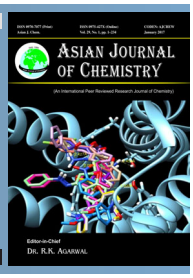
Asian Journal of Chemistry; Vol. 29, No. 2 (2017), 385-392

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2017.20214



Determination and Optimization of Vitamin B Complex (B_1, B_2, B_3 and B_6) in Cellulase Treated Polished Rice by HPLC with UV Detector

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Received: 25 July 2016; *Accepted*: 17 October 2016; *Published online*: 30 November 2016; AJC-18167

A novel processing technology is developed to polish rice in a more selective way with the help of cellulase enzyme of microbial sources. The enzyme were produced from *Trichoderma reese*, MTCC164. Brown rice was treated with enzyme concentration of 60-100 % (40 mL of buffer-undiluted) for 30 to 150 min (with variation of 30 min) at 30 to 50 °C (with variation of 5 °C) to attain a saturated moisture level. Selective degradation of bran layers in enzymatic polished rice (polishing time 20-100 s with variation of 20 s) has facilitated the retention of more thiamine, riboflavin, pyridoxine and niacin nutrients compared to normal milled rice. The retention of vitamin B complex nutrients detected through HPLC with UV detector and optimized by response surface methodology (RSM) with central composite design. The optimized enzyme treated bio-polished rice better sources of thiamine (51 %), riboflavin (48 %), pyridoxine (90 %) and niacin (54 %) concentration over mechanically milled rice.

Keywords: HPLC, Cellulase, Vitamins, Brown rice, Central composite design.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the commonly cereal grains. It ranks as the most widely grown food grain crop and serves as the staple food for almost half of the world's population. Brown rice, which is hulled directly from rough rice, consists of a bran layer (6-7 % of its total weight), embryo (2-3 %) and endosperm (about 90 %) [1]. It contains more nutritional components, such as proteins, lipids, dietary fibres, vitamins B and minerals, than white rice. These nutrients exist mainly in the germ and bran layers of the rice grains, but the germ and bran layers are almost removed during the milling process from brown rice to white rice, which is primarily consumed. Brown rice has lost its appeal due to its dark appearance and poor cooking quality [2,3]. The tough fibrous bran layer of brown rice imparts hard texture and chewiness of the cooked grain. According to nutritionists, brown rice should be preferred because of its high nutritional value. Removal of bran layers in the milling process improves the appearance, cooking quality and palatability of rice though major loss of nutrients and high percentage of brokenness results during mechanical milling. Therefore, producing rice, with minimum breakage, retaining the maximum possible nutrients of brown rice.

Vitamins in food are essential for normal development of body functions and so the absence of vitamins can cause serious physiological problems [4,5]. In foodstuffs, the vitamins B_1, B_2, B_3 and B_6 may be present in free (thiamin, ribofavin, niacin, pyridoxol, pyridoxal and pyridoxamine) and phosphorylated forms (essentially thiamin pyrophosphate, ribofavin-50-phosphate (FMN), riboflavin-50-adenosyldiphosphate (FAD) and pyridoxal phosphate). Furthermore, they may be bound tightly but non-covalently to proteins. Only a very small portion of the FAD is thought to be bound covalently to amino acids in mammalian tissues [4]. In plant tissues, an often considerable percentage of pyridoxol may also be linked to sugars to form conjugate glucosides. Pyridoxol glucosides, just like covalently bound FAD, are vitamin forms of very low bioavailability in man [4].

High performance liquid chromatography with fluorescence detection has been widely used for the determination of thiamine and riboflavin in foodstuffs including cereals and pulses for more than 20 years. As previously mentioned,

different techniques were optimized and published to determine thiamine, riboflavin and pyridoxine in cereals and their derivatives. All of them relied on univariate approaches in order to obtain the better resolution of vitamin B complex. If the fact that univariate methods are often time consuming, it would be advisable to use a multivariate chemometric technique to optimize thiamine by HPLC. Some works have shown a successful determination and optimization riboflavin and thiamine vitamins by HPLC method using of response surface methodology [6-8].

The amount of fibre (non-starch polysaccharides, NSPs) is quite considerable in case of rice bran. The major polysaccharides present in bran are crude cellulose (9.6 g/100 g to 2.8 g/100 g dry basis) and hemicellulose and pentosans (8.6 g/100 g to 10.9 g/100 g dry basis), which constitute the major part of the insoluble dietary fibre. The complex polyphenolic polymer known as lignin (7.7 g/100 g to 12.0 g/100 g dry basis) interacts with the cellulose fibrils creating a rigid structure. The xylan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose imparts the resistant nature of the bran layer [9,10]. Among microorganisms known for their ability to produce plant cell wall polysaccharide degrading enzymes, fungi are the most interesting group. *Trichoderma reesei* Rut C-30 is also known to be one of the best hyper-producing cellulolytic fungal strains, which makes it an ideal test organism for cellulase production. All the components of enzyme system act in a synergism for complete hydrolysis of cellulose [11-13].

The aim of the present study was to optimize and validate a B-complex vitamins: vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_3 (nicotinamide) and vitamin B_6 (pyridoxine) by high performance liquid chromatography with UV detector in bio polished rice through application of fungal enzyme such as cellulases for selective degradation of rice bran polysaccharides giving rise to softer, nutritious bio-polished rice, using a CCRD experimental design has been a novel attempt made in the present work.

