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Thermodynamic characterization of *Aspergillus flavus* KP998209 xylanase with production statistical optimization

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Abstract

This study succeeded in studying the physiochemical, kinetics and thermodynamic characteristics of *Aspergillus flavus* KP998209 xylanase. The *A. flavus* xylanase production utilizing corn cobs as xylan substrate was improved through two steps statistical factorial designs namely Plackett–Burman design and Taguchi L9 (3^4) orthogonal array designs causing 1.52-fold and 2.39-fold increase, respectively in xylanase production compared to un-optimized medium. Moreover, *A. flavus* xylanase kinetics were determined to be 5.68 mg.ml^{-1} for K_m, 1.715 U.mg^{-1} for V_{max} and 73.74 S^{-1} for K_{cat}. Besides, the calculated values for oat xylan hydrolysis thermodynamics including ΔH* (13.41 kJ.mol^{-1}), ΔG* (67.76 kJ.mol^{-1}), ΔS* (−168.27 J.mol^{-1}K^{-1}), $\Delta G^*_{E\text{-}S}$ (4.665 kJ.mol^{-1}) and $\Delta G^*_{E\text{-}T}$ (−6.88 kJ.mol^{-1}). $T_{1/2}$ (half life), K_d (Thermal deactivation constant) and D-values at 50, 55 and 60 ºC were determined to be 143.48, 68.76, 45.35 min, 4.83x10^{-3}, 10.08x10^{-3}, 15.28x10^{-3} min^{-1} and 476.81, 228.47, 150.71 min, respectively. Moreover, *A. flavus* xylanase denaturation thermodynamics including ΔH_d, ΔG_d and ΔS_d at 50, 55 and 60 ºC emphasized its suitability for industrial applications.

Key words: Agricultural wastes; xylan; xylanase; statistical factorial design; thermodynamics. Introduction.

1. Introduction

There is a continuous search for low and safe resources for the production of valuable byproducts. Lignocellulosic wastes are one of these good choices for the production of bioactive compounds, industrial **enzymes**, nanoparticles, improved animal feed materials, organic acids and bioethanol [1-7]. Lignocellulosic wastes composed mainly of lignin (10-25%, a complex polyphenolic structure), hemicelluloses (20-30%, complex polysaccharide network of xylose, glucose, and mannose), and 40–50% cellulose (polysaccharide consisting of β-1,4-linked D-glucose units) of dry weight [8].

Lignocellulosic wastes can serve as fermentation substrate by solid state (SSF) and sub-merged (SMF) fermentation techniques. But the enzymes production by SSF technique is more favorable than SMF because the former has provided quite a lot of advantages in productivity, cost-effectiveness, time and medium components beside some environmental advantages such as less effluents production and waste minimization [9].

Corn cobs, generated worldwide is approximately 144 million tons per year, are rich source of cellulose (27.71%) and hemicellulose (38.78%) but also contain a significant amount of lignin (9.4%) (Fig. 1a). corncobs can serve as a value-added biosorbent for the removal of toxic organic and inorganic pollutants from wastewaters [10-11].

Xylan is a linear β-(1, 4)-D-xylose backbone and substituted with different side chains especially α-L-arabinosyl and α-D-glucuronosyl units. In corn cobs, xylan is composed of 4-O- methyl- D -glucuronic acid, L- arabinose and D- xylose in the ratio 2:07:19 (Fig. 1b) [12]. The conversion of xylan into soluble sugar as an intermediate step in the biofuel production can be achieved, chemically by either acid

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or alkaline, or enzymatically. The latter is more desirable due to the high specificity, low energy consumption, no chemical requirement, and mild environmental conditions thus avoiding sugar degradation and resulting in high sugar yields [13]. The enzymatic hydrolysis of xylan requires the synergistic action of, mainly xylanases (1, 4-β-D-xylan xylanohydrolase, EC 3.2.1.8) [14] and β-xylosidases (1, 4-b-D-xylan xylohydrolase, EC 3.2.1.37) [15].

