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A Thermodynamic Study of Partially-Purified *Penicillium humicola* β-mannanase Produced by Statistical Optimization

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Abstract

A sequential optimization strategy, based on statistical experimental designs, is employed in this study to enhance the β-mannanase production from *Penicillium humicola* in a medium containing coffee waste as a carbon source. A two-level Plackett–Burman design was applied to differentiate between the bioprocess parameters that significantly influence the β-mannanase production followed by fractional factorial design (FFD) to optimize the most significant variables for the highest yield of β-mannanase. Overall, more than a 3.4-fold improvement in enzyme production was achieved due to the statistical optimization. Partially, the purification of β-mannanase was achieved by a 60-70 % saturation of ammonium sulfate with specific activity 27.83IU/mg protein. The partially-purified β-mannanase showed an optimum activity at pH5.5 and retained 80-86 % of its original activity at pH ranging from 4.5 to 6 after 120 minutes. The optimum temperature for the *Penicillium humicola* β-mannanase was 60°C, and the activation energy (Ea) was 16.71kJmol^{-1}. Thermal stability and the thermodynamic parameters of β-mannanase are studied at temperatures ranging between 40 and 60°C.

Keywords: β-mannanase, *Penicillium humicola*, Coffee residues, Statistical designs, Partial purification, Characterization.

1. Introduction

β-Mannan and its heteropolysaccharides are found in many natural products such as copra, guar beans, ivory nuts, locust beans, roots of konjak, palm kernel, aloevera, coffee beans, wheat bran, and the hemicelluloses of soft and hard woods (Kurakake and Komaki., 2001; Keawsompong., 2016) .

A large amount of by-products are generated from the industrial use of these natural products. For example, the coffee industry is responsible for the generation of a large amount of wastes represented mainly in spent coffee ground (SCG); (Mussatto *et al*., 2011; Obruca *et al*., 2015). Thus, taking into consideration that the annual production of coffee beans exceeds eight million tons (Murthy *et al*., 2012) and that one ton of green coffee generates about 650 Kg of SCG (Obruca *et al*., 2015), one becomes well aware of the highly polluting residues of SCG. All of these wastes, arising from agricultural and industrial applications, contain around 30-35 % of polysaccharides (mainly mannan) (Chiyanzu *et al*., 2014 and Ahirwar *et al*., 2016) which may support pathogenic bacteria growth, and cause massive environmental pollution. Therefore, the safe disposal of these wastes or the proper utilization of them are practical methods to be worked on. The application of innovative methods such as enzymatic treatments may also be a promising strategy to transfer these wastes into raw materials for other processes. Moreover, the usage of these wastes to produce valuable products such as enzymes was reported by many researchers (Regalado *et al*., 2000; De Marco *et al*., 2015; Ahirwar *et al*., 2016 and Soni *et al*., 2016).

β-Mannanase (1,4- β-D-mannan mannanohydrolase; 3.2.1.78) is the enzyme that cleaves the β-1, 4-mannosidic linkages in mannans, galactomannans, glucomannans, and galactoglucomannans (El-Naggar *et al*., 2006, Abdel-Fattah *et al*., 2009). Therefore, due to its broad substrate specificity, β-mannanase has a great potential in many industrial applications including foods, feed for animals and plants, pulp and paper, pharmaceuticals and cosmetics, the production of mannan and manooligosaccharids, bioethanol and biodiesel productions, and oil and textile industries (Keawsompong., 2016; Obruca *et al*., 2015; Chiyanzu *et al*., 2014; Ahirwar *et al*., 2016; Soni *et al*., 2016; Kwon *et al*., 2013; and Soni *et al*., 2017). Moreover, β-Mannanase is produced by different microorganisms (Ahirwar *et al*., 2016; Soni *et al*., 2016; Soni *et al*., 2017; Chauhan *et al*., 2012; El-Refai *et al*., 2014; Germec *et al*., 2017).

The goal of the current research is to produce economical β-mannanase from a non-costly production medium using coffee waste. The statistical optimization of the production medium is a promising strategy to increase the yield of enzymes. The Placket-Burman design and Fractional Factorial design are applied in this research to

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optimize the production of β-mannanase from *Penicillium humicola*. A partial purification and characterization of *Penicillium humicola* β-mannanase were also carried out to increase the specific activity of the enzyme.

2. Materials and Methods

2.1. Materials

For this research, locust bean gums (LBG) were purchased from Sigma Chemical, and the Brazilian coffee waste was obtained from a local market. All other chemicals used in this study are analytical grade chemicals.

2.2. Microorganism

The fungal strain *Penicillium humicola* was obtained from the culture collection of the National Research Center in Dokki, Cairo, Egypt, and was morphologically identified by the Micro Analytical center. The culture was maintained on potato dextrose agar and incubated at 30°C for seven days before storage at 4°C with a monthly sub-culturing. The fungal cells were preserved at -80°C in a solution containing 50 % glycerol.

