DOI : 10.5958/0974-0112.2015.00100.0

Indian J. Hort. 72(4), December 2015: 547-552

Optimization of enzymatic maceration for extraction of carotenoids and total phenolics from sweet pepper using response surface methodology

Prerna Nath*, Eldho Varghese and Charanjit Kaur**

ICAR-Division of Food Science and Post-harvest Technology, Indian Agricultural Research Institute, New Delhi 110012

ABSTRACT

Response Surface Methodology (RSM) is used in situations where several input variables potentially influence some performance measure or quality characteristic of a process. In this paper, a three-level, three-factor, Box-Behnken design under response surface methodology (RSM) was used to optimize the conditions of enzyme assisted processing of red capsicum juice. The effects of enzyme concentration (0.2-0.4%), incubation temperature (50-70°C) and time (30-90 min.) on juice yield, total soluble solids, total carotenoids and other bioactives were investigated. Overall, two to three-fold increase in total carotenoids and phenols was observed compared to control. From a response surface analysis of the data, a three-degree polynomial equation was developed which provided the following optimal extraction conditions: T = 50.98°C, extraction time = 32.08 min. and enzyme concentration = 0.351%. Under the optimal conditions, juice extracted from enzyme treated capsicum macerate (CM) had high total carotenoid content (50.43 mg/100 g) and juice yield (77.75%). Results demonstrate that viscozyme is a potential enzyme combination for enhancing recovery of total carotenoids and thereby increases the functional quality of the juice.

Key words: Red capsicum, carotenoids, enzyme assisted extraction; response surface methodology; viscozyme.

INTRODUCTION

Carotenoids have emerged as most promising natural functional ingredients for growing nutraceutical industry (Cazzonelli, 5). Scientific evidences have established their indisputable role in scavenging peroxyl radicals, quenching singlet oxygen and protecting cell against oxidative stress. The most impressive role of carotenoid pigments in the human diet is their pro-vitamin A activity, which is associated with visual acuity and lower incidence of certain types of cancers and cardiovascular diseases (Reische *et al*., 13). Among vegetables, rich in carotenoids, red capsicum has the highest content of total carotenoids (30.37 mg/100 g), which far exceeds that of any other vegetables like carrots (8-10 mg/100 g), sweet potato (5-11.5 mg/100 g), spinach (17.31 mg/100 g), broccoli (1.56 mg/100 g) and cabbage (0.43 mg/100 g) (Ramamoorthy *et al*., 11). Presence of oxygenated carotenoids such as capsanthin, capsorubin and cryptocapsin, which are exclusive to this genus makes it very unique (Nadeem *et al*., 8).

Carotenoids being health promoting compounds their extraction from various plant and animal sources have attracted many investigators and food scientists. However, solvent mediated extraction of these pigments is more common as compared to enzyme assisted extraction. Barzana *et al*. (2), carried out enzyme mediated extraction of carotenoids from marigold flowers, but the pigment were subsequently released by hexane extraction. Recent approach in using enzyme mediated extraction with certain advantages such as the pigment in such extracts remains safe and stable in their natural state, while remaining bound to proteins, thus prevents their oxidation. Further, use of enzyme assisted extraction processes are efficient, economic and green and thus protect the integrity of carotenoids. In addition to these, enzymes also facilitate the release of other secondary metabolites such as phenolics, flavonoids and ascorbic acid, which ultimately result into an extract with high antioxidant activity. In other words, enzyme-mediated processes, exactly fit into the category of food based approach which has a syngeristic mix of all phytochemicals. In contrast, solvent extraction processes are targeted towards pure compounds hence expensive, toxic, labour intensive and are not considered as GRAS (Puri *et al*., 10). Extraction of carotenoids, from carrots as aqueous extracts has been extensively investigated using pectinase mediated extraction (Sun *et al*., 15). Given the matter of fact that capsicum is more promising material in terms of its composition and antioxidant activity, we have for the first time evaluated the potential of enzyme mediated extraction of red capsicum for extraction of both hydrophilic and

*Corresponding author's present address: ICAR-CIPHET, Abohar, Punjab; E-mail: prernenath3185@gmail.com

**ICAR-Indian Agricultural Statistics Research Institute, New Delhi 110012

lipophilic components in an aqueous extract, using a response surface methodology. Keeping above facts in mind, the main intent of the paper was to optimize enzyme assisted extraction process of capsicum juice with enhanced juice yield as well as other bioactive compounds.

