SEPARATION OF FERMENTED INULIN FIBER BY *Lactobacillus acidophillus* USING *Aspergillus clavatus*-CBS_5 THROUGH MICROFILTRATION MEMBRANE

(Pemisahan Serat Inulin Terfermentasi oleh Lactobacillus acidophillus menggunakan Aspergillus clavatus-CBS_5 melalui Mikrofiltrasi Membran)

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ABSTRAK. *Kondisi Sel Filtrasi Berpengaduk (SFB) digunakan sebagai acuan menuju kondisi proses skala besar (modul) terhadap pemisahan serat inulin terfermentasi oleh Lactobacillus acidophillus. Hidrolisat inulin yang digunakan sebagai biomassa dihasilkan dari tahapan hidrolisa inulin oleh enzim inulinase dari kapang Aspergillus clavatus-CBS_5. Pemisahan serat inulin bertujuan untuk mendapatkan serat inulin melalui membran mikrofiltrasi (MF) 0,45 µm pada temperatur ruang, kecepatan putaran pengaduk 400 rpm dan tekanan 40 psia selama 0, 30, 60, 90 dan 120 menit. Hasil penelitian menunjukkan bahwa waktu pemisahan yang lama dapat menahan dan meningkatkan padatan total, serat total, asam total, protein terlarut dan kemampuan pengikat kolesterol (KPK) tetapi menurunkan gula total dalam retentat. Membran mikrofiltrasi melewatkan dan menurunkan gula total, protein terlarut dan KPK tetapi meningkatkan padatan total, serat total, asam total dalam permeat. Berdasarkan KPK optimal, waktu pemisahan terbaik dicapai setelah 120 menit. Pada kondisi ini dihasilkan konsentrat serat inulin terfermentasi dengan konsentrasi gula total 105,21 mg/mL, padatan total 2,11%, serat total 23,36%, asam total 6,66% (berat kering), protein terlarut 4,05 mg/mL dan KPK 13,781 mg/g. Membran MF mampu meningkatkan KPK 23,4% dibandingkan tanpa menggunakan proses pemisahan.*

***Kata kunci**: kemampuan pengikat kolesterol, membran mikrofiltrasi, permeat, retentat, serat inulin*

ABSTRACT. *The condition of Stirred Filtration Cell (SFC) was used as reference to a large-scale process conditions (modules) on the separation offermented inulin fibers by Lactobacillus acidophillus. Inulin hydrolyzate as biomass was produced from inulinase hydrolysis stage by inulinase enzyme from Aspergillus clavatus-CBS_5. Separation of inulinfiber aims to obtain inulin fiber through a microfiltration (MF) membrane 0.45 µm at room temperature, 400 rpm stirrer cycle and 40 psia for 0, 30, 60, 90 and 120 min. The results showed that best separation time was 120 minutes based on optimal CBC (cholesterol binding capacity) which fermented inulin fiber concentrate was produced with a total sugar concentration of 105.21 mg/mL, total solids 2.11%, total fiber 23.36%, total acid 6.66% (dry weight), 4.05 mg of dissolved protein/mL and CBC 13.781 mg/g. MF membrane increased the CBC by 23.4% compared to no separation process.*

Keywords: *cholesterol binding capacity, inulin fiber, microfiltration membrane, permeate, retentate*

