International Journal of Agricultural Science and Technology (IJAST) Volume 2 Issue 4, November 2014 www.seipub.org/ijast
Doi: 10.14355/ijast.2014.0204.01

Optimization of Enzymatic Hydrolysis Parameters of Pigeon Pea for Better Recovery of Dhal

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Abstract

Pigeon pea is mostly consumed in the form of splits and it provides good source of proteins. The pigeon pea grain is considered as most difficult for dehulling as compared to other pulses owing to its seed coat which is more firmly attached with the cotyledons through a layer of gum and mucilage. Pre milling treatments are generally employed to loosen the seed coat to remove husk without losing any edible portion. The effect of four enzymatic hydrolysis parameters, i.e., enzyme concentration (20–60 mg/ 100 g dry matter), incubation time (3-15 h), incubation temperature (40-60 ^{0}C) and tempering water pH (4.0-6.0) on hulling efficiency were optimized using response surface methodology. Three enzymes i.e. xylanase, pectinase, and cellulase were used in combination for enzymatic pre treatment. A quadratic model satisfactorily described the dehulling efficiency with high value for the coefficient of determination R^2 (0.9062). It predicted a maximum dehulling efficiency of 88.37 % at enzyme concentration; 37.80 mg/100 g dry matter, incubation time, 8.69 min, incubation temperature, 48.48 ^{0}C and tempering water pH, 5.49. The results of the predicted optimum conditions were validated experimentally. Dehulling efficiency at optimum condition was observed to be 88.12 % and showed 0.28 % deviation from the predicted values. Results of the study revealed that dehulling efficiency of enzyme treated dhal could be increased 13.81 % compared to the control, i.e., the oil treated sample.

Keywords

Pigeon Pea; Hulling Efficiency; Enzymatic Pre Treatment; Dehulling; Response Surface Methodology

Introduction

Pulses are a valuable source of proteins, minerals and vitamins in daily diets of the people. Pigeon pea (*Cajanus cajan* L.) is one of the important pulse crops of India contributing 20.87 % to the total production of all pulses. India accounts for 90 % of the total world production of pigeon pea (Goyal et al. 2008). It is mostly consumed after dehulling in the form of dhal.

The pigeon pea grain is considered as most difficult for dehulling as compared to other pulses owing to its seed coat which is more firmly attached with the cotyledons through a layer of gum and mucilage (Rout et al. 2007). Due to the presence of gummy layer and hard seed coat, it is difficult to mill. Pre milling treatments are generally employed to loosen the seed coat to remove husk without losing any edible portion. There are many pre milling methods like wet milling, dry milling, CFTRI method, Pantnagar process, CIAE method and IIPR method for pigeon pea milling. All these treatments are time consuming require almost 4 to 7 days for the complete milling of pigeon pea. Moreover, these pre treatments lead to higher processing cost, longer processing time and labour consuming for pigeon pea milling

This necessitated the suitable pre treatment for pigeon pea milling that can shorten the processing time and improve the product quality. The amount of husk removed due to enzymatic pre treatments on different aspects were reported by Verma (1991), Bharodia (2004), Deshpande et al. (2007) and Sreerama et al. (2009). The mechanism of enzymatic activity is governed by four interacting parameters, i.e., seed moisture content, enzyme concentration, incubation time and incubation temperature (Sarkar et al. 1998). Optimum levels of these parameters are necessary to get maximum recovery and better quality of dhal.

Information on the effect of above parameters on dehulled fractions and cooking quality appears to be lacking. No systematically designed research approach for optimization of hydrolysis of process parameters for better recovery of dhal has been attempted. Such an optimization is necessary for the industrial adoption of the technology. Hence, it was considered necessary to optimize the parameters of enzymatic hydrolysis pre-treatment on different aspect, i.e., seed moisture, pH, enzyme concentration, reaction time (incubation time), incubation temperature of pigeon pea to get maximum recovery with good quality of dhal. Hence, the present study was under taken to optimize the milling efficiency of enzyme treated pigeon pea.

Materials And Methods

Selection of variety BDN 2 variety is milled in many of the pulse mills of Gujarat on large scale for getting pigeon pea dhal. In view of this, BDN 2 variety of pigeon pea was selected for the present investigation. The pigeon pea grain, used for the study was procured from the Sagdividi farm, Junagadh Agricultural University, Junagadh. The pigeon pea grains were cleaned manually to remove all foreign matters such as dust, dirt, stones, chaff, and immature grain, insect eaten and broken grains. The clean grains were then graded by manually operated size grader to obtain uniform sized grains (5.27 mm t0 5.38 mm).

Dehusking machine The laboratory scale dehusking machine fabricated (Bharodia 2004) with overall dimensions of 600 mm x 620 mm x 935 mm, capacity 85 kg/h, power unit 1.0 hp electric motor was used for all the milling studies. The optimum operating speed and feed rate of the dehusking machine were 1420 rpm and 64 kg/m^3, respectively.

Selection of Enzymes Each enzyme is catalyzed only a specific reaction involving a specific substrate. Therefore, the selection of enzymes was based on the chemical composition and binding substances present between husk and cotyledon of pigeon pea grain. The xylanase enzyme being widely used as bio-bleaching agent for lignin isolation (Browning, 1967). Cellulase and pectinase break down the cellulose to beta-glucose and pectin to pectinic acid and finally pectic acid. The xylanase, cellulase and pectinase are the key enzymes that rupture the binding materials leading to increase the dehulling efficiency. Hence, commercial food grade enzymes selected for enzymatic treatment were obtained from their manufacturers. The xylanase was procured from Advanced Enzyme Technologies Ltd., Thane (Maharashtra) while Cellulase and Pectinase enzymes were obtained from HiMedia Laboratories Pvt. Limited, Mumbai (Maharashtra).

Standardization of enzyme proportion The preliminary trials were taken to standardize the enzyme proportion of the three enzymes, i.e., xylanase, pectinase and cellulase. Initially, the proportion of all the three enzymes was selected arbitrarily. The effect of selected enzyme combination on husk removal of pigeon pea grain was evaluated keeping the enzyme concentration, incubation time, incubation temperature and tempering water pH constant based on the manufacturer's recommendation. Based on the results obtained, the enzyme proportion of Xylanase : Pectinase : Cellulase as 2 : 1 : 1 (50 %: 25 %: 25 %) gave the maximum husk removal and thereby the maximum hulling efficiency.

Enzymatic pre treatment Three enzymes were used in combination at optimum proportion i.e. two part xylanase, one part pectinase and one part cellulase ranging from 20 to 60 mg/ 100 g dry material, to ascertain the enzyme concentration required to obtain highest recovery and quality dhal. The process flow chart of enzymatic pre-treatment is given in Fig.1 for pigeon pea milling.

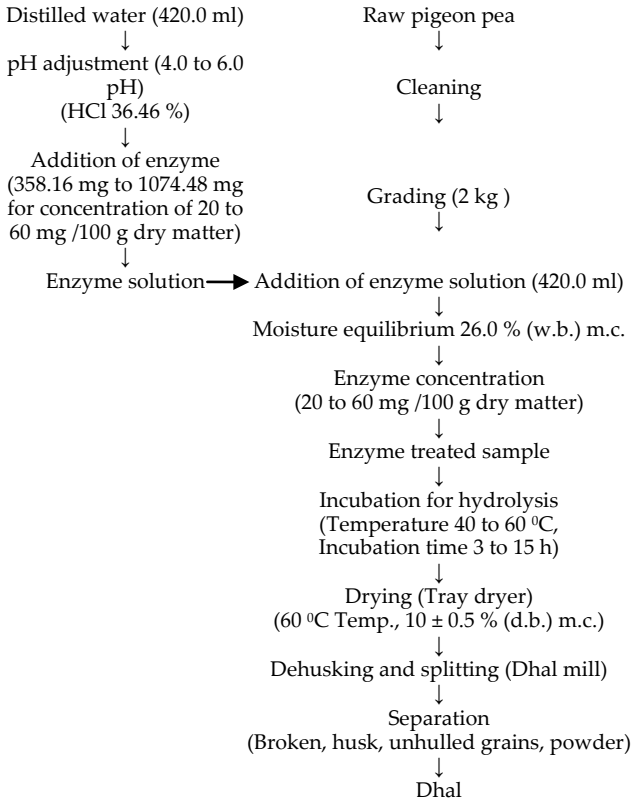

FIG. 1 FLOW CHART FOR ENZYMATIC PRE-TREATMENT MILLING- METHOD OF PIGEON PEA MILLING

pH adjustment The distilled water was mixed with 37% hydrochloric acid to obtain pH 4 to 6 with an interval of 0.5 pH (Yoo, 2007). Digital pH meter (Auto pH system PM-300) was used to adjust required pH range.

Preparation of enzyme solution The required amount of pH water solution was calculated using the formula 1 (Coskun et al., 2005) and required amount of enzymes were weighed and dissolved in pH water solution. The enzymes were dissolved completely before adding to the pigeon pea grains.

$$\text{Weight of water added (g)} = \frac{\text{W1(Mf} - \text{Mi)}}{(100 - \text{Mf})} \qquad (1)$$

Where,

W_1 = Initial weight of sample (g)

Mi = Initial moisture content, % (w.b.)

