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Optimization of Glucoamylase Production by Local Isolate of *Aspergillus niger* Using Agro-Industrial Substrates under Solid State Fermentation

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ABSTRACT

In this work, a local isolate of *Aspergillus niger* was used in the production of glucoamylaseunder Solid state fermentation (SSF).When *A. niger* grown on different agro-industrial wastes, wheat bran was the most promising fermentable substrate for glucoamylase production. Maximum activity of glucoamylase was recorded when the level moisture content was 1.5:1 (v/w) of wheat bran: distilled water. All additive carbon sources decreased the production of the enzyme, while, among organic and inorganic nitrogen sources, addition of 1% (w/w) calcium nitrate gave the best results of the production of the enzyme. The optimum inoculum size, temperature and pH for enzyme production was 1.5ml, 30ºC and 5.5, respectively.The highest activity of the produced enzyme was recorded at 70ºC and pH 5.5. Also, metal salts affected the activity of the enzyme. $CuSO_4$ gave maximum activity, while $HgCl_2$ made a huge decrease in the activity. The enzyme showed a great stability at 50ºC and a moderate stability at 60ºC. Also, the enzyme exhibited great stability at acidic condition of incubation.

INTRODUCTION

Glucoamylase is an important enzyme that used in the production of glucose by hydrolyzing starch and related polymers. Glucoamylase produces glucose by hydrolyzing the glucosidic linkages of starch. It hydrolyzes the non-reducing end of starch by cleaving the α-1,4glucosidic linkage, and by hydrolyzing the α-1,6 glucosidic, resulting in glucose as end product Mertens and Skory (2006). Glucoamylaseis used for glucose production from starch and related polymers, which in form serve as a foodstock in fermentation processes such as production of ethanol or high fructose syrups Pavezzi *et al*. (2008) & Zambare (2010).In beer production, glucoamylase could be used for improving barley mash for the fermentation process Pavezzi *et al.* (2008).

Also, Glucoamylase has applications in pharmaceutical, baking and confectionery industries Pandey *et al.*(2000), in the textile and paper industries Pandey *et al.*(1996). Various species of *Aspergillus* are used for the production of glucoamylase Ellaiah *et al.*(2002); Sutthirak *et al.* (2005) and Nyamful *et al.*(2014). Glucoamylase produced from species of *Aspergillus* widely used in manufacture of glucose syrup are the most able with optimum temperature of 58-65ºC, optimum pH of 4-4.5 for its activity and pH stability in a range of 3.5-5.5 Sauer *et al.* (2000).

A use of submerged fermentation in the production of amylases is quite expensive, because of its high energy consumption and its high costs for operation processing. Solid state fermentation (SSF) is used for reducing production costs. Also, it has low energy requirement, high product concentration, and reduced levels of catabolite repression. Also, it produces less waste water, resolves the problem of solid wastes disposal and requires a lower input in infrastructure and skill Baysal *et al.* (2003). Using of commercial carbon sources such as glucose and starch are not advantageous for amylases production since they are very expensive, so cheap and easily available agricultural wastes may be used. The aim of this research is to evaluate the potential of some agricultural materials as a substrate for production of glucoamylase from a local isolate of *A. niger* using SSF and to optimize the process parameters.

MATERIALS AND METHODS

Microorganism: A fungal isolate of *A. niger* was obtained from Microbiology Dept., Fac. Agric., Mansoura Univ., the fungal isolate was maintained and sub-cultured on potato dextrose agar (PDA) slants, and stored at 4°C.

Inoculum preparation: To each sporulated 5 days old PDA slant culture, 10 ml of sterilized water was added, and the surface of the culture was slightly scraped using a sterile wire loop. Spores per ml of the spore suspension were counted.

Agro-industrial wastes: Five agro-Industrial wastes namely, wheat bran, rice bran, wheat flour, corn flour and rice flour were collected from local markets of Mansoura City, Egypt.

Glucoamylase production in solid state fermentation (SSF): Five grams of each waste(wheat bran, rice bran, wheat flour, corn flour and rice flour) were added with 5 ml of distilled water to 250 ml Erlenmeyer flasks. Flasks were sterilized at 121ºC for 20 min., cooled, inoculated with 1 ml of spore suspension having 1×10^6 spores/ml and incubated at 30ºC for 3, 5 and 7 days.

Enzyme extraction: After SSF process, the fermented fungal substrate was mixed with 50 ml of distilled water and shacked for 30minutes on 150 rpm. Mixture was filtered through double layer gauze. The filtrate was centrifuged at 5000 rpm for 15 min., the clear supernatant was used as crude enzyme.