1. INTRODUCTION

Dahlia (*Dahlia* sp.) tuberis one of potential food plants that contain inulin. Inulin, which is a naturally fructo-oligosaccharide (FOS) composed of a mixture of oligomers with varying molecular weights (MW) that occurs naturally in plants, fruits and vegetables, such as Jerusalem artichoke (*Helianthus tuberosus* L.), chicory (*Chichorium intybus* L.) root, yacon (*Smallanthus sanchifolius*) root, onion (*Allium cepa* L.), garlic (*Allium sativum*) and asparagus (*Asparagus setaceus*) (Mensink, et al., 2015; Kosasih, et al., 2015; Dominguez, et al., 2013; Cassano, et al., 2015). Inulin, which had experience subsequent hydrolysis using β-Amylase enzyme followed by inulinase enzyme of fungi *Aspergillus clavatus*-CBS_5 have major potential as rich sources of soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) to bind cholesterol in digestive system. Inulin hydrolysate, which had been fermented by Lactic Acid Bacteria (LAB) has different characteristic with fermentable hydrolysate by *Lactobacillus acidophillus (L. acidophillus)* on composition, physical properties, organoleptic hedonic index and CBC at pH 2 (related to pH at stomach) and pH 7 (related to colon pH) was able to be hydrolyzed by inulinase enzyme of *Aspergillus clavatus*-CBS_5 fungi to produce FOS for dietary fiber. FOS can not be digested by digestive enzymes. However, it could be fermented by colon bacteria (*Lactobacillus* sp. and *Bifidobacterium*) to produce unsaturated chain fatty acid (USCFA), effecting cholesterol structure and forcing cholesterol synthesis (Tsurumaki, et al., 2015; Younis, et al., 2015).

In chemical engineering, the separation of bioactive compounds from crude fermentation broths or plant extracts requires non-thermal unit operations that different from conventional chemical separation processes because fermentation products are generally targeting compound of interest which more complex, heat sensitive, low concentration and designed for aqueous systems (Alles, et al., 2015). Based on the method of unit operation mentioned above, the separation of inulin fiber from inulin hydrolysate that fermented by *L. acidophillus* using membrane was effective due to the absence of change in phase during process and its operationwas at room temperature. Membrane-based filtration had been selected in order to improve properties over conventional filtration because heat and chemical treatments are not used (Akin, et al., 2012; Drioli, et al., 2001; Cassano, et al., 2015). MF membranes with pore sizes in the range from 0.1-1.0 µm (1000-10.000 nm) and low pressure (1 to 10 bar) was typically able to separate specific compounds in fermentation broth product having particle sizes with linear dimensions in the range of 0.02-10 µm (20-100.000 nm) (Olsen, 2000; Raghavan, et al., 2005; Lee, et al., 2016). The membrane separation mechanism was based on a sieving effect and particles separated according to their dimensions and molecular level (Jegatheesan, et al., 2012; Marchetti, et al., 2014; Kumar, et al., 2013; Saxena, et al., 2009). This research aims to know the best effect of separation condition through different time to produce concentrate (retentate) and inulin hydrolysate extract fermented by *L. acidophillus* as permeate with composition and the best cholesterol binding capacity (CBC).

2. METHODS

Materials

Materials that used in this experiment were inulin from Sukabumi, West Java. Starch and yolk flour from local market. Inulinase enzyme of *Aspergillus clavatus*-CBS_5 and *L. acidophillus* culture purchased from Research Center for Chemistry-Indonesian Institute of Sciences. Skimmed milk powder from Benato, U.S.A., distilled water, reagent-grade chemicals, such as phenol solution, concentrated sulphuric acid, hydrochloric acid (30%), sodium hydroxide, ethanol, acetic acid, O-phtaldehyde, phenolphtalein, aceton, sodium carbonateand copper sulphate that were purchased from E. Merck. Bovine

Serum Albumin from Sigma as standard protein, commercial MF membrane (0.45 µm) that was purchased from DSS AS, Nakskov, Denmark and β-Amylase enzyme.

Main equipments which used in this experiment were high precision analytical balance from Fujitsu. Calibrated pH meter, hydrolysis system, series of fermentation system in laboratory scale, system of laminar flow chamber and incubator were purchased from local. Autoclave from Cheng Yi, China type LS-50L. Waterbath shaker was purchased from Memmert, Stirred Ultrafiltration Cell (SUFC) model 8200 from Amicon, U.S.A. Magnetic stirrer type HI 303N from HANNA Instrument, Japan. Nitrogen cylinder was purchased from local and regulator from Fisher Scientific Company, England. Stop watch from Hanhart, profil 2, Germany, oven, sieve of 200 mesh from Retsch, Germany, UV-vis Spectrophotometer Model RF-550 from Shimadzu and LC-MS from Mariner Biospectrometry.