MATERIALS AND METHODS

Mature uniformly red fruits of sweet pepper (*Capsicum annum* L.) cv. Orebelle at the red ripe stage grown in the field of Indian Agricultural Research Institute, New Delhi were selected. Fruits were washed, cut into pieces (3×2.5 cm^2) and blanched at 90°C for 4 min. followed by crushing in in a domestic blender (Inalsa, India) and subsequently heated at 90°C for 1 min. to inactivate the endogenous polyphenol oxidase activity. The enzymatic liquefaction was performed as described by Khandare *et al*. (6). The crushed macerate was poured in amber coloured bottles to prevent oxidation of carotenoids and subsequently liquefied with viscozyme from *Aspergillus aculeatus* (Novozyme®) at the enzyme/macerate ratio (0.2 to 0.4%). The macerate was mixed thoroughly and placed in a thermostatically controlled incubator with shaker New Brunswick Scientific) and incubated at different temperatures (50 to 70°C) for (30 to 90 min.) (Table 1). The treatments were carried out at 4.5, which falls within optimum activity of enzyme (3.3-5.5) (Alrahmany and Tsopmo, 1). At the end of the incubation period, the liquid and solid phases were separated by pressing in a stainless steel hydraulic press (Johnston Automation, India) using nylon filter bags at 2600 lb/m^2 where the pressure was held for 60 s. The juice was then packed in clean sterilized glass bottles, upturned and sealed. After extraction, the juice samples were immediately heated at 90°C for 30 s in a water bath in order to inactivate the added enzymes. The straight pressed juice served as control. The juice yield was determined by weighing the extracted juice, which was subsequently used for analysis of bioactives. All the treatments were carried out in triplicate.

Total soluble solids (TSS) was measured using a hand held refractometer (Atago, Tokyo, Japan). Total phenolics were estimated spectrophotometrically using Folin-ciocalteu reagent (FCR) (Singleton *et al*., 14). Total carotenoids were estimated as per the method of Lee *et al*. (7), FRAP was performed according to the procedure described by Benzie and Strain (3). DPPH (2, 2-diphenyl picrylhydrazyl) assay was performed as per method described by Brand-Williams *et al*. (4). Antioxidant activity was measured using the ABTS decolouration method using radical ABTS (2, 2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (Re *et al*., 12).

Table 1. Independent variables with coded levels and actual values for fitting response surface model.

Symbol	Independent variable	Unit	Coded level		
			-1	0	+1
A	Enzyme concentration	mg/kg	0.2	0.3	0.4
B	Temperature	°C	50	60	70
C	Time	min.	30	60	90

Response Surface Methodology (RSM) consists of the experimental strategy for exploring the relationship between the response variable and the input variables and to develop an appropriate approximating relationship between them. In present study, a second order response surface model was used as presented below:

$$f(x_u) = \beta_0 + \sum_{i=1}^{v} \beta_i x_{iu} + \sum_{i=1}^{v} \beta_{ii} x_{iu}^2 + \sum_{i=1}^{v-1} \sum_{i'=i+1}^{v} \beta_{ii'} x_{iu} x_{i'u} + e_u,$$

where u = 1,2,…,N, x_{iu} is the level of the i^{th} (i = 1,2,...,v) factor in the u^{th} treatment combination, $f(x_u)$ denotes the response obtained from u^{th} treatment combination and e_u is the random error associated with the u^{th} observation that is independently and normally distributed with mean zero and common variance σ^2, β_0 is a constant, β_i is the i^{th} linear regression coefficient, β_{ii} is the i^{th} quadratic regression coefficient and $\beta_{ii'}$ is the $(i,i')^{th}$ interaction coefficient.

The coded independent variables used in the RSM design have been listed in Table 1. The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 8.0.10 Trial, Delaware, USA Echip, 1993). A Box-behken design was used to conduct the experiment taking three factors, *viz.* enzyme concentration, incubation temperature, and extraction time each at three levels to evaluate the combined effect of these variables on the response. The response values were total carotenoids (TCC), total phenolic content (TPC), juice yield (JY), total soluble solids (TSS), ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) using ABTS and total radical scavenging activity (TRSA) using DPPH. The design consisted of 17 combinations including five replicates of the centre point used to determine the experimental error (Table 2).

