Effect of pores size of microfiltration membrane on separation of savory fraction of autolysate from fermented mung beans (Phaseolus radiatus L.) by Rhizopus-C1 and Aspergillus sp-K3

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Abstract
Separation of savory fraction of autolysate from fermented mung beans (Phaseolus radiatus L.) by Rhizopus-C1 and Aspergillus sp-K3 through membrane of stirred cell microfiltration (MF) with different pores size was an experiment on membrane performance to get retentate product as component source in specific savory (umami) precursor. This separation was conducted using MF membrane having pores size of 0.2, 0.45 and 0.65 µm with stirring speed of cell of 400 rpm and 30 psia. The results of experiment showed that both autolysate suspension of fermented mung beans gave the different performance of MF membrane and retentate composition products. More and more large pores size of MF membrane tend to decrease total solid, total protein, and N-Amino, but increase reducing sugar, while dissolved protein and salt tend to be fix in retentate. Based on the best composition of savory fraction, MF membrane with 0.2 and 0.45 µm in pore size were operated to produce retentate with the optimal composition in autolysate from fermented mung beans by Rhizopus-C1 and Aspergillus sp-K3. The retentate products gave total solids of 6.2415 and 6.3746 %, total protein of 16.0898 and 21.1660 % (dry weight), dissolved protein of 2.1 and 1.4 mg/mL, N-amino of 0.29211 and 0.27713 mg/mL, reducing sugar of 2 and 10.2 mg/mL, and salt of 1.0335 and 1.0335 %, respectively.

Keywords: Microfiltration, pores size, retentate, autolysate, Rhizopus-C1, Aspergillus sp-K3

Introduction
Autolysate from autolysis process with heat and fixed pH is performed to increase savory fraction of fungus declared by Food and Drug Administration (FDA) as natural flavor (Nagodawithana, T. W. 1984 & Spanier, A. M, et al. 1995). Autolysis of vegetable broth from mung beans (Phaseolus radiatus L.), namely product of brine fermentation by inoculum of Rhizopus sp, or Aspergillus sp., is conducted in order to undo cell of fungus and activate proteolytic enzyme so that it will increase amino acids as savory (umami) source and get natural specific savory flavor (Agustine Susilowati, et al., 2006)

In its progress, purification process through membrane system is needed to get savory fraction as savory flavor. The use of microfiltration (MF) membrane enables to separate savory fraction from other components. MF is able to separate makromolecules (> 500,000 g/mol.) or particles (0.1 – 10 µm) at pressure of 0.5 – 5 atm, so that fat (1 – 10 µm), protein (0.04 – 2 µm) and sugar (8 – 20 µm) will retained at membrane surface, while compounds of taste, aroma and flavor formation (< 0.04 µm), colour pigment (0.1 – 10 µm), monosaccharides/lactose (0.001 µm), peptides (0.01 – 0.1 µm) and salt (0.001 – 0.01 µm) will pass as permeate (Anonim. 2005). Difference in molecular weight (MW), particle size, and driving force, such as pressure, enable to pass savory fraction as permeate, whereas other components with particle size larger than pores size will be retained as retentate. The use of membrane with various pores size enable to be indicated characteristic and particles size of components contained in autolysate, such as fat, sugar, protein, amino acids and total solid, and performance of membrane, namely flux influenced by stirring speed and pressure. Autolysate contains also volatile and non-volatile compounds of taste and flavor formation, such peptides, amino acids, nucleotides and sugar during cell lysis. Other organic compounds, such as furan, furanon, alcohol, aldehyde, keton, ester, phenol, tiamin, pirazin or heterocyclic nitrogen compounds generated through thermal reaction (Manley, C. H. 1995).

The objective of this experiment was to find out the effect of pores size of MF membrane on characteristics of permeate and retentate as product of autolysate purification from mung beans broth via brine fermentation by different fungus by means of stirred cell MF at fixed stirring speed and pressure (400 rpm and 30 psia) as savory flavor.
Materials and Methods

Materials and equipments

The materials used in this work was autolysate produced from fermented mung beans on brine fermentation by inoculum of *Rhizopus*-C1 [A] and *Aspergillus* sp-K3 [B] at 30 °C for 18 weeks (vegetable broth), chemical agents for analysis and MF membrane of fluoro polymer (0.2, 0.45 and 0.65 µm in pore size and 30.175 cm² in effective membrane area) manufactured by Danish Separation Systems, Denmark. The equipments utilized in this experimental were process equipment of autolysis of vegetable broth, stirred cell MF (Amicon 8200) equipped with stirrer, and instrument for chemical analysis.

