature. It is also important to maintain that after 4 days, sporulation began to noticeable, which indicates change in the metabolic pattern of the fungus (Smith *et al.*, 1977 and Hours *et al.*, 1988). Similar results were obtained by Pandey (1990) Ramadas *et al.*, 1996, Selvakumar *et al.*, 1996, Selvakumar *et al.*, 1998 while and Bogar *et al.*, (2002) produced α-amylase after 3 days by *Aspergillus oryzae.*

II. Analysis technological and Staling evaluation:

II. 1- Chemical composition of wheat flour and starches:

Table (2) indicate that chemical composition of wheat starches have been determined to estimate the purity of starch separated from different varieties of wheat flours.



(A) Optimized conditions: 30 °C, 65% moisture content and initial pH=5.
(B) Non-optimized conditions: 25 °C, 65% moisture content and initial pH=6.

Fig. (10): Effect of time courses on amylases production in non-optimized and optimized solid substrate fermentation media by *A. niger* M_2.

Table (2): Chemical composition of wheat flour and starches (dry weight basis).

Sample	Starch			Flour		
	Protein (%)	Ash (%)	Oil (%)	Protein (%)	Ash (%)	Oil (%)
Durum wheat:						
Beny Sweif-I. (B_1)	0.25	0.28	0.14	13.4	0.76	1.3
Beny Sweif-3. (B_3)	0.28	0.31	0.16	15.1	0.90	1.5
Sohag-2. (S_2)	0.27	0.26	0.13	13.8	0.80	1.4
Sohag-3. (S_3)	0.30	0.32	0.18	15.3	0.86	1.4
Soft wheat:						
Sakha-93 (SK-93)	0.21	0.22	0.06	10.2	0.70	1.1
Giza-168 (G-168)	0.24	0.25	0.05	11.6	0.75	1.2

It was found that protein, ash and oil were in range of (0.25-0.30%), (0.26-0.32%) and (0.13-0.18%), respectively in durum wheat starches. On the other hand soft wheat starches contained (0.21-0.24%), (0.22-0.25) and (0.05-0.06%) for protein, ash and oil, respectively. This is in accordance with the results of Vonsteelandt & Delcour (1999).

Regarding to wheat flour it was found that durum wheat flour contained protein, ash and oil in range of (13.4-15.3%), (0.76-0.90%) and (1.3-1.5%) respectively while soft wheat flour contained protein, ash and oil in range of (10.2-11.6%), (0.70-0.75%) and (1.1-1.2%), respectively. These results are in agreement with those obtained by Boyacloglu and D'Applonia (1994).

From Table (3) it was found that the starches obtained from durum wheat flours have high ratio of amylopectin (AP) than in soft wheat starches, this starch contained 75-81% AP compared with soft wheat starches which contained 72-73% Ap. (Table 3).

Concerning amylose (Am) it was found that soft wheat starches contained from 27-28% Am, while durum wheat starches contained 19-25% (Am). (Table 3). These results agreed with Boyacloglu and D'Appolonia (1994).

Table (3): The optical densities corresponding to different concentrations of mixtures of amylose and amylopectin fractionated from some wheat starches.

Samples	Composition of mixture		Optical density
	Amylose (%)	Amylopectin (%)	
	0	100	0.038
	10	90	0.053
	20	80	0.068
	30	70	0.086
	40	60	0.100
	50	50	0.115
	60	40	0.131
	70	30	0.146
	80	20	0.162
	90	10	0.178
	100	0	0.193
Durum starch:			
B_1	25.0	75	0.077
B_3	21.0	79	0.070
S_2	23.0	77	0.074
S_3	19.0	81	0.066
Soft starch:			
Sk-93	28.0	72	0.081
G-168	27.0	73	0.079

II. 2- Hydrolysis of wheat flour and starches by *A. niger* M_2 glucoamylase:

From results obtained from Table (4) it is clear that there are different susceptibilities of starch substrate complex within different varieties of durum and soft wheats. Concerning starch it was found that, the susceptibility of durum wheat starches were lower than that of soft wheat starches (Table 4). This may be due to the lower amylose (Am) in durum wheat starches compared with soft wheat starches Table (3) and to high lipid complexes with Am and Ap of durum wheat starch Table (2). Fuwa *et al.,* (1978) reported that. increased amylose content possessed the best susceptibility to enzymatic attack.