Process Steps Hydrolysis of Inulin as FOS

A number of inulin was subsequently added with β-Amylase enzyme at concentration of 0.08% (v/w starch) then adjusted its acidity to pH 5, hydrolyzed at 60ºC and followed by agitating at 140 rpm for 120 minutes to produce hydrolysate I. Further, hydrolysate I was added with inulinase enzyme of *Aspergillus clavatus*-CBS_5 at concentration of 60% (v/w hidrolysate I), adjusted its acidity to pH 5, rehydrolyzed at 30ºC followed by agitating 140 rpm for 120 minutes then inactivated to pH 4 at same temperature for 15 minutes and cooled to produce inulin hydrolysate II as FOS.

Fermentation of Inulin Hydrolysate by *L. acidophillus* Culture

A number of inulin hydrolysate as FOS (ca. 50% w/v water) and skimmed milk (12.5% w/v water) were autoclaved at 121ºC and 1 atm for 20 minutes then cooled and added with culture stock of *L. acidophillus* (15% v/v water) to produce suspension biomass of inulin hydrolysate. This suspension biomass then incubated at 40ºC for 48 hours. In this experimental work, it had been prepared a biomass with volume 2 L (Susilowati, 2014). Further, separation on suspension biomass of inulin hydrolysate was conducted through microfiltration membrane.

Concentrating Inulin Hydrolysate Fermented by *L. acidophillus* through MF Membrane

Concentrating biomass of inulin hydrolysate fermented by *L. acidophillus* was conducted through MF membrane fitted at Stirred Filtration Cell (SFC) with capacity of 180 mL. Prior to utilize, membranes that wetted with deionization water was fitted in SFC having 180 mL capacity and connected to a nitrogen gas cylinder as driving force on fluid. SFC was filled with 50 mL of deionization water and compacted at 40 psi for 5 minutes. SFC then emptied, refilled of inulin hydrolysate fermented by *L. acidophillus* and operated at stirring rotation speed 400 rpm, room temperature and trans-membrane pressure (TMP) was 40 psi for 15 minutes. Volumetric permeate flow rate was measured after each mL of permeate has collected. Samples of permeate were collected and recorded for 15 minutes to determine the permeate flux through the membrane. Permeate and retentate (concentrate) were regularly sampled for analysis. The membrane then rinsed with deionization water for 5 minutes. Equipment and internal model of Stirred Filtration Cell (SFC) utilized were displayed in Figures 1a and 1b, respectively.

Analysis of Total Dietary Fiber (TDF)

Suspension of inulin hydrolysate which fermented by *L. acidophillus* culture (biomass) was sieved through 200 mesh to obtain filtrate and residue. Next, residue homogenized at 4000 rpm for 15 minutes, washed twice with ethanol 95% and aceton at same ratio, dried at higher temperature than 105°C for 3 hours. The dry powder that obtained was pure Total Dietary Fiber (TDF).

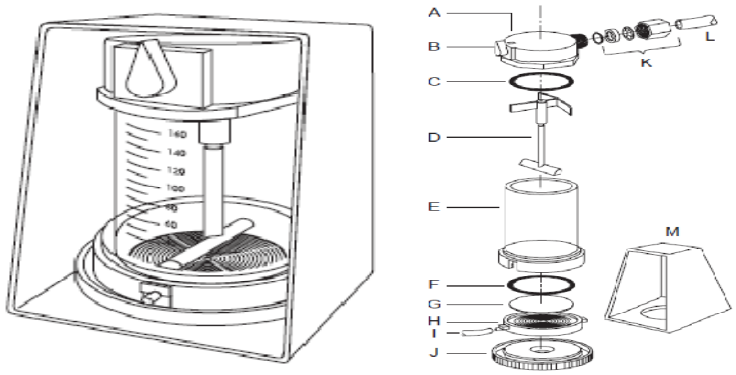

Figure 1. (a) equipment of SFC and (b) internal model SFC, where A=cap assembly; B=pressure relief valve; C=O-ring; D=stirrer assembly; E=body; F=O-ring; G=MF membrane of 0.45 µm; H=membrane holder; I=elastomeric tubing; J=Base; K=tube fitting assembly; L=tubing, plastic and M=stand assembly (Amicon, 2008).