2.3. Cultivation Conditions and Enzyme Production

Inoculum preparation was carried out by transferring the spore suspensions of the well growth slant of the *P. humicola* into a 250 mL Erlenmeyer flask containing 50 mL sterilized medium which consisted of (g/L): locust bean gum, 10.0; peptone, 2.0; urea, 0.3; $(NH_4)_2SO_4$, 1.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 10.0, the pH was adjusted to 5.3 before autoclaving. The inoculated flasks were incubated at 30°C for forty-eight hours in a rotary shaker adjusted at 120 rpm. The culture medium for the enzyme production had the same constituents as the inoculums medium except that the locust bean gums were replaced by coffee residues (El-Refai *et al*., 2014). The culture flasks (50 mL) were inoculated with 10 % (v/v), and were incubated for ten days at 30°C in a rotary shaker at 150 rpm. At the end of the incubation period, the fermented medium was centrifuged at 4000 rpm for fifteen minutes in a cooling centrifuge. The clear supernatant was used as the crude enzyme solution.

2.4. Enzyme Assay and Protein Content

Enzyme assay was performed by incubating 0.5 mL of an appropriately-diluted culture filtrate with 1 mL of 1 % (w/v) locust bean gum (in 50 mM sodium citrate buffer at pH 5.0) for ten minutes at 50°C (Hashem *et al*., 2001). The reducing sugars produced were determined using the Nelson–Smogi reagent (Smogi *et al*., 1952). One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1µM of mannose/ml/min under the assay conditions. The protein content was determined according to Lowry *et al*., (1951) with bovine serum albumin as a standard.

2.5. Optimization of P. humicolaβ-mannanase Production

2.5.1. Plackett–Burman Experimental Design

Plackett–Burman experimental design (PB) (Plackett *et al*., 1946) is used in this study to evaluate the relative importance of culture conditions and medium components for the production of β-mannanase from *Penicillium humicola* in submerged fermentation. Nine different factors were chosen to perform this optimization; these include coffee residues as a carbon source, nitrogen complex (ammonium sulfate: peptone: urea in the ratio of 1:1:1), NaCl, KH_2PO_4, $MgSO_4$, incubation time, initial pH of the culture medium, inoculum size, and agitation speed (rpm) culture. The variables were represented at two levels, high concentration (+1) and low concentration (-1) in elevn trials as shown in Table 1. Each row represents a trial run, and each column represents an independent variable concentration. The Plackett–Burman experimental design is based on the first order linear model:

$$Y = B_0 + \Sigma B_i X_i \quad \text{Eq. (1)}$$

Where Y is the response (β-mannanase biosynthesis), B_0 is the model intercept, and B_i is the variables' estimates. The effect of each variable was determined by the following equation:

$$E(X_i) = 2(\Sigma M_i^{+} - \Sigma M_i^{-})/N \quad \text{Eq.(2)}$$

Where $E(X_i)$ is the effect of the tested variable. M_i^{+} *and* M_i^{-} represent the β-mannanase production from the trials where the variable (X_i) measured was present at high and low concentrations, respectively and N is the number of trials.

The standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (p-value) of each concentration effect was determined using student's t-test:

$$t(X_i) = E(X_i)/SE \quad \text{Eq.(3)}$$

Where $E(X_i)$ is the effect of variable X_i.

Table 1. Coded levels and real values for the Plackett-Berman experiment.

Trial No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	β-mannanase activity (IU/mL)
1	-1(1.8)	-1(0.329)	-1(0.375)	+1(0.06)	+1(12)	+1(6.5)	-1 (120)	-1(8)	-1(0.5)	5.46
2	+1(3.8)	-1(0.329)	-1(0.375)	-1(0.02)	-1(8)	+1(6.5)	+1(180)	+1(12)	+1(1.0)	13.61
3	-1(1.8)	+1(0.862)	-1(0.375)	-1(0.02)	+1(12)	-1(4.5)	+1(180)	+1(12)	+1(1.0)	2.44
4	+1(3.8)	+1(0.862)	-1(0.375)	+1(0.06)	-1(8)	-1(4.5)	-1 (120)	-1(8)	-1(0.5)	23.31
5	-1(1.8)	-1(0.329)	+1(1.25)	+1(0.06)	-1(8)	-1(4.5)	+1(180)	+1(12)	-1(0.5)	7.86
6	+1(3.8)	-1(0.329)	+1(1.25)	-1(0.02)	+1(12)	-1(4.5)	-1 (120)	-1(8)	+1(1.0)	7.84
7	-1(1.8)	+1(0.862)	+1(1.25)	-1(0.02)	-1(8)	+1(6.5)	-1 (120)	-1(8)	-1(0.5)	7.86
8	-1(1.8)	+1(0.862)	-1(0.375)	+1(0.06)	-1(8)	+1(6.5)	-1 (120)	+1(12)	-1(0.5)	3.6
9	+1(3.8)	-1(0.329)	+1(1.25)	-1(0.02)	+1(12)	-1(4.5)	+1(180)	-1(8)	+1(1.0)	5.4
10	+1(3.8)	+1(0.862)	+1(1.25)	+1(0.06)	+1(12)	+1(6.5)	+1(180)	+1(12)	+1(1.0)	7.28

Real values (given in parentheses) are in w/v %. X_1,is coffee residues; X_2,is nitrogen complex [peptone : ammonium sulfate : urea (1:1:1)]; X3, KH_2PO_4; X4, $MgSO_4$; X5, Incubation time (days); X6, pH; X7, shaking speed (rpm); X8, Inoculum size(v/v); X9, NaCl.

