International Food Research Journal 22(3): 1015-1023 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

# Response surface optimization of enzymatic hydrolysis conditions of lead tree *(Leucaena leucocephala)* seed hydrolysate

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**Article history**

*Received: 21 May 2014*
*Received in revised form: 23September 2014*
*Accepted: 25 September 2014*

**Keywords**

*Lead tree seed*
*Hydrolysate*
*Optimization*
*RSM*
*Antioxidant activity*

**Abstract**

The enzymatic hydrolysis of lead tree seed protein with alcalase to obtain Lead Tree Seed Hydrosylate (LTSH) was optimized using response surface methodology (RSM). A three-tiered, factor face centered, central composite design (CCD) was used to study the influence of four independent variables namely: pH (7–9); hydrolysis temperature (50°C, 55°C, 60°C); hydrolysis time (30 min, 60 min, 90 min); and enzyme/substrate (1%, 2%, 3%) ratio on both yield and antioxidant activity. The CCD consisted of twenty-four experimental points and six replicates of central points. All data were analyzed using Design-Expert Software. The optimum conditions obtained from experiments were a pH of 9; an enzyme to substrate ratio of 2%; a hydrolysis time of 90 min; and a temperature of 55°C. Results showed that LTSH derived from optimized hydrolysis exhibited effective ferrous ion chelating activity (92.79%) and strong reducing power ($A_{700}$ = 3.82) at a concentration of 20 mg/ml. LTSH also demonstrated high DPPH radical scavenging activity (76.21%; $IC_{50}$ 1.99 mg/ml), as well as hydroxyl radical scavenging activity (66.72%; $IC_{50}$ 2.45 mg/ml). Superoxide anion scavenging activity was 55.71% ($IC_{50}$ 3.89 mg/ml) at 20 mg/ml. These results suggest that LTSH has potential as a natural antioxidant of functional food and for use in food processing.



## Introduction

Protein hydrolysate has a wide range of applications in various industries including pharmaceuticals, human and animal nutrition, and cosmetics. Food industries use them as milk substitutes for protein supplementation, beverage stabilization and flavour enhancement (Li *et al*., 2007). Protein hydrolysis refers to any process during which protein is broken down by protein-digesting enzymes. Enzymatic hydrolysis is one method used for protein hydrolysis that is influenced by several factors namely: temperature, pH, enzyme/substrate concentration and time. These factors influence enzymatic activity collectively and thus, offer possibilities that allow us to control the process (Liaset *et al*., 2002). Recently, protein hydrolysates extracted from marine by-products and plant sources have become common in the food industry because of their high protein content. Levels and compositions of free amino acids and peptides have also been reported to affect antioxidant activities of protein hydrolysates.

Both natural and synthetic antioxidants are used as food additives to help prolong shelf life and the appearance of many processed foods. However, the use of synthetic antioxidants is strictly controlled due to potentially deleterious health issues. Hence, it became necessary to identify safer food antioxidants in recent years and the search intensely focused on natural antioxidants of plant origin. Several studies on the production of protein hydrolysate from natural sources have been reported (Liu and Chiang, 2008). Hence, this study is focused to develop naturally safe antioxidant from lead tree *(Leucana leucocephalea)* seed due to its high protein content and commercial potential.

Optimization refers to the improved performance of a system, process or product whereby maximum benefit is obtained. Response surface methodology (RSM) optimizes a process when several factors affect a targeted response (Zhen *et al*., 2008). RSM presents optimal conditions using multivariate statistical techniques that obtain a desired response from variables such as pH, temperature and enzyme/substrate concentration. RSM comprises mathematical and statistical techniques based on a polynomial equation that best fits experimental data in order to describe the behavior of the data set with the objective of projecting statistical predictions. Several studies have been reported on the optimization of hydrolysate (Li *et al.*, 2007). However, no studies have been reported on the optimized enzymatic hydrolysis of lead tree seed.

