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Production and Application of Thermostable Glucoamylase from Thermotolerant *Aspergillus fumigatus via* Semisolid State Fermentation

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A THERMOTOLERANT fungus *Aspergillus fumigatus* isolated from agriculture soil, Beheira Governorate, Egypt was screened from other fungal isolates for its high glucoamylase activity. The identification of the fungus based on morphology and sequence analysis of ITS1-5.8S rDNA-ITS2. Five crop wastes (wheat bran, wheat straw, rice hulls, rice straw and broken rice grain) were used to induce the thermostable glucoamylase production by *A. fumigatus via* semisolid state fermentation. Wheat bran is the most suitable waste for glucoamylase production by the tested fungus which further improved using Plackett-Burman and Box-Behnken designs. Optimum conditions for the production of thermostable amylase are reached at pH 6, wheat bran 1gm, yeast extract 6mg/gm, berij 35 40µL/gm, $(NH_4)_2SO_4$ 24mg/gm and an inoculum of 6x 10^5spore/ml with thermostable glucoamylase production titer of 161.11U/gm after 6 days.

$FeCl_3$, $BaCl_2$, and $CaCl_2$ positively affect glucoamylase activity with highest main effect value of $FeCl_3$ ions where $MgCl_2$ has the highest negative main effect on activity. The kinetic constants K_m is 1.37mg/ml with three bands in zymogram. Moreover the enzyme showed good cleaning effect when applied in starch patches in textile. Glucoamylase of *A. fumigatus* exhibited good thermostability as it loss only 28.95% from its original activity after treatment with heat at 80°C for 3hr. Finally, the thermostable glucoamylase from *A. fumigatus* can be produced from cheap agrowaste *via* simple fermentation technique.

Keywords: Thermostable glucoamylase, *Aspergillus fumigatus,* Plackett-Burman, Box-Behnken.

Introduction

Enzymes which are essential in all living cells become also essential in many industrial, agricultural and pharmaceutical applications. The advantage of enzymes based on their properties as natural, mild and safe ingredients. Amylases are the main groups of enzymes used through the world. There are various types of amylases and the most important category of amylases is the glucoamylase (GA) (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3). The importance of this enzyme based on its activity in removing the glucose units from the non-reducing chain-ends of starch and it can also degrade α-1,6 glycosidic linkages of amylopectin (Karim et al., 2016; Ayodeji et al., 2017). Accordingly, GA used in starch hydrolysis and widely used by various food industries such as glucose syrups and bioethanol (Pervez et al., 2014).

Several species of plants, bacteria and fungi can produce amylases (Pasin et al., 2017), however, molds represent the most important source between microorganisms (Riaz et al., 2007) due to large scale production, external secretion,

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organic solvents resistance and thermostability (Ayodeji et al., 2017). In many industrial applications, thermostable enzymes which are resistance to thermal inactivation have steeply increased (Zheng et al., 2010). As it minimise the production of by-products, lower the production time and reduce the risk of contamination (Thorsen et al., 2006).

For enzyme production, solid state fermentation has gained much interest due to the advantages that presents over submerged fermentation such as higher product yields, less energy requirements, easier aeration, less wastewater generation, reduced bacterial contamination and easier product recovery (Pandey et al., 2000). In between, semisolid state fermentation is a sort of solid state fermentation in which the free liquid content has been increased in order to facilitate nutrient availability and fermentation conditions (Economou et al., 2010).

In general, crop wastes are the main source of starchy biomass worldwide (Chaudhary et al., 2012). Agricultural products like rice, wheat and other cereals contain considerable amount of starch and provide excellent resource in numerous fermentation procedures. To the best of our knowledge the production of thermostable GA from agrowastes under semisolid state fermentation conditions has not been reported before this study. Nowadays, Industrial applications need screening for new fungal source of GA with good thermostability on low cost wastes and this is the aim of this work.

Materials and Methods

Isolation and amylolytic screening of thermotolerant strain

Agriculture soil samples were obtained from cultivated soil from Damanhour at Beheira governorate, Egypt. 0.1ml of soil suspension (dilution of 10^{-1}) was inoculated into Czapek starch agar (CSA) which consists of starch (20g); $NaNO_3$ (3g); KH_2PO_4 (1g); $MgSO_4.7H_2O$ (0.5g); KCl (0.5g); $FeSO_4.5H_2O$ (0.01g) and agar (20g) were dissolved in distilled water (1L). The medium was sterilized by autoclaving and chloramphenicol (200mg/l) was added as antibacterial then incubated at 50ºC for 6 days. A clear zone around fungal colonies confirmed amylolytic action. The most prominent fungal colony was transferred on potato dextrose agar (PDA) slants then incubated at 50ºC for 6 days and maintained at 5ºC for further studies.

Identification of fungal isolate

The most prominent isolate was identified based on morphology and microscopic examination after culturing on Czapek Dox agar (CDA) medium at 50°C for 7 days (Raper & Fennell, 1977). In addition, fungal strain was identified also based on sequencing analysis of the amplified ITS1-5.8S rRNA-ITS2 gene (White et al., 1990).

The amplified PCR products of ITS1-5.8SrDNA-ITS2 were sequenced on both strands using ITS1 and ITS4 primers using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., CA, USA) and a Taq FS Dye Terminator Sequencing Kit (ABI, USA). Sequence editing was carried out using Biology Work Bench 3.7 software. The sequence was compared to the available fungal sequences on the NCBI database using the Blastn program and the phylogenetic neighbor-joining tree that reflects evolutionary relationships was constructed using MEGA 4.0.2 software.

Screening some crop wastes for GA production

Crop wastes including wheat straw, wheat bran, rice hulls, rice straw and broken rice grains substrates (1g) were added separately to conical flask (100ml) and wetted by 10 ml distilled water. After sterilization, the substrates were inoculated by 1ml of fungal spore suspension (10^6spore/ml) of the tested fungus and incubated at 50°C for 3 days.

Elution of crude extracellular GA

After fermentation, elution of GA was carried out by saline solution (1:10w/v). After shaking at 200rpm for 30min, the supernatant was separated by filtration and used for GA assay.

Assay of GA activity

The enzyme activity was determined by the quantification of the glucose formed after the incubation of 1 ml of the crude enzyme with 1ml of 1% starch substrate in 100mM sodium phosphate buffer at 50ºC and pH 6.5 for 30min. The amount of glucose released was measured using the dinitrosalicylic acid method (Miller, 1959). One unit (U) of amylase activity was defined as the amount of enzyme required to release one µmol/min of glucose from the soluble starch substrate under the assay conditions.

Optimization of GA production using Plackett-Burman design

Plackett-Burman experimental design (Plackett & Burman, 1946), a fractional factorial design, was used to reflect the relative importance of various factors on GA production. In addition, design was used to determine the near optimum conditions for GA production. Eleven independent variables were screened in twelve trials according to the Plackett-Burman design matrix using Minitab 16 software. For each variable, a high (+), low (-) and basal levels were tested. All trials were performed in duplicates and the average of GA activity results were treated as responses. The main effect of each variable was calculated using Minitab 16 and regression coefficients and analysis of variance for GA activity were calculated for the determination of variable significance.

