Songklanakarin J. Sci. Technol.
33 (4), 389-395, Jul. - Aug. 2011

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Optimizing enzymatic extraction of cereal milk using response surface methodology

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Received 20 April 2011; Accepted 2 August 2011

Abstract

The purpose of this study was to determine the optimal condition of enzymatic production of cereal milk. The commercial enzyme pectinase (Pectinex® Ultra SP-L) assisted in the extraction process and an experimental design using response surface methodology (RSM) was utilized to further optimize the yield. The condition parameters were incubation temperature (X_1; 25, 35, and 45°C) hydrolyzing time (X_2; 60, 120, and 180 min) and concentration of enzyme (X_3; 1, 2, and 3% v/w). The reducing sugar content can be predicted using equation: reducing sugar = $12.292 + 1.776(x_1) + 2.296(x_2) + 0.612(x_2x_3) + 1.159(x_1^2) - 2.533(x_3^2)$ with $R^2 = 0.781$, $p \leq 0.05$). The relationship between incubation temperature, hydrolyzing time, and enzyme concentration on reducing sugar content was shown in terms of linear, quadratic, and interaction. The optimal condition was incubation temperature of 35°C, hydrolyzing time 120 min, and 2% (v/w) pectinase. Under the optimum condition, the reducing sugar, protein, fat, and antioxidant activity of cereal milk increased 3.44, 1.59, 1.14, and 1.03–fold, respectively, compared with control. This research concluded that enzymatic hydrolyzed cereal milk is a potential source of protein, fat, and antioxidants activity.

Keywords: enzymatic, Pectinex® Ultra SP-L, cereal milk, antioxidant, response surface method

1. Introduction

There is growing awareness that the daily diet is an important determinate for a healthy life. Consumers judge food product not only in terms of taste and nutritional needs, but also in terms of the ability to improve their health and well-being. Many food researchers are focus on balanced content of amino acids, led to the development of high nutritional quality beverage.

Soy milk is a popular beverage in Asian countries. Soy milk is a rich source of protein, isoflavones, saponins, and fiber (Bricarello *et al.*, 2004). However, soy protein contains almost all essential amino acids, but it lacks of methionine. Sesame (*Sesamum indicum* L.*)* seed is an excellent protein source for supplementing soybean, peanut, and other vegetable proteins (which lack sufficient methionine) to increase their nutritive qualities (Inyang and Ekanem, 1996). The seed contains appreciable amounts of various bioactive components including tocopherols, phytosterols, resveratrol, and flavonoids, and the lignans sesamin and sesamolin (Namiki, 1990). Maize is a good source of carotenes, precursor of vitamin A. Omueti and Ajomale (2005) reported that adding maize to the soy milk is generally more organoleptically acceptable in texture and appearance than soy milk itself. Therefore, this research produced cereal milk, a protein beverage that is an aqueous extract of a blend of soybean, sesame, and maize.

An antioxidant can be defined as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1999). Antioxidant activities of soybean are due to polyphenolic

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compounds such as isoflvones and genistein (Pratt and Birac, 1979). Sesame seeds contain many antioxidant compounds, including sesamol, gamma-tocopherol, and seasamolinol (Osawa *et al.*, 1985). Maize contains carotenoid and tocopherols that are lipid soluble antioxidants (Egesel *et al.*, 2003).

The plant cell walls are considered to contain as main components cellulose, hemicelluloses, and pectin. Cellulose appears to a key component of the primary cell wall structure of soybeans, whereas pectin seems to be more involved in the secondary cell wall structure and acts as an adhesive between cells (Jung *et al*., 2006). Protein and oil extraction yields were shown to be closely related with cell wall disruption (de Moura *et al*., 2008). Disrupting the integrity of the plant cell wall component network by using cell-wall-degrading enzyme, i.e., enzyme able to degrade celluloses, hemicelluloses and pectin, might increases the extractability of the major soybean component, oil, protein, and antioxidant activity (Dominguez *et al.*, 1995; Rosenthal *et al*., 2001). Enzyme treatment has been used to increase protein and oil yield for several materials such as soybean (Rosenthal *et al.*, 1998), corn germ (Moreau *et al.,* 2004), rice bran (Sharma *et al.*, 2001) and sunflower (Sineiro *et al*., 1998).

