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Characterization and Production of Glucose Oxidase from *Penicillium notatum* by using Response Surface Methodology

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Abstract

Penicillium notatum was used for the production of glucose oxidase emphasizing on optimization of different parameters by using Response Surface. DOE 8.0.7.1 software was used for data analysis and formation of 2D contour and 3D response surface graphs. Different responses were exhibited by glucose oxidase in the condition optimized by RSM. While selecting rice polish as a substrate it was observed that *Penicillium notatum* produced appreciable level of glucose oxidase. Enzyme showed maximum activity at pH 5, temperature 35 ^{o}C, inoculums size 5.50 mL and fermentation time 48 h. Purified glucose oxidase by chromatographic techniques obtained 40.19 U mL^{-1} activity, 446.10 U mg^{-1} specific activity along with 66.9% recovery that was subjected to kinetic and thermal characterization. Km for glucose oxidase was observed to be 52 µM with 61 µM of Vmax. Enthalpy of denaturation and free energy of thermal denaturation for glucose oxidase were 0.89 kJ moL^{-1} and 74.34 kJ moL^{-1}, respectively while entropy of inactivation was negative at each temperature, showing that enzyme is thermodynamically more stable. Based on the data obtained, it is concluded that glucose oxidase produced through RSM, has more yield and is more stable towards pH, temperature and thermal conditions.

Keywords: Glucose oxidase, *Penicillium notatum*, Response Surface Methodology, Production, Charcaterization.

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1. Introduction

Glucose oxidase (GOx) converts glucose into gluconic acid and hydrogen peroxide that makes this enzyme as an antimicrobial agent in most of the pharmaceutical preparations. Glucose oxidase from fungal sources is a dimeric glycoprotein contains identical subunits of polypeptide chain and covalently linked to each other (Bankar *et al.,* 2009). GOx may be harvested from a number of microbes like *Penicillium chrysosporium, Penicillium notatum, Penicillium purpurogenum, Penicillium amagasakiense, Penicillium variabile, Botrytis cinerea* and *Aspergillus niger.* Glucose oxidase from *Penicillium* species is preferred over other after the discovery of notatin that has potential antibacterial activity (Bankar *et al*., 2009; Yogananth *et al*., 2012).

Glucose oxidase achieved a significant commercial importance in the pharmaceutical, chemical, beverage, food, biotechnology, clinical, textile and various household industries due to its large number of applications. GOx is used as a diagnostic agent for glucose estimation and a very important component of glucose biosensors as well (Bankar *et al.,* 2009). In food industry, GOx supplies hydrogen peroxide that act as a preservative because it removes oxygen and glucose to increase the taste and shelf life of products. It is widely used in the baking of food products. By the elimination of oxygen, GOx is in use as color and flavor stabilizer in beer, fish, soft drinks and many other food products (Yogananth *et al*., 2012). GOx is potentially used in wine preparation where it reduces alcohol contents by removal of glucose (Wang *et al*., 2000). GOx also produces gluconic acid that is used as preservative and chelating agent in food, leather and metal industries (Bankar *et al*., 2009).

The objective of this work was to process optimization for GOx by using Response Surface Methodology (RSM). RSM is one of the statistical techniques for process optimization of various enzymes. The salient feature of RSM is to analyze the relationship between variable and fixed experimental factors. The effect of various factors can be monitored in terms of combined interaction in a limited number of trials and in short time. So, we predict that RSM may increase the production of glucose oxidase by saving time, resulting in more cost-effective ratio (Cruz *et al*., 2010).