Concerning wheat flours of durum and soft wheat it was found the susceptibility of durum wheat flours were lower than that of soft wheat flours, see (Table 4). This may be due to the higher contents of protein, ash and oil in durum wheat flour compared with these contents in soft wheat flour Table (2). These results agreed with that of Gaines-Cs *et al.*, (2000).

Table (4): Hydrolysis of different wheat flour and starches by *A. niger* M_2 glucoamlase after (8 hrs) incubated at 60 °C.

Samples	Wheat			
	Flour		Starch	
	MgRS/g* ample	D.H** (%)	MgRS/g* ample	D.H** (%,
Durum starch:				
B1	360.0	36.0	451.4	45.4
B3	275.6	27.6	454.1	45.4
S2	315.0	31.5	509.1	50.9
S3	279.4	27.9	448.9	44.9
Soft starch:				
Sk-93	388.0	38.8	549.8	55.0
G-168	380.0	38.0	528.0	52.8

* mg R.S/mg reducing sugars/g sample.
** D.H / Degree of hydrolysis.

II. 3- Sensory evaluation of bread:

In Table (5) sensory evaluation of bread produced from wheat flours of different wheat varieties cleared that, durum wheat bread have higher total scores (87-97%) compared with soft wheat breads (85-86%). This may be due to the higher amylopectin (AP) relation to amylose (Am) content in durum wheat starch than in soft wheat starch (Table, 3). Dennett and Sterling (1979) reported that significant negative correlation existed between amylose content and fractional volume increase crumb tenderness and crumb hydration capacity. In the same time the freshness depends on water absorption capacity which strongly related to higher protein content, higher (Ap) content, higher molecular weight of starch and higher content of sugar pentoses in durum wheat flour as reported by Boyacloglu and D'Appolonia (1994).

These relation were very indicated by the durum bread in which freshness extended for higher period.

II. 4- Staling evaluation:

Hydrolysis of bread crumb stored at different periods (0, 24, 48 and 72 hrs) at room temperature by *Aspergillus niger* M_2 glucoamylase for 8 hrs of incubation period have been studied in this present work. From Table (6) it is observed that, at zero time (one hour after baking), it was found that the susceptibility of durum bread crumb to be hydrolyzed by *A. niger* M_2 glucoamylase was higher than its corresponding in soft wheat bread crumb (143-179 vs 154-163) mg glucose/1 gm crumb sample. After 24 hrs of storage the susceptibility of drum bread crumb was (134-160 vs 111.6-128) mg glucose /1 gm crumb sample.

Table (5): Sensory evaluation of bread produced from durum and soft wheat varieties.

Samples	Freshness	Layer separation	Crust color	Crumb color	Crumb distribution	Odor	Taste	Total score
	20	10	10	10	20	10	20	100
Durum w. bread:								
B1	19	10	9	9	18	9	18	92
B3	20	10	10	10	19	9	19	97
S2	19	10	8	8	17	8	17	87
S3	20	10	9	9	19	9	18	94
Soft w. bread:								
Sk-93	17	10	8	9	9	7	17	85
G-168	18	10	8	8	8	8	18	86

B_1 = Beny Sweif-1 B_3 = Beny Sweif-3
S_2 = Sohage-2 S_3 = Sohage-3
SK-93 = Sakha-93 G = Giza-168

Table (6): Hydrolysis of different wheat bread crumb by *A. niger* M_2 glucoamylase for 8 hrs. incubation time at 60C° after different storage period.

Samples of bread	Storage time			
	Zero time	24 hr.	48 hr.	72 hr.
	*Mg RS / gm crumb sample			
Durum w. bread:				
B_1	143.0	134.1	129.4	126.6
B_3	176.7	161.4	140.6	135.3
S_2	151.4	150.5	138.6	127.8
S_3	179.1	160.0	147.5	137.7
Soft w. bread:				
Sk-93	154.2	111.6	107.3	94.2
G-168	163.6	128.4	125.2	107.6

* mg reducing sugars as glucose /gm sample.
B_1 = Beny Sweif-1 B_3 = Beny Sweif-3
S_2 = Sohage-2 S_3 = Sohage-3
SK-93 = Sakha-93 G = Giza-168

The same observation was noticed after 48 hrs (129.4-147.5 vs 107.3-125.2) mg glucose/ 1 gm crumb sample. On the other hand after 72 hrs the durum bread crumb susceptibility to *A. niger* M_2 glucoamylase was (126.6-137.7) mg glucose /1 gm crumb sample vs (94.2-107.6) mg glucose/ 1 gm crumb sample of soft wheat crumb. These informations indicated that the

susceptibility of durum bread crumb by *A. niger* M_2 glucoamylase at all storage periods was generally higher than in soft wheat bread crumb. This may be due to the higher gelatinization temperature in durum starch than in soft wheat starch and the higher retention capacity of water in durum starch as reported by Boyacloglu & D'Appolonia (1994) and Eliasson *et al.*, (1995).

