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Optimization of Simultaneous Enzymatic Inactivation and Extraction of Linamarin from Cassava Leaf by UV-assisted Photobioextraction

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Abstract. This research objective was to optimize the linamarin production of cassava leaf through simultaneous linamarase inactivation and osmotic dehydration by applied UV- photobioextractor technology. The optimization of the linamarin production process parameters was conducted by applied Response Surface Methodology (RSM) methods. The fixed parameters were comprised of speed rotary mixer of 75 rpm, chopper blades rotating speed of 125 rpm, drying agent of magnesium sulfate, and dryer temperature of 80^0C. Meanwhile the process variables were comprised of ratio of solvent-cassava leaves (10: 1; 15: 1; 20: 1; 25: 1% (w/w)), the concentration of ethanol (80; 85; 90; 95%), and the concentration of drying agent (5; 7.5; 10; 12.5% (w/w)). The optimization of the linamarin production by applied RSM showed that the concentration of solvent (L) was the most influencing parameter process, meanwhile the ratio of solvent-cassava leaves and the concentration of drying agent had no significant effect to levels of linamarin extraction. The research also showed that along with the increasing of concentration of solvents and solvent-feed ratio, the linamarin yield extract was also increasing. The highest linamarin yield was obtained from the simultaneous linamarase inactivation and osmotic dehydration in 90% of ethanol concentration and solvent feed ratio of 15: 1.

INTRODUCTION

Indonesia is the third of the world largest producer of cassava *(Manihot esculenta Crantz*) after Brazil and Thailand [1]. In Indonesia, cassava tuber is currently used as a raw material for starch production and also developed as a raw material for bioethanol production. However, further utilization of leaf, stem or tuber skin of cassava is still limited. Cassava leaf is rich of cyanogen compounds in form of linamarin (2-β-D-glucopyranosyloxy-2-methylpropanenitrile) and lotaustralin ((2R) -2- glucopyranosyloxy β-D-2-methylbutyronitrile). Linamarin is a derivative of valine whereas lotaustralin is a derivative of isoleucin (Peifan, 2002). Linamarin ratio and lotaustralin on leaves and cassava tubers are approximately 93:7. The linamarin content of cassava leaf is ranged from 25-450 ug equivalents of cyanide/g [2].

Linamarin is stated as a good candidate for antineoplastic or an anti-cancer agent. Further utilization of cassava leaf linamarin is hampered by the enzymatic reaction of linamarin by linamarase, a β-glucosidase enzyme that hydrolyze linamarin into cyanohidrin. Furthermore cyanohidrin decomposes into hydrogen cyanide. Linamarin enzymatic hydrolysis process linamarase mainly occurs due to a mechanical process (raw material preparation process) or as a result of microbial activity (fermentation). Hydrolysis of linamarin as seen on Fig 1 is consists of a

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two-stage reaction involving the formation of intermediate compounds, namely acetonecyanohidrin, which subsequently spontaneously or by the action of the enzyme hydroxynitrilelyase will form *acetone* and hydrogen cyanide [3, 4]

