The Performance of Nanofiltration Membrane in Concentrating Folic Acid from Fermented Mungbean (*Phaseolus radiatus* L.*) by Rhizopus oligosporus* Strain-C_1 and *Rhizopus* sp. for Recovery Efficiency of Natural Folic Acid

Aspiyanto[1,*] *Agustine* Susilowati[1], *Hakiki* Melanie[1], and *Yati* Maryati[1]

[1]Research Centre for Chemistry – National Research and Innovation Agency, KST B.J. Habibie, Serpong, South Tangerang - 15314, BANTEN, Indonesia

Abstract. This research was conducted to evaluate the performance of the nanofiltration (NF) membrane in concentrating permeate obtained from the ultrafiltration (UF) membrane of fermented mung bean. The procedure was performed to determine both the best flux and efficiency of recovering folic acid from the multi-filtration process. Feed A and Feed B were used as ultrafiltered permeate of fermented mung bean (*Phaseolus radiatus* L.) obtained using *Rhizopus oligosporus* strain-C_1 and *Rhizopus* sp., respectively. These feeds were subjected to the NF membrane installed in crossflow filtration (CFF) module system at room temperature, flow rate ~7.5 L/min. and transmembrane pressure (TMP) of 10 bar for 0, 15, 30, 45, 60, 75 and 90 minutes. The results showed that based on the optimum flux, the best performance of the NF membrane for Feed A and B was achieved at 15 and 15 min, with permeate flux of 32.22 and 20.44 L/m^2.h., respectively. Retentate A and B contained folic acid concentrations of 308.51 and 297.53 µg/mL, as well as total solids of 1.39% and 2.20%. Meanwhile, permeate A and B yielded folic acid concentrations of 106.88 and 63.77 µg/mL, with total solids of 0.22 and 0.32%. Under the NF process conditions, retentate A and B showed observed rejection (R_{obs}) rates of folic acid at 65.35 and 78.56%, as well as total solids of 84.34 and 85.36%, respectively. There was also a 9.75 and 3.10% increase in folic acid with a 4.18% and 17.31% rise in total solids compared to the initial condition at 0 minutes. Molecular weight analysis of folic acid in permeate A and B after 15 min. and 15 min. of processing were dominated by monomer with a MW of 442.17 Da. Meanwhile, mass spectra of feed B for a processing time of 15 min. were dominated by monomers with MW of 442.19 and 442.47 Da, both with a relative intensity of 100%.

* Corresponding author: aspiyantolipi@gmail.com

1 Introduction

Mung bean (*Phaseolus radiatus* L.) is an eco-friendly food grain leguminous crop and has been described as a sustainable source of potential nutrients. The biological properties stem from the various complementary, additive, or synergistic interactions of its constituents including high protein (20– 24%), moisture (9.4%), fats (2.05%), fiber (6.4%), oil (2.1%), energy (343.5 kcal/100 g), carbohydrates, a fairly high vitamins A and B content, and significant amounts of bioactive compounds. Among these nutrients, vitamins have gained significant attention due to their integration into functional foods, nutraceuticals, and associated health benefits [1–3]. In recent years, great attention has been focused on investigations into usable and value-added compounds derived from microbial sources such as *Rhizopus oligosporus* strain-C1 (*R. oligosporus* strain-C1) and *Rhizopus* sp. for the conversion or biotransformation of mung bean to folic acid, which is considered a nutraceutical product. This process, which occurs only in plants, bacteria, and fungi [4, 5], is facilitated by the enzymes dihydrofolate synthase (DHFS) and folypolyglutamate synthase (FPGS) to form dihydrofolate and polyglutamate folate, a derivative of folic acid [6, 7].
One of the eight essential water-soluble vitamin B, naturally found in many foods, such as in cereals and legumes or grains (mung bean, corn), green leafy vegetables (GLVs), and fruits is folic acid (FA). Chemically, folic acid, also known as vitamin B9, is characterized by a core molecule consisting of a heterocyclic pterin structure, with a methyl group in the sixth position bound to para-aminobenzoic acid (pABA) and glutamic acids. The molecular formula is $C_{19}H_{19}N_7O_6$ and it has a molecular weight (MW) of 441.4 Da. [8, 9]. Folate (vitamin B9) has various functions in the body, including aiding in the prevention of some birth irregularities, reducing the risk of birth defects related to the brain and spine during early pregnancy, promoting the formation and production of red blood cells, supporting DNA and RNA production, and metabolism of amino acid. It also serves in preventing megaloblastic anemia, neurological disorders, heart-related cardiovascular diseases (especially strokes), certain types of cancers, cognitive impairment, bone-related osteoporosis, neural tube malformations in newborns, as well as the creation of new proteins [10–12].

Membrane-based separation technologies have received significant attention due to their advantages over conventional systems. These advantages include their capability to separate molecules and microorganisms, athermal nature, superior selectivity, no phase changes, chemical agents-free conditions, as well as operation in mild conditions of temperature and pressure. Other advantages include no chemical damage, low energy consumption, easy operation, comprehensive utilization of resources, avoidance of product contamination, preservation of the biological activity of target compounds, reduced pollution, and environmental-related concerns. Consequently, the nanofiltration (NF) membrane is recognized as the best available technology (BAT) [13–15]. NF, a de novo class of pressure-driven membrane separation technology is extensively implemented to separate, purify, and concentrate liquid-phase in biotechnological, nutraceutical, pharmaceutical, dairy, fruit juice, plant extracts, and food-beverage sectors. The potential lies in its ability to separate thermosensitive and degradable compounds with low MW including glucose, amino acid, peptide, folic acid, organic acids, cells, and salts, from the fermented broth [16, 17]. The NF membrane has a pore size of 0.2 – 2 nm or nominal molecular weight cut-off (MWCO) ranging from 200 to 1000 Dalton (Da). The typical operating pressure is between 5 to 35 bar and the selectivity is largely determined by the mechanism of size-sieving and charge (electrostatic) effects [18]. NF process performance is significantly influenced by membrane characteristics including MWCO, porosity, morphology, charge, and hydrophilicity, as well as feed properties composed of molecular weight, size, geometry, charge, hydrophilicity of

the solute, and pH. It is also affected by operational conditions namely temperature, flow rate, operation pressure, and time [19].

