Supplementary Data

Optimization studies for *in vitro* enzymatic hydrolysis

Protocol:

Lentil protein extract was hydrolysed having regard to the assay described in Gonzáles-Montoya *et al*. (2018) with minor modifications based on the optimization study. A Box-Behnken design was generated and conducted with three factors; which were pepsin concentration (X_1) and pancreatin concentration (X_2) ranging for each between (4-10 % w/w protein basis) and duration of gastric digestion (X_3) ranging between 1-2 h for optimization. The response was determined as soluble protein content which was expressed as g soluble protein per g of protein extract (mg/g). Total of 17 experiments was conducted with five center points for optimization experiments. Hydrolysation was stopped in an ice bath at for 10 min. The hydrolysate was centrifuged at 4500 g for 20 min at 4ºC. The supernatant was collected and stored at -20ºC until further analysis. Design Expert Version 11 was used for the statistical experimental design for all the *in vitro* enzymatic hydrolysis experiments with the response as soluble protein content (g soluble protein/g of protein extract). The results were considered statistically significant for *P* values less than 0.05.

Supplementary Tables

Table S1. Box-Behnken design used in the optimization of *in vitro* enzymatic hydrolysis assay concerning the degree of hydrolysis (DH) expressed as soluble protein amount (mg soluble protein per g of protein extract). Optimization of *in vitro* enzymatic hydrolysis was performed according to the Box-Behnken experimental design given with details in the protocol. The concentration of enzymes and duration of gastric phase were chosen as factors important for adequate hydrolysis. The actual levels of these variables and the response were tabulated in Table S1. According to the results, maximum soluble protein (92.4 mg/g) was

obtained at 7% and 4% of pepsin and pancreatin concentration respectively, with 2-hour gastric phase.

	Actual level of variables			Response variable
Run no	**Pepsin concent. (%)**	**Pancreatin concent. (%)**	**Duration of gastric phase (h)**	**Soluble protein amount (mg/g)**
1	7	4	1	68.7
2	7	7	1.5	71.2
3	10	10	1.5	77.0
4	10	7	1	74.8
5	7	10	1	67.1
6	4	7	2	82.7
7	7	7	1.5	73.5
8	10	7	2	88.5
9	4	10	1.5	85.6
10	7	10	2	87.9
11	7	4	2	92.4
12	10	4	1.5	78.8
13	7	7	1.5	73.9
14	4	7	1	82.1
15	7	7	1.5	70.0
16	4	4	1.5	76.8
17	7	7	1.5	70.1

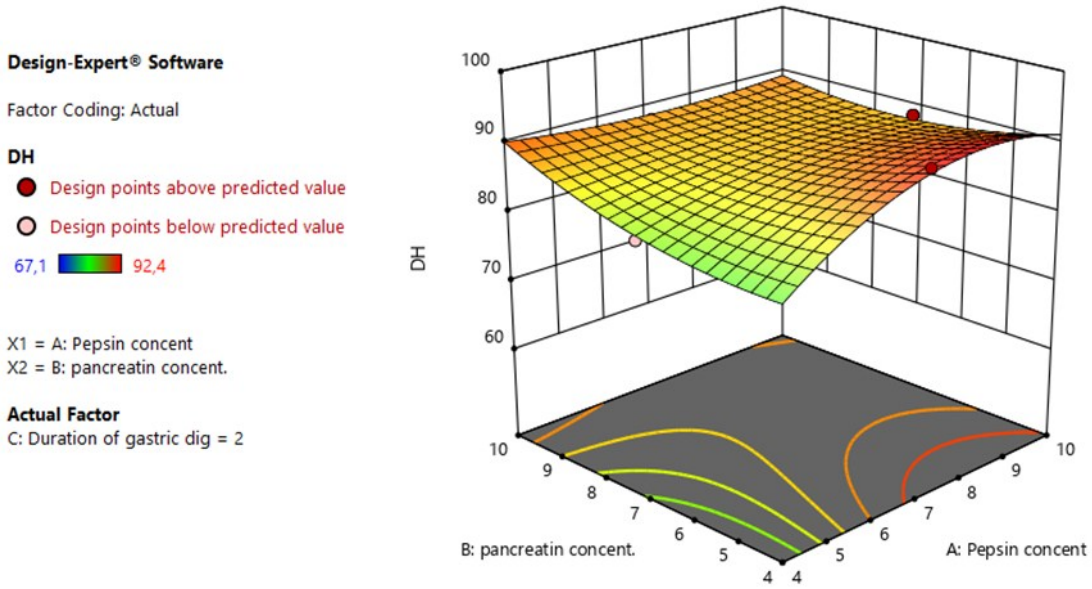
Table S2. Analysis of variance (ANOVA) for the reduced cubic model to determine optimum conditions for *in vitro* enzymatic hydrolysis assay. The optimization results were discussed below, according to the results of the ANOVA presented in Table S2. Since there were some insignificant terms, the full model was reduced by eliminating these terms and re-evaluated. The p-value of the model was 0.0003, which indicated that the constructed model was significant ($p < 0.01$) and P-values less than 0.05 indicated model terms were significant. According to ANOVA duration of gastric phase, the interactions between enzyme concentrations and between pepsin concentration and duration were significant model terms. According to p-values, although concentrations of pepsin and pancreatin were not significant factors whereas some of their interactions were significant, they remained in the model because of the hierarchy principle. The Lack of Fit F-value of 0,76 implies the Lack of Fit is not significant relative to the pure error. Non-significant lack of fit is good because it is desired the model to fit.