Investigation and Analysis

Total protein, dissolved protein, total solid, N-Amino, reducing sugar and salt in autolysate of fermented mung bean by inoculum of *Rhizopus*-C1 [A] dan *Aspergillus* sp-K3 [B] were determined according to Kjeldahl, Lowry, Gravimetry methods, Cu methods, Somogyi-Nelson methods, Salinometri methods and Sohxlet methods, respectively (A.O.A.C. 1995). Investigation of MF process was conducted on flux at stirring speed of 400 rpm and pressure of 30 psia. Contents of total protein, dissolved protein, total solid, N-Amino, reducing sugar, salt and fat in permeate and retentate were determined using the previously methods.

Preparation process and separation of autolysate from fermented mung beans (vegetable broth)

Autolysis Process of mung beans broth

Autolysis process was carried out by pulverizing 2 parts of crude broth and 3 parts water, and adjusting pH to 5.5 by adding HCl/NaOH. This suspension was heated in shaker at 50 °C for 60 hours (A) and 48 hours (B) with agitator speed of 140 rpm. Inactivated enzyme was conducted by heating at 70 °C for 5 minutes and filtrating through sieve of 200 mesh with 1 part of autolysate to 4 part of water ratio so it is produced filtrate (as feed) and autolysate residue.

Separation of autolysate through MF membrane of 0.2 µm

Separation of autolysate was carried out by means of stirred cell MF. Suspension extract placed in cell was operated with stirring speed of 400 rpm, room temperature and pressure of 30 psia. Operating pressure was generated by using nitrogen gas from nitrogen cylinder as driving force of suspension passing pores of membrane so it is produced permeate and suspension retained at membrane surface as retentate. Permeate was collected and recorded its volume in time interval (Anonim, 2000). All processes was showed in Figure 1.

Results and Discussion

Characteristic of autolysate of mung beans broth as feed

Autolysis process with filtration of 200 mesh of mung beans broth from brine fermentation using *Rhizopus*-C1 [A] and *Aspergillus* sp-K3 [B] produced feed A and B with different composition, as indicated in Table 1.

![Figure 1](image-url)
Pulverizing and homogenizing for 15 minutes and filtration through sieve of 200 mesh are performed to decrease particles size of autolysate in order to minimize accumulation of particles during MF process. N-amino concentration in feed A for MF membrane with pores size of 0.2, 0.45 and 0.65 µm, namely 0.6591, 0.2397 and 0.2546 mg/mL, was higher than feed B, namely 0.1890, 0.2771 and 0.2622 mg/mL. This difference is caused not only by composition difference of initial suspension (crude broth) but also by autolysis process. At pH of 5.5, proteolytic activity of Rhizopus-C1 is higher than Aspergillus sp-K3 because autolysis time of Rhizopus-C1 (60 hours) is longer than Aspergillus sp-K3 (48 hours) in hydrolyzing substrate protein to amino acids (Agustine Susilowati, et al., 2006). N-amino in autolysate is an important component as source of savory fraction because total amino content itself is dominated by L-Glutamate acid, while total protein in suspension is accumulation of all proteins (peptides and nucleotide acid). Its high concentration of dissolved protein has indicated its high proteolytic activity of fungus during fermentation. Water soluble protein is generally amino acids with low MW and has high solubility in water (Belitz, H.D. and Grosch, W. 1999). While, salt obtained from initial autolysate as fermentation component is an important component in forming savory flavor to enhance taste, whereas reducing sugar is obtained from amylolitic activity of fungus during fermentation. Amylolitic activity of Aspergillus sp-K3 is higher than Rhizopus-C1 (Hasseltine, C. W. dan Wang, H. L. 1980), so that autolysis process produce higher concentration of reducing sugar than Rhizopus-C1. Autolysis process will increase amyolitic activity of fungus in hydrolyzing carbohydrate to mono saccharides as reducing sugar. Both kind of hydrolysates seem to indicate difference in physical quality in which autolysate from Rhizopus-C1 is a more yellowish suspension than Aspergillus sp-K3 which is savory taste, as showed in Figures 2 a and 2 b, respectively. Different suspension feed color effects also autolysis process (heat, pH and process time) due to occurrence of Maillard reaction in forming brown pigment through reaction between amino acids and sugar (Whiffield, F. B. 1992).

Table 1 Characteristic of feed A and B.