2.5.2. Fractional Factorial Design (FFD)

Based on the results obtained from PB design, the fractional factorial design (FFD) was conducted to gain the optimized levels of the main affecting factors (Rosa *et al*., 2010 and Farid *et al*., 2013). The FFD design of nineteen experiments and the coded and un-coded levels of the four investigated independent variables are listed in Table 3. The second-order empirical model can be obtained from the experimental data, the relationship between the response yield (β-mannanase activity) and the variables through polynomial regression analysis. The form of the second-order polynomial mode is as follows:

$$Y_{Activity} = ß_0 + ß_1 X_1 + ß_2 X_2 + ß_3 X_3 + ß_4 X_4 + ß_{11} X_1^2 + ß_{22} X_2^2 + ß_{33} C_3^2 + ß_{44} C_4^2 + ß_{12} X_1 X_2 + ß_{13} X_1 X_3 + ß_{14} X_1 X_4 + ß_{23} X_2 X_3 + ß_{24} X_2 X_4 + ß_{34} X_3 X_4$$ Eq. (4)

Where $Y_{Activity}$ is the predicted production of β-mannanase (IU/ml) and X_1, X_2, X_3 and X_4 are the independent variables corresponding to the chosen affecting factors. $ß_0$ is the intercept; $ß_1$, $ß_2$, $ß_3$, and $ß_4$ are linear coefficients; $ß_{11}$, $ß_{22}$, $ß_{33}$ and $ß_{44}$ are quadratic coefficients; $ß_{12}$, $ß_{13}$, $ß_{14}$, $ß_{23}$, $ß_{24}$ and $ß_{34}$ are cross-product coefficients.

2.5.3. Statistical Analysis

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the determination of multiple coefficients for each variable. The quadratic models were represented as contour plots (3D), and the response surface curves were generated by using STATISTICA (0.6).

2.6. Partial Purification of P. humicola β-mannanase

All of the purification steps were carried out at 4°C. The crude enzyme solution *of P. humicola* (100 mL) was partially-purified by fractional precipitations with ammonium sulfate (20 –100 %, w/v), acetone and ethanol (20–100 %, v/v). The precipitated protein was obtained by centrifugation (5,000 rpm for twenty minutes) and was re-suspended in a minimum volume of 50mM sodium citrate buffer (pH 5.5). All the fractions of ammonium sulfate were dialyzed against the same buffer overnight. The enzyme activity and the protein content were estimated for each fraction.

2.7. Biochemical Characterization of Partially-Purified P. humicola β-mannanase

2.7.1. Effect of pH on Enzyme Activity and Stability

The effect of pH on the activity of partially-purified *P. humicola* β-mannanase, was detected at pH ranging between 4 and 6 with sodium citrate buffer (50mM). The pH stability was determined by incubating the enzyme in the abovementioned buffer at pH ranging between 4 and 6, without the substrate, at room temperature and for different periods of time. The residual enzyme activity was estimated under the standard assay condition.

2.7.2. Effect of Temperature on Enzyme Activity

The effect of temperature on the activity and stability of the partially-purified *P. humicola* β-mannanase was studied at temperatures ranging from 40 to 60 °C. The activation energy (E_a) for β-mannanase was calculated by Arrhenius plot (log V (logarithm of % residual activity) versus reciprocal of temperature in Kelvin (1000/T)), as given in the following equation:

$$\text{Slope} = -E_a/R$$ Eq. (5)

2.7.3. Thermal Stability and the Thermodynamic Parameters

The partially-purified enzyme solution was incubated with sodium citrate buffer (50mM) with pH at 5.5 with the temperature ranging from 40 to 60 °C for different periods of time. The residual enzyme activity was estimated under the standard assay condition. Results were expressed as first-order plot. Thermal deactivation rate constants (k_d), half-lives ($T_{1/2}$) and *D*-values (decimal reduction time or time required to pre-incubate the enzyme at a given temperature to maintain 10 % residual activity) at each temperature were determined. The k_d was determined by regression plot of log relative residual activity (%) versus time (min). The $T_{1/2}$ and *D-value* of the partially-purified β-mannanase were determined by the following equations:

$$T_{1/2} = \ln 2/k_d$$ Eq.(6)

$$D\text{-}value = \ln 10/k_d$$ Eq. (7)

The increase in the temperature necessary to reduce *D-value* by one logarithmic cycle (*z value*) was calculated from the slope of graph between log D versus T (°C) using the equation:

$$\text{Slope} = -1/z$$ Eq. (8)

The denaturation energy (E_d) for β-mannanase was determined by a plot of log denaturation rate constants (lnk_d) versus the reciprocal of the absolute temperature (*K*) using the equation:

$$\text{Slope} = -E_d/R$$ Eq. (9)

The change in enthalpy ($\Delta H°$, kJ mol^{-1}), free energy (ΔG°, kJ mol^{-1}), and entropy (ΔS°, J $mol^{-1}k^{-1}$) for the thermal denaturation of β-mannanase were determined using the following equation:

$$\Delta H° = E_d - RT$$ Eq. (10)

$$\Delta G° = -RT\ln((kdXh))/((kb \cdot \square))$$ Eq. (11)

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T}$$ Eq. (12)

Where *T* is the corresponding absolute temperature in Kelivn, (*K*), *R* is the gas constant (8.314 $Jmol^{-1}k^{-1}$), h is the Planck constant (11.04×10^{-36} J min), and k_B is the Boltzman constant (1.38×10^{-23} Jk^{-1}).

3. Results and Discussion

3.1. Statistical Optimization of β-mannanase Production

For multivariable processes such as biochemical systems, in which numerous potentially influential factors are involved, it is necessary to analyze the process with an initial screening design prior to optimization. A sequential optimization approaches were applied in this study. The first approach deals with screening for nutritional factors affecting the growth of *P. humicola* with respect to β-mannanase production. The second approach is to optimize the factors mostly affecting the enzyme-production process.