Assay of enzyme activity: Enzyme activity was assayed by measuring the amount of glucose released after 5 minutes of enzymatic reaction by using 3,5-dinitrosalicylic acid (DNS) method Miller (1959). 0.1 ml of crude enzyme was added to 0.9 ml of starch solution 4% in 0.1 mM citrate buffer on pH 5.5 and incubated for 5 minutes at 50ºC. The reaction was stopped byadditionof 1.5 ml DNS solution, boiled for 5 minutes, cooled in ice water, and then read on spectrophotometer on 540nm. Glucose is used as standard curve for glucoamylase activity, one unit of glucoamylase defined as amount of enzyme released 1µmol of glucose per minute.

Factors affecting glucoamylase Production

Effect of initial moisture content on enzyme production: Wheat bran medium were adjusted by ten levels of moisture ranging from 0.5:1 to 5:1 (distilled

water : substrate, v/w), at interval 0.5. Fermentation medium were incubated at 30ºC for 3, 5 and 7 days.

Effect of additional carbon sources on enzyme production: Production of glucoamylase by *A. niger* on wheat bran medium was optimized by supplementation with (1% w/w) of different sources of carbon; glucose, fructose, lactose, mannitol, starch, sucrose, sorbitol, arabinose, galactose and maltose. Control flask contained wheat bran medium without any addition of carbon source. Flasks were incubated at 30ºC, and glucoamylase activity was measured after 3, 5 and 7 days.

Effect of additional nitrogen sources on enzyme production: Wheat bran medium was supplemented with (1% w/w) of organic and inorganic additional nitrogen sources. Control flask contained basal medium without addition of nitrogen source. Flasks were incubated at 30ºC, and enzyme activity was measured after 5 and 7days.

Effect of inoculum size on enzyme production: Fermentation media was inoculated by 0.5, 1.0, 1.5, 2.0, 2.5 and 3 ml of spore suspension (1ml contains 1× 106 fungal spores). Flasks were incubated at 30ºC for 5 and 7 days.

Effect of incubation temperature: It was studied by incubating the fermentation media at 25, 30, 35 and 40ºC for 5 and 7 days, to select the optimum temperature for glucoamylase production.

Effect of initial pH: The fermentation media having different pH ranging from 3-7 with pH 0.5 interval were incubated at 30ºC for 5 days.

At all parameters, the optimum factor achieved by the previous tests was kept constant for the consecutive experiment.

Factors affecting enzyme activity:

Optimum temperature: It was determined by measuring theactivity of the enzyme at pH 5.5 at temperature varying from 30 to 90ºC.

Optimum pH: Enzyme activity was measured at 70ºC in range of pH from3-7 using phosphate buffer and citrate buffer.

Effect of metal salts: It was evaluated by adding some metal salts in concentrations of 5 and 10 µmole to reaction mixture.

pH stability was measured by incubating the enzyme at pH range from 3 to 7 at 4ºC and then the residual enzyme activity was measured at different times interval between 0 and 72 hours.

Thermal stability was determined by pre-incubation of glucoamylase in presence and absence of $CaCl_2$ at different temperature degrees50, 60 and 70ºC at pH 5.5 in absence of substrate. Aliquots were removed at different times intervals between 0 and 72 hours, and immediately cooled on ice. Residual enzymatic activities were assayed in the treated enzyme solutions.

RESULTS AND DISCUSSION

The variation of glucoamylase production with SSF using five agro-industrial wastes as nutrient medium anchorage for *A. niger* under SSF through 3, 5 and 7 days of incubation at 30ºC shown in data in Fig. (1). Maximum production of glucoamylase by *A.niger* was recorded when wheat bran was used as agro-industrial waste followed by rice bran, wheat flour, rice flour and corn flour. Similar results were obtained by Anto *et al.* (2006); Kaur *et al.* (2003) and Nyamful *et al.* (2014), they reported that wheat bran is suitable agro-industrial waste for the production of glucoamylase. Also results showed that the maximum production of glucoamylase by *A. niger* under SSF using wheat bran was451.29 IU/g substrate after 5 days of incubation. Ezugwu *et al.* (2015) reported that the highest peaks of glucoamylase were obtained after 4 days of incubation. The decrease in the production, observed after 5days may be because of the reduced consumption of nutrient materials, toxic substances accumulation and the proteolysis of glucoamylase. From data it can concluded that wheat bran could be a rich source of nutrients for the production of glucoamylase by *A. niger*, and it could be helpful in reducing the production cost.