Mf = Final moisture content, % (w.b.)

For each experiment, 2 kg grains were taken in glass bottle and calculated amount of enzyme solution was mixed to increase the initial moisture content, i.e., 10.46 % (w.b.) to the desired level moisture content 26 % (w.b.). The glass bottle after its closing was shaken with enzyme solution for 3 minutes for equilibrium moisture. The samples at 26 % (w.b.) moisture content were incubated in humidity oven (Electroquip, Ahmedabad) at experimental temperature (40-60 ^{0}C) and incubation time (3-15 h). After incubation, the samples were dried in mechanical dryer at 60 ^{0}C to inactive enzyme and also to bring down the moisture content of grain to 10 ±0.5 % (d.b.). Enzyme treated samples were spread in wire mesh tray and dried under thin layer drying using the tray dryer.

Milling of sample: Enzymatic pre treated and control samples of size 2 kg having about 10 ± 0.5 % moisture content (d.b.) were milled using laboratory dehusking machine/dhal mill. Samples were milled at the standard settings of the machine, i.e., 1420 rpm operating speed and 64 kg/h feed rate. After milling, all the fractions were collected in polyethylene bag. Each of the samples were milled separately, care was taken to obtain all the fractions without any loss using the cleaning brush.

Dehulled sample separation: The different fractions of the milled product were separated by suitable sieves and hand picking such as whole dehulled grains, split dehulled grains, partly dehulled and unhulled grains, broken, husk and powder. A grain was considered completely dehulled when there was no husk adhering to it.

Husk content The husk (seed coat) content in whole grain was determined by soaking approximately 2 g of pigeon pea grain in distilled water (2 h at 50 ^{0}C). The seed coats were separated manually from the cotyledons, dried in a hot air oven at 100 ± 5 ^{0}C and weighed. The husk content was calculated using the formula 2.

$$\text{Husk content, \%} = \frac{\text{Wt. of Husk}}{\text{Wt. of pigeon pea grain}} \text{ X } 100 \qquad (2)$$

Calculations on the dehulling fractions All the fractions were weighed accurately using digital weighing balance with an accuracy of 0.01g. (Mettler model PE 3600). According to Singh *et al.* (2004), the following calculations were made on dehulling fractions obtained by dehulling treatments

$$\text{Husk removed (HR), \%} = \frac{\text{HRd}}{\text{Ht}} \text{ X } 100 \qquad (3)$$

$$\text{Coeeficient of hulling (Ch)} = 1 - \frac{\text{Wuh}}{\text{Wth}} \qquad (4)$$

$$\text{Coeeficient of wholeness of kernel (Cwk)} = \frac{\text{Wfp}}{\text{Wfp} + \text{Wbr} + \text{Wpo}} \qquad (5)$$

Where,

HRd = Husk removed during dehusking,(g)

Ht = Total husk content (g)

= Husk content in fraction X weight of grain used for milling (g)

Wuh = Weight of unhulled grain after milling (g)

Wth = Weight of total grain used for milling (g)

Wfp = Weight of finished product (g) (Splits and whole dehulled kernel)

Wbr = Weight of brokens (g)

Wpo = Weight of powder (g)

The hulling efficiency could be determined as,

$$\text{Hulling efficiency (HE)} = \text{Ch X Cwk X } 100 \qquad (6)$$

Experimental design The effect of four independent variables viz. enzyme concentration, incubation time, incubation temperature and tempering water pH on hulling efficiency were studied with variables coded as X_1, X_2, X_3 and X_4, respectively. Three response variables viz. hulling efficiency, protein content and cooking time were determined for optimization of the

process. Response Surface Methodology (RSM) was used for designing the experiments. A Central Composite Rotatable Design (CCRD) of 4 variables at 5 levels each with 6 centre point combinations were used (Khuri and Cornell 1987). Altogether 30 combinations (including 6 replications at the centre point and single observation at other points) were chosen according to a central composite rotatable design. The coded and uncoded variable values of the design are presented in Table 1.

TABLE 1 CODED AND UNCODED VARIABLES LEVELS

Variables		Coded variables				
		-2	-1	0	+1	+2
Enzyme concentration, mg/100 g dry sample	(X_1)	20	30	40	50	60
Incubation time, h	(X_2)	3	6	9	12	15
Incubation temperature, ^{0}C	(X_3)	40	45	50	55	60
Tempering water pH	(X_4)	4.0	4.5	5.0	5.5	6.0

Data analysis and optimization: The CCRD design was used to conduct experiments and the Response Surface Methodology (RSM) was applied to the experimental data using a commercial statistical package, Design Expert – version 8.0.0.6 (Stat-ease, 2009). Analysis of variance (ANOVA) was conducted for fitting the model represented by Eq^n. 7 to examine the statistical significance of the model terms. Model analysis with respect to lack-of fit test and R^2 (co-efficient of determination) was done for determining adequacy of model. The co-efficient of variation (CV) was calculated to find the relative dispersion of the experimental points from the prediction of the model. Response surfaces were generated and by using the same software, numerical optimization was done. The most commonly used model for optimization using response surface methodology is a second order polynomial equation (Bas and Boyaci, 2007). The model is of the form:

$$Y_k = b_{k0} + \sum_{i=1}^{3} b_{ki} X_i + \sum_{i=1}^{3} b_{kii} X_i^2 + \sum_{i \neq j=1}^{3} b_{kij} X_i X_j \quad (7)$$

(k=0,1,2,3….)

Where,

Y_k is the response, b_{k0}, b_{ki}, b_{kii}, and b_{kij} are the constant, linear, quadratic and cross-product regression coefficients, respectively and X_i's are the coded independent variables.

Validity test: The optimum conditions obtained through statistical analysis was verified by conducting an experiment in triplicate. The average value of dehulling efficiency was considered for the validation.

Results And Discussion

Effect of enzymatic treatment on hulling efficiency The analysis of variance (ANOVA) was made for the experimental data and the significance of enzyme concentration, incubation time, incubation temperature and tempering water pH as well as their interactions on hulling efficiency was analyzed. The response surface quadratic model was fitted to the experimental data and statistical significance of linear, interaction and quadratic effects were analyzed for hulling efficiency response (Table 2).

TABLE 2 ANOVA FOR EFFECT OF ENZYMATIC TREATMENT VARIABLES ON HULLING EFFICIENCY

Source	df	Sum of Squares	Mean Sum of Square	F Value	p-value Prob>F
Model	14	247.94	17.71	2.58*	0.0395
X_1: Enzyme concentration	1	17.61	17.61	2.57	0.1300
X_2: Incubation TTtime	1	4.42	4.42	0.64	0.4348
X_3: Incubation temperature	1	29.70	29.70	4.33*	<0.0450
X_4: Tempering water pH	1	70.52	70.52	10.28**	<0.0059
X_1X_2	1	0.49	0.49	0.071	0.7930
X_1X_3	1	0.15	0.15	0.022	0.8851
X_1X_4	1	0.16	0.16	0.023	0.8807
X_2X_3	1	0.13	0.13	0.019	0.8910
X_2X_4	1	0.37	0.37	0.054	0.8190
X_3X_4	1	1.97	1.97	0.29	0.5996
X_1^2	1	62.78	62.78	9.15*	<0.0085
X_2^2	1	6.83	6.83	1.00	0.3342
X_3^2	1	61.54	61.54	8.97**	<0.0091
X_4^2	1	31.21	31.21	4.55*	<0.0499
Residual	15	102.95	6.86	6.86	
Lack of Fit	10	100.25	10.02	18.53**	0.0024
Pure Error	5	2.70	0.54		
Correlation Total	29	350.89			

* and ** indicate significant at 5 % and 1 % level of significance, respectively

The results showed that among linear effects, incubation temperature and tempering water pH had significant effect on hulling efficiency ($p<0.05$) at 5% and ($p>0.01$) 1 % level of significance, respectively. However, linear effects of enzyme concentration, incubation time and interaction effects of enzyme concentration, incubation time, incubation temperature and tempering water pH were found to be non-significant. Quadratic effect of enzyme concentration and incubation temperature had significant effect on hulling efficiency ($p<0.01$) at 1%

level of significance while effect of tempering water pH on hulling efficiency ($p<0.05$) at 5% level of significance. The incubation time was found to be non-significant on hulling efficiency.

The hulling efficiency varied from 76.95 to 88.95 % (Table 3). The minimum hulling efficiency was found in treatment number 17 having the combination of enzyme concentration of 60 mg/100g dry matter, 9 h incubation time, 50 ^{0}C incubation temperature and 5.0 tempering water pH, while the maximum hulling efficiency found in treatment number 8 having the combination of enzyme concentration of 30 mg/100g dry matter, 6 h incubation time, 45 ^{0}C incubation temperature and 5.5 tempering water pH. The quadratic response surface model data indicated the results as significant. The coefficient of determination (R^2) was 0.9062 for enzymatic pre-treatment which indicated that the model could fit the data for enzyme activity very well for all the four variables, i.e., enzyme concentration, incubation time, incubation temperature and tempering water pH.