GA production was carried out in 100ml Erlenmeyer conical flasks under static conditions at 50°C. After autoclaving for 20min, the flasks were inoculated with 1ml of freshly prepared spore suspension inoculum. At the end of incubation, the crude enzyme was separated from culture medium by filtration through a Whatman No. 1 paper and the filtrates were centrifuged at 10,000rpm for 10min at 4°C. For preparation of inocula, fungal isolate was grown in PDA medium at 28ºC for 6 days. The fungal spores were gently scraped from the PDA surface into sterile distilled water and used as spore suspension. The pH was adjusted using freshly prepared 0.1N HCl and 0.1N NaOH solutions to obtain the required pH before autoclaving by using digital pH meter (Hanna Instruments, Italy).

Optimization of GA production using Box-Behnken design

In the second phase of medium formulation for optimum GA production, the Box-Behnken experimental design (Box & Behnken, 1960) was applied using Minitab 16 software. Experiment was carried out using 40ml broth culture media in 100ml Erlenmeyer flasks at pH 6, under static conditions and at 50°C. The culture media containing: wheat bran 1gm; and Yeast extract 6mg/gm. After sterilization, the flasks were inoculated with 1ml of 6x 10^5spores/ml freshly prepared inoculum. At the end of incubation, the enzyme activity was measured.

In this model, the most significant independent variables, are examined at the three different levels, low (-), high (+) and basal (0). Thirteen combinations were examined and their observations were fitted to the second order polynomial Equation (1) as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad \text{.....(1)}$$

where, Y is the dependent variable (GA activity, µg/min/gm wheat bran); X_1, X_2 and, X_3 are the independent variables; b_0 is the regression coefficient at center point; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second order interaction coefficients; b_{11}, b_{22} and b_{33} are quadratic coefficients. The second order polynomial equation was used to find out the relationship between independent variables and response.

The optimum concentrations were predicted using the analysis of variance (ANOVA) and in order to ensure a good model, the quality of the fit of the polynomial model equation was expressed by R^2, the coefficient of determination. The value of R^2, which is closer to 1.0, indicates the better fitness of model in the experimental data.

Thermostability of crude GA

Thermostability were determined by incubating the enzyme for 3hr at 80°C the remaining GA activity was then assayed in its optimum conditions.

Effect of some ions on GA activity

The effect of some ions ($CoCl_2$, $NiCl_2$, $CuCl_2$, NH_4Cl, $MgCl_2$, $ZnSO_4$, $BaCl_2$, $CaCl_2$, $FeCl_3$, NaCl and the chelating agent EDTA) on GA activity were determined according to Plackett-Burman design. The ions (5mM) were incorporated into enzyme assay mixture in 100mM sodium phosphate buffer at 50ºC and pH 6.5 for 30min using 1ml of the crude enzyme with 1ml of 1% starch substrate.

Gel electrophoresis analysis of GA

Crude GA was analysed using 1% (w/v) agarose gel electrophoresis in non-denaturing conditions. The electrophoresis was done at room temperature in a buffer consisting of 100mM sodium phosphate buffer, pH 6.5, under an electric current of 40mA and 120V. In order to identify the amylolytic activity after electrophoresis, the gel was immersed in a solution of 1% starch (w/v) in 100mM sodium phosphate buffer, pH 6.5, incubated in a water bath at 50ºC for 30min. After that, the gel was immersed in a solution of iodine (10mM I_2 and 14mM KI).

Kinetics of GA

The kinetic constants (K_m and V_{max}) values were calculated according to Lineweaver and Burk plot. Assays were performed under the optimal conditions of assay asset in previous experiments. Using different concentrations (0.04 to 3.75g/L) of soluble starch in 100mM sodium phosphate buffer, pH 6.5, incubated in a water bath at 50ºC for 30min.

Statistical analysis

Experimental data are presented as mean± standard error and the analysis of variance (ANOVA) of data was conducted and means property values were separated ($P \leq 0.05$) with Student-Newman-Keuls (SNK) test by the SPSS program (ver. 21.0, USA).

Results

Isolation, identification of thermotolerant fungal strain

Among many isolated fungal species, the most active amylolytic and thermotolerant fungal isolate was identified based on morphological and molecular basis using ITS1-5.8S rDNA-ITS2 sequences. The nucleotide sequence determined was obtained and recorded in Gen Bank database under the accession number MG711601. According to 99% similarity index of BLASTN search results (Fig. 1), the present strain was identified as *A. fumigatus*.

Screening of crop wastes for GA production by A. fumigatus

Maximum GA activity (88.19U/ml) was obtained in filtrate of semisolid state fermentation of the fungus culture grown on wheat bran compared with other crop wastes substrates (Fig. 2). Therefore, wheat bran was selected for further investigation.

Optimization of GA production using Plackett-Burman design

As shown in Table 1, trials 3 and 9 recorded the highest GA activity (135.10 and 145.09U/gm wheat bran, respectively), whereas, trial 4 and 12 have the lowest GA activity values (45.51 and 55.40U/gm, respectively). The main effects of each variable upon GA activity were estimated and expressed graphically in Fig. 3. Among the eleven examined variables, five factors; $(NH_4)_2SO_4$, incubation period, surfactant, yeast extract and water content showed positive effects on GA activity. However, wheat bran, NH_4Cl, inoculum, $NaNO_3$, pH and peptone had negative main effects on GA activity. $(NH_4)_2SO_4$, incubation period and surfactant have the highest positive main effect on GA activity.

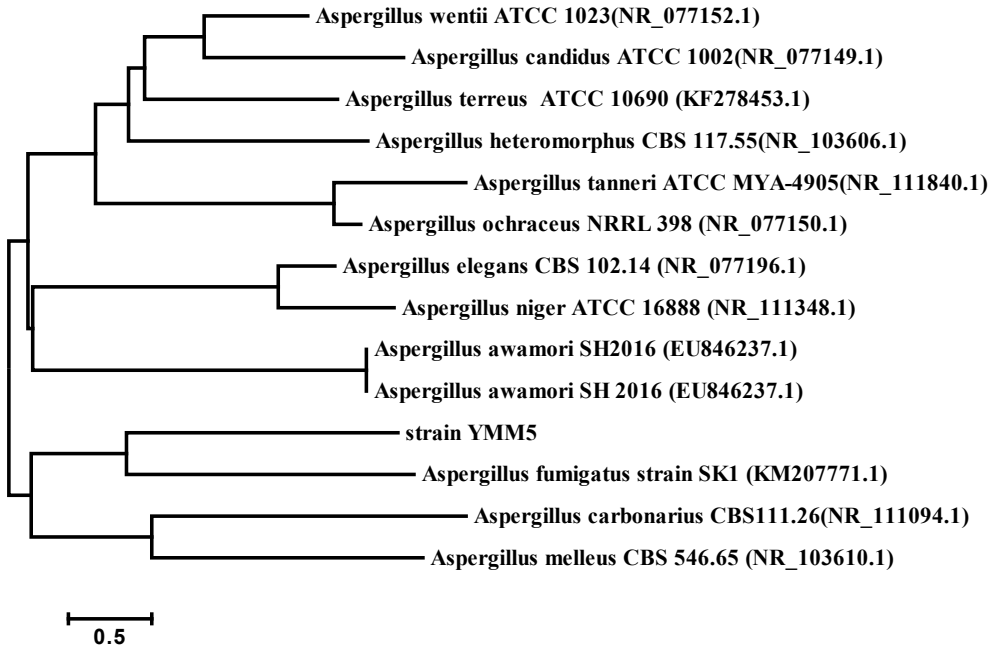

Fig. 1. A neighbor-joining phylogenetic tree constructed based on the alignment of the ITS sequences of *Aspergillus genotypes* using MEGA 4.0.2 software [The branch length is proportional to the number of substitutions per site, letters and numbers written before the strains are the accession numbers].