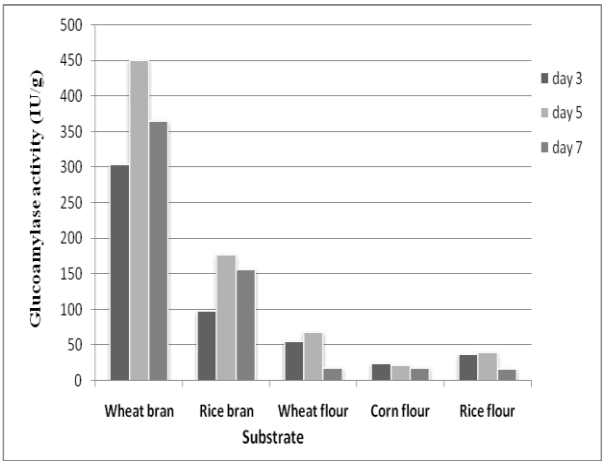

Fig. 1. Effect of different agro-industerial wastes on glucoamylase production by *A.niger*under SSF process.

Effect of initial moisture content on glucoamylase production

Results in Fig.(2) showed that the optimal moisture level with wheat bran to achieve highest enzyme production was found to be 1.5: 1 (v/w), which is 60.28% of moisture, the enzyme achieved 498.7 UI/g substrate after 5 days of incubation. The enzyme production decreased below and above. Chimata *et al.* (2010) reported that 70% of moisture level gave the highest production of amylases by *Aspergillus* sp. using wheat bran. Kundu and Das (1970) mentioned that enzyme production was inhibited when moisture content is below determined optimal moisture level, and there was great enzyme diffusion from the substrate if moisture content was above the optimum level. The enzyme decreases in production with high level of moisture is due to the decrease in the porosity of the substrate, resulting in decrease in gas exchange, while the decrease of enzyme production at low moisture levels is due to the fungal weak growth and lower swelling degree of substrate which resulting in a decreases in enzyme production. The subsequent experiment for the enzyme production was carried out with initial moisture content of 1.5: 1 of moisture ratio.

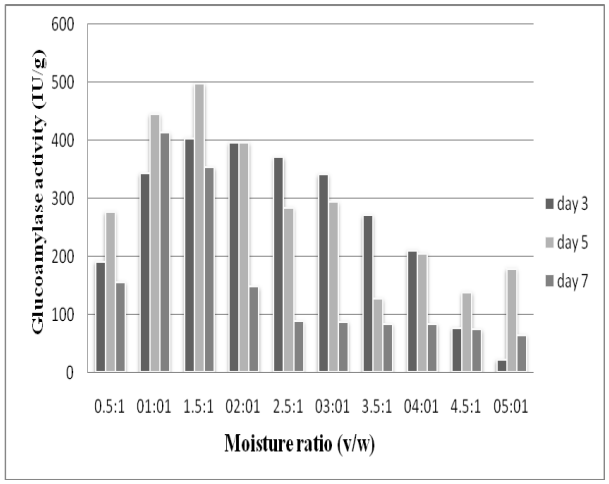

Fig. 2. Effect of moisture content on glucoamylase production by *A. niger* using wheat bran medium under SSF process.

Effect of additive carbon sources on glucoamylase production

Fig. (3)showed the effect of ten carbon sources addition on glucoamylase production. Data showed that all additive sources of sugars decreased the enzyme production through different incubation periods. Wheat bran without any additive of carbon source gave highest enzyme production 509.08 IU/g at 5 days of incubation.

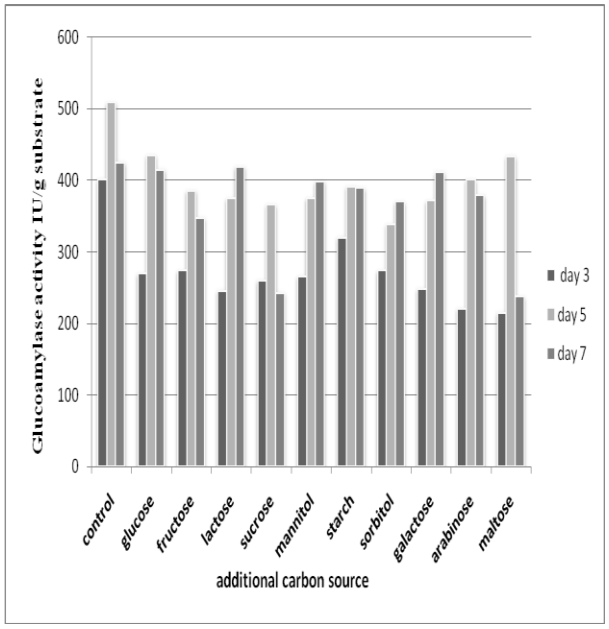

Fig. 3. Effect of carbon sources addition on glucoamylase production by *A. niger* using wheat bran under SSF process.