Analysis of Cholesterol Binding Capacity (CBC)

A number of egg yolk (ca. 2.5 g) and 2 g of fermented inulin fiber were mixed and shaked with 50 mL of deionization water then adjusted its acidity (pH 7) and shaked (80 rpm, 2 hours) in water bath at 37°C. This mixture was added with 16 mL ethanol (absolute) and centrifuged at 4000 rpm for 20 minutes to produce supernatant and residue. Supernatant 1 mL was taken, diluted 5 times using acetic acid 90% and evaporated at 40°C for 1 hour. Evaporation result 1 mL was subsequently added with 0.1 mL of O-phthaladehyde reagent (50 mg in 100 mL acetic acid 90%) and 2 mL of sulphuric acid, homogenized and allowed for 20 minutes. Analysis on sample was conducted using UV-vis Spectrophotometer at λ 550 nm and compared with the blank one. Cholesterol concentration in sample was determined by comparing standar curve of standard cholesterol solution.

3. RESULTS AND DISCUSSION

Characteristic of Materials

The characteristic and comparison of preparation of inulin hydrolysate (FOS) that fermented by *L. acidophillus* from inulin were inulin, inulin hydrolysated by β-Amylase enzyme and ferment inulin hydrolysate by *Aspergillus clavatus*-CBC5 inulinase enzyme (FOS) as a feed in separation processed by MF membrane with size 0.45 µm. Components that contained in inulin were total solids 65.17%, total sugars 525.8 mg/mL, total dietary fiber (TDF) 20.75% (dry matter) and CBC at pH 2 was 15.58 mg/g. On the other hand, inulin hydrolysate by β-Amylase enzyme contains components of total solids 41.47%, total sugars 407.21 mg/mL, total dietary fiber (TDF) 30.67% (dry matter) and CBC 17.9 mg/g at pH 2. Whereas, inulin hydrolysate in fermented inulin hydrolysate by *Aspergillus clavatus*-CBC_5were contains of total solids 22.02%, total sugars 148.85 mg/mL, dissolved protein 2.45 mg/mL, total dietary fiber (TDF) 5.91% (dry matter) and CBC 11.67 mg/g at pH 2. Characteristic of materials were summarized in Table 1.

The data presented in Table 1 showed that inulin had higher concentration of both total solids and total sugars than inulin that hydrolysated by β-Amylase enzyme and fermented inulin hydrolysated by *Aspergillus clavatus*-CBC_5 inulinase enzyme. Meanwhile, inulin hydrolysate by β-Amylase enzyme gave a higher concentration of both total dietary fiber (TDF) and CBC at pH 2 than inulin and fermented inulin hydrolysated by *Aspergillus clavatus*-CBC_5 inulinase enzyme. Those happened because TDF in fermented inulin hydrolysate by *Aspergillus clavatus*-CBC_5 inulinase enzyme was a result of degrading macromolecules to simpler and smaller

Table 1. Characteristic of inulin, inulin hydrolysated by β-Amylase enzyme and fermented inulin hydrolysated by *Aspergillus clavatus*-CBC_5 inulinase enzyme

Type of components	Type of material		
	Inulin	Inulin hydrolysate by β-Amylase enzyme	Fermented inulin hydrolysate by *Aspergillus clavatus*-CBC_5 inulinase enzyme
Total solids (%)	65.17	41.47	22.02
Total sugars (mg/mL)	525.80	407.21	148.85
Dietary fiber TDF (% dry matter)	20.75	30.67	5.91
CBC at pH 2 (mg/g)	15.58	17.90	11.67
Dissolved protein (mg/mL)	-	-	2.45
Total acids (%)	-	-	1.94

molecules. Meanwhile, CBC in fermented inulin hydrolysate by *Aspergillus clavatus*-CBC_5 inulinase enzyme was still associated with macromolecules.

