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APPLICATION OF DHURRIN FOR KINETICS AND THERMODYNAMIC CHARACTERIZATION OF LINAMARASE (B-GLUCOSIDASE) GENETICALLY ENGINEERED FROM *SACCHAROMYCES CEREVISIAE*.

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ABSTRACT:

Recombinant* Saccharomyces cerevisiae *cells at the stationary phase of growth were recovered, homogenized and centrifuged to obtain crude extracts designated as $GELIN_0$. Carboxy methyl cellulose, diethyl amino-ethyl-sephadex and diethyl amino-ethyl-cellulose were used to purify the crude extracts of $GELIN_0$ resulting in $GELIN_1$, $GELIN_2$ and $GELIN_3$, respectively. The ability of the enzyme extracts and a commercial native linamarase (CNLIN) to hydrolyse cyanogenic glucosides was challenged using dhurrin from sorghum as substrate. Precisely, the actions of commercial native linamarase (CNLIN) and the genetically engineered linamarase (β-glucosidase) from* Saccharomyces cerevisiae *on dhurrin as influenced by degree of its purification, pH (6.8) and temperature(30-45^oC) were investigated and the data derived were applied for kinetics and thermodynamic characterization of the enzymes. Enzymic degradation kinetics of the dhurrin were evaluated using a 4 x 6 x 8 B/W design comprising of 4 enzyme types (GELINo, $GELIN_1$ $GELIN_2$ and $GELIN_3$,, 6 temperatures (30, 32, 35, 37, 40, 45 °C) and 8 time intervals(0-70 min.). Data obtained from the residual HCN with time were fitted into zero, first and second order kinetics models to derive reaction rate constant ($Kmin^{-1}$) values which were analyzed using the Arrhenius and absolute reaction rate models Thermodynamic parameters were obtained including; activation energies (E_a), frequency factor(K_o), enthalpy (ΔH) and entropy ($\Delta S^{\#}$) that characterized the reactions on dhurrin catalyzed by commercial native linamarase (CNLIN) and the genetically engineered linamarase (β-glucosidase) from* Saccharomyces cerevisiae. *The results showed that the best fitted order based on higher coefficient of linear regression (r^2) values > 0.998 and linearity of curves was the first order kinetics model and not either zero or second order models. $Kmin^{-1}$ values ranged from $GELIN_O$- $GELIN_3$ 0.03-0.07 µmol/min while the derived D-values from K-values were in the range of 24-65 min. The frequency factors (Ko) increased with enzyme purity from $GELIN_0$ to $GELIN_3$ corresponding to K_o (min^{-1}) of 22.585 to 56.462. The energy of activation Ea (KJ/mol) generated 60.0995 to150.6900 corresponding to enzymes $GELIN_0$ to $GELIN_3$ followed the same pattern with frequency factor for breaking of bonds in dhurrin molecules. At pH 6.8 CNLIN showed no action on dhurrin. The high correlation coefficient values of (r^2= 0.97 to 0.99) indicated the best fit of the Arrhenius and the absolute reaction rate models. The entropy change (ΔS) increased with enzyme purity from 0.588 J/mol.deg. to 1.4625Jmol degree. The enthalpy change KJ/mol followed the same pattern whereby increases influenced by enzyme purity ranged from 1892 KJ/mol to 13104KJ/mol.

Keywords: kinetics, thermodynamic, characterization, dhurrin, genetically engineered β-glucosidase, *Saccharomyces cerevisiae*.

