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Response Surface Approach for Optimization of Protein Hydrolysis from *Reutealis trisperma* Cake as Potential Animal Feedstock

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Abstract

The conditions for protein hydrolysis were optimized to prepare Reutealis trisperma cake for potential animal feedstock. The cake's content was 34.03 % protein, 6.32 % moisture, 18.56 % total sugar, 15.58 % lipid and 25.51% others. Other components in cake could be fibre and lignin. The cake is a byproduct of mechanical pressing process of the seeds and contains high protein content (34.03%). It was ground prior the hydrolysis process. A central composite design including concentration of NaOH, ratio of cake to NaOH, time and temperature were used to develop second order model to predict protein content under various experimental conditions. Protein yield was primarily affected by ratio pressed cake to NaOH and concentration of NaOH. Based on the Response Surface Methodology (RSM) model, maximum yield of protein was 11.33% which was obtained at cake/solvent ratio 1: 50; 1.5 % w/v NaOH; 15 minutes of hydrolysis at 40^{o}C. The actual maximum protein yield from the experiment was obtained at cake/solvent ratio 1: 40; 1.5 % w/v NaOH; 20 minutes of hydrolysis at 45^{o}C which was 21.28 %.

Keywords: *animal feedstock; protein hydrolysis; response surface; Reutealis trisperma*

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INTRODUCTION

Reutealis trisperma is a tropical plant found in the Java island of Indonesia, especially in the western parts of Java. It can grow up until 15 m height, at altitude up to 1000 m from sea level (Holilah, 2013). *Reutealis trisperma* trees can prevent erosion and increase soil water absorption. Although the plants are abundantly available, the seeds are yet to be developed for good industrial uses. The seed of *Reutealis trisperma* has about 52% oil based on dry seed weight (Anggraini *et.al*, 2013). The seed oil has tung oil-like properties (Barley, 1950; Kataren, 1986). The oil is inedible but has been studied for making biodiesel (Holilah, 2013). The cake after oil extraction contains about 34% protein, so it is potential to develop the seed cake as a protein source for animal feedstock. On the other hand, the *Reutealis trisperma* seed cake can be considered for value-added non-food uses (Manurung *et.al*., 2016).

The potential of using seed cakes, such as those from castor and sunflower,for enzyme production has been reported (Castro *et.al*., 2016). Pressed cakes (biomass) as residues remaining after mechanical extraction of the oils from the seed kernels of *Reutealis trisperma* can be used for animal and fermentation feedstock for enzyme production. Many biomasses were characterized using some approaches which resulted in inorganic matter, carbohydrates, protein, lipids, moisture and ash content (Vassilev et.al, 2012, Martin et.al, 2010). The biomass has been characterized using NREL method. Since the protein is high, the biomass will be hydrolised to reduce the protein content. There has been a study to optimize the extraction condition for protein in watermelon seed using sodium hydroxide solution for food application. There are several studies in biomass hydrolysis as potential feedstock which are optimized using Response Surface Method (RSM). Response Surface Methodology was applied to obtain protein yield as 86.08 % using 1.3 %w/v sodium hydroxide with ratio of 70: 1 sodium hydroxide to watermelon seed at 40ºC for 15 minutes (Wani et.al, 2006). Apiwatanapiwat *et.al*. (2009) produced 71.69% protein hydrolysate from *Jatropha curcas* cake. The experiment was carried at 50ºC, using 2.5% NaOH solution and 0.0125% reaction volume for 45 minutes. Cassales *et.al*. (2011) showed the potential use of soybean hulls as a substrate for several bioprocesses after acid hydrolysis. The best conditions for recovering sugar were 153ºC and mass fraction of 1.7% H_2SO_4 for 60 minutes with hydrolysis efficiency of 87%. This study aimed to hydrolyse protein in *Reutealis trisperma* cake using NaOH with minimal breakdown of protein and optimize the hydrolysis condition using RSM. Once the hydrolysed protein is gained, it can be further processed to yield protein concentrate as potential animal feedstock.

