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Full Length Research Paper

Statistical optimization of L–glutaminase production by *Trichoderma* species under solid state fermentation using African locust beans as substrate

Musa Olatunji Olarewaju* and Humphrey Chukwuemeka Nzelibe

Department of Biochemistry, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria.

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Six strains of *Trichoderma* species isolated from soil and air samples were screened for extracellular L-glutaminase production. The highest enzyme producing isolate, *Trichoderma longibrachiatum* was subjected to screening and nutrients optimization experiments under solid state fermentation using a constant weight of African locust beans as substrate. A specific activity of 54.96 U/mg was achieved at 1% (w/v) glutamine, 0.5% (w/v) glucose, 1% (w/v) NaCl, pH 3.0 and 1.4 ml inoculums size respectively after 3 days of incubation at 27°C as the optimum condition for L–glutaminase production. Asides glutamine, other nitrogen sources; malt extract and ammonium sulphate had a negative influence on enzyme production likewise the supplemented sources of metal ions; zinc and iron (II) ions.

Key words: *Trichoderma*, L-glutaminase, fermentation, African locust beans.

INTRODUCTION

L-glutaminase (E.C. 3.2.1.5) is an amidohydrolase that catalyzes the deamination of L-glutamine, producing glutamate and ammonia as reaction products. The enzyme is important in nitrogen metabolism and has a wide distribution in cells of microorganisms, plants and animals (El-Ghonemy, 2015).

L-glutaminase has received tremendous research interest, with focus on characterization and production optimization of the enzyme from different microbial sources. L-glutaminase's promising therapeutic alternative to the treatment of leukaemia and retroviral diseases justifies this research attention (Binod et al., 2017).

L-glutaminase's applications span across the food and medical industries. The L-glutaminase /asparaginase complex is used in the treatment of acute leukaemia and is one of the most important therapeutic enzymes in the medical industry (El-Ghonemy, 2015). In the food industries, L-glutaminase is used as flavour enhancing agent (Sarada, 2013). Several microorganisms have been reported to secrete L-glutaminase into fermentation media. Among the studied organisms, fungi are reported as the most potent producers of the enzyme (Binod et al., 2017). Several species, from the genus of *Penicillium* (El–Shafei et al., 2014) *Saccharomyces* (Iyer and Rekha, 2010) and *Aspergillus* (Dutt et al., 2014) have hence

*Corresponding author. E-mail: akomst6@gmail.com.

Table 1. Medium components of the Plackett–Burman design.

Parameter	Low (–)	High (+)
Temperature (°C)	37.0	27.0
pH	9.0	3.0
Incubation period (days)	7.0	3.0
Inoculum sizes (mL)	1.0	0.5
Glutamine (% w/v)	1.0	0.5
Ammonium sulphate (% w/v)	0.4	0.2
Malt extract (% w/v)	0.7	0.3
Glucose (% w/v)	1.0	0.5
$FeCl_2$ (% w/v)	0.05	0.02
$ZnSO_4$ (% w/v)	0.4	0.2
NaCl (% w/v)	2.0	1.0

been studied in this regard. However, the industrially important *Trichoderma* genus has received relatively fewer attentions with regards to L-glutaminase production among the species. In addition, the need to explore low-cost carbon sources as the substrate for biochemical production is highly desirable for a better market competitiveness of the products.

African locust bean tree (*Parkia biglobosa*) has a wide distribution across the West Africa. A mature tree is capable of producing over 1 metric ton of beans per annum. Despite the abundance, its usage is restricted to the production of a local condiment called “daddawa” (Kayode, 2012). Hence, Elemo et al. (2011) characterized African locust bean is an under-exploited tropical legume.

MATERIALS AND METHODS

Isolation and species identification

Soil samples were collected separately from decaying woods, plants and leaves, open vegetative fields and garden bed soil within the Ahmadu Bello University main campus Samaru, Zaria, Kaduna State. Soil samples were sprinkled on separate potato dextrose agar (PDA) plates and incubated at laboratory temperature (28 ± 1°C). Three other PDA plates, not inoculated with soil samples, were exposed to laboratory air throughout the incubation period.

Fungal growth on each plate was after 3 to 7 days examined for morphological characteristics of *Trichoderma* species. Suspected *Trichoderma* species were picked from the isolation plates, sub cultured on fresh PDA plates and incubated at laboratory temperature. Pure colonies of suspected *Trichoderma* were identified to species level on 2% malt extract agar as described by Gams and Bissett (2002).

Qualitative plate screening assay for L–glutaminase production

Isolated *Trichoderma* species were screened for L-glutaminase production by the plate assay method using Minimal Glutamine Agar (MGA) medium with the following composition (g/L); 0.5 KCl, 0.5 $MgSO_4$, 1.0 KH_2PO_4, 0.1 $FeSO_4$, 0.1 $ZnSO_4$, 25 NaCl, 2.0 glucose, 20.0 agar, 10.0 L-Glutamine and 2.5% phenol red as an indicator. L-glutamine serves as a carbon and sole nitrogen source growth in the media. Development of pink zone around individual colony was indicates extracellular L-glutaminase activity. Level of activity base on the pink intensity is reported as low (+), moderate (++), high (+++) and very high intensity (++++) as described by El-Shafei et al. (2012).

Maintenance of fungi and conidia harvesting

The selected *Trichoderma* specie was maintained on malt extract agar and periodically sub cultured. Prior to conidia harvesting, the culture was grown in the dark and under light at 28±1°C for 3 and 6 days, respectively. Conidia suspension from 9 days old culture was prepared as described by Ahmed and El-Katatny (2007). The conidia concentration was determined using hemocytometer and diluted to 8×10^5 conidia/ml.

Screening of fermentation condition for L–glutaminase production

Eleven physicochemical parameters potentially affecting L-glutaminase yield were screened for effect at two levels; a high (+1) and a low (-1) level settings using Plackett–Burman Design as shown in Table 1. The negligible levels of zinc and iron in African locust bean (Ouoba et al., 2003) justified their inclusion in the design, while other physiochemical parameters were selected on the basis of their potential effect on L-glutaminase production in some previously published studies (El–Sayed, 2009; Iyer and Rekha, 2010; El–Shafei et al., 2012).

Optimization using response surface methodology

Response Surface Methodology (RSM), using Central Composite Design (CCD) was used to optimize crucial parameters selected from the screening experiment and to build a polynomial model that relates factors and response. JMP Statistical Discovery (2013) v. 11.0.0 was used to generate experimental run matrix based on 3-level settings of a high (+1), medium (0) and a low (–1) levels of individual parameters. The relationship between dependent and independent variables was related by the following second-order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_1 AE + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} ED + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2$$

Table 2. Scale of colour intensity from qualitative plate screening assay for extracellular L–glutaminase production.

Trichoderma species	Colour intensity*	Source
T. longibrachiatum	++++	Air
T. citrinoviride	+++	Air
T.harzianum	++	Soil
T. inhamatum	++	Soil
T. pseudokoningii	++	Soil
T. viride	+	Soil

*++++ (very high intensity), +++ (high intensity), ++ (moderate intensity), + (low intensity).

Where Y is the dependent variable (L-glutaminase production); β_0 is the intercept in the design ; β_1, β_2, β_3, β_4 and β_5 are the linear coefficients in the design ; β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35} and β_{45} are the interaction coefficients in the design ; β_{11}, β_{22}, β_{33}, β_{44} and β_{55} are the squared coefficients in the design ; and A, B, C, D, E, AB, AC, AD, AE, BC, BD, BE, CD, CE, ED, A^2, B^2, C^2, D^2 and E^2 are independent variables in the design.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The analysis included the Fisher's F–test (overall model significance), its associated probability values, coefficient of determination R^2 which measures the goodness of fit of regression model. The fitted polynomial equation was then expressed in the form of 2D contour plots in order to illustrate the relationship between the factors and responses.