2. Material and Methods

2.1 Process optimization for GOx production

Pure culture of *Penicillium notatum* was procured from Enzyme Biotechnology Lab., Dept. of Biochemistry, University of Agriculture, Faisalabad, that was maintained on potato-dextrose-agar (Rasul *et al*., 2013). To prepare inoculums, spores of *Penicillium notatum* were transferred into 250 mL flask which contains 50 mL sterile Krick-basal media. To get spore suspension, media were incubated on rotatory shaker at 120 rpm for 36 h. after adjusting pH at 5.6 (Yogananth *et al*., 2012). The composition of enzyme production media was (g L^{-1}) glucose 20, diammonium titrate 0.22, KH_2PO_4 0.21, $MgSO_4.7H_2O$ 0.05, $CaCl_2.2H_2O$ 0.01, thiamine 0.001, tween 80 (10%) 10 mL, vertyl alcohol 10 mL, trace elements 10 mL, chloromophenol 1 mL and rice polishing 5 g. Composition of trace element solution (g L^{-1}) was $CuSO_4$ 0.08, $NaMoO_4$ 0.05, $MnSO_4.4H_2O$ 0.07, $ZnSO_4.7H_2O$ 0.0438 and $Fe_2(SO_4)3$ 0.05.

Production of glucose oxidase was carried out by optimizing different parameters by using Response Surface Methodology like pH (2-8), temperature (20-50 ^oC), inoculum size (1-10 mL), fermentation time (20-72 h), rice polish 5 g and agitation speed 100-250 rpm (Liu *et al*., 2003). After completion of fermentation, biomass was homogenized by using pestle and mortar and filtered. Cell debris was removed by centrifugation (10,000 rpm at 4 ^oC) and supernatant was used for enzyme assay (Zia *et al*., 2012a). Activity of GOx was noted by o-dianisidine-peroxidase coupled reaction where 100 µL enzyme was mixed with dianisidine buffer solution containing peroxidase and glucose and absorbance was noted at 460 nm (Zia *et al*., 2012b).

2.2 Purification and Characterization of GOx

Crude enzyme was isolated by using 60 - 80% ammonium sulfate where centrifugation was carried out at 10,000 rpm at 4 oC for 15min. to salt out GOx (Shin *et al*., 1993). After desalting, enzyme fraction was applied on DEAE-cellulose column. Bound protein was eluted and fractions of 2 mL were collected for enzyme assay and protein estimation by Biuret method (Zia *et al*., 2013). Fraction having maximum specific activity was applied on Sephadex G-150 column where again fractions of 2 mL were collected (Umbreen *et al*., 2013). Active fractions were used for thermodynamic and kinetic characterization.

To seek the optimum pH, GOx was assayed at various pH ranging from 4-8. To get the optimum temperature, GOx was assayed at different temperatures (20, 25, 30, 35, 40, 45, 50 and 60 ^oC) by keeping the pH 6. Energy of activation was found by assaying GOx at temperatures ranging from 20-60 ^oC (Simpson *et al*., 2007). The Mechalis-Menton kinetic constants (Km and Vmax) were determined by assaying the different concentrations of glucose (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20%) (Eremin *et al*., 2001; Rasul *et al*., 2013). Purified GOx was subjected to thermal denaturation and reactivation by incubating at different temperatures for 15 min. and placed in ice for 30 minutes. Rate constants for thermal denaturation and activation energy for denauration were obtained from first order plot and thermostability parameters were calculated by Eyring's absolute rate equation (Umbreen *et al*., 2013; Arshad *et al*., 2014).

3. Results and Discussion

Conventional techniques for process optimization are least capable to represent the combined effects of all factors involved in fermentation. These techniques are tedious, time consuming, laborious and involve a large number of experimental trials. So, Response surface method (RSM) is a prominent experimental design to identify the presentation of composite systems. RSM can analyze the interaction and observed response between factors involved in process optimization and is one of the widely used statistical techniques of process optimization for the production of various enzymes. One of the advantages of RSM is to study the relations/interaction between many factors in small set of experiments (Liu *et al*., 2003).This technique is complimentary than one-factor-at-a-time (OFAT) for process optimization in terms of cost, time, accuracy and analysis. So, we found an increased production of glucose oxidase by using RSM in terms of yield and processing cost, as well (Cruz *et al*., 2010). Design expert 8.0.7.1 software was used for data analysis and formation of 3D response surface and 2D contour graph. The response variable was glucose oxidase activity for central composite design (CCD). While selecting rice polish as a substrate it was observed that *Penicillium notatum* produced appreciable level of glucose oxidase. In this design, 5 factors were studied in 32 runs and the results of values are the means of triplicate cultures (Table 1). Maximum activity of GOx was obtained at pH 5, temperature 35 ^oC, fermentation time 48 h and inoculum size of 5.5%. Full quadratic multiple regression analysis given up the subsequent regression equation for the

production of glucose oxidase. Mathematical relationship of the response Y to significant independent variable A, B, C, D and E can be approximated by the quadratic model equations (Gunny *et al*., 2013).