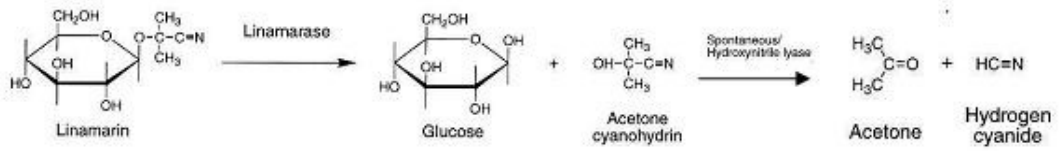

FIGURE 1. The reaction of hydrogen cyanide formation of linamarin

Linamarin conventional extraction were conducted by utilized an organic solvent of acetone [5, 6], and by applied water as the extraction medium. Unfortunately, the linamarin hydrolysis process is still occur due to the activity of linamarase. Basically, linamarin production development is done by inhibiting the activity of hydrolase enzymes (US Patent No. 7,033,618) [7, 8]. Hydrolase enzyme activity is believed to be inhibited by the addition of polar compounds such as ethanol (US Patent No. 2002/0031562 A1) [9]. The enzyme could also inactivated by applied UV. Meanwhile the addition of drying agent such as gelatin, calcium chloride, and sodium sulfate could serves as a drying agent by osmosis. The use of ultraviolet light and its combination with the addition of drying agent are very promising since it inactivate the enzyme and simultaneously extract the bioactive compounds in the extractor. Theoretically, development of UV-photobioextractor and its combination with the addition of drying agent are very likely to be applied. This is because the technology is able to summarize the three phases in a single stage process as follows: linamarase inactivation treatment, the extraction process, and the process of osmotic dehydration [10]. Therefore, the purpose of the study was to determine the optimum process of linamarin production of cassava leaf through simultaneous linamarase inactivation and osmotic dehydration in a UV- photobioextractor technology by applied Response Surface Methodology (RSM) methods [11].

METHODS

Cassava leaves were collected from local farming area in Gunungpati Semarang. The main equipment used in this study are UV-photobioextractor, filtration apparatus, centrifuges, and vacuum separating funnel. As seen on Fig. 2, UV-photobioextractor is comprised of a stirred tank equipped with knife chopper as in the blender and it is also equipped with UV light. Photobioextractor tool set-UV and osmosis dehydration of the stages used for the production of active compounds linamarin. The circuit consists of an extractor tool enzymatic inactivation UV light-based motorcycle equipped mixers, a chopper blade, temperature controllers, round stirrer, and a pH controller.

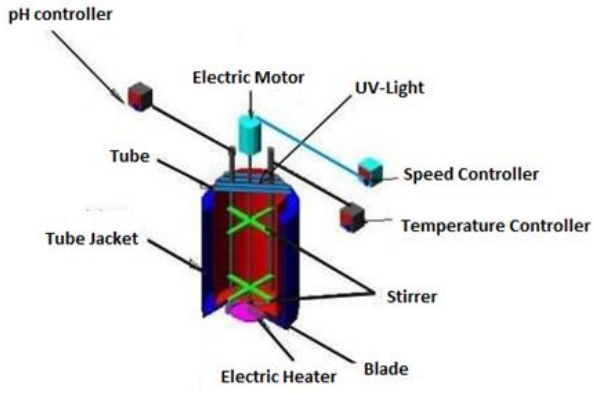

FIGURE 2. Photobioextractor-UV

EXPERIMENTS

Experiments were performed to obtain data useful in determining optimum process conditions. The data have been measured are interpreted using Response Surface Methodology (RSM). Measurement data is done in Laboratory of Chemical Engineering Vocational School Separation Diponegoro University for 4 months.

Process Variables

The fixed parameters were comprised of speed rotary mixer of 75 rpm, chopper blades rotating speed of 125 rpm, drying agent of magnesium sulfate, and dryer temperature of 80^0C. Meanwhile the process variables were comprised of ratio of solvent-cassava leaves (10: 1; 15: 1; 20: 1; 25: 1% (w / w)), the concentration of ethanol (80; 85; 90; 95%), and the concentration of drying agent (5; 7.5; 10; 12.5% (w / w)).

Experimental Procedure

Enzyme inactivation and simultaneous extraction was performed using UV-Photobioextractor with ethanol as the solvent. UV-photobioextractor equipped blades at the bottom and comes to UV light at the top. Cassava leaves and ethanol at a certain temperature and a particular concentration ratio were fed into the extractor. Prior to the extraction process, UV-photobioextractor is also set at a certain temperature. The pH in the enzymatic inactivation of the extraction process is conditioned by addition of phosphate buffer. After the extraction process is complete, the solids are then separated from the extract using a filter or centrifugation. The extract was separated from the solids (supernatant) was purified using activated carbon and tested for its linamarin content by using a Genesys visible UV-spectrophotometer. The experiment method used the UV-Photobioextractor and osmosis dehydration (add the drying agent such a ethanol) for inactivation the linamarse and the linamarin would be extracted.