African locust bean seeds preparation and fermentation

An African locust bean was sourced from Zaria main markets, Sabon Gari Local Government, Kaduna State. They were identified and validated as *Parkia biglobosa* in the family of *Fabaceae* at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, and a voucher with number 7064 was issued. The seeds were prepared for fermentation by means of steaming for four and a half hours to soften the hard testa protecting the cotyledon. The steamed seeds were dehulled to expose the cotyledon by applying friction between the beans and a rough surface as described by Sadiku (2010).

Solid state fermentation (SSF) was carried out on the prepared cotyledon in a 150 ml Erlenmeyer flask. For every experiment, thirty grams of cotyledon was mixed with nutrients and pH set at their low and high values based on individual experimental runs and according to the experimental design. The mixture was then autoclaved at 121°C for 30 min, cooled to room temperature and under aseptic conditions, excess fluid was drained out of each flask before each experimental set-up was inoculated with volumes of 8 × 10^5 conidia/ml of *Trichoderma* sp. conidia suspension and incubated.

Crude enzyme extraction

Crude L-glutaminase was extracted from the fermented cotyledon through simple contact method, using 0.05 M citrate-phosphate buffer (pH 6.0) according to the method of Kashyap et al. (2002). The fermented beans were thoroughly mixed with 15 ml of the buffer solution using a rotary shaker set at 150 rpm for 30 min. The entire contents of the flask were squeezed through a mesh, the pooled extract was then centrifuged for 10 min at 10,000 rpm (4°C) and the collected supernatant was used as the crude enzyme for subsequent assays.

Protein quantification

Protein concentration was estimated with Folin–Ciocalteu reagent using Lowry modified protocol as describe by Hartree (1972) with bovine serum albumin (BSA), as standard protein. Protein concentration was expressed as mg/ml of crude enzyme.

L-glutaminase assay

L-glutaminase was assayed by direct Nesslerization according to the method of Imada et al. (1973). The enzymatic reaction mixture contains 0.5 ml of 1% L-glutamine in 0.5 ml of 0.5 M citrate-phosphate buffer (pH 6.0) and 0.5 ml of the crude enzyme incubated at 37°C for 30 min. Enzymatic reaction was stopped with 0.5 ml of 1.5 M trichloroacetic acid. The reaction mixture was then centrifuged at 3500 rpm for 30 min to remove protein precipitates. The released ammonium was quantified by the addition of 0.5 ml Nessler reagent and absorbance was recorded at 480 nm with a spectrophotometer. Substrate and enzyme blank were used as control. One unit (U) of L-glutaminase is defined as the amount of enzyme that liberates 1 µmol of ammonia under assay conditions.

Experimental model validation

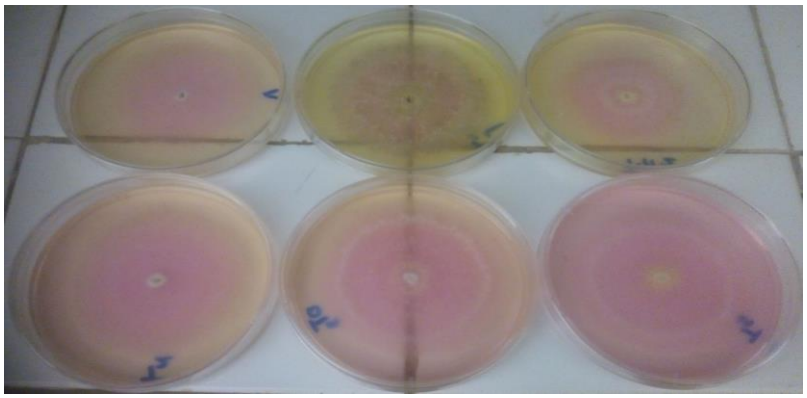
The predicted optimized condition of fermentation for maximal yield of L–glutaminase by *T. longibrachiatum* according to the polynomial model was experimentally validated by setting parameters at their individual optimized levels.

RESULTS AND DISCUSSION

This study undertook the isolation and identification of *Trichoderma* species in a preliminary step to identify a high extracellular glutaminase producing genus member. All the collected soil samples failed to yield *Trichoderma* specie isolates except garden bed soil samples, representing about 16% of the sampled areas. In total, four isolates of *Trichoderma* species were recovered from the soil sample plates and additional two isolates from the "air sample" plates as shown in Table 2. The poor recovery of *Trichoderma* from soil samples has previously been noted by other researcher and among other factors, soil nutrient and pH are major determinants of isolation success rate (Mirkhani and Alaei, 2015).

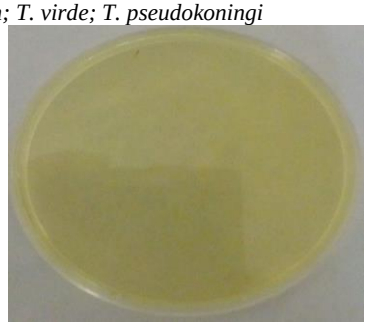
Qualitative plate screening experiment of the six isolates as reported in Table 2 and Figure 1 indicated *Trichoderma longibrachiatum* as the highest extracellular enzyme producing isolate adjudge from the pink colour intensity around its colony, this was followed in colour intensity by *Trichoderma citrinoviride* while *Trichoderma viride* showed the least extracellular L-glutaminase activity as compared to moderate activity exhibited by *T. harzianum* and *T. inhamatum*.

Previous researchers (El–Shafei et al., 2012) have



A

From left bottom; *L. Iongibrachiatum; T. citrinoviride; T. harzianum*
From left top; *T. inhamatum; T. virde; T. pseudokoningi*



B

Figure 1. Qualitative plate screening assay of six *Trichoderma* species for extracellular L-glutaminase production. A: Test plates, B: Control plate.

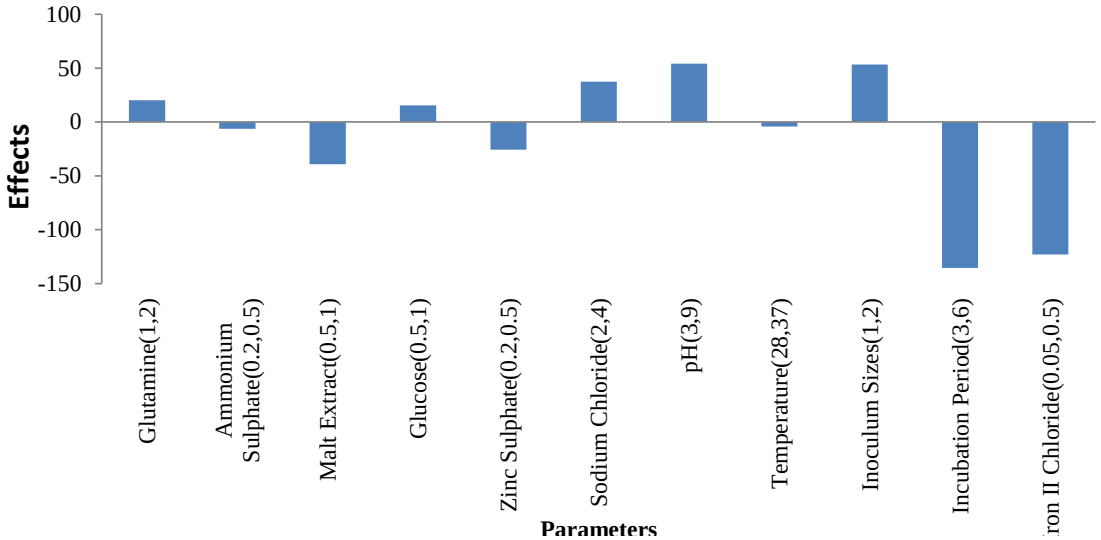


Figure 2. Effect of different parameters on L–glutaminase production by *T. longibrachiatum*.

likewise used plate screening assay method to select a high L– glutaminase producing organism prior to optimization works, thus demonstrating the efficiency of the method for candidate strain selection for optimization.