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The lead tree belongs to the tropical and subtropical legume family and is a small leguminous tree originally from Central America. It is considered an important crop and is encouraged under social forestry schemes in drought-prone areas and semi-arid tracts in India because it provides useful timber as well as leaves for fuel, energy and feed. It is, therefore, a nitrogen-fixing multipurpose tree that serves as foliage for livestock, as vitamin K and β-carotene supplements, as fuel (wood), as green manure, and for drought resistance (Lalitha *et al*., 1993). A study using extraction solvent, prior dechlorophyllisation, and drying methods was conducted by Benjakul et al. (2012) who found that lead brown seed extract was also a natural antioxidant. Additionally, lead tree seeds and leaves have been consumed as human food as it is rich in protein and essential amino acids such as isoleucine, leucine, phenylalanine and histidine that likely account for the antioxidant activity of its hydrolysate. The trunk of the lead tree is used for fuel and generally the seed is wasted so that potential food industry profits are lost. Hence, in view of current and increasing attention focused on plant protein hydrolysates with antioxidative properties, this study focused on the production of Lead Tree Seed Hydrosylate (LTSH).

Since enzymatic hydrolysis alters the lead tree seed to produce a naturally sourced hydrolysate with antioxidative properties, the objectives of the study were to determine the chemical composition of lead tree seed in order to optimize conditions for the yield of its enzymatic hydrolysis and antioxidant activity by altering temperature, enzyme/substrate ratio, pH and hydrolysis time. In addition, the mechanism of antioxidant activity for optimized LTSH was also determined.

## Materials and Methods

*Materials*

Lead tree seeds were collected at the Universiti Malaysia Terengganu, Malaysia. Liquid Alcalase® 2.4 L (2.4 AU/g) was purchased from Novo Industry (Denmark). All chemicals used were of analytical grade.

*Sample preparation*

The pods of collected lead tree seeds were removed and the seed was sealed in a polyethylene bag and then stored at -40°C for further use.

*Chemical analysis*

Chemical analysis of the seed included crude protein, crude fat, moisture, ash and carbohydrate content; all determined according to AOAC (2000).

*Preparation of the LTSH*

The LTSH was prepared by following the protocol of Jamdar *et al.* (2010) with slight modification. About, 33 g of lead tree seed (LTS) was added to 110 g of distilled water. Alcalase, at an enzyme/substrate ratio of 1%, 2% or 3%, respectively, was added and the mixture was homogenized by stirring for 5 mins. This mix was then placed in a water bath and stirred until reaching temperatures of 50°C, 55°C and 60°C, respectively, as the pH for each was adjusted to specified levels for hydrolysis at 7, 8 or 9, also respectively. Sodium Hydroxide, 0.1N (NaOH) was used for the process of hydrolysis for 30, 60 and 90 min runs, respectively. After incubation, the enzyme was inactivated at 90°C for 10 min and the liquid was centrifuged at 5000 x *g* (4°C) for 20 min. The supernatant was then freeze dried to obtain the LTSH in powdered form.

*Optimization of enzymatic hydrolysis conditions of LTSH by response surface methodology (RSM)*

RSM was used to predict optimal hydrolysis conditions for LTS using alcalase. Optimized hydrolysis conditions were previously accomplished by employing RSM by Amiza *et al.* (2011). Thirty hydrolysis trials were randomly run per Central Composite Design (CCD). Four independent variables including temperature (A: 50, 55, 60°C); time (B: 30, 60, 90min); enzyme to substrate concentration (C: 1, 2, 3% v/w); and pH (D: 7, 8, 9) were employed at three equidistant levels (−1, 0, +1).

*1, 1- diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity*

With some modification, we used Yen and Wu's method (1999) to measure DPPH radical-scavenging activity for all 30 hydrolysate runs by vigorously mixing 4 ml (20 mg/ml) of each sample solution with 1.0 ml of 0.2 mM DPPH. A spectrophotometer measured absorption at 517 nm after incubating each mix for 30 min. A control sample was similarly processed with distilled water instead of a sample. DPPH radical scavenging activity was calculated as a percentage: (1- $A_{517}$ of sample) / ($A_{517}$ of control) x 100.