3.1.1. Plackett–Burman Design

In the first approach, the Plackett–Burman design was applied to reflect the relative importance of nine different

factors. The averages of β-mannanase activity for the different trials were given in IU/mL and shown in Table 1.

Data indicated a wide variation from 2.44 to 23.31IU/mL for the β-mannanase activity. The main effects of the examined factors on the enzyme activity were calculated and presented graphically in Figure 1. It was offered the view for the ranking of factor estimates obtained by Plackett–Burman design. This variation reflected the importance of medium optimization to attain high productivity. The data analysis from the Plackett–Burman experiments involved a first-order (main effects) model.

The regression coefficients analyses of the tested variables for the β-mannanase were: coffee residues, $MgSO_4$, Nitrogen complex, and NaCl which all showed a positive effect on the β-mannanase activity. On the other hand, medium initial pH, KH_2PO_4, incubation time, inoculums size, and the shaking speed contributed negatively. The first-order model describing the correlation of the nine factors and the β-mannanase activity can be presented as follows:

$Y_{Activity}$= 18.460+4.971X_1–5.792X_2+550.850X_3 +1.45X_4– 2.314 X_5–0.0044.733 X_6 – 0.09X_7 +0.180X_8 + 18.460X_9

Table 2 shows the *t* test, *p* effect, and confidence level. The variables showed a high confidence level that was above 90 % (Coffee residues, incubation period, agitation speed (rpm) and inoculum size) were selected for further optimization (Farid *et al*., 2013).

Table 2. Statistical analysis of Plackett-Burman design showing coefficient values, effect, *t*- and *P*- values for each variable on β-mannanase analysis.

	β- mannanase analysis				
Variables	Coefficient	Effect	*t*-test	*P*-value	Confidence Level (%)
Intercept	18.460				
Coffee residue	4.971	7.6575	3.160751	0.008	99.2
Nitrogen complex	-5.792	1.6925	0.698605	0.2537	74.63
KH_2PO4	550.850	-3.386	-1.3□762	0.0574	84.26
$MgSO_4$.$7H_2$ o	-2.314	2.15525	0.889613	0.2016	79.84
NaCl	1.45	1.234	0.4356	0.2145	68.87
Incubation time	0.004	-6.9475	-2.86769	0.012	98.8
pH	-0.092	-2.3025	-0.95039	0.1868	81.32
Rpm	0.180	-3.54225	-1.46212	0.0936	90.64
Inoculum size	18.460	-3.54225	-1.46212	0.0936	90.64

Nitrogen complex: (peptone+ ammonium sulfate+ urea).

3.1.2. Fractional Factorial Design (FFD)

Fractional factorial design (FFD) for four independent variables was applied to reach to the optimum concentration for the most significant medium components obtained from the PB design (shown above). Table 3 shows the observed and the predicted values of the produced β-mannanase. Multiple regression analysis of the experimental data gave the following second- order polynomial equation:

$Y_{activity}$=–3.405+6.432X_1+ 0.932X_1^2+ 0.116X_2^2+ 0.012X_3^2+ 0.099X_4^2– 0.352X_1X_2–0.104X_1X_3 +1.256X_1X_4+0.015X_1X_4–0.370X_1X_4– 0.005X_1X_4

The results obtained by ANOVA analysis (Tables 4 and 5) show a significant F–value (11.106) which indicates the significance of the model. Model terms having values of prob > F (0.002) being less than 0.05 are considered significant. The determination of coefficient (R^2) was calculated as 0.946 for β-mannanase (value of R^2 >0.75 indicates the aptness of the model) which shows that the statistical model explains 94.6 % of the variability in the response. The efficient of the model can be checked by the determination of R^2; the closer R^2 is to1; the more suitable the model will be, and the better the predicted response will become. The value of R (0.973) being close to1 indicates a close agreement between the experimental results and the theoretical value predicated by the model equation.

The highest value of β-mannanase (35.2 IU/mL), which is higher than the predicated value (32.66), was produced with a 3.8 % coffee residue, and inoculum size of 8 % at 120 rpm for twelve days. The results indicated that the statistical optimization increased the biosynthesis of β-mannanase about 3.4 fold of that of the basal medium (10.3 IU/mL). The influence on the yield of β-mannanase imposed by the factors and reciprocity between them is represented in Figure 2 A-F. It was reported by many researchers that the statistical optimization model for the fermentation process could overcome the limitations of classic empirical methods. Moreover, it was proved to be more significant for the optimization production of β-mannanase (Mohamad *et al*.,2011; Rashid *et al*., 2011; Ahirwar *et al*., 2016; Janveja *et al*.,2016; Soni *et al*., 2017).

Table 3. Fractional factorial design (FFD) and response of β-mannanase activity from *Penicillium humicola*.

