Separation of Cellulase Enzyme from Fermentation Broth of Palm Oil Mill Effluent by Ultrafiltration Process

Rashid S S[1], Alam M Z[2], Fazli M B F A[3]

Abstract—Cross-flow ultrafiltration (UF) was used to investigate the separation process of cellulase enzyme, a bioconversion product of palm oil mill effluent (POME) by *Trichoderma reesei* RUT C-30. Microfiltration (MF) was used as a pretreatment process for the ultrafiltration of the cellulase enzyme broth to discard the suspended solids and unwanted macromolecules. A 0.45 µm membrane of molecular weight cut-off (MWCO) with the transmembrane pressure (TMP) of 1.025 bar was used. During Ultrafiltration, the maximum cellulase enzyme concentration (9-fold) was achieved when 10 KD membrane was used under 1.025 (bar) TMP with the protein retention 34.67%, permeation 43.89%, fouling 21.44%, chemical oxygen demand (COD) removal 30.78% and the initial and final permeate flux was 30.72 L/m^2-h and 8.12 L/m^2-h, respectively, during this operation. The protein concentration and cellulase enzyme activity in the permeate were 1.47 mg/ml and 3.5 CMC (U/ml), respectively, during the separation process with this specific operational condition.

Keyword—Cellulase; Palm oil mill effluent (POME); *Trichoderma reesei* RUT C-30; Ultrafiltration (UF); Molecular weight cut-off (MWCO)

I. Introduction

CELLULASE is one of the important enzymes for the different commercial interests such as textile and garment industry, paper and pulp industry, food processing, detergent industry and bio-ethanol industry. Recently, the market for the stable, highly active and substrate specific enzyme is growing rapidly. Since 1995 to 2005, demand of the industrial enzymes has been doubled to 2.0 billion US dollar and it is ever increasing [1]. Cellulases accounted for approximately 14% of the world's industrial enzyme market and the value of which was approximately 190 million US $ [2] and around half of the enzymes marketed for textiles are cellulases [3].

Many researchers reported of using the synthetic medium containing several organic and inorganic constituents for the production of cellulase enzyme, such as glucose, yeast extract, peptone, urea, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$, $CoCl_2$, $CaCl_2$ etc [4]. Many wastes have been reported to be used, as well, in the sludge, DWS [6], dairy manure [7] production of cellulase enzyme through bioconversion, as for example POME [5], domestic wastewater, banana wastes [8], wood-based media [9], oil palm empty fruit bunches [10], corn stover [11] etc. Many insoluble matters, unwanted compounds and metals are contained in the bioconverted enzyme broth. Recently, UF has been used for the separation and purification technique for a wide range of products and processes, such as separation of proteases [12], purification and concentration of collagen [13], production of pure water [14], ultrafiltration of wastewater from isolated soy protein production [15], fractionation and concentration in the food [16], pharmaceutical and biotechnological industries [17].

Efficient large scale separation/purification technique is inevitable to meet the increasing demand in the various application scopes of the enzyme. Chromatography or affinity purification techniques are widely used in a laboratory scale for enzyme purification [18]. These techniques are employed for small quantity because of the instrumentation complicacy, lower yield and high production cost, though these techniques have high resolution capacity [19]. On the contrary, ultrafiltraion (UF) is a cost effective technique with high productivity and reasonable product purity. Due to these features UF has been established as one of the main separation and purification techniques for the enzyme industry in addition to the salt precipitation, spray drying, lyophilyzation, etc. [20]. However, fouling and concentration polarization are the major problems in cross-flow ultrafiltration faced by the enzyme industries [21]. Apart from the reduction of permeate flux, increase in cleaning cost and membrane damage is very frequent due to the membrane fouling [22]. There is no linear relation of fouling with the type of membrane, hydrophobic or hydrophilic, used in the UF system [23]. Considering all the above mentioned shortcomings, an efficient UF system can retain at least 90% of the macromolecules such as enzymes by using an appropriate membrane with an effective cut-off and trans-membrane pressure [24].

There is no report has been published regarding the separation/concentration of cellulase enzyme from the fermentation broth of organic waste especially POME as a basal medium. Colloidal nature of POME and high concentration of protein and other macromolecule are going to affect the flux of the separation process. In this study, membrane with different cut-off and TMPs was used through crossflow ultrafiltration to investigate the enzyme separation/concentration efficiency.

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II. MATERIALS and METHODS

A. Sample Preparation

Sample of crude cellulase enzyme was collected from the environmental engineering laboratory stock of International Islamic University Malaysia (IIUM). Crude cellulase are the broth of the bioconversion product of Palm Oil Mill Effluent (POME) by *Trichoderma reesei* RUT C-30 using optimized media and process conditions in a 30L of bioreactor. POME with 2% (w/v) total suspended solids (TSS) was used as a basal medium includes the supplementary nutrients such as peptone (0.5%, w/v), cellulose (0.5%, w/v) and tween 80 (0.2%, v/v). A 7.5% culture inoculum was inoculated into the bioconversion medium and incubated for 5 days where temperature, aeration, agitation and pH were 30ºC, 1.5 VVM, 200 rpm and 7 respectively .The fermentation broth of cellulase was first filtered using the bag filter with porosity of 250µm and stored in -20ºC and thawed at 4ºC overnight before micro and ultrafiltration were started. After thawing, the enzyme solution was centrifuged at 4ºC with 10,000g. The supernatant was used as feed in the cross flow micro and ultra filtration system.