Purification process

About 60 grams cassava leaf extract dissolved in 250 ml of *distilled water* and added to 80 grams of activated carbon and put in a shaker at 190 rpm for 30 minutes at room temperature to obtain a colorless solution. Furthermore, the solution was filtered using Whatman paper filter with a vacuum flask. Further samples were analyzed for its linamarin content by using a Genesys visible UV-spectrophotometer.

RESULTS AND DISCUSSION

Extraction of linamarin from cassava leaves by applied polar compounds such as ethanol is having dual function, i.e. ethanol is inactivate the linamarase enzyme and ethanol is extract the active compound, and found that linamarase activity is inhibited by the addition of polar compounds [9]. Diffusion of ethanol into cassava leaf cells is depicted in Fig.3. It is intended to make the linamarase located in the cytoplasm penetrated with solvent which caused the inhibitionof the enzyme activity. The next mechanism is that ethanol will infiltrate and penetrate walls of the tonoplast membrane and then comes in contact with the active compound. The polar solvent will diffuse out of the cell by bringing the linamarin out of the cassava leaves.

Enzymes are giant molecules with a molecular weight that varies between 5000-5 million Da. The enzyme belongs to a group that is larger macromolecular protein and consists of a series of linear chains of specific amino acids. In optimum conditions the enzyme will undergo a folding process (Fig.4). Folding process of an enzymes is a process that involves the inclusion of amino acid chains that are hydrophobic to the side inside of the enzymes and process out or shifting chain hydrophilic amino acid gets out of the three-dimensional arrangement of the enzyme.

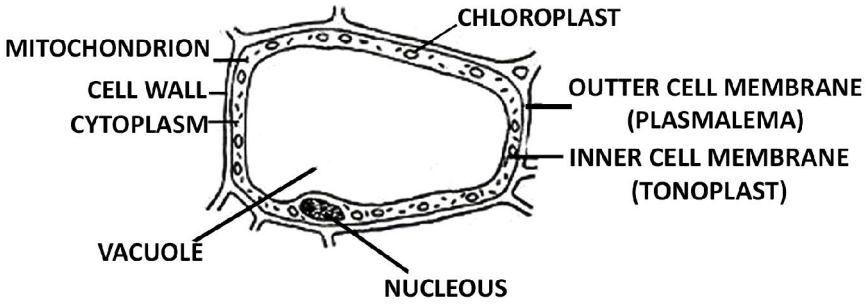

FIGURE 3. Cassava leaf cells

The circuit of amino acids in the enzyme will form a three-dimensional arrangement of certain, specific to each type of enzyme (tertiary structure). Part of the tertiary structure of the enzyme that is responsible for the catalytic activity of the enzyme called the active site. The number of the active site of an enzyme reaches 10-20% of the total volume of the enzyme. The active site of an enzyme usually a hydrophilic gap consisting of a series of amino acid chains that binds the substrate (Fig. 5.a) or bind a cofactor (Fig. 5.b) and catalyzes the reaction.