This lead to lower crystallization ability of durum starch, therefore staling ability of durum bread was much lower than soft wheat bread.

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استخدام بعض المخلفات الزراعية الثانوية في إنتاج إنزيمات الأميليز من فطر الأسبرجلس نيجر إم، بطريقة التخمير الصلب واستخدامها في دراسة ظاهرة البيات في الخبز

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أتجه العلماء فى الأونة الأخيرة نحو التحلل البيولوجى للنشا باستخدام إنزيمات الأميليز الميكروبية بدلاً من التحلل الكيماوى وإستخدام الحرارة العالية حيث تلعب هذه الإنزيمات دورا حيويا فى عمليات التسكر للمواد النشوية بالإضافة إلى ذلك فقد تم إستخدام إنزيمات الأميليز الميكروبية فى الحكم على جودة الخبز ودراسة ظاهرة البيات فيه ولذلك فقد هدفت هذه الدراسة إلى دراسة العوامل المؤثرة على الإنتاج العالى لهذه الإنزيمات بإستخدام مصادر رخيصة الثمن بغرض خفض تكاليف إنتاجها وكذلك إستخدامها فى تحليل الدقيق والنشا الناتجين من القمح الصلب واللين وكذلك إستخدامها فى الحكم على جودة الخبز البلدى وظاهرة البيات فيه.

وقد أوضحت هذه الدراسة النتائج التالية:

أولاً: فى مجال إنتاج إنزيمات الأميليز:

(١) إستخدم (Spent brewing grain) "مخلف زراعى صناعي ناتج من صناعة البيرة عديمة الفائدة كمصدر للكربون فى البيئة التخميرية الصلبة بواسطة فطرة أسبرجلس نيجرام (٢) لإنتاج انزيمات

الأميليز وأجريت تجارب عديدة لإستبدال المواد الصناعية المكونة للبيئة الأصلية المستخدمة في الإنتاج بمواد أخرى رخيصة الثمن وشملت الدراسة الظروف البيئية والغذائية المثلى المؤثرة على الإنتاج.

(٢) بدراسة الظروف البيئية وجد انه عند مستوى رطوبة ٦٥%، كمية لقاح ١٠^٦ جرثومة لكل واحد جرام من مخلف صناعة البيرة عند درجة تركيز أيون الهيدروجين ٥,٠ ودرجة حرارة ٣٠°م أعطت هذه العوامل مجتمعة أعلى إنتاج من الإنزيمات الثلاثة.

(٣) بدراسة العوامل الغذائية وجد أنه بإضافة بعض مصادر الكربون الصناعية والطبيعية إلى بيئة التخمير بنسبة ١% إتضح أن إضافة عسل الجلوكوز كمصدر طبيعي للكربون بنسبة تركيز ٣% حث الفطر موضع الدراسة على زيادة إنتاج إنزيمات الأميليز بنسبة ١٠٣,٣ ، ١٠٥,٣ ، ١١٢,٨% بالنسبة لإنزيم ألفا ، بيتا ، جلوكوأميليز على التوالي مقارنة بالبيئة الأصلية المستخدمة.

(٤) بدراسة إضافة مصادر النيتروجين الصناعية والطبيعية (سائل نقيع الذرة) وجد أنه بإضافة سائل نقيع الذرة كمصدر نيتروجيني طبيعي بنسبة ٠,٢١٠جم% كنيتروجين عنصرى في بيئة التخمير أعطى زيادة في إنتاج إنزيمات الأميليز بلغت ١٢٢,٦ ، ١٣٣,٨ ، ١١٥% لكل من الألفا ، البيتا والجلوكوأميليز على التوالي بمقارنة بنترات الأمونيوم المصدر الرئيسي لبيئة التخمير "بيئة المقارنة".

(٥) بدراسة الأملاح المضافة إلى بيئة التخمير وجد أن عدم إضافة هذه الأملاح ليس له تأثيرا سلبيا على كفاءة الفطر المستخدم في إنتاج إنزيمات الأميليز.