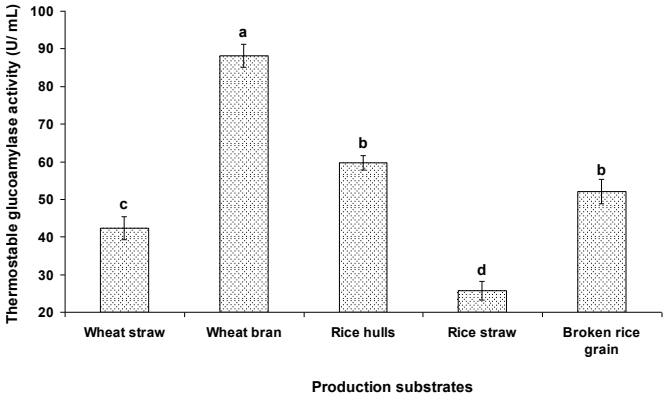

Fig. 2. Screening for production of thermostable GA against different substrates; wheat straw, wheat bran, rice hulls, rice straw, and broken rice grains, using semisolid state fermentation at 50°C for 3hr [Data are the average and standard error of three replicates, different letters on the bars indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05)].

TABLE 1. Independent variables and their levels examined in the Plackett-Burman experiment for *A. fumigatus* thermostable GA.

Trials	Independent variables											GA activity (U/ gm)± SE
	Wheat bran (gm)	Water volume (ml)	pH	$NaNO_3$ (mg/gm)	$(NH_4)_2SO_4$ (mg/gm)	NH_4Cl (mg/gm)	Peptone (mg/gm)	Yeast extract (mg/gm)	Incubation (days)	Inoculum (10^5 spore/ml)	Surfactant (µL/gm)	
1	+1 (3)	-1 (20)	+1 (8)	-1 (0)	-1 (0)	-1 (0)	+1 (6)	+1 (6)	+1 (4)	-1 (6)	+1 (25)	88.18^{b}± 2.31
2	+1 (3)	+1 (40)	-1 (6)	+1 (6)	-1 (0)	-1 (0)	-1 (0)	+1 (6)	+1 (4)	+1 (10)	-1 (0)	75.65^{c}± 1.76
3	-1 (1)	+1 (40)	+1 (8)	-1 (0)	+1 (6)	-1 (0)	-1 (0)	-1 (0)	+1 (4)	+1 (10)	+1 (25)	135.10^{a}± 4.73
4	+1 (3)	-1 (20)	+1 (8)	+1 (6)	-1 (0)	+1 (6)	-1 (0)	-1 (0)	-1 (2)	+1 (10)	+1 (25)	45.51^{e}± 1.33
5	+1 (3)	+1 (40)	-1 (6)	+1 (6)	+1 (6)	-1 (0)	+1 (6)	-1 (0)	-1 (2)	-1 (6)	+1 (25)	91.47^{b}± 2.0
6	+1 (3)	+1 (40)	+1 (8)	-1 (0)	+1 (6)	+1 (6)	-1 (0)	+1 (6)	-1 (2)	-1 (6)	-1 (0)	76.21^{c}± 2.5
7	-1 (1)	+1 (40)	+1 (8)	+1 (6)	-1 (0)	+1 (6)	+1 (6)	-1 (0)	+1 (4)	-1 (6)	-1 (0)	70.09^{c}± 2.44
8	-1 (1)	-1 (20)	+1 (8)	+1 (6)	+1 (6)	-1 (0)	+1 (6)	+1 (6)	-1 (2)	+1 (10)	-1 (0)	93.45^{b}± 2.3
9	-1 (1)	-1 (20)	-1 (6)	+1 (6)	+1 (6)	+1 (6)	-1 (0)	+1 (6)	+1 (4)	-1 (6)	+1 (25)	145.09^{a}± 4.67
10	+1 (3)	-1 (20)	-1 (6)	-1 (0)	+1 (6)	+1 (6)	+1 (6)	-1 (0)	+1 (4)	+1 (10)	-1 (0)	89.53^{b}± 2.67
11	-1 (1)	+1 (40)	-1 (6)	-1 (0)	-1 (0)	+1 (6)	+1 (6)	+1 (6)	-1 (2)	+1 (10)	+1 (25)	79.89^{c}± 1.69
12	-1 (1)	-1 (20)	-1 (6)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (2)	-1 (6)	-1 (0)	55.40^{d}± 1.67
13	0 (2)	0 (30)	0 (7)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (8)	0 (12.5)	78.71^{c}± 1.73

- Independent variable values are between brackets.
- Response values are mean of three replicates and given as mean± standard error.
- Different letters in the same column indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05).

