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

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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

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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

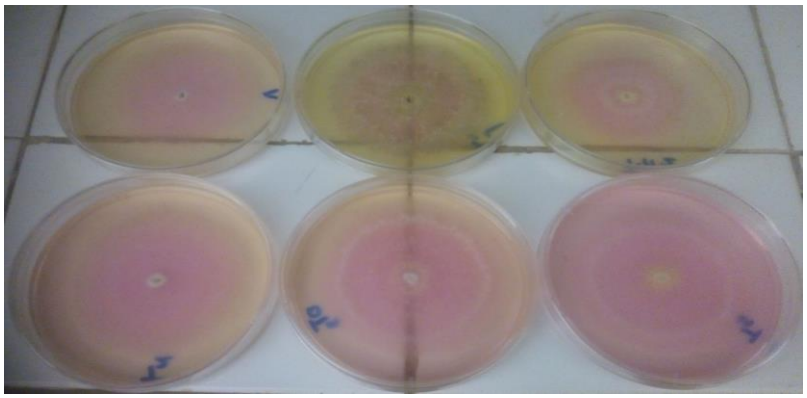
The predicted optimized condition of fermentation for maximal yield of L–glutaminase by *T. longibrachiatum* according to the polynomial model was experimentally validated by setting parameters at their individual optimized levels.

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).

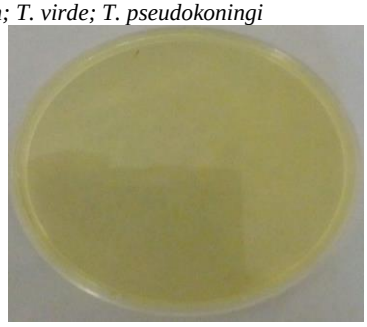
Qualitative plate screening experiment of the six isolates as reported in Table 2 and Figure 1 indicated *Trichoderma longibrachiatum* as the highest extracellular enzyme producing isolate adjudge from the pink colour intensity around its colony, this was followed in colour intensity by *Trichoderma 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.

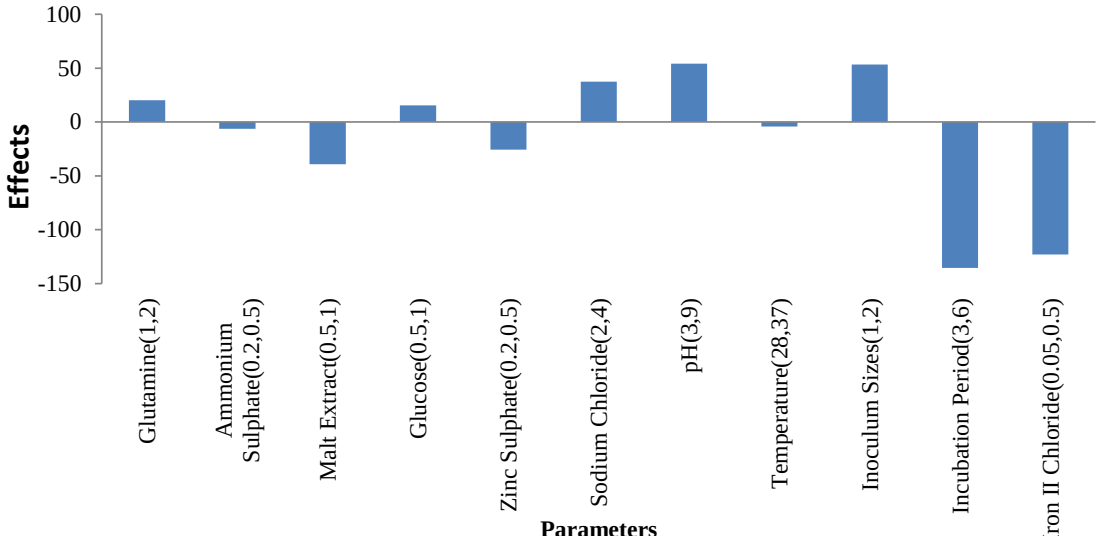


Figure 2. Effect of different parameters on L–glutaminase production by *T. longibrachiatum*.