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INTRODUCTION

Cyanogenic glucosides are toxic chemical substances (Conn, 2005) found in some food crops and used for human and animal nutrition are dhurrin , amygdalin, prunasin, taxiphyllin, lotaustralin and linamarin in sorghum, almond, stone fruits, bamboo shoots, lima beans and cassava respectively (Selmar *et.al.,*2005.,Thayer and Conn, 2001., Frehner and Conn, 2009). When ingested some of the substances react with the gut linamarase (β-glucosidase) resulting in the release of hydrocyanic acid (HCN) causing acute and chronic cyanide poisoning (Nok and Ikediobi, 1999). The chronic cyanide toxicity associated with prolonged consumption and accumulation of residual cyanide in the body produce syndromes such as ; Konzo, stunted growth, tropical ataxic neuropathy (TAN), calcifying pancreatitis (CAP), pancreatic diabetes (PAD), endemic goitre (ENG), cretinism (CT), congenital malformation (CMF) and other mal-functional problems especially protein malnutrition and crown gail disease (Conn, 2005). These syndromes are of serious public health concern, especially in immune -compromised populations in African countries such as; Nigeria, Congo, Mozambique, Rwanda, South Africa and Tanzania (Akintonwa and Tunwashe, 2009). Cyanogenic glucosides undergo degradation reaction catalyzed by specific β-glucosidases which were characterized by various authors who used both kinetics and thermodynamic parameters including; order of reaction, rate constant, frequency factor, activation energy, enthalpy, entropy and the isokinetic temperature respectively from mathematical models (Ariahu *et al*., 2010., Ariahu *et al*., 1997., .Doungkamol *et al*., 2006., Fan *et al*., 1985., Legras *et al*., 1989., .Mkpong *et al*., 2000 and Ikya, *et al*.,2012a). The enzyme catalyzed reactions can be described adequately by various best fit mathematical models initially with kinetic models which generate the reaction rate constants that are fitted into the thermodynamic models of Arrhenius and absolute rate models to derive the thermodynamic parameters (Ikya, *et al*.,2012a). According to Ikya, *et al.*(2012a) the values of the kinetics and thermodynamic parameters in the enzyme catalyzed reactions depends on many variables including; the mathematical model used for data evaluation, substrate, environmental pH, temperature, enzyme type including its genetic make-up and the degree of purity. The authors also stated that certain enzymes exhibit absolute specificity showing a narrow spectrum while a few number genetically engineered perform the action of group specificity with a wider spectrum capable of reacting with a good number of substrates(Petrucciioli. *et al.*, 1999). The native linamarase from cassava(*Manihot esculenta crantz*) according to Onyike *et al.*(2001), hydrolysised linamarin to release hydrocyanic acid at neutral pH 7 and optimum temperature of 35^{o}C but cannot at the same conditions act on other cyanogenic glucosides. The indifference is due to the natural genetic sequencing in amino acids influenced by sources of the enzymes(Hesel and Bart, 2005., .Keresztessy *et al*., 2010., Keresztessy *et al.*, 1994 and .Keresztessy *et al*., 1996). A genetically engineered linamarase (β-glucosidase) from *Saccharomyces cerevisiae* by Ikya, *et al.*(2012b), degraded linamarin from cassava(*Manihot esculenta crantz*) and results of activities of the genetically modified enzyme samples compared favourably with those of the commercial native linamarase used as control in their study. Cicek and Essen, (1998) studied the structural characteristics of dhurrianse and expression it from sorghum. The analogue form of dhurrianse was genetically engineered (genetically engineered linamarase (β-glucosidase) from *Saccharomyces cerevisiae*) by gene manipulation techniques methods of Old and Primrose, (1985) Gruenbaum,.(1981) and CBRT, (2008). In the challenged test the enzyme degraded dhurrin , amygdalin, prunasin, taxiphyllin, lotaustralin and linamarin in sorghum, almond, stone fruits, bamboo shoots, lima beans and cassava respectively, Ikya, *et al*.(2012b). However, the kinetics and thermodynamic parameters to describe the degradation of dhurrin by the genetically engineered linamarase (β-glucosidase) from *Saccharomyces cerevisiae* is lacking. The objective of this study was to evaluate the kinetics and thermodynamic parameters that can be used to describe adequately the degradation of the toxic dhurrin by the genetically engineered linamarase (β-glucosidase) from *Saccharomyces cerevisiae* for processing safe to consume products from sorghum in the food industry.