The regression equation is:

Response = (- 47.0) + (- 6.3 A) + (2.53 B) + (- 0.51 C) + (-0.05 D) + (0.415 E) + (0.0097 AB) + (0.0144 AC) + (0.0042 AD) + (0.00137 AE) + (0.008 BC) + (-0.00023 BD) + (-0.00007 BE) + (-0.0150 CD) + (0.00365 CE) + (-0.00124 DE) + (0.55 A2) + (0.0371 B_2) + (0.035 C_2) + (0.0043 D_2) + (-0.00115 E_2).

Table. 1. Process optimization using RSM under central composite design

Runs	Temperature (°C)	pH	Incubation time (hrs.)	Agitation speed (rpm)	Inoculum size (ml)	Response
1	32.50	5.00	48.00	162.50	5.50	0.332
2	32.50	5.00	48.00	125.00	5.50	0.33
3	26.25	5.00	48.00	125.00	5.50	0.329
4	32.50	5.00	48.00	125.00	5.50	0.331
5	45.00	8.00	24.00	50.00	10.00	0.441
6	45.00	2.00	24.00	200.00	10.00	0.44
7	***32.50***	***5.00***	***60.00***	***125.00***	***5.50***	***0.665***
8	20.00	2.00	24.00	50.00	10.00	0.55
9	32.50	5.00	36.00	125.00	5.50	0.334
10	20.00	8.00	24.00	50.00	1.00	0.553
11	32.50	5.00	48.00	125.00	3.25	0.331
12	20.00	2.00	72.00	200.00	10.00	0.552
13	20.00	8.00	72.00	200.00	1.00	0.551
14	20.00	8.00	24.00	200.00	10.00	0.55
15	38.75	5.00	48.00	125.00	5.50	0.332
16	45.00	2.00	72.00	200.00	1.00	0.442
17	45.00	8.00	72.00	200.00	10.00	0.441
18	32.50	5.00	48.00	125.00	5.50	0.334
19	32.50	5.00	48.00	125.00	5.50	0.333
20	32.50	5.00	48.00	125.00	5.50	0.335
21	20.00	2.00	24.00	200.00	1.00	0.554
22	32.50	5.00	48.00	125.00	5.50	0.329
23	32.50	6.50	48.00	125.00	5.50	0.331
24	45.00	8.00	72.00	50.00	1.00	0.443
25	45.00	2.00	24.00	50.00	1.00	0.442
26	20.00	8.00	72.00	50.00	10.00	0.554
27	32.50	5.00	48.00	87.50	5.50	0.336
28	45.00	8.00	24.00	200.00	1.00	0.445
29	45.00	2.00	72.00	50.00	10.00	0.441
30	32.50	5.00	48.00	125.00	7.75	0.334
31	20.00	2.00	72.00	50.00	1.00	0.551
32	32.50	3.50	48.00	125.00	5.50	0.335

Validation of variability in experimental response values by investigational factors and their interactions is calculated by R2 which is called as coefficient of determination and its values should be between 0 and 1. It was reported that closer the R2 values to 1 means model is more reliable and stronger and it can better predicts the response (Liu *et al*., 2003). The predicted R2 value of 0.707 by the model for glucose oxidase production is comparable with the adjusted R2 values of 0.173. The actual R2 value was corrected by adjusted R2 for given sample size and number of factors in the model.

Table. 2. Purification summary of glucose oxidase from *Penicillium* notatum

Purification step	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Fold purification	%age Recovery
Crude	60	3.53	16.99	1	100
Desalted	50.32	1.65	30.49	1.79	83.86
DEAE-Cellulose	45.23	0.21	215.11	12.69	75.38
Sephadex	40.19	0.09	446.10	26.28	66.9

A large number of factors/parameters may apply in the model with small sample size then value of adjusted R_2 will be smaller than the R_2. Here the value is smaller than R_2, which showed satisfactory representations of the process optimization by using this model. Precision and accuracy of trials was analyzed by coefficient of variation that obtained 45.33%. The value of standard deviation (0.072) proved the model to comply with response.