Experimental design was employed by using response surface methodology (RSM) and the effects of several process parameters and their interactions on response variables were evaluated. RSM is an efficient tool for finding optimal conditions; it is a collection of statistical and mathematical techniques for analyzing and solving multivariable equations. RSM uses contour plots to find the response surface and locate optimal parameters, which are useful to assess optimizing conditions clearly. Hence, RSM has been successfully used for developing, improving, and optimizing biochemical processes including those related to enzyme systems (Rai *et al*., 2004; Liew Abdullah *et al.,* 2007; Boonsawang and Wongsuvan, 2010; Senklang and Anprung, 2010). The aim of this study was to evaluate the effect of enzyme treatment (Pectinex® Ultra Sp-L) on the increase in the nutritional components of cereal milk and on the optimization of the process conditions by response surface methodology.

2. Materials and Methods

2.1 Enzyme Source

Pectinex® Ultra SP-L enzyme from *Aspergillus aculeatus* was purchased from Novozyme Company (East Asiatic, Thailand), which had an activity of 10292 PGU/ml in liquid form, and was brown in color.

2.2 Reagents

3,5 dinitrosalicylic acid, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid, L-ascorbic acid, potassium persulphate, potassium sodium tartate, ethyl alcohol, methanol, boric acid, and glucose anhydrous were purchased from Sigma-Aldrich. All reagents used for analysis were of analytical grade.

2.3 Preparation of cereal milk

The processing of cereal milk was based on preliminary experiments. Soybeans were thoroughly washed and soaked three hours at room temperature. Maize was separated from cob and cleaned remove hairs and other extraneous materials. Sesames were separated from dust and roasting at 150°C for 3 min. The ratios of cereal mixture were according to the preliminary studied by sensory evaluation method. The optimum ratio of the mixture was using soybean : maize : sesame : water (13.5 : 7.25 : 4.25 : 75). The cereals were mixed by using a blender. These cereal milk samples were treated with the enzyme at different levels of hydrolyzing temperature, incubation time, and enzyme concentration for optimization of the extraction condition. At the end of the enzyme treatment, the enzyme was inactivated by heating at 90°C for 10 min.

2.4 Determination of reducing sugar (Miller, 1959)

To monitor the degradation of cell wall components after enzyme treatment the reducing sugar released from cereal milk were quantified by DNS method. 1.0 mL of DNS reagent (ready prepared; 3,5-dinitrosalicylic acid and sodium potassium tartrate dissolved in dilute sodium hydroxide) was added to sample (1.0 mL). The mixtures were placed in boiling water for 10 min. Then rapidly cool in ice to room temperature, distilled water (9 mL) was added to the mixtures, and read absorbency at 530 nm. A standard curve is prepared by using the glucose ranged of 0-1000 mg/mL and results were expressed in g glucose/g sample.

2.5 Proximate analysis

The methods for sample treatment and analyses were the standard procedures recommended by Association of Official Analytical Chemists (AOAC, 1990). Moisture content was assayed by loss in weight on drying at 105°C in a hot air oven. The ash was determined by incineration of known weights of the samples in a muffle furnace. The crude fat was determined by exhaustively extracting a known weight of sample in petroleum ether in a Soxhlet extractor. The ether was volatilized and the dried residue quantified gravimetrically and calculated as percentage of fat. Protein was determined by the Kjeldahl method (conversion factors: soybean, 5.71, maize 6.25, and sesame 3.38). Crude fiber was determined after digesting a known weight of fat-free sample in refluxing 1.25% sulphuric acid and 1.25% sodium hydroxide. The available carbohydrate was obtained by the difference method (subtracting the percent crude protein, fat, fiber, moisture, and ash from 100% dry matter).

2.6 Assessment of ascorbic equivalent antioxidant capacity (AEAC)

Evaluation of scavenging properties using long-lived $ABTS^{\cdot+}$ radical was performed by using the method of Arnao *et al.* (2001). A 7.4 mM $ABTS^{\cdot+}$ solution was used to react with 2.6 mM of potassium persulfate solution in a ratio 1:1 and then the solutions were allowed to react and form free radicals for 12 hrs at room temperature in the dark (the output chemical should be used within 4 hrs). Following this, 1 ml of $ABTS^{\cdot+}$ solution was mixed with 60 ml of methanol to obtain an absorbance of 0.75±0.2 units at 734 nm ($ABTS^{\cdot+}$ should be prepared everyday). Subsequently, cereal milk (150 mL) were allowed to react with 2,850 mL of the $ABTS^{\cdot+}$ solution for 2 hrs in the dark and absorbance values were measured at 734 nm. The standard curve was prepared by using *L*-ascorbic acid as a standard substance in a concentration range of 25–600 mM and results were expressed in mM ascorbic acid equivalent capacity/g fresh mass (AEAC/g fm).