*DPPH radical scavenging activity*

The ability of LTSH to scavenge free radicals was measured by using the synthetic free radical compound 1,1- diphenyl-2-picrylhydrazyl (DPPH) according to the method employed by Bersuder *et al.* (1998). 500 µl from each sample was mixed

with 500 µl of ethanol and 125 µl (0.02%, w/v) of DPPH in 99.5% ethanol. The mixture was shaken vigorously and incubated in the dark. After 60 min, the absorbance was measured at 517 nm with a spectrophotometer. The DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical-scavenging activity} = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where $A_{blank}$ and $A_{sample}$ were control and sample, respectively, and the $IC_{50}$ value determined the inhibition of DPPH radical formation at 50%. The synthetic antioxidant reagent, BHT, was used as positive control.

*Reducing power*

The ability of LTSH to reduce iron (III) was methodically determined as described by Yildirim *et al*. (2001). Briefly, 500µl of hydrolysate at five concentrations (0.5, 1, 5, 10 and 20 mg/ml) were mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 30 min followed by the addition of 2.5 ml of 10% (w/v) trichloroacetic acid. The mixture was then centrifuged at 1650 x *g* for 10 min. Finally, 1.25 ml of the supernatant solution was mixed with 1.25 ml of distilled water and 250 µl of 0.1 % (w/v) ferric chloride. After 10 min reaction, absorbance was measured at 700 nm. BHT was used as positive control. Increased absorbance of the reaction mixture indicated increased reducing power and all values are presented as the mean of three trials (±SD).

*Chelating effects on ferrous ion*

LTSH chelation of ferrous ions was determined according to the method described by Dinis *et al.* (1994) using 1.6 ml of distilled water and 50µl of 2 mM $FeCl_2$ added to samples at five concentrations (0.5, 1, 5, 10, 20 mg/ml). After 15 min, 0.1 ml 5 mM ferrozine was added. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance was then measured spectrophotometrically at 562nm. LTSH metal chelating activity was calculated as follows:

$$\text{Metal chelating effect (\%)} = [(Abs_1 - Abs_2)/Abs_1] \times 100$$

Where $Abs_1$ is the absorbance of the control and $Abs_2$ is the absorbance of LTSH.

*Scavenging effect on superoxide anions*

LTSH scavenging of superoxide anion radicals was measured by following the method of Liu *et al.* (1997). Superoxide anions were generated in 3 ml of Tris-HCL buffer (100 mM, pH 7.4) containing 750 µl of NBT (300 µM), 750 µl of NADH (936 µM) and 300 µl of LTSH at five concentrations (0.5, 1, 5, 10, 20 mg/ml). BHT was used as positive control. The reaction was initiated by adding 750 µl of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, absorbance was measured at 560 nm with a spectrophotometer. Decreased absorbance of the reaction mixture indicated an increase in superoxide anion scavenging activity.

$$\text{Inhibition (\%)} = (A_0 - A_1/A_0) \times 100$$

Where $A_0$ is the absorbance of the negative control (Blank) and $A_1$ is the absorbance of the reaction mixture. The $IC_{50}$ value was determined and results are reported as a mean of three trials (±SD).

*Hydroxyl radical scavenging activity*

LTSH scavenging of hydroxyl radicals was measured by the Fenton Reaction according to the method described by Yu *et al.* (2004). The reaction mixture contained 60 µl of 1.0 mM $FeCl_3$; 90 µl of 1mM 1,10-phenanthroline; 2.4 ml of 0.2 M phosphate buffer (pH 7.8); 150 µl of 0.17 M $H_2O_2$; and 1.5 ml of LTSH at five concentrations (0.5, 1, 5, 10, 20 mg/ml). The reaction was started with the addition of $H_2O_2$ and absorbance at 560 nm was measured with a spectrophotometer after incubation at room temperature for 5 min. The hydroxyl radical scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = (A_0\text{-}A_1) / A_0 \times 100)$$

Where $A_0$ is the absorbance of the control (blank) and $A_1$ was the absorbance in the presence of the sample. The $IC_{50}$ value was determined and results are reported as a mean of three trials (± SD).