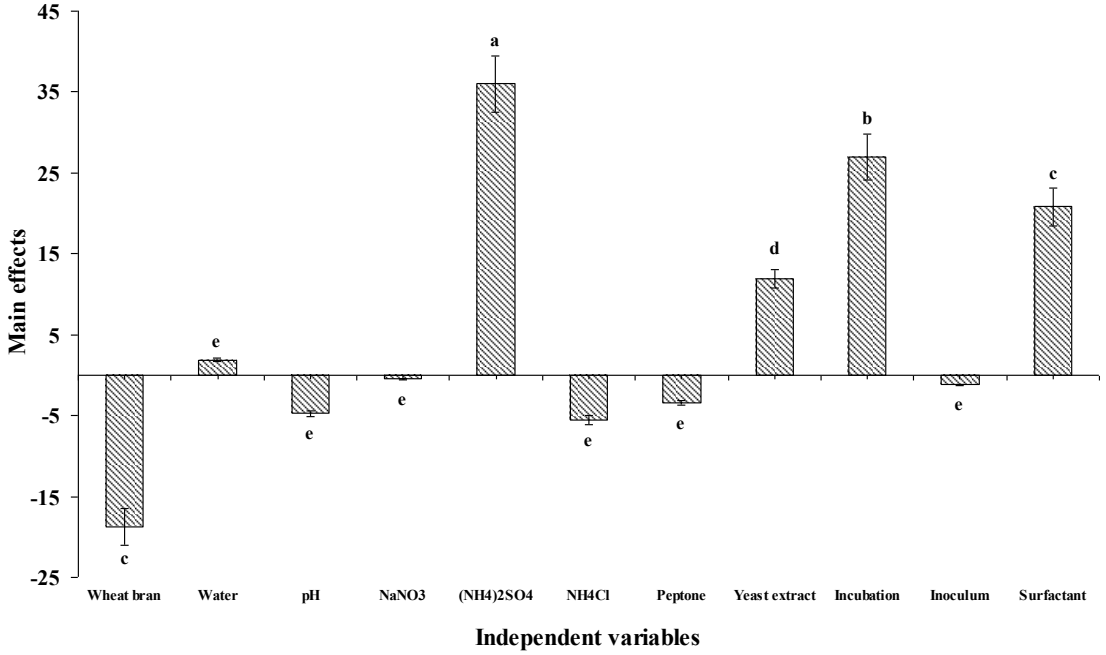

Fig. 3. Main effect of independent variables examined in Plackett-Burman design of thermostable GA activity [Data are the average and standard error of three replicates, different letters on the bars indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05)].

Positive effect explains that if a higher concentration was used, a better response was achieved, while a negative effect means lower concentrations are favored for better results. Figure 4 shows the Pareto chart of the effects displaying the relative levels of significance and is a convenient way to view the results of a Plackett–Burman design.

Optimization of GA production using Box-Behnken design

The main aim of response surface analysis is to investigate the interaction among the independent variables and to determine the optimum concentration of each factor for maximum GA activity. In order to approach the optimum response area of GA activity, the independent variables including incubation period (X_1; days), $(NH_4)_2SO_4$ (X_2; mg/gm) and surfactant (X_3; µl of Berij35/gm) were investigated, each at three different levels (-, 0 and +) according to the Box and Behnken design (Box & Behnken, 1960). The other components of the production mixture were added in their optimum concentration obtained from Plackett-Burman and treated as constant factors.

Table 2 represents the design matrix of the coded variables together with the experimental results of GA activity. Data revealed a considerable variation in enzyme activity depending on the levels of the three independent variables.

A second order regression equation showed the dependence of GA activity on the reaction mixture constituents. The parameters of the equation were obtained by multiple regression analysis of the experimental data. A second order polynomial function was fitted to the GA activity results of the applied Box-Behnken experiment. According to the obtained statistical analysis results, the empirical relationship between the response and the three independent variables can be described by the following equations; Equation 2 (with out heat treatment) and Equation 3 (with heat treatment at 80°C for 3hr).

$$Y = 159.49 - 1.39x_1 - 0.69x_2 + 2.26x_3 + 4.86x_1x_2 - 1.39x_1x_3 - 2.43x_2x_3 - 10.65x_1^2 - 16.20x_2^2 - 9.95x_3^2 \quad \text{(2)}$$

$$Y = 110.19 + 0.09x_1 - 1.22x_2 + 5.12x_3 + 3.47x_1x_2 - 4.34x_1x_3 - 3.47x_2x_3 - 14.90x_1^2 - 23.41x_2^2 - 12.12x_3^2 \quad \text{(3)}$$

The goodness of fit of the model was checked by determination coefficient (R^2). In the present study R^2 value indicating that 92.42% and 97.48% of the total variability in the response could be

explained by the model with out heat treatment and with heat treatment at 80°C for 3hr, respectively. Therefore, the present R^2-values reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for predicting GA activity.

Representation of the experimental results in the form of surface plots reflects the interactive effects of examined variables. The three dimensional response surface plots is the graphical representations of the regression equation, from which the GA activity is generated for the pair wise combination of the three factors, with one variable kept constant at its optimum level and with variation of the other two variables within the experimental range. The optimum value of each variable was located based on the hump in the three-dimensional plot.

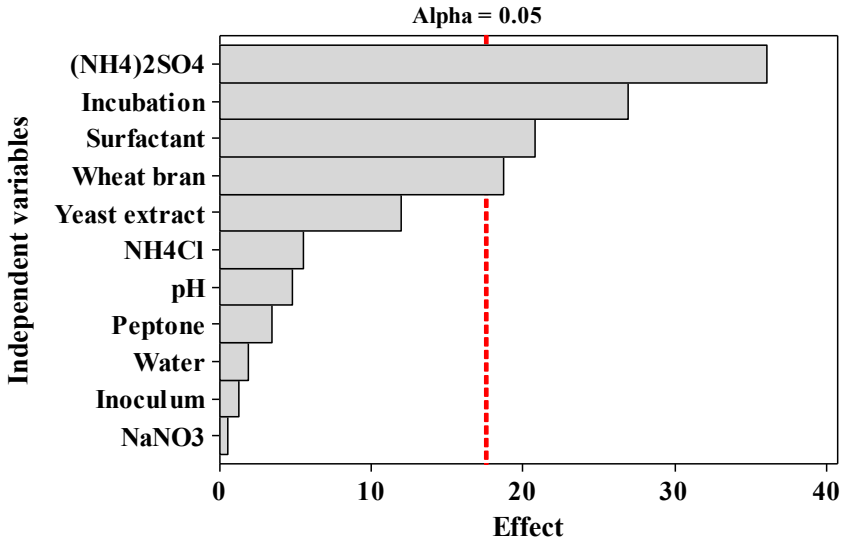

Fig. 4. Pareto chart of standardized effects of thermostable GA activity in Plackett-Burman design [The point at which the effect estimates were statistically significant is indicated by vertical line].

TABLE 2. Thermostable GA activity using Box-Behnken design.

Trial	Independent key variables			GA activity (U/gm)± SE		% loos of activity
	X_1: Incubation (days)	X_2: $(NH_4)_2SO_4$ (mg/gm)	X_3: Surfactant (µl/gm)	Without heat treatment	Heat treatment	
1	-1 (5)	-1 (12)	0 (40)	$143.06^{b} \pm 3.67$	$78.47^{c} \pm 2.50$	45.15
2	+1 (7)	-1 (12)	0 (40)	$123.61^{d} \pm 3.87$	$66.67^{e} \pm 2.08$	46.06
3	-1 (5)	+1 (36)	0 (40)	$131.94^{bcd} \pm 1.82$	$70.14^{de} \pm 2.78$	46.84
4	+1 (7)	+1 (36)	0 (40)	$131.94^{bcd} \pm 1.84$	$72.22^{cde} \pm 1.39$	45.26
5	-1 (5)	0 (24)	-1 (30)	$135.42^{bcd} \pm 2.41$	$72.92^{cde} \pm 1.20$	46.15
6	+1 (7)	0 (24)	-1 (30)	$142.36^{b} \pm 2.50$	$86.81^{b} \pm 1.39$	39.02
7	-1 (5)	0 (24)	+1 (50)	$138.19^{bc} \pm 2.20$	$88.19^{b} \pm 1.84$	36.18
8	+1 (7)	0 (24)	+1 (50)	$139.58^{bc} \pm 5.51$	$84.72^{b} \pm 1.84$	39.30
9	0 (6)	-1 (12)	-1 (30)	$127.08^{cd} \pm 2.41$	$65.97^{e} \pm 0.69$	48.09
10	0 (6)	+1 (36)	-1 (30)	$130.56^{bcd} \pm 2.30$	$69.44^{de} \pm 1.84$	46.81
11	0 (6)	-1 (12)	+1 (50)	$140.97^{b} \pm 2.48$	$86.81^{b} \pm 1.39$	38.42
12	0 (6)	+1 (36)	+1 (50)	$134.72^{bcd} \pm 1.84$	$76.39^{cd} \pm 2.78$	43.30
13	0 (6)	0 (24)	0 (40)	$159.03^{a} \pm 2.35$	$109.03^{a} \pm 1.84$	31.44
14	0 (6)	0 (24)	0 (40)	$158.33^{a} \pm 3.18$	$112.50^{a} \pm 1.20$	28.95
15	0 (6)	0 (24)	0 (40)	$161.11^{a} \pm 1.84$	$109.03^{a} \pm 1.84$	32.33