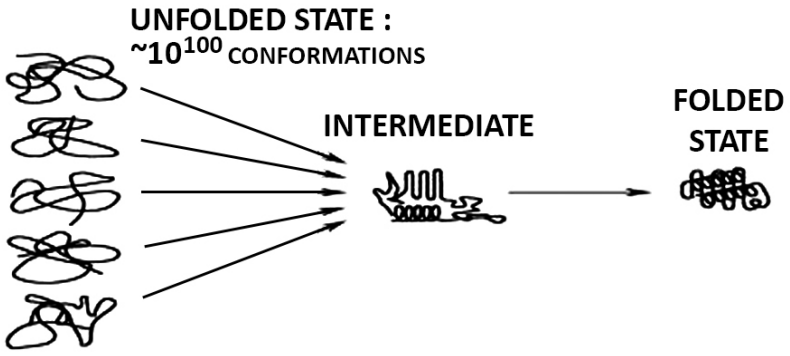

FIGURE 4. The process of folding

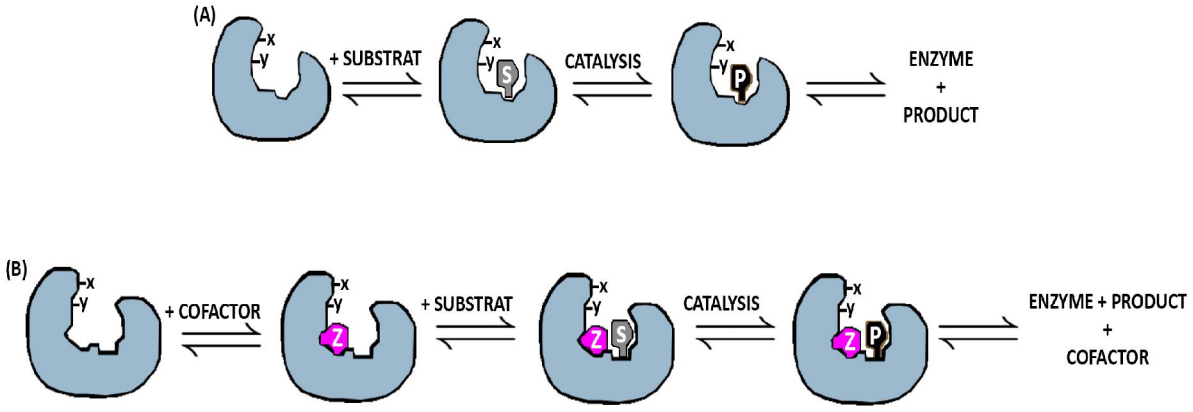

FIGURE 5. The active side of the enzymes

Response Surface Methodology

RSM (Response Surface Methodology) is a statistical method for the design of experiments, mathematical modeling, optimization and statistical analysis in the study. By using RSM, a quadratic polynomial equation developed to estimate the results of the experiment as a function of the interaction between the independent variables. The coefficient of the empirical model estimated using regression analysis techniques multidirectional present in RSM. The data of the linamarin extraction results obtained at various feed ratios of solvent, solvent concentration and drying agent designed using Central Composite Design of RSM is presented in Table 1.

TABLE 1. Transmittance of the linamarin extract

Ratio	Solvent Concentration(L)	Drying agent(L)	Transmittance(DV)
10.00000	85.00000	7.50000	86.1
10.00000	85.00000	12.50000	97.2
10.00000	95.00000	7.50000	85.2
10.00000	95.00000	12.50000	85.2
20.00000	85.00000	7.50000	85.5
20.00000	85.00000	12.50000	95.7
20.00000	95.00000	7.50000	81.4
20.00000	95.00000	12.50000	81.4
6.59104	90.00000	10.00000	91.4
23.40896	90.00000	10.00000	88.6
15.00000	81.59104	10.00000	97.2
15.00000	98.40896	10.00000	84.7
15.00000	90.00000	5.79552	97.2
15.00000	90.00000	14.20448	85.9
15.00000	90.00000	10.00000	89
15.00000	90.00000	10.00000	89

The data generated from the experiments were then be analyzed to obtain the estimated value of the main effects and interactions as well as the mathematical model equations. The main effects of price data and interaction effects are presented in Table 2.

