Fermented Beetroot (Beta vulgaris L.) Extract by Kombucha as Energy Booster Produced via Cross-Flow Microfiltration Membrane

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Abstract. Beetroot extract (permeate) fermented by kombucha culture separated through cross-flow microfiltration (CFMF) with pore size 0.15 μm at TMP 2 and 6 bar for 0, 5, 15, 25, and 35 minutes, with a flow rate of 7.5 L/m3.hour, at room temperature potentially serves as a natural energy booster. The research results based on soluble protein show that the optimization process at TMP 2 and 6 bar was achieved at 15 minutes with permeate flux of 32.52 and 28.05 L/m3.h, respectively, and soluble protein content of 0.72 and 0.38 mg/mL, total solids of 3.40% and 3.36%, total sugar of 45.91 and 47.47 mg/mL, and acetic acid of 0.93 and 0.97%. Under these conditions, the CFMF system increased the soluble protein in permeate by 123.53% or 1.23 times, indicating partial rejection, and 22.58% or complete rejection. The identification of amino acids in the optimum condition was dominated by three threonine monomers with molecular weights of 120.11, 120.37, and 120.90 Da (M+), and six tryptophan monomers with molecular weights of 205.11, 205.21, 205.44, 205.60, 205.88, and 205.94 Da (M+). The permeate under optimum conditions had average particle sizes of 2788.0 nm and 2922.0 nm, with particle index of 1.523 and 1.795. The particle distribution in permeate at TMP 2 and 6 bar was less than 10,000 nm in size .

1 Introduction

Protein, as an essential building block of the body, serves as a fundamental component in obtaining energy. The caloric value of protein is equivalent to 4 calories per 1 gram of protein (whether plant-based or animal-based), making it a potential source of energy. Daily calorie requirements for activities range from 1,600 to 2,400 calories for women and 2,000 to 3,000 calories for men [1]. High-protein plant-based beverages contribute as an alternative for energy enhancement [2]. These can take the form of concentrates, hydrolysates, or powders, available as supplements or ready-to-drink beverages.

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Beetroot (*Beta vulgaris* L.) is a type of root vegetable that comes in shades of purple to dark purple, rich in nutrients with energy content (180 kJ or 43 kcal), carbohydrates (9.56 g), protein (1.6 g), folate (vitamin B9) (109 μg), and a variety of pigments (betacyanin, anthocyanin, beta carotene), vitamins (B1, B2, B5, B6, C), minerals (Zn, Ca, Mn, Mg, Fe, K, Na, and P), fats, and dietary fiber [3, 4]. It serves multiple special functions, including acting as an antioxidant and preventing conditions such as hematuria (blood in the urine), hematochezia (bleeding in the stool), and rectal bleeding [5, 6]. Its role as an energy booster due to the presence of amino acids and organic acids produced during kombucha culture activity has not yet been fully utilized.

Fermentation of beetroot using kombucha culture is an effort to enhance its function as a source of amino acids, including carnitine, lysine, threonine, methionine, and tryptophan, which play a role in energy acquisition. Commercial energy drink products typically rely on synthesized components to formulate an energy source. Therefore, using natural amino acid sources for this functional beverage is an alternative and a diversification of natural energy-enhancing beverage products. *Acetobacter* sp and *Saccharomyces cerevisiae* are the dominant microbes in kombucha culture, along with *Saccharomyces ludwigii*, *Saccharomyces bisporus*, *Zygosaccharomyces* sp, and several yeast species (*Torolupsis* sp). Sometimes, lactic acid bacteria are also found, which are believed to be capable of degrading sugars in beetroot into simple sugars (glucose, fructose, xylose, etc.) and degrading proteins into amino acids [7]. The biomass also contains organic acids, including glucuronic acid, acetic acid, and malic acid, with a specific fresh aroma [8, 9].

The separation process of soluble protein from biomass through a cross-flow microfiltration system (CFMF) is to obtain fermented beetroot extract as an energy-booster beverage on a semi-pilot scale (9 L). The selection of a cross-flow microfiltration (CFMF) system is because it differs from conventional filtration systems (dead-end filtration or centrifugation systems) based on rotational speed [10, 11]. CFMF system will reduce the accumulation of solute particles, which causes concentration polarization and minimizes fouling. However, this is also influenced by material characteristics (particle size, molecular weight, adhesion properties between components), operational conditions (pressure, flow rate, temperature, time), and membrane material types. This is further improved because amino acids are expected to pass through the membrane surface more readily than being retained on the membrane surface. This allows to determine the extent of the increase in soluble protein as permeate compared to soluble protein in biomass or feed, or at the initial process (with an accumulation time of approximately 2-3 seconds when the CFMF system is installed). The MF membrane with a pore size of 0.15 μm is chosen for its capability to separate components based on differences in particle size and molecular weight. It is capable of filtering particles with sizes ranging from >0.1 - 10 μm at pressures between 0.3 – 3.3 bar [12]. Amino acids with molecular weights between 190 to 500 Da, which have sizes between 0.001 - 0.01 μm, will pass through as permeate [12, 13, 14]. However, fouling can occur, which may retain these compounds in the retentate [15].

This study aims to determine the characteristics of the optimum permeate resulting from the cross-flow microfiltration of fermented beetroot, at TMP of 2 and 6 bar for different time intervals, under constant conditions of a flow rate of ~7.5 L/min and room temperature. The overall composition of the permeate will be evaluated, particularly soluble proteins, the characteristics of the molecular weights of monomers such as carnitine, lysine, threonine, methionine, and tryptophan, as well as their distribution and particle sizes for a natural energy booster drink. In comparison, retentate is obtained as a concentrated product for fermented beetroot powder.

2 Materials and Methods

2.1 Materials and Equipments

The main materials used in this experimental work included fresh beetroot (*Beta vulgaris* L.) and sucrose (both obtained from the local market), kombucha culture (Research Center for Chemistry – BRIN), commercial microfiltration (MF) membrane (pore size of 0.15 µm, diameter of 20 cm, fluoropolymer, FSM-0.15-PP, Alfa Laval, Denmark), and all chemicals used for the analysis of total solids, acetic acid, dissolved proteins, and total sugar. The process equipment used consisted of fermentation equipment (autoclave, laminar air flow, incubator), a cross-flow membrane filtration module (Lab Unit M20, DSS, Denmark) and analytical instruments including a UV-Vis Spectrophotometer (Model RF-550, Shimadzu, Japan), Liquid Chromatography (Hitachi L 6200) coupled with Mass Spectrometry (LC-MS) (Mariner Biospectrometry), and Particle Size Analyzer (PSA) using PSA Coulter SZ 100 (Horiba Nano Partica).