There is an increasable demand for xylanases due to their enormous industrial applications, as a biobleaching agent in paper industry [16], in animal feed, production of xylo-oligosaccharides as food additive and degumming [17-18], fruit Juice clarification and baking industry [19], Most recently, there have been reports of the use of xylanases to biofabricate silver, gold, and silver-gold alloy nanoparticles for biomedical and environmental applications in the emerging field of nanobiotechnology [20-22].

Xylanolytic enzymes have been reportedly produced by many microorganisms, including bacteria, fungi, actinomycetes, and yeasts. Filamentous fungi are most interesting producers of xylanases owing to the fact that they produce extracellular xylanase [23].

Optimization of fermentation conditions using conventional methods is time consuming and costly, especially for a large number of variables. Statistical experimental designs are alternative strategies that involve a minimum number of experiments for a large number of factors. These methods have been employed to improve the production of enzymes.

The object of this study was to utilize agro-wastes for the production of xylanase by fungal isolate through by two techniques (SSF and SMF) with production enhancement through two steps statistical factorial designs (Plackett–Burman design and Taguchi L9 (3^4) orthogonal array designs). Besides, a full physiochemical, kinetic and thermodynamic characterization of the produced xylanase.

2. Materials and methods

2.1. Isolation and identification

The fungal isolate was isolated from soil by suspending ten grams of fresh soil in 100 ml of sterile water; the mixture was shaken for 30 min at 200 rpm, and 0.1 ml of the suspension was spread on xylan agar medium prepared by dissolving g.l^{-1}: 10 xylan and 20 agar. Colonies showing good growth were selected, purified and screened for xylanase production. The most potent xylanase producer fungal isolate was genetically identified as *Aspergillus flavus* isolate NERMEEN12 with accession number KP998209 in Genbank by Sigma scientific services company, Egypt. The strain was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured at monthly intervals.

2.2. Lignocellulosic wastes

Agro-residues (rice straw (RS), corn cobs (CC), wheat bran (WB), saw dust (SD)) were collected from local market in Egypt. They were washed with distilled water to remove unwanted dust particles and then dried in an oven (70 °C for 24 h). The dried materials were ground in an electric grinder, separated by 1 cm sieve and packed in air-tight containers for use as the substrate for xylanase production by SSF and SMF techniques.

2.3. Determination of hemicelluloses content of agro-wastes

The hemicelluloses contents of rice straw, wheat straw, wheat bran, saw dust and corn cobs were determined as shown in Table (1) according to Chen and Anderson [24] as follows:

The agro-waste (10 g) was suspended in 100ml 10% NaOH (w/v) for 24 h at 30ºC followed by filtration and washing with distilled water. The filtrate pH was adjusted to 5 with HCl followed by adding 150 ml 95% ethanol allowed for standing for 24 h. The precipitate was washed with 70% ethanol to remove lignin followed by dehydration with 95% ethanol then filtration and vacuum drying at 50ºC.

2.4. Xylanase production

Two techniques (SSF and SMF) utilizing different lignocellulosic wastes (WS, RS, CC, WB and SD) as xylan substrate were used for xyalnase production.

In SSF, lignocellulosic wastes were added on equal hemicellulose content (0.366 g hemicelluloses/flask) in 250 ml Erlenmeyer flasks were moistened with 10 ml of mineral salts solution contained (g/l) according to Li et al. [25]: KH_2PO_4, 1.0, NaCl, 1.0, $MgSO_4.7H_2O$, 1.0, $CaCl_2.2H_2O$, 0.5, yeast extract, 5.0 and pH 5.0. After sterilization the flasks were inoculated with 1 ml of spore suspension containing $1x10^6$ spores of 5 days old culture which was prepared by harvesting the slant of the fungus in 20 ml sterile distilled water. The inoculated flasks were incubated for different fermentation period (1,

3, 5, 7 and 11 days) at 30°C under static conditions at the end of the fermentation period the xlanase was extracted by the addition of 50 ml distilled H_2O to the flasks for 60 min on shaker 150 rpm followed by centrifugation at 4000 rpm for 20 min, and the supernatant was used as crude xylanase.