EXPERIMENTAL

Freshly harvested, Pant Sugandha dhan, long and slender variety of paddy has been procured from CRC in pantnager, India. Paddy was harvested in October 2013. After 24 h from harvesting, it was sun-dried to about 14 g/100 g moisture content (dry basis) and was stored in airtight containers for 30 days at ambient temperature (30 ± 2 °C). Paddy was dehulled and broken grains were removed using laboratory grader and head brown rice was used for further experimentation. Brown rice was polished in abrasive polisher for 20-100 s with DOM ≥ 10 % to get fully milled white rice. The enzymatically polished rice was also dried to same moisture level (14 g/100 g of grain). Samples of brown rice, enzymatically polished rice and fully milled rice were packed in plastic containers and kept at ambient temperature (30 ± 2 °C).

Chromatographic conditions: A PU-2080 plus chromatography pump (JASCO, Tokyo, Japan) equipped with a UV-2075 plus ultraviolet detector (JASCO) was used. The HPLC column was a CAPCELL PAK C18MG II (250 mm × 4.6 mm i.d., Shiseido, Tokyo, Japan). This column is provided with packing material made of totally porous spherical silica coated with a silicone polymer monolayer with octadecyl (C18) groups. Solvent used for mobile phase has (buffer/methanol 70:30, 5 mm sodium salt of hexane sulphonic acid as buffer) with a flow rate of 1 mL per min under pressure 77 bars at 27 °C. Sample injections at the rate of 20 µL with column effluent being monitored at 254 nm wavelength [13]. A wash-step with 100 % mobile phase can be initiated optionally after each sample. Data acquisition was done with Varian Star Software.

Vitamin standards (thiamine-HCl, riboflavin, nicotinic acid and pyridoxine-HCL) and methanol (HPLC grade) for chromatographic analysis were obtained from Sigma-Aldrich (rudrapur, pantnager, India). Glacial acetic acid, calcium carbonate and hexane sulphonic acid sodium salt (analytical grade) were obtained from Sigma-Aldrich (Rudrapur, Pantnager, India). Other reagents used were of the highest purity available. Aqueous solutions were prepared with deionized water.

Enzyme preparation: Cellulase (endoglucanase) enzyme was produced through solid-state fermentation by growing *Trichoderma reese*, MTCC164 Ocimum gratissimum seeds and sawdust [13]. The optimized endo-glucanase activity was 21 IU/mL, whereas cellobiohydrolase and b-glucosidase activities of the same crude extract of 6-day-old fermented culture were 0.23 IU/mL and 0.15 IU/mL, respectively. The crude enzymes were centrifuged at 10,000 rpm and supernatant enzymes were concentrated through ultra filtration unit of 10 kDa cut-off membrane. The endoglucanase activity was determined according to [14], using 1 % Na-carboxymethyl cellulose (CMC). The amount of reducing sugars released was determined using dinitrosalicylic reagent [15]. The activity was expressed in International Units (IU). Cellobiohydrolase (CBH) and b-glucosidase (BG) were assayed in a reaction mixture (1 mL) containing 5 mM *p*-nitrophenol, b-D cellobioside and 5 mM *p*-nitrophenol b-D glucoside, respectively, 50 mmol/L acetate buffer, pH 5.0 and appropriately diluted enzyme solutions [16].

Selection of enzyme dilutions for brown rice treatment: Concentrated crude preparations of cellulase enzymes were taken and serial dilutions were made in acetate buffer at pH 5.0. These dilutions of cellulase enzyme were having in the ratio 100 % (undiluted), 90 % (90 mL undiluted enzyme + 10 mL buffer), 80 % (80 mL undiluted enzyme + 20 mL buffer) and 70 % (70 mL undiluted enzyme + 30 mL buffer), 60 % (60 mL undiluted enzyme + 40 mL buffer). Enzyme activities of the enzymes were expressed in International Units (IU). One IU was defined as one µmol. After preparation of dilution of enzymes should be pre-treatment to the brown rice [17].

Enzymatic pre-treatment of brown rice: Brown rice grain (100 g) was soaked in 50 mL water for 24 h. Water was changed at regular intervals for minimizing bacterial contamination. Rice grain was soaked for another 1 h in 50 mL fresh water with 2.5 g of calcium carbonate at 60 °C (10 °C below gelatinization temperature of the rice grain) to saturate the grains to a moisture content of 37-39 %. The absorbed calcium ions acted as an inducer for the enzyme activity [18]. Rice grain was strained out and washed in water to remove excess calcium carbonate and then with 150 mL of cellulase enzyme solution prepared from different concentration in the ratio 100 % (undiluted), 90 % (90mL undiluted enzyme +

10 mL buffer), 80 % (80 mL undiluted enzyme + 20 mL buffer) and 70 % (70 mL undiluted enzyme + 30 mL buffer), 60 % (60mL undiluted enzyme + 40 mL buffer) respectively at different temperatures 30, 35, 40, 45 and 50 °C for different time 30-150 min (30, 60, 90, 120,150 min). Rice grain was strained out and washed to remove the enzymes [13].