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**p-value**	
Model	921.8	10	92.13	28.40	0.0003	*Significant*
X_1	8.20	1	8.20	2.53	0.1629	
X_2	9.30	1	9.30	2.87	0.1413	
X_3	495.6	1	495.6	152.63	< 0.0001	
X_{12}	28.09	1	28.09	8.66	0.0259	
X_{13}	42.90	1	42.90	13.23	0.0109	
X_1^2	123.5	1	123.5	38.06	0.0008	
X_2^2	24.56	1	24.56	7.57	0.0332	
X_3^2	100.6	1	100.6	31.04	0.0014	
$X_1^2X_2$	21.45	1	21.45	6.61	0.0422	
$X_1^2X_3$	114.0	1	114.0	35.15	0.0010	
Residual	0.1946	6	3.24			
Lack of Fit	0.0535	2	2.68	0.7590	0.5255	*not significant*

Pure Error	0.1411	4	3.53		
Cor Total	940.73	16			
Std. Dev.	1.80			R-Squared	0.9793
Mean	77.71			Adj R-Squared	0.9448
C.V. %	2.32			Pred R-Squared	0.7196
				Adeq Precision	17.464

Supplementary Figures

A)



B)

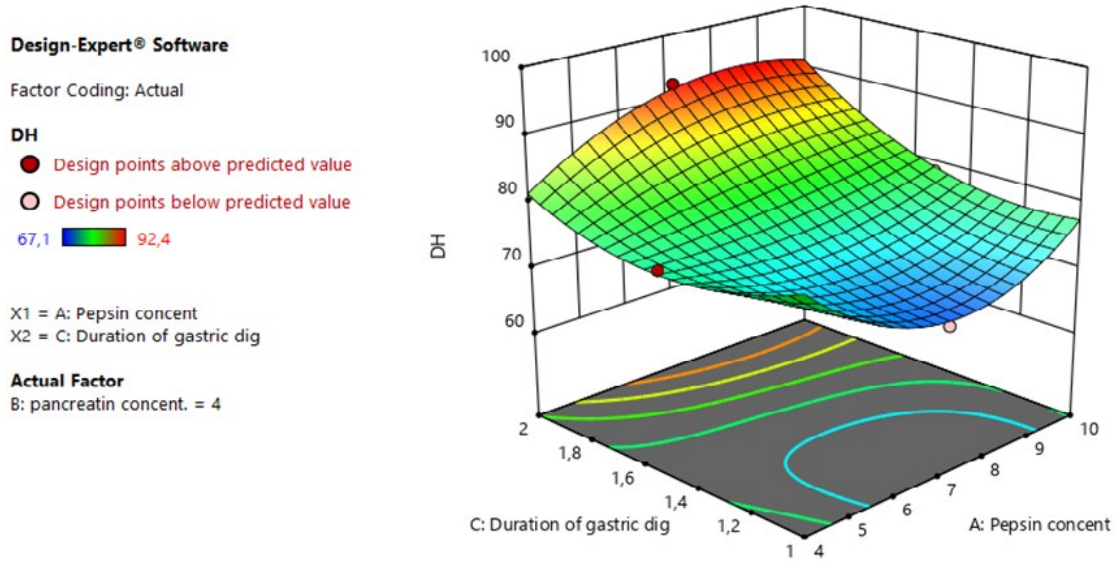
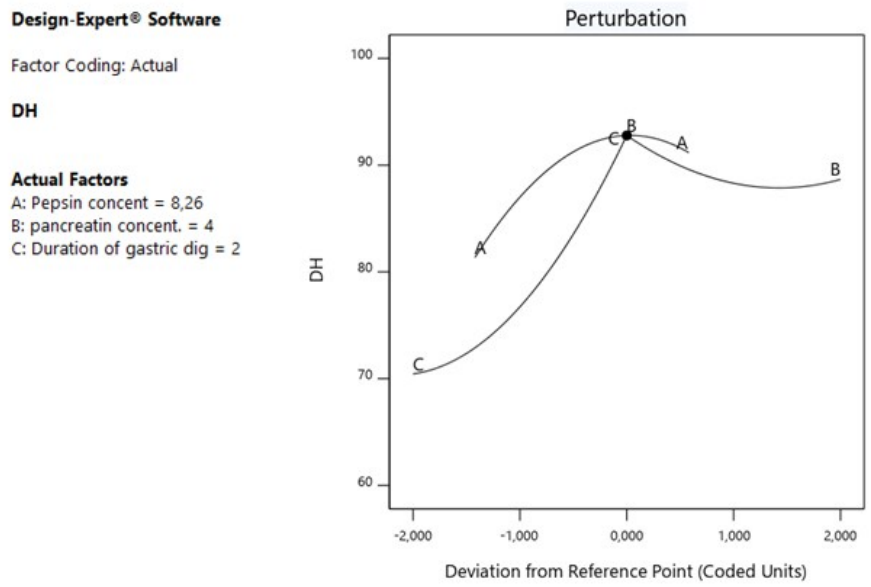