<table>
<thead>
<tr>
<th>Kind of materials (Feed)</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total protein, % (dry matter)</td>
</tr>
<tr>
<td>Rhizopus-C1 (A)</td>
<td>0.2 µ (A₁)</td>
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<tr>
<td></td>
<td>0.45 µ (A₂)</td>
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<td></td>
<td>0.65 µ (A₃)</td>
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<tr>
<td>Aspergillus sp-K3 (B)</td>
<td>0.2 µ (B₁)</td>
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<tr>
<td></td>
<td>0.45 µ (B₂)</td>
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<td></td>
<td>0.65 µ (B₃)</td>
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Figure 2 Autolysate of mung bean broth-Rhizopus-C1 (a) and autolysate of mung bean broth-Aspergillus sp-K3 (b) as Feed A and Feed B.
At pore size of membrane of 0.2 and 0.45 µm, permeate fluxes of autolysate of A and B do not indicate a significant difference, but it becomes different at membrane with pore size of 0.65 µm. It is possibly that average higher concentration of total solid in autolysate B causes higher permeate flux of autolysate A. Total solid component is a product of accumulation from all components having various particle size.

At fixed stirring speed of cell agitator and pressure (400 rpm and 30 psia) causes more and more high permeate flow rate for wider pore size of membrane so that permeate flux become more and more high. Components having smaller particle size than pore size of membrane will pass via membrane as permeate, while components with larger particle size than pore size of membrane will be retained at membrane surface as retentate. From this difference in permeate flux showed that autolysates of A and B are probably dominated by components with average larger or same (0.65 µm) because they have the highest permeate flux value, 0.9648 and 0.0466 mL/cm².second, respectively. This difference is probably caused by particle size of amino acids, 0.01 – 0.1 µm (Woemer, I.G. 2004) so that MF membrane with pore size of 0.2, 0.45 and 0.65 µm will pass them as permeate, except amino acids with same particle size or larger than 0.2, 0.45 and 0.65 µm will be rejected at the membrane surface. From this discussion can be indicated that autolysate A (Rhizopus-C1) contains more amino acids with larger particle size of 0.45 µm than smaller particle size of 0.45 µm. Different trend will see at autolysate B in which difference in particle size of amino acids in retentate and permeate at membrane with pore size of 0.45 µm (0.2771 and 0.2771 mg/mL) and membrane with pore size of 0.65 µm (0.2622 and 0.2622 mg/mL) are not sufficient significant, but they are enough different for membrane with pore size of 0.2 µm (0.2472 and 0.2322 mg/mL). Thus, it is said that at autolysate B contains same quantity of amino acids in retentate and permeate for membrane with pore size of 0.45 and 0.65 µm, as demonstrated in Figure 4. Autolysate is a suspension containing rich amino acids with MW and various particle size as a result of cell degrading of fungus and protease enzyme activity in hydrolyzing of broth substrate of mung bean (Agustine Susilowati et al., 2008). Difference in N-amino in feed is a process basic to generate N-amino in purification process, in which N-amino concentration in feed A is higher than feed B, so that it is produced same high concentration of N-amino for same particle size of membrane.

**MF effect on retentate and permeate compositions**

**N-amino**

Separation of autolysate via MF membrane with more and more wider pore size produce N-amino concentration in retentate and permeate which is different for both kind of autolysates, as displayed in Figure 4.

At autolysate A, MF membrane of pore size of 0.2 µm is able to retain particles of amino acids in retentate (0.2921 mg/mL) more than particles of amino acids passing via membrane as permeate (0.2022 mg/mL), and the same concentration of amino acids in retentate and permeate (0.2846 mg/mL) at use of MF membrane with pore size of 0.45 µm. At the largest pore size of membrane (0.65 µm), MF membrane is not able to retain more much amino acids in retentate (0.2546 mg/mL) and pass via membrane as permeate (0.2996 mg/mL). This difference is probably caused by particle size of amino acids (0.01 – 0.1 µm) so that MF membrane with pore size of 0.2, 0.45 and 0.65 µm will pass them as permeate, except amino acids with same particle size or larger than 0.2, 0.45 and 0.65 µm will be rejected at the membrane surface. From this discussion can be indicated that autolysate A (Rhizopus-C1) contains more amino acids with larger particle size of 0.45 µm than smaller particle size of 0.45 µm. Different trend will see at autolysate B in which difference in particle size of amino acids in retentate and permeate at membrane with pore size of 0.45 µm (0.2771 and 0.2771 mg/mL) and membrane with pore size of 0.65 µm (0.2622 and 0.2622 mg/mL) are not sufficient significant, but they are enough different for membrane with pore size of 0.2 µm (0.2472 and 0.2322 mg/mL). Thus, it is said that at autolysate B contains same quantity of amino acids in retentate and permeate for membrane with pore size of 0.45 and 0.65 µm, as demonstrated in Figure 4. Autolysate is a suspension containing rich amino acids with MW and various particle size as a result of cell degrading of fungus and protease enzyme activity in hydrolyzing of broth substrate of mung bean (Agustine Susilowati et al., 2008). Difference in N-amino in feed is a process basic to generate N-amino in purification process, in which N-amino concentration in feed A is higher than feed B, so that it is produced same high concentration of N-amino for same particle size of membrane.