This study focused on the recovery of folic acid from a suspension of fermented mung bean using *R. oligosporus* strain-C1 and *Rhizopus* sp. The process was performed through a series of multi-filtration steps, including purification with an ultrafiltration membrane (UF, 100 kDa.) at a pump motor frequency (PMF) of 20 Hz and operating pressure of 4 and 6 bar for 90 minutes. Moreover, observations were carried out at an interval of 15 minutes. This process divided the feed solution into a permeate fraction containing all components that permeated the membrane, and a retentate fraction composed of rejected compounds. The retentate was further processed through the drying process to obtain concentrate powder. Meanwhile, the permeate was concentrated by nanofiltration (NF-99-PE) membrane to obtain the NF concentrate, effectively separating all folic acid components in the biomass. These series of processes produced folic acid with maximum separation efficiency [20–23]. This research activity aimed to determine the performance of NF membrane (NF-99-PE) installed in the CFF module system for the separation and concentration processes of UF permeate obtained from fermented mung bean using *R. oligosporus* strain-C1 and *Rhizopus* sp. The parameters assessed included process time, flux, composition of solutes, observer rejections (R_{obs}) of folic acid and total solids, as well as characteristics of folic acid monomer.

2 Materials and Methods

2.1 Materials and Equipments

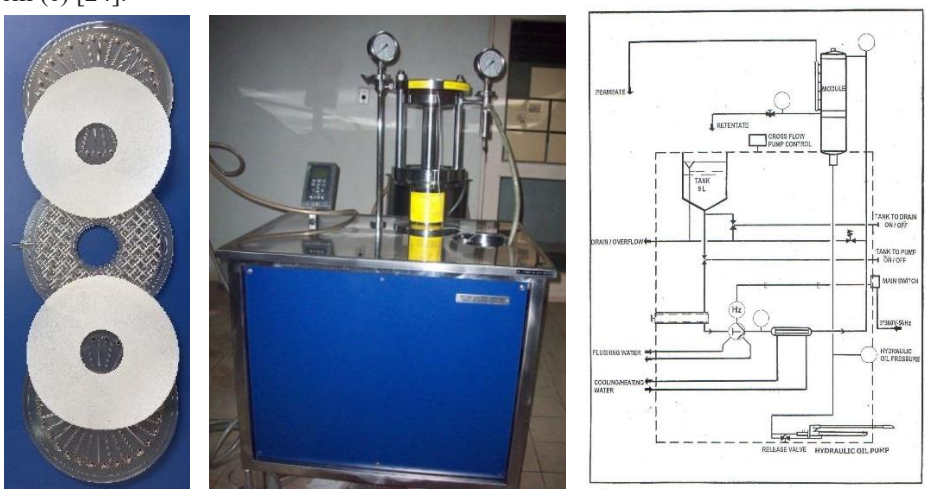
The main materials used in this study included mung bean procured from the local market (South Tangerang), fungi of *R. oligosporus* strain-C1 (Center Research for Chemistry, BRIN), fungi of *Rhizopus* sp. (commercial, PT Aneka Fermentasi Indonesia, Bandung), standard folic acid (Aldrich/SIGMA), 2 sheets of commercial UF membrane (FS-40-PP, Fluoro polymer, MWCO 100 kDa., Alfa Laval, Denmark), 2 sheets of commercial NF membrane (NF-99-PE, Thin Layer Composite on Polyester, MWCO ≤ 200 Da., Alfa Laval, Denmark). Meanwhile, the equipment used was laminar flow chamber (local), incubator (local), pulverizer (local), homogenizer (Ultra-Turrax, Ika Labortechnik, T50, Janke & Kunkel, Germany), High-Frequency Separation, sieve of 100 mesh, plate & frame type cross-flow membrane filtration in module (LabUnit M20, DSS, Nakskov, Denmark) [24], and glass wares for chemical analysis. The main instruments of analysis were UV-vis Spectrophotometer (Model RF-550, Shimadzu, Japan) [25], Liquid Chromatography coupled with Mass Spectrometer (LC-MS) (Mariner Biospectrometry) with LC (Hitachi L 6200) [26].

2.2 Research Design

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 typical process started by preparing fermented mung bean using A (*R. Oligosporus* strain-C_1) and B (*Rhizopus* sp.) inoculated at 0.2%, w/w with a pH of 5 and left overnight. Furthermore, steeped mung bean grains were packaged in perforated plastic, and incubated at 30°C for 48 hours until fermented mung bean of A and B with the growth of uniform yeast were produced. To prepare feed A and B, 1 part of fermented mung bean was pulverized by adding 4 parts of clean water, homogenized (8000 rpm, 15 – 25 min.), and sieved using 100 mesh. Feed-A was introduced to a UF membrane (100 kDa.) installed in plate and frame type cross-flow membrane filtration in module system at room temperature, PMF of 20 Hz (flow rate ~7.5 L/min) and TMP of 6 bar for 90 min, producing both retentate and permeate. Similar concentration processes by NF membrane and conditions were applied to feed B (*Rhizopus* sp.), resulting in UF retentate-A and UF permeate-B. Subsequently, UF permeate-A and UF permeate-B were introduced to NF membrane (NF-PE-99) equipped with CFF module system at room temperature, a flow rate of ~7.5 L/min., and TMP of 10 bar for 0, 15, 30, 45, 60, 75, and 90 minutes. Sampling was conducted by interval time of 15 min to produce retentate and permeate. Cleaning was carried out sequentially by rinsing with distilled water followed by chemical cleaning with 0.1 N NaOH solution at room temperature. Subsequently, distilled water was filtered at 6 bar until the pH of the feed was neutralized. Figure 1 shows a spacer-membrane-support plate-membrane-spacer sandwich (a), a photo of the Module of LabUnit M20 (b), and the schematic diagram of the membrane filtration system (c) [24].