The reduction in enzyme production caused by sugars addition may be due to catabolite repression. Similar results were obtained by Alva *et al.* (2007), they found that glucose and other metabolizable substrates have repressed glucoamylase production by catabolite repression. Anto *et al.* (2006) reported that addition of maltose, glucose, lactose and starch to wheat bran media decreased glucose production, while sucrose addition caused slightly increase.

Effect of additive nitrogen source on glucoamylase production

Some nitrogen additives decreased the production of glucoamylase such as beef extract and ammonium nitrate, while most of nitrogen additives increased the enzyme production as it shown in Fig.(4). Calcium nitrate, peptone and ammonium sulphate were the highest nitrogen source additives. After 5 days of incubation, calcium nitrate gives the maximum increase glucoamylase, it recorded 676.39 IU/g, increasing the enzyme production by 32.2%, while peptone increased the production by 26.5% recording 647.05 IU/g, and ammonium sulphate recorded 625.74 IU/g increasing the enzyme production by 22.3%.Anto*et al.* (2006) found that addition of peptone and ammonium sulphate to wheat bran medium increased enzyme production, while casein and ammonium chloride decreased enzyme production.

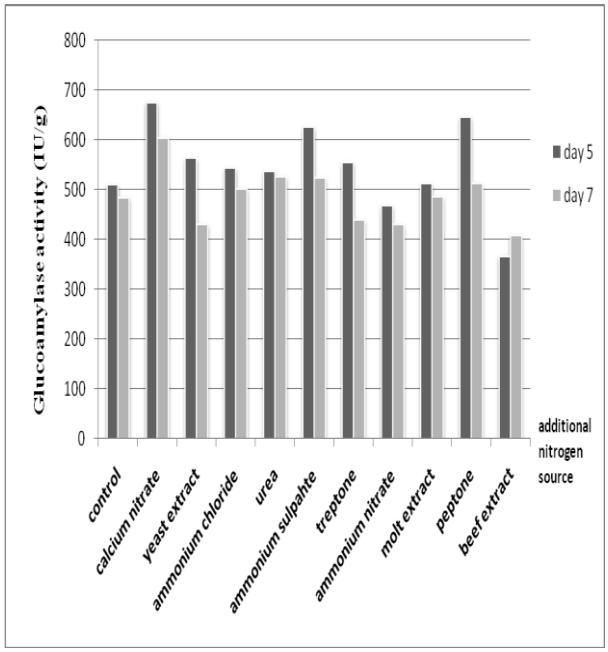

Fig. 4. Effect of nitrogen sources addition on glucoamylase production by *A. niger* using wheat bran under SSF process.

Effect of inoculum size on glucoamylase production

Flasks were inoculated with different inoculum size (0.5, 1.0, 1.5, 2.0, 2.5 and 3 ml) to study the inoculum size effect on glucoamylase production. Data in Fig.(5) showed that glucoamylase production increased with increasing the inoculum size and reached its maximum with 1.5 ml of inoculum. Further increasing of inoculum led to decrease the glucoamylase production. However, the higher inoculum size may cause an increase in moisture level causing a decrease in enzyme production. On the other hand, with low size of inoculum, it may prolongthe time needed for the fermentation process to produce a desired product Murthy *et al.*(2009).

Effect of incubation temperature on glucoamylase production

Data in Fig. (6) showed that production of glucoamylase recorded the highest value at 5 days of incubation on 30ºC and recorded 756.46 IU/g, then decreased at 7 days to511.16IU/g, while enzyme production decreased with incubation at 25ºC, whereas

incubation at temperature over 30ºC caused a big fall in the production of glucoamylase. The optimum temperature of the enzyme production was 30ºC for 5 days. These results are in agreement with Uma and Nasrin (2013), they found that 30ºC was optimum for the production of glucoamylase from *A. niger*.

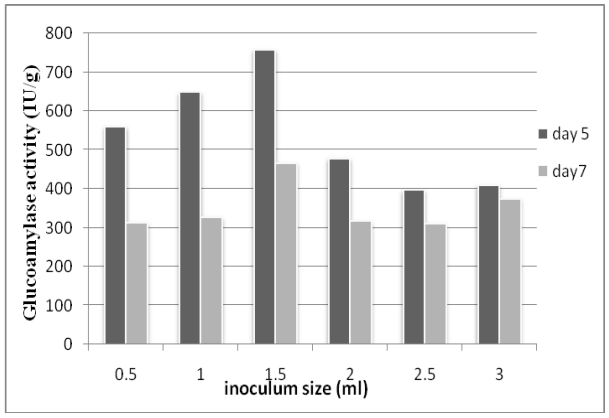

Fig. 5. Effect of inoculum size on glucoamylase production by *A. niger* by using wheat bran under SSF process.