Screening experiment

T. longibrachiatum was selected for optimization studies for L-glutaminase production. As shown in Figure 2, only

five of the parameters; inoculum sizes, pH, NaCl, glucose and glutamine had positive impact on enzyme yield. The impact of glucose and glutamine on enzyme yield is quite in agreement with the findings of some researchers. El–Shafei et al. (2012) found glucose among ten other sugars as having the most impact on L-glutaminase yield by *Penicillum brevicompactum,* the researchers also found glutamine as suitable nitrogen source in achieving a high glutaminase yield. Similar observations was reported by El-Sayed (2009) and in addition found pH and inoculums sizes as having significant effect on L-glutaminase yield by *Trichoderma koningii.* Also, El-Shafei et al. (2012) and Abdallah et al. (2013) found significant effect for NaCl on L-glutaminase yield and determined the optimal concentrations for the organisms.

The different physiochemical parameters having negative impact on enzyme yield also shown in Figure 2 are incubation period, iron (II) chloride, zinc sulphate, malt extract, ammonium sulphate and temperature. Other researchers have also studied the effect of incubation period on L-glutaminase yield and found different optimized incubation periods for the organisms under study (Iyer and Rekha, 2010; El–Shafei et al., 2012). The presence of zinc and iron (II) ions in the medium was detrimental to enzyme production or may have caused the inhibition of available enzyme in the fermentation medium leading to overall negative impact. Jesuraj et al. (2013), studied the effect of four divalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+}) supplementation on L-glutaminase yield from three bacteria isolates and found only Mg^{2+} and Mn^{2+} ions to slightly improve enzyme yield, while both Zn^{2+} and Fe^{2+} ions had negative impact on enzyme yield. Similar observation is also reported in this study.

The negative impact of nitrogen supplements, both malt extract and ammonium sulphate on enzyme yield could indicate the preference and sufficiency of glutamine as both an L-glutaminase inducer and a nitrogen source for cell growth. The repression of glutaminase secretion in the presence of malt extract and ammonium salts as was observed in this study, is in agreement with the findings of El-Sayed (2009) and Abdallah et al. (2013). The impact of temperature on L–glutaminase yield was the most minimal, only slightly having a negative effect on enzyme production. Similar observation was reported by El-Sayed (2009) and El-Shafei et al. (2012). These researchers noted slight and gradual decrease in enzyme yield for every 5°C rise in temperature of the fermentation medium and thus found 30 and 28°C respectively as optimum temperature for L-glutaminase production.

Placket–Burman Design (PBD) is a valuable tool in screening of large number of factors likely to influence product yield. The use of the design in this study has allowed the identification of five key variables and elimination of six others not eligible for further optimization with regards to L-glutaminase production. However, the design provides no details about variables interaction and how they affects products yield. Hence, a design that offers higher resolution of main effect interaction was used for further optimization studies.

Optimization experiment

Central composite design (CCD) under response surface methodology (RSM) was used to optimize L-glutaminase yield using the selected factors from the screening experiment and to build a polynomial model relating factors and response. The experimental design with respect to the real values of the independent variables and attained values is presented in Table 3. A total of 28 experimental runs were modeled on a polynomial equation:

$$\text{Specific Activity} = 18.27 - 1.69A - 2.83B - 1.97C + 0.39D + 2.67E - 1.77AB - 2.83AC + 7.26BC - 6.44AD + 0.10BD - 1.44CD + 3.74AE + 0.69BE + 2.48CE - 1.72DE + 5.05A^2 + 10.19B^2 + 6.29C^2 + 2.40D^2 - 15.01E^2$$

Where A= [(Glutamine – 0.6) / 0.4], B= [(Glucose – 1) / 0.5], C= [(NaCl – 1.5) / 0.5], D= [(pH – 6) / 3], E= [(Inoculum Sizes – 1) / 0.5

The adequacy of the model was investigated using Analysis of Variance (ANOVA) which was tested using Fisher's statistical analysis, as shown in Table 4. The model p–value of <0.0001 imply that the model is significant, suggesting that chances of the model F–value of 40.12 occurring due to noise is 0.01%, likewise, the non-significant lack of fit of the model is evidence that the experimental response fits with the model adequately (Salihu et al., 2011). Also the high R^2 of 0.9913 being the measure of the goodness of fit of the model suggest that 99.13% variation in the response can be accounted for by the model equation.

Significance of individual model coefficient values, shown in Table 5 indicates the strength of each terms effect towards overall L-glutaminase yield. The smaller the p-values, the greater the significance level of the corresponding coefficient to the response, thus, the glucose-NaCl and glutamine–pH interaction term and the inoculum–inoculum quadratic terms with p–values <0.0001 exert the most influence on the system. Other interaction terms were also significant as shown in Table 5.

In order to better navigate the design space and offer a graphical representation of how interactions among variables could be used to determine response, the 2D contour plot of variable concentrations is shown in Figure 3. The plot is based on a function of two variable concentrations while others are kept constant. Figure 3A represent the interaction between glutamine and pH, as glutamine concentration increases for any particular pH, there is a corresponding increase in enzyme yield, however, as the pH tends towards minimum and glutamine concentration tends towards maximum, the

Table 3. Experimental design and results of CCD of response surface methodology for the optimization of L–glutaminase production.

Pattern	Glutamine	Glucose	NaCl	pH	Inoculum sizes	Specific activity	Pred. specific activity
a0000	0.2	1	1.5	6	1	24.05	24.99
A0000	1	1	1.5	6	1	22.10	21.61
000a0	0.6	1	1.5	3	1	19.18	19.36
−−+−+	0.2	0.5	2	3	1.5	21.34	20.21
+−+++	1	0.5	2	9	1.5	17.18	17.21
−+−−+	0.2	1.5	1	3	1.5	10.46	9.68
0A000	0.6	1.5	1.5	6	1	28.00	25.60
−−−−−	0.2	0.5	1	3	0.5	31.17	30.43
+−−+−	1	0.5	1	9	0.5	36.06	36.48
0a000	0.6	0.5	1.5	6	1	28.43	31.27
++−−−	1	1.5	1	3	0.5	16.22	16.31
++++−	1	1.5	2	9	0.5	7.20	8.58
00A00	0.6	1	2	6	1	23.55	22.57
++−++	1	1.5	1	9	1.5	16.73	17.12
+++−+	1	1.5	2	3	1.5	41.88	41.58
00000	0.6	1	1.5	6	1	19.39	18.36
+−−−+	1	0.5	1	3	1.5	52.63	51.38
−+−+−	0.2	1.5	1	9	0.5	32.29	33.19
−−−++	0.2	0.5	1	9	1.5	39.29	38.85
0000A	0.6	1	1.5	6	1.5	3.829	6.80
−++++	0.2	1.5	2	9	1.5	41.62	42.13
+−+−−	1	0.5	2	3	0.5	15.70	15.43
0000a	0.6	1	1.5	6	0.5	2.20	1.66
00a00	0.6	1	1	6	1	25.10	26.52
−−++−	0.2	0.5	2	9	0.5	29.79	30.34
−++−−	0.2	1.5	2	3	0.5	26.12	26.33
00000	0.6	1	1.5	6	1	19.10	18.36
000A0	0.6	1	1.5	9	1	21.68	20.93