Effect of MF Membrane Process on Composition of Material

Micro Filtration is one of membrane-based separation unit operations. This separation process is physical that depends on the low pressure difference between the two sides of the porous membrane called permeable solids and non permeable solids (retentate or concentrate). MF is characterized by the ability to separate molecules of different sizes and physical characteristics (Baker, 2012). Inulinase enzyme across MF membrane (0.45 µm) had been given retentate (concentrate) and permeate through separating total solids, total sugars, TDF, CBC at pH 2, dissolved protein and total acids contained in fermented inulin hydrolysate by *Aspergillus clavatus*-CBC_5 (Muro, et al., 2012).

Total Acids and Dissolved Protein

Effect of time on total acids and dissolved protein in permeate and retentate as a result of separation of inulin fiber fermented by *L. acidophilus* from inulin hydrolysate using *Aspergillus clavatus*-CBS_5 inulinase enzyme through MF membrane (0.45 µm) were shown in Figure 2.

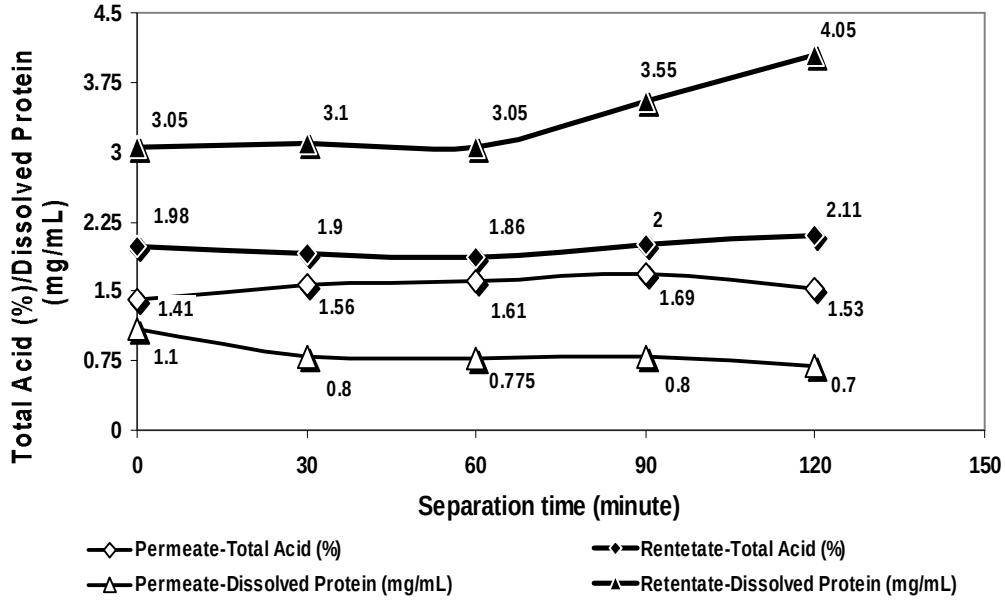

Figure 2. Effect of time on total acids and dissolved protein

In this research, the whole total acids content in permeate was lower than in retentate (concentrate). Total acids content in permeate increased gradually within 90 minutes then decreased from 90 to 120 minutes and gave total acids content initiated by 1.41, 1.56, 1.61, 1,69 and 1.53% (dry matter), respectively. Total acids produced from fermentation of FOS (inulin hydrolysate II) by *L. acidophillus* as major organic acids (100-500 MWCO, 0.4-0.8nm) were accumulated in the feed medium (O'Sullivan, et al., 1984).

Due to high porosity of the membrane, MF membrane was commonly inadequate to separate and retain total acids (100-500 MWCO, 0.4-0.8 nm) which has smaller particle size than pore size of membrane (0.45 µm). Whereas, amount of total acids was retained on the top membrane surface as retentate (concentrate), because of their association with macromolecules. Hence, the content of smaller MW total acids remain was relatively constant on both permeate and retentate (concentrate) sides of the membrane.