Degree of rice polishing: For polishing the retreated samples at different time of 20 s interval (20, 40, 60, 80 and 100 s) respectively by using abrasive type Satake laboratory polisher. The bran adhering to the polished white rice was removed by sieving. After weighing the white rice and the bran their percentages were calculated. The whole kernels were separated from the brokens by sizing of length [13].

Extraction procedure: After polishing of cellulase enzyme treated rice having different lots was analyzed. Rice samples (10 g) were cooked in deionized water (20 mL) with 20-25 min, after proper cooking of sample to make paste by piston mortar. The paste of cooked rice (0.5 g) dissolved in 2 mL of mobile phase in centrifugation tube. the mixture was homonized in centrifuge with 6000 rpm at 15 to 20 min. The supernatant was taken and filtered through Millipore membrane filter and was used for chromatographic separation [13].

Preparation of standard solutions: All standard solutions were prepared with 0.5 % hexane sulphonic acid double-distilled water as buffer. A preliminary standard of four water-soluble vitamins was created by weighing out 10 mg of each thiamine, pyridoxine, niacin and riboflavin into a 25 mL volume flask. The flask was brought up to 25 mL by adding 5 mL of buffer solution. Then properly vortexed to ensure proper dissolution, after which the flask was made up to 25 mL with buffer and filtered through Millipore membrane filter and was used for chromatographic separation [13].

Optimization procedure: A central composite rotatable design (CCRD) using four factors at five levels (coded levels –2, 0 and 2) was used for optimization of a B-complex vitamins: vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_3 (nicotinamide) and vitamin B_6 (pyridoxine) determination in enzyme treated rice by HPLC. CCRD consisted of 30 experiments (chromatographic runs) under different conditions. Five replicates in the central point were included in order to estimate the experimental error. Factors and its levels were selected taking into account previous literature on this topic where evolution vitamin content in enzymatic treatment of brown rice and milled rice by HPLC [13,17,19-21]. The independent variables levels are coded for experimental design. The main advantage of the design is that it enables the study of one or more variables simultaneously in a single experimental design of practical size [22,23]. Experimental data were analyzed using design expert 8.0.6 statically software and the second-order polynomial model predicted for optimization of dependent variables (Y) was:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} A.B + \beta_{13} A.C + \beta_{14} A.D + \beta_{23} B.C + \beta_{24} B.D + \beta_{34} C.D + \varepsilon \quad (1)$$

where by β_0 (constant), β_1, β_2, β_3, β_4 (coefficients for linear effects); β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} (coefficients for interaction effects); β_{11}, β_{22}, β_{33}, β_{44} (coefficients for quadratic effects); and ε (random error).The response surfaces and contour plots for these models were plotted as a function of two variables, while keeping the other variable at the optimum level [24]. Using the presented experimental design, each experiment was conducted by making duplicate injections of the extracted vitamin B complex such as vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_3 (nicotinamide) and vitamin B_6 (pyridoxine).

RESULTS AND DISCUSSION

In recent years, chemometric tools have been frequently applied to the optimization of different analytical methods considering their advantages such as reduction in the number of experiments resulting in lower reagent consumption and considerably less laboratory work [6,7,19]. A five levels central composite rotatable factorial design was used for the optimization of concentrated vitamin B complex: vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_3 (nicotinamide) and vitamin B_6 (pyridoxine) in enzymatic treated rice. The kind of response surface methodology (RSM) uses second-order polynomial models to describe responses in the experimental design (Table-1). The experimental data of all the 30 test points of enzyme treated polished rice are shown in Table-2.

TABLE-1
FINAL EQUATIONS IN TERMS OF CODED FACTORS

Response	Equation			
Y_1	0.19-7.917E-003 A+2.500E-004 B-2.417E-003C+7.417E-003D-4.958E-003A^2-8.208E-003B^2-0.014C^2-5.833E-004D^2-8.375E-003A B-5.875E-003A C-7.500E-004AD-6.875E-003B C-5.250E-003BD+5.000E-004CD R^2adj. = 0.8314	(2)	R^2 = 0.8732	F = 7.38
Y_2	1.90+0.013A+0.028B+0.036 C+0.011D-0.029A^2-0.045B^2-0.085C^2-0.038 D^2-0.036AB-0.016A C+0.013AD-0.067BC-0.063 B D+5.813E-003CD R^2adj. = 0.7866	(3)	R^2 = 0.8896	F = 8.63
Y_3	0.044+5.625E-003A-7.625E-003 B+2.917E-004C+6.792E-003D+9.062E-004A^2 + 1.562E-004B^2-1.469E-003 C^2+7.812E004$D^2$1.438E003AB+1.812E-003A C-1.938E-003AD+2.688E-003B C-1.312E-003B D-1.062E-003CD R^2adj. = 0.8179	(4)	R^2 = 0.8644	F = 6.83
Y_4	1.40-0.024A-0.023B+0.026C+0.060D+0.049A^2+0.057B^2+0.041C^2+0.028D^2-8.250E-003AB+3.625E-003AC-7.500E-003A D-2.375E-003B C+1.000E-003BD+1.250E-004D R^2adj. = 0.7981	(5)	R^2 = 0.8781	F = 7.72

Y_1 = Thiamine, Y_2 = Niacin, Y_3 = Riboflavin, Y_3 = Pyridoxine; R^2 = Coefficient of determination; Adj R^2 = Adjusted; R^2Pred = Predicted.