Table 4. Analysis of variance of model for L–glutaminase production by *T. longibrachiatum.*

Source	DF	Sum of squares	Mean square	F ratio
Model	20	3653.6293	182.681	40.1251
Error	7	31.8696	4.553	**Prob > F**
C. Total	27	3685.4989		<.0001*

R^2 = 0.956, Lack of fit (p–value) = 0.0701 *Indicates that the model is significant.

enzyme yield is further increased as opposed to a rise in pH. Similar trend can be observed for the glutamine–NaCl and glutamine– glucose interaction as depicted in Figure 3B and C. The glutamine–inoculum size interaction follows similar pattern, however, an optimum is reached at around 1 ml inoculums size (Figure 3D) as could be observed also for the pH and glucose–inoculum size interaction after which there is a decline in enzyme yield for any further increase or decrease in inoculums size (Figure 3E and F). Figures 3G and H showed that, there is a corresponding increase in enzyme yield as glucose and other variables (NaCl and pH) levels tends toward minimum, similar trend could be observed in the NaCl–pH interaction as shown in Figure 3I. Thus, it can be seen that the various parameters under optimization study exhibit strong synergistic effects towards L-glutaminase productivity by *T. longibrachiatum* strain.

Experimental model validation

An optimal level of each of these parameters was hence

Table 5. Model coefficients estimated by multiple linear regression and significance of regression coefficient for glutaminase yield from *T. longibrachiatum*.

Term	Estimate	Prob>|t|
Intercept	18.27	<.0001*
Glutamine(0.2,1)	–1.69	0.0121*
Glucose(0.5,1.5)	–2.8	0.0008*
NaCl(1,2)	–1.97	0.0057*
pH(3,9)	0.39	0.4567
Inoculum Sizes(0.5,1.5)	2.67	0.0011*
Glutamine*Glucose	–1.77	0.0126*
Glutamine*NaCl	–2.83	0.0011*
Glucose*NaCl	7.26	<0.0001*
Glutamine*pH	–6.44	<0.0001*
Glucose*pH	0.10	0.8485
NaCl*pH	–1.44	0.0301*
Glutamine*Inoculum Sizes	3.74	0.0002*
Glucose*Inoculum Sizes	0.69	0.2335
NaCl*Inoculum Sizes	2.48	0.0023*
pH*Inoculum Sizes	–1.72	0.0143*
Glutamine*Glutamine	5.05	0.0076*
Glucose*Glucose	10.19	0.0001*
NaCl*NaCl	6.29	0.0024*
pH*pH	2.40	0.1214
Inoculum sizes*Inoculum Sizes	–15.01	<0.0001*

Table 6. Experimental model validation for the production of L–glutaminase from *T. longibrachiatum.*

Experiment	Glutamine (w/v)	Glucose (w/v)	NaCl (w/v)	pH	Inoculums size (ml)	Specific activity (U/mg)	
						Experimental	Predicted
Test	1.0	0.5	1	3	1.4	54.96±0.18	55.504
Control	0.0	0.0	0.0	0.0	1.4	32.05±0.25	

suggested by the software and thus validated experimentally. As shown in Table 6, 1% glutamine, 0.5% glucose, 1% NaCl, pH 3 and 1.4 ml inoculums sizes were suggested as optimal levels of parameters with a predicted specific activity of 55.50 U/mg which was hence validated experimentally in triplicates with an output value of 54.96 U/mg achieved.

This result represents a 1.7 fold increase in L-glutaminase production when compared with the non-supplemented, un–optimized condition of L-glutaminase production using African locust as substrate.

The result of the optimized L-glutaminase production achieved in this study compares favourably with those of previous researchers. Using optimized conditions, El–Shafei et al. (2012) recorded a maximum specific activity of 5.21 U/mg using *Penicillium brevicompactum* while 23.2 and 40.32 U/mg were obtained by El-Sayed (2009) and Bülbül and Karakuş (2013) using *T. koningii* and *Hypocrea jecorina* (anamorph of *Trichoderma reesei*) respectively.

Conclusion

Enzyme productivity obtained in this study, using *T. longibrachiatum* compares favourably with some reported productivity from other glutaminase producing organisms. The high productivity achieved in this study also underscores the potential of African locust beans as substrate and basal medium for the production of biochemicals, including enzymes. African locust beans remains an underexploited tropical legume, thus, this work has demonstrated that African locust beans could be exploited in enzyme production by microorganisms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

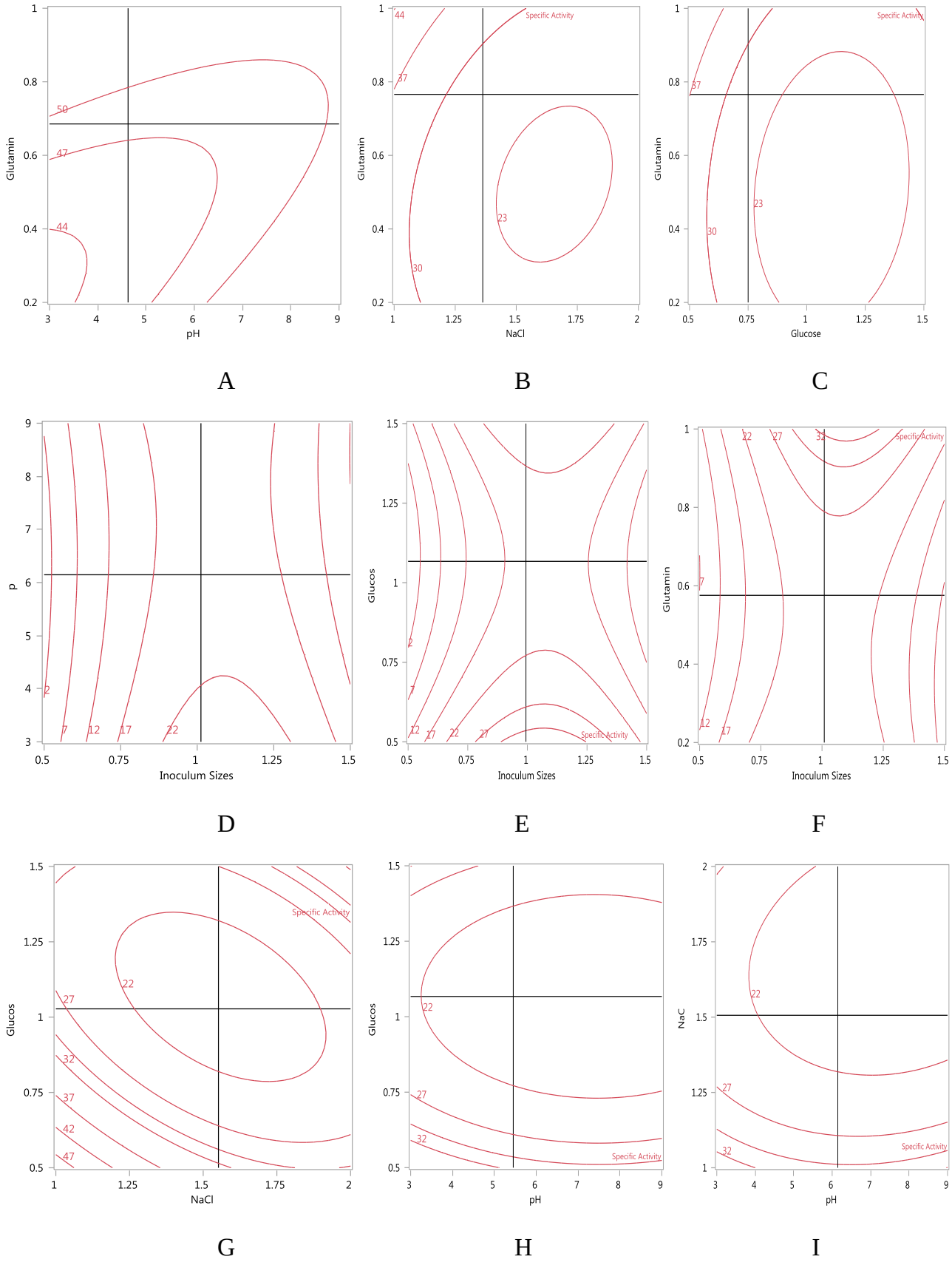


Figure 3. Contour plot interactive effects for among factors influencing L–glutaminase yield.