As a result of experimental work, all dissolved protein content in permeate was lower than in retentate (concentrate). Dissolved protein in permeate have tendency to increase gradually within 120 minutes and gave dissolved protein content of 1.1, 0.8, 0.775, 0.8 and 0.7 mg/mL respectively. Meanwhile, dissolved protein in retentate (concentrate) rose slowly within 60 minutes then sharply lowered from 60 to 120 minutes and gave dissolved protein content of 3.05, 3.1, 3.05, 3.55 and 4.05 mg/mL, respectively. Most proteins produced from fermentation of FOS (inulin hydrolysate II) by *L. acidophillus* have particle size of 2-10 nm (10.000-1.000.000 MWCO) (O'Sullivan, et al., 1984). Due to high porosity of the membrane, MF membrane was commonly insufficient to separate and retain dissolved protein (10.000-1.000.000 MWCO, 2-10 nm) which has smaller particle size than pore size of membrane (0.45 µm). A possible reason was the amount of dissolved proteins which were associated with macromolecules retained and rejected by MF membrane.

Total Sugars and CBC

Effect of time on total sugars content and CBC with acidity to 2 (pH 2) in permeate and retentate as a result of separation of inulin fiber fermented by *L. acidophilus* from inulin hydrolysate using *Aspergillus clavatus*-CBS_5 inulinase enzyme through MF membrane of 0.45 µm were demonstrated in Figure 3.

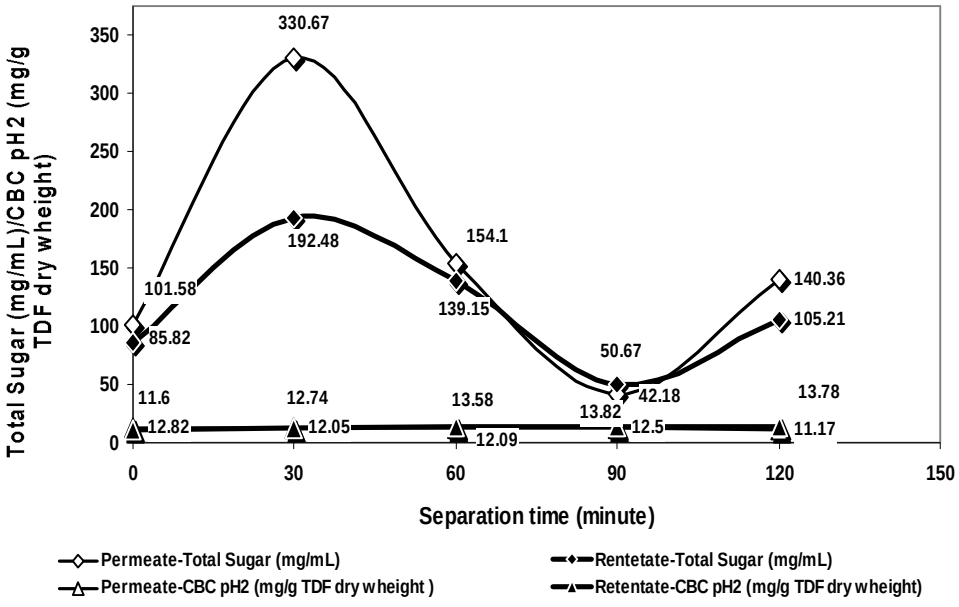

Figure 3. Effect of time on total sugars and CBC at pH 2

In this research, total sugars content in permeate was relatively higher than in retentate (concentrate). Total sugars content in permeate sharply increased within 30 minutes then strongly decreased from 30 to 90 minutes while hardly increased from 90 to 120 minutes and gave total acids content of 101.58, 330.67, 154.10, 42.18 and 140.36 mg/mL. On the other hand, total sugars content in retentate (concentrate) was lightly raised within 30 minutes, drastically declined from 30 to 90 minutes then slightly increased from 90 to 120 minutes and gave total acids content of 85.82, 192.48, 139.15, 50.67 and 105.21 mg/mL, respectively. Total sugars generated from fermentation of FOS (inulin hydrolysate II) by *L. acidophillus* were mono- and di-saccharides having particle size of 0.8-1 nm (200-400 MWCO) (O'Sullivan, et al., 1984).