*Experimental design*

The enzymatic hydrolysis of LTS was optimized by employing RSM with a three-tiered, factors face centered, central composite design (CCD). The CCD consisted of 24 experimental points and six replicates of the central points. There were 30 runs for samples with each run optimally designed and validated via different independent variables such as temperature, time, pH and enzyme/substrate ratio. Yield and antioxidant activity (DPPH) were dependent variables.

*Statistical Analysis*

For chemical analysis, all treatments and analyses

were done in triplicate and the T-test was used to determine the chemical composition of lead tree seed. To optimize enzymatic hydrolysis conditions, the RSM Design–Expert 6.0.10 software (Stat-Ease 2003) was used. Results were expressed as a mean (±SD) for each analysis. Comparative statistical analysis between means with ANOVA was calculated with the Minitab 14.0 to assess significant differences between treatments.

## Results and Discussion

*Chemical composition of lead tree seed (LTS)*

The chemical composition of lead tree seed between immature and mature seed were determined (Table 1). Due to the higher protein content (40.77%) of the immature seed—mature seed protein content (38.99%)—it was chosen to produce LTSH. The moisture, ash and carbohydrate content for the mature lead tree seed were higher, however. Hence, the chemical composition indicated that LTS is a rich source of protein and can be utilized to produce value added products. Nevertheless, factors such as species, age, season and environmental conditions may also affect the composition of lead tree seed.

*Optimization of enzymatic hydrolysis conditions on yield and antioxidant activity by response surface methodology (RSM)*

The RSM method was used to optimize enzymatic hydrolysis conditions for the preparation of LTSH. Data from 24 experimental and six central point runs using the central composite design (CCD) showed that six central point values ranged between 7.97–10.29% for yield, and 38.04–55.57% for LTSH antioxidant activity. LTSH yields obtained in this study ranged from 5.72–13.59%, lower than those from fish protein hydrolysates (10–15%) as reported by Quaglia and Orban in 1990. This difference may be due to losses during the freeze-drying process. Observed values of antioxidant activity in this study ranged from 13.88–91.90%. The range of values for LTSH yield and antioxidant activity differed from Quaglia and Orban's study as well and may due to hydrolysate source, the type of enzyme used, and different conditions for enzymatic hydrolysis.

*Model of summary statistics for LTSH yield*

The quadratic model is the model summary suggested for LTSH yield. A study by Qi *et al*. (2009) also reported a quadratic model for the optimization of wheat straw enzymatic hydrolysis.

*Analysis of variance (ANOVA) for yield of LTSH*

The analysis of variance (ANOVA) for the Response Surface Quadratic Model for LTSH yield after model reduction is shown in Table 2. Fisher's test (F-test) of the experimental data made it possible to estimate the statistical significance of the proposed model (Maache-Rezzoug *et al*., 2011). The response surface quadratic model was tested for significance with the lack-of-fit test, which indicated the model exhibited a non significant lack-of-fit and therefore could be used to predict optimal conditions within the range of input variables as evaluated in our experimental design (Karki *et al.*, 2011). The 'Lack of Fit F-value' for this model was 3.75 which implied a 7.45% possibility that a "Lack of Fit F-value" this large would occur due to noise. That the "Lack of Fit" was not significant was fortunate as the model was fitted to data that identified the variables' as input values, as per design, which then led to the highest estimates for LTSH yield.

The coefficient variation ($R^2$) was 0.5714. Although a coefficient variation ($R^2$) closer to 1 is desirable, it can, due to experimental error such as yield loss during freeze-drying, contribute to a coefficient variation ($R^2$) value of only 0.5714. However, a "Pred R-Squared" of 0.2835 is within reasonable agreement with the "Adj R-Squared" value of 0.4596. The adjusted $R^2$ value is particularly useful when comparing models with a different number of terms. This comparison was, however, done against a background where model reduction took place. While "Adeq Precision" measures signal to noise ratio, an "Adeq Precision" ratio of 8.929 indicates an adequate signal. Ratios greater than four indicate adequate model discrimination. Hence, this model qualified to navigate the design space.