MATERIALS AND METHODS

Enzyme samples: Commercial Native Linamarase (CNLIN) used as the control for this study was purchased from Sigma Co. Lousina, USA. β-glucosidase (GELIN) was obtained from the Department of Food Science and Technology, University of Agriculture, Makurdi. Crude GELIN extract from *Saccharomyces cerevisiae* (GELIN0) was further purified using Carboxy methyl cellulose ($GELIN_1$), Diethyl-amino-ethyl- sephadex ($GELIN_2$) and Diethyl-amino-ethyl-cellulose ($GELIN_3$).

Purification was done following the standard mthods described by Wilson and Walker, (1995) and *Jakobi (1971).*

Production of Dhurrin: Sorghum seeds were obtained from Tse-Akaa Village, Mbalagh, Makurdi, Benue State, Nigeria for the production of dhurrin using the method described by Ikediobi *et al.,* (1985). Sorghum seeds (1kg) were washed with tap water and promptly frozen overnight at -10 oC. and homogenized with 160 ml of chilled 0.1M phosphoric acid solution. The resultant slurry was filtered rapidly using glass wool and the filtrate centrifuged (1000 rpm) for 5 min. The resultant filtrate was centrifuged at 5000 x g for 5 min and the supernatant adjusted to pH 8.0 followed by re-centrifugation at 5000 x g for 8 mins. After decanting, the solid residue was air-dried to obtain about 50g of white dhurrin of mp.143 ^{o}C. This was stored at 4 ^{o}C and subsequently used for kinetics and thermodynamic characterization.

Buffer Solutions and Analytical grade Reagents: Buffer solutions and reagents were prepared for the study using standard methods.

Approaches for kinetics and thermodynamic characterization of linamarases (β-glucosidases) genetically engineered from Saccharomyces cerevisiae

Kinetic studies approach requiring an experimental design:

By use of the 5x8x6 B/W experimental design comprising 5 levels of purification ($GELIN_O$- $GELIN_3$ and CNLIN), 8 time intervals (0,10,20,30,40,50,60 and 70 min.) and 6 temperature regines (30, 32, 35, 37, 40 and 45^{0}C) enzyme catalysed degradation of **dhurrin** was studied.

The ability of Commercial Native Linamarase (CNLIN) purchased as control and the GELIN extracts from *Saccharomyces cerevisiae* to hydrolyze dhurrin extracted from sorghum to hydrocyanic acid (HCN) released within a fixed time was challenged. Spectrophotometric method was used for the estimation of hydrocyanic acid (HCN) released within a fixed time as described by Onyike *et al.* (2001).

The time dependency of linamarase degradation of the dhurrin was verified using the Zero and the first order kinetics models (Eqn.1) and (Eqn.2), respectively. $C=C_o-Kt$ (Eqn.1) and $lnC= lnC_o-Kt$ (Eqn.2)

Thermodynamic activated complex approach:

The temperature dependency of linamarase degradation of the dhurrin was verified using the Arrhenius (Eqn.3) and Absolute reaction rate (Eqn.4) models, respectively.

$\ln K= \ln K_o- Ea/R(1/T)$ (Eqn. 3) where Ea is activation energy, R is the universal gas constant (0.008314 kJ/mole oC), T is absolute temperature and ko is the frequency factor. Activation energy and ko values were determined from slopes and intercepts, respectively, of lines generated by regressing the ln k versus 1/T by use of least square linear regression as described by Ariahu and Ogunsua (2000).

Regressional analysis of ln K versus inverse absolute temperature for GELIN extracts degradation of dhurrin:

Thermodynamic absolute rate theory approach:

$\ln k/T = (Ks/hc+ \Delta S /R)- \Delta H /R (1/T)$ (Eqn. 4) where Ks and hc are Boltzmann and Planck constants, respectively. From the slopes and intercepts of the lines obtained by regressing ln k/T versus 1/T relationship, ΔH # (activation enthalpy) and ΔS # (activation entropy), respectively, were evaluated.

Statistical Analysis: The least square linear regression analysis of the kinetics thermodynamics (Arrhenius and transition state model parameters) were calculated as described by Van-Boekel (1996) and Gupta (1979).