MATERIALS AND METHODS

Characterization of Pressed Cake

Pressed cake *Reutealis trisperma* was characterized for sugar, protein, moisture, and lipids contents. Nitrogen content of the biomass sample was measured by Kjeldahl method and the protein content was estimated using an appropriate Nitrogen Factor (NF). Sugar content was measured using procedure to determine extractives in biomass by NREL method.

Reutealis trisperma Pressed Cake Pre-treatment

De-oiled *Reutealis trisperma* pressed cake was kindly provided by CV. Energi Baru Sejahtera. It was ground to fine powder and screened through a 40-mesh screen. Fine defatted *Reutealis trisperma* pressed cake then stored in a vacuum condition at room temperature.

Hydrolysis of *Reutealis trisperma* Pressed Cake

A 5 gram of *Reutealis trisperma* pressed cake was hydrolysed with selected 31 combinations of independent variables which are NaOH concentration (0.6-1.8%w/v), temperature (40-60ºC), hydrolysis time (15-35 minutes) and cake/solvent ratio (1:10 to 1:50 w/v). The hydrolysis process was carried out in a flask bottle maintained at selected temperature by connecting them to a shaking water bath for certain period of time. At the end of hydrolysis treatment, a Buchner funnel containing filter paper was used to separate supernatant and solid component. The quantity of soluble protein was determined in the supernatant solution using a Bio-Rad protein assay. The cake was then removed from the solvent and dried in an oven at 50ºC for 2 hours and was kept in a sealed vacuum container. All the experiments were carried out in duplicate.

Experimental Design and Statistical Analysis

The effect of four independent variables X_1 (NaOH concentration), X_2 (hydrolysis time), X_3 (temperature) and X_4 (cake/solvent ratio) at five levels on protein yield (dependent variable) were investigated using central composite design (Table 1) and *Response Surface Methodology* (RSM). The predicted model is described in Equation (1).

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_{12} + \beta_6X_{22} + \beta_7X_{32} + \beta_8X_{42} + \beta_9X_1X_2 + \beta_{10}X_1X_3 + \beta_{11}X_1X_4 + \beta_{12}X_2X_3 + \beta_{13}X_2X_4 + \beta_{14}X_3X_4 \quad (1)$$

Where Y is the response and β_0 is the value of the fixed response at central point of the experiment. β_nX_m and $\beta_{nn}X_nX_m$ are the linear, quadratic, and cross product coefficients, respectively, where n and m are indexes for β and X.

Analysis of data was carried out for variance and regression models using a commercial statistical package Mini Tab (MiniTabInc, USA). A second-order polynomial was fitted to the data to obtain regression equations. Statistical significance of the terms in the regression equation was examined.

Table 1. Independent variables and their levels used for central composite rotatable design

Independent variables	Symbol	Coded Variable Levels				
		-2	-1	0	+1	+2
NaOH concentration (%w/v)	X_1	0.6	0.9	1.2	1.5	1.8
Hydrolysis time (min)	X_2	15	20	25	30	35
Temperature (ºC)	X_3	40	45	50	55	60
Cake/solvent ratio (%w/v)	X_4	1:10	1:20	1:30	1:40	1:50

Table 2. Characteristics of *Reutealis trisperma* pressed cake

Composition	Percentage (w/w)
Protein	34.03 %
Moisture	6.32%
Total sugar	18.56 %
Lipid	15.58 %
Others	25.51%

RESULTS AND DISCUSSION

Characteristic of *Reutealis trisperma* Cake

The cake used in this study was ground into 40 mesh. It was then dried at 50^{o}C for 1 hour. The characteristic of cake can be seen in Table 2.

Other components could be lignin and fibre. The protein, sugar, lignin and fibre contents in *Aleurites trisperma* is 38.7%, 15.4%, 5%, and 40.1% respectively (Martin et.al, 2010). The mass fraction of protein content in *Reutealis trisperma* found in this work was lower than that reported in *Aleurites trisperma*.