Trials	Independent variable				Observed response				
	X_1 Coffee residue (g/l)	X_2 Inoculum (%)	X_3 rpm Cycle	X_4 Incubation period (Days)	Final pH	Dry wt (g/flask)	Protein (mg/mL)	β-mannanase (IU/mL)	
								Observed	Predicted
1	(+1)3.8	(+1)12	(-1)120	(-1)8	5.85	2.15	7.52	19.47	14.18
2	(+1)3.8	(+1)12	(+1)180	(-1)8	5.59	1.2	8.54	10.43	11.4
3	(-1)1.8	(+1)12	(-1) 120	(-1)8	6.2	1.07	6.1	8.43	6.4
4	(0)2.8	(0)10	(0)150	(0)10	6.9	1.86	7.38	10.3	13.65
5	(-1)1.8	(+1)12	(+1)180	(-1)8	6.26	1.02	6.39	7.92	7.23
6	(+1)3.8	(+1)12	(+1)180	(+1)12	5.46	2.24	11.49	14.14	14.6
7	(-1)1.8	(-1)8	(+1)180	(+1)12	6.33	1.22	6.78	6.04	4.96
8	(+1)3.8	(-1)8	(+1)180	(-1)8	5.56	1.71	7.32	15.19	4.18
9	(+1)3.8	(-1)8	(-1)120	(-1)8	5.72	1.52	8.1	17.47	14.74
10	(-1)1.8	(+1)12	(+1)180	(+1)12	6.31	1.69	6.32	7.68	7.68
11	(-1)1.8	(-1)8	(-1)120	(-1)8	6.25	1.06	6.09	6.44	7.44
12	(-1)1.8	(-1)8	(-1)120	(+1)12	6.37	1.13	6.63	6.44	6.68
13	(+1)3.8	(-1)8	(+1)180	(+1)12	5.4	1.75	12.08	16.55	7.55
14	(0)2.8	(0)10	(0)150	(0)10	6.9	1.86	7.38	10.3	13.65
15	(+1)3.8	(-1)8	(-1)120	(+1)12	5.52	2.05	12.6	35.2	32.66
16	(-1)1.8	(-1)8	(+1)180	(-1)8	6.13	1.92	6.42	7.42	4.69
17	(-1)1.8	(+1)12	(-1)120	(+1)12	6.3	1.14	6.6	7.22	7.01
18	(0)2.8	(0)10	(0)150	(0)10	6.9	1.86	7.38	10.3	13.65
19	(+1)3.8	(+1)12	(-1)120	(+1)12	5.63	2.01	9.63	23.2	21.11

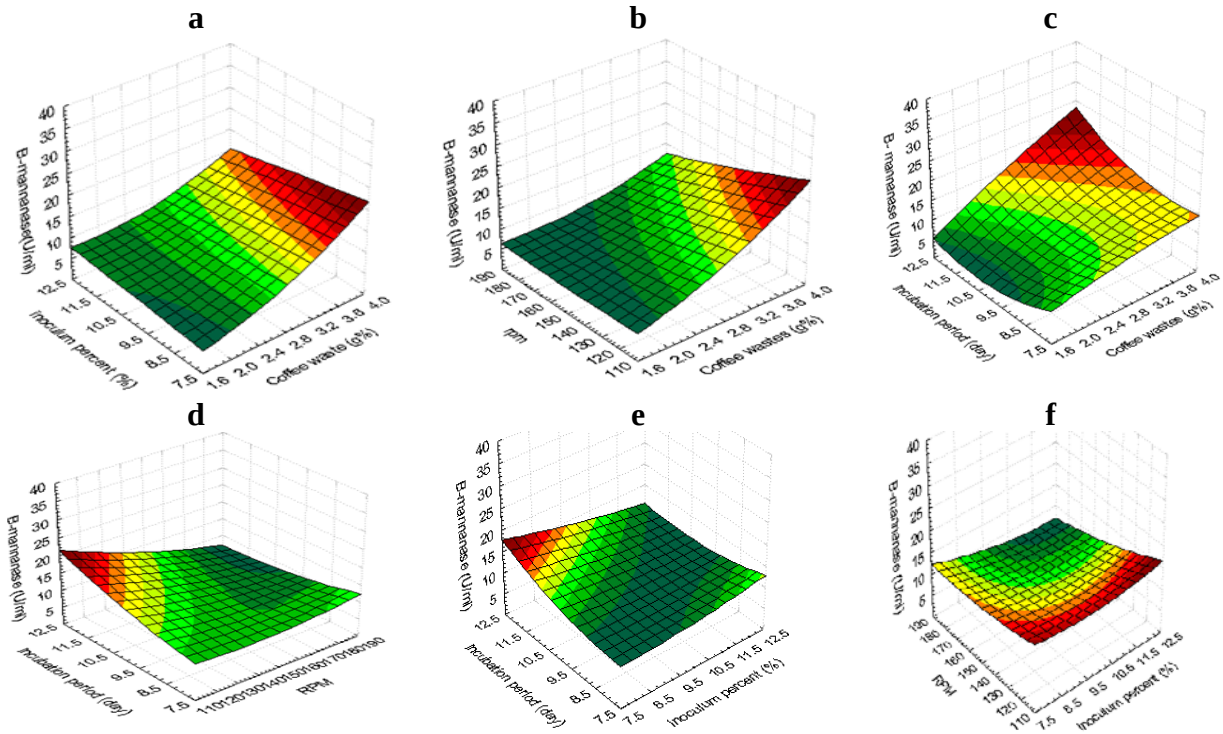

Figure 2A-F. Effect of culture conditions and medium composition on β- mannanase (IU/mL) Produced from *Penicillium humicola*.