In SMF technique, the lignocellulosic waste was moistened with 50 ml of the mineral salts solution previously mentioned. The inoculated flasks were incubated for different fermentation period 1, 3, 5, 7 and 11 days at 30°C in a shaking incubator at 150 rpm. At the end of the fermentation period, the cell-free culture filtrate, obtained after filtration and centrifugation at 4000 rpm and 4 °C for 20 min was used as xylanase enzyme.

1.1. Xylanase assay

Xylanase activity was determined by incubating reaction mixture that contained 0.5 ml of enzyme solution and 0.5 ml of 1% xylan (0.05 M acetate buffer, pH 5.0) for 30 min at 50 ºC according to Warzywoda et al. [26]. The amount of reducing sugar liberated was quantified by the methods of Neish [27] using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute under assay conditions.

1.2. Determination of protein:

The protein content of the enzyme preparation was estimated according to Lowry et al. [28].

1.3. Agricultural waste quantity

Different weights of corn cobs were added depending on xylan content (0.25, 0.366, 0.5 and 1.0 g/flask) according to Table 1. Enzyme assay was carried out after 168h of incubation under optimized conditions.

1.4. Statistical optimization of *A. flavus* KP998209 xylanase production

1.4.1. Plackett–Burman design (PB)

In this step, we tested the qualitative effect of nineteen factor namely; Initial pH (a), Moisture content (B), fructose (C), carboxymethyl cellulose (CMC) (D), yeast extract (E), peptone (F), urea (G), $MgSO_4.7H_2O$ (H), KH_2PO_4 (J), $CaCl_2.2H_2O$ (K), NaCl (L), KCl (M), $FeCl_2.4H_2O$ (N), $MnSO_4.H_2O$ (O), K_2HPO_4 (P), Tween 80 (Q), $ZnCl_2$ (R), $CoCl_2.6H_2O$ (S) and $CuSO_4$ (T) at two levels (+1 and -1) on *A. flavus* KP998209 xylanase production resulting in 20 run.

1.4.2. Taguchi methodology

In this design the quantitative effect of the most effective factors predicted ($CaCl_2.2H_2O$, $CuSO_4$, CMC and NaCl) from the PB design by Design-Expert®8 software from Stat-Ease, Inc. were studied by testing these factors with three levels.

1.5. Physiochemical characterization of *A. flavus* KP998209 xylanase

The optimum temperature and substrate concentration conditions for maximam xylanase activity were determined by carrying out the reaction at, different temperature degrees 30, 40, 50, 60 and 70 ºC, different xylan concentrations 0.25- 4% for maximum xylanase activity were examined. The thermostability of xylanase was investigated by incubating enzyme solutions without substrate at different temperatures (40 to 60 °C) for different incubation periods (15, 30, 45 and 60 min), after which the residual activity was determined under optimum assay conditions

Effect of metal ions and inhibitors on xylanase activity were studied by pre-incubating 5 mM of different metal ions including Ca^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Hg^{2+}, Ba^{2+} and EDTA with the enzyme at 30 °C for 30 min. Then, the reaction was carried out under optimum conditions and residual activities were determined with considering the activity in the absence of metal ion as 100 %.

1.6. Kinetic and thermodynamic characterization of *A. flavus* KP998209 xylanase

The Michaelis-Menten constant K_m and maximum velocity V_{max} are critical enzyme parameters that determine the enzyme sensitivity and were determined from Lineweaver-Burk plot.