*Independent variable values are between brackets. Response values are mean of three replicates and given as mean ± standard error. Different letters in the same column indicate significant differences according to the Student-Newman-Keuls (SNK) test ($P \leq 0.05$).

Figures 5 and 6 represents the surface plot of independent variables that affect the GA activity based on the results of the Box-Behnken experiment. (A) Represents the interaction between incubation and $(NH_4)_2SO_4$, (B) represents the interaction between incubation and surfactant, and (C) represents the interaction between surfactant and $(NH_4)_2SO_4$. From these figures, it can be suggested that, optimum condition for GA activity was obtained at values of independent variables very closer to the basal levels.

Solving the model according to the data obtained from Table 2 revealed an optimum response at X_1= 6 days, X_2= 24mg/gm and X_3= 40µl/gm of Berij35 with a predicted GA activity of 112.50 and 161.11U/mg wheat bran, respectively for heat treated and no heat treated crude enzyme.

Thermostability of crude GA

The thermostability of crude GA was examined and data in Fig. 7 revealed that GA activity loss (%) after 3hr of heat exposure at 80°C, are ranged from 28.95 at optimum condition to 48.09 in other trails.

Effects of some ions on GA activity

Generally, ions either inhibit or induce GA activity (Table 3). The heat treated GA activity (80°C for 3hr before assay) was stimulated by $NiCl_2$, NH_4Cl, $MgCl_2$, $BaCl_2$ and NaCl. In addition, $NiCl_2$ has the highest positive effect on activity. On the other hand, it was inhibited by $CoCl_2$, $CuCl_2$, $ZnSO_4$, $CaCl_2$, $FeCl_3$, and EDTA. Furthermore, EDTA has the highest negative main effect on activity (Fig. 8).

Similarly, only $BaCl_2$, $CaCl_2$ and $FeCl_3$ positively affect GA activity (without pre-treatment with heat) with highest main effect value of $FeCl_3$ ions where $MgCl_2$ showed the highest negative main effect on the enzyme activity (Fig. 9).

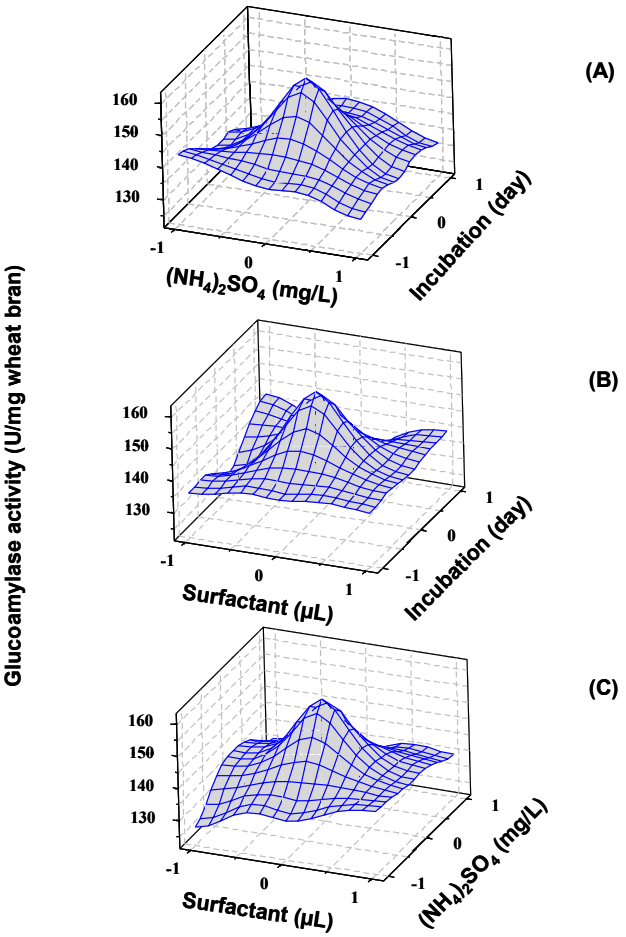

Fig. 5. Surface plot of independent variables that affect the GA activity based on the results of the Box-Behnken experiment vs. incubation and $(NH_4)_2SO_4$ (A), incubation and surfactant (B) and surfactant and $(NH_4)_2SO_4$ (C).

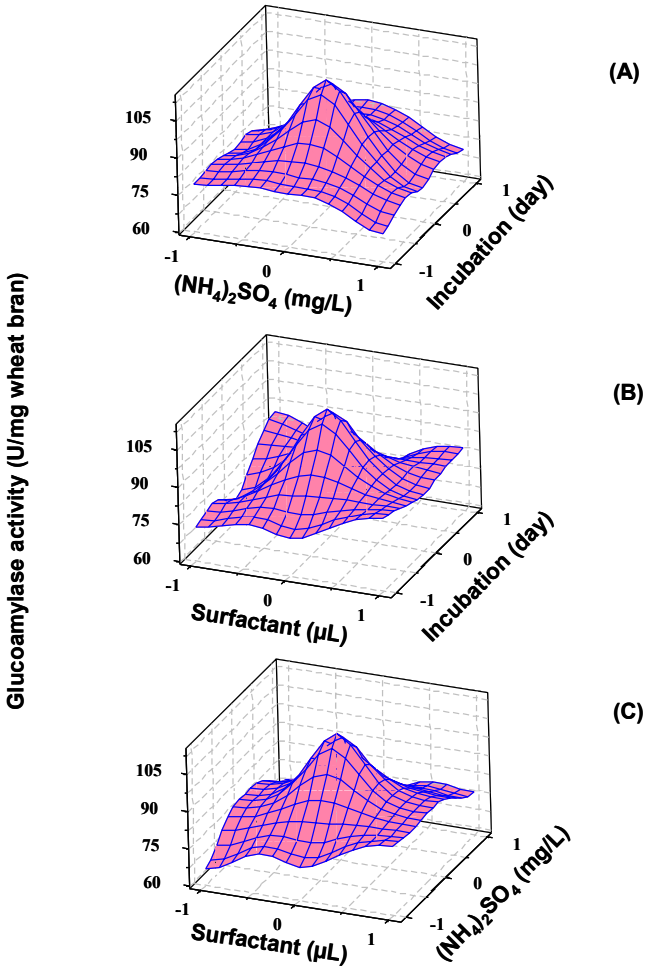

Fig. 6. Surface plot of independent variables that affect the GA activity after heat treatment at 80°C for 3hr based on the results of the Box-Behnken experiment vs. incubation and $(NH_4)_2SO_4$ (A), incubation and surfactant (B), and surfactant and (NH4)2SO4 (C).