2.2 Research Design

Fermented beetroot biomass was separated through a cross-flow microfiltration (CFMF) system at room temperature, pump motor frequency (PMF) of 20 Hz (flow rate of ~7.5 L/min.), and TMP of 2 bar and TMP of 6 bar for 0, 5, 15, 25 and 35 minutes. Analysis was conducted on the initial biomass, feed, retentate, and permeate resulting from the MF membrane process on soluble protein (Lowry method) [16, 17], total solids (Gravimetric method) [18, 19], acetic acid (Titrimetric method), and total sugar (Phenol Sulfate method) [20, 21]. Identification of amino acids, including carnitine, lysine, threonine, methionine, and tryptophan, was performed using LCMS (Liquid Chromatography tandem Mass Spectrometry) [22, 23, 24], and particle size distribution was determined using PSA (Particle Size Analyzer) [25, 26, 27]. All processes and analyses were conducted in duplicate, and the data presented in this description are based on the average analysis results.

This research was carried out using mung bean fermented by inoculum *R. oligosporus* strain-C1 (A) and *Rhizopus* sp. (B). NF membrane process was carried out using permeate obtained from UF of mung bean fermented by *R. oligosporus* strain-C1 as feed. The conditions ensured included room temperature, PMF of 20 Hz (flow rate ~7.5 L/min), and TMP of 10 bar, as well as a duration of 90 minutes. The permeate and retentate were collected at intervals of 0, 15, 30, 45, 60, 75, and 90 minutes.

2.3 Process Steps

The process begins with the preparation of beetroot (*Beta vulgaris* L) inoculum, which involves sorting, size reduction, blanching for 5 minutes at 80°C, pulverizing with distilled water at a ratio of 1:4, filtration through a 60-mesh sieve, the addition of 10% sucrose (w/v, filtrate), followed by pasteurization at 90 - 95°C. After cooling, it was inoculated with a 10% kombucha culture (v/v, filtrate). This mixture was then fermented for 7 days in a closed container in a dark room to obtain fermented beetroot inoculum. The fermentation process was initiated similarly, but the beetroot was pulverized at a ratio of 1 part beetroot to 8 parts distilled water. Filtration was done through a 100-mesh sieve, followed by the addition of 10% sucrose (w/v, filtrate of beetroot) and a 10% inoculum (v/v, filtrate of beetroot). This mixture was fermented for 30 days at room temperature in a closed container in a dark room and homogenized at 8000 rpm for 15-30 minutes. The resulting biomass was kept as

fermented beetroot extract or feed for the cross-flow microfiltration (CFMF) membrane process.

The separation process was conducted by placing feed liquid in the feed tank (volume 9 L) with overflow/drain, and then feed liquid was circulated through the system consisting of pre-filtered in 200 μm filter, cooling/heating water, and MF membrane module until the experimental conditions were stable. The filtration of feed liquid was carried out in total recirculation mode, i.e., both permeate and retentate were recycled back to the feed tank. The operation condition of the MF process was performed at room temperature, pump motor frequency (PMF) of 20 Hz (flow rate ~7.5 L/min.), and TMP of 6 bar. The amount of samples was taken from permeate and retentate for chemical analysis during the experiment for 0, 15, 25, and 35 min. A similar MF membrane process procedure was performed at room temperature, PMF of 20 Hz (flow rate ~7.5 L/min.), and TMP of 2 bar for 0, 15, 25, and 35 min. For each experiment condition, permeate flux was measured twice to achieve an average value.

3 Results and Discussion

3.1 Characteristic of materials

The fermentation process for 30 days resulted in differences in composition, with a significant increase in soluble protein and acetic acid in the biomass by 65.96% and 94.54%, respectively. However, there was a decrease in total sugar and total solids by 34.98% and 51.86%, respectively, compared to the initial substrate, which was beetroot pulp obtained by pulverization at a ratio of 1:4. The increase in acetic acid is possibly caused by the activity of the kombucha culture, particularly *Acetobacter* sp and *Saccharomyces cerevisiae* [7, 8], which degrade sucrose into simple sugars and organic acids during fermentation. The increase in soluble protein is driven by the protein lysis of beetroot (1.6%) due to the presence of organic acids formed during fermentation. The decrease in total sugar is a result of its utilization as a source of microbial metabolism, while the decrease in total solids is caused by converting partial total solids to dissolved metabolites. The preparation of the feed also reduces the composition due to homogenization at 8000 rpm for 15-30 minutes to prevent blockage in the CFMF system. Table 1 shows the overall composition of the material before the separation process through the MFCF system, while Figures 1a, 1b, 1c, and 1d display the overall materials.

Table 1. Composition of materials in separating amino acid as energy booster through microfiltration (MF) membrane.

Type of material	Soluble protein (mg/mL)	Acetic acid (%)	Total sugars (mg/mL)	Total solids (%)
Pulp of beetroot	0,47	0,06	65,02	10,22
Fermented beetroot (30 days)	0,78	1,10	43,32	6,73
Feed	0,31	0,53	36,64	3,42

[a] [b] [c] [d]

Fig. 1. (a) Fresh beetroot, (b) beetroot pulp, (c) fermented beetroot for 30 days, and (d) feed of fermented beetroot

3.2 Influence on process condition of microfiltration membrane

Flux (J) is one of the criteria of membrane performances besides the selectivity reflected as solute rejection. The permeate flux was defined as the number of permeate volumes (L) passing through the effective membrane area unit (A) collected over the measured time intervals (t), J = L/(A x t). It is usually given in terms of volume per unit membrane area per unit time, e.g., Liters/m2.hour (L/m2.h) [28, 29, 30]. The separation process of fermented beetroot by means of NF membrane shows a decreasing flux over time, as depicted in Figure 2.

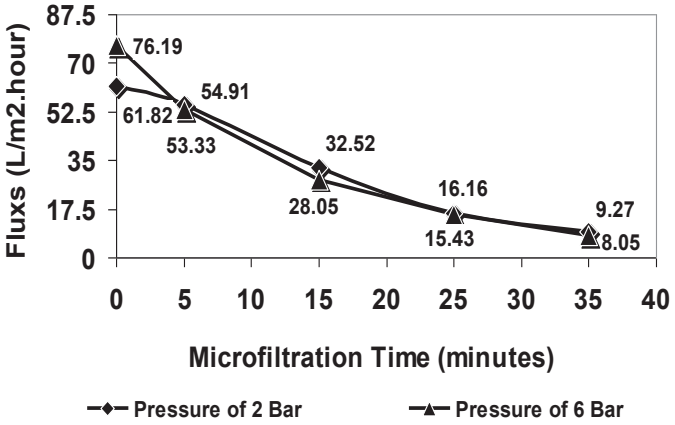


Fig. 2. The relationship between TMP and time on flux in separating fermented beetroot by MF membrane at room temperature and flow rate of ~7.5 L/min.

3.3 Influence on process condition of microfiltration membrane on composition and rejections

The optimization process at TMP 2 and TMP 6 bar is obtained for 25 min and yield fluxes of 15.43 and 16.16 L/m2.h., respectively. This difference of flux is possibly caused by higher driving force (TMP of 6 bar) compared with TMP of 2 bar so that permeate passing becomes lower due to its occurrence of thick suspension of feed until a steady state is achieved. Optimization at TMP 2 and 6 bar was achieved at 25 minutes, resulting in fluxes of 15.43 and 16.16 L/m2.hour, respectively. This difference in flux is possibly caused by the higher driving force at TMP 6 bar compared to TMP 2 bar, resulting in a lower permeate flow rate due to the thickening of the feed suspension. This eventually reaches a point of a steady state.