(a) (b) (c)

Fig. 1. Visualization of modules used in the research activity. (a). Spacer-membrane-support plate-membrane-spacer like sandwich, (b). A photo of Module of LabUnit M20 and (c) The schematic diagram of the membrane filtration system [24].

2.4 Characterization

The collected samples were then analyzed for total solids (Gravimetric method) **[27]** and folic acid (UV-vis Spectrophotometer) [28] to determine the observed rejection (Robs) of solutes. The volume of the permeate was measured to calculate permeate flux. Similar NF membrane processes and conditions were applied to fermented mung bean by *Rhizopus sp*. (B) as feed, and the experiment data was carried out in triplicates.

3 Results and Discussion

3.1 Characteristic of materials

The fermentation process of mung bean using *R. oligosporus* strain-C1 (A) and *Rhizopus* sp. (B) resulted in a better composition compared to the unfermented sample. Folic acid increased significantly by 412.38% (4.12-fold) and 1,168.5% (11.68-fold) for *R. oligosporus* strain-C1 (A) and *Rhizopus* sp. (B), respectively. The process also elevated the concentrations of dissolved protein, total sugars, and reducing sugars, while the total solids decreased. The enzymatic activity of protease played a role in degrading mung bean to folic acid and dissolved protein, while amylolytic activity raised the concentration of reducing sugars, contributing to the general increase in total sugars. The reduction in the total solids may be due to the water adsorption that occurred during fungi metabolism in the fermentation process. In general, *Rhizopus sp.* similar to tempeh fungi comprises various species such as *R. oryzeae*, and *R. oligosporus*, while biomass A was a single culture of *R. oligosporus* strain-C1. The pulverizing process was carried out by adding 1 part of water to 6 parts of the sample, followed by filtration through a 100 mesh sieve to obtain filtrate and residue. The filtrate was used as feed in the concentration process using a UF membrane (UF feed). UF feed-A and UF feed-B served as feeds of fermented mung bean obtained using *R. oligosporus* strain-C1 and *Rhizopus* sp., respectively. Based on the results, UF feed-A yielded a higher concentration of folic acid (597.53 µg/mL) compared to UF feed-B (323.84 µg/mL). This was caused by the interaction among the pulverization, homogenization, and filtration processes, with the fermented mung beans. Mung bean fermented using *R. oligosporus* strain-C1 (A) had a less compact appearance with fungi growth compared to those fermented using *Rhizopus* sp. which had widely dispersed mycelium on the surface of substrates. This resulted in mung bean grains fermented with *R. oligosporus* strain-C1 (A) being softer and easier to pulverize, making the particles and folic acid more soluble [29–30]. Figure 2 shows various products, including mung bean (a), fermented mung bean by *R. oligosporus* strain-C1 (b) and *Rhizopus* sp. (c), as well as the feed of fermented mung bean by *R. oligosporus* strain-C1 for UF feed-A (d), and *Rhizopus* sp. for UF feed-B (e).

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

Fig. 2. (a) mung bean, (b) fermented mung bean by *R. oligosporus* strain-C1 (A), (c) fermented mung bean by *Rhizopus* sp. (B), (d) feed of fermented mung bean by *R. oligosporus* strain-C1 for UF (UF feed-A), and (e) feed of fermented mung bean by *Rhizopus* sp. for UF (UF feed-B).

Feed-A and feed-B, respectively were introduced to the UF membrane (MWCO of 100 kDa.) installed in the cross-flow filtration (CFF) module system. This was the initial step to separate the folic acid compound from a suspension of fermented mung bean at room temperature, flow rate of ~7.5 L/min. and TMP of 4 and 6 bar, with a duration of 0 – 90 minutes. The optimum conditions of both feed-A and feed-B were achieved at TMP of 4 bar for 75 minutes and TMP of 4 bar for 45 minutes, respectively. The retentates were then dried to obtain concentrate powder, while the permeates were further processed using NF membrane. Table 1 shows the composition of materials in NF process using the result of fermented mung bean by *R. oligosporus* strain-C_1 (A) and *Rhizopus* sp. (B) [31].

Table 1. Composition of materials in NF process using UF permeate from result of biomass of fermented mung bean by *R. oligosporus* strain-C1 (A) and *Rhizopus* sp. (B).

Kind of materials	Concentration		Kind of Materials	Concentration	
	Folic Acid (µg/mL)	Total Solids (%)		Folic Acid (µg/mL)	Total Solid (%)
Mung bean Fermented mung bean–A*	31.43 161.04	39.53 34.28	UF permeate (4 bar, 75 min)–A	479.22	1.97
Fermented mung bean–B*	398.70	38.89	UF retentate (4 bar, 45 min)–B	496.75	4.98
UF Feed–A**	597.53	2.85	UF permeate (4 bar, 45 min)–B	479.22	1.97
UF feed–B**	323.84	4.59	NF feed–A***	328.31	1.12
UF retentate (4 bar, 75 min)–A	649.74	3.23	NF feed–B***	302.99	1.11

Legend: *Fermented mung bean A: by *R. oligosporus*-C_1 at room temperature, 36 hours,
B: by *Rhizopus* sp. at room temperature, 48 hours;
**UF feed as a result of pulverizing fermented mung bean at ratio of 1 : 6;
***permeate from mixture of UF permeate at room temperature, flow rate of ~7.5 L/min. and TMP of 6 bar for 90 min. as feed of NF.