RESULTS AND DISCUSSION

Kinetics parameters derived from first order kinetics models:

The results showed that the first order kinetic model had an advantage over the zero and second order kinetics. This was validated by the linearity of the curves and higher r^2 – values (0.998-0.998) determined for each degradation at different temperatures and pH. The first order plots for action of genetically engineerd linamarase on dhurrin at 30-45^{0}C and pH 6.8 is shown in Figures 1 and to Table 1. The regression parameters and derivatives from the corresponding figure1 are those presented for the experimental dhurrin. The reaction rate constants K (µmol/min.) for the degradation of dhurrin by the engineered linamarase as influenced by purity level ranged from 0.03-0.07 µmol/min. The derived D-values from K-values in the range of 24-67 min. were influenced by enzyme purity, pH and fermentation temperatures . The plots were linear with $r^2 \geq 0.98$. **A** typical plot showing the linearity of the curve in Figures 1 confirms the first reaction order kinetics. All degredation curves showed the linear first order reaction kinetic for all enzymes showing dhurrin degradation at 30, 32, 35, 37, 40 and 45^{0}C within 10 -70 min in a bench fermentor containing 1ml each of genetically engineered linamarase from yeast (*Saccharomyces cerevisiae)* $GELIN_0$, $GELIN_1$, $GELIN_2$ and $GELIN_3$ at pH6.8. The degree of purification ranging from 1st, 2nd and 3rd

producing purified fractions from $GELIN_0$ into $GELIN_1$, $GELIN_2$ and $GELIN_3$ respectively influenced faster dhurrin degradation as shown in the corresponding K-values.

K values ranging from 0.0252moles per second to 0.92 for all enzymes, are in agreement with the work of Lionel *et al.*(2008). The mean K values are normally used in computing E_a energy and K_o, and also in the computation of ΔH and ΔS values. Pseudo first order reaction kinetics of enzymes used for the degradation of substrate were said to be temperature dependent (withers *et al.,* 200). These authors calculated there K values for the degredation of vitamins and obtained K values in the range of 0.025 – 0.25. In this study the value of 0.025 – 0.09 per minute in GELIN catalyzed reaction and 0 – 0.02 per minutes for CELIN catalyzed reaction are in agreement with the studies of Wither *et al.*(2002). The result shows that biomolecules are degraded under the same mechanism.