3.3.1 Soluble protein (mg/mL) and its rejection (%)

The CFMF system exhibits fluctuating rates of separation for soluble proteins as the separation time increases. The treatments at TMP 2 and 6 bar each resulted in the optimization of soluble protein in permeate for 15 minutes, yielding concentrations of 0.72 and 0.38 µg/mL, respectively. At the same time, soluble protein of 0.35 and 0.52 µg/mL were produced in retentate (concentrate), as shown in Figure 3a. Under these conditions, the CFMF system was able to pass soluble protein in permeate at TMP 2 and 6 bar by 132.26% or 1.32 times and 22.58% compared to the soluble protein in the feed (0.31 mg/mL).
Regarding the rejection of soluble proteins at TMP 2 bar for 5, 15, and 25 minutes, it shows that more soluble protein passed into the permeate (Cp>Ck) compared to being retained on the membrane surface, indicating partial separation. However, further into the process (between 25 and the end of the process at 35 minutes), more soluble protein was retained in the retentate (Cp<Ck). At TMP 6 bar, successful separation was achieved throughout the separation time. In other words, TMP of 6 bar overall resulted in better rejection of soluble proteins (26.36%) compared to TMP 2 bars (-106.79%) or unsuccessful separation, as shown in Figure 3b.

Rejection refers to the solute component which did not penetrate through the membrane. The rejection (R) of solute was calculated based on the equation, R (%) = [1 – Cp/Cr] x 100%, in which Cp is the concentration of solute in permeate, and Cr is the concentration of solute in feed or retentate [31, 32]. This difference in rejection is caused by the difference in driving force. In cases with a higher driving force (6 bar), fouling occurs due to interactions with other components, leading to the formation of a 'cake' that is retained on the membrane surface. Conversely, at TMP 2 bar, the driving force is not strong enough, allowing more soluble proteins to pass into the permeate. The particle size of amino acids, as soluble protein (0.001 – 0.01 µm) is smaller particle sizes than the pore size of MF membrane (0.15 µm) [33, 34, 35]. Soluble protein encompasses all amino acids, peptides, and all compounds of protein derivatives detected according to the Lowry method [16, 17].

3.3.2 Total solids (%) and its rejection (%)

Optimization on recovery of total solids in permeate at TMP of 2 bar and 6 bar are achieved for 15 min and 5 min, yielding total solids of 3.41 and 3.44%, respectively. At the same time, total solids retained on retentate (concentrate), namely 3.36% and 3.50%. This difference is caused by different driving forces. A higher driving force (TMP of 2 bar) seems to require a longer process time (15 min) compared with a higher driving force (TMP of 6 bar), requiring faster time (5 min) to get the optimum total solids. In this condition, the MF membrane system is insufficiently able to pass total solids in permeate, namely –0.29% and 0.58%, respectively, compared with total solids in feed (3.42%). MF membrane system displays successful separation of total solids, in which the whole ratio of the concentration of total solids in retentate (concentrate) is higher compared with permeate (Ck > Cp) or rejection of total solids is 0.16% and 9.49%, as demonstrated in Figure 4b.

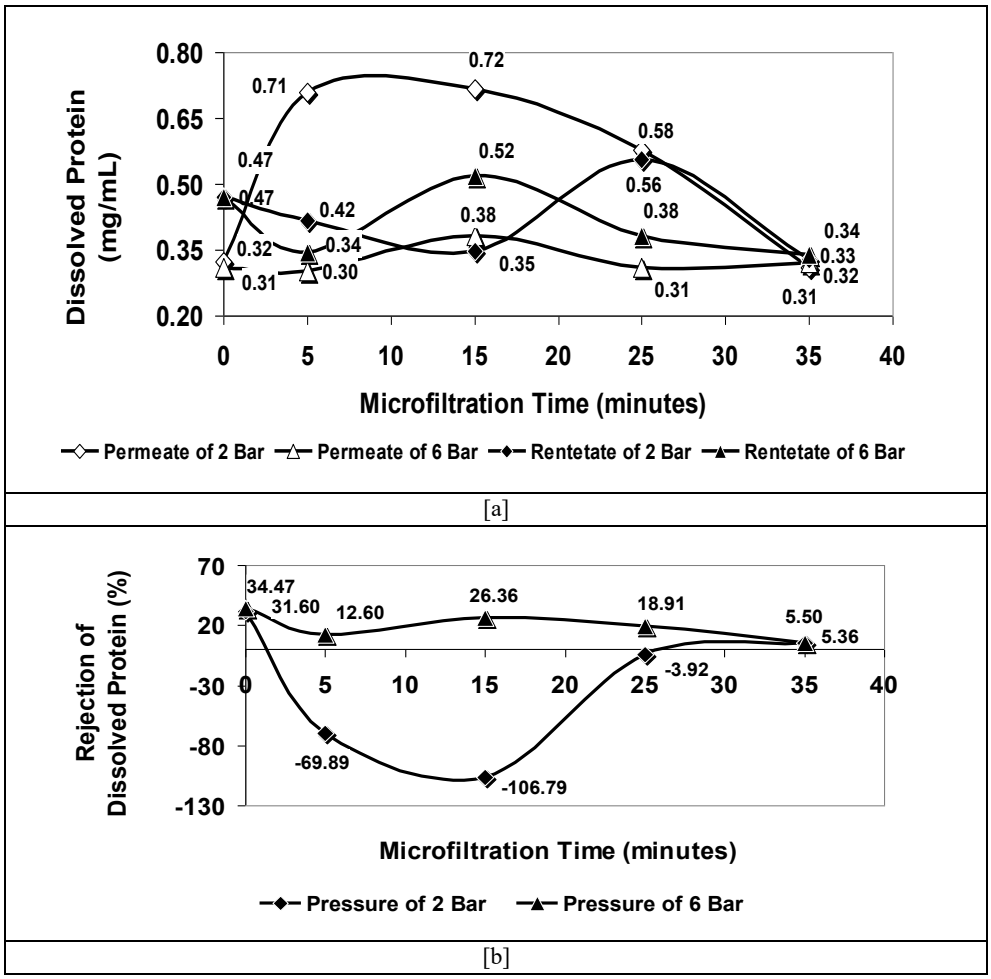


Fig. 3. The relationship between TMP and time on (a) dissolved protein and (b) rejection of dissolved protein in separating fermented beetroot by MF membrane at room temperature and flow rate of ~7.5 L/min.