**Dissolved protein**

Use of MF membrane with wider particle size will give a different separation of dissolved protein for both kind of autolysates, as showed in Figure 5. Dissolved protein is an amino acids with low MW.
produced through brine fermentation and autolysis as savory flavor. Solubility of dissolved protein as amino acids in water at room temperature (25 °C) is enough high, in which glutamic acid (0.7 g/100 mL) and aspartic acids (0.4 g/100 mL) (Belitz, H.D. and Grosch, W. 1999), are precursor of savory flavor explored in high quantity in permeate as a result of stirred cell MF on mung bean broth by inoculum of Rhizopus-C1, and Aspergillus sp-K3 (Aspiyanto & Agustine Susilowati. 2006. The solubility property affects on dissolved protein concentration in MF process. Soluble peptides have a range of particle size of 0.01 – 0.1 µm (Anonim. 2005 and Agustine et al., 2008) so that all kinds of MF membrane in this experiment are tend to pass as permeate, except for peptide with larger particle size of 0.2 µm will rejected at membrane surface.

Figure 5 Relationship between pore size of membrane and dissolved protein concentration in retentate and permeate as a result of MF of autolysate-Rhizopus-C1 [A] and autolysate-Aspergillus sp-K3 [B] at stirring speed of cell agitator of 400 rpm, room temperature 30 psia.

At autolysate A, dissolved protein concentrations in retentate and permeate decrease with wide pore size of membrane, while dissolved protein concentration in autolysate B is tend to be constant in permeate and fluctuates in retentate. This difference is relating with dissolved protein concentration in feed, in which dissolved protein concentration in feed A is higher than feed B. From above discussion, concentration of dissolved protein in retentate (0.21 mg/mL) with larger size of particle of 0.2 µm is retained more much at membrane surface than autolysate B (0.16 mg/mL).

Total protein
Protein has a range of particle size of 0.04 – 2 µm [4, 13] with high MW and is a polypeptides consisting of much amino acids. Protein of vegetable broth from fermented mung bean is relating with initial autolysate of mung bean (56 % of total autolysate) in preparating vegetable broth (Agustine Susilowati, et al., 2006). Proteolytic activity of fungus causes its hydrolyzing protein to simpler peptides unit, especially amino acids with lower MW, nevertheless a number of polipeptides are hydrolyzed partial so that MF process with different size of pores produces retentate and permeate with different concentration of total protein, as displayed in Figure 6.

Figure 6 Relationship between pore size of membrane and total protein concentration in retentate and permeate as a result of MF of autolysate-Rhizopus-C1 [A] and autolysate-Aspergillus sp-K3 [B] at stirring speed of cell agitator of 400 rpm, room temperature and pressure of 30 psia.

Autolysate A gives separation of protein with particle size of 0.2 µm in permeate (20.8399 %, dry matter) which is higher than in retentate (16.0898 %, dry matter). This total protein concentration in permeate drop, when it is used MF membran with pore size of 0.45 and 0.65 µm, namely 8.8134 and 14.982 % (dry matter), and in retentate, namely 12.5198 and 14.1778 % (dry matter), respectively. Different trend will be shown on autolysate B, in which use of MF membrane with more and more wide pore size will increase both total protein concentration in permeate and retentate. More much total protein in retentate is rejected than in permeate. Utilize of MF membrane having pore size of 0.2, 0.45 and 0.65 µm are yielded total protein concentration in permeate, namely 5.5130, 15.2857 and 15.1519 % (dry matter) and in retentate, namely 20.1361, 21.1660 and 27.4909 % (dry matter). Thus, use of MF membrane with the smallest pore size (0.2 µm) gave the optimal result in retaining total protein in retentate (20.1361 %, dry matter) of autolysate B which is higher than total protein in retentate (16.0898 %, dry matter) of autolysate A.