It was showed that independent variables/parameters (pH, temperature, fermentation time, agitation speed and inoculums size) corresponded positively to glucose oxidase production from *Penicillium* notatum. The model and the error sum of square were divided by the respective degrees of freedom to obtained mean square. The model F-value of 1.3241 denotes that model is significant. Linear (A, B, C, D, E), interactions (AB, AC, AD, BC, BD, BE, CD, CE, DE) and quadratics (A_2, B_2, C_2, D_2, E_2) are significant model with p-value. F-values of Lack of Fit (1030) indicate that it as a non-significant relative to the pure error so non-significant Lack of Fit confirmed the strength of response surface methodology.

Fisher's variance ratio or F-value is defined as a ratio of mean square of regression to the mean square of error. This can be defined as a determination of variation in mean of calculated data. The tabulated values must be less than calculated F-value for a better prediction of the model. The p-values provide an indicator for the determination of every coefficient. High F-values and non-significant lack of fit conclude that model was good fit (Cruz *et al*., 2010). Regression coefficient response for glucose oxidase production revealed that a big value of positive or negative coefficient of a factor or independent variable showed that it has strong effect on glucose oxidase production. The large values of factors in the linear expression like temperature showed the highly noteworthy effect on glucose oxidase production. The negative linear expression of fermentation time in the table showed that at high level of fermentation time activity of glucose oxidase would be decreased (Gunny *et al*., 2013).

3.2 Interactions among variables

While considering the temperature and pH, graph indicated the presence of glucose oxidase with their differential production at varied temperature and pH at widely different isoelectric points. During optimization, pH and temperature obtained same and opposite responses as well. Optimum temperature for glucose oxidase was observed at 35 ^{o}C. It was noted that at high temperature, enzyme showed denaturation due to disturbing the structure of glucose oxidase (Fig. 1).

Response surface illustrated a high glucose oxidase activity at central point of both factors of pH vs inoculum size. The contour plot of glucose oxidase showed that a pH of 5 and 5 mL inoculum increased the production of glucose oxidase. Response surface plot showed that pH more than 5 slowed down the activity of enzyme. Fig. 1 indicated that pH and inoculum size increased the production of glucose oxidase but very high pH and inoculum size inhibited it. Because at high pH the concentration of hydrogen ion increased which inhibit the production of glucose oxidase from *Penicillium* notatum.

Response surface graph indicated that maximum yield of glucose oxidase was high at fermentation period of 36 h and pH 5, when subjected to combined effect of pH vs fermentation time. This study shows contour plot of pH alongside fermentation time with 3 other factors of different levels. The graph also predicts that factor had strong interaction with the growth of *Penicillium* notatum. pH and agitation speed both have positive effect on glucose oxidase production. Fig. 1 shows the effect of pH vs. fermentation time at fixed levels of other three factors and showed maximum production of enzyme at pH 5 and 250 rpm agitation speed. Temperature vs. inoculum greatly affected the production of glucose oxidase as shown in Fig. 1.