*Response surface plots and effects of factors for LTSH yield*

According to the model's regression analysis, the best explanatory model equation was given as follows:

LTSH Yield = +8.57 +1.17A +0.33 C + 0.22 D -1.02 $A^2$ + 2.46 $D^2$ + 0.71 AC.

A 3D response surface with graphical representations of the regression equation is presented in Figure 1 that shows the response surface plot for hydrolysate yield as a function of time and pH. Results indicated that the maximum yield for LTSH at optimized enzymatic activity would occur within a pH range of 8.5–9, after which decreases is leading to lower LTSH yield. Protein extraction increases at a higher pH due to higher electrostatic repulsion between amino acids causing higher protein

Table 1. Chemical composition of lead tree seed

| | Crude Protein (%) | Moisture (%) | Ash (%) | Crude Fat (%) | Carbohydrate (%) |
|---|---|---|---|---|---|
| Mature lead tree seed | 38.99 ± 0.13[b] | 42.77 ± 0.07[a] | 4.08 ± 0.09[a] | 0.69 ± 0.01[a] | 13.47 ± 0.02[a] |
| Immature lead tree seed | 40.77 ± 0.17[a] | 27.29 ± 1.22[b] | 2.72 ± 0.05[b] | 0.46 ± 0.01[a] | 28.76 ± 1.09[a] |

[a,b] different letters indicate significant difference in sample means in the same column ($p < 0.05$)

Table 2. Analysis of variance (ANOVA) after choosing significant model for LTSH yield

| Source | Sum of Squares | DF | Mean Square | F Value | Prob>F |
|---|---|---|---|---|---|
| Model | 60.69 | 6 | 10.12 | 5.11 | 0.0018 significant |
| A | 24.73 | 1 | 24.73 | 12.50 | 0.0018 |
| C | 1.97 | 1 | 1.97 | 1.00 | 0.3284 |
| D | 0.88 | 1 | 0.88 | 0.44 | 0.5115 |
| $A^2$ | 3.56 | 1 | 3.56 | 1.80 | 0.1929 |
| $D^2$ | 20.90 | 1 | 20.90 | 10.56 | 0.0035 |
| AC | 8.02 | 1 | 8.02 | 4.05 | 0.0559 |
| Residual | 45.52 | 23 | 1.98 | | |
| Lack of Fit | 42.38 | 18 | 2.35 | 3.75 | 0.0745 not significant |
| Pure Error | 3.14 | 5 | 0.63 | | |
| Cor Total | 106.21 | 29 | | | |

solubility in an alkaline medium. This study's actual trials showed that LTSH yields decreased around a pH of 9. During hydrolysis, the enzyme broke down the peptides of protein into smaller chains. The most active peptides breakdown was shown at pH 8. This might have been due to optimal pH of alcalase was at pH 8. When pH increased to pH 9, the enzyme activity became slower, hence, lower the yield of LTSH. Besides that, results also demonstrated that longer incubation time yielded higher percentages of LTSH. Hence, it appears that increasing incubation time allows for increased proteolysis (Haslaniza *et al*., 2010). Proteolysis is the breakdown of proteins into simpler, more soluble substances such as peptides and amino acids (digestion) which may have enhanced LTSH yield.

*Model summary statistics for an antioxidant activity of LTSH*

The model summary suggested for LTSH antioxidant activity was the quadratic model similar to a previous study on the hydrolysate optimization of sunflower shells (Czerniak *et al*., 2011).

*Analysis of variance (ANOVA) for LTSH antioxidant activity*

The backward elimination protocol was selected to automatically reduce insignificant terms. The resulting ANOVA table for the reduced quadratic model for antioxidant activity is shown in Table 3 and indicates that the model was significant as per the model's F-value (6.60). Only a 0.46% chance exists that a "Model F-Value" this large would occur due to noise. Hence, the model's terms significantly affect responses.