TABLE-2
EXPERIMENTAL CONDITIONS AND RESPONSE VALUES OBTAINED FOR EACH OF THE 30 TEST POINTS OF THE CENTRAL COMPOSITE DESIGN

Run	Actual values				Dependent variables			
	Enzyme concentration (%)	Treatment time (s)	Polishing time (s)	Treatment temp. (°C)	Thiamine (mg/100 g)	Niacin (mg/100 g)	Riboflavin (mg/100 g)	Pyridoxine (mg/100 g)
					Y_1	Y_2	Y_3	Y_4
1	70	60	40	35	0.0362	1.4231	0.0362	1.4986
2	90	60	40	35	0.0562	1.5352	0.0562	1.5025
3	70	120	40	35	0.0293	1.8453	0.0293	1.5112
4	90	120	40	35	0.0324	1.7571	0.0324	1.4362
5	70	60	80	35	0.0432	1.5991	0.0432	1.6722
6	90	60	80	35	0.0511	1.6681	0.0511	1.4872
7	70	120	80	35	0.0254	1.7123	0.0254	1.4782
8	90	120	80	35	0.04456	1.6723	0.04456	1.5564
9	70	60	40	45	0.0642	1.5583	0.0642	1.6347
10	90	60	40	45	0.0515	1.6364	0.0515	1.623
11	70	120	40	45	0.0446	1.6825	0.0446	1.6727
12	90	120	40	45	0.0347	1.7636	0.0347	1.4873
13	70	60	80	45	0.0517	1.7853	0.0517	1.6728
14	90	60	80	45	0.0523	1.8724	0.0523	1.6772
15	70	120	80	45	0.0415	1.6754	0.0415	1.7114
16	90	120	80	45	0.0526	1.5914	0.0526	1.5895
17	60	90	60	40	0.0285	1.7714	0.0285	1.6216
18	100	90	60	40	0.0516	1.6714	0.0516	1.5729
19	80	30	60	40	0.0544	1.7733	0.0544	1.6828
20	80	150	60	40	0.0292	1.7455	0.0292	1.5722
21	80	90	20	40	0.0341	1.4986	0.0341	1.5332
22	80	90	100	40	0.0365	1.6947	0.0365	1.5925
23	80	90	60	30	0.0296	1.7827	0.0296	1.3895
24	80	90	60	50	0.0511	1.7898	0.0511	1.6394
25	80	90	60	40	0.0421	1.7941	0.0421	1.3884
26	80	90	60	40	0.0421	1.7941	0.0421	1.3884
27	80	90	60	40	0.0421	1.7941	0.0421	1.3884
28	80	90	60	40	0.0421	1.7941	0.0421	1.3884
29	80	90	60	40	0.0421	1.7941	0.0421	1.3884
30	80	90	60	40	0.0421	1.7941	0.0421	1.3884

Diagnostic checking of the fitted model: Regression coefficients significant at 95 % levels were selected for developing the models representing the final equations in terms of coded factors. The resulting polynomial, after removing the non-significant terms and all model term values, was calculated and is presented in Table-3.

TABLE-3
ANALYSIS OF VARIANCE (ANOVA) FOR SECOND-ORDER POLYNOMIAL MODEL FITTED TO RESPONSE SURFACE

Response	Source	df [a]	SS	MS	F-value	p
Y_1	Model	14	0.013	8.987E-004	7.38	0.0002*
	Lack-of- fit	10	1.494E-003	1.494E-004	2.14	0.1930**
	Pure error	5	3.333E-004	6.667E-005		
	Residual	15	1.828E-003	1.219E-004		
	Total	29	0.014			
Y_2	Model	14	0.47	0.033	8.63	≤ 0.0001*
	Lack-of- fit	10	0.049	4.944E-003	2.67	0.1208**
	Pure error	5	8.333E-003	1.667E-003		
	Residual	15	0.058	3.852E-003		
	Total	29	0.52			
Y_3	Model	14	3.686E-003	2.633E-004	6.83	0.0003*
	Lack-of- fit	10	4.584E-004	4.584E-005	1.91	0.2462**
	Pure error	5	1.200E-004	2.400E-005		
	Residual	15	5.784E-004	3.856E-005		
	Total	29	4.264E-003			
Y_4	Model	14	0.29	0.021	7.72	0.0002*
	Lack-of- fit	10	0.035	3.544E-003	3.50	0.898**
	Pure error	5	5.070E-003	1.014E-003		
	Residual	15	0.041	2.701E-003		
	Total	29	0.33			

Y_1 = Thiamine, Y_2 = niacin, Y_3 = Riboflavin, Y_3 = Pyridoxine; respectively. *Significant at $P \leq 0.0005$; **Non-significant $P \geq 0.5$; SS: sum of square; MS: mean of square. [a]Degrees of freedom.