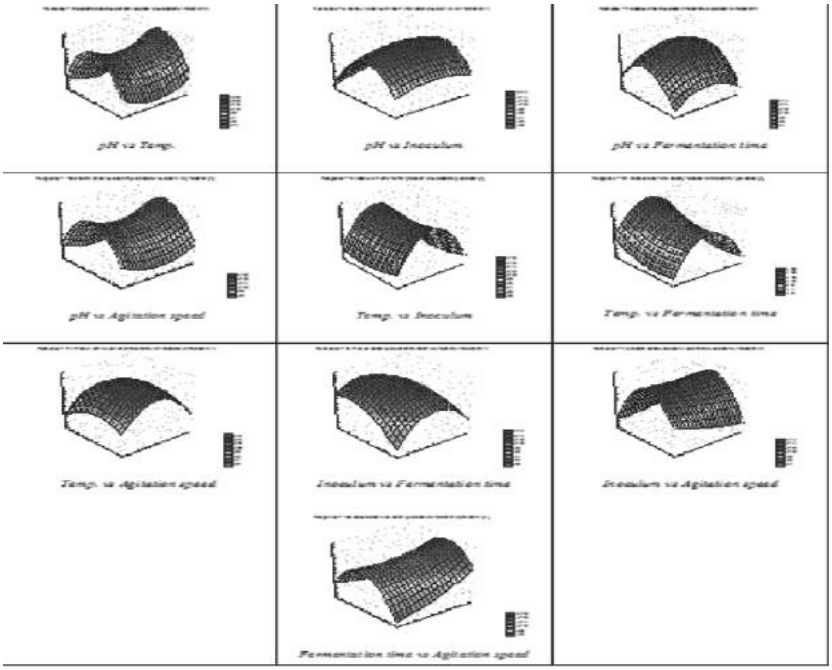

Fig. 1. Response surface plots showing the interactive effect of various parameters on glucose oxidase production: (a) pH vs. temperature, (b) pH vs. inoculum, (c) pH vs. fermentation time, (d) pH vs. agitation speed, (e) temperature vs. inocu lum, (f) temperature vs. fermentation time, (g) temperature vs. agitation speed, (h) inoculum vs. fermentation time, (i) inoculum vs. agitation speed, (j) fermentation time vs. agitation speed.

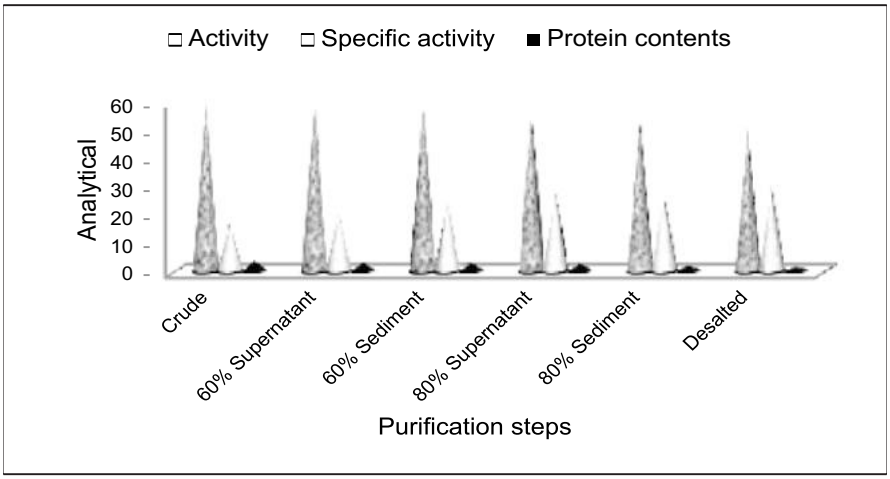

Fig. 2. Isolation of glucose oxidase from *Penicillium notatum* by ammonium sulfate precipitation

The relations between fermentation time and temperature for glucose oxidase production indicated that with increased temperature and fermentation time resulted into improved enzyme activity. Increased temperature than optimum one (35ºC) resulted in a gradual decrease in enzyme activity. Glucose oxidase showed maximum activity at temperature 35 ºC and fermentation time 48 h. Agitation speed alongwith temperature has encouraging effect on GOx production. Contour and response surface plot indicated a maximum activity at the middle of the temperature (15 -50) and agitation speed (30 -200 rpm). Interaction between inoculum size and fermentation time may be responsible here. Fig. 1 predicted a strong interaction among the two variables for GOx biosynthesis from *Penicillium* notatum. The detailed results of these factors including inoculum vs. agitation speed and fermentation time vs.

agitation speed are shown in Fig. 1.