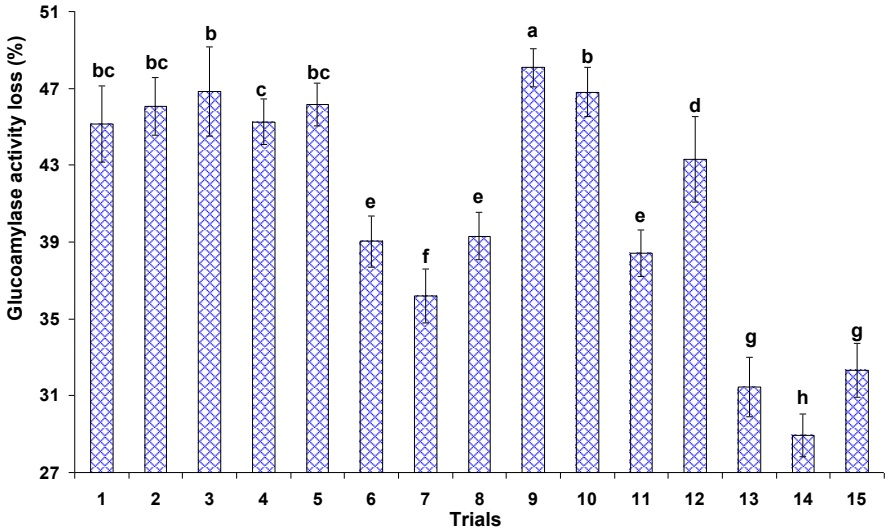

Fig. 7. Activity loss (%) of GA; due to heat treatment at 80°C for 3hr against different trials of Box-Behnken design [Data are the average and standard error of three replicates, different letters on the bars indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05)].

TABLE 3. Independent variables and their levels examined in the Plackett-Burman experiment for effect of ions (0 or 5mM) on thermostable glucoamylase (U/ml) at 50°C for 30min using 1ml enzyme and 1ml starch (1%) at pH 6.5.

Trials	Independent variables (mM)											Amylase activity ± SE
	$CoCl_2$	$NiCl_2$	$CuCl_2$	NH_4Cl	$MgCl_2$	$ZnSO_4$	$BaCl_2$	$CaCl_2$	$FeCl_3$	NaCl	EDTA	
1	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	171.1[a] ±0.77
2	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	155.1[c] ±0.90
3	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	110.2[e] ±0.74
4	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	110.0[e] ±1.34
5	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	107.1[e] ±1.14
6	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	91.0[f] ±0.53
7	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	-1 (0)	122.4[d] ±0.66
8	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	-1 (0)	122.5[d] ±0.74
9	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	110.6[e] ±0.77
10	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	-1 (0)	111.4[e] ±0.72
11	-1 (0)	+1 (5)	-1 (0)	-1 (0)	-1 (0)	+1 (5)	+1 (5)	+1 (5)	-1 (0)	+1 (5)	+1 (5)	119.4[d] ±1.26
12	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0)	161.0[b] ±1.40

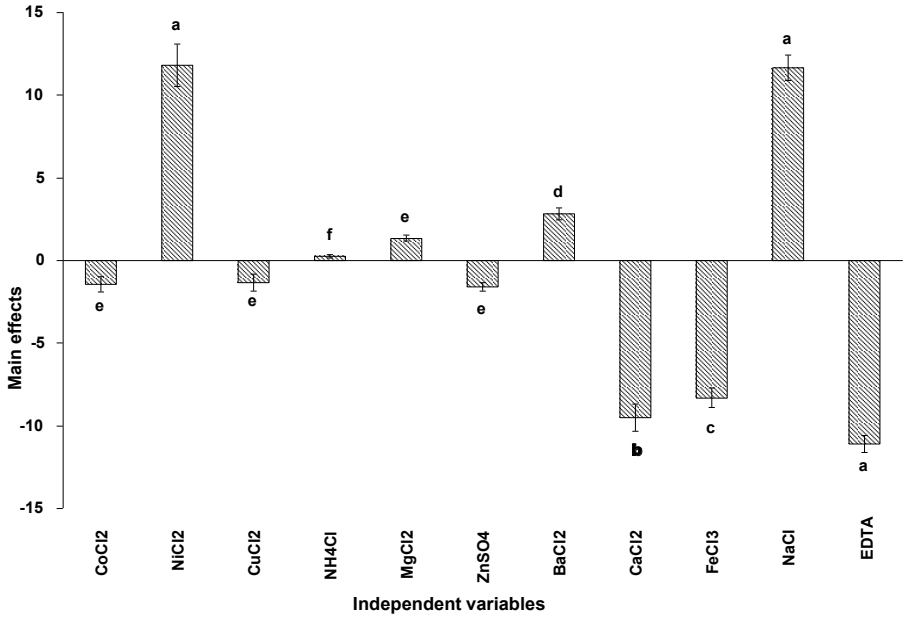

Fig. 8. Main effect of independent variables examined in Plackett-Burman design of ions effect on heat treated thermostable GA activity [Data are the average and standard error of three replicates, different letters on the bars indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05)].

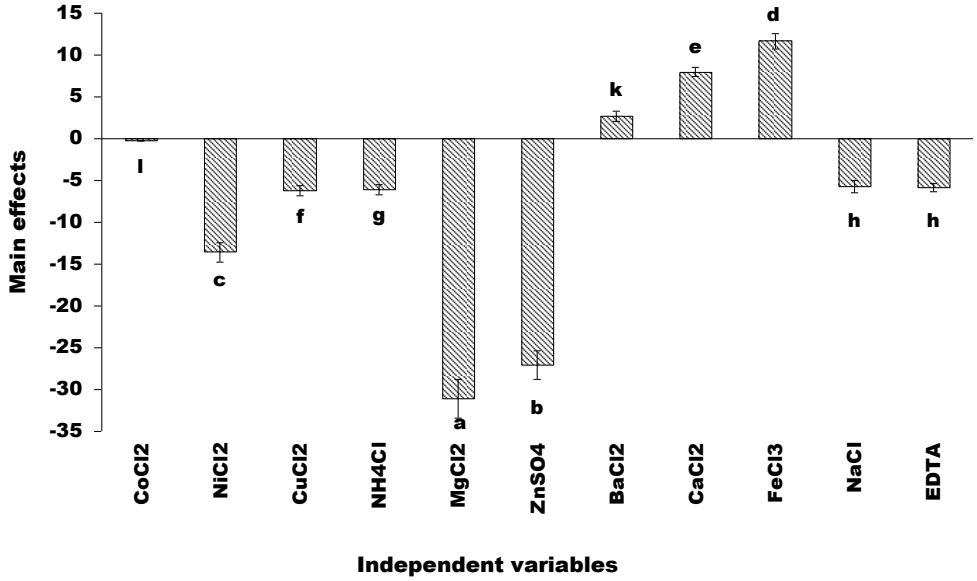

Fig. 9. Main effect of independent variables examined in Plackett-Burman design of ions effect on thermostable GA activity [Data are the average and standard error of three replicates, different letters on the bars indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05)].