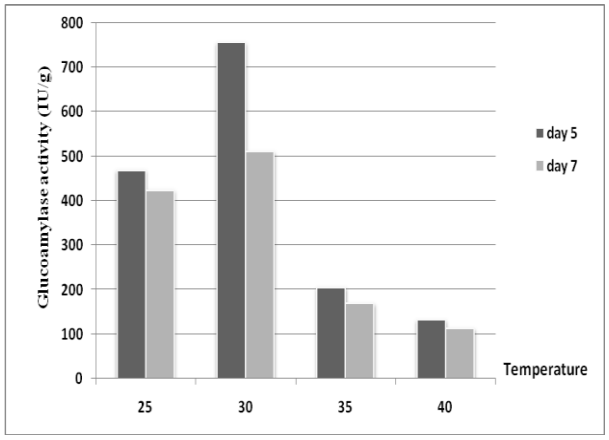

Fig. 6. Effect of incubation temperature on glucoamylase production by *A. niger* by using wheat bran under SSF process.

Effect of initial pH on glucoamylase production

pH is one of the most important factors which affecting the growth and enzyme production during solid state fermentation processes. Results in Fig.(7) showed that pH 5.5 was the optimum initial pH for the enzyme production, which the enzyme activity recorded 799.4 IU/g after 5 days of incubation. Enzyme production decreased if initial pH is higher or lower the optimum pH value; this is due to the high sensitivity of metabolic activities of *A. niger* to changes in pH. Anto *et al.* (2006) reported that initial pH 5 was optimum for glucoamylase production by *Aspergillus* sp. Isolate HA-2. Also, Parbat and Singhal (2011) found that the optimum initial pH of glucoamylase produced by A. *oryzae* grown on agro-industrial products was 5.0, while Kumar *et al.* (2013) mentioned that pH 6.0 is the optimum pH for glucoamylase production by *A.oryzae* on wheat bran.

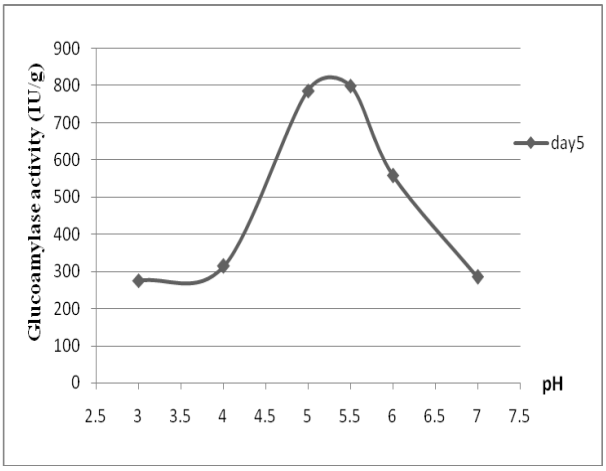

Fig. 7. Effect of initial pH on glucoamylase production by *A. niger* by using wheat bran under SSF process.

Factors affecting enzyme activity

Optimum pH of enzyme activity

As indicated in Fig. (8), it could be noticed that the optimum pH of enzyme activity was 5.5. Enzyme activity decreased with lower or higher pH values, even though the enzyme do well at pH range of 4.5 - 6.5, while enzyme activity highly decreased at very low and at high pH values. Bagheri *et al.* (2014) found that glucoamylase produced by *A. niger* was gave highest enzyme activity at pH 5.0. Sutthirak *et al.* (2005) reported that pH 4.5 was the optimum for the activity of glucoamylase produced by *A. niger*, the enzyme lost its activity rapidly after 5 min of incubation at 60°C and pH 5.5, and completely inactivated at pH 6.5. Koç and Metin (2010) and Kumar *et al.*(2013) reported optimum pH 6.0 for glucoamylase produced by *A. flavus* and *A. oryza*. Optimum pH for glucoamylase produced by *A. oryzae* was 6.0 Zambare (2010), while Nahid *et al.* (2012) found acidic pH range 4.5-4.7 for glucoamylase produced by *A. niger*.