Statistical Analysis for Protein Hydrolysis

Since the protein is quite high, the hydrolysis was carried out to extract the protein which will be used for feedstock. Parameters used in protein hydrolysis of *Reutealis trisperma* cake were solvent concentration, hydrolysis time, temperature, and ratio of solvent used to pressed cake (Table 3).

Table 3. Central composite rotatable design and responses for the optimization of protein hydrolysis from *Reutealis trisperma* cake

Run	Coded variables				Uncoded variables				Protein *yield* (Y), % w/w
	x_1	x_2	x_3	x_4	NaOH concentration (X_1), % w/v	Hydrolysis time (X_2), minutes	Temperature (X_3), °C	Cake/solvent ratio (X_4), %w/v	Experimental
1	1	-1	1	1	1.5	20	55	1/40	13.11
2	2	0	0	0	1.8	25	50	1/30	4.98
3	1	1	1	-1	1.5	30	55	1/20	13.25
4	0	0	0	0	1.2	25	50	1/30	11.63
5	-1	1	-1	-1	0.9	30	45	1/20	9.13
6	0	0	0	0	1.2	25	50	1/30	15.83
7	-1	-1	1	1	0.9	20	55	1/40	17.37
8	-1	-1	-1	-1	0.9	20	45	1/20	8.56
9	0	2	0	0	1.2	35	50	1/30	13.41
10	-1	-1	1	-1	0.9	20	55	1/20	12.32
11	0	-2	0	0	1.2	15	50	1/30	12.47
12	-1	1	-1	1	0.9	30	45	1/40	7.88
13	1	1	1	1	1.5	30	55	1/40	10.61
14	0	0	0	0	1.2	25	50	1/30	18.98
15	-1	1	1	-1	0.9	30	55	1/20	14.36
16	-2	0	0	0	0.6	25	50	1/30	7.92
17	1	1	-1	1	1.5	30	45	1/40	11.71
18	0	0	0	0	1.2	25	50	1/30	15.55
19	1	1	-1	-1	1.5	30	45	1/20	9.06
20	0	0	0	-2	1.2	25	50	1/10	6.98
21	1	-1	-1	1	1.5	20	45	1/40	21.28
22	0	0	0	2	1.2	25	50	1/50	6.89
23	1	-1	1	-1	1.5	20	55	1/20	10.35
24	-1	1	1	1	0.9	30	55	1/40	16.79
25	1	-1	-1	-1	1.5	20	45	1/20	9.29
26	0	0	0	0	1.2	25	50	1/30	15.55
27	0	0	0	0	1.2	25	50	1/30	15.83
28	0	0	2	0	1.2	25	60	1/30	13.58
29	0	0	0	0	1.2	25	50	1/30	14.95
30	0	0	-2	0	1.2	25	40	1/30	11.86
31	-1	-1	-1	1	0.9	20	45	1/40	15.62

Based on regression equation coefficients of independent and response variables as shown in Table 3, the model for protein yield was as follows:

$$Y = 14.947 - 0.383\ X_1 - 0.552\ X_2 + 0.795\ X_3 + 1.162\ X_4 - 1.659\ X_1X_1 - 0.038\ X_2X_2 - 0.093\ X_3X_3 - 1.540\ X_4X_4 - 0.232\ X_1X_2 - 1.480\ X_1X_3 + 0.091\ X_1X_4 + 1.179\ X_2X_3 - 1.604\ X_2X_4 - 0.803\ X_3X_4 \quad (2)$$

Where Y = protein yield, X_1 is concentration of NaOH, X_2 is hydrolysis time, X_3 is temperature and X_4 is the ratio of cake/NaOH solution.

The values of protein yields in the optimum condition were calculated using the regression model. The value for coefficient of determination R^2 was 0,688. Other studies have shown R^2 ranging from

0.710-0.952 for flaxseed, pigeon pea, tomato and watermelon seeds (Wanasundara & Sahidi 1996, Mizubuti et.al, 2000, Sogi *et.al*, 2003, Wani et.al, 2006).