The response surface equation was obtained for the model of second degree in terms of coded factors is as under.

$$\text{Hulling efficiency, \%} = 87.44 - 0.86X_1 - 0.43X_2 - 1.11X_3 + 1.71X_4 - 0.18X_1X_2 - 0.096X_1X_3 - 0.10X_1X_4 + 0.091X_2X_3 - 0.15X_2X_4 + 0.35X_3X_4 - 1.51X_1^2 - 0.50X_2^2 - 1.50X_3^2 - 1.07X_4^2 \quad (8)$$

Where,

X_1 = Enzyme concentration (mg/100g dry matter),

X_2 = Incubation time (h), X_3 = Incubation temperature (^{0}C) and X_4 = Tempering water pH

Effect of enzyme concentration and incubation time on hulling efficiency: The effect of enzyme concentration and incubation time on hulling efficiency was determined keeping incubation temperature and tempering water pH constant at 50 ^{0}C and 5.0, respectively which is shown in Fig. 2. It could be observed that with increase in incubation time, the hulling efficiency increased at a particular enzyme concentration. It also confirms the findings that hulling efficiency first increases with incubation time and enzyme concentration and then decreases. The reduction in activity at higher enzyme concentration might be due to saturation of active sites of enzymes with substrate leading to lower hulling efficiency. However, the effect of enzyme concentration on hulling efficiency was found to be non- significant. Higher incubation time might have produced inhibitor substances for enzyme action resulting in lower hulling efficiency.

The minimum hulling efficiency of 76.95 % was obtained for the combination of enzyme concentration of 60 mg/100g dry matter, 9 h incubation time, 50 ^{0}C incubation temperature and 5.0 tempering water pH whereas, the maximum hulling efficiency was found for the combination of enzyme concentration of 30 mg/100g dry matter, 6 h incubation time, 45 ^{0}C incubation temperature and 5.5 tempering water pH. This showed that incubation temperature and tempering water pH played prominent role than the enzyme concentration and incubation time on hulling efficiency.

TABLE 3 EFFECT OF ENZYMATIC TREATMENT VARIABLES ON HULLING EFFICIENCY

Treat. No.	Enzymatic treatment variables				Responce
	Enzyme concentration (mg/100 g dry matter)	Incubation Time (h)	Incubation Temperature (^{0}C)	Tempering water pH	Hulling Efficiency (%)
1	50	12	55	5.5	83.63
2	30	12	55	5.5	85.93
3	50	6	55	5.5	85.54
4	30	6	55	5.5	86.49
5	50	12	45	5.5	85.32
6	30	12	45	5.5	88.02
7	50	6	45	5.5	87.52
8	30	6	45	5.5	88.95
9	50	12	55	4.5	78.77
10	30	12	55	4.5	80.98
11	50	6	55	4.5	79.52
12	30	6	55	4.5	81.41
13	50	12	45	4.5	82.75
14	30	12	45	4.5	83.52
15	50	6	45	4.5	83.67
16	30	6	45	4.5	84.58
17	60	9	50	5.0	76.95
18	20	9	50	5.0	80.65
19	40	15	50	5.0	82.47
20	40	3	50	5.0	83.24
21	40	9	60	5.0	77.70
22	40	9	40	5.0	80.02
23	40	9	50	6.0	81.82
24	40	9	50	4.0	79.35
25	40	9	50	5.0	86.21
26	40	9	50	5.0	87.07
27	40	9	50	5.0	88.07
28	40	9	50	5.0	87.54
29	40	9	50	5.0	88.24
30	40	9	50	5.0	87.53

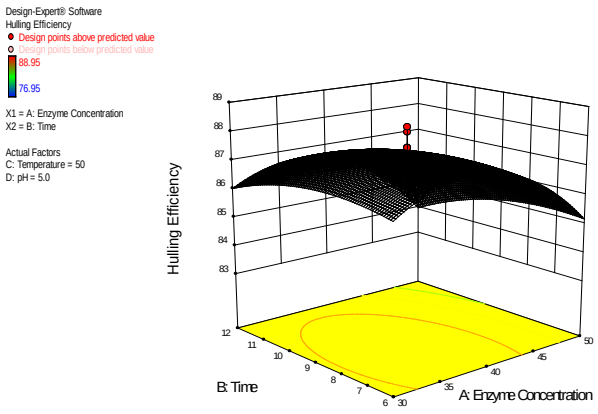

FIG. 2 EFFECT OF ENZYME CONCENTRATION AND INCUBATION TIME ON HULLING EFFICIENCY

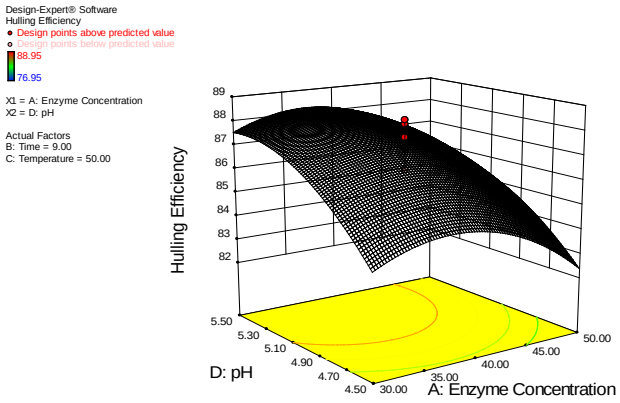

FIG. 3 EFFECT OF ENZYME CONCENTRATION AND INCUBATION TEMPERATURE ON HULLING EFFICIENCY

Effect of enzyme concentration and temperature on hulling efficiency The effect of enzyme concentration and incubation temperature on hulling efficiency was determined keeping incubation time and tempering water pH constant at 9 h and 50 ^{0}C, respectively which is shown in Fig. 3. Three dimensional responses for hulling efficiency of enzyme treated samples were generated. From these surfaces, it could be evident that hulling efficiency initially increased with increase in incubation temperature and enzyme concentration and then started decreasing, thereby indicating the existence of optimum levels of hydrolysis parameters within the selected range. Incubation temperature had shown significant effect on hulling efficiency. It was observed that with increase in incubation temperature, the hulling efficiency increased at a particular enzyme concentration. The reduction in enzymatic activity at above optimum temperature was due to denaturing of enzyme, resulting in the reduction of the hulling efficiency. It also confirmed the facts that maximum enzymatic reaction occurred at optimum temperature levels.

Effect of enzyme concentration and tempering water pH on hulling efficiency The effect of enzyme concentration and tempering water pH on hulling efficiency was determined keeping incubation time and incubation temperature constant at 9 h and 5.0, respectively as shown in Fig. 4. From three dimensional responses surfaces, it could be evident that hulling efficiency initially increased with increase in tempering water pH and enzyme concentration and then started decreasing, thereby indicating the existence of optimum levels of hydrolysis parameters within the selected range. Tempering water pH had significant effect on hulling efficiency and a sharp increase in hulling efficiency up to 5.49 pH value. It could be observed that with increase in tempering water pH, the hulling efficiency increased at a particular enzyme concentration. The reduction in enzymatic activity at above optimum pH was due to denaturing of enzymes, resulting in a decrease in the hulling efficiency.

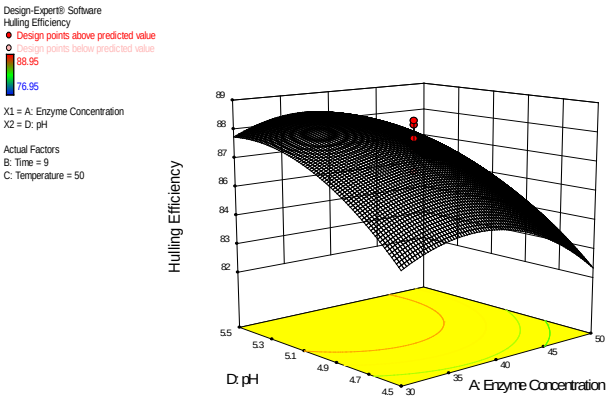

FIG. 4 EFFECT OF ENZYME CONCENTRATION AND TEMPERING WATER PH ON HULLING EFFICIENCY

Effect of incubation time and incubation temperature on hulling efficiency: The effect of incubation time and incubation temperature on hulling efficiency at constant enzyme concentration (40 mg/100g) and tempering water pH (5.0) is shown in Fig. 5. It could be evident that hulling efficiency initially increased with increase in incubation time and incubation temperature and then started decreasing, thereby indicating the existence of optimum levels of hydrolysis parameters within the selected range. Incubation temperature was showing significant effect on hulling efficiency. However, it was observed that the hulling efficiency increased with increase in incubation time was small as compared to incubation temperature. It was also observed that with increase in incubation temperature, the hulling efficiency increased at a particular incubation time. The reduction in enzyme activity at above optimum

incubation temperature would denature the enzymes, resulting in a decrease in the hulling efficiency.