Table 4. Analysis of Fractional factorial design for β- mannanase activity from *P. humicola.*

Term	Regression coefficient	Standard error	t- test	*P*-value
Intercept	-3.405	19.809	- 0.172	0.868
X_1	6.432	14.520	0.443	0.671
X_1^2	0.932	2.185	0.426	0.683
X_2^2	0.116	0.135	0.860	0.418
X_3^2	0.012	0.001	0.459	0.660
X_4^2	0.099	0.135	0.735	0.486
X_1X_2	-0.352	0.353	-0.998	0.352
X_1X_3	-0.104	0.024	-4.410	0.003
X_1X_4	1.256	0.353	3.562	0.009
X_2X_3	0.015	0.012	1.309	0.232
X_2X_4	-0.370	0.176	-2.097	0.074
X_3X_4	-0.005	0.012	-0.412	0.693

Table 5. ANOVA's Analysis of Fractional Factorial design for *P. humicola* β-mannanase activity.

ANOVAs					
	Df	*SS*	*SM*	*F test*	*P*
Regression	11	971.907	88.355	11.106	0.002
Residual	7	55.687	7.9551		
Total	18	91027.594			

df Degree of freedom; *SS* Sum of squares; *MS* Mean sum of squares; *F* Fishers's function; *P* corresponding level of significance , R^2 = 0.946. The value of adjusted R^2 was 0.861.

3.2. Partial Purification of P. humicola β-mannanase

Of all the fractions obtained by the three precipitants, the fraction obtained with ammonium sulphate (60-70 % w/v) displayed the highest specific β-mannanase activity (27.83 IU/mg protein) which represented 60.7 % of the recovered activity. The results of the present study agree with those obtained by Mudau and Setati, (2008) who found that β-mannanase from *Scopularipsis candida* was partially-purified by ammonium sulfate.

3.3. Biochemical Characterization of Partially-purified P. humicola β-mannanase

3.3.1. pH Optima and Stability

The results in Figure 3 indicate that the optimum pH of the partially-purified *P. humicola* β-mannanase was 5.5 similar to that of the crude enzyme in an earlier work by the same authors (El-Refai *et al*., 2014). It was noted by some researchers that the fungal β-mannanase was active at acidic pH (Ahirwar *et al*., 2016; De Marco *et al*., 2015; Abdel-Fattah *et al*., 2009; Blibech *et al*., 2010). The pH stability of the tested enzyme is represented in Figure 4. It indicates that the enzyme was almost stable for two hours at pH 6. Moreover, the enzyme had a great stability at the pH range of 4.5 to 5.5, and lost only 20 % of its activity after a two-hour incubation in buffer within this pH range. The tested enzyme had a half-life-time of fifty minutes at pH 4. Abdel- Fattah *et al*., 2009 showed that the high pH stability was at pH ranging between 4 and 6 for two hours.

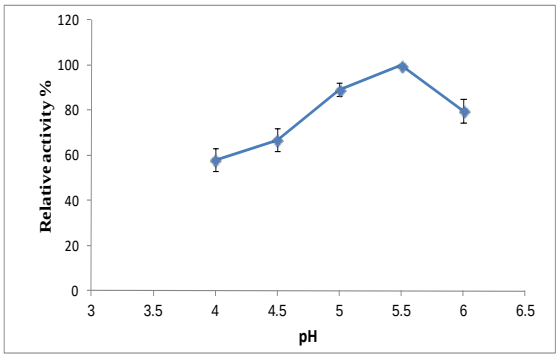

Figure 3. Effect of the pH value of the reaction mixture on the activity of the partial pure *P. humicola* β-mannanase. Reaction was carried out at 50˚C with 1% (w/v) locust bean gum.

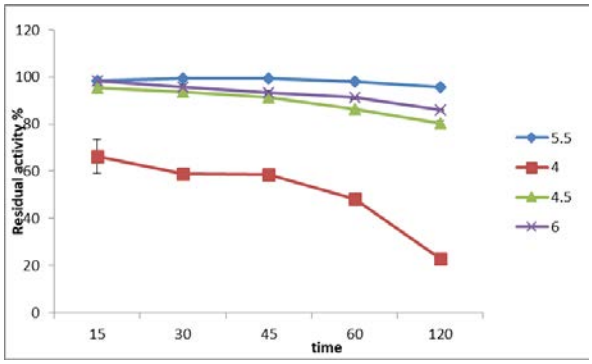

Figure 4. pH stability of partially pure *P. humicola* β-mannanase. The enzyme solution was incubated at different pHs for different periods of times, and the residual activity was determined under the optimum conditions.

3.3.2. Effects of temperature on the Enzyme Activity

Figure 5-A represents temperature dependence of the activity of the partially-purified *P. humicola* β-mannanase at temperatures ranging from 40 to 65°C at pH 5.5. The results indicate that the optimum temperature for β-mannanase is 60°C. From a biotechnological point of view, high temperature is preferred to improve conversion rates, decrease microbial contamination, and allow greater solubility of the substrate. In this study, the optimum temperature *of P. humicola* β-mannanase is within the range recorded for other fungal β-mannanase (Howard *et al*., 2003; De Marco *et al*., 2015; Ahirwar *et al*., 2016).