Effect of NaOH Concentration, time, temperature and ratio pressed cake/ NaOH on Protein Yield

Effect of reaction time, NaOH concentration, temperature and ratio of hydrolysis reagent and pressed cake on protein yield was studied. The four parameters used in this study showed the influence of those parameters on protein yield.

Fig.1. shows protein yield by varying temperature and NaOH concentration at 25 minutes time of hydrolysis and ratio cake to NaOH 1: 30. Higher concentration of NaOH will lead to higher protein yield, but at concentration exceed than 1.2% w/v the yield of protein was lower. It is due to the protein breakdown to small peptides was carried out at higher NaOH concentration as will be explained later in this paper. Higher temperature with higher concentration NaOH will degrade the protein. Hence, to get an optimum yield of protein, the hydrolysis has to be carried at low concentration of NaOH (0.6%w/v) if we used high temperature (60ºC).

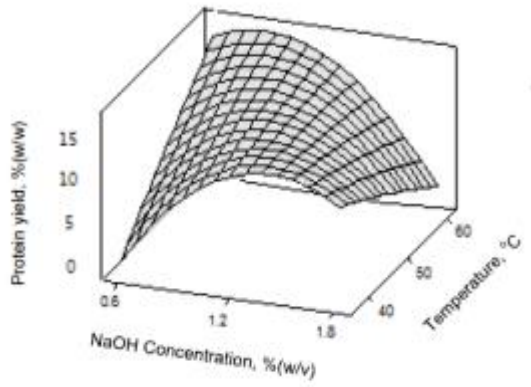

Figure 1. Effect of NaOH concentration and temperature on protein yield for 25 minutes and ratio cake to NaOH 1: 30

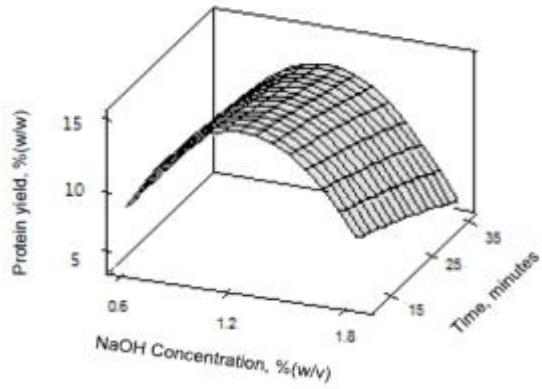

Figure 2. Effect of NaOH concentration and time on protein yield with ratio cake: NaOH 1:30 and hydrolysis temperature of 50ºC

The protein yield was also investigated by varying hydrolysis time and NaOH concentration. As shown in Fig.2, with increase of % NaOH, protein yield increases and later decreases which may be because of the protein degradation at higher NaOH concentration. Reaction time doesn't have such significant effect up to 1.2 % NaOH. However, further increase of reaction time also probably due to the protein degradation for longer time reaction. This result showed that the concentration of NaOH gave more impact than hydrolysis time.

Higher volume of NaOH (up to 1.2 % w/v) increases protein yield. It is consistent with higher ratio cake to NaOH increases protein yield. However, the protein yield started decreasing when the ratio cake to NaOH further increased as shown in Fig.3.

Protein yield was obtained from various hydrolysis times and ratio of pressed cake to solvent is shown in Fig.4. At shorter time, volume of NaOH has very significant effect to increase protein yield as the mechanism for this behavior is explained in Fig.6. Increase of hydrolysis time will not give significant impact in protein yield at certain ratio of solvent to pressed cake. It is clear that the ratio of cake to solvent is significantly impact on protein yield rather than that of hydrolysis time.