The variables were coded according to the equation: $X_i = \frac{A_i - \text{Mean}(A_i)}{\Delta A_i}$, (i =1, 2 and 3), where Xi is the i^{th} coded value of X, Ai is the corresponding actual value of A, ΔAi is the increment of Ai corresponding to a change of 1 unit of A.

Table 2. Box-Behnken design and experimental data for TCC, TPC, JY, TSS, FRAP, TEAC, TRSA.

Experiment No.	Independent variable			Dependent variable						
	A	B	C	TCC (mg/100 g)	TPC (mg/100 g)	JY (%)	TSS (°Brix)	FRAP (µmol TE/ml)	ABTS (µmol TE/ml)	DPPH (µmol TE/ml)
1	-1	-1	0	42.69	73.39	70.75	8.15	1.63	2.91	0.68
2	0	-1	-1	50.43	75.27	77.25	8.35	1.68	3.0	0.70
3	0	-1	1	42.69	70.96	73.25	9.13	1.51	2.81	0.63
4	1	-1	0	48.13	77.03	74.25	9.3	1.70	3.0	0.70
5	0	0	0	40.22	67.55	74	8.9	1.49	2.72	0.63
6	1	0	-1	39.76	69.13	73.5	8.85	1.50	2.75	0.65
7	0	0	0	40.01	79.74	77.75	8.65	1.72	3.31	0.78
8	0	0	0	36.23	85.68	76.5	8.63	1.76	3.41	0.80
9	1	0	1	35.02	70.87	76.25	8.55	1.51	2.85	0.66
10	0	0	0	41.22	78.86	77.5	8.58	1.71	3.21	0.77
11	-1	0	-1	40.51	80.13	67.5	8.68	1.72	3.24	0.78
12	0	0	0	40.92	76.23	69.25	9.05	1.69	3.12	0.72
13	-1	0	1	39.39	73.05	64.5	8.7	1.65	3.02	0.69
14	0	1	1	43.30	78.59	74	8.63	1.70	3.20	0.77
15	-1	1	0	42.54	84.13	68.75	8.5	1.75	3.40	0.80
16	1	1	0	33.43	101.90	79	8.85	1.86	3.61	0.83
17	0	1	-1	41.43	96.43	65.5	8.65	1.82	3.52	0.81

RESULTS AND DISCUSSION

Optimization of enzymatic prepress maceration of capsicum was performed using the multi component, viscozyme preparation produced by *Aspergillus* (Table 1). Viscozyme is a commercial multi enzyme preparation from *A. aculeatus* containing a wide range of carbohydrases, including arabanase, cellulase, Q-glucanase, hemicellulase, xylanase and pectinase activity (NCBE, 9). Viscozyme can degrade the non-starch polysaccharide and branched pectin like substances in plant cell wall, which can decrease the viscosity of the plant extract and increase the juice yield. A significant variation in juice yields was found in response of different enzymatic maceration treatments and the results are presented in Table 2. The obtained juice yields ranged from 64.50-77.50%. The juice yield data were consistent with the interpretation that increased juice extraction is a result of enzyme catalyzed degradation of the pectin in the plant cell wall matrix and in the middle lamella that acts as putty between the cells and binds water. Significant pectin degrading activity due to the presence of pectin esterase, pectin lyase, and polygalacturonase catalyze the degradation of the smooth regions of the pectic substance. In addition, the cellulase and hemicelluase, present in viscozyme resulted in breakdown of insoluble matrix leading to complete disintegration and release of bound water. Overall synergistic action of various enzymes in viscozyme results in enhanced juice yields. Our results are in agreement with the reports of previous researchers where viscozyme has been shown to increase juice yield, quercetin content and oil recovery (Alrahmany and Tsopmo, 1).