Effect of incubation time and tempering water pH on hulling efficiency: The effect of incubation time and tempering water pH on hulling efficiency at constant enzyme concentration (40 mg/100g dry matter) and incubation temperature (50 ^{0}C) is shown in Fig. 6. It could be observed that with increase in tempering water pH, the hulling efficiency increased at a particular incubation time. From these surfaces, it is evident that hulling efficiency initially increased with increase in incubation time and tempering water pH and then started decreasing, thereby indicating the existence of optimum levels of hydrolysis parameters within the selected range. Effect of pH on hulling efficiency was found significant. However, incubation time and interaction of these two factors were found to be non- significant. It was also observed from the Fig.6 that the increase in hulling efficiency with the increase in incubation time within the range tested was small.

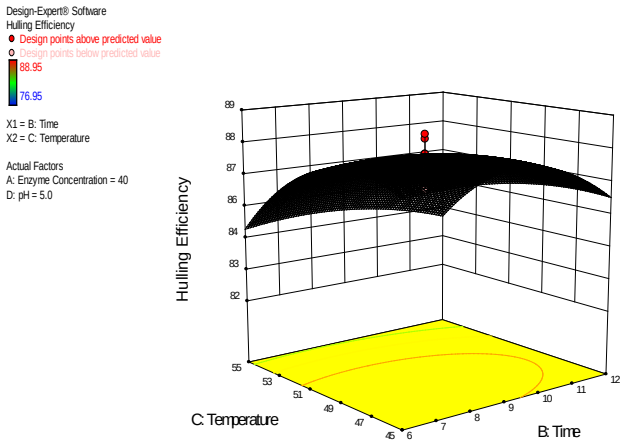

FIG. 5 EFFECT OF INCUBATION TIME AND INCUBATION TEMPERATURE ON HULLING EFFICIENCY

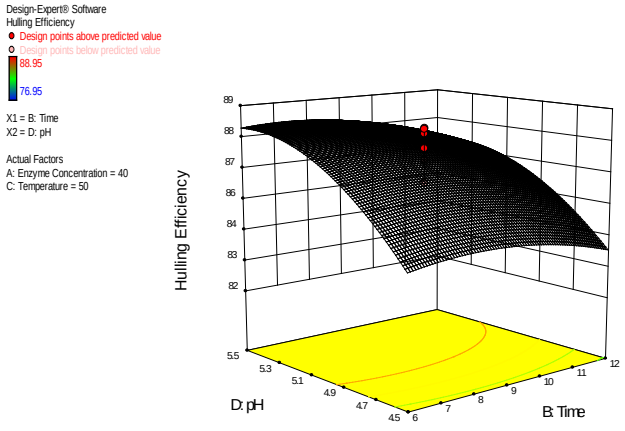

FIG. 6 EFFECT OF INCUBATION TIME AND TEMPERING WATER PH ON HULLING EFFICIENCY

Effect of incubation temperature and tempering water pH on hulling efficiency: The effect of incubation temperature and tempering water pH on hulling efficiency at constant enzyme concentration (40 mg/100g dry matter) and incubation time (9 h) is shown in Fig. 7. It was observed that with increase in tempering water pH, the hulling efficiency increased at a particular incubation temperature. From these surfaces, it could be evident that hulling efficiency initially increased with increase in incubation temperature and tempering water pH and then started decreasing, thereby indicating the existence of optimum levels of hydrolysis parameters within the selected range. Effect of tempering water pH and incubation temperature on hulling efficiency was found significant. However, interaction of these two factors were found to be non- significant

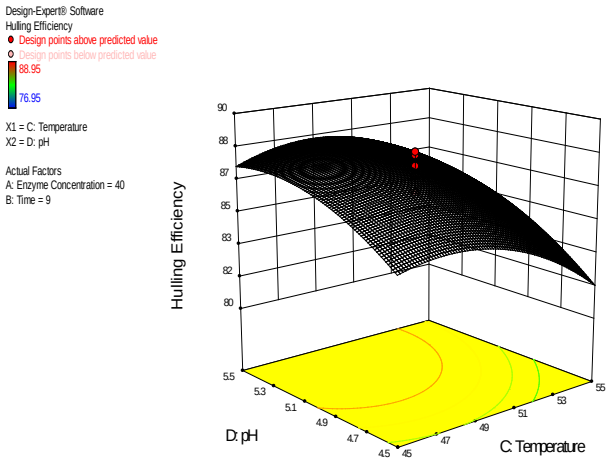

FIG. 7 EFFECT OF INCUBATION TEMPERATURE AND TEMPERING WATER PH ON HULLING EFFICIENCY

Optimization of enzymatic treatment variables Software Design Expert version 8.0.0.6 was used for the optimization of responses. A stationary point, i.e., a point at which the slope of the response surface was zero in all directions was calculated by partially differentiating the model with respect to each variable, equating these derivatives to zero and simultaneously solving the resulting equations. The optimum values of enzymatic hydrolysis pre-treatment were evaluated using equation 8. The multiple regression package was used for this purpose. The response surface quadratic model optimized the pre-treatment as enzyme concentration of 37.80 mg/100 g dry matter, incubation time 8.69 h, incubation temperature 48.48 ^{0}C(≅48.5 ^{0}C) and tempering water pH 5.49 which gave the predicted values of hulling efficiency 88.37 %. The optimum values for different variables and their predicted responses thus obtained are given in Table 4.

TABLE 4 OPTIMIZED VARIABLES AND THEIR RESPONSES FOR ENZYMATIC PRE TREATMENT OF PIGEON PEA GRAINS

Variables	Optimized values	Response	Predicted value
Enzyme concentration mg/100 g dry matter	37.80	Hulling efficiency, %	88.37
Incubation time, min	8.69		
IncubationTemperature,^{0}	48.48		
Tempering water pH	5.49		
R^2	0.9062		

It may be mentioned that the optimum values of different variables for enzymatic pre-treatment were within the range considered in the study.

The hulling efficiency of oil treated (control) sample was found 76.16 % while the observed value of hulling efficiency of enzymatic treatment sample at optimum condition was 88.12 %. Hence, there was an increase in hulling efficiency of 13.47 % over oil treated sample.

Validity of the model The performance of this model was also verified by conducting an experiment for the validation. In order to validate the optimum conditions of enzymatic pre-treatment variables, the experiment was conducted in triplicate at derived conditions. The predicted values of hulling efficiency was 88.37 and this was experimentally verified in the laboratory and observed value of hulling efficiency was found to be 88.12 %. The predicted value of hulling efficiency obtained from equations showed 0.28 % deviation from the experimental value. It could reveal that the experimental value was very close to the predicted value which confirmed the optimum conditions.

Conclusion

For enzymatic pre-treatment, the enzyme solution having 2: 1: 1 proportion of xylanase, pectinase and cellulase enzymes should be prepared using 5.49 tempering water pH. The enzyme solution should be applied at the rate of 37.80 mg/ 100 g of dry pigeon pea grain. The enzyme treated pigeon pea grains should be kept at 48.5 ^{0}C incubation temperature for 8.69 h incubation time. The observed value of hulling efficiency at the suggested conditions of enzymatic pre-treatment variables was 88.12 %.

Mathematical model predicted a maximum dehulling efficiency of 88.37 % at optimum enzyme concentration of 37.80 mg/100 g dry matter, incubation time 8.69 min., incubation temperature 48.5 ^{0}C and 5.49 tempering water pH. Dehulling efficiency at optimum condition was experimentally observed to be 88.12 % and were close to the predict value.

ACKNOWLEDGMENT

Authors are grateful to Head, Industrial Application Development Laboratory, Advanced Enzyme Technologies Ltd. 106, Dhanlaxmi Industrial Estate, Gokulnagar, Thene(w)- 400 601 for providing the required quantity of xylanase enzyme for conducting the above research work.

REFERENCES

Bas D, Boyaci IH (2007). Modelling and optimization I: Usability of response surface methodology. J Food Eng 78: 836-841.

Bharodia NR (2004). Studies on pre treatments for loosening of seed coat of pigeon pea grains. M.Tech. (Agril.Engg.) Thesis, Gujarat Agricultural University, Junagadh

Browning BL (1967). Methods of Wood Chemistry, Interscience Publishers, A Division of John Wiley & Sons.

Coskun MB, Yalcin I, Ozarslan C (2005). Physical properties of sweet corn seed (zea mays saccharatasturt) J Food Eng 74: 523-525

Deshpande SD, Balasubramanya RH, Khan S, Bhatt DK (2007). Influence of pre milling treatments on dhal recovery and cooking characteristics of pigeon pea. J Agric Eng 44: 53-56

Goyal RK, Vishwakarma RK, Wanjari OD (2008). Optimisation of pigeon pea dehulling process. Biosystems Eng 99: 56-61

Khuri AI, Cornell JA (1987). Response surface design and analysis. Marcel Dekker, Inc., New York, NY.