Among the most important criteria of any industrial enzyme is the activation energy (E_a), and activation energy (E_d) for denaturation were determined by an Arrhenius plot of log denaturation rate constants (ln k_d) versus reciprocal of the absolute temperature (K) using the following equation:

$$Slope = {}^{-E_d}/_{R}$$

The thermodynamics of substrate hydrolysis including specificity constant (V_{max}/K_m), the turnover number (k_{cat}), catalytic efficiency (K_{cat}/K_m), free energy of transition state binding (G^*_{E-T}) and free energy of substrate binding (G^*_{E-S}) were determined as described by Abdel-Naby et al. [29] as follows:

$$K_{cat} = ({k_b T}/{h}) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)}$$

$$\Delta H^* = E_a - RT$$

$$\Delta G^* = -RT \ln\left(k_{cat}\, {h}/{k_b} \times T\right)$$

$$\Delta S^* = {(\Delta H^* - \Delta G^*)}/{T}$$

Free energy of substrate binding

$$\Delta G^*_{E-S} = -RT \ln K_a$$

, where $K_a = {1}/{k_m}$

Free energy for transition state formation

$$\Delta G^*_{E-T} = -RT \ln\left({k_{cat}}/{k_m}\right)$$

The thermodynamics of xylanase denaturation including the half-life ($T_{1/2}$, min^{-1}), D-values, enthalpy (ΔH_d, $kJ.mol^{-1}$), free energy (ΔG_d, $kJ.mol^{-1}$) and entropy (ΔS_d, $J.mol^{-1}.K^{-1}$) for thermal denaturation of xylanase are important thermostable parameters that were determined from the following equations:

$$T_{1/2} = {ln2}/{k_d}$$

$$D - value = {ln10}/{k_d}$$

$$\Delta H_d = E_d - RT$$

$$\Delta G_d = -RT.\ln\left({k_d.h}/{K_b.T}\right)$$

$$\Delta S_d = {(\Delta H_d - \Delta G_d)}/{T}$$

Where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J. mol^{-1}. K^{-1}), h is the Planck constant (6.626 X 10^{-34} J. s), K_b is the Boltzman constant (1.38 X 10-23 J. K^{-1}) and K_d is the deactivation rate constant (min^{-1}).

2. Results and discussion

2.1. Xylanase production by *A. flavus* KP998209

The cost of carbon source plays significant role in the economics of xylanase production. Recently the use of lignocellulosic materials as substrates instead of the expensive pure xylan is gaining ground to reduce the cost of xylanase production. The xylanase production profile by SSF and SMF for different fermentation periods as shown in Fig. (2a, 2b) indicated many points as following: firstly, *A. flavus* KP998209 xylanase can be produced by both SSF and SMF techniques but more effectively by SSF technique. Secondly, *A. flavus* KP998209 was capable of utilizing all the fermented lignocellulosic wastes for xylanase production. The order of xylanase production by SSF on different lignocellulosic wastes was as following CC>RS>WS>WB>SD. This can be attributed to high hemicelluloses content of CC (21.25%), RS (18.28%) and WS (21.09%). The highest xylanase production was obtained on CC by SSF (0.70 $U.ml^{-1}$) and SMF (0.68) after 3 and 7 days of fermentation, respectively and prolonged incubation resulted in a decline in xylanase production. Also, Elegbede and Lateef [30] reported the highest *Aspergillus fumigatus* SD5A xylanase after 7 days by SMF. Our results were higher than that obtained by Sharma et al. [31] for xylanase production by *Fusarium* sp. XPF5 (0.55 $U.ml^{-1}$) after 4 days of fermentation. The decrease in production may be due to the depletion of available nutrients or due to the proteolysis [32]. These results coincide with that reported by Oliveira et al. [33], Alves-Prado et al. [34] and Da Silva et al. [35] from *Penicillium janthinellum CRC 87M-115*, *Neosartorya spinosa* and *Thermoascus aurantiacus*, respectively on corn cobs. This can be attributed to its lowest lignin content according to Nigam et al. [36] which act as strong barrier for contact between enzyme and xylan in agricultural waste. However, Liao et al. [37] reported that wheat straw was the most effective for xylanase production among the lignocellulosic materials (corn stover, rice straw, wheat bran, and wheat straw)

Furthermore, xylanase production in SSF increased with an additional concentration of corn cobs from 2.5% (0.562 $U.ml^{-1}$) to 5% (w/v) (0.758 $U.ml^{-1}$) (data not shown). The use of 5% corn cobs caused 8.28% enhancement in xylanase activity. Further increase in corn cobs concentration did not show positive effect. Sepahy et al. [38] reported maximum xylanase production using 2% (w/v) oat bran as xylan source.