Feeds used for NF membrane process were permeate UF mung bean fermented using *R. oligosporus* strain-C_1 (NF feed-A) and *Rhizopus* sp. (NF feed-B) with a total solid concentration of 1.12 and 1.11%, respectively. This high concentration directly affected the permeate flux. For NF feed-A, the initial permeate flux (38.89 L/m^2.h) decreased rapidly at 15 minutes (32.22 L/m^2.h). This was attributed to the colloids, macromolecules, and undesirable solute particles that accumulated on the surface of the membrane and were adsorbed, resulting in effective membrane pore blocking (irreversible pore blocking). There was a gradual decrease in the flux as follows: 30 (31.66 L/m^2.h), 45 (31.66 L/m^2.h), 60 (31.11 L/m^2.h), 75 (30.55 L/m^2.h), and 90 minutes (28.39 L/m^2.h), eventually reaching a steady state depending on the condition of the kind of feed.

3.2 Effect of nanofiltration process condition on flux, concentrations of folic acid (µg/mL) and total solids (%), and rejections of folic acid (%) and total solids (%)

The performance of NF membrane system was assessed based on its ability to generate high productivity (permeate flux) within a short period while maintaining a high solute rejection rate relative to the concentration. The permeate flux was determined by measuring the volume passing through the effective membrane area unit collected over the measured time intervals. It is usually expressed in volume per unit membrane area per unit time, or Liters/m^2.hour (L/m^2.h) [32]. Feeds used for NF membrane process were permeate UF mung bean fermented using *R. oligosporus* strain-C_1 (NF feed-A) and *Rhizopus* sp. (NF feed-B) with a total solid concentration of 1.12 and 1.11%, respectively. This high concentration directly affected the permeate flux. For NF feed-A, the initial permeate flux (38.89 L/m^2.h) decreased rapidly at 15 minutes (32.22 L/m^2.h). This was attributed to the colloids, macromolecules, and undesirable solute particles that accumulated on the surface of the membrane and were adsorbed, resulting in effective membrane pore blocking (irreversible pore blocking). There was a gradual decrease in the flux as follows: 30 (31.66 L/m^2.h), 45 (31.66 L/m^2.h), 60 (31.11 L/m^2.h), 75 (30.55 L/m^2.h), and 90 minutes (28.39 L/m^2.h),

eventually reaching a steady state depending on the condition of the kind of feed. For NF feed-A, cross-flow helped to gradually remove solutes on the membrane surface from 30 minutes to 90 minutes of the filtration process. This occurred due to the reversible pore blocking caused by solutes in the feed. For NF feed-B, the initial permeate flux of 25.02 L/m^2.h reduced gradually as follows: 15 (20.44 L/m^2.h), 30 (19.66 L/m^2.h), 45 (18.44 L/m^2.h), 60 (17.66 L/m^2.h), stabilizing after 75 (16.77 L/m^2.h), and 90 minutes (16.44 L/m^2.h). The gradual decrease may be due to the formation of a polarization concentration layer on the membrane surface, which was directly related to the increased solute concentration at the interface between the membrane and the feed suspension. This layer was responsible for the decrease in the permeate flux towards a steady state value of approximately 16.44 L/m^2.h. The decline was also attributed to the increasing feed suspension viscosity caused by the water mass transfer passing freely through the membrane. Under conditions of constant cross-flow rate and TMP, permeate flux was reduced to a steady-state value, which could be lower than the initial flux value. The effect of NF process time on permeate flux in the concentration process of UF permeate-A (NF feed-A) UF permeate-B (NF feed-B) at the fixed operating conditions of room temperature, flow rate ~7.5 L/minutes, and TMP of 10 bar for 15 minutes is shown in Figure 3.

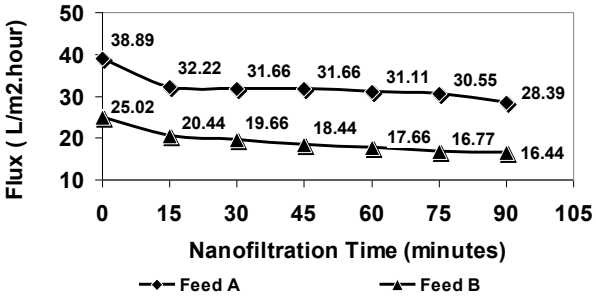
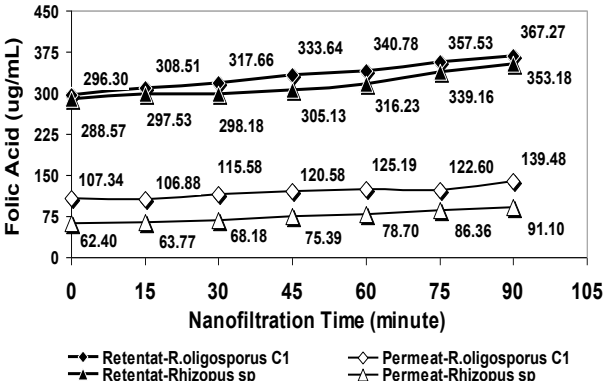
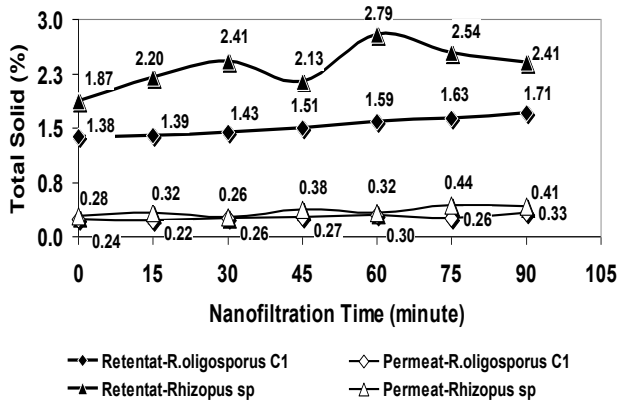


Fig. 3. The relationship between NF process time and flux at various feeds of UF permeate from fermented mung bean by *R. oligosporus* strain-C1 and *Rhizopus sp.* at room temperature, flow rate ~7.5 L/min. and TMP 10 bar.