Due to high porosity of the membrane, MF membrane was not able enough to separate and retain total sugars (200-400 MWCO, 0.8-1 nm) that have smaller particle size than membrane pore size (0.45 µm). On the other hand, amount of total sugars was retained on the top membrane surface as retentate (concentrate) which caused by their association with macromolecules.

Physical property testing was conducted on permeate and retentate (concentrate) and gave almost similar CBC with acidity to 2 (pH 2). Prior to experimental run, CBC with acidity (at pH 2) on initial permeate was 12.82%, while CBC with acidity to 2 (pH 2) on permeate for 30, 60, 90 and 120 were 12.05, 12.09, 12.50 and 11.17, respectively. Meanwhile, before experimental run, CBC with acidity to 2 (pH 2) on retentate (concentrate) was 11.60% and CBC with acidity to 2 (pH 2) on retentate (concentrate) for 30, 60, 90 and 120 were 12.74, 13.58, 13.82 and 13.78, respectively. As expected result, CBC with acidity to 2 (pH 2) on retentate (concentrate) was slightly higher than on permeate because their association with macromolecules in retentate (concentrate).

Total Solid and Total Dietary Fiber

Effect of time on total solids and TDF in permeate and retentate (concentrate) as a result of separation of inulin fiber fermented by *L. acidophilus* from inulin hydrolysate using *Aspergillus clavatus*-CBS_5 through MF membrane 0.45 µm was shown in Figure 4.

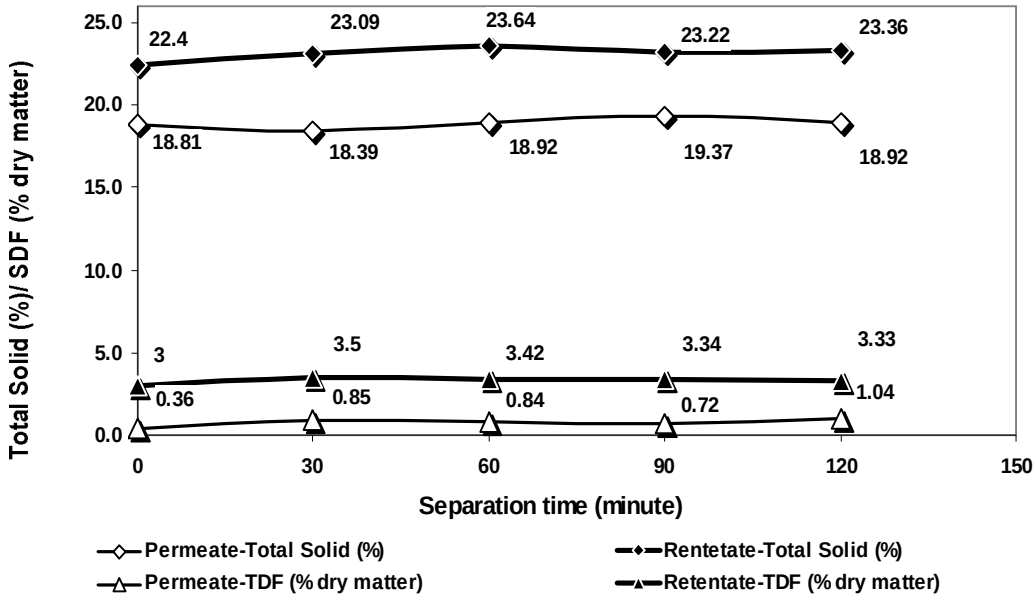

Figure 4. Effect of time on total solids and TDF

In this research, total solids content in permeate was lower than in retentate (concentrate). Total solids content in permeate was gradually increased during the third 30 minutes of microfiltration then slowly decreased from 90 to 120 minutes and gave total solids content of 18.81, 18.39, 18.92, 18.37 and 18.92% (dry matter), respectively. Meanwhile, total solids content in retentate (concentrate) gently lowered during the second 30 minutes then raised gradually from 60 to 120 minutes and gave total solids content of 22.4, 23.09, 23.64, 23.22 and 23.36% (dry matter), respectively.