Regression analyses showed that thiamine was significantly ($p<.05$) affected by quadratic (x_1^2, x_2^2 and x_4^2) and interaction (x_1x_3, x_1x_4 and x_2x_3) terms only. Riboflavin and pyridoxine were affected by linear term (x_4), interaction (x_2x_4) term and quadratic (x_1^2, x_2^2, x_3^2 and x_4^2) terms. Linear term (x_1), interaction (x_3x_4) term and quadratic terms (x_1^2, x_2^2, x_3^2 and x_4^2) influenced niacin.

Analysis of variance (ANOVA): The goodness-of-fit of the models was evaluated using the correlation coefficient R^2, the R^2 adj. [25], the Fisher F test, as well as the derived P values and the results are presented in Tables 1 and 3, respectively. In addition, significance of the lack of fit term was used to judge adequacy of model fit. Regression models fitted to experimental results showed good correlation coefficients (> 86 %) for all vitamins retention in enzyme treated rice. For a response surface, these correlation coefficients were quite high. Table-3 shows that the F-values for Y_1, Y_2, Y_3 and Y_4 were significant at the 95 % level. However, the lack of fit was not significant for all Y_1, Y_2, Y_3 and Y_4 ($P > 0.05$), while the F-values significant for all Y_1, Y_2, Y_3 and Y_4 it should lower than ($P < 0.05$). Quantitative estimation of major B complex vitamins and analysis of retention time, area and peak in optimum enzyme treated cooked rice are shown in Table-4.

TABLE-4
QUANTITATIVE ESTIMATION OF MAJOR B-VITAMINS IN OPTIMUM ENZYME TREATED COOKED RICE THROUGH HPLC

Vitamin (mg/100 g) in rice	Brown rice	Optimized enzyme treated rice	Polished rice
Thiamine	0.194 ± 0.041	0.177163	0.069 ± 0.021
Niacin	1.945 ± 0.119	1.841420	0.784 ± 0.134
Riboflavin	0.0691 ± 0.011	0.055710	≤ 0.02
Pyridoxine	1.741 ± 0.312	1.558910	0.156 ± 0.032

Thiamine: It is evident from eqn. 2 that Y_1 depends on the three factors. The negative coefficient of linear term (A and D), quadratic terms (A^2, B^2, C^2 and D^2) and interaction terms (AB,AC,AD and BC) decrease Y_1, whereas positive linear terms (B and C) increase Y_1. However among all terms A, D, A^2, B^2, C^2, AB and BC were significant within the level of confidence selected.

Selected thiamine values of the products are reported in Fig. 1 as a function of independent variables and the standard chromatogram. The response surface graphs obtained from this model show that the thiamine concentration were increased by increasing treatment temperature and enzyme concentration frequently but highest retention of thiamine were obtained at 85-90 % enzyme concentration, 40-45 °C treatment temperature, polishing time 30-35 % and treatment time 100-105 min due to the fact that enzyme at optimum level highly acting on brown layer even after cooking because brown rice contain xylan covalently bonded to lignin and non-covalently bonded to cellulose exhibits important role in maintaining cellulose integrity *in situ*, which controlling the overall speed of the xylolytic hydrolysis reaction, exhibiting a crucial effect on the polymer's enzymatic degradation [10,26]. This results less loss of water soluble fractions during cooking and soaking of rice because all nutrients moves to inner to the endosperm. However, further increasing all parameters (except treatment temperature) the thiamine retention should be decreased due to conversion of xylan into monomers which cannot controlled the xylolytic hydrolysis reaction and hemicelluloses integrity which make more losses. The present observations corroborate those reported earlier [9,10].

Niacin: It is evident from eqn. 2 that Y_4 depends on the three factors. The negative coefficient of all quadratic terms (A^2, B^2, C^2 and D^2) and interaction terms (AB, BC and BD) decrease Y_1, whereas positive coefficient of all linear terms (A,B,C and D) and interaction terms (AD and CD) increase Y_1. However among all coefficients all quadratic and linear coefficients significantly affected the niacin retention concentration. The standard retention time of niacin is evaluated by chromatography (Fig. 2).

Niacin retention concentration values in the response surface experiments varied widely between 1.423 and 1.874 mg/100 g. Also during enzymatic treatment process the retention of niacin concentration in enzyme treated rice by HPLC increased by increasing all dependent variables (enzyme concentration, treatment temperature, treatment time and