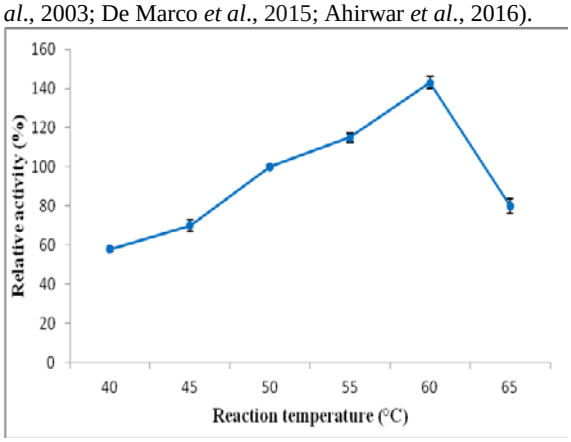

Figure 5 A. Effect of temperature of the reaction on the activity of the partial pure *P. humicola* β-mannanase. Reactions were carried out at pH 5.5 with 1% (w/v) locust bean gum at different temperatures.

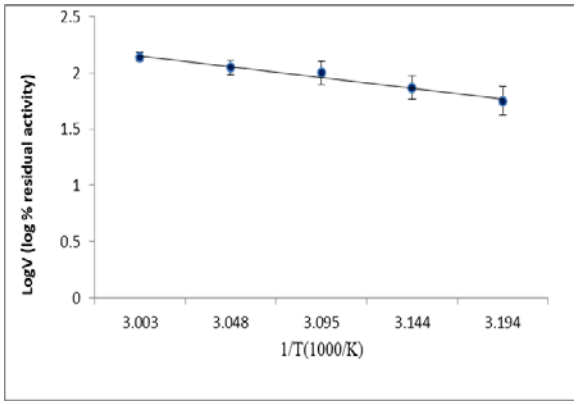

Figure 5 B.: Arrhenius plots to calculate activation energy (E_a) for partially pure *P. humicola* β-mannanase.

3.3.3. Thermal Stability and Thermodynamic Parameters

The Arrhenius equation has an important application in the calculation of the energy of activation and for the determination of the rate of chemical reactions. When a reaction has a rate constant which obeys Arrhenius' equation, a plot of ln (k) versus T^{-1}(K) gives a straight line, whose gradient and intercept can be used to determine the energy of activation (E_a).

The apparent activation energy (E_a) of catalysis for the partially-purified β-mannanase was calculated using Arrhenius plot (Figure 5 B). The regression equation for Arrhenius plots of β-mannanase was: y = –2.010 + 8.1893.

E_a of the *Penicillium humicola* β-mannanase was calculated to be 16.71114 kJmol^{-}1 which is less than the E_a recorded by Regmi *et al.* (2016) for the *Bacillus* sp.CSB39 β- mannanase (26.85 kJmol^{-}1). The lower value of E_a indicates that the conformation of the active site for the enzyme substrate complex requires less energy. These criteria make *P. humicola* β-mannanase more suitable for industrial applications; hence it needs a low value of activation energy which will be reflected on the total cost of industrial processing.

Thermostability of the enzymes increases their economic values, and provides potential benefits to different industrial processes; hence they are gaining more biotechnological attention. The results represented in Figure 6-A show that the enzyme retained 96 % of its activity when sustained in 45°C for 120 minutes and retained 66.8 % of its activity when incubated at 50ºC for sixty minutes, but lost about 80 % of its activity when incubated at 60˚C for sixty minutes. Blibech *et al*., (2010, 2011) found that *P. occitanis* β-mannanase retained 80 % of its original activity for thirty minutes when incubated at 50˚C.

Temperature enhances the rate of catalytic reaction of the enzyme, and it also affects its stability and may even lead to a complete deactivation. The thermodynamic data interpret the relation between the thermo parameter and the catalytic and other physical parameters of the enzyme. The thermostability parameters of the enzyme are summarized in Table 6. The rate of the heat of inactivation of *P. humicola* β-mannanase was investigated in the temperature range between 40ºC and 60ºC. The plots of log % residual activity versus time were linear, indicating the first- order kinetics of the enzyme (Figure 6-B). The half-lives and D-values of β- mannanase prolonged stability remarkably at temperatures between 40 and 45 °C. The half-life time ($T_{1/2}$) and the calculated D value of the β-mannanase were found to be 6931.4–135.9 min and 23025.85– 451.48 min respectively at the temperature range between 40 and 60°C. The higher values of $T_{1/2}$ and D were very important in industrial applications. The denaturation energy (E_d) was determined by applying the Arrhenius plot (Figure 6-C).The E_d for the partially-purified enzyme was 109.277 kJmol^{-1}, which means that the energy required for denaturing the tested enzyme was high (Arrhenius *et al*., 1889). The catalytic efficiency of the tested enzyme under different conditions was evaluated by the investigation of the thermodynamic parameters as: ΔH°, ΔG°, and ΔS°. The enthalpy change (ΔH°) of the partial-purified mannanase was 69.5669–68.18 kJ mol^{-1} (at the tested temperature range of 40°C –60°C) which is higher than that reported for β-mannanase by Regmi *et al.* (2016) (24 kJ mol^{-1}) and Panwar *et al.*, (2017) (29.9– 48.9 kJ mol^{-1}), but is lower than that reported bySrivastava *et al.* (2016) (86.7 kJ mol^{-1}). The observed change in ΔH° indicates that β-mannanase exhibits considerable conformational change at higher temperatures (Ortega *et al.*, 2009). The Gibbs free energy (ΔG°) is related to the enzyme stability and reveals the thermal unfolding of the enzyme structure, and apparently increases with the increase of temperature. ΔG° of β-mannanase was slightly decreasing (111.41 kJ mol^{-1}– 107.8 kJ mol^{-1}) with the increase of the temperature (40–60°C) which means that the enzyme was stable over the tested temperatures. The changes in entropy (ΔS°) measure the extent of the disorder (Blibech *et al*., 2011). So it represents the variation between the disorder in the ground and the transition state. β–mannanase had a negative entropy (–133.67 J mol^{-1}k^{-1} to –118.996 J mol^{-1}k^{-1}which indicates that the entropy decreases upon forming the transition state (Regmi *et al*., 2016) (Table 6). In general, the higher values of ΔG°; ΔH° and ΔS° reflect the higher thermostability of the tested enzyme. The z–value of β–mannanase, which represents the increase in the temperature necessary to reduce D *–value* by one logarithmic cycle, was 12.33°C (Figure 6-D).

