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UTILIZATION OF MEMBRANE MICROFILTRATION IN PREPARATION OF HYDROLYZED VEGETABLE PROTEIN FROM FERMENTED RED BEAN *(Phaseolus vulgaris* L.*)* EXTRACT AS FORTIFICATION AGENT

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ABSTRACT

Preparation of Hydrolyzed Vegetable Protein (HVP) as savory flavor from fermented red bean broth through stirred membrane cell using micro filtration membrane with pore size of 0.45 µm was performed to get fortified agent utilized in preparation of beans sauce. The objective of this work was to study an effect of pressure and kind of red bean broth extract on content of total protein, soluble protein and dry solid in the retentate and permeate as hydrolyzed vegetable protein used for fortified agent of red bean sauces. Preparation process of hydrolyzed vegetable protein was done using fixed rotary speed of 400 rpm, pressure of 20, 25 and 30 psi at room temperature. To investigate the effect of pressure on this separation, the feed were red bean broth extract fermented for 6, 8, 10 and 12 weeks, respectively. Fermentation process were conducted using salt fermentation with inoculum of Rhizopus-C_1, *salt and red bean ratios of 30:10:60%. The analysis of flux and contents of total protein, dissolved protein and dry solid in the retentate and permeate was carried out, and the result of experiment showed that interaction of Red bean broth extract with 6, 8, 10 and 12 weeks of fermentation and operation condition of microfiltration membrane separation tends to affect on flux and content of total protein, dissolved protein and dry solid in retentate and permeate. Red bean broth extract for 6 weeks fermentation resulted higher protein content in permeate as hydrolyzed vegetable protein than in retentate. Permeate at pressure of 25 psi gives flux value of 0.0217 mL/cm^2.minute and contents of total protein of 1.31 %, dissolved protein of 6.9 mg/g, and dry solid of 2.6%, while retentate as hydrolyzed vegetable protein or fortified agent indicate contents of total protein of 1.52%, dissolved protein of 4.15 mg/g, and dry solid of 3.64%. It was found that micro filtration process was able to increase dissolved protein content of about 3 times.*

Keywords: *Microfiltration, membrane, Hydrolyzed Vegetable Protein (HVP), fermented red bean (*Phaseolus vulgaris L.*).*

INTRODUCTION

Legumes have been a staple part of diets for thousands of years and remain the main source of protein in many countries today. They are high in complex carbohydrates, protein, and fiber, yet are extremely low in fat and also contain bioactive chemicals and many volatile components, such as anti-oxidants [1]. Legumes are an important and inexpensive source of vegetable protein that might be used as an alternative of dietary protein in human nutrition. Recently, legume protein products such as hydrolysates, concentrates and isolates have been considered as a potential ingredient in the food industry. Hydrolyzed Vegetable Protein (HVP) is a valuable ingredient in food because of their high nutritional value and possible health benefits. Legumes possess the ability to reduce oxidative damage associated with many diseases, including cancer, cardiovascular disease, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases, aging and brain dysfunction. One of the kinds of legumes utilized in preparation of hydrolysates vegetable protein as fortified agents in various food applications is red beans. They possess a well-established history, greater nutrition, healthful properties and functional characteristics [2,3].

Preparation of hydrolysates vegetable protein from red bean broth extract is related with process of salt fermentation. Salt fermentation on red beans are the basic process in which role of inoculum and fermentation conditions have affect on the product of crude broth. An optimum fermentation time is required for the production of red bean broth. Longer fermentation will results in the production of too high levels of amino acids and volatile compounds when consumed. Fermentation processes in the food industry are being used for several purposes. One of these purposes is in preserving against spoilage by spontaneous fermentation. The product of this fermentation process can be consumed within several weeks. The red bean fermentation process is a spontaneous process caused by the fungi present on the starter. It is initiated by hydrolysis of protein, thereby liberating nutrients for the fungi, allowing then