The 'lack of fit' test is designed to determine whether a selected model adequately describes observed data or whether a more complicated model should be utilized. Table 3 shows a "Lack of Fit F-value" for this model at 10.52, which also implied significance as only a 0.79% possibility existed that an "F-value" this large would occur due to noise. The coefficient of variation ($R^2 = 0.5282$) indicated that 47.18% of total variations which showed a low $R^2$ was due to experimental error in controlling the parameter such as temperature and pH during the preparation of LTSH. While the "Pred R-Squared" value of (0.1764) was in reasonable agreement with the "Adj R-Squared" value of 0.2783, adequate precision is a measure of the range of predicted responses relative to associated errors; in other words, a single-to-noise ratio above four is considered desirable. This model's ratio was 4.552 and demonstrated an adequate signal. Overall, the model was validated for navigating the design space.

*Response surface plots and the effects of factors on LTSH antioxidant activity*

According to the model's regression analysis, the best explanatory model equation given in terms of

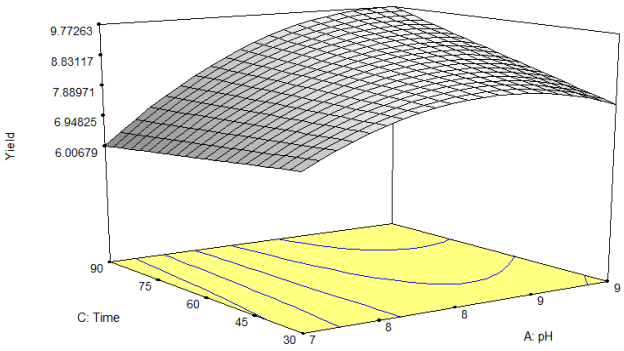


Figure 1. Response surface graph for yield (%) as function of time and pH

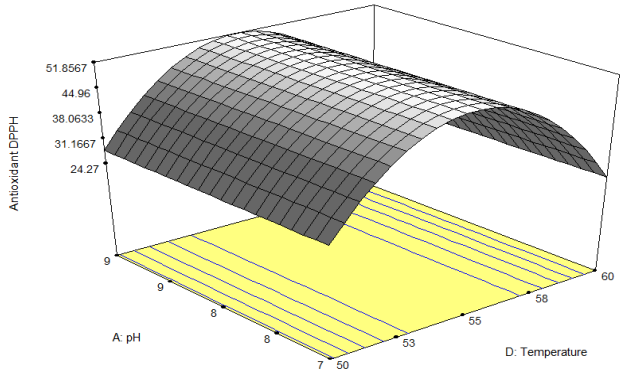


Figure 2. Response surface graph for antioxidant activity (DPPH) (%) as function of temperature and pH

coded factors was:

$$\text{LTSH antioxidant activity} = +51.86 - 1.80D - 25.78D^2$$

The equation shows that the largest value of the estimated regression coefficient was the second-order effect of temperature ($D^2$), which indicated that temperature was the most important linear variable influencing antioxidant activity. On the basis of increased antioxidant activity, Figure 2 shows that pH and temperature also played dominant roles in the process. The 3D surface graphs show that antioxidant activity increased as the temperature increase until reaching 55–58°C, after which it decreased. Based on the result, increasing the temperature resulting in the increase of LTSH antioxidant activity may due to the increases of peptide content in the supernatant. This result was in the same agreement with the study conducted by Haslaniza *et al*. (2010) on cockle hydrolysate.

In addition, it was noted that when pH values ranged from 7–9, LTSH antioxidant activity values remained linear; most likely because peptides with shorter chains and amino acids in the protein hydrolysate were less affected by the charge modifications governed by pH changes. Basically, protein hydrolysates are soluble over a wide pH range and show little effects, whereas native proteins with tertiary and quaternary structures are considerably affected by pH (Gbogouri *et al*., 2004). Based on the response surface graph obtained, the present study indicated that antioxidant activity (by DPPH) was influenced by both pH and temperature.