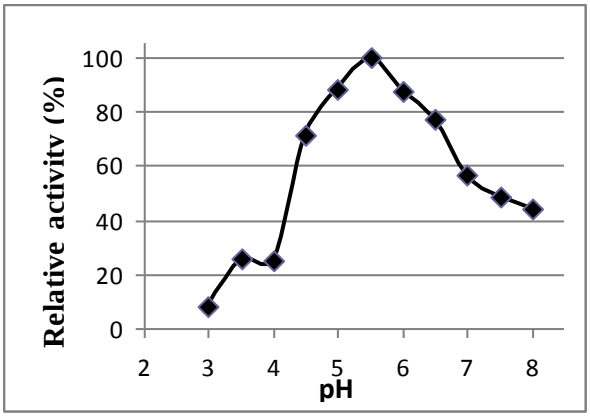

Fig. 8. Effect of pH of reaction mixture on the activity of glucoamylase produced by *A. niger* by using wheat bran under SSF process.

Optimum temperature of enzyme activity

Fig. (9) showed the effect of reaction mixture incubation temperature on glucoamylase activity. Data showed that glucoamylase activity increased within the increases in temperature from 30ºC to 70ºC, then decreased with the rises of temperature degree. The

optimum temperature of enzyme activity was 70ºC. Results are in agreement with Bagheri *et al.* (2014), they found that glucoamylase produced by *A. niger* exhibited optimal activity in a narrow range of temperature around 70ºC and it lost its activity rapidly above this temperature. Results were in agreement with those obtained by Norouzian *et al.*(2006), they found that 70ºC was the optimum temperature of glucoamylase activity. Results demonstrated that at 60ºC the activity of the enzyme lost about 9.33% compared with activity at optimum temperature. There was huge decrease in enzyme activity with raising the temperature degree over 70ºC, at 80ºC the enzyme activity dropped 57.84% compared to the activity on optimum temperature, whereas there is almost no activity at 90ºC. The increase in glucoamylase activity with the increases of temperature may happen because of the change in the enzyme conformation which brings the essential residues to close proximity for catalysis. Koç and Metin (2010) mentioned that temperature of 60°C was the optimum for glucoamylase produced by *A. flavus*, Sutthirak *et al.*(2005) reported that 60ºC is optimum for glucoamylase produced by *A. niger*. Ezugwu *et al.* (2015) found that optimum temperature of GluAgCSV4 and GluCSV11 were 50ºC and 55ºC. On the other hand, Optimum temperature for gluamylase produced from *A. niger* was 40ºC Jebor *et al.* (2014). El-Gendy (2012) reported that the optimum temperature range of glucoamylase produced by *A. niger* was observed at 50-60ºC.

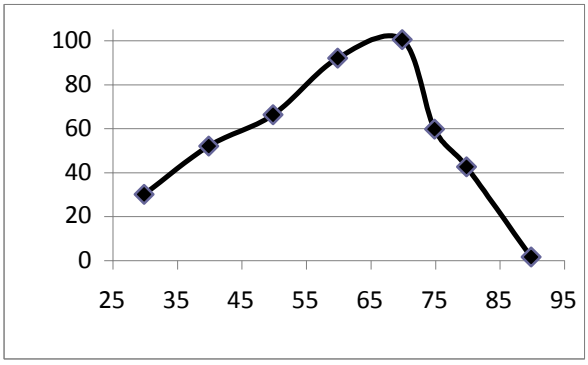

Fig. 9. Effect of reaction mixture incubation temperature on the activity of glucoamylase produced by *A. niger* by using wheat bran under SSF process.

Effect of metal salts on glucoamylase activity:

Adding of some metal salts enhance the activity of group amylases, whereas others may reduce enzyme activity. Data in Fig. (10) showed the effect of some metal salts with concentrations of 5 and 10 µmol on the activity of glucoamylase. Data showed that most of metal salts increased enzyme activity in concentration of 5 µmol, while the effect of 10 µmol vary from salt to another. $CuSO_4$ gave highest increase in the enzyme activity in concentration of 5 µmol, it raised the activity by 53.97%. Also addition of $CaCl_2$ and $FeSO_4$ made good increases in the activity of the enzyme, while $HgCl_2$ and EDTA caused a huge decrease in the activity of the enzyme in both 5 and 10 µmol. Deb *et al.* (2013) reported that most of amylases are known to be metal dependant. Ezugwu *et al.* (2015) found that Mn^{+2}, Zn^{+2}, Fe^{+2} and Ca^{+2}enhanced the activity of GluAgacsV4, while Co^{+2} inhibited the enzyme activity. On the other hand, the activity of GluAgSV11 was enhanced by Ca^{+2} and Co^{+2}, while Fe^{+2}, Zn^{+2}, $Pb^{+2}$and Mn^{+2}completely inactivated the enzyme. Glucoamylase produced by *A. flavus*was activated by $Mn^{+2,}$ Ca^{+2} and Co^{+2} and inhibited by Fe^{+2} and Zn^{+2} Koç and Metin (2010). Activity of glucoamylase produced by *Rhizopus nigricans* was enhanced by Mn^{+2} and significantly reduced by K^{+2}, Fe^{+2}, Zn^{+2}, Cu^{+2} and Ca^{+2} supplementation. Supplementation of Mn^{+2}, Ca^{+2} and Fe^{+2} increased the activity of *Rhizopus oligosporus* SK5 mutant Kareem *et al.*(2014). The inhibition of some metal salts on glucoamylase may be due to the formation of complexes between the enzyme and metals preventing the enzyme from binding to the substrate Aziz and Ali (2012).