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to proliferate under aerobic facultative conditions. During the fermentation process, the carbohydrate is converted into glucose, the protein is converted into amino acids, especially glutamate acid. The fat is converted into fatty acids. All of them are accumulated to form volatile compounds, such as flavor. In red bean broth preparation, proteolytic enzymes from *Rhizopus*-C_1 play the major role of digestion of bean proteins as the main substrate. Prolonged fermentation time in preparation of crude broth affect on the composition of protein, especially soluble protein. Protein is main parameter for content amino acids, such as glutamate acid and compounds of aroma, taste and flavor. During salt fermentation process, enzymes yielded by fungi will degrade and change carbohydrate, protein, fat and other compounds to simple compounds in which they affect on end products, such as aroma, taste and flavor [4]. Prolonged time of fermentation, degradation of protein of beans and interaction with the other processes (salt content, kind and concentration of inoculum) will increase contents of soluble protein and N-amino [5].

The application of protein (peptides) as functional and nutritional ingredients in food industry has been gaining interest during the last decades. Hydrolysates vegetable protein often must be fractionated to obtain the peptides with a higher functionality or higher nutritional value in a more purified form. Since the differences in physico-chemical properties of these peptides are often small, a separation technology which can discriminate small differences in size and hydrophobicity of species is needed. Potential applications of membrane technology in the food industry are numerous and include microfiltration, ultrafiltration, nanofiltration and reverse osmosis. Microfiltration is a pressure driven process with microporous membranes for separating particles from solid/liquid suspensions. Although the exact size range of particles which can effectively be remove by microfiltration is a matter of debate, microfiltration is generally applied to suspensions containing colloidal or fine particles with linear dimensions in the range of 0.02 – 10 μm. Most of the components in red bean broth extract fall into this size range. Red bean broth extract contain protein (amino acid), carbohydrate, fat, sugar (fructose, glucose & sucrose) and volatile compounds to form taste, flavor, aroma, minerals, fungi spore and bacteria. Protein has a particle size of 0.04 – 2 μm, particle size of fat was more than 0.5 μm, the particle size of sugar was between 8 – 20 Å and particle size of volatile compounds, such as taste, aroma and flavor was less than 0.04 μm. The separation is primarily based on size, although adsorption able to play a role in some applications. Among the many interesting applications of microfiltration is protein separation from fermentation broths, for which high permeate flux and high protein transmission through membrane are desired. In operation, a pressure differential drives the fluid through the membrane and the particles or suspended matter are trapped by the membrane and accumulate on the surface. Particles are retained by different mechanisms, depending on the type of membrane and the nature of the particles. If the membrane pores are smaller than the particles, the solids are retained by sieving or surface filtration mechanisms. Depth filtration processes occur when the pores of the membrane are larger than the particles [5-7].

In the present work, we investigated influence of pressure with constant stirrer speed of 400 rpm at various kind of red bean broth extracts fermented for 6, 8, 10 and 12 weeks on content of total protein, dissolved protein and dry solid in retentate as hydrolysates vegetable protein. Such a product could be used for sauce, instant soup, paste products, mayonnaise, flavor enhancer and a variety of food uses.

EXPERIMENTAL SECTION

Material

The materials used in this work was fresh red beans (*Phaseolus vulgaris* L.) purchased from a local market, broth inoculum from *Rhizopus* -C_1 [Research Center for Chemistry, Indonesian Institute of Sciences], chemical agents for analysis and micro filtration membrane of fluoro polymer (0.45 μm in pore size and 30.175 cm^2 in effective membrane area) manufactured by Danish Separation Systems, Denmark.

Instruments

The equipments utilized in this experimental was process equipment of fermentation and preparation of broth, trays, autoclave, dead-end cell (Amicon 8200) equipped with stirrer and instrument for chemical analysis.

Procedure

Preparation process of red bean broth extract

Preparation of red bean broth inoculum. Red beans were sorted, washed, soaked for 18 - 22 h and dehulled of skin. The soak water was discarded and beans leaked. The beans were then sterilized using autoclave at 121 °C for 15 min. Inoculation process was conducted by adding *Rhizopus* -C_1 (0.6%, w/w). The mixture of substrates were sprayed on tray, incubated at 30 °C for 64 h, dried using cabinet oven at 50 °C for 24 h, milled into fine flour and sieved through 80 mesh stainless steel screens. The flour of substrate was sealed into plastic bags and further used.