Rout B, Sahoo S, Senapati PK (2007). Effect of pre milling treatment on protein and carbohydrate content in tribal pulses. Indian J Traditional Knowledge 6:69-71

Sarkar BC, Singh BPN, Agrawal YC, Gupta DK (1998). Optimization of enzyme treatment of rapeseed for enhanced oil recovery. J Food Sci Technol 35:183-186

Singh SK, Agrawal US, Saxena RP (2004). Optimization of processing parameters for milling of green gram (Phaseolus aureus), J Food Sci Technol 41: 124-130

Sreerama YN, Shashikala VB, Pratape VM (2009). Effect of enzyme pre-dehulling treatments on dehulling and

cooking properties of legumes. J Food Eng 92: 389-395

Stat-Ease Inc. (2009). Design Expert User's Guide, The Stat-Ease Inc., USA

Verma P (1991). Enzymatic pre treatment of pigeon pea (Cajanus cajan L.) grain and its interaction with milling, M.Tech. (Agril.Engg.) Thesis, G.B. Pant University of Agril. & Technology, Pantnagar.

Yoo J (2007). Effect of enzyme application in temper water on wheat milling, M.Sc. thesis submitted in College of Agriculture, Kansas State University, Manhattan, Kansas.

doi: 10.14355/ijast.2014.0204.02

Effects of Temperature on Growth and Survival of Pathogenic Vibrio Parahaemolyticus

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Abstract

This study investigated the effects of temperature on the survival of *Vibrio parahaemolyticus* during cold storage and heating process. *V. parahaemolyticus* enriched in sterile alkaline peptone water (APW) supplemented with 1.5% NaCl (APW-salt broth) at 37°C overnight (12-16h) was subjected to cold storage (-30, -18, 0, 5, 10, 15 and 20°C) and heating treatment (50, 55, 60, 70, 80, and 90 °C) at various temperatures. The populations of *V. parahaemolyticus* were determined before and after the treatment. Results showed that *V. parahaemolyticus* in APW-salt broth increased rapidly when temperature was higher than 15 °C, while decreased gradually at 0 and 5 °C. Freezing treatment could greatly decrease the population of *V. parahaemolyticus* in APW-salt broth. Storage at -18 °C was more effective in inactivating *V. parahaemolyticus* than that at -30°C. Keeping *V. parahaemolyticus* frozen at -18 and -30 °C from 15 to 30 days could reduce the pathogen to non-detectable levels (<3 MPN/g). Heating at 60 °C for 5 min, 70 °C for 2 min, or 80 °C or higher for 1 min also reduced *V. parahaemolyticus* from 10,000 MPN/g to non-detectable level. In conclusion, *V. parahaemolyticus* did not survive well during frozen treatment or low temperature storage (<5 °C). In addition, heating treatment (at 60 °C) is also effective to inactivate *V. parahaemolyticus*. This study can be applied to reduce risks of infections associated with foodborne pathogen *V. parahaemolyticus.*

Keywords

Vibrio Parahaemolyticus; Survival; Cold Storage; Heating Treatment

Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium (Baumann and Schubert, 1984) and widely distributed in marine environments including water, sediment and seafood (Liston, 1990). This organism is the most prevalent foodborne pathogen in China (Chen *et al.,* 2007), which accounted for 31.1% of all bacteria food poisoning events in thirteen provinces between 1992 and 2001 (Liu *et al.,* 2005). It has also been recognized as a leading cause of diarrhea associated with seafood consumption throughout the world including Japan, Canada and the United States (Su and Liu, 2007). Thermal treatment and cold storage (including refrigeration, icing and freezing) are the most effective means to destroy or limit the growth of foodborne pathogens including *V. parahaemolyticus.* This study investigated the fate of both virulent and non-virulent strains of *V. parahaemolyticus* in a multi-culture strain cocktail during chilled and frozen storage, and heating treatment at different temperatures.

Materials and Methods

Bacteria Cultures.

Two pathogenic strains of *V. parahaemolyticus* possessing tdh gene (VP33846 and VP 33847) were used in this study. The strains obtained from Microbiology Institute of Chinese Academy of Sciences, Beijing, China. Each culture was individually grown in sterile alkaline peptone water (APW) supplemented with 1.5% NaCl (APW-salt broth) at 37°C overnight (12-16h) and a two-strain cocktail culture suspension of *V. parahaemolyticus* was prepared according to our previous study (Shen *et al.,* 2009; Thompson and Thacker, 1973).

Effects Of Temperatures On The Populations Of V. Parahaemolyticus During Chilled And Frozen Storage

V. parahaemolyticus in APW-salt broth was stored at the different temperatures (-30, -18, 0, 5, 10, 15 and 20 °C). Populations of *V. parahaemolyticus* in the broth were determined before and after storage using the pour-

plate method.

Survival Of V. Parahaemolyticus During Heating Treatment

0.1 mL of fresh prepared *V. parahaemolyticus* cocktail suspension was added into a tube containing 9.9 mL of sterile APW-salt broth which was tempered at the set temperature (50, 55, 60, 70, 80, or 90 ˚C) in a water bath, and then continuously was incubated at the same temperature for a certain period of time (1, 2, 3, 5, 10 min). After the required contact time, the tubes were transferred to iced water to stop the action of the heat as quickly as possible. Populations of *V. parahaemolyticus* in the broth were determined before and after heating by using the pour-plate procedures.

Microbiological Analysis

V. parahaemolyticus counting in culture suspension was detected by the pour-plate method using nutrient agar (Shanghai Reagent Providing and Research Center for Diarrheal Disease Control, Shanghai, China) supplemented with 1.5% NaCl.

Results and Discussions

Effects Of Chilled Storage On Populations Of V. Parahaemolyticus At Different Temperatures

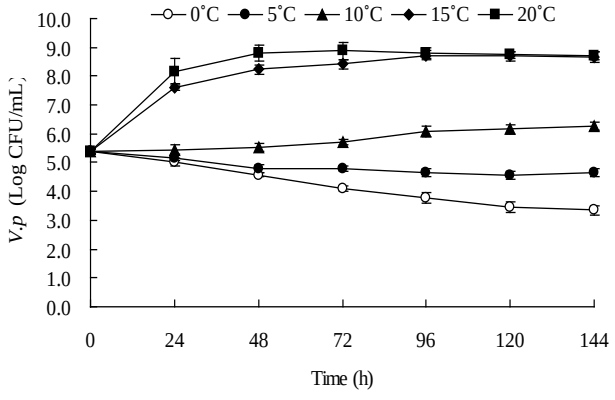

FIG.1 CHANGES IN POPULATION OF *V. PARAHAEMOLYTICUS* DURING STORAGE AT VARIOUS TEMPERATURES (0-20˚C)

Figure 1 showed that population of *V. parahaemolyticus* in APS broth increased rapidly when temperature was higher than 15 ˚C, but decreased gradually at 0 and 5 ˚C. The initial number of *V. parahaemolyticus* in APS broth was 5.37 Log10 CFU/mL, which increased by 3.28 and 3.34 Log10CFU/mL after storage for 144 hours at 15 and 20 ˚C, but decreased by 2.01 and 0.71 Log10CFU/mL after storage at 0 and 5 ˚C, respectively. At 10˚C, *V. parahaemolyticus* multiplied slowly and its density increased by only 0.71 Log10CFU/mL after 144h storage that could be considered static for the most time. These results were partly supported by previous studies. *V. parahaemolyticus* in oyster homogenates could decrease rapidly from the initial level (10^3 cells) to non-detectable level after storage at 0-4 ˚C; while multiplication was found to occur at 10-12 ˚C, but not at 8 ˚C (Thompson and Thacker, 1973). *V. parahaemolyticus* in crab meat decreased rapidly at 5 ˚C, with 5 log reduction observed after storage for 14 days (Ray, Hawkins and Hackney, 1978) and that on fish fillets could decline by 1-2 log10 CFU/fillet after storage at 4-8 ˚C for 9 days (Vasudevan *et al.*, 2002). Therefore, keeping cold (˂ 10 ˚C) was an effective method to limit the growth of *V. parahaemolyticus* in foods. For reducing *V. parahaemolyticus* contamination in foods, the cold temperatures should be maintained from shipping, retail and home refrigeration.