Total solids produced from fermentation of FOS (inulin hydrolysate II) by *L. Acidophillus* were major organic acids (100-500 MWCO, 0.4-0.8 nm) (O'Sullivan, et al., 1984), such as acetic acid, citric acid (210 MWCO) and lactic acid. Result of total solids content in permeate was smaller than in retentate (concentrate). This condition was caused by high porosity of the membrane, MF membrane was commonly inadequate to separate and retain total solids (100-500 MWCO, 0.4-0.8 nm) that have smaller particle size than pore size of membrane (0.45 µm). On the other hand, amount of total solids was retained on the membrane surface as retentate (concentrate), due to their association with macromolecules

Dietary Fiber (DF) has important role in nutrition source and health because its ability to induce a number of physiological effects which increases fecal bulk and improves large bowel function, as well as reduces levels of blood cholesterol and sugar also prevents colon cancer. The purpose for DF determination is to measure the content of fraction traditionally defined as fiber (non starch polysaccharides and lignin) (Schneeman, 1986). In the experiment, TDF content in permeate was lower than in retentate (concentrate). TDF content in permeate increased gradually within 90 minutes then slowly decreased from 90 to 120 minutes and gave TDF content of 0.36, 0.85, 0.84, 0.72 and 1.04% (dry matter), respectively. Meanwhile, TDF solids content in retentate (concentrate) dropped gradually within 60 minutes thengently increased from 60 to 120 minutes and gave TDF content of 3.0, 3.5, 3.42, 3.34 and 3.33% (dry matter), respectively.

TDF produced from fermentation of FOS (inulin hydrolysate II) by *L. Acidophillus* were consists of major organic acids (100-500 MWCO, 0.4-0.8 nm) (O'Sullivan, et al., 1984), such as acetic acid, citric acid (210 MWCO) and lactic acid. Due to high porosity of the membrane, MF membrane was commonly inadequate to separate and retain TDF (100-500 MWCO, 0.4-0.8 nm) which has smaller particle size than pore size of membrane (0.45 µm). The amount of TDF was retained on the membrane surface as retentate (concentrate), due to their association with macromolecules. Concentrate and permeate of fermented inulin hydrolysate as a result of SFC at optimum condition (stirrer rotation of 400 rpm, room temperature and pressure of 40 psi for 120 minutes of process) were shown in Figures 5a and 5b.

(a) (b)

Figure 5. Concentrate (a) and permeate (b) as a result of separation fermented inulin fiber by microfiltration membrane.

4. CONCLUSIONS

Microfiltration (MF) in this research could be considered as membrane-based non-thermal unit operation in chemical engineering field to separate, purify and recovery fermentation products. SFC mode had potential use in separation and concentration processes of inulin hydrolysate fermented by *L. acidophillus* to produce extract (permeate) and concentrate (retentate) as functional food for cholesterol binder. Long separation time using SFC mode retained and increased total solids, Total Dietary Fiber (TDF), total acids, dissolved protein and CBC also decreased total sugars in retentate. SUFC mode passed and decreased total sugars, dissolved protein and CBC but increased total solids, TDF, total acids in permeate, as well. Based on the optimum CBC, the best time of separation was achieved at pressure 40 psi for 120 minutes. This condition generated fermented inulin fiber concentrate with concentrations of total sugar 105.21 mg/mL, total solids 2.11%, TDF 23.36%, total acids 6.66% (dry weight), dissolved protein 4.05 mg/mL and CBC 13.781 mg/g. MF membrane was able to increase CBC 23.4% compared to a process with no separation. Process condition on SUFC was a principle reference on separation of fermented inulin fiber by *L. acidophillus* toward condition in larger scale (module).

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