Total solids represent the accumulation of all components, both soluble and insoluble, according to the Gravimetric method [18, 19]. The CFMF system at TMP 6 bar shows a sharp decrease of total solids in permeate between 0 - 25 minutes but increases at 35 minutes. At TMP 2 bar, the decrease in total solids continues until the end of the process. Meanwhile, the trend in the total solids concentration in retentate (concentrate) at TMP 2 bar is nearly the same as that in the retentate at TMP 6 bar. This suggests that as the processing time increases, total solids become more polarized and trapped on the membrane surface, making it increasingly difficult for them to pass through the membrane. This is reflected in the decreasing flow rate of concentrate between 0 - 15 minutes. Subsequently, the total solids increase towards the end of the process at both TMP 2 and 6 bar, although this increase is generally >30% due to the driving force of the membrane system [38, 39], as shown in Figure 4a.

The optimization of total solid recovery in permeate at TMP 2 and 6 bar was achieved at 15 minutes and 5 minutes of processing, respectively, resulting in total solids of 3.41% and 3.44%, respectively. At this point, the total solids retained in retentate (concentrate) were 3.36% and 3.50%, respectively. This difference is attributed to the varying driving forces, where a less high driving force (2 bar) appears to require a longer processing time (15 minutes) compared to a higher driving force (6 bar), which requires a shorter processing time (5 minutes) to achieve optimal total solids. Under these conditions, the CFMF system was less capable of passing total solids in permeate, resulting in -0.29% and 0.58%, respectively, compared to total solids in feed (3.42%). However, the CFMF system exhibited successful separation of total solids, with the overall concentration of total solids in retentate (concentrate) being higher than in permeate (Ck>Cp) or rejection of total solids was 0.16% and 9.49%, as shown in Figure 4b.

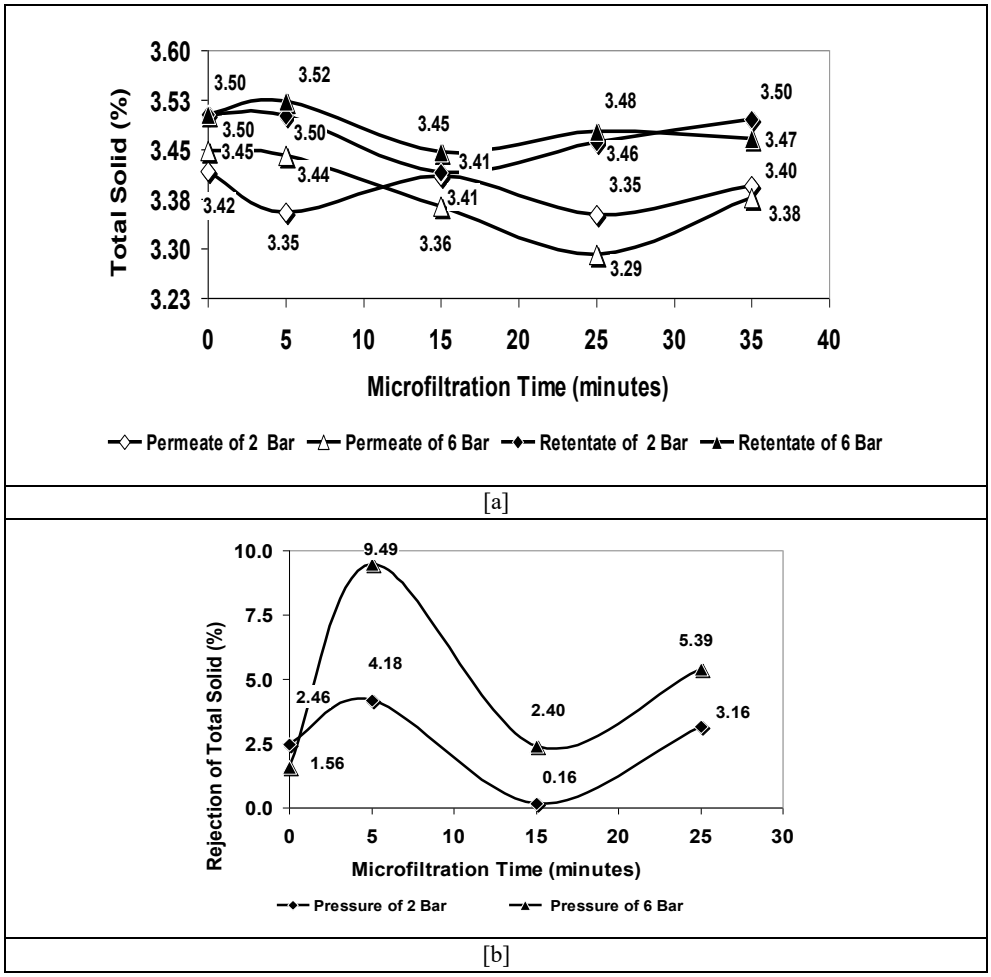
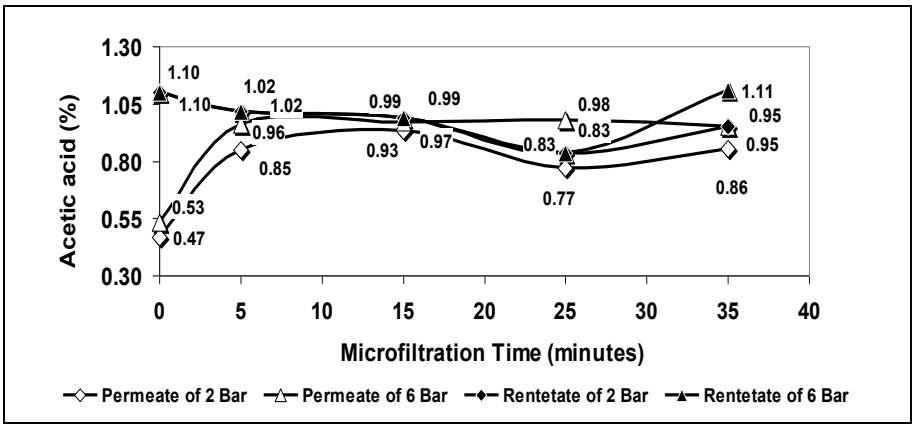


Fig. 4. The relationship between TMP and time on (a) total solids and (b) rejection of total solids in separating fermented beetroot by MF membrane at room temperature and flow rate of ~7.5 L/min.