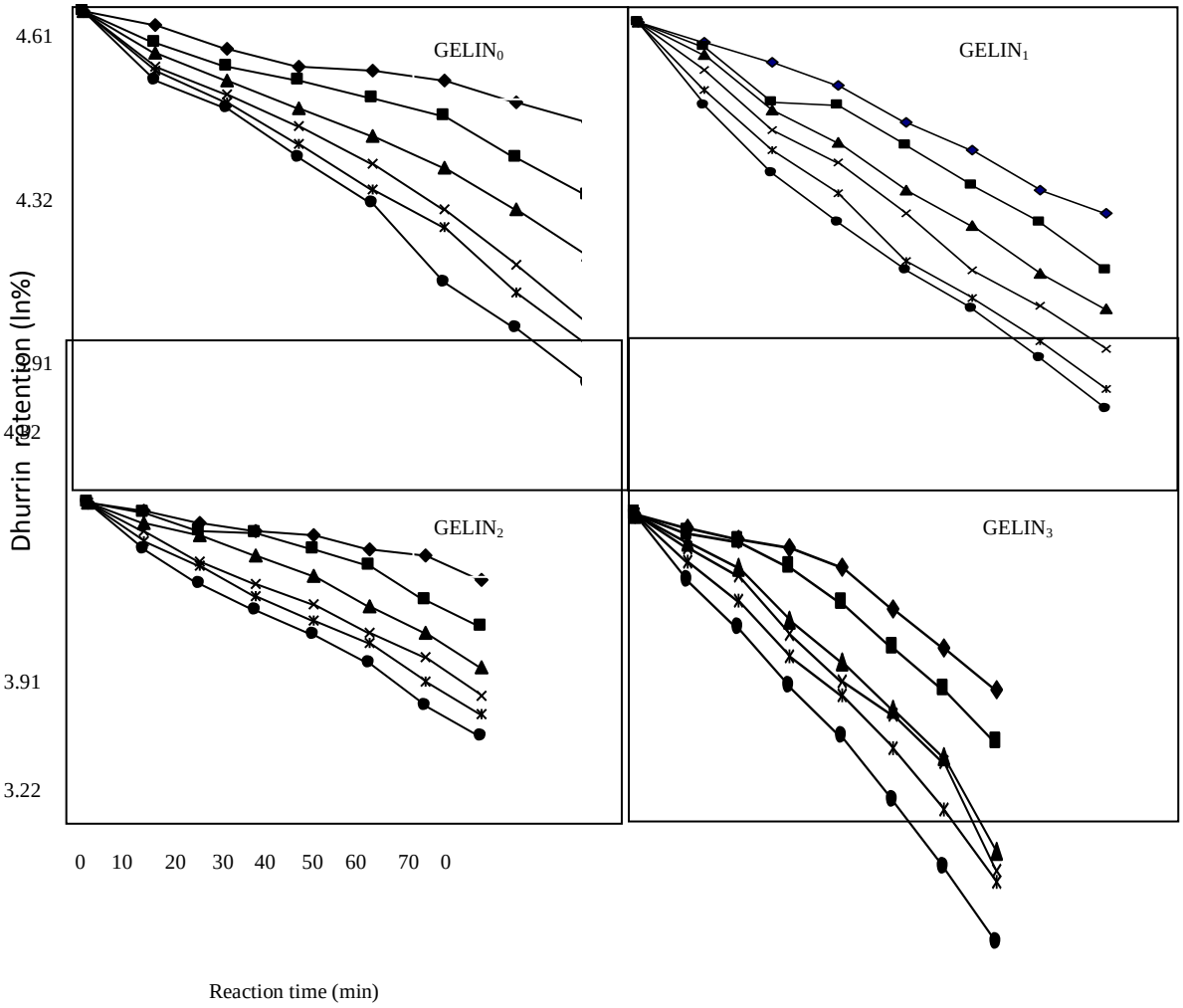

Table 1: First Order Kinetics Parameters for Dhurrin Degradation by Genetically Engineered Linamarase from Yeast (*Saccharomyces cerevisiae*).

Enzymes	Parameters	pH 6.8					
	Temperature ^{0}C	30	32	35	37	49	45
	r^2	0.99	0.99	0.99	0.99	0.99	0.99
$GELIN_0$	S.E	0.17	0.22	0.37	0.43	0.51	0.68
	Intercept	4.7	4.7	4.8	0.49	4.9	4.7
	Gradient	-0.03	-0.03	-0.04	-0.05	-0.05	-0.06
	$\therefore$(K Min^{-1})	0.03	0.03	0.04	0.05	0.05	0.06
	D(min)	65	64	48	44	40	35
	r^2	0.99	0.99	0.99	0.99	0.99	0.99
$GELIN_1$	S.E	0.37	0.43	3.8	0.46	0.24	0.25
	Intercept	4.9	4.8	4.9	4.9	4.9	4.7
	Gradient	-0.04	-0.04	-0.05	-0.05	-0.05	-0.07
	$\therefore$(K Min^{-1})	0.04	0.04	0.05	0.05	0.05	0.07
	D(min)	48	48	45	43	39	29
	r^2	0.99	0.96	0.95	0.94	0.94	0.94
$GELIN_2$	S.E	0.07	0.17	0.21	0.22	0.24	0.27
	Intercept	4.6	4.7	4.7	4.7	4.7	4.8
	Gradient	-0.03	-0.03	-0.04	-0.04	-0.04	-0.05
	$\therefore$(K Min^{-1})	0.03	0.03	0.04	0.04	0.04	0.05
	D(min)	47	46	43	42	38	26
	r^2	0.99	0.98	0.98	0.98	0.98	0.97
	S.E	0.07	0.11	0.12	0.12	0.15	0.18
$GELIN_3$	Intercept	4.6	4.6	4.6	4.6	4.6	4.7
	Gradient	-0.03	-0.04	-0.04	-0.04	-0.04	-0.05
	$\therefore$(K Min^{-1})	0.03	0.04	0.04	0.04	0.04	0.05
	D(min)	48	45	41	41	35	24