TSS in red capsicum juice was found to be 8.15-9.12°Brix, whereas Sun *et al*. (15) reported 5.9-6.9°Brix in carrot. Probably viscozyme might have caused a greater degree of tissue breakdown thereby releasing more soluble components contributing higher TSS. Carotenoids are the most potential bioactive compounds present in capsicum fruits. Optimizing a process for their maximum recovery is of prime importance. TCC ranged from 33.43-50.43 mg/100 ml. Enzymes hydrolyze cell wall components such as pectin and cellulose, thereby increasing the cell wall permeability, which results in higher extraction yields of bioactives like carotenoids from cell wall and increase extraction efficiency. The central value for enzyme concentration was determined based on preliminary experiment. A report

on carrot processing (Sun *et al*., 15) have reported, β-carotene content ranging from 34.30-59.12 mg/ kg, which is considerably low, in comparison to enzyme assisted liquefaction by viscozyme carried in the present study. The capsicum macerate (CM) obtained through straight processing was viscous and thus difficult to press. EAP, accelerated the liquefaction of the CM resulting in a significant increase (P<0.05) in total carotenoids content and juice yield. Phytochemicals such as phenolics and carotenoids contained in vegetables and fruit matrix appear to be entangled with the plant cell wall polysaccharide *via* hydrophilic and hydrophobic bonds. The release of these phytochemicals can be enhanced *via* enzyme catalyzed degradation of the cell wall polysaccharides.

TPC ranged from 54.34-101.9 mg/100 ml. Both enzyme dosage and temperature is very crucial factors as excessive dosage can lead to breakdown of the phenols. TPC varied insignificantly with the parameters such as enzyme concentration, incubation temperature and time. Increased antioxidant activity is attributed to the increased contents of total carotenoids, phenolics content and other bioactive components. FRAP content ranged between 1.49-1.86, ABTS content ranged between 2.72-3.61, DPPH content ranged between 0.63-0.83.

The fitted models for different responses, *viz.*, *TCC(R_1), TPC(R_2), JY(R_3), TSS(R_4), FRAP (R_5), ABTS (R_6), DPPH (R_7)* along with the standard error (in parenthesis) of the parameter estimates is as follows:

$R_1 = 39.72 - 1.09\ X_1 - 2.90^{*}\ X_2 - 1.46\ X_3 - 1.90\ X_1^2 + 3.88^{*}\ X_2^2 + 0.85^{*}\ X_3^2 - 3.63^{*}\ X_1X_2 - 0.90\ X_1X_3 + 2.40^{*}\ X_2X_3$

(0.78) (0.62) (0.62) (0.62) (0.85) (0.85) (0.85) (0.87) (0.87) (0.87)

(R^2 = 0.92)

$R_2 = 77.61 + 1.02\ X_1 + 8.05^{*}X_2 - 3.53X_3 - 0.26\ X_1^2 + 6.76\ X_2^2 - 4.05\ X_3^2 + 3.53\ X_1X_2 + 2.20\ X_1X_3 - 3.38X_2X_3$

(3.22) (2.55) (2.55) (2.55) (3.51) (3.51) (3.51) (3.61) (3.61) (3.61)

(R^2 = 0.73)

$R_3 = 75.00 + 3.93^{*}\ X_1 - 1.03\ X_2 + 0.53\ X_3 - 1.93\ X_1^2 + 0.12\ X_2^2 - 2.62\ X_3^2 + 1.68\ X_1X_2 + 1.43\ X_1X_3 + 3.12\ X_2X_3$

(1.49) (1.18) (1.18) (1.18) (1.63) (1.63) (1.63) (1.67) (1.67) (1.67)

(R^2 = 0.75)

$R_4 = 8.76 + 0.019X_1 - 0.037X_2 + 0.059X_3 - 0.026X_1^2 - 0.033X_2^2 - 0.039X_3^2 - 0.2\ X_1X_2 - 0.08\ X_1X_3 - 0.2\ X_2X_3$

(0.13) (0.10) (0.10) (0.10) (0.14) (0.14) (0.14) (0.14) (0.14) (0.14)

(R^2 = 0.54)

$R_5 = 1.67 + 0.022X_1 - 0.076X_2 + 0.043X_3 - 0.010X_1^2 + 0.071X_2^2 - 0.068X_3^2 - 0.01X_1X_2 - 0.02\ X_1X_3 - 0.012X_2X_3$

(0.05) (0.03) (0.03) (0.03) (0.05) (0.05) (0.05) (0.05) (0.05) (0.05)

(R^2 = 0.55)

$R_6 = 3.51 - 0.045X_1 + 0.251^{*}X_2 - 0.078X_3 - 0.045X_1^2 + 0.121X_2^2 - 0.143X_3^2 + 0.03X_1X_2 + 0.08X_1X_3 - 0.032X_2X_3$