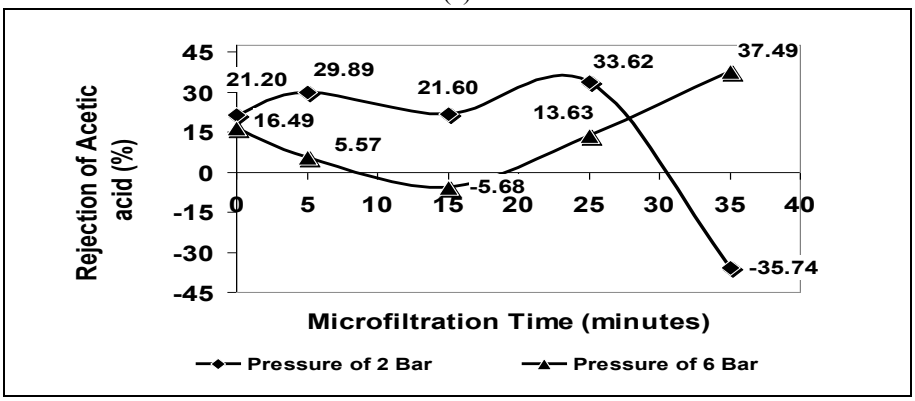
3.3.3 Acetic acid (%) and its rejection (%)

The use of a kombucha culture with a predominance of *Acetobacter* [7, 8] is suspected to result in total acids dominated by acetic acid, detected using the titrimetric method [20, 21]. In addition to other organic acids such as lactic acid, malic acid, and glucuronic acid, interactions with other metabolites, especially monosaccharides, create a specific aroma and flavor resembling fruits. The CFMF system at TMP 2 and 6 bar shows that the separation rate of acetic acid in permeate tends to sharply increase between 0 - 5 minutes, then becomes stagnant (linear) but fluctuates in retentate (concentrate), and finally increases until the end of the process, as shown in Figure 5a.

The optimization of acetic acid in the permeate was achieved at TM 2 and 6 bar for 15 and 25 minutes, resulting in acetic acid concentrations of 0.93% and 0.98%, respectively. This difference is attributed to the lower driving force (2 bar), where the system is not able to accumulate acetic acid, thus requiring a shorter time (15 minutes) to pass through the permeate. On the other hand, with a higher driving force (6 bar), the substance accumulates more quickly, leading to greater retention in retentate (concentrate) due to fouling, requiring a longer time to pass through the permeate (25 minutes). This phenomenon is also evident in the fluctuating rejection of acetic acid at both TMP settings. At TMP 6 bar for 15 minutes, Cp (permeate concentration) > Ck (concentrate concentration), and then rejection increases until the end of the process. Meanwhile, at TMP 2 bar, the decrease in rejection occurs at 35 minutes or Cp > Ck. Under these optimum conditions, the CFMF system can pass acetic acid in the permeate, resulting in 75.47% and 84.90% compared to acetic acid concentration in the feed (0.53%), with rejection of 21.6% and 13.63%, respectively, or successful separation of acetic acid (Cp<Ck), as shown in Figure 5b.



(a)



[b]

Fig. 5. The relationship between TMP and time on (a) acetic acid and (b) rejection of acetic acid in separating fermented beetroot by MF membrane at room temperature and flow rate of ~7.5 L/min.

3.3.4 Total sugars (mg/mL) and its rejection (%)

Kombucha culture is a mixture of microorganisms that use sucrose as a carbon source for their metabolism [7, 8]. Not all of the sugar is utilized, so a portion remains in the biomass, along with sugar produced through metabolism and from the beetroot itself. All of these sugars can be detected using the Phenol Sulfate method [20, 21]. The CFMF system at TMP 2 bar achieves optimum total sugar in permeate at 5 minutes (60.01 mg/mL), followed by a decrease until the end of the process. Meanwhile, at TMP 6 bar, the increase of total sugar in permeate occurs at 35 minutes (61.56 mg/mL). At the same time, the separation rate of total sugar in retentate occurs inversely to that in permeate, as shown in Figure 6a.
The particle size of sugar (monosaccharides) is approximately 0.01-0.001 μm [33, 34, 35, 36, 37], which is smaller than the pore size of MF membrane (0.15 μm). This leads to the prediction that the sugar particles have a higher likelihood of passing through the membrane and ending up in permeate rather than being retained in retentate. This is evident in the rejection of total sugar, where, for most of the process duration, both pressure conditions show a higher recovery of total sugar in permeate compared to the total sugar retained in retentate (concentrate), or Cp>Ck. At TMP of 6 bar, the higher driving force results in lower rejection, allowing the system to concentrate total sugar more effectively, thereby reducing the passing grade in permeate. Under these optimum conditions, the CFMF system is capable of passing total sugar in permeate by 73.78% and 68.01% compared to the total sugar in the feed (36.64 mg/mL). The rejection of total sugars was -53.59% and -37.85%, indicating partial separation of total sugar (Cp>Ck), as shown in Figure 6b.

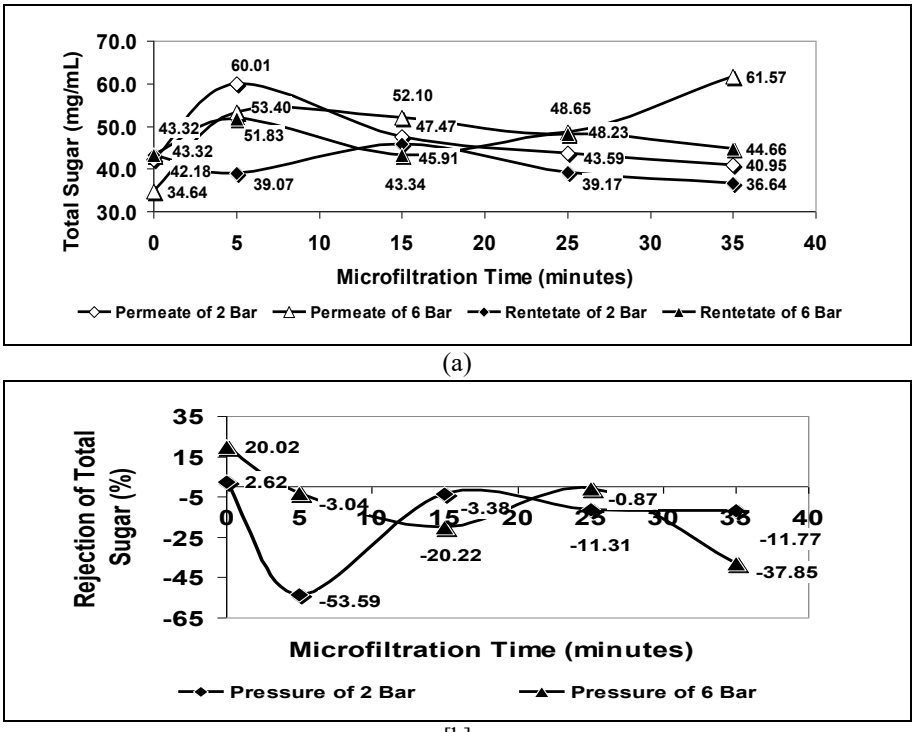


Fig. 6. The relationship between TMP and time on (a) total sugars and (b) rejection of total sugars in separating fermented beetroot by MF membrane at room temperature and flow rate of ~7.5 L/min.