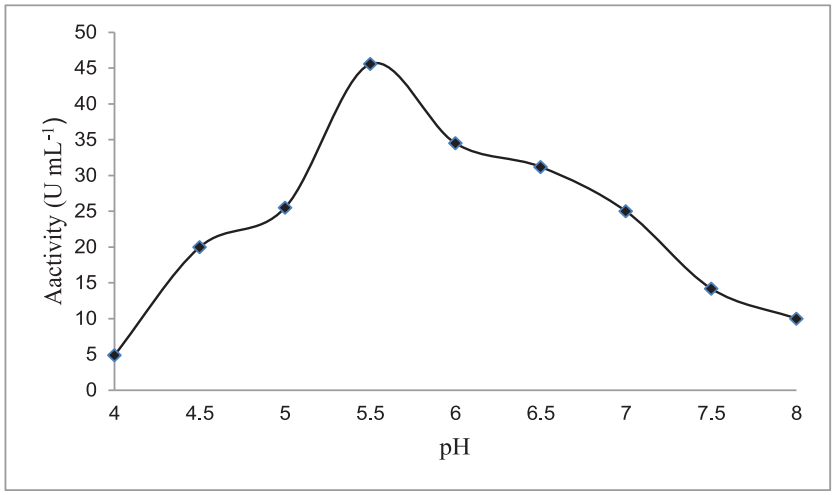

Fig. 3. Effect of pH on glucose oxidase purified from *Penicillium* notatum.

3.3 Purification of glucose oxidase

The crude enzyme produced from *Penicillium notatum* by using optimized media showed activity and specific activity of 60 U mL^{-1} and 16.99 U mg^{-1}. This crude enzyme was subjected into ammonium sulfate precipitation and enzyme activity obtained by 60% supernatant and sediment was 58.91 U mL^{-1} and 56.83 U mL^{-1}, respectively. While enzyme exhibited an activity of 53.66 U mL^{-1} and 52.47 U mL^{-1} after 80% supernatant and sediment, respectively. The results indicate that purification fold or specific activity reached their maximum when subjected to precipitation at 80% saturation (Fig. 2). It was further dialyzed against phosphate buffer of pH 6 that resulted into increased specific activity of 30.49 U mg^{-1} with an enzyme activity of 50.32 U mL^{-1} (Table 2). Such a higher index of purity represented a good basis for improved kinetic and thermodynamic parameters. It was further reported that dialyzed sample of glucose oxidase from *Aspergillus niger* showed their maximal activity and specific activity of 18.18 U mL^{-1} and 7.73 U mg^{-1}, respectively (El-Sherbeny *et al*., 2005).

Diethyl-aminoethyl cellulose is a positively charged resin used in ion exchange chromatography, used in protein and nucleic acid purification. Gel matrix beads are derivatized with diethyl aminoethyl (DEAE) and lock negatively charged proteins or nucleic acids into the matrix, until released by increasing the salt concentration of the solvent. Suspension having glucose oxidase was eluted and 30th fraction obtained an activity of 45.23 U mL^{-1} with 215.11 U mg^{-1} specific activity, 12.69 purification fold and 75.38% recovery (Table 2). Literature reported that glucose oxidase from *Aspergillus niger* showed maximum activity 15.82 U mL^{-1} with 21.67 U mg^{-1} specific activity (Zia *et al*., 2012b). A %age yield of 56.2 with 127 fold degree of GOx purification by using *Penicillium* sp. after DEAE-cellulose treatment is also observed (Sukhacheva *et al*., 2004). Moreover another findings showed 99% recovery with 7 fold purification for purification of GOx from Botrytis cinerea (Liu *et al*., 1998). Our results indicate that increase in specific activity and fold purification indicates that enzyme is better purified after this treatment. Purification of glucose oxidase from *Penicillium notatum* by Sephadex column in 14th fraction increased the specific activity of enzyme to 446.10 U mg^{-1} with 26.28 fold purity and 66.9% of its recovery (Table 2). After gel filtration, maximum impurities were removed so a marked increase in fold purification (255.23) and yield (61.9%) of GOx was achieved (Zia *et al*., 2012b). Literature also showed that after gel filtration, specific activity of *P. notatum* glucose oxidase was 59.37 U mg^{-1} and 65.203 U mg^{-1}, respectively (Zia *et al*., 2013) (El-Sherbeny *et al*., 2005).