(0.11) (0.09) (0.09) (0.09) (0. 12) (0.12) (0.12) (0.12) (0.12) (0.12)

(R^2 = 0.64)

$R_7 = 0.74 - 0.013X_1 + 0.062^{*}X_2 - 0.023X_3 - 0.01X_1^2 + 0.02X_2^2 - 0.035X_3^2 + 0.002X_1X_2 + 0.025X_1X_3 + 0.007X_2X_3$

(0.02) (0.02) (0.02) (0.02) (0.03) (0.03) (0.03) (0.03) (0.03) (0.03)

(R^2 = 0.66)

* = Significant at P<0.05 level

Among the fitted models, model corresponding to R_1 was found to be best fitted model clearly indicating a significant influence of the three input variables, *viz.*, enzyme concentration, temperature and time on TCC. Responses R_4 (TSS °Brix) and R_5 (FRAP) were not found to be significantly influenced by the input variables as none of the parameter estimates were found to be significant.

Responses (*viz. TCC (R_1), TPC (R_2), JY (%)(R_3), TSS (°Brix)(R_4), FRAP(R_5), ABTS (R_6), DPPH (R_7)*) were optimized using multi-response optimization technique by taking all the responses together and giving restriction on X_1, X_2 and X_3 (*i.e.*, enzyme concentration, temperature and time) in the interval (0.2-0.4%), (50-70°C) and (30-90 min.) respectively. The optimum values for X_1, X_2 and X_3 are obtained as X_1 = 0.351%, X_2 = 50.98°C, X_3 = 32.08 min. In order to verify the predictive capability of the model, optimum conditions were established by RSM and comparisons between predicted results and the practical values were done by experimental rechecking using those presumed optimal conditions.

The best combination of process variables for the best set of response properties included an enzyme concentration 0.351% (v/w), incubation temperature of 50.98°C and incubation time of 32.08 min. The responses calculated at optimal extraction conditions were total carotenoid content (R_1) = 50.67 ± 1.43 (mg/100 g), total phenolic content (R_2) = 70.49 ± 5.87 (mg/100 g), juice yield (%) (R_3) = 75.93 ± 2.73 (%), TSS (°Brix) (R_4) = 8.72 ± 0.23 (°Brix), FRAP (R_5) = 1.62 ± 0.09 (µmol TE/ml), ABTS (R_6) = 2.86 ± 0.20 (µmol TE/ml), DPPH (R_7) = 0.68 ± 0 (µmol TE/ ml) and has been shown by cube plots in Fig. 1 to 7. The results obtained suggested that the change in the above three input variables (Viscozyme

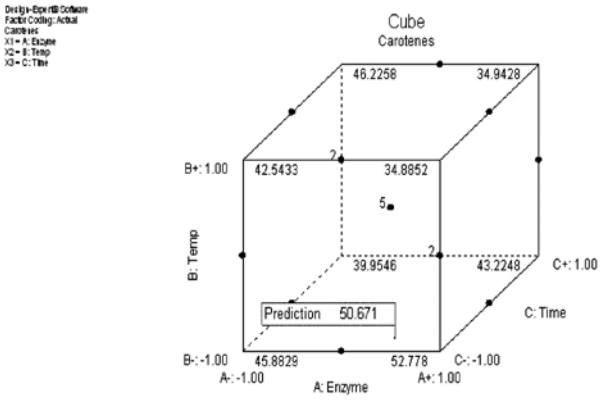

Fig. 1. Predicted response of total carotene content from capsicum extracted through EAP.

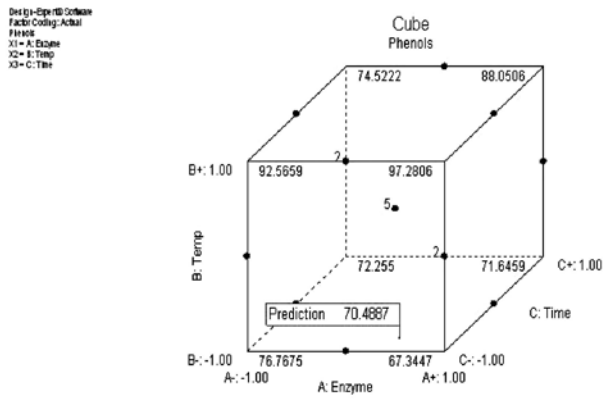

Fig. 2. Predicted response of total phenols content from capsicum extracted through EAP.