Fermentation of red bean broth and preparation of red bean broth extract. Red beans were sorted, washed, soaked for 18 – 22 h, dehulled,

sterilized using autoclave at 121 °C for 15 min, cooled at about 35 °C and mixtured with broth inoculum and salt in aseptic condition. This substrate was prepared with red beans, inoculum and salt ratios of 60:30:10%, w/w. The red beans as substrates 150 g (60%, w/w) were filled into glass jars (about 350 mL). To the jars were further added 25 g (10%, w/w) NaCl and inoculum 75 g (30%, w/w). A total of 8 glass jars were thus filled and covered with lined screw caps. All 8 jars were cap-sealed, shaken to mix thoroughly, and incubated at 30 °C for 6, 8, 10 and 12 weeks including 2 folds of replicate. During the fermentation, the products were sampled each 2 weeks for analyses, such as total protein, dissolved protein and dry solid. The fermented samples were sealed in a plastic bag. The mixture of substrates was fermented in glass jar with agitation 1 – 2 times/week at room temperature for 6, 8, 10 and 12 weeks. The product of fermentation was then extracted with an additional tap water 80 °C for 20 min in fermented red beans broth and water ratios of 1:7 (w/w), homogenized with a homogenizer (Ultra Turrax T50) operated at 4000 rpm for 15 min and filtered through 200 mesh stainless steel screens so resulted supernatant I and insoluble residue. The insoluble residue was reextracted in hot water for 10 min residue and water ratios of 1:1 (w/w), homogenized with a homogenizer operated at 4000 rpm for 15 min and filtered by 200 mesh stainless steel screens so resulted supernatant II and insoluble residue. Supernatant I and II were mixtured. The mixed supernatant was designated as the Red bean broth extract and used as feed during micro filtration processing.

Separation of Red bean extract using micro filtration membrane. Microfiltration experiments were performed at room temperature using an Amicon 8200 stirred cell of 180 mL in capacity. The stirred cell was initially filled with pure water. This membrane was flushed with at least 50 mL of pure water prior to use to remove any wetting agents. The water flux was then measured as a function of time at a constant pressure until a steady flux was attained. The stirred cell was then emptied and refilled with a red bean broth extract solution. The red bean broth extract liquid in the cell was stirred at a speed of 400 rpm and pressure of 20, 25 and 30 psi provided by nitrogen gas from a cylinder, and the permeate solution was then collected and measured at appropriate intervals. Permeate and feed/retentate samples were collected for subsequent analysis. At the end of the experiment, the stirred cell and membrane were gently rinsed with pure water, and the water flux through the fouled membrane was reevaluated at the same pressure used during the Red bean broth extract filtration.

Investigation and Analysis

Total protein, dissolved protein and dry solid in red bean broth fermented for 6, 8, 10 and 12 weeks, fermented red bean extract of 6, 8, 10 and 12 weeks after extraction process (1:8 crude red bean broth to total water ratio, filtration of 200 mesh) were determined according to Kjeldahl, Lowry and gravimetry methods, respectively [8]. Investigation of filtration process was conducted on flux at stirrer speed of 400 rpm with pressure of 20, 25 and 30 psi. Contents of total protein, dissolved protein and dry solid in permeate and retentate were determined using the previously methods.

RESULT AND DISCUSSION

Effect of activity of proteolytic and amilolytic broth inoculum & fermentation time on crude broth and characteristic of red bean broth extract.