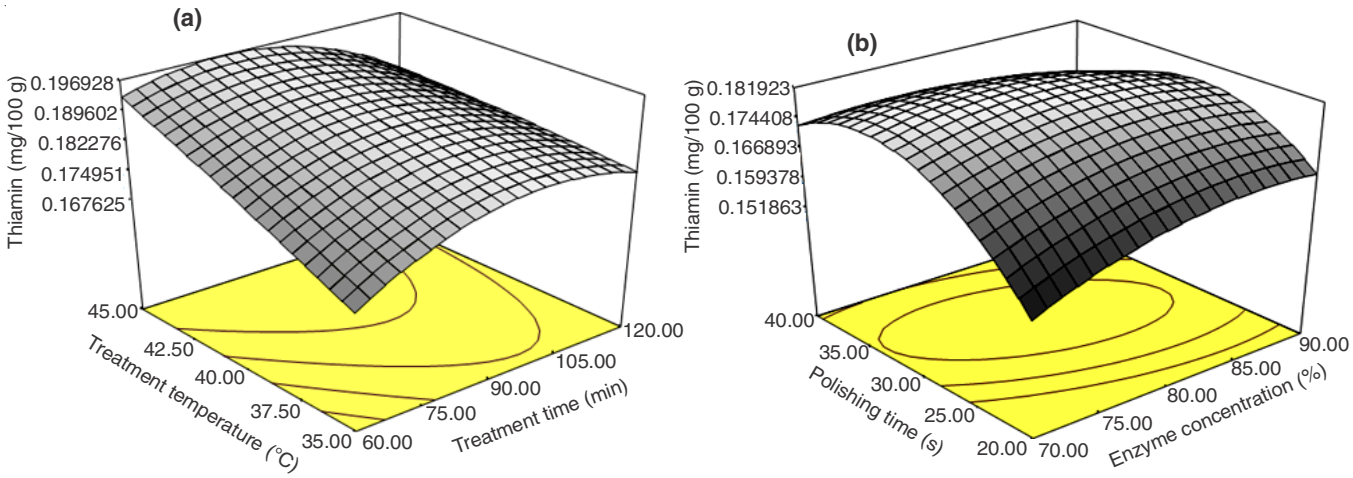

Fig. 1. 3D surface plot for effect of enzymatic treatment parameters on thiamine content a) treatment temperature *vs*. treatment time. b) polishing time *vs*. enzyme concentration

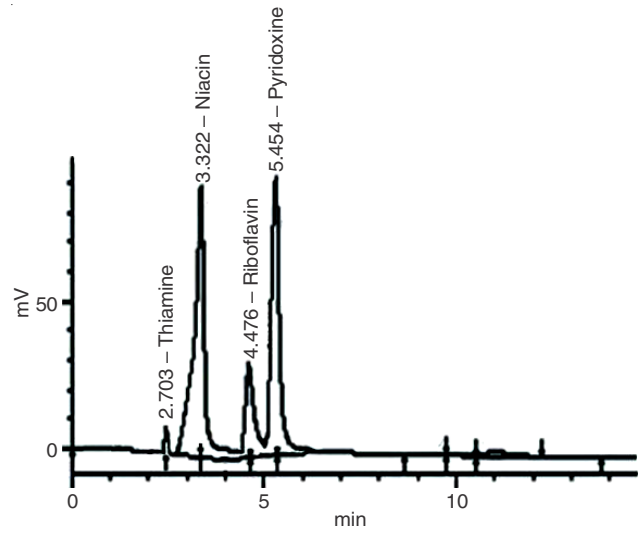

Fig. 2. Typical chromatograms for B complex vitamins (thiamine, niacin, pyridoxine and riboflavin) in optimized enzyme treated rice samples

polishing time) initially thereafter decreased due significantly affect of all quadratic and linear coefficients. Among all dependent variables treatment time and temperature significantly affected. The highest value of niacin retention concentration was obtained at optimum value of 85-90 % enzyme concentration, 40-45 °C treatment temperature, polishing time 20-40 s and treatment time 100-108 min observed in Fig. 3 due to the fact that parallel cellulose and xylonaose chains interact with bran layer through hydrogen bonds and van der Waals forces, resulting in microfibriles, which are very extensive and crystalline aggregates which makes less loss water soluble fractions to outside allure on layer resulting this forces cusses nutrients to inaner side chain of endosperm [27,28].

Riboflavin: The model equation predicting this response is given by eqn. 3. Comparing the magnitude of the coefficients, it is evident from eqn. 3 that the enzyme concentration had a very strong effect on Y_2 as it had the largest quadreiatic coefficient, followed by treatment temperature, treatment time and polishing time. The positive linear coefficients(A, C and D), quadratic coefficients (A^2, B^2 and $D^{2)}$ and (AC and BC) interaction terms contributed to the increase of Y_2, whereas remaining negative linear (B), interaction (AC, BD and CD) and quadratic coefficients (C^2) had a reverse trend on Y_2. However, among all terms only liners coefficients (A, B and D) considered were significant within the level of confidence selected. The standard retention time of riboflavin is evaluated by chromatography (Fig. 2).

Riboflavin increased from (0.025 to 0.71 mg/100 g) as a function of both enzymatic concentration and temperature (Fig. 4). Also, during enzymatic treatment process, higher treatment time and polishing time lead to low retention of riboflavin concentration. Generally, enzyme concentration and temperature increases the riboflavin retention in enzyme treated rice increased frequently due to swollen in action of xylanase which causes uncontrolled of hydrolytic reaction makes breaking of covalent and non-covalent bonds among xylan, lignin and cellulose. this results bran layer become loose and pours structure causes more lose of water soluble riboflavin in cooking and soaking process [29]. However polishing time and treatment time increased retention of riboflavin reverse trend due to development of hard xylolytic layer during long time by partial heating which causes stagnant the water soluble nutrients [30-32].

Pyridoxine: Eqn. 4 shows that on Y_3 positively all quadratic coefficients(A^2, B^2, C^2 and D^2) along with that C and D linear coefficients and (AB, AC, BD and CD) interaction coefficients respectively indicates, to increased Y_3. Negative coefficients of Aand B linear terms AD and BC interaction effects that decreased Y_3. However, all quadratic coefficients considered were significant within the level of confidence selected. The standard retention time of pyridoxine is evaluated by chromatography (Fig. 2).