3.4 Optimum process condition

The separation process of fermented beetroot based on the soluble protein at TMP 2 and 6 bar was achieved within 15 minutes and resulted in permeate compositions with soluble protein of 0.72 mg/mL and 0.38 mg/mL, total solids of 3.40% and 3.36%, total sugar of 45.91 mg/mL and 47.47 mg/mL, and acetic acid of 0.93% and 0.97%. Meanwhile, retentates (concentrates) had soluble protein of 0.35 mg/mL and 0.52 mg/mL, total solids of 3.41% and 3.45%, total sugars of 43.34 mg/mL and 52.10 mg/mL, and acetic acid of 0.99% and 0.99%. Under these conditions, there was an increase in soluble protein in the permeate by 123.53% or 1.23 times and 22.58%, total sugar by 25.30% and 29.56%, and acetic acid by 75.47% and 83.02%. However, total solids were retained in retentate at 0.58% and in permeate of 1.75% compared to their respective components in the feed.
In this condition, the performance of the CFMF system at TMP 2 and 6 bar showed flux rates of 32.52 L/m3.hour and 28.05 L/m3.hour, with complete separation or rejection of total solids at 2.85% and 2.58%, but partial rejection of soluble protein, total sugar, and acetic acid were retained in the retentate at -106.79% and 26.36%, -3.38% and -20.22%, and 21.56% and -5.68%, respectively. Figure 7a and 8b show permeate from treatments with TMP 2 and 6 bar with a flow rate of 7.5 L/hour at room temperature for 15 minutes.

[a] [b]

Fig. 7. Permeates of fermented beetroot as a result of the MF membrane process at room temperature, flow rate of ~7.5 L/min with (a) TMP of 2 bar for 15 min and (b) TMP of 6 bar for 15 minutes.

3.5 Identification of amino acids as stamina booster

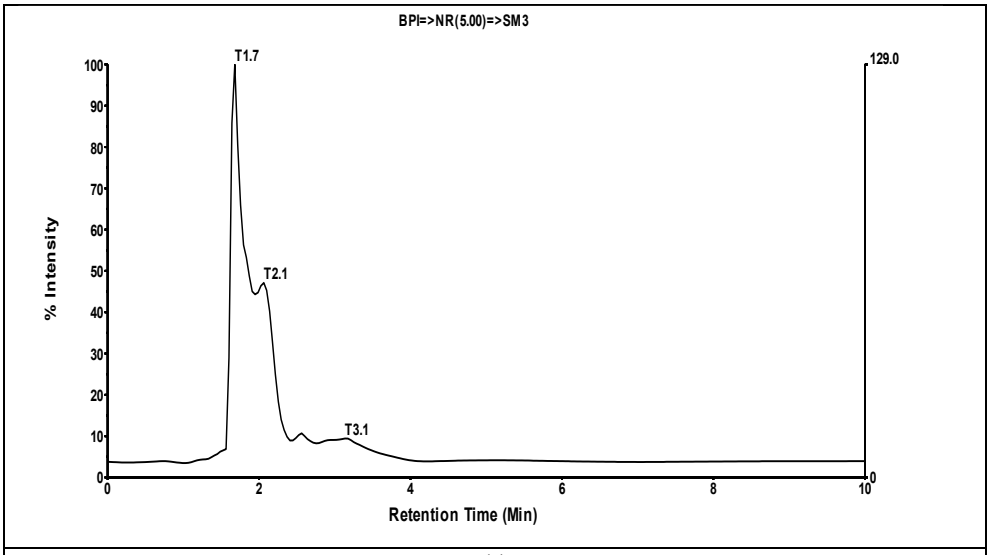
Identification of soluble proteins as amino acids in permeate was carried out through LC-MS from samples at the optimum TMP of 2 and 6 bar for 15 minutes of microfiltration process. Amino acids such as carnitine (M+162), lysine (M+147), threonine (M+120), methionine (M+150), and tryptophan (M+205) were obtained in the mass spectra from LC-MS operating conditions with an injection volume of 5 µL, flow rate of 0.2 mL/minute, using an eluent mixture of methanol and water at a ratio of 80:20, and a C-8 column (15 mm x 2 mm). In the analysis of these amino acid monomers, MW (Molecular Weight) was used as M+ [22, 23, 24]. Table 2 shows the types of amino acids, the number of dominant amino acid content, and the molecular weight of amino acids in permeate at the optimum conditions obtained from the CFMF process.
In the permeate of fermented beetroot obtained from CFMF for 15 minutes at TMP of 2 bar, a chromatogram with 3 peaks of retention times (RT) was observed, namely T1.7, T2.1 at RT 0 - 10 minutes, with relative intensities of 100%, 50%, and 10%, as shown in Figure 8a. Meanwhile, the mass spectra of the dominant amino acid in this sample, which is threonine, exhibited 3 threonine monomers with molecular weights of 120.11, 120.37, and 120.90 Da, as shown in Figure 8b. In the permeate of fermented beetroot obtained from CFMF for 15 minutes under the treatment of 6 bar, a chromatogram with 3 peak retention times (RT) was observed, namely T1.7, T2.1, and T3.2 with relative intensities of 100%, 35%, and 4%, as shown in Figure 8c. The mass spectra of the dominant amino acid in this sample, which is

tryptophan, exhibited 6 tryptophan monomers with molecular weights of 205.11, 205.21, 205.44, 205.60, 205.88, and 205.94 Da, as shown in Figure 8d. Based on the analysis of monomer amino acid characteristics, it is known that, in terms of dominant amino acid types and the number of monomers obtained, the permeate obtained under the treatment of TMP of 2 and 6 bar is dominated by threonine and tryptophan, respectively, with molecular weights of 120 (M+) and 205 (M+), although other amino acids such as carnitine, lysine, and methionine were also found. The CFMF system demonstrates that at higher pressures, it can pass through the feed in such a way that it produces a wider variety of amino acids with a greater number and a broader range of molecular weights compared to the treatment with TMP of 2 bar.

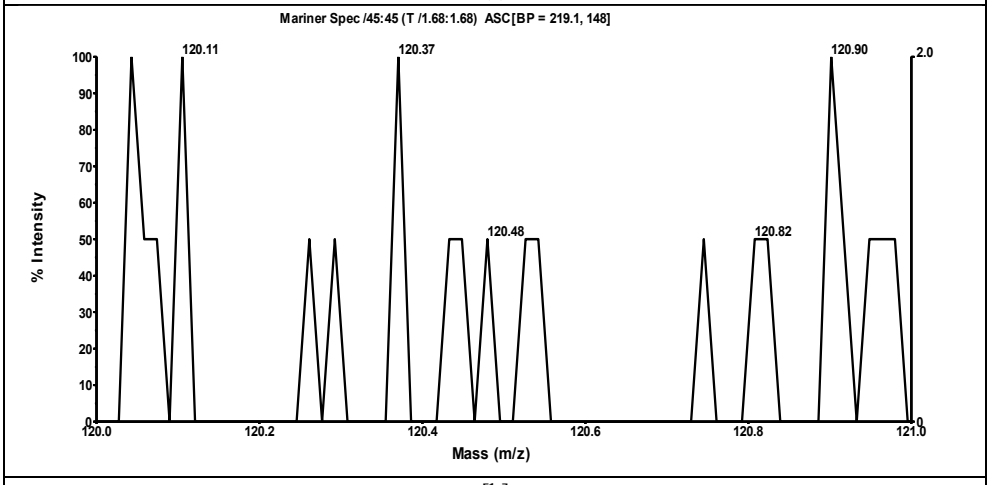
Table 2. Types of predominant amino acid and molecular weight (M+) in permeate from MF membrane process at TMP 2 and 6 bar identified by LC-MS.