A broth inoculum prepared using red bean substrate and inoculated with the activated *Rhizopus* -C_1 at 0.6% (w/w), incubated at 30 °C for 64 h and allowed to dry at 50 °C for 24 h showed that proteolytic and amilolytic activity was high. Components in this broth inoculums were total protein of 30.8%, dissolved protein of 7.7 mg/g, N-amino of 1.47 mg/g, reduction sugar of 35 mg/g and water of 9.88%. While activities of protease and amylase enzymes were 0.95 and 7.5 Unit/g, respectively. In fermentation process, broth inoculum is generally fungi seeds which are able to produce enzymes utilized in hydrolysis of components in substrate. At this fermentation process is used inoculum of red bean substrate incubated with *Rhizopus* -C_1 so its composition is almost similar with red bean composition. Inoculum of red bean owns higher composition (total protein and dissolved protein) than rice substrate so that proteolytic and amilolytic activity in red bean inoculum is higher in rice substrate. These activities relates with intrinsic factors (level of fungi purity, fungi species) and extrinsic factors (incubation time, starter concentration, aeration, kind of substrate and drying temperature and time). Inoculum of broth using *Rhizopus* -C_1 showed higher amylase activity (7.5 Unit/g) than proteolytic activity (0.95 Unit/g). This condition enables to yield product of specific taste broth because of ability of amylase activity to degrade carbohydrate in red bean is more effective than protein. This ability is also affected by other factors, such as inoculum, salt and red bean ratios and prolonged fermentation time. At 6, 8, 10 and 12 h of red bean fermentation produced broth and its characteristics are summarized in Table 1.

All process showed that long fermentation time will increase content of dissolved protein. The Changes in this component was related to degrading of protein component in red bean by protease enzyme during fermentation process and ratios of inoculum, salt and red bean. Utilizing of high broth inoculum concentration (60%) enables to contribute increasing total protein so

Table 1. The characteristics of crude Red bean broth inoculated with *Rhizopus* -C_1 and Red bean broth extract filtered through 200 mesh stainless steel screen.

No	Kind of material	Total Protein, %	Dissolved protein, mg/mL	Dry solid, %
1	Rb-6	28,38	5,8	84,73
2	Rb-8	26,53	8,2	83,75
3	Rb-10	26,94	6,9	78,85
4	Rb-12	35,7	7	85,37
5	RbE-6	2,72	2,4	5,45
6	RbE-8	2,9	2,4	4,77
7	RbE-10	2,57	2,3	7.61
8	RbE-12	2,36	2,9	4,65

Rb-6 = Red bean broth after 6 weeks of fermentation.
Rb-8 = Red bean broth after 8 weeks of fermentation.
Rb-10 = Red bean broth after 10 weeks of fermentation.
Rb-12 = Red bean broth after 12 weeks of fermentation.
RbE-6 = Red bean broth extract (Mixed supernatant) after 6 weeks of fermentation.
RbE-8 = Red bean broth extract (Mixed supernatant) after 8 weeks of fermentation.
RbE-10 = Red bean broth extract (Mixed supernatant) after 10 weeks of fermentation.
RbE-12 = Red bean broth extract (Mixed supernatant) after 12 weeks of fermentation.

that dissolved protein content of broth is also high due to its proteolytic activity in degrading complex protein molecule to simple peptides. Red bean fermentation product is semi solid material. This product is extracted by adding water at total ratio of 1:8 at 80 ºC for 15 min, and homogenized at 4000 rpm for 15 min and allowed to filter through 200 mesh stainless steel screens**.** For all kinds of broth extract showed decreasing of component concentration, and this was caused by adding water and filtering through the screens. The aim of extraction, homogenization and filtration of red bean broth was to obtain components in broth suspension as much as possible so that it will be easy to separate soluble components using microfiltration membrane.

Effect of micro filtration membrane pressure and kind of red bean broth extract on composition permeate and retentate

The effect of fermentation time of Red bean broth extract on flux using microfiltration membrane of 0.45 µm at pressure of 20, 25, and 30 psi and stirrer speed of 400 rpm is shown in Figure 1. Separation process of the needed components in red bean broth extract produced by fermentation of 6, 8, 10 and 12 weeks using microfiltration membrane showed that fluxes increased from 6 to 8 weeks but decreased up to 12 weeks. For fermentation product of 8 weeks at all various pressure gives high flux. The fermentation product of 8 weeks at pressure of 30 psi results the highest flux (0.0283 mL/cm^2.min), whereas at pressure of 20 and 25 psi results the flux of 0.0216 and 0.026 mL/cm^2.min, respectively, whereas fermentation product of 12 weeks results low flux.