Effects Of Freezing Treatment On The Survival Of V. Parahaemolyticus

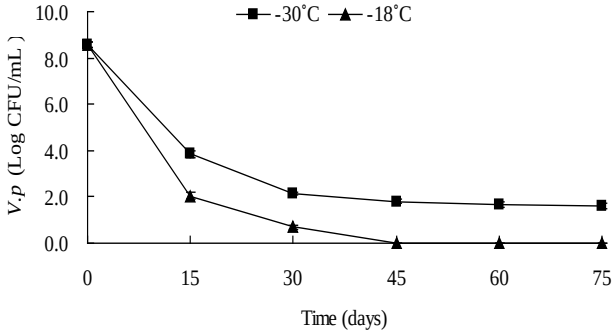

FIG.2 SURVIVAL LEVELS OF *V. PARAHAEMOLYTICUS* DURING FROZEN STORAGE AT DIFFERENT TEMPERATURES (-18,-30˚C)

In addition to chilling, freezing is also a common method used to preserve food quality and enhance food safety. Figure 2 showed freezing treatment could decrease the population of *V. parahaemolyticus* in APS broth. Freezing the culture suspension of *V. parahaemolyticus* at -18 and -30 ˚C for 15 days could decrease the bacteria population from the initial level 8.59 to 2.04 and 3.84 Log10CFU/mL (with 6.55 and 4.75 log reductions), respectively, and continued to decline at a slower rate even to undetectable level during the storage. Storage at -18 ˚C was more effective in inactivating *V. parahaemolyticus* than that at -30˚C. This might be explained by the fact that the bacterial cell damage caused by ice crystals at -18 ˚C was more serious than that at -30 ˚C since intracellular ice crystals formed in bacterial cells at a higher freezing temperature (-18 ˚C) were bigger than those at a lower temperature (-30 ˚C) (Jay, Loessner and Golden, 2005). Similar phenomena were also found in inoculated oysters (Liu and Su, 2009) and fish fillet (Vasudevan *et al.*, 2002). These results suggested that frozen storage

should also be an effective post-harvest treatment for reducing *V. parahaemolyticus* contamination in foods.

Effects Of Heating Treatment On Survival Of V. Parahaemolyticus

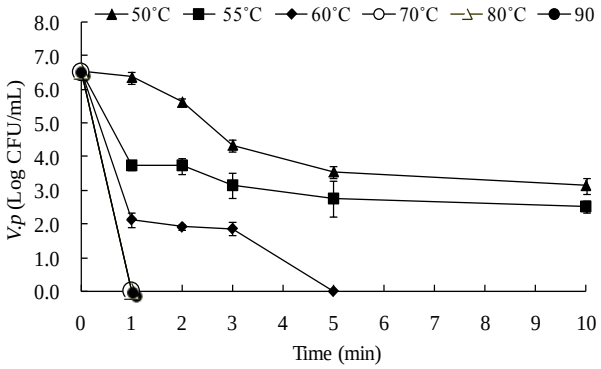

FIG.3 INACTIVATION OF *V. PARAHAEMOLYTICUS* DURING HEATING TREATMENT AT VARIOUS TEMPERATURES (50-90˚C)

Figure 3 showed heating treatment was very effective to reduce *V. parahaemolyticus* level in APW-salt broth and the number of *V. parahaemolyticus* decreased rapidly upon heating at ≥50 °C. However, heating the culture broth at 50 or 55 ˚C for 10 min could not completely inactivate this organism, for $10^{3\text{-}4}$ CFU/mL viable cells were detected after the 10 min heat treatment. Heating the culture cocktail at higher temperatures (60, 70, 80, 90 ˚C) could achieve greater than 6-log reduction, no viable cells were detected in the culture broth at 60 °C for 5 min and at 70 °C or higher for 1 min. Although Andrews *et al* (2000) (Andrews, Park and Chen, 2000) reported the 50 ˚C water heat treatment of inoculated shellstock oysters for 10 min could reduce the numbers of *V. parahaemolyticus* from 10^5MPN/g to non-detectable level, the result of this study demonstrated that *V. parahaemolyticus* possessed high heat resistance. Heating *V. parahaemolyticus* in APW-salt broth at 50 ˚C for 10 min in our study could only achieve 3.41 log reduction. More than 10^3 viable cells of *V. parahaemolyticus* were still detected both in culture broth at 50 ˚C for 10 min. Vanderzant and Nickelson (1972) also reported that *V. parahaemolyticus* in shrimp homogenates showed higher heat resistance (Vanderzant and Nickelson, 1972) than that in shellstock oysters (Andrews, Park and Chen, 2000). Therefore, for full inactivation of *V. parahaemolyticus* in foods, cooking in hot water at 60 °C for 5 min, 70 °C for 2 min, 80 °C or higher for 1 min was recommended.

Although eating raw or cooked food is a matter of personal preference, there are still a lot of people who like eating seafood raw or undercooked due to the special flavors of raw seafood. For example, in the United States today, there is a high demand for raw oysters on the half-shell (shooters) typically served at oyster bars. In China, clams are most often cooked before consumption, but undercooked is highly possible since the clams are just quickly boiled in hot water. These eating habits pose health risk because food borne pathogens including *V. parahaemolyticus* may survive in seafood. It is estimated that >60% of seafood-associated illness could be avoided if consumers would stop eating raw or undercooked molluscan shellfish (Liston, 1990). People at high risk groups, for example, young children, the elderly, pregnant women, and those in poor health conditions, should not eat raw or undercooked seafood including oysters and clams to reduce the potential risks of life-threatening disease caused by Vibrio infection.

Conclusions

In summary, cooling foods down to < 10 ˚C quickly after harvest and keeping them below 10 ˚C was effective to prevent *V. parahaemolyticus* from rapid multiplication. For reducing *V. parahaemolyticus* contamination in foods, cold storage at 0-5 ˚C was highly recommended. In addition, freezing was also an effective treatment to inactivate *V. parahaemolyticus*. Keeping foods at the range between -18 ˚C for 15-45 days could reduce *V. parahaemolyticus* contamination from 100 MPN/ml to non-detectable levels. Finally, cooking at 60 °C for 5 min, 70 °C for 2 min, 80 °C or higher for 1 min could reduce *V. parahaemolyticus* contamination from 10^6 MPN/ml to non-detectable level.

ACKNOWLEDGEMENTS

The authors would like to thank the project (No. 2014T05) supported by special research fund for the national non-profit institutes (East China Sea Fisheries Research Institute) and the Yangfan program (14YF1408100) from science and technology commission of Shanghai municipality.

REFERENCES

Andrews, L.S., Park, D.L., and Y.P. Chen. "Low temperature pasteurization to reduce the risk of Vibrio infections from raw shell-stock oysters." Food Additives and Contaminants 19 (2000): 787–91.

Baumann, P., and R.H.W. Schubert. "Family II. Vibrionaceae." In Bergey's Manual of Systematic Bacteriolog, edited by N.R. Krieg and J.G. Holt, 516–550. Baltimore: Williams and Wilkins Co., 1984.

Chen, R.Y., Liu., J.Z., Su, Y.C., and C.C. Liu. "Review on Risk Analysis, Detection and Control of *Vibrio parahaemolyticus* in Food. " Food Science 28 (2007): 341–346.

Jay, J.M., Loessner, M.J. and D.A. Golden. Modern Food Microbiology. New York: Springer Science & Business Media, Inc., 2005.

Liston, J. "Microbial hazards of seafood consumption." Food Technol. 44 (1990): 56–62.

Liu, C.C., Lu, J.Z., and Y.C. Su. "Effects of flash freezing, followed by frozen storage, on reducing *Vibrio parahaemolyticus* in Pacific raw oysters." Journal of Food Protection 72 (2009): 171–174.

Liu, X.M., Cheng, S.Y., Chen, Y., Yuan, B.J., Dai, J.H., Ma, Q.F., Dai, C.F., and J.W. Yan. "Active surveillance on *Vibrio parahaemolyticus* in retail seafoods from coastal areas of China in 2003." Chinese Journal of Food Hygiene 17 (2005): 97–99.

Ray, B., Hawkins, S.M., and C.R. Hackney. "Method for the detection of injured *Vibrio parahaemolyticus* in seafoods." Applied and Environmental Microbiology 35 (1978): 1121–1127.

Shen, X.S., Cai, Y.Q., Liu, C.C., Liu, W.W., Hui, Y.H., and Y.C. Su. "Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (Crassostrea plicatula)." International Journal of Food Microbiology 136 (2009): 129–132.

Su, Y.C., and C.C. Liu. "*Vibrio parahaemolyticus*: A concern of seafood safety." Food Microbiolog 24 (2007): 549–558.

Thompson, W.K., and C.L. Thacker. "Effect of temperature on *Vibrio parahaemolyticus* in oysters at refrigerator and deep freeze temperatures." Canadian Institute of Food Science and Technology Journal 6 (1973): 156–158.

Vanderzant, C., and R. Nickelson. "Survival of *Vibrio parahaemolyticus* in shrimp tissue under various environmental conditions." Applied Microbiology 23 (1972): 34–37.

Vasudevan, P., Marek. P., Daigle, S., Hoagland, T., and K. Venkitanarayanan. "Effect of chilling and freezing on survival of *Vibrio parahaemolyticus* on fish fillets." Journal of Food Safety 22 (2002): 209–217.

doi: 10.14355/ijast.2014.0204.03

Content Analysis of Shikimic Acid in the Masson Pine Needles and Antiplatelet-aggregating Activity

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Abstract

Shikimic acid content was measured by high-performance liquid chromatography (HPLC) in the Masson pine needles as one potential source of shikimic acid. The antiplatelet-aggregating activity of shikimic acid was characterized by turbidimetry with an animal model. The shikimic acid level was compared with Oriental arborvitae leafytwigs and Star anise. The results showed that the shikimic acid content in Masson pine needles, Oriental arborvitae leafytwigs and Star anise was 5.71%, 1.74% and 8.95%, respectively. The yield of shikimic acid was the highest when the powder of Masson pine needles was ultrasonically-treated for 60 min in 3.5 mmol/L phosphoric acid. Shikimic acid, when separated by HPLC, exhibited a dose-dependent inhibitory effect on platelet aggregation induced by adenosine diphosphate and collagen in rabbits. Because of the relative high content and good antiplatelet-aggregating activity of shikimic acid, the Masson pine needles can be used as a potential source of shikimic acid.