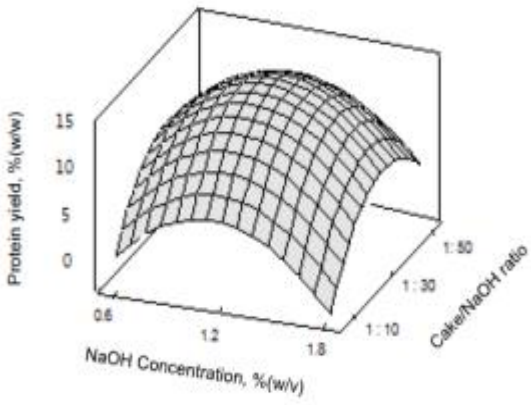

Figure 3. Effect of NaOH concentration and ratio of cake/solvent on protein yield at 50ºC, 25 minutes

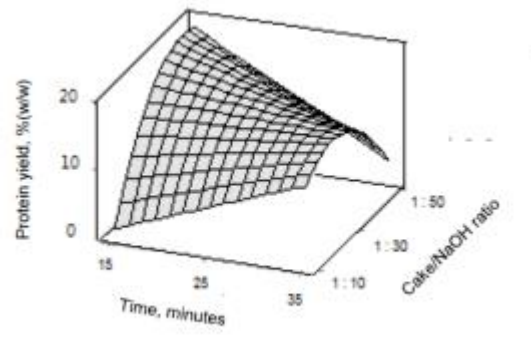

Figure 4. Effects of time and ratio of pressed cake to solvent on protein yield at 50ºC with NaOH concentration of 1.2% w/v.

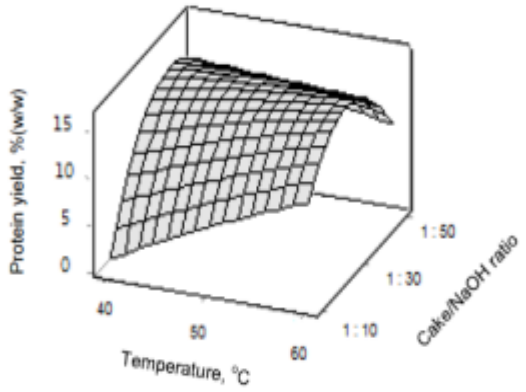

Figure 5. Effects of temperature and ratio of pressed cake to solvent on protein yield with NaOH concentration of 1.2% w/v and 25 minutes of hydrolysis.

Figure 5 shows that yield of protein increased when the hydrolysis of protein was carried out at higher temperature and at lower ratio of cake to NaOH. These phenomena can be explained by the reaction as shown in Figure 6.

The reaction followed the Le Chatelier principle which showed that increase in temperature will make the equilibrium move to endothermic state since the protein hydrolysis is exothermic (Bettelheim, *et. al*, 2014).

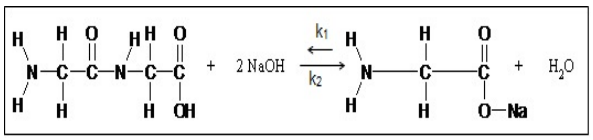

Figure 6. Reaction between pressed cake and alkali solution

The increased of temperature gave less impact for the protein yield at any ratio of cake to solvent, whereas the ratio cake to solvent influenced the protein yield as can be seen from Fig.5 which showed that at various ratio cake to solvent from 1:10 up to 1:30 at various temperature (40ºC – 60ºC), the yield increased. The yield decreased when the ratio cake to solvent is more than 1:30. More solvent in the hydrolysis process will extract more protein. Other study showed the similar result which is higher ratio of solvent to meal will extract more protein. The optimum condition was achieved at 50ºC; 1.5% NaOH and ratio of solvent to meal was 70:1 (Wani, *et.al*, 2006). Our results showed the optimum condition was fulfilled when the temperature was 45ºC, NaOH concentration was 1.5 % and ratio solvent to cake was 40:1.