TABLE 2. Summary effect

Factor	Effect	Std.Err.	t(6)	P	-95% Cnf.Limt	+95% Cnf.Limt	Coeff	Std.Err. Coeff	-95% Cnf.Limt	95% Cnf.Limt
Mean	89.36010	3.699326	24.15578	0.000000	80.3082	98.41203	89.36010	3.699326	80.30818	98.41203
(1)Ratio (L)	-2.11015	2.839665	-0.74310	0.485489	-9.0586	4.83826	-1.05508	1.419832	-4.52928	2.41913
Ratio (Q)	-1.03161	3.447784	-0.29921	0.774874	-9.4680	7.40482	-0.51580	1.723892	-4.73402	3.70241
(2)Solvent Concentration(L)	-7.66244	2.839665	-2.69836	0.035654	-14.6109	-0.71403	-3.83122	1.419832	-7.30543	-0.35702
Solvent Concentration(Q)	-0.35985	3.447784	-0.10437	0.920275	-8.7963	8.07657	-0.17993	1.723892	-4.39814	4.03828
(3) Drying Agent(L)	0.33620	2.839665	0.11840	0.909618	-6.6122	7.28461	0.16810	1.419832	-3.30610	3.64231
Drying Agent (Q)	0.06441	3.447784	0.01868	0.985701	-8.3720	8.50083	0.03221	1.723892	-4.18601	4.25042
1L by 2L	-1.37500	3.710200	-0.37060	0.723667	-10.4535	7.70353	-0.68750	1.855100	-5.22677	3.85177
1L by 3L	-0.22500	3.710200	-0.06064	0.953613	-9.3035	8.85353	-0.11250	1.855100	-4.65177	4.42677
2L by 3L	-5.32500	3.710200	-1.43523	0.201229	-14.4035	3.75353	-2.66250	1.855100	-7.20177	1.87677

The estimation values presented in Table 2 show the effect of the influence of variable feed rate: solvent, solvent concentration and *drying agent* used against linamarin levels. The greater the estimated effect of a variable price indicates the greater influence on the process variable extract obtained. The effect of variable feed rate of solvent, solvent concentration and *drying agent* as well as the interaction of variables can be seen from the Pareto chart (Fig. 6) which describes the relationship between the estimated effects of standardized variables. Price effects of variables through the line p = 0:05 is the most influential variable, while the price effects of variables that do not cross the line p = 0:05 is not an influential variable so that it can be ignored. RSM analysis results against linamarin extraction experiments showed that the most influential variable is the concentration of the solvent (L). Variable ratio (L) are also influential but not as much the effect of variable concentrations of solvent (L).

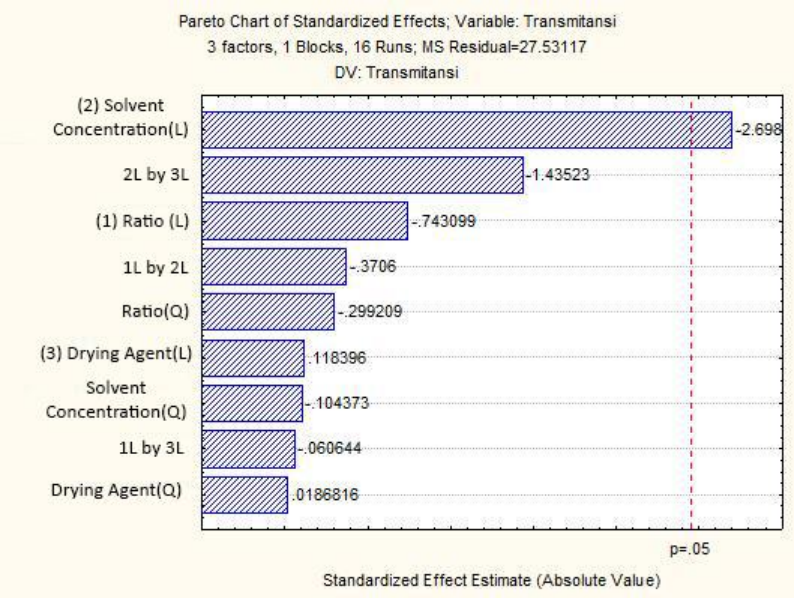

FIGURE 6. Pareto chart

Based on the ANOVA results (Table 3) shows that the concentration of solvent (L) significantly (p-value less than 0.05). By changing the feed ratio of solvent and additional *drying agent* had no significant effect (p-value greater than 0.05) to levels linamarin extracted. more than 0.05 (not significant).