Key:
GELIN = Genetically engineered linamarase
$GELIN_0$ = Crude
$GELIN_1$ = 1st degree of purification using carboxy-methyl cellulose (CMC),
$GELIN_2$ = 2nd degree of purification using diethyl amino ethyl-sephadex (DEAE-sephadex)
$GELIN_3$ = 3rd degree of purification using diethyl amino ethyl-cellulose (DEAE-cellulose)

Table 2: Activation Energy (E_a) and Frequency Factor (K_o) Derived from Arrhenius Plot in the Degradation of Dhurrin

Kinetic parameter	Genetically engineered linamarase			
	$GELIN_0$	$GELIN_1$	$GELIN_2$	$GELIN_3$
N	6	6	6	6
r^2	0.997	0.992	0.992	0.995
S.E	0.099	0.099	0.099	0.099
Intercept	3.117	3.523	3.810	4.034
$\therefore K_o$(min^{-1})	22.585	33.877	45.169	56.464
Gradient	-7228. 71	-1084.67	1451.60	-1812.80
$\therefore E_a$(KJ/mol)	60.0995	90.1460	120.6415	150.6900

Key:
$GELIN_0$ = Crude
$GELIN_1$ = 1st degree of purification using carboxy-methyl cellulose (CMC),
$GELIN_2$ = 2nd degree of purification using diethyl amino ethyl-sephadex (DEAE-sephadex)
$GELIN_3$ = 3rd degree of purification using diethyl amino ethyl-cellulose (DEAE-cellulose)
SE = Standard error
K_o = Frequency factor
E_a = Activation energy

T**hermodynamic parameters derived from absolute temperature dependent Arrhenius model:**

Table2 shows the absolute temperature versus reaction rate constant (K) relationship(Arrhenius model) for evaluation of Activation Energy(E_a) and Frequency Factor(K_o), as the thermodynamic parameters involved in the degradation of dhurrin at pH 6.8 in the presence of genetically engineered enzymes $GELIN_0$, $GELIN_1$, $GELIN_2$, and $GELIN_3$. The temperature dependent of the heat degradation rate constants (K) were related to the Arrhenius plot for the generation of the activation energy (E_a) and frequency factor (K_o).

The frequency factors (Ko) increased with enzyme purity from $GELIN_0$ to $GELIN_3$ corresponding to K_o (min^{-1}) of 22.585 to 56.462. The energy of activation Ea (KJ/mol) generated 60.0995 to150.6900 corresponding to enzymes $GELIN_0$ to $GELIN_3$. It followed the same pattern with frequency factor for breaking of bonds in dhurrin molecules. At pH 6.8 CNLIN showed no action on dhurrin. The high correlation coefficient values of (r^2= 0.97 to 0.99) indicated the best fit of the Arrhenius model in the characterization of the activation energy involved in the degradation of dhurrin. Larger magnitude of E_a is associated with higher temperature dependence. Dhurrin was therefore, more sensitive to temperature at pH, 6.8 and higher enzyme purity. This implies that a small change in temperature in the presence of purified enzyme at optimum pH produces a larger change in the rate of degradation of dhurrin. In the thermodynamic calculations the frequency factors (K_o) indicated the higher effective collision of dhurrin molecules to enzyme interactions. This provided enough energy for the degradation of dhurrin. The changes in Ea were accompanied by parallel increase of the collin factors (K_o). According to Speroni *et al.* (1985), whenever the E_a or K_o values differ between two systems, it is implies dissimilarity in mechanism of heat required in the degradation reaction. In this study the E_a or K_o-values did not differ significantly between all the genetically engineered enzymes, $GELIN_0$ to $GELIN_3$ for degradation of dhurrin. However, the frequency factors were determined by extrapolation to values well outside the range of temperatures used experimentally with minor changes in the E_a resulting in substantial changes in K_o. The E_a values described by heating in the present study were outside the ranges of 210-630KJ/mol for negative microbial cells destruction heat (Rhim *et.al.*,1990). Thermodynamics parameters (E_a, K_o, ΔS ΔH) are the values of heat degradation of linamarase catalysed degradation of dhurrin. The influence of enzyme purity, PH, variation and activation temperatures on the rate constant (K) associated with the degradation of dhurrin were derived on the basis of the first order reaction kinetics (Toledo, 1987), showing high value of (r^2=0.998).