Optimization Condition for Protein Hydrolysis

The effects of reaction time, NaOH concentration, temperature and ratio of cake to solvent on protein yield showed that 2 out of 4 parameters significantly affected the hydrolysis of *Reuteatlis trisperma* cake. The parameters were ratio pressed cake to NaOH and concentration of NaOH and temperature (P value < 0.05). Yield of protein is linear to NaOH concentration and quadratic function to ratio of cake/NaOH. Based on equation (2), the most optimum condition for hydrolysis of *Reutealis trisperma* cake was at 1.5% w/v NaOH, 15 minutes, 40ºC and the ratio of cake to NaOH concentration was 1: 50 with the highest predicted protein yield was 11.33 %. According to experimental result, the highest soluble protein yield was 21.28% using 1.5 % w/v of NaOH at 45ºC, 20 minutes hydrolysis time and ratio cake to solvent of 1: 40. The protein extracted from the hydrolysis process was detected to have high molecular weight (larger than 11 kDa/kiloDalton) which is more important than small peptide (Fig 7). It was confirmed that the protein breakdown during the hydrolysis process was minimized at lower concentration of NaOH. High concentration of NaOH (1.8% w/v) will result in breakdown of protein to small peptide (less than 11 kDa) which is less valuable for animal feedstock. Hence, the soluble protein was decreased at higher concentration of NaOH which were shown in Fig 1-3. *Reutealis trisperma* plant is one of Indonesia biodiversity. High protein content (34.03%) was detected in the cake which is a positive feature for animal feedstock. The cake itself is a by product of non-edible oil seeds pressing process which is used for biodiesel production. The protein hydrolysis of *Reutealis trisperma* cake was never studied, hence the optimization condition was investigated to get the optimum condition to produce protein hydrolysed.

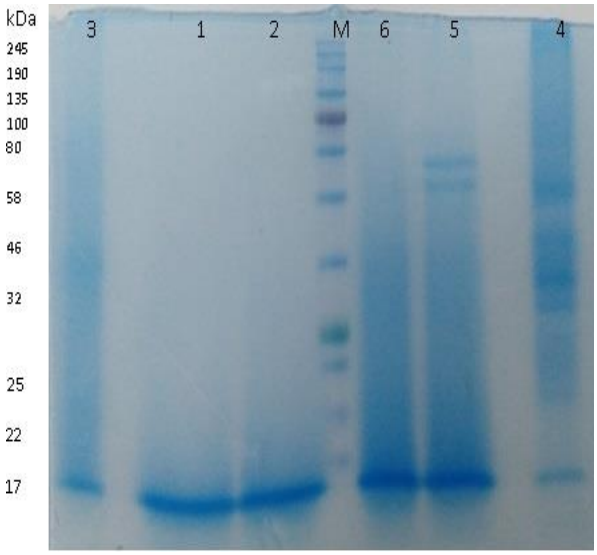

Figure 7. Profile of protein hydrolysate at 50ºC using SDS-PAGE. (1) 1:50, 1.8% NaOH, 15 minutes; (2) 1:50, 1.8% NaOH, 25 minutes; (3) 1:10,1.8% NaOH, 25 minutes.(4) 1:10, 0.6% NaOH, 15 minutes;(5) 1:50, 0.6% NaOH,15 minutes; 1:50, 0.6% NaOH 25 minutes.

CONCLUSION

The present study demonstrates the potential use of *Reutealis trisperma* pressed cake as potential animal feedstock. The characteristic of the biomass showed that high content protein can be considered for value-added feed uses. Based on the experimental result, the optimum variable process for protein hydrolysis was obtained at 1.5% w/v of NaOH, ratio of cake/solvent 1: 40, 45ºC and 20 minutes time of hydrolysis with the protein yield of 21.28%. The RSM model showed that the optimum hydrolysis was obtained at 1.5% w/v of NaOH, ratio of cake/solvent 1: 50, 40ºC and 15 minutes time of hydrolysis with 11.33 % protein yield.