Fig. 5 shows the selected response surface for pyridoxine against the independent variables. The pyridoxine concentration of all enzyme treated rice ranged from (1.388 to 1.711

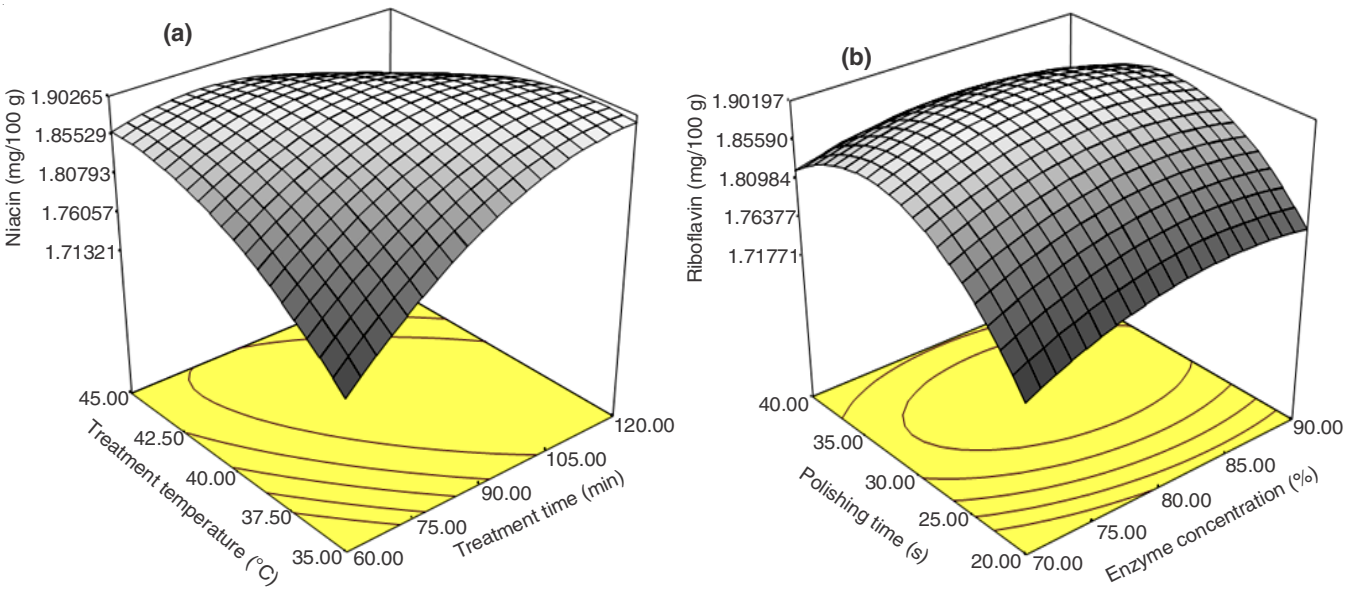

Fig. 3. 3D surface plot for Effect of enzymatic treatment parameters on niacin content: (a) treatment temperature *vs*. treatment time (b) polishing time *vs*. enzyme concentration

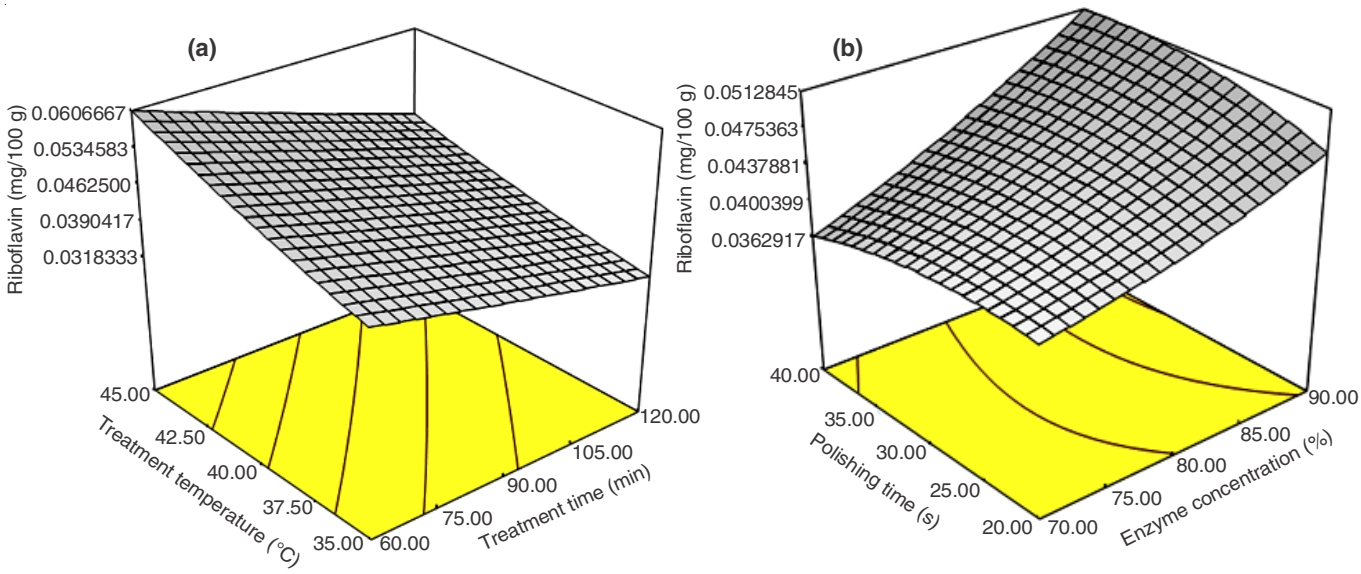

Fig. 4. 3D surface plot for effect of enzymatic treatment parameters on riboflavin: (a) treatment temperature *vs*. treatment time (b) polishing time *vs*. enzyme concentration