(٦) وجد أن الفترة المثلى للإنتاج العالى من إنزيمات الأميليز التى بلغت ٧٧٠ ، ٣٩٠ ، ٩٥٠ وحدة إنزيم لكل واحد جرام من مخلف صناعة البيرة من الفا ،بيتا،جلوكو أميليز على التوالي هى أربعة أيام تحضين.

ثانيا: فى مجال إستخدام أنزيم الجلوكوأميليز فى الحكم على جودة الخبز ودراسة ظاهرة البيات فيه أوضحت الدراسة ما يلى:

(١) نشا القمح الصلب "ديورم" إحتوى على ٠,٢٥-٠,٣٠% بروتين ، ٠,٢٦-٠,٣٢% رماد، ٠,١٣-٠,١٨ زيت بينما نشا القمح اللين إحتوى على ٠,٢١-٠,٢٤% بروتين، ٠,٢٢-٠,٢٥ رماد ، ٠,٠٥-٠,٠٦% زيت على الترتيب وبالنسبة لدقيق القمح الصلب فقد إحتوى على ١٣,٤-١٥,٣% بروتين ، ٠,٧٦-٠,٩% رماد، ١,٣-١,٥% زيت بينما دقيق القمح اللين كان محتواه من البروتين ١٠,٢-١١,٦% ، ٠,٧٠-٠,٧٥% رماد، ١,١-١,٢% زيت.

(٢) كذلك أوضحت الدراسة أن محتوى نشا القمح الصلب ١٩-٢٥% أميلوز ، ٧٥-٨١% أميلوبكتين بينما نشا الأقماح اللينة احتوت على ٢٧-٢٨% أميلوز ،٧٢-٧٣% أميلوبكتين.

(٣) بمعاملة دقيق ونشا القمح الصلب "الديورم" بإنزيم أسبرجلس نيجر أم٢ جلوكوأميليز نجح الإنزيم فى تحليل دقيق الديورم بأصنافة المختلفة بنسب تحليل تراوحت ما بين ٢٧,٦-٣٦% بينما في نشا الديورم ما بين ٤٤,٩-٥٠,٩% بعد ٨ ساعات تحليل على درجة ٦٠مئوية بينما بلغت نسبة التحليل في دقيق ونشا القمح اللين بأصنافه المختلفة بعد ٨ ساعات تحضين على درجة ٦٠مئوية ما بين ٣٨,٠-٣٨,٦% وكانت فى النشا ما بين ٥٢,٨-٥٥% وهذا يرجع إلى إرتفاع محتوى دقيق ونشا القمح الصلب من معقد الأميلوز والليبيدات.

(٤) أوضحت دراسة البيات في الخبز معبرا عنها بدرجة تحلل لبابة الخبز بإنزيم الجلوكوأميليز المنتج من فطرة أسبرجلس نيجرأم٢ أنه بعد (صفر ، ٢٤ ، ٤٨، ٧٢ساعة) من التخزين على درجة حرارة الغرفة (٢٣-٢٥م) أن درجة تحلل الخبز الناتج من الأصناف المختلفة للأقماح الصلبة كانت ١٢,٦-١٧,٩% بعد ٨ساعات تحليل على درجة ٦٠مئوية بينما تحليل لبابة الخبز الناتج من الأصناف اللينة بعد ٨ساعات تحليل على درجة ٦٠مئوية كانت ٩,٤-١٦,٤%. وهذا يعنى ان خبز الديورم كانت مدة صلاحيته طازجا أطول بمعدل بيات أقل من خبز الأقماح اللينة وهذا يعود إلى إرتفاع محتواه من الأميلوبكتين ومعقد الأميلوز والليبيدات.

وتجدر الإشارة إلى أن خاصية ثبات هذه الإنزيمات تجاه درجات الحرارة العالية فضلا عن التوافق العالى بين الإنزيم ومواد التفاعل التسوية مما يؤكد أهمية هذه الإنزيمات الثابتة حراريا فى مثل هذه الصناعات البيوتكنولوجية الهامة.

(٥) أوضحت الإختبارات الحسية أن خبز الأقماح الصلبة حاز على درجات جودة حسية وطزاجة أعلى من خبز الأقماح اللينة بعد ساعة واحدة من الخبيز حيث كانت درجات الجودة الحسية فى خبز الأقماح الصلبة "الديورم" من ٨٧-٩٧% ، الطزاجة ٩٥-١٠٠% مقابل ٨٥-٨٦% ، ٨٥-٩٠% لدرجات الجودة الحسية والطزاجة فى خبز الأقماح اللينة على الترتيب.