Total solid
Use of MF membrane with more and more wider pore size is tend to give the optimal separation result on total solid. Retentate contains higher concentration of total solid than permeate for both kind of autolysate, as shown in Figure 7.
More and more wide pore size in MF membrane is tend to give more and more effective separation, in other words particles in permeate will pass freely via membrane, and more and more small possibility of particles are retained at membrane surface. Nevertheless, this trend for both autolysate is not same. Both total solid concentrations in retentate and permeate of autolysate A become more and more high relating with more and more wide pore size of membrane. At MF membrane with pore sizes of 0.2, 0.45 and 0.65 µm result concentration of total solid in retentate, namely 6.2415, 8.6582, and 8.5038 %, and in permeate, namely 2.6668, 4.7873, and 4.9614 %, respectively. While autolysate B, more and more wide pore of membrane will indicate a decrease of total solid concentration in retentate and permeate. For MF membrane with pore sizes of 0.2, 0.45 and 0.65 µm give total solid concentrations in retentate of 6.5166, 8.6582, and 8.5038 %, and in permeate of 4.8890, 4.8932, and 4.9614 %, respectively. Difference on total solid in this MF result is possibly caused by a difference in each higher composition of components (amino acids, reducing sugar, etc.) in total solid A than B (see Tabel 1) so that more much total solid of feed A are rejected by membrane as retentate than passing in permeate of total solid of feed B. This possibility is caused by the presence of fouling, namely the presence of accumulation of particles of autolysate at membrane surface, and “cake” layer which will cut off solutes diffusion (Munir Cheryan, 1986), so that it might increase total solid concentration in retentate, in spite of particles size of autolysate is smaller than 0.2 µm for both feed A and B.

Salt
Pore size in MF membrane affect also on salt concentration in retentate and permeate of feed A and B, as displayed in Figure 8.
in permeate and retentate of autolysate A in which membrane with pore size of 0.2, 0.45 and 0.65 µm give reducing sugar in permeate, 60, 76, and 118 mg/mL, and in retentate, 20, 33, and 74 mg/mL, respectively. At autolysate B, this system will fluctuate, in which at this same condition gives sugar reducing concentration in permeate, namely 90, 102 and 73 mg/mL. Because average concentration of reducing sugar of feed B is higher than autolysate A (see Tabel 1), MF system in this process is interaction among stirring speed of cell agitator (400 rpm), pressure (30 psia) and material so that process is able to convert sugar molecule to simpler sugar with lower MW of autolysate B.

While, MF process of autolysate A pass reducing sugar with smaller particles size or equal to 0.65 µm. For all processes, this purification generates permeate and retentate with physical property, such as different color of suspension at both kind of autolysates. Difference in suspension color is influenced by composition of initial autolysate and ability of membrane in separating components, especially color pigment of melanoidin as a result of Maillard reaction in initial fermentation and autolysis. Use of MF membrane of autolysate A with the smallest pore reaction (0.2 µm) produces permeate with more yellow color than autolysate B, and retentate is thicker suspension than permeate, as showed in Figure 10 a, b, c and d, respectively.

Conclusions
1. Pore size in MF membrane and process conditions affect on retentate and permeate compositions. Smaller particles size in autolysates than pore size in membrane will pass freely as permeate, while larger particles size in autolysates than pore size in membrane will rejected at membrane surface.
2. More and more wide pore size of membrane will increase permeate flux value, concentrations of N-amino, total solid, reducing sugar and salt, but decrease total protein and dissolved protein in retentate. Permeate is clear liquid and retentate is suspension, both of them are umami taste.
3. Based on the optimal pore size of membrane, MF membrane with pore size of 0.2 µm is able to retain N-amino, dissolved protein and reducing sugar, MF membrane with pore size of 0.45 µm is able to retain total solid and salt, and MF membrane with pore size of 0.65 µm is able to reject total protein and the highest reducing sugar in retentate of autolysate A.
4. Based on the optimal pore size of membrane, MF membrane with pore size of 0.2 µm is able to retain solid, reducing sugar and salt, MF membrane with pore size of 0.45 µm is able to retain total protein and dissolved protein, and MF membrane with pore size of 0.65 µm is able to retain the highest dissolved protein in retentate of autolysate B.

Figure 9 Relationship between pore size of membrane and reducing sugar concentration in retentate and permeate as a result of MF of autolysate-Rhizopus-C1 [A] and autolysate-Aspergillus sp-K3 [B] at stirring speed of cell agitator of 400 rpm, room temperature and pressure of 30 psia.

Figure 10 Permeate (a) and retentate (b) as a result of MF on autolysate of Rhizopus-C1 [A], permeate (c) and retentate (d) as a result of MF of autolysate of Aspergillus sp-K3 [B] at stirring speed of agitator cell of 400 rpm and pressure of 30 psia using MF membrane of 0.2 µm.
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