2.7 Optimizing enzymatic extraction of cereal milk

A statistical experimental design based on Central Composite Design (CCD) was planned. The three independent variables were incubation temperature (X_1), hydrolyzing time (X_2), and enzyme concentration (X_3). The coded values of the independent variables were -1 (lowest level), 0 (middle level), and 1 (highest level). Correspondences between these coded and actual values are presented in Table 1. The end and central values were chosen from the results of preliminary tests. The experimental design consisted of 19 points, including five replications at the central point, and was carried out in a random order. The result of reducing sugar was evaluated.

2.8 Statistical analysis

The experiments were based on a central composite design with the quadratic model employed to study the combined effects of three independent variables (i.e., incubation temperature, hydrolyzing time, and enzyme concentration).

The dependent variable measured was reducing sugar of the cereal milk. The results obtained were used to create quadratic equations to determine the correlation of data by SPSS program version 13 and to make response surface curves and contour plots by STATISTICA program version 5.5.

3. Results and Discussion

3.1 Effect of enzyme on reducing sugar

The effect of hydrolyzing temperature, incubation time and enzyme concentration on the reducing sugar content are shown in Table 2. Kapchie *et al.* (2010) suggested that to monitor the degradation of cell wall components by determined the amount of reducing sugar. From this research, it was clear that the reducing sugar content in all enzyme treated samples was higher than control. Similar result was reported by Jung *et al.* (2006), which reported that enzyme pectinase and cellulase were identified as effective in reducing sugar and protein extractability from defatted soy flakes. Kasai *et al.* (2004) reported that cellulase treatment of soybean fiber

Table1. Matrix of experimental central composite design (CCD) for enzymatic extraction of cereal milk.

Treatment No.	Dependent		
	Temperature (code/actual level)	Time (code/actual level)	Enzyme (code/actual level)
1	-1(25)	0(120)	0(2)
2	+1(45)	0(120)	0(2)
3	0(35)	-1(60)	0(2)
4	0(35)	+1(180)	0(2)
5	0(35)	0(120)	-1(1)
6	0(35)	0(120)	+1(3)
7	+1(45)	+1(180)	+1(3)
8	-1(25)	+1(180)	+1(3)
9	+1(45)	-1(60)	+1(3)
10	-1(25)	-1(60)	+1(3)
11	+1(45)	+1(180)	-1(1)
12	-1(25)	+1(180)	-1(1)
13	+1(45)	-1(60)	-1(1)
14	-1(25)	-1(60)	-1(1)
15-19	0(35)	0(120)	0(2)

Table 2. Effect of incubation temperature, hydrolyzing time, and enzyme concentration on reducing sugar.

Treatment No.	Dependent variables			Independent variables [A]
	Temperature (code/°C)	Time (code/min)	Enzyme (code/%)	Reducing Sugar (mg/ml)
1	-1(25)	0(120)	0(2)	$11.75^{cde} \pm 1.50$
2	+1(45)	0(120)	0(2)	$16.09^{a} \pm 0.74$
3	0(35)	-1(60)	0(2)	$9.01^{fg} \pm 1.74$
4	0(35)	+1(180)	0(2)	$15.15^{ab} \pm 1.76$
5	0(35)	0(120)	-1(1)	$11.24^{cdef} \pm 1.13$
6	0(35)	0(120)	+1(3)	$9.22^{efg} \pm 1.35$
7	+1(45)	+1(180)	+1(3)	$14.06^{bc} \pm 1.05$
8	-1(25)	+1(180)	+1(3)	$11.43^{cdef} \pm 1.86$
9	+1(45)	-1(60)	+1(3)	$8.32^{g} \pm 1.99$
10	-1(25)	-1(60)	+1(3)	$7.35^{gh} \pm 0.53$
11	+1(45)	+1(180)	-1(1)	$13.00^{bc} \pm 0.43$
12	-1(25)	+1(180)	-1(1)	$9.34^{defg} \pm 2.82$
13	+1(45)	-1(60)	-1(1)	$10.89^{cdef} \pm 0.20$
14	-1(25)	-1(60)	-1(1)	$5.62^{h} \pm 0.99$
15	0(35)	0(120)	0(2)	$12.00^{c} \pm 0.94$
16	0(35)	0(120)	0(2)	$11.94^{cd} \pm 0.92$
17	0(35)	0(120)	0(2)	$11.76^{cde} \pm 0.97$
18	0(35)	0(120)	0(2)	$11.92^{cd} \pm 1.02$
19	0(35)	0(120)	0(2)	$11.91^{cd} \pm 0.97$
control	-	-	-	$3.46^{i} \pm 0.11$