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The pro-oxidant effect of dextran sodium sulphate on oxidative stress biomarkers and antioxidant enzymes in *Drosophila melanogaster*

Augustina O. Akinsanmi[1,2]*, Opeyemi Balogun[3], Johnson O. Titlayo[2], Ishaya Y. Longdet[2] and Aguiyi C. John[1]

[1]African Centre of Excellence in Phytomedicine Research and Development, Nigeria.
[2]Department of Biochemistry, Faculty of Medical Sciences, University of Jos, Jos, Nigeria.
[3]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, University of Jos, Jos, Nigeria.

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This study sought to evaluate the behavioural and biochemical effects of Dextran Sodium Sulphate (DSS) on the oxidative stress biomarkers and antioxidant enzymes. *Drosophila melanogaster* (both sexes) of 3 days old were exposed to various DSS incorporated diets at concentrations, 0.5 - 3.0% for a period of 14 days (Survival rate). The second phase comprised of 3 groups; Group I- Control (normal diet), Group II-0.5% DSS, and Group III – 1.0% DSS and fed for 5 days. Climbing activity and biochemical assays were then determined. The survival rates of the flies with concentrations above 1.0% were highly reduced. The induced oxidative stress caused by DSS showed an impaired climbing activity, a significant ($p<0.05$) increase in the catalase enzymatic activity and malondialdehyde content in both Groups II and III in relation to the flies in Group I (control). Also there was significant ($p<0.05$) inhibition of GST activity and reduction of total thiol contents in group III in comparison to the control. In conclusion, the DSS dose- dependent toxicity effect was revealed by the increase in the malondialdehyde contents and catalase enzymatic activity.

Key words: *Drosophila melanogaster*, oxidative stress, pro-oxidant, antioxidant enzymes, dextran sodium sulphate.

INTRODUCTION

Drosophila melanogaster is a dipteran insect with two wings; typical characteristic of the “true flies”. Since one hundred years ago, these flies have been introduced as a research model in the studies of genetics and other related aspects of molecular biology. Of recent, Drosophotoxicology has been introduced, which is a toxicology study in which parts or whole of the fly is used (Rand, 2010; Chifiriuc et al., 2016). It meets the standard of the European Centre for the Validation of Alternative Methods (ECVAM): Reduction, Refinement and Replacement (3Rs) of laboratory animal usage (Festing et al., 1998). The use of *D. melanogaster* in toxicity studies addresses the problem of obtaining ethical clearance for the animals, and has many advantages over vertebrates, such as; it is 3-4 mm long in size, wholesome presentation for toxicological testing, smaller required reagents for assays, and has 75% of homologs of human disease genes, with about 90% nucleotide

*Corresponding author. E-mail: tinamakeens@gmail.com.

sequence identified in some of its species (Festing et al., 1998; Chifiriuc et al., 2016). One of the tools in toxicological testing in *D. melanogaster* involves an assessment of the oxidative stress biomarkers and its antioxidant enzymes activity.

Invertebrates and vertebrates have no control over the challenges posed to them from the environment, so they have evolved mechanisms to control their metabolic pathways as to manage the oxidative stress and other challenges (Hermes-Lima et al., 2001; Costantini, 2018). Oxidative stress is an imbalance between the free radical levels (both from endogenous and exogenous sources) and the antioxidant system of any living organism (Valko et al., 2006, Halliwell, 2007). Pro-oxidants can be endobiotic or xenobiotic that is capable of inducing oxidative stress. This they do by either generating reactive oxygen/ nitrogen species; or by their mechanism of expulsion or inhibition of antioxidant enzymes activity (Halliwell, 1991; Sies, 2018). Antioxidant enzymes are the first and primary defense system from cellular attack by free radicals. The resultant molecular mechanism of pro oxidants – oxidative stress is the basis for many life threatening diseases, which includes, diabetes, cardiovascular, inflammation, cancer and neurodegenerative disease conditions. Reactive oxygen species (ROS) a by-product of the oxidative phosphorylation that takes place in the electron transport chain of the mitochondria and this is the main producer of energy (ATP) (Valko et al., 2007; Sies, 2018). These signalling molecules are important at physiological concentration, but at a higher concentration they overwhelm the antioxidant defense system. Consequently, this may result in DNA damage, protein carbonylation and lipid peroxidation of biomolecules (Valko et al., 2007; Sies, 2018).

Dextran Sodium sulphate (DSS), $C_6H_7Na_3O_{14}S_3$, is a polyanionic sulphated derivative of a selected Dextran fraction. DSS acts as a chemical incitant for the inducement of inflammation (colon) because it is toxic to the mucosa epithelial cells. It also causes damage to its barrier integrity. DSS because of its polyanionic nature is pro-oxidant in its mechanism of action. It complexes with biomolecules and forms a nanometer sized vesicles which will activate inflammatory signal pathways (Amchelslavsky et al., 2009; Jianming et al., 2010; Liberti et al., 2017). The activated inflammatory signal pathways bring about the production of reactive oxygen species. During the process of inflammation, the phagocytes such as neutrophils and macrophages generates a large amount of ROS, and reactive nitrogen/chlorine species due to the rolling and frictional forces produced by the movement of these signalling molecules (Feany and Bender, 2000; Mittal et al., 2014). DSS has been used to induce inflammation in the intestinal stem cells of the *D. melanogaster* (Amchelslavsky et al., 2009; Apidianakis and Rahme, 2011; Hairul et al., 2014), but there is paucity of any research work done on the dose-dependent pro-toxicant effect of DSS in *D. melanogaster.* So we sought to investigate the DSS pro-oxidant induced toxicological effect on the oxidative stress biomarkers and antioxidant enzymes in *D. melanogaster*.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in this research work were of analytical grade and the water used was glass distilled (Milli-Q Direct 8/16 System, Molsheim, France). Dextran sodium sulphate was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Drosophila melanogaster strain and culture

D. melanogaster (Harwich strain) from National Species Stock Center (Bowling Green, OH, USA), was obtained from department of Biochemistry, College of Medicine, University of Ibadan, Nigeria. Flies were maintained at constant temperature and humidity (23 ± 1°C; 60% relative humidity, respectively) under 12 h dark/light cycle conditions in the *D. melanogaster* fly laboratory of the African Center of Excellence in Phytomedicine Research and Development, (ACEPRD), University of Jos, Nigeria.

Drosophila melanogaster feed formulation and its handling

The flies were fed with the standard formulated diet corn meal medium, which contained brewer's yeast (1%w/v), sucrose (2%w/v), powdered milk (1%w/v), agar (1%) and Nipagin (0.08%). The water used for making the diet was double distilled water (Milli-Q water system). Flies were randomly selected from vials containing 1-3 days old. Caution was taken when counting the flies and an appropriate brush with soft ends was used. Much care was taken in handling the flies as to prevent "handling stress".