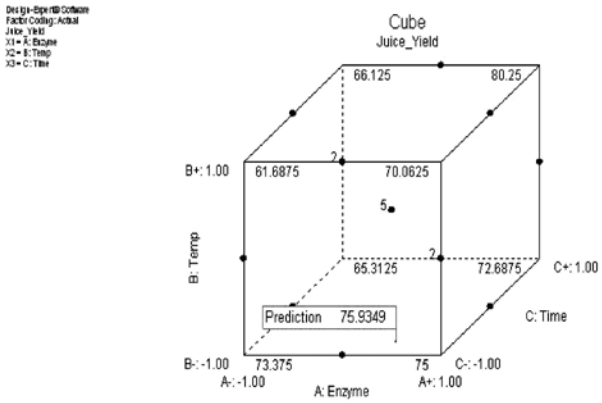

Fig. 3. Predicted response of juice yield from capsicum extracted through EAP.

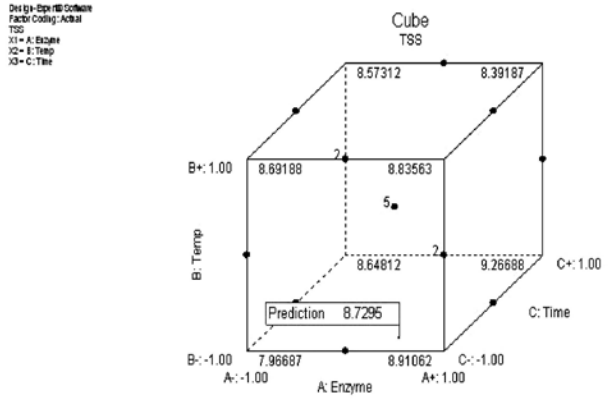

Fig. 4. Predicted response of total soluble solids (TSS) from capsicum extracted through EAP.

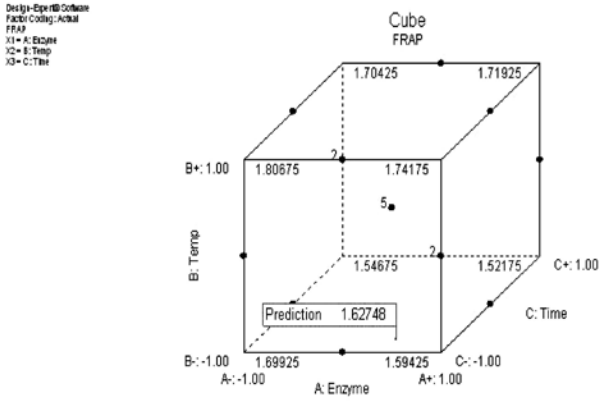

Fig. 5. Predicted response of FRAP from capsicum extracted through EAP.

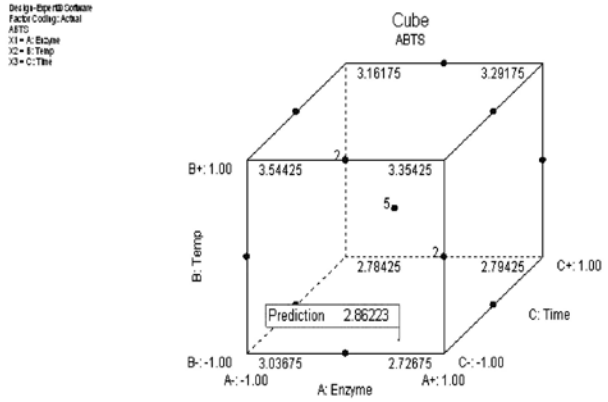

Fig. 6. Predicted response of ABTS from capsicum extracted through EAP.