Both NF feed-A and NF feed-B yielded significant folic acid retained on the surface of the NF membrane compared to the permeate. The folic acid concentration increased in line with the longer NF process time. NF feed-A generated a higher concentration both in retentate and permeate compared to NF feed-B. In this optimum process condition, NF system is able to increase folic acid concentrations in NF retentate-A (19.20%) and NF retentate-B (27.27%) compared to initial NF process of 7.57% (296.3 µg/mL) and 21.21% (288.57 µg/mL), respectively and folic acid concentration in feed, namely 328.31 µg/mL and 302.99 µg/mL, respectively, as shown in Figure 4a. Furthermore, both samples had significantly high total solids retained on the surface of NF membrane compared to those passing as permeate. The total solids concentration also increased consistently with higher NF process time. NF feed-A generated higher concentrations of total solids in retentate and permeate compared to NF feed-B. In this optimum process condition, NF system is able to raise total solids concentration in retentate-A (41.96%) and retentate-B (151.35% or 1.51-fold) compared to concentrations of total solids in feeds, namely 1.12% (34.06%) and 1.11% (49.20%) compared to starting NF process, namely 1.38% and 1.87%, as shown in Figure 4b.



(a)



(b)

Fig. 4. Effect of nanofiltration time on concentrations of folic acid (a) and total solids (b) present in retentate and permeate in concentration process of UF permeate of fermented mung bean by *R. oligosporus* strain-C1 and *Rhizopus* sp. at room temperature, flow rate ~7.5 L/min. and TMP 10 bar.

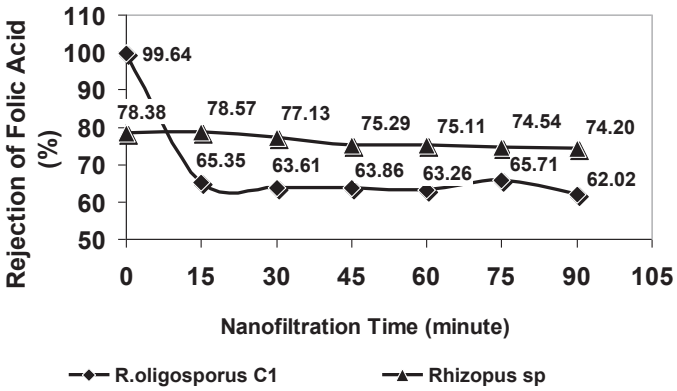
The term "rejection" referred to the solute component that did not pass through the membrane, but was instead retained or rejected. The observed rejection (R_{obs}) was calculated based on the equation, R_{obs} (%) = [1 – Concentration of solute in permeate/Concentration of solute in feed or retentate] x 100%. The selectivity, reflected in the R_{obs} of solute values ranged from 0% (for solutes with the highest probability of passing freely through the membrane) to 100% (when solutes were completely retained on the surface) **[33–34].** The effects of process time on R_{obs} of folic acid and total solids in the concentration process of both NF feed-A and NF feed-B at room temperature, flow rate ~7.5 L/min., and TMP of 10 bar are shown in Figures 5a and 5b.

For NF feed-A, the R_{obs} of folic acid reduced significantly from the initial value of 99.64% as follows: 15 (65.35%), 30 (63.61%), 45 (63.86%), and 60 minutes (63.26%). There was a gradual increase at 75 minutes (65.71%) and a slow decline at 90 minutes (62%). Meanwhile, for NF feed-B, the R_{obs} of folic acid indicated a gradual increase from the initial value of 78.38% to 78.57% at 15 minutes. There was a slight drop at 30 (77.13%), 45 (75.29%), 60 (75.11%), 75 (74.54%), and 90 minutes (74.00%). The R_{obs} of total solids in concentration process of NF feed-A appeared to increase from an initial value of 83.53% to 84.34% after 15 minutes, followed by a subsequent sharp drop at 30 (81.99%), slow increase

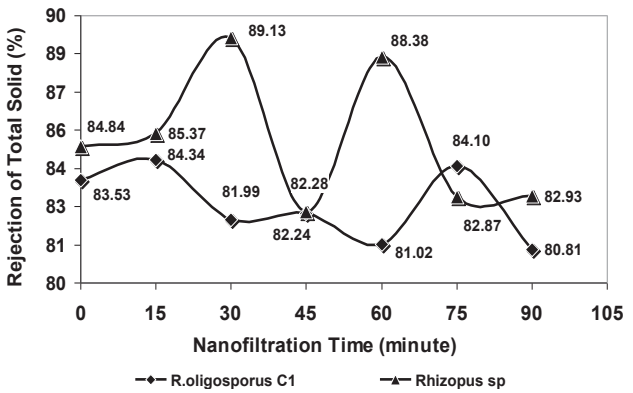
at 45 (82.24%), decrease at 60 (81.02%), sharp increase at 75 (84.10%), and a sharp decline at 90 minutes (80.81%). On the other hand, the R_{obs} of total solids generated from concentration process of NF feed-B showed a gradual increase from the initial value of 84.84% to 85.37% after 15 minutes, followed by a sharp increase at 30 (89.13%), strong decline at 45 (82.28%), sharp increase at 60 (88.13%), significant decline at 75 (82.87%), and a slight increase at 90 minutes (82.93%). For NF feed-A, the performance of NF membranes in terms of average R_{obs} for folic acid and total solids were 69.06% and 82.57%, while NF feed-B was 76.17% and 85.11%, respectively, depending on the size of the pores. The lower R_{obs} was attributed to the hydrophilic behaviors and the low molecular weights of the component. NF system is able to show successfully separation of components from NF feed-A and NF feed-B with R_{obs} of folic acid in the range of 74.20% - 78.38% and in the range of 62.02% - 63.78%, respectively. There was a higher ratio of total solids concentration in permeate and retentate of NF feed-B compared to NF feed-A. Meanwhile, NF membrane equipped the CFF module system produced a successful separation of NF-Feed A with R_{obs} between 83.53%-80.81%, as well as NF-Feed B with R_{obs} ranging from 84.84%-82.93%, respectively. The higher R_{obs} was attributed to the interaction amongst operation condition (flow rate, pressure, time) and sensitivity of NF membrane characteristic on each feed causing optimum selectivity as one of the membrane performances [35]. NF system is able to show successfully separation of components from NF feed-A and NF feed-B with R_{obs} of folic acid in the range of 74.20% - 78.38% and in the range of 62.02% - 63.78%, respectively. Meanwhile, NF membrane equipped the CFF module system produced a successful separation of NF-Feed A with R_{obs} between 83.53%-80.81%, as well as NF-Feed B with R_{obs} ranging from 84.84%-82.93%, respectively. The effect of NF time on R_{obs} of folic acid and total solids in the concentration process of UF permeate obtained from mung beans fermented by *R. oligosporus* strain-C1 and *Rhizopus sp.* at room temperature, flowrate ~7.5 L/minutes and TMP of 10 bar is shown in Figures 5a and 5b.