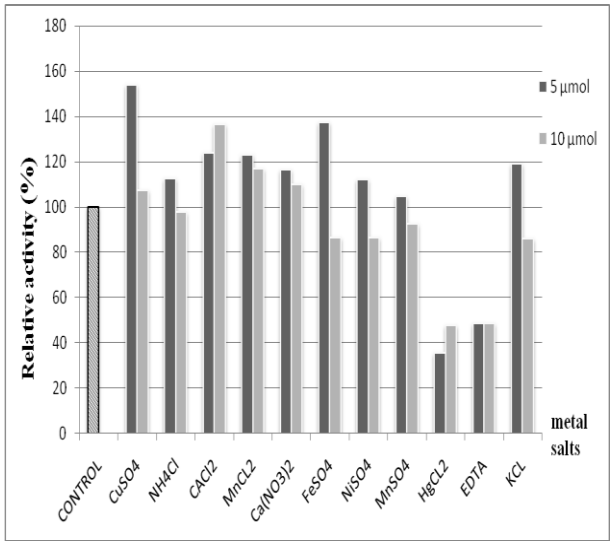

Fig. 10. Effect of different metal salts on *A. niger* glucoamylase activity. The activity was measured at 70ºC and pH 5.5 for 5 min.

pH stability of glucoamylase

The residual enzyme activities were assayed after incubating the enzyme at different values of pH from 3-7 for 72 hours. Data illustrated in fig (11) showed that the enzyme activity dropped with time at different pH values. Data showed that glucoamylase enzyme is stable at pH 3 and 4 for 24 hours. At pH 3, the enzyme was very stable; it lost just 10.10% of its activity after 24 hours, 14.98% after 48 hours and 20.09% after 72 hours. The enzyme has reasonable stability at pH values of 5.5, 6 and 7.

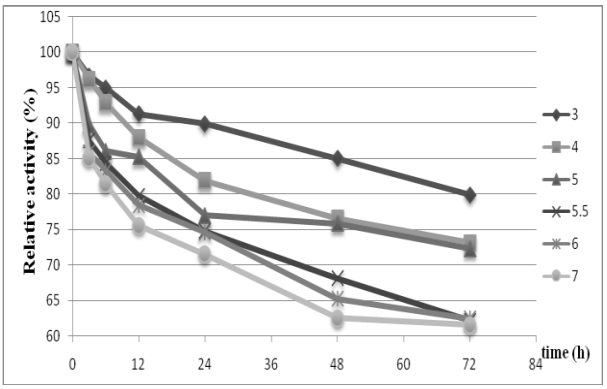

Fig. 11. Effect of pH on the stability profiles of glucoamylase produced by *A. niger*.

Temperature stability of glucoamylase

The enzyme solution, in the presence and absence of $CaCl_2$ were exposed to different temperature range between 50 and 70ºC for different intervals.