Type of sample*	**Type of amino acids**	**Number of monomers (**)**	**Molecular Weight (Dalton)**
TMP 2 bar	Carnitine	2	162,27 ; 162,40
	Lysine	1	147,00
	Threonine	3	120,11 ; 120,37 ; 120,90
	Methionine	2	150,21 ; 150,42
	Tryptophan	1	205,30
TMP 6 bar	Carnitine	1	162,53
	Lysine	3	147,03 ; 147,15 ; 147,22
	Threonine	1	120,90
	Methionine	1	150,20
	Tryptophan	6	205,11 ; 205,21 ; 205,44 ; 205,60 ; 205,88 ; 205,94

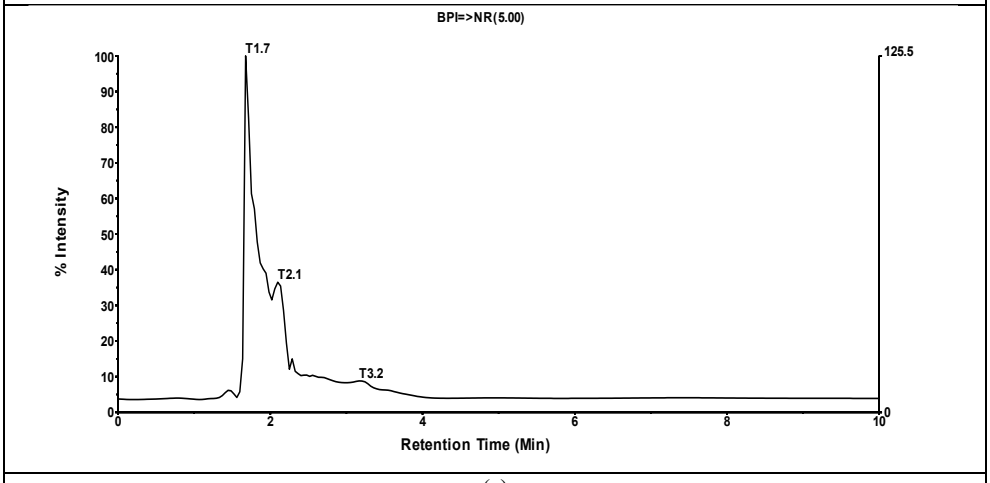
Legend: (*) room temperature and flow rate of ~7.5 L/min for 15 min. of process;
(**) from a number of monomers obtained with a relative intensity of 100%.


BPI=>NR(5.00)=>SM3
T1.7
T2.1
T3.1
129.0
% Intensity
Retention Time (Min)

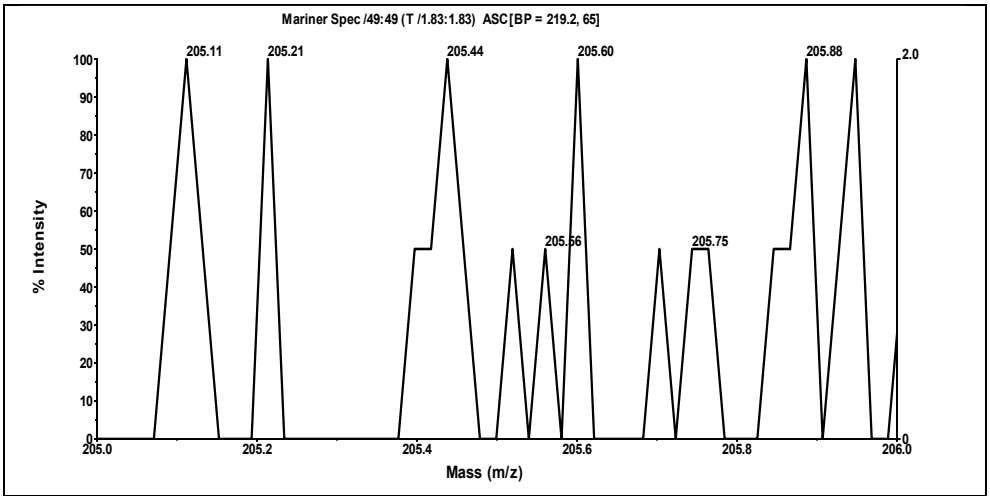
(a)


Mariner Spec /45:45 (T /1.68:1.68) ASC[BP = 219.1, 148]
120.11
120.37
120.48
120.82
120.90
2.0
% Intensity
Mass (m/z)

[b]


BPI=>NR(5.00)
T1.7
T2.1
T3.2
125.5
% Intensity
Retention Time (Min)

(c)



(d)

Fig. 8. (a) Chromatogram and (b) mass spectra of threonine in permeate at TMP of 2 bar and (c) chromatogram and (d) mass spectra of tryptophane in permeate of fermented beetroot yielded from MF membrane process at room temperature, flow rate of ~7.5 L/min, and TMP of 2 and 6 bar for 15 minutes.

3.6 Identification of particle size and particle size distribution (PSD)

The permeate of fermented beetroot from the optimal conditions of CFMF process at TMP of 2 bar and 6 bar at room temperature and flow rate of ~7.5 L/min for 15 minutes, appears as a clear reddish-purple suspension. On the other hand, the retentate (concentrate) is a thicker suspension. The biomass resulting from fermentation and the feed are both the initial material and the result of filtration through a 100-mesh filter. The particle size of the feed (5693.6 nm) appears larger than that of the biomass (5351.4 nm), even though it has undergone filtration through a 100-mesh filter, which is due to the homogenization process (8000 rpm, 30 minutes) prior to filtration. The CFMF process at TMP of 6 bar produced larger particle sizes in retentate (concentrate) (5181.3 nm) compared to the particle sizes in the concentrate at TMP of 2 bar (5656.4 nm). The higher driving force (6 bar) is suspected to compress the concentrate particles more, leading to greater accumulation and binding compared to the lower driving force (TMP of 2 bar). This also affects the particle size of the permeate, with the permeate at TMP of 6 bar being larger (2922.0 nm) compared to the treatment at TMP of 2 bar (2788.0 nm), as shown in Table 3.

Table 3. Average particle size and particle index (PI) from initial material, permeate, andretentate (concentrate) generated from separation of fermented beetroot through MF membrane process.