[A] Values are means and ± standard deviation of triplicate determination. Different superscripts in the same column indicate significant difference ($p \leq 0.05$).

was effective in digesting the primary cell wall, whereas pectinase treatment was effective in digesting the secondary cell wall. The increase in reducing sugar product was a synergistic effect on degrading the cell wall when these enzymes were use together.

3.2 Model fitting

The application of RSM offers, on the basis of parameter estimate, an empirical relationship between the response variable and the test variable under consideration (Rostogi and Rashmi, 1999). By applying multiple regressions analysis on the experimental data, the response variable and the test variables is related by the following second-order polynomial equation:

$$\text{Reducing sugar} = 12.292 + 1.776x_1 + 2.296x_2 + 0.612x_2x_3 + 1.159x_1^2 - 2.533x_3^2 \quad (R^2 = 0.781, p \leq 0.05) \quad (1)$$

where x_1 is the code for incubation temperature (-1, 0, +1), x_2 is the code for hydrolyzing time (-1, 0, +1), and x_3 is the code for enzyme concentration (-1, 0, +1). The regression coefficient equation of color reducing sugar presented in Table 3, showed that the coefficient values of determination (R^2) was 0.781 ($p \leq 0.05$). Results indicated that these equations had a model fit with experimental data (Rastogi and Rashmi, 1999), which may be used to estimate reducing sugar after varying temperature, incubation time and enzyme concentration.

From the reducing sugar equations, the relationship between incubation temperature, hydrolyzing time, and enzyme concentration was shown in terms of linear, quadratic, and interaction. An increase or decrease in one of this parameter could affect the others. Reducing sugar was negative related to the quadratic term of enzyme concentration. The results indicated that a higher enzyme concentration provided not enough substrate for enzyme-substrate complexes. When considering coefficient values from Table 3, the influence of the incubation time (β_2=2.269) of treatment on reducing sugar was higher than the incubation temperature (β_1= 1.776) and enzyme concentration (β_3=0.118).

Incubation time is an important process parameter because it may influence the extraction reducing sugar and protein yield, i.e., the longer the enzyme can react with substrate, the greater the hydrolysis of cell wall components. However, a longer hydrolysis time will also increase the processing costs and risk of microbial growth (Jung *et al*., 2006).

Table 3. Regression coefficient and coefficient of determination (R^2) of reducing sugar response of enzymatic extraction of cereal milk.

Regression coefficient	Reducing sugar
β_0	12.292*
β_1	1.776*
β_2	2.269*
β_3	0.118
$\beta_1\beta_2$	0.120
$\beta_1\beta_3$	-0.552
$\beta_2\beta_3$	0.612*
β_{1^2}	1.159*
β_{2^2}	-0.680
β_{3^2}	-2.533*
R^2	0.781

Note: Subscripts; β_0 = constant, β_1 = temperature(x_1), β_2 = Incubation time(x_2), β_3 = enzyme concentration(x_3); * significant at 0.05 level.