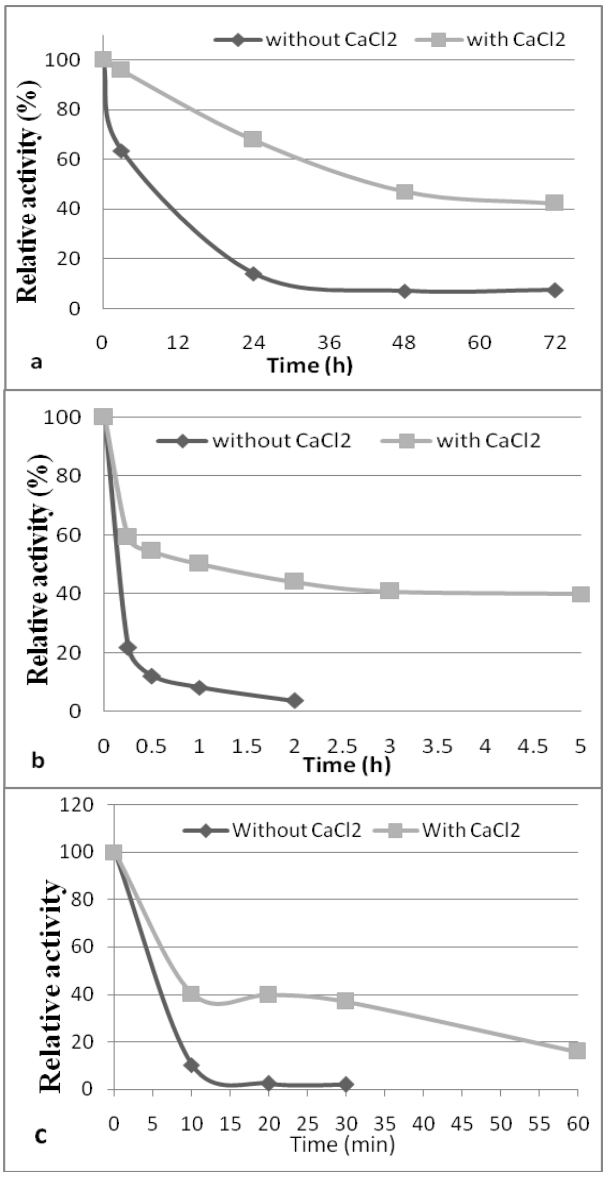

Fig. 12. Effect of storage at 50ºC (a), 60ºC(b) and 70ºC(c) on glucoamylase stability.

From data illustrated in Fig (12) it could be noticed that the enzyme exposed to 50ºC did not lose pronounced activity during the first 2.5 hours in the prescence of $CaCl_2$. It lost 4.16% of its activity after 3 hours and 32.20% after 24 hours. The loss in enzyme activity increased by increasing time of exposure. The enzyme solution exposed to 60ºC lost 40.86% and 45.60% of its activity after 15 and 30 min of exposure. On the other hand, enzymes exposed to 70ºC for 10, 20 and 30 min lost 96.30, 97.67 and 98.00% of its activity. It could be stated that glucoamylase showed high thermal stability at temperatures below 50ºC and the enzyme was sensitive to temperature above 60ºC. Also data showed that the presence of $CaCl_2$ (10µmol) has made the enzyme more stable at tested temperature. Similar results were obtained by Sutthirak *et al.* (2005), they found that *A. niger* glucoamylase activity decreased during prolonged incubation at 60ºC

Interesting observation was that the enzyme showed and attained maximum activity at 70ºC and showed optimum activity under acidic conditions. This make the produced glucoamylase as useful for various industrial applications like starch liquefaction.

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إنتاج إنزيم الجلوكوأميليز بواسطة عزلة محلية من فطر أسبيرجيلس نيجر بواسطة تخمر الحالة الصلبة على ردة القمح

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تم استخدام عزلة محلية من الفطر أسبيرجيلس نيجر فى إنتاج إنزيم الجلوكوأميليز بواسطة تخمر الحالة الصلبة. وبعد تجربة الإنتاج على عدة مخلفات كان أعلى إنتاج للإنزيم على مخلف ردة القمح بعد 5 أيام من التخمر. وكانت نسبة الرطوبة المثلى 1,5:1 مقدرة بوزن : حجم من الردة والماء. انخفض إنتاج الإنزيم باستخدام كل مصادر الكربون الإضافية المستخدمة، فى حين تمت تجربة مصادر نيتروجين عضوية ومعدنية بنسبة 1% وكانت نترات الكالسيوم أكثر مصادر النيتروجين زيادة فى إنتاج الإنزيم. فى حين كان حجم اللقاح الأمثل لإنتاج الإنزيم 1,5 مل من اللقاح ودرجة الحرارة المثلى كانت 30º م والأس الهيدروجينى عند 5,5. وقد أعطى الإنزيم المنتج أكثر نشاط عند 70º م وعند أس هيدروجينى 5,5. وقد لوحظ تأثر الإنزيم بإضافة الأملاح المعدنية ففى حين كانت كبريتات النحاس أكثر الأملاح زيادة فى نشاط الإنزيم، كان كلوريد الزئبقيك أكثرها فى تثبيط الإنزيم. وقد أعطى الإنزيم درجة ثبات حرارية عالية عند تحضينه على درجة حرارة 50º م وثبات جيد عند تحضينة على درجة حرارة 60º م. كما لوحظ أن للإنزيم ثبات عالى عند تحضينه فى درجات الحموضة العالية.