Type of material	Z-Average (nm)*	PI**
Biomass, fermentation 30 days	5351.4	0.526
Feed	5693.6	0.563
Retentate, TMP 2 bar	5656.4	0.370
Permeate, TMP 2 bar	2788.0	1.523
Rentetate, TMP 6 bar	5181.3	0.905
Permeate, TMP 6 bar	2922.0	1.795

Legend: *average particle diameter size; **Particle Index (PI)

The PSA analysis system indicated the Particle Index (PI) ranging from 0.37 to 1.795 for all materials. In other words, particle size will be homogeneous if PI <1, while if PI>1, it indicates that particle size tends to be non-homogeneous. This is influenced by the initial particle size of the materials and the processes they have undergone [25, 26, 27]. Regarding particle distribution, the distribution of fermented beetroot biomass resulted in particle sizes with diameters of <500 nm, <1000 nm, and <10,000 nm, with frequencies of 2.481%, 3.38%, and 3.31%, respectively, and cumulation of 3.55%, 22.43%, and 100%. Particle distribution for other materials is shown in Table 4, while Figure 9 shows the particle distribution for biomass during 30 days of fermentation, the feed, retentate (concentrate), and permeate from TMP 2 bar treatment, and the retentate (concentrate) and permeate from TMP 6 bar treatment.

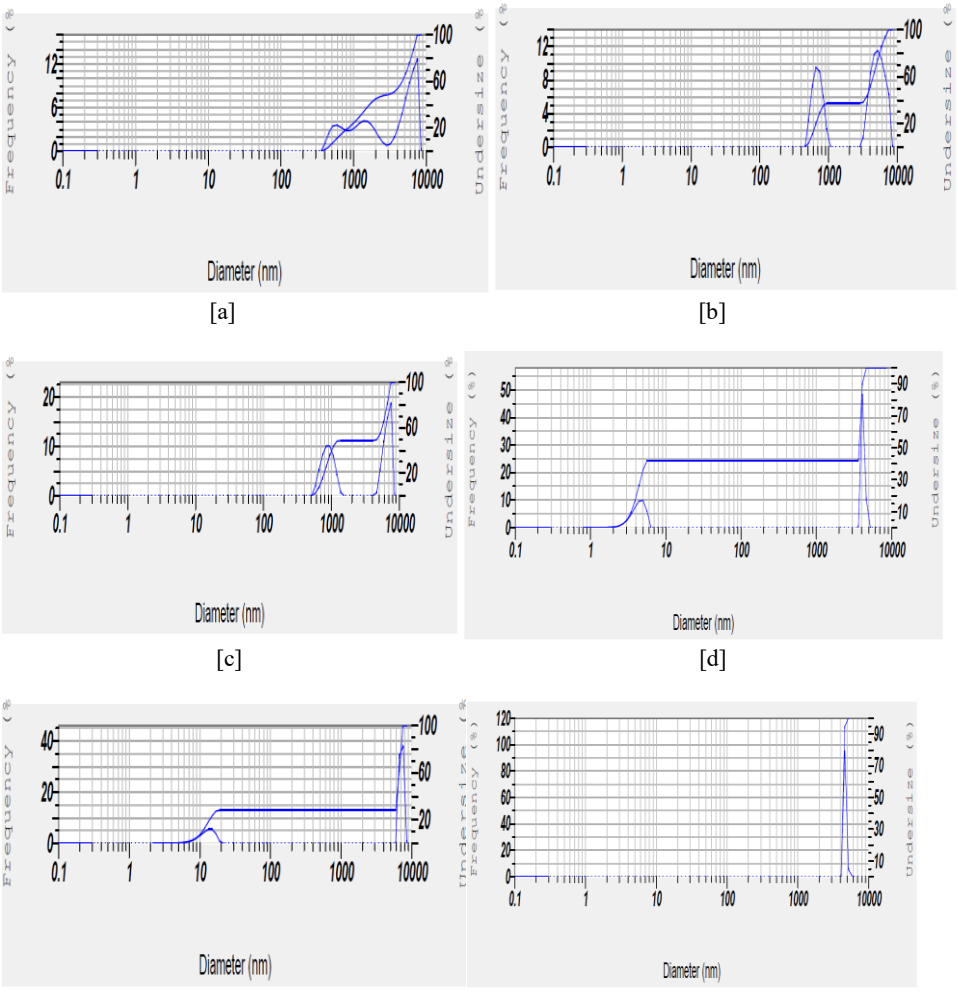


Fig. 9. Particle size distribution (PSD) of (a) biomass for 30 days of fermentation, (b) feed, (c) retentate (concentrate) at TMP of 2 bar, (d) permeate at TMP of 2 bar, (e) retentate (concentrate) at TMP of 6 bar, (f) permeate at TMP of through MF membrane process at room temperature and flow rate of ~7.5 L/min.

Table 4. Particle size distribution (PSD) of biomass, feed, permeate, and retentate (concentrate) through MF membrane at room temperature, flow rate of ~7.5 L/min.

Type of material	Ø (nm)	Frequency (%)	Cumulation (%)
Biomass	<500	2,481	3,55
	<1000	3,38	22,43
	<10000	3,31	100
Feed	<500	0	0
	<1000	3,27	37,04
	<10000	6,29	100
Retentae, TMP 2 bar	<500	0	0
	<1000	10,04	35,69
	<10000	7,93	100
Permeate, TMP 2 bar	<500	0	41,90
	<1000	41,90	41,90
	<10000	0	100
Retentate, TMP 6 bar	<500	0	0
	<1000	0	27,86
	<10000	37,90	100
Permeate, TMP 6 bar	<500	0	0
	<1000	0	0
	<10000	5,07	100

4 Conclusions

The optimization of the CFMF system in the separation of fermented beetroot permeate by kombucha culture based on soluble proteins at TMP 2 and TMP 6 bar was achieved for 15 minutes of processing, a flow rate of 7.5 L/m2.h at room temperature. Under these conditions, each TMP treatment produced permeate with soluble proteins of 0.72 and 0.38 mg/mL, total solids of 3.40% and 3.36%, total sugars of 45.91 and 47.47 mg/mL, and acetic acid of 0.93 and 0.97%, respectively. The CFMF system increased the soluble protein in permeate by 123.53% or 1.23 times that of the feed, with soluble protein rejections of -106.79% (partial separation) and 26.36% (complete separation), at flux rates of 32.52 and 28.05 L/m2.hour, respectively.

The identification of amino acids in the optimum conditions revealed the dominance of three threonine monomers with molecular weights of 120.11, 120.37, and 120.90 Da (M+), and six tryptophan monomers with molecular weights of 205.11, 205.21, 205.44, 205.60, 205.88, and 205.94 Da (M+). Permeate under optimum conditions had average particle sizes of 2788.0 nm and 2922.0 nm, with particle index of 1.523 and 1.795, respectively. The particle size distribution of permeate at TMP 2 and 6 bar produced particle diameters of <500 nm, <1000 nm, and <10,000 nm with frequencies of 0%, 41.9%, 0%; and 0%, 0%, 100%, and cumulative percentages of 41.90%, 41.90%, 100%; and 0%, 0%, 100%, respectively. In other words, permeate at TMP 2 and 6 bar had a particle size distribution consisting of 100%

particles smaller than 10,000 nm. The concentrate has the potential to be used as a concentrated powder for antioxidant-infused beverages, energy and stamina boosters.

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