Gel electrophoresis analysis of GA

Electrophoresis analysis of the crude enzyme (Fig. 10) revealed three bands were observed corresponding to the GA zymoforms.

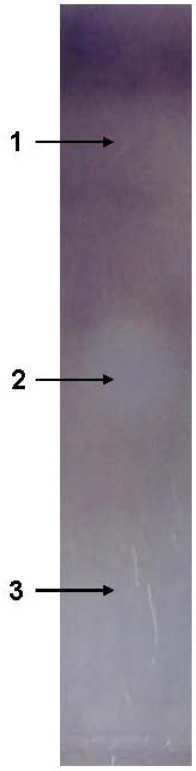

Fig. 10. Glucoamylase gel electrophoresis showing zymogram, under standard assay condition, arrows indicate GA zymoforms.

GA kinetic parameters

The enzyme kinetics is fundamental for large scale industrial applications. The kinetic constants K_m and V_{max} determined are 1.37mg/ml and 121.95U/ml, respectively (Fig. 11). Obvious small K_m value resulted from the high affinity of GA for starch. In addition, lower K_m allows quicker and easier industrial potential. Figure 12 showing the GA action on starch stains.

Discussion

In our continuous search for new sources of enzymes, fungi have their special importance as a wide field for this mission. One of the most important families of enzymes is amylases which account about 25 % of total enzyme sales worldwide. On the other hand, many of produced amylases and GAs are stable just under mild experimental and environmental conditions and their function are limited due to their inactivation by extreme temperatures, pH or, and their short half-lives.

Thermophilic and thermotolerant fungi are expected as a good source of highly thermostable GA. In this study, thermotolerant *A. fumigatus* was isolated from Egyptain soil and selected from other isolates as a good GA producer and as the authors are aware this is the first report about this fungus for GA production.

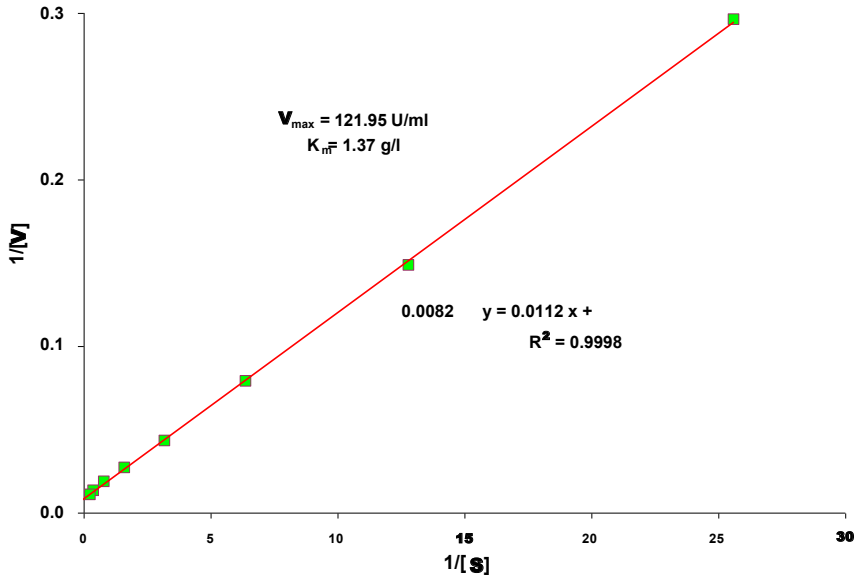

Fig. 11. The GA kinetic constants (K_m and V_{max}) calculated according to Lineweaver and Burk plot [Using different concentrations (0.04 to 3.75g/L) of soluble starch in 100mM sodium phosphate buffer, pH 6.5, incubated in a water bath at 50°C for 30min, data are the average and standard error of three replicates].

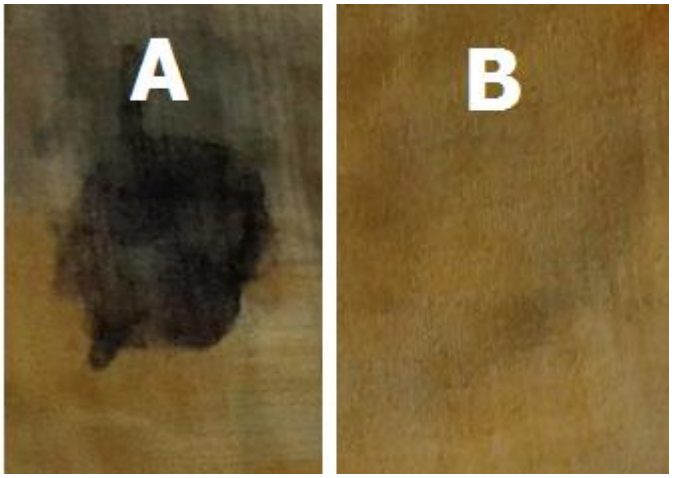

Fig. 12. The GA action: (A) Cloth piece without GA treatment, (B) Cloth piece with a starch stain with GA treatment for 1hr.

To maximize enzyme yield with a starch stain without increasing the cost of production, some agrowastes were tested as a substrates for GA production. Among five agrowastes tested, wheat bran was the most suitable one as it have large pores for mycelial penetration and passage (Kumar & Satyanarayana, 2004). In this connection, wheat bran was used by El-Gendy (2012) to improve rice bran properties for GA production. Fujio & Morita (1996) and Ramadas et al. (1996) recommended wheat bran for the enzyme production by *Rhizopus* SP.A-1 and *Aspergillus niger* based on its more compatibility with semisolid fermentation technique.