Optimum permeate fluxes were achieved by using UF permeate-A (as NF feed-A) and UF permeate-B (as NF feed-B) for 15 minutes with a yield of 32.22 L/m^2.h and 20.44 L/m^2.h. Under these conditions, NF retentate-A produced folic acid of 308.51 µg/mL and total solids of 1.39%, while NF retentate-B values of 297.53 µg/mL and 2.20%. Meanwhile, permeate NF A yielded folic acid of 106.88 µg/mL and total solids of 0.21%. NF permeate-B had folic acid of 63.77 µg/mL and total solids of 0.32%. These optimum permeate fluxes resulted in R_{obs} of folic acid of 65.35% and R_{obs} of total solids of 84.34% for NF feed-A as well as 78.56% and 85.36% for NF feed-B, respectively. NF membrane showed increased folic acid content in NF retentate-A and NF retentate-B, namely 4.12% and 3.1%, compared to NF feed-A and NF feed-B at the initial time, which were 296.30 and 288.57 µg/mL. On the other hand, a decrease in folic acid occurred in NF permeate-A and NF permeate-B amounting to 6.03% and 1.80% compared to NF feed-A and NF feed- for the initial time, at 328.31 and 302.99 µg/mL, respectively.

Studying process of optimisation on folic acid and total solids in retentate and permeate demonstrate that the optimum permeate fluxes are achieved by using UF permeate-A (as NF feed-A) for 15 min. with flux yielded 32.22 L/m^2.h., and UF permeate-B (as NF feed-B) for 15 min. with flux yielded 20.44 L/m^2.h. In this condition, it is produced NF retentate-A with folic acid of 308.51 µg/mL and total solids of 1.39%, and NF retentate-B with folic acid of 297.53 µg/mL and total solids of 2.20%, while NF permeate-A with folic acid of 106.88 µg/mL and total solids of 0.21%, and NF permeate-B with folic acid of 63.77 µg/mL and total solids of 0.32%.



(a)



(b)

Fig. 5. Effect of nanofiltration time on R_{obs} of (a) folic acid and (b) total solids in concentration process of UF permeate of fermented mung bean by *R. oligosporus* strain-C1 and *Rhizopus sp.* at room temperature, flow rate ~7.5 L/min. and TMP of 10 bar

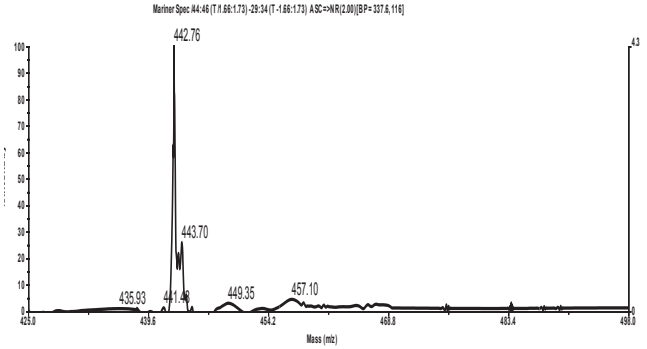
In this optimum permeate fluxes are generated R_{obs} of folic acid 65.35% and R_{obs} of total solids 84.34% for NF feed-A and R_{obs} of folic acid 78.56% and R_{obs} of total solids 85.36% for NF feed-B. In this condition, NF membrane displays increase of folic acid in NF retentate-A and NF retentate-B, namely 4.12% and 3.1%, respectively as compared with NF feed-A and NF feed-B for initial time, namely 296.30 and 288.57 µg/mL, respectively. On the other hand, it occurs a decrease of folic acid in NF permeate-A and NF permeate-B, namely 6.03% and 1.80% as compared with NF feed-A and NF feed-B for initial time, namely 328.31 and 302.99 µg/mL. In this condition, NF system is able to raise folic acid concentrations in retentate A (4.12%) and retentate B (3.10%) compared with folic acid concentrations in initial NF process for retentate A (296.30 µg/mL) and retentate B (288.57 µg/mL), however, it takes place a decline of folic acid concentrations in retentate A (6.03%) and retentate B (1.80%) compared with folic acid concentrations in NF feed-A (328.31 µg/mL) and NF feed-B (302.99 µg/mL). Figures 6a, 6b, 6c and 6d visualize NF retentate-A, NF permeate-A, NF retentate-B and NF permeate-B at the optimum condition based on permeate flux for 15 min.