TABLE 3. ANOVA of Extraction linamarin

Factor	SS	df	MS	F	p
(1)Ratio(L)	15.2026	1	15.2026	0.552196	0.485489
Ratio (Q)	2.4647	1	2.4647	0.089526	0.774874
(2)Solvent Concentration (L)	200.4587	1	200.4587	7.281154	0.035654
Solvent Concentration(Q)	0.2999	1	0.2999	0.010894	0.920275
(3)Drying Agent(L)	0.3859	1	0.3859	0.014018	0.909618
Drying Agent(Q)	0.0096	1	0.0096	0.000349	0.985701
1L by 2L	3.7812	1	3.7812	0.137344	0.723667
1L by 3L	0.1013	1	0.1013	0.003678	0.953613
2L bz 3L	56.7112	1	56.7112	2.059893	0.201229
Error	165.1870	6	27.5312		
Total SS	445.0094	15			

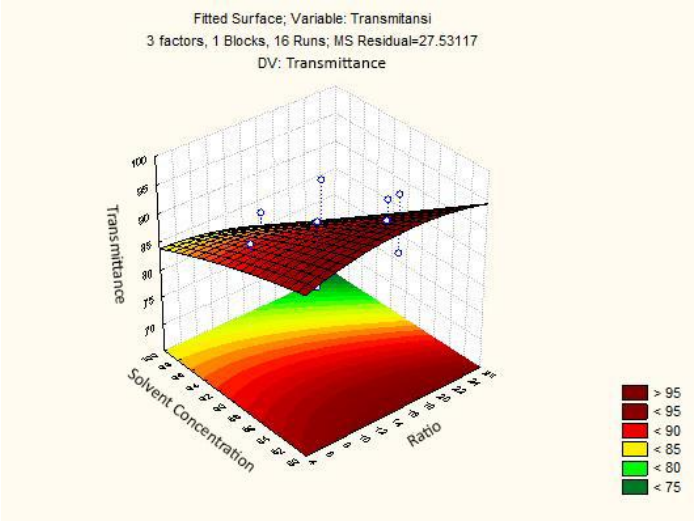

FIGURE 7. 3-dimensional optimization of solvent concentration ratio curve

A suitable coefficient model, coefficient of determination (R^2) should have a proportion at least 80 of% the variation . Response the associated value of R^2 of the model generated from this study was 0.9823. These results indicate that the resulting model is very appropriate because it has a rate of R^2 is more than 80%. The value of R^2, which exceeds 80% indicates the proportion of the high variety.

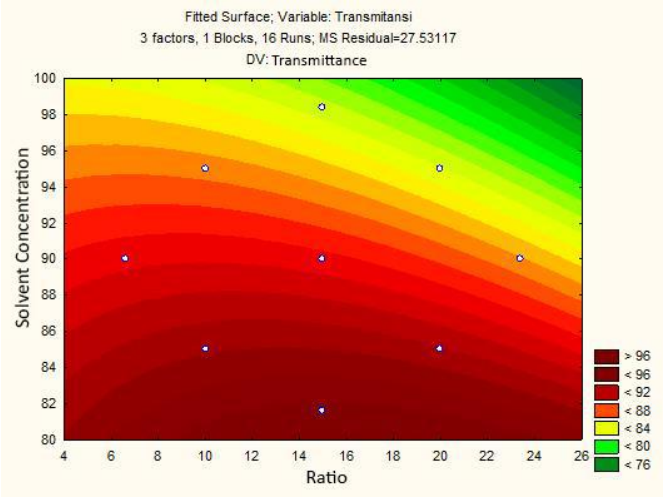

FIGURE 8. 3-dimensional Concentration vs. the ratio of surface contours solvent curve

Furthermore, 3-dimensional optimization curve and surface contour graph which is a graph of concentration and solvent-solvent to feed ratio linamarin level (transmittance) is presented in Figure 7-10. The greater the concentration of solvents and solvent-feed ratio, the greater the linamarin extracted.