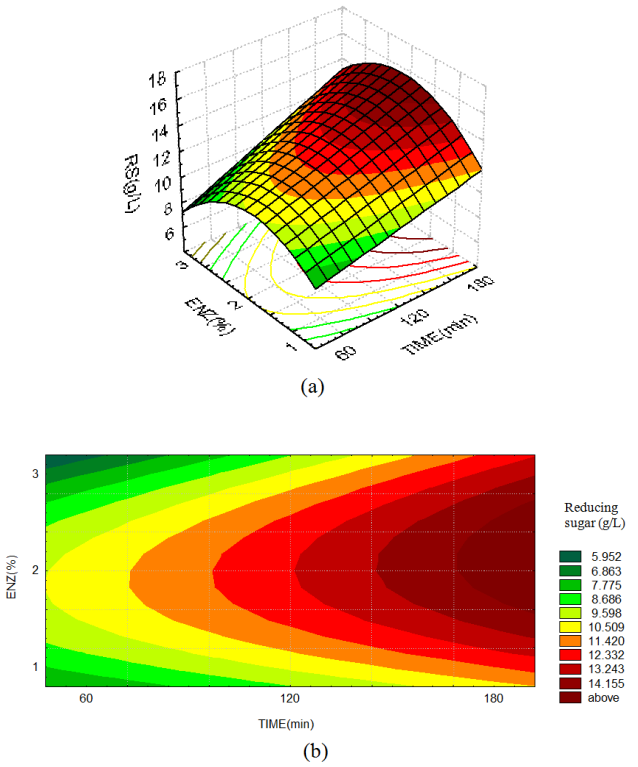

Figure 1. Response surface (a) and contour plots (b) for the effects of enzyme concentration (%) and incubation temperature (°C) at constant temperature 35°C on the yield of reducing sugar (g/L).

3.3 Response surface plotting

Figure 1 shows the response surface curves of the relationship between incubation time and enzyme concentration on the reducing sugar contents at 35°C. At lower incubation time and enzyme concentration there was almost a linear increase in reducing sugar. However, at a higher level (+1, +1, +1), a nonlinear behavior was observed as also evidenced from the sign of the quadratic terms of the response. The result indicated that enzyme concentration displayed a negative quadratic effect on the extracted reducing sugar; hence extracted reducing sugar increased up to about 2% enzyme, followed by a decline and then with a further increase. The optimum condition for enzymatic assisted cereal milk was incubation at 35°C for 120 min and 2% (v/w) of enzyme concentration.

3.4 Comparisons of control and optimization for enzymatic extraction

The proximate analysis of enzyme-assisted and non

Table 4. Proximate analysis of cereal milk (control) and enzyme-assisted cereal milk.[A]

Compositions	Control	Enzyme-assisted
Reducing sugar (g/L)	3.46 ± 0.11*	11.91 ±0.82
Total soluble solid (%)	4.00±0.02	4.000.00
pH	6.50±0.13	6.50±0.04
Crude protein (%)	12.72±0.15*	20.20±0.13
Fat (%)	5.83±0.10*	6.65±0.25
Fiber (%)	1.23±0.03	1.24±0.00
Ash (%)	1.45±0.03	1.32±0.05
Carbohydrate (%)	4.22±0.05*	1.91±0.03
Moisture	74.55±0.30*	68.68±0.34
Antioxidant activity (μmol/g AEAC)	2692.85±24.00*	2865.43±89.65

[A] Values are means and ± standard deviation of triplicate determination. Within in a row, each control value with an asterisk is significantly different from enzyme assisted value ($p \leq 0.05$).

enzyme-assisted (control) cereal milk samples are shown in Table 4. It was found that the enzymatic assisted method resulted in a higher amount of reducing sugar, protein, fat, and antioxidant activity. The reducing sugar content increased almost 3.44–fold compared with control. Moreover, the range of protein, fat, and antioxidant activity increased 1.59, 1.14 and 1.03-fold, respectively, over control. Similar, many studies reported that enzyme treatment has been used to increase protein and oil yield for several materials such as soybean (Rosenthal *et al.*, 1998), corn germ (Moreau *et al.,* 2004), rice bran (Sharma *et al.*, 2001) and sunflower (Sineiro *et al.*, 1998).

It would have been interesting to investigate the effects of other pectinase or pectinolytic enzyme such as polygalacturonase, pectate lyaese, or pectin methyl esterase to determine their potential in increasing protein extractability without causing protein degradation (Jung *et al.,* 2006). Enzyme Pectinex Ultra SP-L had activities including polygalacturonase, pectinlyase, pectin esterase, β-galactosidase, chitinase, and transgalactosidase (Mutlu *et al.*, 1999; Liew Abdullah *et al.*, 2007). Thus, disrupting the plant cell wall component network by using cell wall degrading enzymes can increase extractability of protein, fat, and antioxidant activity in cereal milk.

4. Conclusions

The optimal conditions for extraction of cereal milk were incubation temperature of 35°C, 120 min of hydrolyzing time, and 2% (v/w) of enzyme concentration. Under the optimum conditions, the cereal milk contained reducing sugar, protein, fat, and the antioxidant activity increased 3.44, 1.59, 1.14, and 1.03–fold, respectively, when compared with control. It confirms that this enzyme is more efficient in hydrolyzing the cell wall constituent of cereals.

Acknowledgements

The authors would like to acknowledge the Nakhon Ratchasima Rajaphat University for financial support.

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