*Optimal response conditions*

After limitations were set, optimization tests were evaluated by the Design Expert 6.0.10 software system and solutions for optimization conditions were further evaluated. The optimized condition for LTSH hydrolysis selected comprised the following parameters: a pH of 9; an enzyme substrate ratio of 2%; a hydrolysis time of 90 min; and a temperature of 55 oC with the desirability accounted by RSM was 0.499. Optimum conditions obtained were very similar to those reported by Yuan et al. (2008) for Gourd (*Momordica charantia* L.) hydrolysate.

*Validation test*

RSM has been successfully used to optimize parameters affecting protein hydrolysis. The optimal conditions for a predicted yield obtained was 9.77% and for antioxidant activity was 51.87%. To confirm the suggested mathematical model's validity, an additional experiment was conducted under predicted optimal conditions and the trial was repeated three times. The results obtained for yield were 9.92% and 53% for antioxidant activity. Experimental values for both responses were quite close to predicted values (data not shown). T-test analysis also showed no significant difference between experimental and predicted values. These results confirmed the model as robustly suitable for estimating experimental values.

*DPPH radical scavenging activity*

The DPPH radical scavenging activity of LTSH showed that the activity increased as LTSH concentration increased (data not shown). In addition, there were significant differences ($p < 0.05$) between LTSH and the positive control (BHT) for each concentration. LTSH exhibited excellent DPPH radical-scavenging activity (76.21%) at a concentration of 20 mg/ml. However, this was much lower than that of BHT. The DPPH method was based on the reduction of an ethanolic DPPH solution in the presence of a hydrogen donating antioxidant due to the reaction's formation of the non-radical form (DPPH-H). This study showed that LTSH reduced the stable radical (DPPH) to yellow-colored diphenylpicrylhydrazine, which indicated that LTSH was capable of donating hydrogen and thus, acted as an antioxidant. This finding was similar to that

Table 3. Analysis of variance (ANOVA) after choosing significant model for LTSH antioxidant activity

| Source | Sum of Squares | DF | Mean Square | F Value | Prob>F |
|---|---|---|---|---|---|
| **Model** | 4844.95 | 2 | 2422.48 | 6.60 | 0.0046 significant |
| **D** | 58.54 | 1 | 58.54 | 0.16 | 0.6929 |
| **$D^2$** | 4786.42 | 1 | 4786.42 | 13.03 | 0.0012 |
| **Residual** | 9915.60 | 27 | 367.24 | | |
| **Lack of fit** | 9705.96 | 22 | 441.18 | 10.52 | 0.0079 significant |
| **Pure Error** | 209.64 | 5 | 41.93 | | |
| **Cor Total** | 14 760.55 | 29 | | | |

reported by Wang *et al.* (2009), who found that DPPH free radical scavenging by wheat bran hydrolysate was due to its hydrogen-donating ability. In this work, the $IC_{50}$ value for LTSH was expressed as 1.99 mg/mL, lower than chick-pea's $IC_{50}$ of 1.0 mg/mL (Li *et al*., 2008). The differences in radical scavenging ability value may be attributed to differences in protein composition and surface hydrophobicity values for the respective hydrolysates. This studied demonstrated that LTSH can terminate a radical chain by converting free radicals to more stable products.

*Reducing power*

The reducing power of LTSH were showed increased with increasing concentrations of the LTSH solution ($p < 0.05$). For this assay, the presence of antioxidants caused the reduction of the $Fe^{3+}$/ ferricyanide complex to its ferrous form showing LTSH's excellent ability to change the yellow test solution to green depending on each concentration's reducing power. This greater reducing power indicated that the hydrolysate could donate an electron to a free radical leading to prevention or retardation of propagation. In the propagation phase, free radicals react with oxygen to form peroxy radicals that react with more unsaturated lipids to form hydroperoxide. The lipid free radical so formed can react with oxygen to form a peroxy radical. Lower values of reducing power for hydrolysates prepared from other sources like roselle seed ($A_{700}$ = 0.3-0.7) (Tounkara *et al*., 2013) have also been reported. The higher value for LTSH could be associated with specific amino acids and peptide sequences as reported by He *et al.* (2013a). In this study, however, LTSH showed an excellent ability to donate electrons by converting free radicals to more stable products, thereby terminating chain reactions initiated by free radicals. This may also be due to lead tree seed's higher content of hydrophobic amino acids.