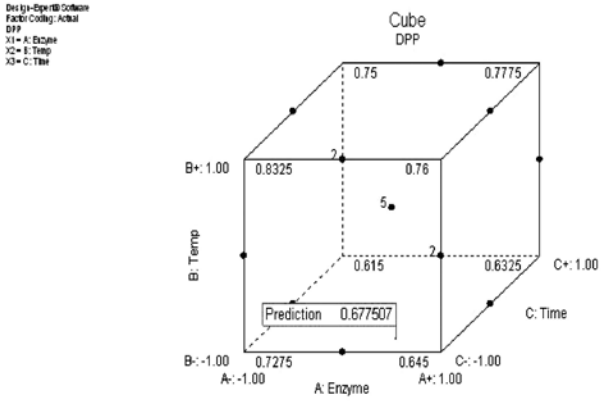

Fig. 7. Predicted response of DPPH from capsicum extracted through EAP.

concentration, temperature and incubation time) had a significant effect on the TCC and JY, whereas other responses were found to be insignificant. With an increase of enzyme concentration, temperature and incubation time, the juice yield and TCC increased sharply. Therefore, we conclude that both enzyme concentration and extraction temperature are important factors for attaining an efficient extraction of total carotenoids from red capsicum. Under these optimized conditions, the experimental maximum juice yield and TCC was 76.00% and 51.0 mg /100 g, respectively. There is an excellent agreement of the experiment values with the predicted values indicating the suitability of the models developed and the success of RSM in optimizing the extraction conditions in red capsicum.

REFERENCES

1. Alrahmany, R. and Tsopmo, A. 2012. Role of carbohydrates on the release of reducing sugar, total phenolics and on antioxidant properties of oat bran. *Food Chem.* **132**: 413-18.
2. Barzana, E., Rubio, D., Santamaria, R.I., Garcia-correa, O., Garcia, F., Ridaura sanz, V.E. and Munguiaa, L.A. 2002. Enzyme-mediated solvent extraction of carotenoids from marigold flower (*Tagetes erecta*). *J. Agric. Food Chem.* **50***:* 4491-96.
3. Benzie, I.F.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: the FRAP assay. *Anal. Biochem.* **239**: 70-76.
4. Brand, W.W., Cuvelier, M.E. and Berset, C. 1995. Use of free radical method to evaluate antioxidant activity. *LWT Food Sci. Tech*. **28**: 25-30.
5. Cazzonelli, C.I. 2011. Carotenoids in nature: insights from plants and beyond. *Funct. Plant Biol.*, **38**: 833-47.
6. Khandare, V., Walia, S., Singh, M. and Kaur, C. 2011. Black carrot (*Daucus carota* ssp. *sativus*) juice: Processing effects on antioxidant composition and color. *Food Bioprod. Process.* **89**: 482-86.
7. Lee, H.S. 2001. Characterization of carotenoids in juice of red navel orange (Cara Cara). *J. Agril. Food Chem.* **49**: 2563-68.
8. Nadeem, M., Anjum, F.M., Khan, M.R., Saeed, M. and Riaz, A. 2011. Antioxidant potential of bell pepper (*Capsicum annum* L.)- A review. *Pakistan J. Food Sci*. **21**: 45-51.
9. National Center for Biotechnology Education (NCBE). 2006. Carbohydrase mix: Viscozyme Available from: http://www.ncbe.reading.ac.uk/NCBE/MATERIALS/ENZYMES/viscozyme.html. Accessed August 10, 2006.
10. Puri, M., Sharma, D. and Barrow, C.J. 2012. Enzyme-assisted extraction of bioactives from plants. *Trend. Biotech.* **30**: 37-44.
11. Ramamoorthy, K., Bhuvaneswari, S., Sankar, G. and Sakkaravarthi, K. 2010. Proximate composition and carotenoid content of natural carotenoid sources and its colour enhancement on marine ornamental fish amphiprionocellaris. *World J. Fish Marine Sci.* **2**: 545-50.
12. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorisation assay. *Free Radical Biol. Med*. **26**: 1231-37.
13. Reische, D.W., Lillard, D.A. and Eitenmiller, R.R. 2002. Antioxidants. **In**: *Food Lipids: Chemistry, Nutrition, and Biotechnology* (2nd Edn.); Akoh, C.C. and Min, D.M. (Eds.), CRC Press, Boca Raton, FL, pp. 489-516.
14. Singleton, V.L., Orthofer, R. and Lamuela-Ranventos, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **299**: 152-78.
15. Sun, Y.Y., Wang, Z., Wu, J., Chen, F., Liao, X. and Hu, X. 2006. Optimising enzymatic maceration in pretreatment of carrot juice concentrate by response surface methodology. *Int. J. Food Sci. Tech.* **41**: 1082-89.

Received : September, 2014; Revised : November, 2015; Accepted : November, 2015