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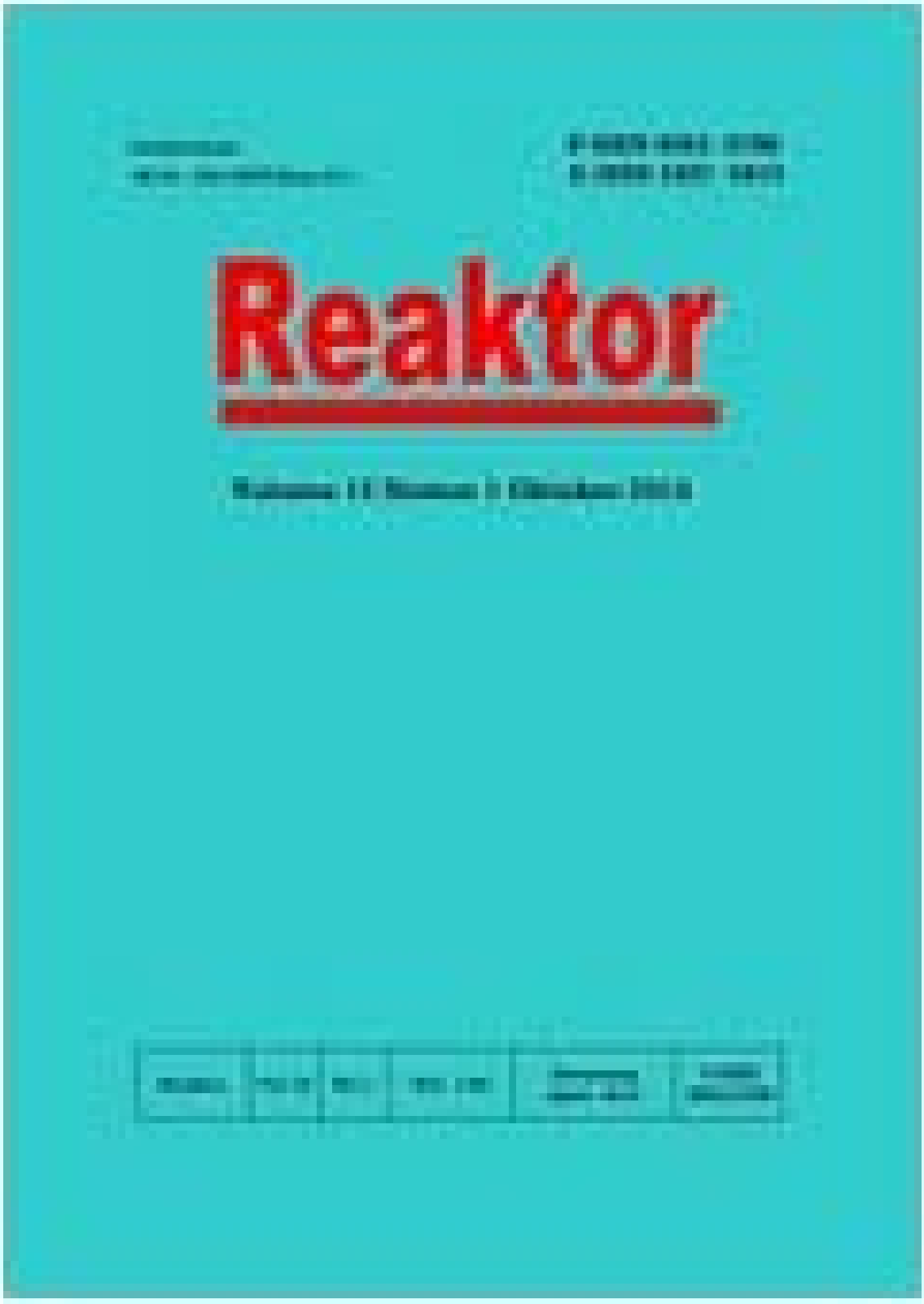
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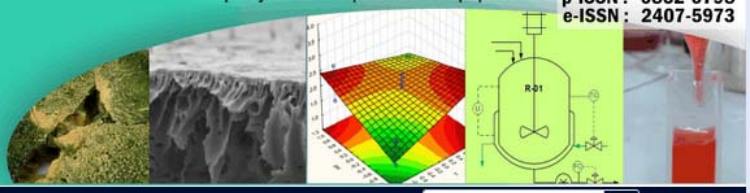

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