Keywords

Masson Pine Needles; Shikimic Acid; HPLC; Antiplatelet-aggregating Activity.

Introduction

Shikimic acid is an intermediate product of the shikimic acid pathway in many advanced plants and microorganisms (Herrmann, 1995; Pittard and Wallace, 1966; Liu *et al.*, 2014). Shikimic acid is known for its analgesic and anti-thrombotic effects, and it is also known as an ingredient in the anti-bird flu drug (Tamiflu). Some related derivatives with biological activities are synthesized from shikimic acid (Krämer *et al*., 2003; Foye, Wang and Wang, 1997; Huang *et al*., 2002). Thus far, Chinese star anise is the major source for shikimic acid extraction, but it cannot satisfy the market demand at present (Li *et al*., 2010). The current experiments were designed to identify a new source for shikimic acid and to provide data for developing new anti-thrombotic drugs. We determined the shikimic acid content in the Masson pine needles by high-performance liquid chromatography (HPLC). Comparison was made between Oriental arborvitae leafytwigs and Star anise with respect to shikimic acid content. Turbidimetry (Foye, Wang and Wang, 1997) was used to determine the antiplatelet-aggregating activity of shikimic acid from Masson pine needles. The prospect of large-scale production of shikimic acid with Masson pine needles as raw material was evaluated.

Materials and Methods

Reagents and Animals

A standard solution of shikimic acid was prepared by dissolving shikimic acid standard (purity 99%; Sigma, St. Louis, MO, USA) in 3.5 mmol/L phosphoric acid to achieve a concentration of 0.635 mg/ml. Adenosine diphosphate (ADP) and collagen were purchased from Sigma. Masson pine needles and Oriental arborvitae leafytwigs samples were collected from Hubei Province, China. Star anise was purchased from a commercial market. After being dried at 70℃ for 12 h, the samples were passed through a 20-mesh sieve. Rabbits weighing 2.3±0.5 kg were provided by the Experimental Animal Center of Guangzhou Medical University, China.

Shikimic Acid Eextraction and Determination

One hundred milligrams of Masson pine needles, Oriental arborvitae leafytwigs and Star anise powders were dissolved in a 3.5 mmol/L H_3PO_4 solution. After ultrasonic treatment for 60 min, the solution was diluted to 100 ml. The sample solution was further diluted and passed through a 0.45-μm polypropylene membrane. HPLC was performed with a Waters HPLC System (Water Corp., Milford, MA, USA) under the following conditions: Diamonsil C18 column (5 μm, 4.6 mm ×250 mm); flow rate, 0.8 ml/min; injection volume, 10 μl; detection wavelength, 215 nm;

room temperature; and mobile phase, 3.5 mmol/L aqueous solution of phosphoric acid (pH=2.8). Before sample injection, the column was eluted with methanol for 30 min. The sample was analyzed with an external standard method to measure the peak area and to identify the target component by retention time. The procedure was repeated 3 times for each sample, and the average was taken as the final result.

Spike and Recovery of Shikimic Acid

One hundred milligrams of Masson pine needles powder was weighed and dissolved in a 3.5 mmol/L H_3PO_4 solution. Then 0, 6, 8, and 10 mL of a 0.635 mg/ml standard solution of shikimic acid was added. The sample solutions were ultrasonically-treated for 60 min, and the volume was set at 100 ml. The sample solution was further diluted and passed through a 0.45-μm polypropylene membrane. The samples were injected 3 times into the column and the **s**hikimic acid content was measured.

Separation of Shikimic Acid and Platelet Plasma

Shikimic acid was extracted by HPLC under the above-mentioned conditions. The target components were identified by the retention time and the shikimic acid was prepared in a small amount. Carotid artery blood samples were collected from rabbits and treated with 3.28% sodium citrate to prevent blood coagulation (blood-to-anticoagulant ratio = 9:1). The blood samples were centrifuged at 1000 r/min for 5 min to separate platelet-rich plasma (PRP). The remaining plasma was further centrifuged at 3000 r/min for 10 min to obtain platelet-poor plasma (PPP).

Antiplatelet-aggregating Activity Experiments

Purified shikimic acid was made into solutions of different concentrations, and 20 μl of the solution was added to 180 μl of PRP to incubate the cells at 37℃ for 4 min. PPP was used as the zero reference. The 11 μl of collagen and ADP were added to the cuvette, and the 5 min platelet aggregation rate in vitro after drug administration was measured by turbidimetry (Foye, Wang and Wang, 1997) with a TYXN-91 Intelligent Blood Agglutometer (Shanghai, China). The platelet aggregation inhibition rate was calculated by the following formula: platelet aggregation inhibition rate (%) = (percentage of platelet aggregation in the control tube - percentage of platelet aggregation in the experimental tube) / percentage of platelet aggregation in the control tube×100.

Results and Analysis

Establishment of HPLC Procedures for Shikimic Acid

Standard solutions (4, 8, 12, 16, and 20 μl of 15.52 μg/ml) of shikimic acid were injected into the column, and the peak area was determined. The injected amount of the standard reference was plotted on the X-axis, and the peak area was plotted on the Y-axis. The regression equation was as follows: y = 26691 x - 16975; R2=0.9995. Ten microliters of the solution of Masson pine needles powder was injected 5 times. The peak area of shikimic acid was determined to be 347991.5, 345958.5, 348130.5, 347869.5, and 349861. 5 for the 5 standard solutions, respectively. The relative standard deviation (RSD) was 0.4%, indicating good precision. In the spike-and-recovery experiment of shikimic acid from Masson pine needles, the average recovery rate was 96.4% and the RSD was 0.79%, indicating a high recovery effect.

Comparison of Shikimic Acid Extraction Conditions

In the previous section, two methods were used to extract shikimic acid from Masson pine needles (ultrasonic treatment and reflux at 90℃). When the time was fixed, the efficiency of ultrasonic treatment was higher than the efficiency of reflux, with shikimic acid content reaching the highest amount at 60 min (Fig. 1). The increase of shikimic acid content was not significant, compared with the extraction for 15 min. Because shikimic acid has strong polarity and water dissolubility, both extraction methods achieved good results on the premise that the samples were fully smashed. Prolonging the extraction time only increases the extraction efficiency in a mild degree.

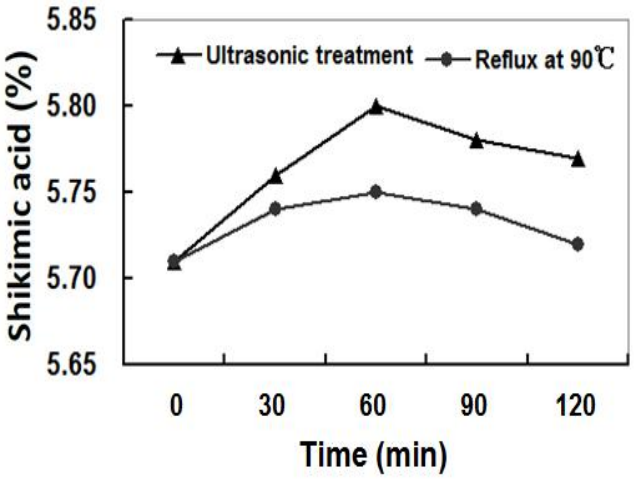

FIG. 1. THE EFFECTS OF TWO EXTRACTING CONDITIONS ON THE SHIKIMIC ACID FROM MASSON PINE NEEDLES.

TABLE 1. EFFECT OF SHIKIMIC ACID FROM MASSON PINE NEEDLES ON THE RABBIT PLATELET AGGREGATION INDUCED BY THE ADDITION OF ADP AND COLLAGEN IN VITRO (N=10)

Group	Concentration (mmol/L)	Aggregation rate (%)		Inhibition rate (%)	
		ADP	Coll	ADP	Coll
Control	PB.	46.9 ± 4.1	45.7 ± 5.7		
Shikimic acid	0.50	28.9 ± 4.5 *	30.9 ± 3.3 *	38.4	32.3
	1.00	22.3 ± 3.7**	22.1 ± 6.4**	52.5	51.5
	2.00	20.1 ± 2.6**	14.5 ± 3.8**	57.1	68.3
	4.00	11.9 ± 3.2**	9.2 ± 2.0**	74.6	79.9

PB: Phosphate Buffer, used as control; ADP: Adenosinediphosphate; Coll: Collagen. Compared with controls, * P<0.05, ** P<0.01 (Analysis of variance, followed by Games-Howell comparison).