2.2. Statistical Optimization of *A. flavus* xylanase production

2.2.1. Plackett–Burman design (PB)

As shown in Table (2a) there was a remarkable variation in xylanase activity (0.515-1.153 $U.ml^{-1}$) achieving 1.52-fold increase in xylanase activity in run 20. *A. flavus* xylanase activity can be calculated from the following equation

Xylanase activity ($U.ml^{-1}$)= 0.859 – 0.011*moisture content– 0.007*fructose + 0.022*CMC + 0.007*peptone – 0.062*Urea + 0.022*KH_2PO_4 + 0.171*$CaCl_2.2H_2O$ + 0.103*NaCl + 3.94*$MnSO_4.H_2O$ + 0.045*K_2HPO_4 –0.0199* Tween80– 1.752* $ZnCl_2$ – 74.4* $COCl_2.6H_2O$ + 0.762* $CuSO_4$.

The effect of the tested factors on *A. flavus* xylanase production as shown in Pareto chart (Fig. 3) indicated that, fourteen factors of the tested nineteen factors were significant and were distinguished into, eight positive factors (CMC (D), peptone (F), KH_2PO_4 (J), $CaCl_2.2H_2O$ (K), NaCl (L), $MnSO_4.H_2O$ (O), K_2HPO_4 (P), $CuSO_4$ (T)) coinciding with that reported by Long et al. [39] for the positive effect of peptone and $CaCl_2$ on *Trichoderma orientalis* xylanase production. While Rathee et al. [40] reported the enhancement effect of fructose on *Bacillus tequilensis* xylanase production. Irfan et al. [41] found that supplementation of medium with xylose enhanced *Trichoderma viride*-IR05 xylanase production while arabinose showed low enzyme productivity. Modi et al. [42] reported the maximum *Aspergillus awamori* xylanase production with mixture of NH_4NO_3 and urea supplementation. Long et al. [39] reported the positive effect of tween 80 on *T. orientalis* xylanase production.

The other five factors including initial pH (A), yeast extract (E), $MgSO_4.7H_2O$ (H), KCl (M) and $FeCl_2.4H_2O$ (N) were not significant on *A. flavus* xylanase production. However, Tallapragada and Venkatesh [43] and Yadav et al. [44] reported the stimulation effect of yeast extract on *A. niger* and *Anoxybacillus kamchatkensis* xylanase production.

The success of the design was emphasized by, the analysis of variance (ANOVA) as shown in Table (2b), and the values of R^2 (0.9934) was so close to 1.00, adjusted R^2 (0.9748) and predicated R^2 (0.8938) were close to each other. The value of R^2 means that 99.34% of the results can be explained by the design.

2.2.2. Taguchi L9 (3^4) orthogonal array design

The four variables chosen from the PB design, $CaCl_2.2H_2O$, $CuSO_4$, CMC and NaCl were optimized by Taguchi design. As shown in Table (3a) the maximum xylanase production was obtained in run 7 (1.816 $U.ml^{-1}$) causing 2.39-fold improvement in xylanase production compared to un-optimized medium (0.758 U/ml). *A. flavus* xylanase activity can be calculated from the following equation

Xylanase activity ($U.ml^{-1}$)= 1.010889 + 0.517778* $CuSO_4$ [1] – 0.06322* $CuSO_4$ [2] – 0.02589* CMC [1] + 0.415444* CMC [2] + 0.108111* NaCl [1] + 0.027778* NaCl [2]

These results were similar to that obtained by Gowdhaman et al. [45] using Box–Behnken design causing 2.42- fold enhancement. Maciel et al. [46] using statistical experimental designs reported 1.5-fold increase. Zhang et al. [47] using RSM reported 1.99-fold increase, while Valte et al. [48] using statistical methods of media optimization documented 1.31 enhancement. The maximum xylanase production was obtained when the medium contained ($g.l^{-1}$): CMC, 10, yeast extract, 1, peptone, 10, urea, 0.3, $MgSO_4.7H2O$, 0.5, KH_2PO_4, 0.25, $CaCl_2.2H_2O$, 1; K_2HPO_4, 1.5, NaCl, 10, $CoCl_2.6H_2O$, 0.001, $CuSO_4$, 0.2, Tween 80, $2ml.l^{-1}$, pH 5.