Table 3: Absolute Reaction Rate Parameter for Dhurrin Degradation Genetically Engineered Linamarase from Saccharomyces c*erevisiae*

Kinetic parameter	$GELIN_0$	$GELIN_1$	$GELIN_2$	$GELIN_3$
N	6	6	6	6
r^2	0.995	0.993	0.992	0.983
S.E	0.095	0.023	0.014	0.013
Intercept	0.087	0.122	0.175	0.218
ΔSJ/mol deg.	0.587	0.823	1.172	1.463
Gradient	-1227	-1350	-1463	-1576
ΔH (KJ/mol)	1892.00	11232	12168	13104

KEY:

$GELIN_0$ = Crude

$GELIN_1$ = 1st degree of purification using CMC,

$GELIN_2$ = 2nd degree of purification using DEAE-sephadex

$GELIN_3$ = 3rd degree of purification using DEAE-cellulose

ΔH = Activation Enthalpy change

ΔS = Activation Entropy change

SE = Standard error

Thermodynamic parameters derived from absolute reaction rate model:

Table 3 shows the thermodynamic data including; entropy change (ΔS) in the units of J/mol.deg and enthalpy change ΔH (KJ/mol) which followed the same pattern where increases were influenced by enzyme purity in order of $GELIN_0$, $GELIN_1$, and $GELIN_3$. The entropy change (ΔS) increased with enzyme purity from 0.588 J/mol.deg. to 1.4625Jmol degree. The enthalpy change KJ/mol followed the same pattern where increases influenced by enzyme purity ranged from 1892 KJ/mol to 13104KJ/mol. The high correlation coefficient r^2 value of to .999 suggest adequate fit of the absolute reaction rate model in describing and characterizing enzymes in the degradation of dhurrin (Arriahu and Ogunsua, 2000). Corresponding increases in entrophy (ΔS) and enthalpy (ΔH) values suggest greater sensitivity of the reaction with temperature and enzyme purity. The entrophy (ΔS) being a non zero parameter assumed that the heat degradation of dhurrin was spontaneous and irrevisible (Ariahu and Ogunsua, 2000). The positive values of entrophy (ΔS) indicated a decrease in structural order of molecules in the activated complex. Higher rates suggests faster degradation rates as a result of the empirical nature of the Arrhenius model, it was proposed that absolute reaction rate model be applied in the analysis of the thermal rate data, (Arriahu and Ogunsua, 2000). The value of entropy (ΔS) also includes the degree of steric and orientation requirements and also solvent (Sodium acetate buffer) effects. The absolute reaction theory, provides a better insight into the roles of these factors in the degradation of dhurrin than the less definite probalility factor of the collision theory (Ariahu and Ogunsua, 2000). The enthalpy change ΔH (KJ/mol) values indicated measure of the internal energy of the activated complex formed first before the transition state is reached (Atkins 1980). In general, the derived enthalpy change ΔH (KJ/mol) values being a measure of the energy barrier must be reduced by reacting molecules. These values are related to the strength of the bonds in dhurrin which are broken and made in the formation of the transition sate from the reactants. The enthalpy change ΔH (KJ/mol) values is related to how molecules with the appropriate energy can actually react. (Ariahu and Ogunsua, 2000). These values are related to the strength of the bonds in dhurrin which are broken and made in the formation of the transition state from the reactants.

CONCLUSION:

In this study genetically engineered linamarase (β-glucosidase) from *Saccharomyces cerevisiae* acted on dhurrin. The results showed that the best fit order was the first order kinetics model and not either zero or second order models. The study also indicated that the best fit of the Arrhenius and the absolute reaction rate models described degradation of dhurrin by genetically engineered linamarase (β-glucosidase) from *Saccharomyces cerevisiae. The enzyme is recommended for use in food processing.*

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