Behavioural experiments

Survival rate analysis

For the determination of the concentration to be used in the research, an initial experiment was carried out using various concentrations of Dextran Sodium sulphate (DSS %). This was done to observe the sum of dead and surviving flies during the fourteen days period. For the survival assay, flies (both genders) of 1-3 days old were divided into seven groups, with each group having 3 vials each. Each vial contained 50 flies.

Group I – Control (Normal diet), Group II – 0.5% DSS, Group III – 1.0% DSS, Group IV -1.5% DSS, Group V – 2.0% DSS, Group VI – 2.5% DSS, Group VII -3.0% DSS

The survival assay was carried out in three replicates of each concentration. The diet was changed every four days, during the period of this experiment. The survival rate was determined with all the concentrations, and both the live and dead flies were recorded daily. By the end of this experiment (14 days), the data obtained were 2ulphate and plotted as percentage of live and dead flies. Two concentrations were then selected Group II and III (0.5% and 1.0%), this is because their survival rate was comparable with that of the control group.

Climbing activity

The climbing performance of the flies was carried out using

negative geotaxis method in the second phase, last (5^{th}) day (Adedara et al., 2016). Briefly, ten flies from the two selected DSS concentrations and control were put in a mild static position, by placing it in a dry fitted filter paper on a petri dish, with dried ice underneath it. Consequently, they were placed respectively in empty labelled vertical glass columns measuring, length 11 cm and diameter 3.5 cm. Within a period of 15 to 20 mins, flies recovered from the mild anaesthesia and the bottom of the column was tapped gently to return them back to the bottom. The number of flies that climbed above the 6 cm mark of the column in 6 s, as well as those that remained below the 6 cm mark was recorded. This procedure was repeated three times at 1 min interval. The scores represent the mean of the number of flies at the top which was expressed as a percentage of the total number of flies.

Tissue homogenate preparation for biochemical assay

For the determination of biochemical assays, a second group experiment was carried out, were DSS of 0.5 and 1.0% were introduced to the flies' diet relatively for a period of five days. Each group had five vials containing 50 flies (both gender). At the end of the treatment period, flies were anaesthetized in ice, weighed, and homogenized in cold 0.1M phosphate buffer, Ph 7.0 (1:10 w/v), and centrifuged at 4000 x g for 10 min at 4°C (Allegra X-15R Centrifuge, Beckman Coulter, USA). Then the supernatants were separated into labelled Eppendorf tubes, and used for the various biochemical assays. All the assays were carried out in five replicates for the three groups and relative absorbance read using Jenway spectrophotometer 7315, by Bibi Scientific Ltd, UK.

Determination of oxidative stress biomarkers

Determination of total thiol concentration

Total thiol content was estimated by the method of Ellman (1959). Briefly, the reaction mixture was made up of 170 µl of 0.1 M potassium phosphate buffer (Ph 7.4), 20 µl of sample, and 10 µl of 10 Mm 5', 5'- dithios – 2-nitrobenzoic acid (DTNB). This was followed by 30 min incubation at room temperature, and the absorbance was measured at 412 nm. A standard curve was plotted for each measurement using GSH as standard (expressed as µmol/mg protein).

Thiobarbituric acid reactive substances (TBARS)

Using the Ohkawa et al., (1979) method, briefly fly samples were homogenized (50 flies per vial) in cold 0.1 M phosphate buffer at Ph 7.4 in a ratio of 1:5 (w/v). The stock reagent contained equal volume of TCA (10% w/v), and 2- Thiobarbituric acid (0.75% w/v) in 0.1M HCl. 100 µl of tissue supernatant and 200 µl of stock reagent were incubated at 95°C for 60 min. After cooling for a period of 30 min, they were centrifuged at 8000 x g for 10 min and the absorbance measured at 532 nm. The TBARS levels were expressed as mmol MDA/ mg tissue.

Determination of antioxidant enzyme levels

Determination of catalase enzymatic activity

Catalase activity was measured according to a modified method of Aebi (1984) and Abolaji et al. (2015) by monitoring the clearance of H_2O_2 at 240 nm at 25°C in a reaction medium containing 1800 µl of 50 Mm phosphate buffer (Ph 7.4), 180 µl of 300 Mm H_2O_2, and 20 µl of sample. The kinetics mode was used and monitored for 120 s (2 min) at 10 s intervals, at 240 nm, (expressed as U/mg protein).

Determination of glutathione-S-transferase enzymatic activity

Glutathione-S-transferase (GST; EC 2.5.1.18), activity was estimated by the Habig and Jakoby (1981) method. Briefly, 1-chloro- 2, 4-dinitrobenzene (CDNB) was used as the substrate. The assay reaction mixture was made up of 270 µl of a solution containing (20 µl of 0.25 M potassium phosphate buffer, Ph 7.0, with 2.5 Mm EDTA, 10.5 µl of distilled water, and 500 µl of 0.1 MGSH at 25°C), 20 µl of sample, and 10 ml of 25 Mm 1-chloro-2,4-dinitrobenzene (CDNB). The reaction was monitored for 5 min (30 s intervals) at 340 nm and the data were expressed as mmol/min/mg protein using the molar extinction coefficient (ε) of 9.6 $Mm^{-1}cm^{-1}$ for CDNB conjugate.

Protein determination

Protein concentrations in the whole fly homogenates were determined as described by Lowry (1951), using bovine serum albumin (BSA) as the standard.

Statistical analyses

All data were expressed as mean ± standard deviation. The statistical analysis used was one-way ANOVA, followed by the post hoc Tukey's multiple comparison tests, where $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS

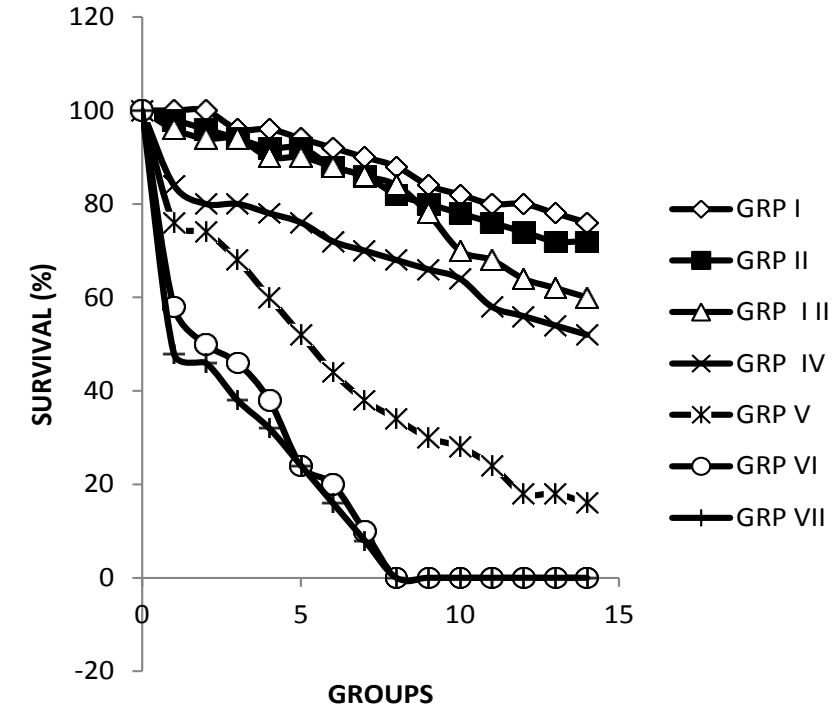
Effect of Dextran sodium sulphate (DSS) incorporated into the diet on survival rate and alive score in *D. melanogaster*

Figure 1A shows the survival rate of the seven Groups I – VII (0.5-3.0%). It revealed that 0.5% DSS incorporated into the feed revealed that, Group II is not significantly ($p>0.05$) different from the control Group (I). Groups III – VII is significantly ($p<0.05$) lower than the control (Group I). Also, DSS incorporated into the diet caused a drastic fall in the survival rate of Groups VI and VII (DSS 2.5 and 3.0%).