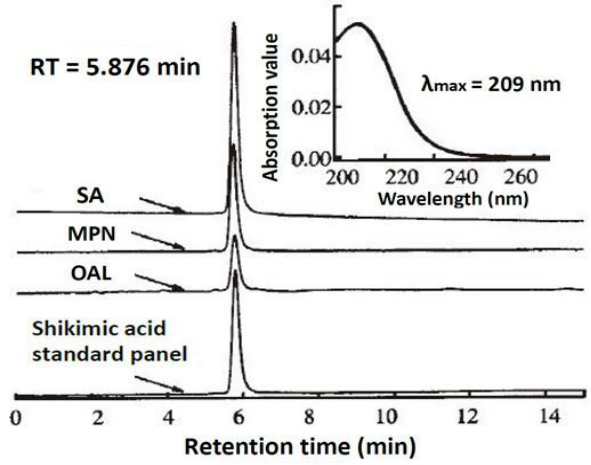

FIG. 2. THE HPLC SEPARATION AND ULTRAVIOLET ABSORPTION SPECTRA OF SHIKIMIC ACID FROM MASSON PINE NEEDLES (MPN), ORIENTAL ARBORVITAE LEAFYTWIGS (OAL) AND STAR ANISE (SA).

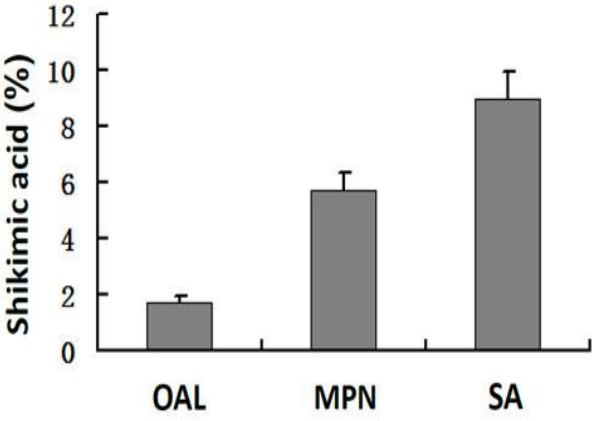

FIG. 3. THE COMPARISION OF SHIKIMIC ACID CONTENT FROM MASSON PINE NEEDLES (MPN), ORIENTAL ARBORVITAE LEAFYTWIGS (OAL) AND STAR ANISE (SA).

Quantification of Shikimic Acid in Three Kinds of Plants

The HPLC separation spectra, the ultraviolet absorption spectra of shikimic acid from Masson pine needles, Oriental arborvitae leafytwigs and Star anise are shown in Fig. 2. When 3.5 mmol/L of phosphoric acid was the mobile phase, the retention time of shikimic acid was 5.876 min. All of the absorption peaks appeared at this point for the three species. Similar to the shikimic acid standard, shikimic acid from Masson pine needles, Oriental arborvitae leafytwigs and Star anise showed the maximum absorption peak at 209 nm. The area of the absorption peak was calculated. The shikimic acid content of Masson pine needles, Oriental arborvitae leafytwigs and Star anise was 5.71%, 8.95% and 1.74%, respectively (Fig. 3).

Antiplatelet-aggregating Activity of Masson Pine Needles

The vitro effects of shikimic acid from Masson pine needles on the platelet aggregation and platelet aggregation inhibition rates in rabbits were analyzed. Platelet aggregation was induced by the addition of ADP and collagen (Table 1). As the content of SA from Masson pine needles increased, the platelet aggregation rate decreased (r=0.966), whereas, the platelet aggregation inhibition rate increased (r=0.980); there were differences in statistical significance, compared to the normal control (P<0.01). Thus, shikimic acid from Masson pine needles inhibited platelet aggregation induced by ADP and collagen in a dose-dependent manner.

Conclusions and Suggestions

Conditions for Shikimic Acid separation and Purification

The difference between the extraction efficiency of ultrasonic treatment and reflux at 90℃ was not significant if the sample was fully smashed. The optimal extraction time with the two methods was 30-60 min. A prolonged extraction time causes the loss of shikimic acid and the increases of related resource consumption. In our experiments, the HPLC column was eluted with methanol for 30 min before and after each analysis. Low concentration phosphoric acid (3.5 mmol/L) as the mobile phase prevented damage from the fillers of the column, while ensuring the best

separation effect. It has been reported that the maximum ultraviolet absorption wavelength has some changes for shikimic acid in different molecular state (Zelaya *et al*., 2011). We used a diode-array detector to detect the absorption spectra. The maximum ultraviolet absorption wavelength of shikimic acid in phosphoric acid solution (3.5 mmol/L) was 209 nm. Under these conditions, the absorption peak shape of shikimic acid was symmetric and the detection sensitivity of shikimic acid was high.

Effects of Anti-coagulation from Masson Pine Needles

Our research showed that the shikimic acid extraction from Masson pine needles significantly inhibited platelet aggregation, which is suggestion of an anti-thrombotic effect. Recent studies have shown that the Masson pine needles contain rich nutrients and many bioactive substances, such as flavonoids, hormones, and terpenoids. In addition to the anti-oxidant and anti-inflammatory effects, the extract of Masson pine needles can also inhibit thrombotic formation in arteries and veins, hence it can prevent cerebral thrombosis (Ma *et al*., 1999; Kong, Liu and Ding, 1995; Kwak, Moon and Lee, 2006). Therefore, Masson pine needles can serve as a candidate material for developing anti-thrombotic drugs or functional foods with anti-thrombotic effects.

Prospect of Shikimic Acid Production from Masson Pine

The shikimic acid content in Masson pine needles was 5.71%, which is slightly lower than the content of star anise; however, star anise has a short harvest season and the yield is low. Thus, star anise cannot supply sufficient raw materials for the production of shikimic acid, but Masson pine needles is extensively grown in central and south China (Xu *et al*., 2012). Masson pine needles have a rapid growth speed, and a large amount of young-aged trees, so they can provide sufficient needle resources. In addition to easy availability and higher shikimic acid content of needles, shikimic acid extraction from Masson pine needles is also less expensive. Masson pine needles offer a good solution to the deficiency of raw materials for shikimic acid production, and their development prospects are bright.

ACKNOWLEDGMENT

The research was funded by the Science and Technology Planning Project of Guangdong Province, China (83047 to C.X.); Collaborative Innovation Center for Environmental Toxicity (L.Y.)

REFERENCES

Foye WO., Wang X., and Wang H. "Synthesis and platelet aggregationinhibitory effects of harman and phthalide derivatives related to ligusticum chuanxion (hort) conatituents". Med Chem Res 7 (1997): 180-191.

Herrmann KM. "The shikinmte pathway: early steps in the biosynthesis of aromatic compounds". Plant Cell 7 (1995): 907-919.

Huang F., Xiu Q., Sun J., and Hong E. "Anti-platelet and anti-thrombotic effects of triacetylshikimic acid in rats". J Cardiovasc Pharmacol 39 (2002): 262-270.

Kong Z., Liu Z., and Ding B. "Study on the antimutagenic effect of pine needle extract". Mutat Res 347 (1995): 101-104.

Krämer M., Bongaerts J., Bovenberg R., Kremer S., Müller U., Orf S., Wubbolts M., and Raeven L. "Metabolic engineering for microbial production of shikimic acid". Metab Eng 5 (2003): 277-283.

Kwak CS., Moon SC., and Lee MS. "Antioxidant, antimutagenic, and antitumor effects of pine needles (Pinus densiflora)". Nutr Cancer 56 (2006): 162-171.

Li G., Sun Z., Xia L., Shi J., Liu Y., Suo Y., and You J. "Supercritical CO(2) oil extraction from Chinese star anise seed and simultaneous compositional analysis using HPLC by fluorescence detection and online atmospheric CI-MS identification". J Sci Food Agric 90 (2010): 1905-1913.

Liu X., Lin J., Hu H., Zhou B., and Zhu B. "Metabolic engineering of Escherichia coli to enhance shikimic acid production from sorbitol". World J Microbiol Biotechnol 30 (2014): 2543-2550.

Ma Y., Xu QP., Sun JN., Bai LM., Guo YJ., and Niu JZ. "Antagonistic effects of shikimic acid against focal cerebral ischemia injury in rats subjected to middle cerebral artery thrombosis". Acta Pharmacol Sin 20 (1999): 701-704.

Pittard J., and Wallace BJ. "Gene controlling the uptake of shikimic acid by Escherichia coli". J Bacteriol 92 (1966): 1070-1075.

Xu Y., Zhang Y., Li Y., Li G., Liu D., Zhao M., and Cai N.

"Growth promotion of yunnan pine early seedlings in response to foliar application of IAA and IBA". Int J Mol Sci 13 (2012): 6507-6520.

Zelaya IA., Anderson JA., Owen MD., and Landes RD. "Evaluation of spectrophotometric and HPLC methods for shikimic acid determination in plants: models in glyphosate-resistant and -susceptible crops". J Agric Food Chem 59 (2011): 2202-2212.