3.4 Characterization of glucose oxidase

Tertiary structure of enzymes is in stress by a minute change in pH, temperature and substrate concentration. In view of the fact that pH shows a prevailing task as *A. niger* GOx loosed activity by 12% when kept at pH higher than 8 in a stability test (Bankar *et al*., 2009). So a broad range of pH from 4-8 was settled for GOx and the results possessed an optimum activity of 45.57 U mL^{-1} at pH 5.5 (Fig. 3). It was observed that glucose oxidase from *Penicillium notatum* was maximally active at pH 5.4 that decreased sharply after this point (El-Sherbeny *et al*., 2005). So, it can be stated that pH and temperature my affect the state of active

site of GOx so changing the mode of interaction with substrate. Change in temperature causes the change in activity of the enzyme. It was observed that GOx from *P. notatum* reflected an excellent stability at 45 °C (Fig. 4) with activation energy (Ea) of 26.52 kJ mol^{-1}. This is in accordance with the findings where an optimum temperature for *Penicillium* pinophilum glucose oxidase activity was in the range of 30-40 °C (Rando *et al*., 1997). It is clear from Arrhenius plot that the enzyme had a single conformation up to the transition temperature. The enzyme exhibited low Ea, which makes our glucose oxidase more stable and superior to those from other sources.

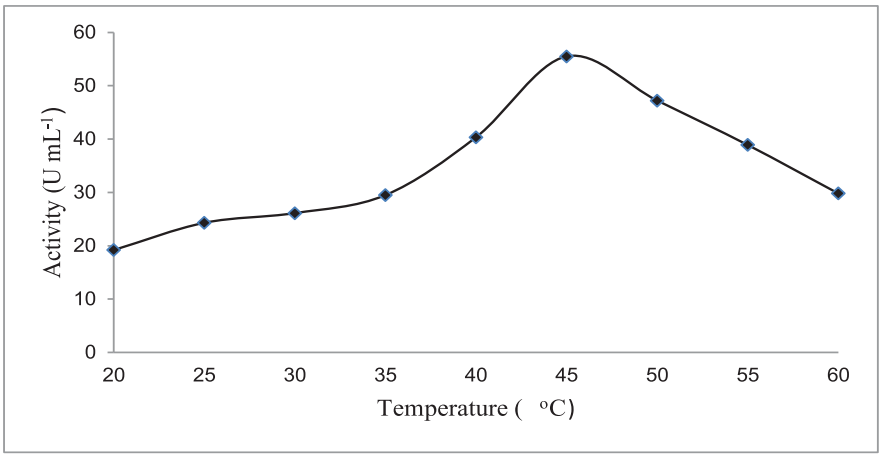

Fig. 4. Effect of temperature on glucose oxidase purified from *Penicillium* notatum.

Km and Vmax for glucose oxidase were resoluted by when using enzyme with varying concentration of glucose and the findings were graphed for activity and substrate. Activity of enzyme is directly proportional to the rate of decomposition of substrate and with further addition of substrate have no impact on the rate of reaction. The association involving rate of reaction and depends upon the affinity of enzyme for substrate. Which is typically articulated as Km that for glucose oxidase was obtained to be 52 µM from Michealis-Menten calculations and in line weaver burk plot it was 61 µM. Thermostability is an ability of enzyme molecule that resist against thermal unfolding in absence of substrate but thermophilicity refers to enzyme catalysis at high temperatures. Enthalpy of denaturation and free energy of thermal denturation for glucose oxidase were 0.89 kJ moL^{-1} and 74.34 kJ moL^{-1}, respectively at various temperatures. A constant trend in value of thermal denturation of free energy was observed with increasing temperature. Entropy of inactivation was negative at each temperature, showing that enzyme is thermodynamically more stable.

Conclusion

Glucose oxidase has wide range of applications especially in clinical diagnosis, food and other industries. We used response surface methodology for the production of glucose oxidase from *Penicillium notatum* resulting into more yield by using rice polish as a substrate at pH 5, temperature 35 °C, fermentation time 36 h and inoculum size of 5.5%. Moreover, with improved kinetic and thermal stability, this GOx may serve as a biotechnological and industrial solution.

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