(a) (b) (c) (d)

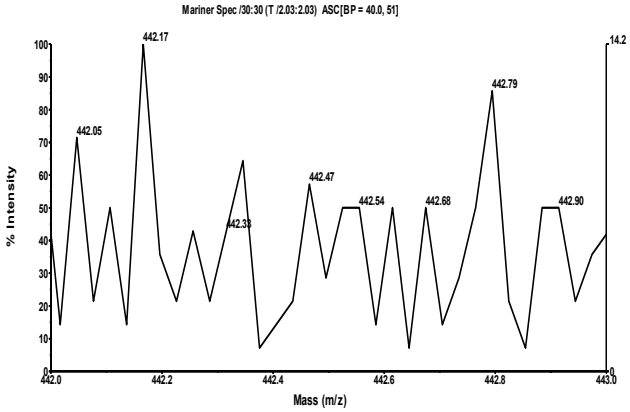
Fig. 6. (a) NF retentate-A, (b) NF permeate-A, (c) NF retentate-B and (d) NF permeate-B as a result of NF concentration process by using feed of UF permeate of fermented mung bean by *R. oligosporus* strain-C_1 (A) and *Rhizopus* sp. (B) at room temperature, flow rate ~7.5 L/min. and TMP of 10 bar for 15 min

3.3 Identification of folic acid monomer

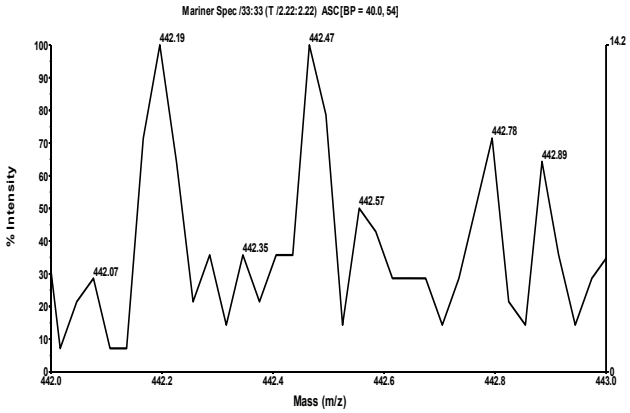
The identification of folic acid monomer was determined using Liquid-chromatography mass spectrometry (LC-MS) with a Mariner Biospectrometry instrument. Samples were prepared from standard folic acid, and permeate was collected from the best treatment, based on fluxes for both 15 and 15 minutes of processing. Folic acid, with a molecular weight (MW) of 441 Da. was analyzed through LC-MS to identify compounds that might exhibit differences in MW, with the possibilities being M^+, M^+Na^+, $2M^{++}$ or $2M^+$, Na^+. In this analysis, folic acid monomers were identified as M^+**.** The operation conditions of LC-MS were an injection volume of 5 µL and a flow rate of 0.2 mL/min. Meanwhile, the types of solvents used included an eluent mixture containing 80 parts of methanol and 20 parts of water with column C-8 (15 mm x 2 mm)**.** For standard folic acid, mass spectra in a range of 425 – 498 m/z predominantly showed folic acid monomer with MW of 442.76 Da and relative intensity of 100%, as shown in Figure 7a. The identification of folic acid monomer was performed on NF feed-A and NF feed-B from the optimum flux treatment at room temperature, flow rate of ~7.5 L/minutes, and TMP of 10 bar for 15 and 15 min. These fluxes were 30.55 and 20.44 L/m^2.h respectively. Mass spectra of NF feed-A with a range of 425 – 498 m/z yielded 8 monomers of folic acid with MW ranging from 442.05 – 442.90 Da. The most predominant monomer had a MW of 442.17 Da. Meanwhile, the mass spectra of NF feed-B with a range of 425 – 498 m/z showed 7 monomers with MW between 442.07 and 442.89 Da. Two monomers were more common with MW of 442.1948 and 442.4747 Da. as well as relative intensities of 100%, as shown in Figures 6b and 6c.



(a)



(b)



(c)

Fig. 7. (a) mass spectra of standard folic acid, (b) mass spectra of NF feed-A and (c) mass spectra of NF feed-B in separating fermented mung bean through NF membrane process at room temperature, flow rate of ~7.5 L/min. and TMP of 10 bar Hz.

4 Conclusions

The performance of NF membrane equipped with a cross-flow filtration (CFF) module system to concentrate the result of UF permeate obtained from mung bean fermented by *R. oligosporus* strain-C_1 (A) and *Rhizopus* sp. (B) at fixed process condition (room temperature, flow rate of ~7.5 L/min, and transmembrane pressure (TMP) of 10 bar for 0 – 90 min. showed a decline in permeate flux and observed rejection (R_{obs}), However, an increase was observed in the composition of folic acid and total solids, with extended process duration. The optimum performance was achieved at 15 minutes for both NF feed-A and NF feed-B, with permeate fluxes of 32.22 and 20.44 L/m^2.h. Under these conditions, the folic acid and total solid concentrations were 308.51 and 297.53 µg/mL, as well as 1.39% and 2.20%. Meanwhile, both permeate samples yielded values of 106.88 and 63.77 µg/mL as well as 0.21 and 0.32%, respectively. NF feed-A and NF feed-B produced folic acid R_{obs} of 65.35 and 78.56%, while total solids were 84.34 and 85.36%. An increase in folic acid was observed in the NF retentate-A and NF retentate-B, namely 4.12% and 3.10% compared to the initial process

time. NF permeate-A was dominated by folic acid monomers with MW of 442.17 Da, while NF permeate-B predominantly contained two monomers having MW of 442.19 and 442.47 Da, as well as relative intensity of 100%.