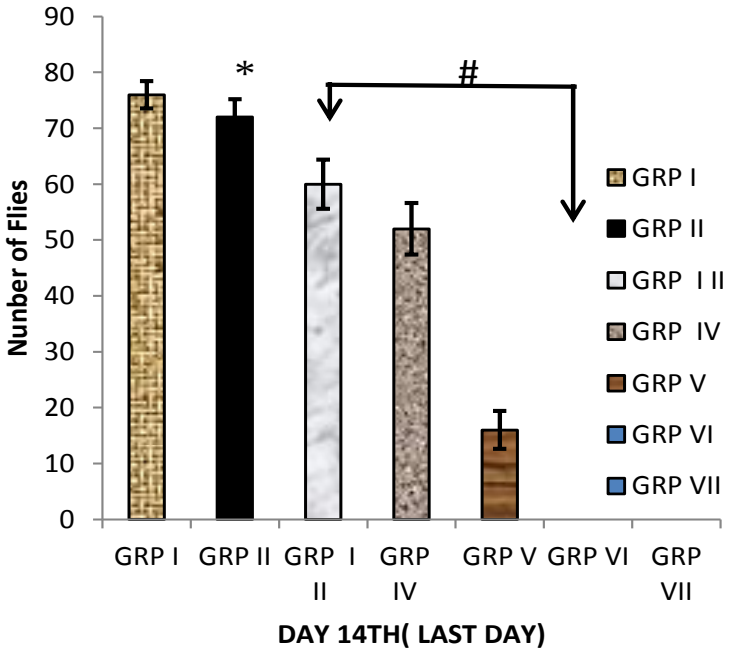
Figure 1B shows a representation of all the alive flies on the last (14^{th}) day of the experiment. The effect of DSS incorporated into the diet at (0.5%) Group II on the flies, was not significantly ($p>0.05$) different from the control group. Groups III- VII (1.0 - 3.0%) flies were significantly reduced ($p>0.05$) in comparison to the control. The reduction was more in the Groups VI and VII treated with 2.5 -3.0% DSS respectively.

Effect of Dextran sodium sulphate (DSS) incorporated into the diet on negative geotaxic (climbing) activity of *D. melanogaster*

Figure 2 represents the climbing activities of the two selected Groups I and II (0.5-1.0% DSS), it revealed that



(A)



(B)

Figure 1A and B. Dextran sodium sulphate incorporated into the diet caused reduction in survival rate of *D. melanogaster* after 14days (A) Survival curve analysis and (B) number of alive flies (both sexes) after 14 days of administration to *D. melanogaster* flies to Groups I – VII. $^{\#}p<0.05$ vs control and $^{*}p>0.05$ vs control. Data are presented as mean ± SD (n=3). Group I-Control, Group II-0.5%DSS, Group III-DSS 1.0%, Group IV- 1.5%DSS, Group V-2.0% DSS, Group VI-2.5% DSS, Group VII-3.0% DSS.

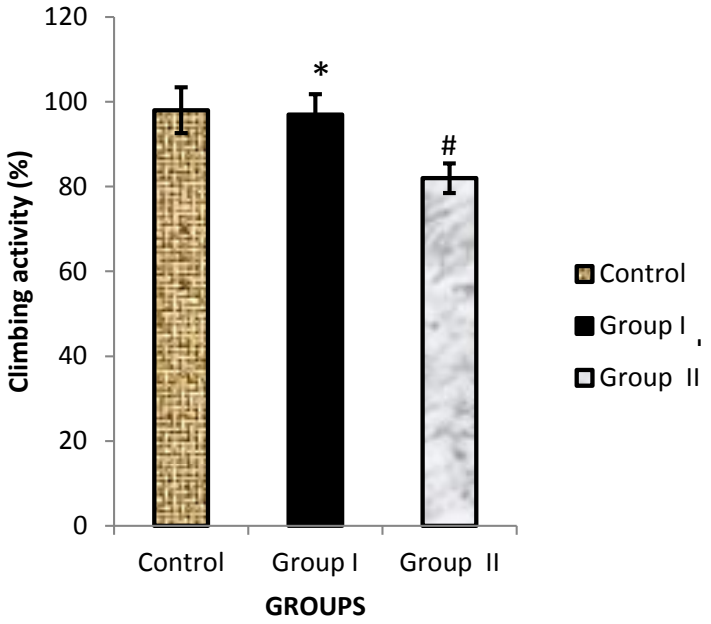


Figure 2. Dextran sodium sulphate incorporated into the diet decreases the climbing rate (%) after 5 days in Control, Group I (0.5%) and Group II (1.0%).

Table 1. The Catalase, Glutathione S-transferase enzymatic activities, total thiol and malondialdehyde contents of control, group I (0.5%) and group II (1.0%) flies.

Group	Catalase activity (U/ mg protein)	GST activity (mmol/min/mg protein)	Total thiol content (µmole/mg protein)	Malondialdehyde content (mmol MDA/mg protein)
Control	49.70 ± 0.82	0.71 ± 0.031	196.68 ± 2.56	2.47 ±0.06
Group II	52.72 ± 1.50*	0.67 ± 0.02*	182.44 ± 2.82*	3.64±0.14*

[a]On the same column, shows there is no significant difference (*p>0.05) from the control, and [b] shows that there is significant ([#]p<0.05) difference from the control.

After 5 days of exposure to the DSS, the effect of 0.5% DSS upon the flies in Group I was not significantly (p>0.05) different from the control group. Whereas the effect of 1.0% DSS upon the flies in Group II climbing activity was significantly (p<0.05) reduced in comparison to the control.

Effect of Dextran sodium sulphate (DSS) incorporated into diet on Total thiol and Malondialdehyde content in *D. melanogaster*

Table 1, also gives a representation of both the total thiol and malondialdehyde contents of the three groups studied, control, I (0.5%) and II (1.0%). The level of total thiols in flies exposed to 1.0% DSS Group II was significantly (p<0.05) reduced in comparison to the control group. The total thiol content in flies in Group I (0.5%DSS) were not significantly (p>0.05) different when compared to the flies in the control group. The quantification of malondialdehyde produced from the activity of thiobarbituric acid reactive species in Group II flies was significantly (p<0.05) increased in comparison to those in control group. Group I also revealed that there was no significant (p>0.05) difference in MDA content in comparison to the control group.

Effect of Dextran sodium sulphate (DSS) incorporated into the diet on catalase, and glutathione S-transferase enzymatic activities in *D. melanogaster*

Table 1 also revealed that the catalase activity was significantly (p<0.05) enhanced in flies exposed to 1.0% DSS in Group II in comparison to the control. While there was no significant (p>0.05) change in Group I (0.5% DSS) in comparison to group control. There was no significant (p>0.5) difference in the GST activity of the

flies in Group I in comparison to those in control group.

DISCUSSION

D. melanogaster is one of the alternative invertebrate models useful in toxicological testings. It meets the standard of the ECVAM, Reduction, Refinement and replacement (3Rs) of the usage of Laboratory animals (Festing et al., 1998). DSS has been used over a period of time to induce inflammation- pro-oxidative mechanisms (Amcheslavsky et al., 2009; Jianming et al., 2010; Mittal et al., 2014). In this experiment, incorporation of varying concentration of DSS into the diet was used for the first phase, to investigate if its pro-oxidant effect can induce oxidative stress using *Drosophila melanogaster* as a model. From the survival rate and numberof flies alive, 0.5% and 1.0% DSS was chosen for use in the next phase for antioxidant enzyme activities and oxidative stress determination. The result (Figure 1A and B) showed that the flies in Group II survival rate (%) was not significantly ($p>0.05$) different from those in the control Group I. Though Group III flies were significantly ($p<0.05$) reduced in comparison to those in the control group, but 60% survived (in Group III) having more surviving flies when compared to those in Groups IV- VII treated with 1.5 -3.0% DSS. The reduction in their survival rate correlates with the level of dose (toxicity) administered in the diets. This can be attributed to the pro-oxidant activity of DSS because of its ability to induce inflammation and its consequent signalling pathways which brings about the production of reactive oxygen species (Amcheslavsky et al., 2009; Mittal et al., 2014).