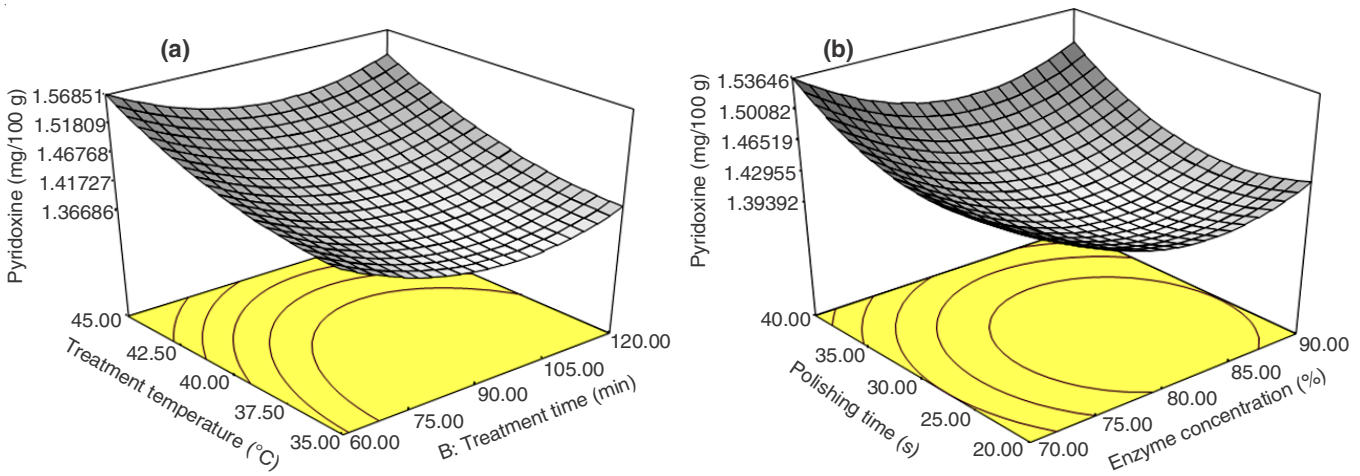

Fig. 5. 3D surface plot for Effect of enzymatic treatment parameters on pyridoxine content: (a) treatment temperature *vs*. treatment time (b) polishing time *vs*. enzyme concentration

mg/100 g). In fact, during enzymatic treatment process, initially the retention of pyridoxine concentration decreased by increasing all parameters (enzyme concentration, treatment temperature, treatment time and polishing time) thereafter increases. Among them treatment temperature significantly affected which continually increased. The reason because due to bonding nature of xylan, lignin and cellulose in bran layer. generally xylan covalently bonded to lignin and non-covalently bonded to cellulose exhibits important role in maintaining integrity of bran layer which will effects during removal of bran layer in polishing time [10,13] demonstrated that by increasing all parameters (enzyme concentration, treatment temperature, treatment time and polishing time) of enzyme treatment process increases retention of pyridoxine concentration initially due to significant of quadratic coefficients.

Conclusion

In this study, the statistical methodology, CCRD, Response surface design is demonstrated to be effective and reliable in finding the optimal conditions for retention of thiamine, riboflavin, pyridoxine and niacin concentration in enzyme treated rice, brown rice and milled rice by HPLC with UV detector. The results showed that the enzyme treatment parameters have significantly effect on retention of vitamins in all rice samples. The second order mathematical model was developed by regression analysis of the experimental data obtained from 30 batch runs. Applying the method of the desirability function, optimization of parameters for enzymatic treatment (enzyme concentration: 90.00 %, treatment temperature: 43.90 °C, treatment time: 91.92 min and polishing time: 75.7 s) obtained maximum retention vitamins concentration during polishing reported in Table-4. It is noteworthy that significant loss of thiamine (65 %), pyridoxine (91 %), niacin (60 %) and riboflavin (> 90 %) was observed due to mechanical milling over brown rice. This study demonstrated selective bio polishing of rice has helped to retain the major vitamins of brown rice even after cooking, with minor loss in thiamine (8.7 %), niacin (5.3 %), pyridoxine (10 %) and riboflavin (21 %) mostly due to the loss of water soluble fractions during soaking of rice. Finally enzyme treated polished rice more

nutritious compared to normal milled rice, which is better method prevention vitamin loss during polishing.

ACKNOWLEDGEMENTS

The financial support received from the Technical Education Improvement Programme-II, Pantagar, College of Technology, G.B. Pant University of Agriculture & Technology is gratefully acknowledged.

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