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.

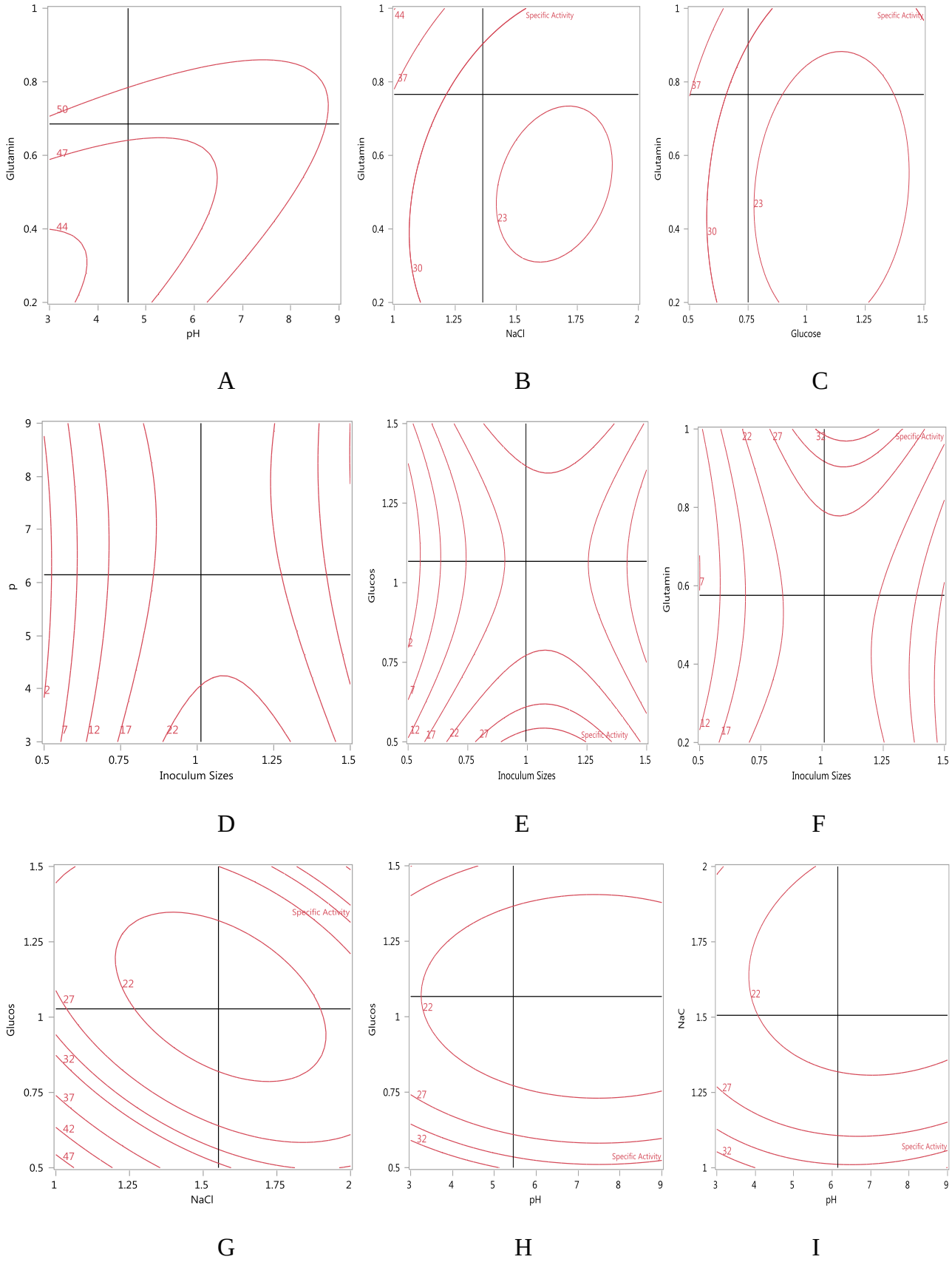


Figure 3. Contour plot interactive effects for among factors influencing L–glutaminase yield.

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