Substances that can act as pro-oxidants are generally toxic in nature, expressing their damaging effect by affecting the Redox balance, consequently affecting both the survival rates of the flies and the negative geotaxic (vertical climbing) activity. From Figure 1A and B, the survival rate (%) of flies in Group IV – VIII was reduced by the toxic effect of the high concentration used. On the 7^{th} day the flies in Group VI and VII were all dead. Naturally flies have explorative tendencies which depend basically on the novelty of the situation and the levels by which their motor function has been altered by the toxicant (Durier and Rivault, 2002). DSS, an anionic polymer, has been used as one of the incitants for inflammatory bowel diseases in animal models. DSS because of its polyanionic nature is pro-oxidant in its mechanism of action. It complexes with biomolecules and forms a nanometer sized vesicles which will activate inflammatory signal pathways. The process of inflammation in itself favours a large production of reactive oxygen species; due to the rolling and friction forces produced by the signalling molecules (Mittal et al., 2014, Adedara et al., 2016). The ability to induce oxidative stress is a hallmark for the measurement of the toxicity of any substance in the *D. melanogaster*. This was shown in the survival activity, in which an increase in the dose level of DSS, increased toxicity, consequently a decrease in survival rate (%). This observation agrees with Oboh et al. (2018), which shows a reduction in survival rates observed in flies fed with 0.5-1.0% dietary inclusion of *Garcinia kola*. This showed that an increase in dose level also increases reactive oxygen species (ROS) level would have led to the decrease in survival rate of flies in Groups IV – VII.

Malondialdehyde is one of the products of lipid peroxidation, which is one of the biomarkers for oxidative stress (Habig and Jakoby, 1981; Ghani et al., 2017). When the antioxidant system is overwhelmed, there is a mitochondrial dysfunction which leads to accumulated oxidative damage and an increase in reactive species generation. In this study we observed an increase in the level of oxidative damage (MDA) highest in the homogenate of flies from Group (II) fed with 1.0% of DSS. The increase in ROS levels and reactive nitrogen species is measured by a method based on the ROS–dependent oxidation of 2'7' dichlorodihydrofluorescin diacetate (DCFH-DA) to dichlorodihydrofluorescin (DCF) – a general index of oxidative stress measurement (Pérez-Severiano et al., 2004). In comparison, TBARS assay is known to estimate lipid damage from cells and tissues and an increase in its level is an indirect indication of high ROS production. Some scientific scholars are predisposed about the use of TBARS assay in the assessment of oxidative damage in the Harwich strain of *D. melanogaster* fly. This is because of the interference of the red pigment in its eyes with the generated pink colour of TBARS assay. But in spectrophotochemical readings, the sample blank (contains all the assay constituents except the analyte), and using this practice, the blank is treated as identical to the sample as possible). Thus by taking the sample blank reading, the level of interference is taken into consideration and brought to level zero before other readings are determined (Ingle and Crouch, 1988). Some researchers have determined both the level of malondialdehyde produced from lipid peroxidation (Ohkawa et al., 1979), a standard oxidative damage indicator and level of reactive oxygen species via oxidation of fluorescent dye 2,7-dichlorofluorescein diacetate (DCF-DA) a general index of ROS levels (Pérez-Severiao et al., 2004) using the Harwich strain of *D. melanogaster* (Paula et al., 2016; Saraiva et al., 2018; Poetini et al., 2018). This is supported by our findings from Table 1, in which the inhibition of GST activity may be due to the increase in the toxicity effect of 1.0% DSS. This increase in the malondialdehyde level agrees with the result of Paula et al. (2016), Saraiva et al. (2018) and Colpo et al. (2018) which showed an increase in the MDA levels of the `Harwich strain of *D. melanogaster.*

Total thiols contain a sulfhydryl group which are among the major portion of the total body antioxidants and they play a significant role in the defense against reactive

oxygen species (ROS). So the quantification of the total thiols group (indicates the chemical effects in the thiol group of proteins and peptides) is an indirect measurement of oxidative stress biomarkers. Under oxidative/nitrosative stress condition, S-Glutathionylated proteins are reduced to free thiol groups by (thioltransferases) glutaredoxins (McDonagh, 2017). The total thiol level is an indirect marker of oxidative stress biomarker, because it gives an indication of any chemical changes in thiol groups of proteins and peptides (Durier et al., 2002; Abolaji et al., 2014; Ghani et al., 2017; Oboh et al., 2018). The total thiol content in this study was showed that group II was significantly ($p>0.05$) different from the control, this could be as a result of thiol consumption in reaction to the presence of reactive oxygen species.

The antioxidant enzymatic network consist of, superoxide dismutase (SOD), catalase, glutathione peroxidase (Gl-Px), glutathione reductase (Gl-Red) and glutathione-s-transferase (GST) that play an important role in the prevention and management of ROS generated endogenously e.g., during inflammation. They represent the adaptive response to most toxic substances (Weydert and Gullen, 2010). Catalase enzymes react efficiently with hydrogen peroxide by reducing it to water and molecular oxygen. This enzyme also plays an essential role in activating the tolerance level in relationship to adaptive response of cells in oxidative stress. This result agrees with the reported effect of catalase enriched transfected cells that were able to prevent drug induced damage by either destroying the hydrogen peroxide moiety or by interaction with the drug (Mittal et al., 2014). This activity of catalase enzyme is very important as a first line defense antioxidant enzyme. The concept of maintaining low cellular level of hydrogen peroxide (H_2O_2), is gaining increasing recognition because of its damaging effect (Mittal et al., 2014). The result from this study showed that the increasing concentration of DSS (pro oxidant) led to an enhanced catalase enzymatic activity.

Glutathione S-transferases (GST) are phase II detoxification enzyme that catalyse the conjugation of glutathione with electrophilic centres of both endogenous and exogenous electrophiles. They also function in the regulation of some cellular processes involved in oxidative stress in nature (Heydel et al., 2013). The result from this study showed that GST enzymatic activity of flies in Group I were not significantly ($p>0.05$) different from those in the control group. But due to the toxicity level of flies in Group II the antioxidant system was overwhelmed, as revealed in the MDA result in Table 1. The inhibition of the GST activity in the flies in Group II may be due to both the overwhelming nature of toxicity and the *D. melanogaster* GST (DmGSTs) may not have been expressed fully, because it develops much better and effective as the fly matures into the adult stage (Gonzalez et al., 2018). In this experiment the flies used were juveniles (3 days old). This result is in agreement with Abolaji et al. (2014), which showed an inhibition in the activity of GST using 3 days old flies. The findings of this study demonstrated that the administration of 0.5% DSS produced a reduced state of oxidative stress. At higher concentrations of 3.0 -3.5% DSS the flies were not able to survive.

RECOMMENDATIONS AND FUTURE DIRECTIONS

It is recommended that all experiment for the enzymatic assays be done under cold environment and where possible the Eppendorf test-tube holder should be placed on ice. It is necessary for further study to be carried out to determine the relative age and sex factor in the cause of oxidative stress in *D. melanogaster* using DSS as a pro-oxidant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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