The interactive effect of two factors on the xylanase production is shown in Figs. 4 (a,b,c). The analysis of variance (ANOVA) as shown in Table (3b) indicated that the model was significant. The fitting of model was confirmed by the determination of the R^2 (0.998), indicating that 99.8 % of total response variability could be explained by the model. As the value of R^2 was closer to 1, the better was the correlation between the experimental and predicted values. The predicted R^2 (0. 9624) was in reasonable agreement with the adjusted R^2 0. 9925.

2.3. Characterization of *A. flavus* KP998209 xylanase

As shown in Fig. (5a), the maximum *A. flavus* KP998209 xylanase activity was obtained at 50 ºC similar to that reported for xylanases from *Penicillium oxalicum GZ-2* [37], *Trichoderma inhamatum* [49], *Aspergillus parasiticus* URM 5963 [50] and *Penicillium chrysogenum* P33 [51] and higher than that reported for xylanases from *Aspergillus niger* DFR-5 [52], *Fomes fomentarius* [53] and *Bacillus* sp. [54]. The thermostability profile of *A. flavus* KP998209 xylanase at 55 and 60 °C (data not shown) revealed that the enzyme was thermostable with approximately 54.6 and 21.12% residual activity at 55 and 65 ◦C, respectively, after 1 h of heat pretreatment nearly similar to *Penicillium oxalicum GZ-2* xylanase [37]. As shown in Fig. (5b) the maximum *A. flavus* KP998209 xylanase activity was obtained at pH 4 similar to xylanase produced by *Penicillium oxalicum* GZ-2 [37] suggesting that it may be of great value in fruit juice processing where the acidic pH is favored. The maximum *A. flavus* KP998209 xylanase activity was obtained with 1.5% oat xylan (data not shown) afterwhich any increase in xylan concentration did not cause more enzyme activity, this may be due to the full saturation of enzyme active sites. As shown in Fig. (5c) *A. flavus* KP998209 xylanase activity was negatively influenced by many metal ions with different degrees. It was strongly inhibited by Hg^{2+} and Cu^{2+}causing 88.01, 68.33%, respectively reduction in the activity similar to xylanases from *Trichoderma inhamatum* [49], *Penicillium janczewskii* [55]. Inhibition by Hg^{2+} seems to be a common characteristic of xylanases, demonstrating the existence of cysteine thiol groups near or in the $_{active}$ site of the enzyme [56]. It was also obvious that the effect of metal ion differs according to the enzyme microbial source that *A. flavus* KP998209 xylanase was activated by Ca^{2+} causing 22.4% increase in enzyme activity similar to its effect on *P. janczewskii* xylanase [55]. Co^{2+} caused 79.67% reduction in the activity in contrast to that reported by Terrasan et al. [55] for *P. janczewskii* xylanase.