*Chelating effects on ferrous ion activity*

The $Fe^{2+}$ chelating ability of some compounds is related to their antioxidant activity. It was estimated by the reduction absorbance (red) of ferrozine-$Fe^{2+}$ complexes after the addition of hydrolysate. The LTSH's chelating effects on ferrous ion activity showed an increased with the concentrations increased. The results also indicated significant differences ($p < 0.05$) between concentration levels. LTSH showed strong chelating activity (92.80%) on ferrous ion compared to the positive control (BHT) at 20 mg/mL (higher than BHT). Carboxyl and amino groups in the side chains of the acidic and basic amino acids are thought to play an important role in chelating metal ions. Presumably, peptide bond cleavages lead to enhanced $Fe^{2+}$ binding due to increased concentrations of carboxylic ($COO^-$) and amine groups in acidic and basic amino acids, thus removing pro-oxidative free metal ions from the hydroxyl radical system. Suetsuna *et al*. (2004) also reported that the presence of histidine residue was thought to be responsible; an activity largely related to its imidazole ring. It is also believed that acidic and/or basic amino acids play important roles in the chelation of metal ions by their side chains on the hydrolysate. Hence, high amino acid levels may have contributed to LTSH's strong iron chelating effects when compared to BHT. This effect on the ferrous ion was higher than wheat bran hydrolysates (64.05 %) (Wang *et al.*, 2009). As an addendum, LTSH may also serve to prevent metal ion-dependent oxidative damage to food lipids and thus, serve as a food preservative.

*Scavenging effects on the superoxide anion*

LTSH scavenging effect on superoxide radicals was observed to drastically increased as the LTSH concentration increased (from 0.5–20 mg/ml) (data not shown). This correlation was linear ($R^2$=0.9974) ($p < 0.05$). The scavenging of superoxide anions of LTSH obtained was 39.81% with an $IC_{50}$ at 3.89 mg/ml; lower than the BHT positive control (41.42%) at 1 mg/ml (data not shown) but without significance difference ($p > 0.05$) and higher than that reported for African yam bean seed protein hydrolysate (30% at 1 mg/ml) (Ajibola *et al*., 2013). This difference

in values was possibly due to singular hydrolysis conditions and the types of proteases employed.

*Hydroxyl radical scavenging activity*

Although lower than the positive control (BHT) at 1 mg/mL (data not shown), LTSH's hydroxyl radical scavenging activity increased as its concentration increased (data not shown) and measured between 43.68–66.72% with an $IC_{50}$ of 2.45 mg/ml. These results were most likely caused by the antioxidant activity of intrinsic LTSH amino acids and peptide sequences, especially hydrophobic amino acids that react with peptide and fatty acids which inhibit oxidation. LTSH's hydroxyl radical inhibition also approximated that of soy protein hydrolysate fractions (molecular weight 30–50 kDa) whose hydroxyl radical scavenging capacity was 69.75% (Moure *et al*., 2006). In addition, LTSH ($IC_{50}$, 2.45 mg/ml) showed higher free radical scavenging ability compared to walnut protein hydrolysate ($IC_{50}$, 5.04 mg/ml) (Chen *et al*., 2012). The findings therefore indicate that LTSH can be used as a hydroxyl radical scavenger in food processing.

## Conclusion

Immature lead tree seed has been chosen in this study due to its higher protein content compared to mature lead tree seed. The study showed that both yield and antioxidant activity (DPPH) of LTSH was significantly influenced by time, temperature, pH and enzyme concentration. LTSH also showed an excellent ability to donate an electron or hydrogen atom and was also able to scavenge superoxide, hydroxyl and DPPH radicals. In addition, LTSH acted as both a primary and secondary antioxidant due to its higher reducing ability and its chelation of ferrous ion.

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