Fluctuated fluxes relates with composition of extract suspensions which directly affect on extract

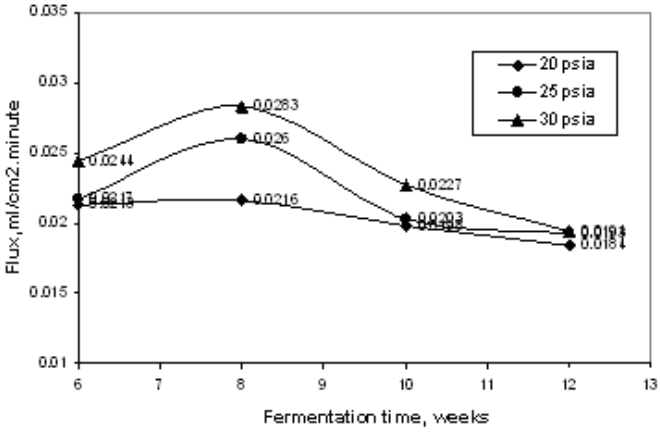
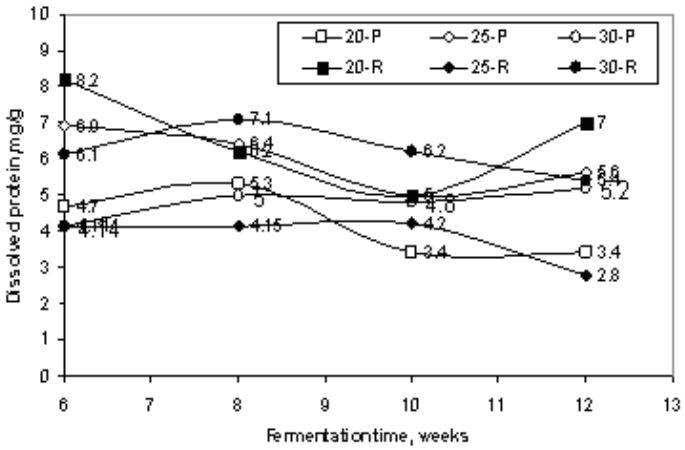


Figure 1. Effect of fermentation time of red bean broth extract on flux

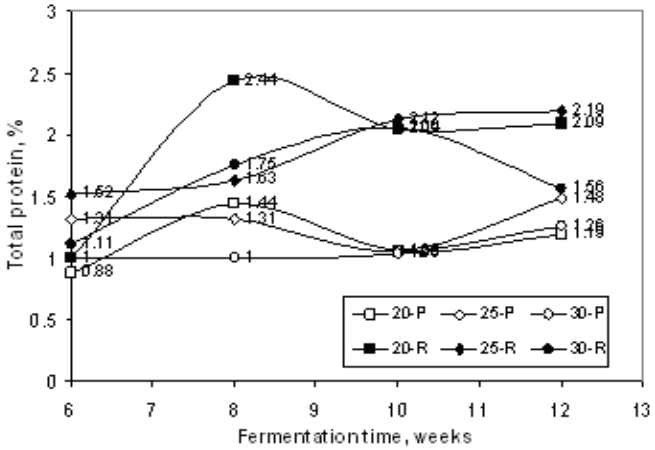
suspension viscosity and interaction between stirrer speed and pressure. For constant stirrer speed (400 rpm), the high pressure enables to increase flux value [9]. Red bean broth extract produced by fermentation of 8 weeks contains of 2.9% total protein, 2.4 mg/g dissolved protein and 4.77% dry solid, and the total protein content is higher than that in broth extract produced by fermentation of 6, 10 and 12 weeks. It can be explained that the dissolved protein and solid permeation through the membrane depend not only on physico-chemical properties of the membrane and the fluid, but also on the operating conditions, such as pressure, stirrer speed, temperature and membrane pore size. In this work, the analysis of the experimental results was focused on the effect of operating pressure on flux and contents of total protein, dissolved protein and dry solid in permeate and retentate.