In general, statistically based experimental designs have proven to be valuable tools in optimizing microbial reactions (Mohammed & Badawy, 2017). One of the advantages of applying multi-factorial experiments is that such an approach considers the interaction between the non-linear natures of the responses in short experiments. The factorial design without losing information about the main effect of variables could reduce the number of experiments (Elibol, 2004). In the present study, environmental

factors affecting GA production by *A. fumigatus* has been investigated using the Plackett-Burman statistical design (Plackett & Burman, 1946). The results revealed that, ammonium sulfate, incubation period and surfactant (Berij 35) have the highest positive main effect on GA activity. For ammonium sulfate the supplementation of rice barn with nitrogen sources (organic or inorganic) enhance production of amylolytic enzymes by fungi (Pandey et al., 2005). On the other hand organic nitrogen sources negatively affected production of raw starch degrading enzymes by *Rhizopus sp*. MKU 40 since supplementation with organic nitrogen sources may induce the formation of protease, which resulted in the proteolysis of the enzyme degrading starchy materials (Morita & Fujio, 2000). For positive enzyme induction, Berij35 was used in this study. Hypothetically, surfactants can influence the production and secretion of raw starch degrading enzymes through changing the permeability of cell membrane. El-Gendy (2012) using Tween-80 and Triton X-100 which stimulated GA activity by 32 and 25%.

In this study, the approach of ions effect was carried out in different manner as it carried by matrix of Plackett-Burman. Ba, Ca and Fe positively affect GA activity with highest main effect value of Fe. Calcium ions have been implicated in the mechanisms involving thermal inactivation of *Bacillus* amylases. Almost all of the α-amylases require a certain quantity of calcium ions in the application, because their thermostability depends on the presence of structural calcium ions (Chiang et al., 1979). Mg, Zn, Ni, Cu, EDTA, Na showed negative main effect on activity. This inhibitory effect has also been reported for other GAs (Tosi et al., 1993; Kumar & Satyanarayana, 2003; Michelin et al., 2008). On the other hand, Bagheri et al. (2014) found that GA activity was enhanced slightly in the presence of EDTA, but strongly inhibited by Cu, Fe, Al and Hg. It is noteworthy that various enzymes from different fungal sources exhibit considerable differences of GA activity in the presence of metal ions.

The thermostability of crude GA from *A. fumigatus* was examined and data revealed that the enzyme lost only 28.95% of its initial activity after 3 h of heat exposure at 80°C. Comparing with other published data (Table 4) this is good property which could be due to higher concentration of charged residues on the surface of thermostable proteins and considerable increase in the proportion of Arg, Glu, Lys and Val whereas, Asn, Gln, Ser and Thr are less (Cambillau & Claverie, 2000). Moreover, surface salt bridges residues at the surface of proteins tend to be flexible and showed free intra protein interactions. These interactions enhance the thermotolerance of proteins from thermophilic organisms (Loladze et al., 1999).

The enzyme kinetics is fundamental for large scale industrial applications. In addition, lower Km allows quicker and easier industrial potential. The kinetic constants Km is 1.37mg/ml which is higher than that reported for *A. niger* NCIM (Selvakumar et al., 1996), *Sclerotium rolfsii* (Kelkar & Desphande, 1993) and *A. niveus* (Da Silva et al., 2009). Where it is similar to that reported for *R. oryzae* (Yu & Hang, 1991) and much lower than that of *A. niger* ATCC 10864 (4.76 mg/ml) (Silva, 2005).

TABLE 4 . Thermostability of GA from different fungal sources.

Fungus	Thermal stability	Ref.
Aspergillus fumigatus MG711601	Loss 28.95% after 3hr of heat exposure at 80°C	This study
Scytalidium thermophilum	Half-life around 30 rain at 60°C	Aquino et al. (2001)
Aspergillus sp. AS-2	Half-life of more than 2hr at 50°C	Soni et al. (2003)
F. solani	Half-life of 26min at 60°C	Bhatti et al. (2007)
Paecilomyces variotii	Half-life 45min at 60°C	Michelin et al. (2008)
Aspergillus niger B-30	Half-life 4min at 75°C and 2.8min at 80°C.	Yang et al. (2013)
Aspergillus niger	Half-life from 20–25min at 60°C to less than 3min at 80°C	Bagheri et al. (2014)
Aspergillus flavus	Retaining about 62% and 45% of the initial activity after 1hr of incubation at 60 and 70°C, respectively	Ayodeji et al. (2017)
Aspergillus japonicas	Lost 90% of its initial activity at 55°C.	Pasin et al. (2017)

Conclusion

The present study reveals that thermotolerant fungus *A. fumigatus* significantly produces thermostable GA suitable for biotechnological application. The fungus produce GA on wheat bran as a cost effective substrate and its production was considerably enhanced with $(NH_4)_2SO_4$ and Berij35 after 6 days. In addition, the enzyme showed good thermal stability. More studies must be conducted in improving this stability in both physiological and molecular levels.

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إنتاج وتطبيق انزيم الجلوكوآميليز متحمل للحرارة من فطرة اسبرجلس فيوميجاتس المتحملة لدرجة الحراره المرتفعه عبر التخمر شبه الصلب

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تم عزل فطرة اسبرجلس فيوميجاتس كسلالة متحمله لدرجات الحراره المرتفعه من تربة زراعية بمحافظة البحيرة بمصر. واختير من ضمن عزلات أخرى نظرا لنشاطه العالى فى انتاج انزيم الجلوكواميليز. تم تعريف الفطره ظاهريا بناءا على الصفات المورفولوجيه وجزيئيا بتحديد تسلسل ITS1-.5.8S rDNA-ITS2. لإنتاج إنزيم الجلوكواميليز استخدمت خمس مخلفات زراعيه هي نخالة القمح، قش القمح، قش الأرز، سرس الأرز وحبوب الأرز المكسرة عن طريق التخمر شبه الصلب. أوضحت النتائج أن نخالة القمح هي الأكثر ملاءمة للفطره لإنتاج الجلوكوأميليز والذي تحسن بشكل أكبر باستخدام تصميمات بلاكت بيرمان و بوكس بنكن. وجد أن الظروف المثلى لإنتاج الإنزيم هي نخالة القمح 1 جم، الرقم الهيدروجيني 6، ومستخلص الخميرة 6 ملج/جم، برج (35) 40 ميكرولتر/جم، كبريتات الأمونيوم 24 ملج/جم ولقاح 6 × 10^5 كونيده/مل مع عيار حيث ينتج الفطر 161.11 وحده دوليه/جم بعد 6 أيام من التحضين. تؤثر أيونات $FeCl_3$ و $BaCl_2$ و $CaCl_2$ تأثيرا إيجابًا على نشاط الجلوكواميليز مع أعلى قيمة تأثير رئيسية لأيون $FeCl_3$ بينما يكون لـ $MgCl_2$ التأثير السلبي الأعلى على النشاط . الثوابت الحركية للإنزيم هي 1.37 ملغم/مل. تطبيقيا أظهر الإنزيم تأثير تنظيف جيد عند معاملته لبقع النشا في النسيج كما اظهر ايضا قدرة جيده على التحمل الحرارى حيث خسر 28.95% فقط من نشاطه الأصلي بعد معاملته حراريا عند 80 درجة مئوية لمدة 3 ساعات. وأخيراً، يمكن إنتاج انزيم الجلوكواميلز من فطرة اسبرجلس فيوميجاتس بإستخدام مخلفات زراعيه رخيصة وعن طريق تقنية تخمير بسيطة مع ميزات كيميائية حيوية جيدة تجعله مفيدا فى كثير من تطبيقات التكنولوجيا الحيوية.