Figure S1. 3D response surface plots showing A) the interaction of pepsin and pancreatin concentration, B) the interaction of duration of gastric phase (digestion) and pepsin concentration with the response (degree of hydrolysis; DH) as soluble protein content (g soluble protein/g of protein extract). A variety of graphs were examined to optimize and fix the

conditions (Fig.S1). It was observed through 3D response surface plots that high amount of pepsin and low amount of pancreatin (Fig.S1A) with 2-hour incubation in the gastric phase (Fig.S1B) led to effective digestion.

A)



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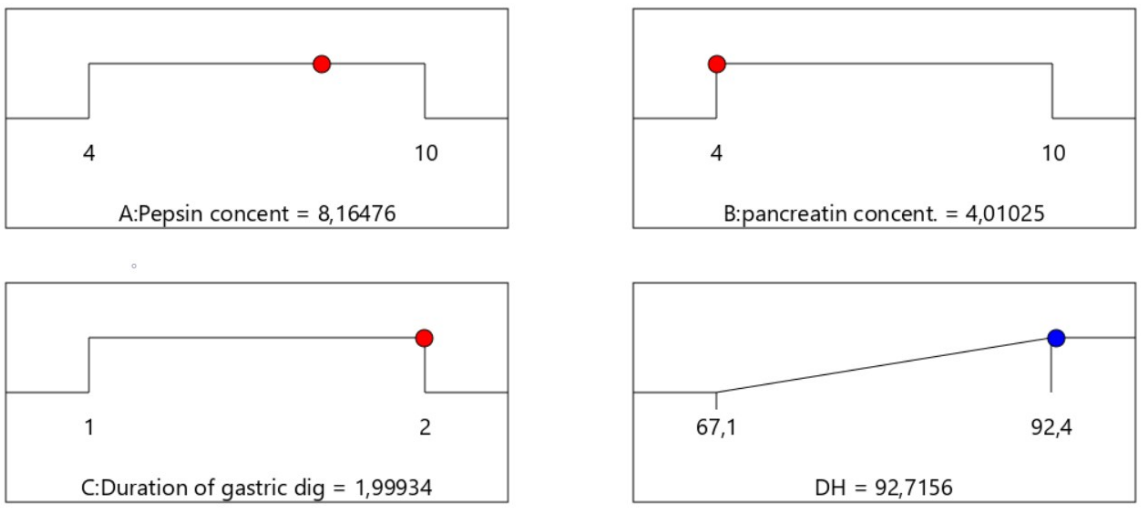

Figure S2. Optimization with maximizing the response A) perturbation plot showing the interaction between enzyme concentrations and duration of gastric phase; B) numerical optimization. The response (degree of hydrolysis; DH) was expressed as soluble protein content (g soluble protein/g of protein extract). In order to fix the hydrolysis parameters, numerical optimization was applied with maximizing the response (Fig.S2). Ramps graphs (Fig.S2A) presented a good visual of the best factor settings with the highest overall desirability of the predicted response. Analyses and graphs predicted higher hydrolysis efficiency with high desirability if the conditions were set as 8% pepsin concentration, 4% pancreatin concentration with 2-h gastric incubation (Fig.S2B). The adequacy of the model equation and a total of three verification experiments were carried out at the predicted optimum conditions. The model was validated (data not shown) and the conditions were fixed as 8% pepsin (w/w, protein basis), 4% pancreatin (w/w, protein basis) with 2-h gastric incubation.