Table 6. Kinetic and thermodynamic parameters for thermal inactivation of purified *Penicillium humicola* β-mannanase.

		Temperatures				
Parameters		40°C	45°C	50°C	55°C	60°C
Deactivation rate constant	$K_d 10^{-3}$	0.1	0.8	1.8	3.5	5.1
Half-life time (min)	$T_{1/2}$	6931.472	866.434	385.0818	198.0421	135.9112
Decimal reduction time (min)	*D Value*	23025.85	2878.231	1279.214	657.8815	451.4873
The change in enthalpy	ΔH°	69.5669	69.22128	68.87567	68.53006	68.18444
Free energy	ΔG°	111.4055	107.7293	107.2874	107.1767	107.8101
Entropy	ΔS°	-133.67	-121.095	-118.92	-117.825	-118.996

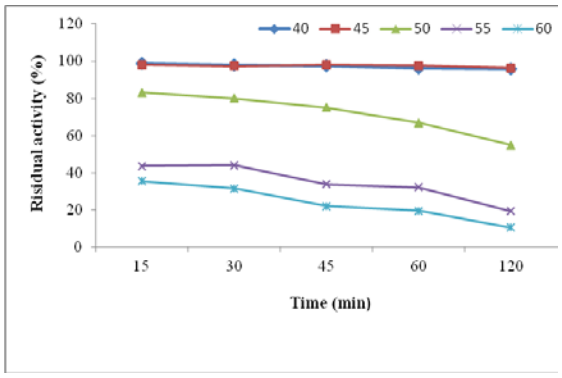

Figure 6 A. Thermal stability of partially pure *P. humicola* β-mannanase. The enzyme solution was held at different temperatures for different periods of times, and the residual activity was determined under the optimum conditions.

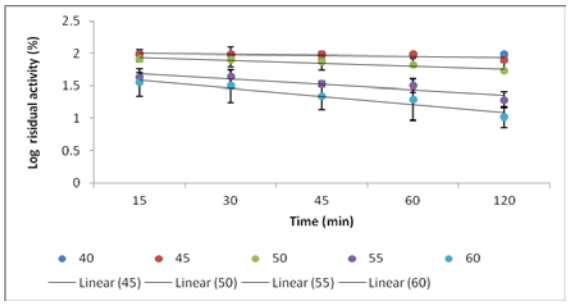

Figure 6 B. Effect of different temperatures on the rate of denaturation of the partially pure *P. humicola* β-mannanase.

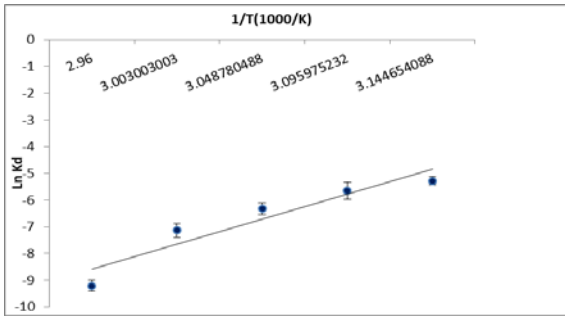

Figure 6 C. Arrhenius plot to calculate deactivation energy (E_d) for denaturation of the partially pure *P. humicola* β-mannanase.

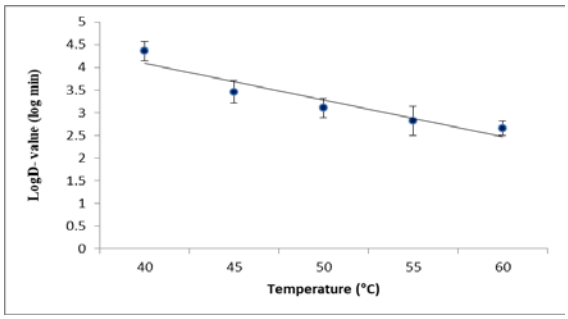

Figure 6 D. Temperature dependence of the decimal reduction of partial purifiedβ-mannanase to calculate z-values.

4. Conclusion

This research evaluates the application of the statistical optimization strategy based on Plackett- Burman and Fractional Factorial designs to maximize the production of *P. humicola* β-mannanase using coffee waste. The current study improved the enzyme production by 3.4 fold. *P. humicola* β-mannanase was fractionally purified with ammonium sulfate. The fraction obtained at 60 –70 % ammonium sulphate saturation displayed the highest specific activity of β-mannanase (27.83 IU/mg protein) and was stable at pH ranging from 4.5 to 6 for more than two hours. The partially- purified enzyme has optimal activity at pH 5.5 and 60°C. The thermodynamic study proved that this enzyme has future prospects for bioremediation in many industrial applications.

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