References

1. T. Dinsa, U. Balcha, F. Benya, M, Fufa, *J. Sci. Agric.,* **6,** 1 **(2022)**.
2. K. Kaysha, D. Shanka, M. Bibiso, *Cogent Food Agric.,* **6,** 1771112, 2 **(2020).**
3. Z. Shi, Y. Yao, Y. Zhu, G. Ren, *Crop J.,* **4**, 398 **(2016).**
4. P. M. Hauser, I. G. Macreadie, FEMS Microbiol Lett., **256** (2), 244–250 **(2006)**.
5. V. Gorelova, O. Bastien, O. De Clerck, S. Lespinats, F. Rébeillé, D. Van Der Straeten, Sci. Rep., **9**, 5731, 1 **(2019).**
6. N. D. Karakousis, K. Gourgoulianis, O.S. Kotsiou, J. Pers. Med. **13,** 561, 2 **(2023)**.
7. V. Crécy-Lagard, B. El Yacoubi, R. Díaz de la Garza, A. Noiriel, A. D. Hanson, BMC Genom., **8**:245, 2 **(2007).**
8. S. H. Zafar, M. Umair, M. Akhtar, Food Chem. Advances*,* **2**, 100160, 1 **(2023).**
9. Y. Shulpekova, V. Nechaev, S. S. Kardasheva, A. Sedova, A. Kurbatova, E. Bueverova, A. Kopylov, K. Malsagova, J.C. Dlamini, V. Ivashkin. Molecules, **26**, 3731, 2 **(2021).**
10. J. Singh, Vitamin B9 in Dark Green Vegetables: Deficiency Disorders, Bio-Availability, and Fortification Issues, 1-2 **(2021)**.
11. E. Gujska, M. Czarnowska, J. Michalak, Towaroznawcze Problemy Jakosci**, 1,** 38, 111 **(2014)**.
12. L. Kaldygulova, T. Ukybassova, G. Aimagambetova, A. Gaiday, A. Tussupkaliyev, Biomedicines, **11**, 272, 2-3 **(2023).**
13. C. Quezada, H. Estay, A. Cassano, E. Troncoso, R. Ruby-Figueroa, Membranes, **11,** 368, 2 **(2021).**
14. C. Yang, C. Guoqiang, W. Yinhua, L. Jianquan, Eng. Life Sci., **21,** 405 **(2021)**.
15. C. Conidi, E. Drioli, A. Cassano, Biomolecules, **10**, 2. **(2020)**.
16. D. Yadav, S. Karki, P. G. Ingole, Food Eng. Rev. **14**, 580 **(2022)**.
17. M. G. Eloffy, D. M. El-Sherif, M. Abouzid, M. Abd Elkodous, H. S. El-nakhas, R. F. Sadek, M. A. Ghorab, A. Al-Anazi, G. S. El-Sayyad, Nanotechnol. Rev., 11, 10 (2022).
18. A. M. Nasir, M. R. Adam, S. N. E. A. M. Kamal, J. Jaafar, M. H. D. Othman, A. F. Ismail, F. Aziz, N. Yusof, M. R. Bilad, R. Mohamud, M. A. Rahman, W. N. W. Salleh, Sep. Purif. Technol., **286,** 120454, 1,5 **(2022).**
19. A. Cassano, C. Conidi, R. Ruby-Figueroa, R. Castro-Muñoz, Int. J. Mol. Sci., **19,** 35, 6 **(2018)**.
20. S. Jafarinejad, M.R. Esfahani, Separations, **8**, 206, 2 **(2021).**
21. Y. Liao, C. H. Loh, M. Tian, R. Wang, A.G. Fane, *Prog. Polym. Sci.,* 77, 69–94 **(2018).**
22. D. Rehman, Y. D. Ahdab, V. J. H. Lienhard, Water Res., **199,** 3 **(2021).**
23. L. Xian hui, T. Sheng, L. Jian quan, M. Pinelo, Chem. Sci. Eng., **15**, 4, 841 **(2021).**
24. O. J. Olsen, Operating Manual of DSS LabUnit M20, Danish Separation Systems AS, Nakskov, Denmark, January **(2000).**
25. W. Ruengsitagoon, N. Hattanat, Simple Spectrophotometric Method for Determination of Folic Acid, in The 4th. Annual Northeast Pharmacy Research Conference of Pharmacy Profession in Harmony, 11-12 February 2012, Khon Kaen University, Thailand **(2012).**

26. P. Eichhorn, T. P. Knepper, J. Mass Spectrom., **36** (6), 677 – 684 **(2021).**

27. AOAC International, 16th Edition, Volume 1 **(1995).**

28. L. C. P. Marieta, M. L. M.F.S. Saraiva, *Measurement,* **135,** 896 **(2019).**

29. R. Andreas, S. Reggie, Int. J. Gastron. Food S., **26**, 100413, 3 **(2021).**

30. A. D. Ahnan-Winarno, L. Cordeiro, F. G. Winarno, J. Gibbons, H. Xiao, Compr. Rev. Food Sci. Food Saf., **20,** 1717–1767 **(2021).**

31. H. L. Xian, T. Sheng, Q. L. Jian, P. Manuel, Front. Chem. Sci. Eng., **15,** 4, 841 **(2021).**

32. Y. Diksha, H. Swapnali, G. I. Pravin, 2022 , Emergent Mater., **5**, 1314 **(2022)**.

33. M. S. Christian, S. Matthias, L. Ronny, H. Jorg, Sep. Purif. Technol., **239,** 116550, 4 **(2020).**

34. R. Epsztein, Front. Membr. Sci. Technol., **1**, 1048416, 02 **(2022).**

35. E.H.H. Al-Qadami, A. Ahsan, Z. Mustaffa, A.S. Abdurrasheed, K.W. Yusof, S.M.H. Shah. J. Desalination and Water Purification, **18**, Issue : 31 March, pp.6 **(2020)**.