3.4. Kinetics and thermodynamics characterization of *A. flavus* KP998209 xylanase

A. flavus KP998209 xylanase kinetics including K_m, V_{max} were determined from lineweaver-burk plot (Fig. 6a) to be 5.68 $mg.ml^{-1}$ and 1.715 $U.mg\ protein^{-1}$, respectively. K_m value reflects the sensitivity of the enzyme to the substrate as the value decreases as the sensitivity increases. *A. flavus* KP998209 xylanase K_m on oat xylan was nearly similar to that of *Bacillus* (5.26 $mg.ml^{-1}$) [57] and *Paenibacillus* sp. NF1 (5.64 $mg.ml^{-1}$) [58], and lower than that reported for xylanases from *Trichoderma inhamatum* (14.5 $mg.ml^{-1}$) [49] and *Trichoderma longibrachiatum* (20 $mg.ml^{-1}$) [59]. As shown in Table 4, catalytic efficiency (K_{cat}/K_m) which reflects the ability of the enzyme to hydrolyze the substrate was determined to be 12.98 S^{-1}.mg and as this value increases as the efficiency in degrading xylan increased. Also, The V_{max}/K_m value was taken as the criterion to evaluate the substrate specificity according to Abdel-Naby et al. [29]. Moreover ΔH^*, ΔG^*, ΔS^*, ΔG_{E-S}, ΔG_{E-T} for xylan hydrolysis were also calculated which are very characteristic for the enzyme as mentioned by Abdel-Naby [60]. ΔG^* is a measure for conversion of an enzyme-substrate complex into a product. Thus, the low ΔG^* value suggests that the conversion of a transition state of enzyme-substrate complex into a product was more spontaneous. Q10 value is a term used to determine whether or not the metabolic reactions are mainly controlled by temperature or by some other factors [61] meaning that the reaction is controlled by factors other than the temperature if the value exceeds 1 [62].

Thermodynamics are the best indicator for the suitability of the enzyme for industrial application. The E_a (activation energy) and E_d (activation energy of denaturation) of *A. flavus* KP998209 xylanase were determined from Arrhenius plots (Figs. 6b, 6c) to be 16.1 and 98.68 $KJ.mol^{-1}$, respectively. The E_a and E_d are very important criteria for thermostable enzyme meaning that thermostable enzyme is characteristic with low E_a and high E_d. *A. flavus* KP998209 xylanase E_a was nearly similar to that of *T. longibrachiatum* (15.79 $KJ.mol^{-1}$) [59] and lower than that of *Melanocarpus albomyces* (150.1 $KJ.mol^{-1}$) [63] and *Arthrobacter* sp. GN16 (27.08 $KJ.mol^{-1}$) [64]. As shown in Table (5) $T_{1/2}$ values at 60 and 65 °C were 45.35 and 26.74 min, respectively higher than those, for *P. janczewskii* 16 and 6 min [55] and *M. albomyces* 12.2 and 10.1 min [63] at the same temperatures. The values of ΔH_d at 55, 60 and 65 °C were very close to that at 50 °C indicating high resistance of *A. flavus* xylanase towards thermal unfolding at higher temperatures [65]. The lower values of ΔS_d for enzyme suggests an increased compactness and high resistance for thermal

inactivation [52]. *A. flavus* xylanase ΔS_d values at 50, 55, 60 and 65 °C (7.27, 8.60, 7.42 and 7.31 $J.mol^{-1}K^{-1}$) were much lower than those for xylanase from *M. albomyces* (165, 163, 172 and 167 $J.mol^{-1}K^{-1}$)

3. Conclusion

Corn cobs succeeded as a xylan substrate for the production of industrial potent xylanase by SSF and SMF techniques without any pretreatment by the fungal isolate *A. flavus* KP998209. After the optimization by using Plackett–Burman and Taguchi designs, the optimal medium composition for the maximum xylanase production was found to be (g/l): fructose, 5.0; CMC, 10.0, urea, 0.3, yeast extract, 10.0, peptone, 10.0, $MgSO_4.7H_2O$, 5.0, KH_2PO_4, 0.25, $CaCl_2.2H_2O$, 1.0, NaCl, 10.0, K_2HPO_4, 1.5, $CoCl_2.6H_2O$, 0.001, $CuSO_4$, 0.2 and Tween80, 2ml added to 2.35 $g.flask^{-1}$ corn cobs causing 2.39-folds increase in xylanase production compared with the un-optimized medium. Moreover, the kinetics and thermodynamics of *A. flavus* KP998209 xylanase emphasized its suitability for saccharification of many agro-wastes for hydrolyzing their hemicellulose into reducing sugar which is a critical step in biofuel industry.

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Conflict of interest

The authors confirmed there is no conflict of interest.

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