The effect of fermentation time of Red bean broth extract on dissolved protein in retentate and permeate



Legend : P: Permeate, R: Retentate

Figure 2. Effect of fermentation time of red bean broth extract on dissolved protein in retentate and permeate



Legend : P: Permeate, R: Retentate

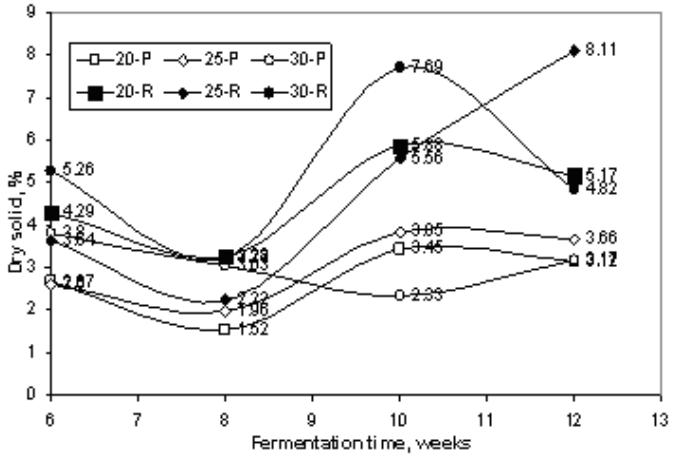
Figure 3. Effect of fermentation time of red bean broth extract on total protein in retentate and permeate

using microfiltration membrane of 0.45 µm at pressure of 20, 25, and 30 psi and stirrer speed of 400 rpm is shown in Figure 2.

It can be seen from Figure 2 that the curves are non linear, which indicates that content of dissolved protein in retentate are higher than in permeate because of accumulation of protein particles on the membrane. The rate of increasing of dissolved protein content in the retentate during fermentation of 6, 8, 10 and 12 weeks and pressure of 20, 25 and 30 psi fluctuated but tend to decrease slowly. Retentate of broth extract with fermentation of 6 weeks and pressure of 20 psi showed that dissolved protein content (8.2 mg/g) is higher than at pressure of 25 psi (6.9 mg/g) or 30 psi (6.1 mg/g). The similar tendency is indicated on permeate, in which prolonged fermentation time reduced dissolved protein content for 6 weeks of fermentation with pressure of 20 and 30 psi but tended to constant at pressure of 25 psi. The broth extract with 10 weeks of fermentation gives the lowest dissolved protein content in retentate and permeate than fermentation of 6, 8 and 12 weeks. This decrease is caused by high dissolved protein content in initial feed and prolonged fermentation time so that it occurred fouling on the membrane surface. At stirrer speed of 400 rpm and pressure of 25 psi, the membrane was not able to allow the particles passed through its pores because of high total protein content in initial process (2.72%), therefore the flux was low. This condition caused dissolved protein accumulated on the membrane surface as retentate and protein particles with lower molecular weight and smaller than 0.45 µm which were able to pass through pores as permeate. This condition gave the difference at pressure of 30 psi, in which high driving force at constant stirrer speed (400 rpm) enabled to form equilibrium of flux so that dissolved protein content in retentate was similar to permeate. From the above observation indicated that there was a relation between kinds of broth extract and fermentation time and separation process condition at the difference pressure which can reduce yield of protein hydrolysate or prolonged fermentation time would yield retentate as protein hydrolysate with low dissolved protein content because many protein particles with lower molecular weight would pass through the membrane pores. Protein hydrolysate with high dissolved protein content which is yielded through microfiltration membrane of 0.45 µm at stirrer speed of 400 rpm and pressure of 25 psi was suitable for its function as HVP which was produced by fermentation process of 6 weeks, in which dissolved protein content of 2.4 mg/g increased to be 4.15 and 6.9 mg/g in the retentate and permeate, respectively. Red bean broth extract with 6 weeks of fermentation using separation of microfiltration membrane and giving high dissolved protein content (8.2 mg/g) as retentate at pressure of 20 psi represented the recovery of dissolved protein which equivalent to dissolved protein from red bean broth through spontaneous fermentation of 8 weeks (8.2 mg/g).