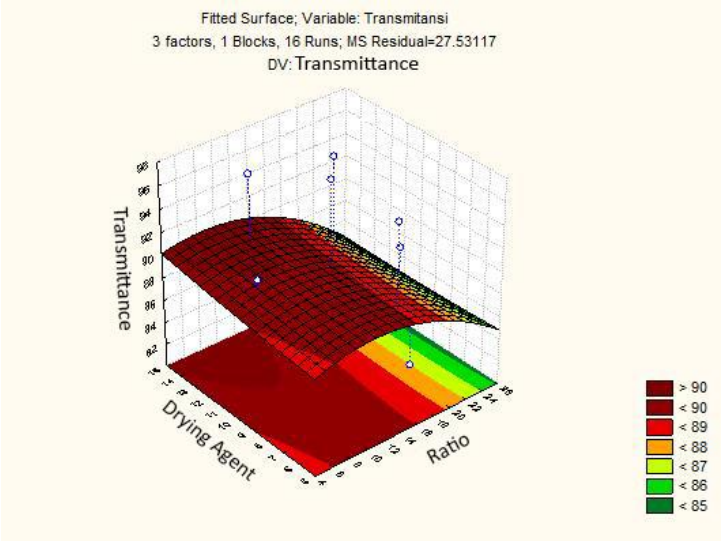

FIGURE 9. 3-dimensional optimization versus the ratio of drying agent curve

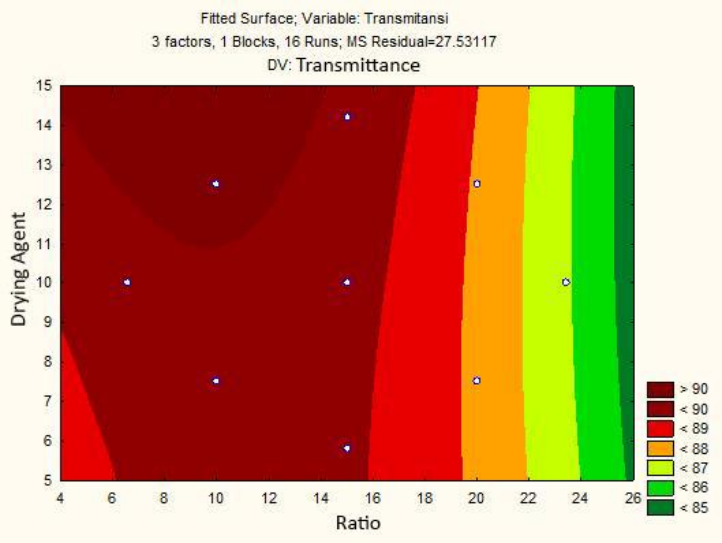

FIGURE 10. Contour versus the ratio of drying agent curve

CONCLUSION

The optimization of the linamarin production by applied RSM showed that the concentration of solvent (L) was the most influencing parameter process, meanwhile the ratio of solvent-cassava leaves and the concentration of drying agent had no significant effect to levels of linamarin extraction. The research also showed that along with the increasing of concentration of solvents and solvent-feed ratio, the linamarin yield extract was also increasing. The highest linamarin yield was obtained from the simultaneous linamarase inactivation and osmotic dehydration in 90% of ethanol concentration and solvent feed ratio of 15: 1.

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