The effect of fermentation time of red bean broth extract on total protein in retentate and permeate using microfiltration membrane of 0.45 µm at pressure of 20, 25, and 30 psi and stirrer speed of 400 rpm is shown in Figure 3.

The separation process using microfiltration membrane with pressure of 20, 25 and 30 psi indicated that total protein content in retentate was higher than in permeate. For all separation processes occurred, total protein content in retentate and permeate increased but this increasing of total protein content in retentate or permeate was not indicate the increase of dissolved protein content. Separation of microfiltration membrane of 0.45 µm showed that at lower pressure, kinds of broth extract and prolonged fermentation time will yield higher total protein. At prolonged fermentation time, protein particles were accumulated on membrane



Legend : P: Permeate, R: Retentate

Figure 4. Effect of fermentation time of red bean broth extract on dry solid in retentate and permeate.

surface so that total protein content in retentate will be higher than in permeate. This is shown that broth extract with 8 weeks of fermentation, pressure of 20 psi would produce retentate as protein hydrolysate with the highest total protein content (2.44%) than at 25 psi (1.63%) and at 30 psi (1.75%). High total protein in retentate of broth extract was caused by the highest total protein content (2.9%) in the feed so that lower flux (0.0216 mL/cm^2.min) enabled the particles accumulated on membrane surfaces, therefore the particles were difficult to pass through membrane pores. This condition would result higher total protein content for other retentate as well.

The effect of fermentation time of red bean broth extract on dry solid in retentate and permeate using microfiltration membrane of 0,45 µm at pressure of 20, 25, and 30 psi and stirrer speed of 400 rpm in Figure 4. All processes indicated that prolonged fermentation time would increase dry solid content in retentate and permeate but at certain condition it started decrease. The highest dry solid content (8.11%) in retentate was taken place at fermentation of 12 weeks, and pressure of 25 psi. Dry solid of broth extract was an accumulation of all components in extract which were still in crude broth. Through fermentation process, extraction with an additional hot water and separation using microfiltration membrane, all components agglomerated to form solids containing protein, carbohydrate, fat, salt, mineral and other organic compounds. The possibility of prolonged fermentation time of broth extract contains components passing through membrane pores with water as permeate due to their low molecular weight as an effect of fermentation process. Although high operating pressure could still affect fouling on membrane surface so that it would be difficult in separation. For all processes, dry solid content in retentate is higher than in permeate.

The separation process using microfiltration membrane has potential role as separation method of vegetable broth extract due to its ability to produce retentate and permeate as fortification agent.

CONCLUSION

Microfiltration membrane of 0.45 µm owns technically potency for separating of red bean broth extract to be retentate as protein hydrolysate and permeate as seasoning agent. Interaction between crude red bean broth extract, difference fermentation time and operating condition at microfiltration membrane tends to influence on composition of total protein, dissolved protein and dry solid in retentate and permeate. Operating pressure and composition of broth extract tend to effect on flux. High pressure and low dry solid content increase flux. Red bean broth extract for 6 weeks fermentation resulted higher protein content in permeate as hydrolyzed vegetable protein than in retentate. Permeate at pressure of 25 psi gave flux value of 0.0217 mL/cm^2.min and contents of total protein of 1.31%, dissolved protein of 6.9 mg/g, and dry solid of 2.6%, while retentate as hydrolyzed vegetable protein or fortified agent indicated contents of total protein of 1.52%, dissolved protein of 4.15 mg/g, and dry solid of 3.64%. It was found that microfiltration process was able to increase dissolved protein content of about 3 times. Red bean broth extract fermented for 8 weeks gave the highest flux of 0.0283 mL/cm^2.min. To obtain the needed components in retentate as protein hydrolysate optimally, operating pressure in stirred microfiltration cell has to be increased (> 30 psi) and the pore size of microfiltration membrane is greater than 0.45 µm. The use of ingredients complementing the application of protein hydrolysate without affecting the final product makes fortifying protein hydrolysate an attractive way to promote this product.

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