B. Micro and Ultrafiltration System for Separation of Cellulase

The separation processes on cellulase enzyme were conducted by sartoflow slice crossf-low system of Biolab Technical Services. The transmembrane pressure (TMP) and cross-flow rate were adjusted by manual valve and pump controller. The pressure was measured by a standard pressure gauge. All the experiments were carried out at ambient temperature (~25ºC).

The membrane used was the sartocon slice cassette of sartorius (Sartorius AG, Germany). Hydrosart membranes of 0.2 and 0.45 µm of MWCO with an effective surface area 0.1 m^2 were used for microfiltration (MF). The polyethersulfone membranes of 1, 5, 10 and 30 KDa of MWCO with an effective surface area 0.1 m^2 were used for ultrafiltration (UF).

In case of new membrane, it was soaked overnight in distilled water to remove the impurities of manufacturing process and stabilizing additives left over and flush out the filter cassettes according to the manufacturer specifications before the initial use. This procedure prevents membrane compaction during permeation or retention.

Cleaning of the membranes is an important step in ensuring that all the runs had similar starting conditions. There are many different types of cassette membranes were used in this study and flushing and cleaning strategy is different for different types of membranes. The cleaning, flushing, integration checking and the clean water flux determination were carried out according to the operating manual provided by the manufacturer.

C. Operating Conditions for MF of the Fermentation Broth

To select the most viable membrane with the best operating conditions, TMP of 0.625, 0.825 and 1.025 bar were used for cross-flow microfiltration process. The highest and lowest feed pressure was studied at 1.8 and 1.0 bar, respectively, and the retentate pressure was kept constant at 0.25 bar for all the conditions applied. Six (6) liters of crude cellulase (after removal of debris from fermentation broth) was used to study each of the operating conditions. Collected retentate and permeate were used for different physical and chemical analysis. The permeate volume was recorded using graduated cylinder and stopwatch.

D. Effects of Membrane MWCO and Operating Conditions during UF

To study the effects of membrane MWCO and the operating conditions during UF, 0.625, 0.825 and 1.025 bar were used for cross-flow ultrafiltration. The highest and lowest feed pressure and the retentate pressure were maintained same as referred to the microfiltration system. Based on the filtration rate of the individual membrane against the applied TMP, 2 to 7.5 liter of crude cellulase were used for each experiment. Collected retentate and permeate were used for different physical and chemical analysis. The permeate volume was recorded using graduated cylinder and stopwatch.

E. Experimental Procedures for Cross-flow Micro and Ultrafiltration Processes

Fouling of the membranes was only examined during the study of the effects of membrane MWCO and operating conditions during cross-flow micro-filtration. Before starting each experiment, distilled water flux, $J_\circ$, was measured with the clean membrane at the same TMP (value) until it reached equilibrium. After microfiltration was completed, the holding tank was emptied, briefly washed, refilled with distilled water and measured the flux, J_f, with the fouled membrane at the same TMP. Then the fouling percentage was calculated by using the following equation:

$$Fouling(\%) = \left(1 - \frac{DWP_a}{DWP_b}\right) \times 100 \quad (1)$$

where DWP_a (L/m^2hMPa) and DWP_b (L/m^2hMPa) are the distilled water permeability after and before of microfiltration, respectively.

TMP, the average pressure experienced by the membrane surface between the feed and retentate ports, was calculated using the following equation:

$$TMP = \frac{P_{in} + P_{out}}{2} \quad (2)$$

where P_{in} is the feed pressure (bar) and P_{out} is the retentate pressure (bar).

Permeate flux (L/m^2h), measure of the flow of sample through the surface area of the membrane, is calculated using the following equation:

$$J = \frac{1}{A} \cdot \frac{dV}{dt} \quad (3)$$

where J is the permeate flux (L/m^2h), A is the area of the membrane (m^2), V is the filtrate volume (L) and t is the unit of time(h).

The protein retention ratio (R) was estimated with the following equation:

$$R = 1 - \frac{C_P}{C_F} \quad (4)$$

where C_F is the concentration of protein in feed stream and C_P is the concentration of protein in permeate.

F. Analytical Methods

Cellulase activity of thepermeate and retentate samples of the micro and ultrafiltration experiments was determined by CMC (Carboxy methyl cellulose) assay (CMCase) where CMC was used as a substrate [25]. Protein estimation of the permeate and retentate samples of ultra-filtration was carried out by the Lowry method [26] using bovine serum albumin as a standard. Chemical oxygen demand (COD) of the ultrafiltration permeate sample was analyzed according to the standard method of APHA [27].

III. Result And Discussion

A. Microfiltration (MF) Process for Pretreatment of Cellulase Enzyme

MF has been used as a pretreatment for the ultrafiltration (UF) process. Three parameters, namely flux of permeate, membrane fouling (%) and enzyme permeation, were examined to select the membrane size for the removal of suspended matters of the enzyme broth sample. During the study, it was found that TMP of 0.625 bar was not enough pressure to carry out the filtration using 0.2 µm membrane, whereas TMP of 1.025 bar was too much for the 0.45 µm membrane (data not shown). The results in Fig. 1 show that the fluxes of permeate for 0.45 µm were faster than the 0.2 µm. No retentate was found during microfiltration with 0.45 µm membrane. These results made the scenario more complex to select a functional membrane, because it was most likely to have leftover of the suspended matter as retentate during microfiltration [28].

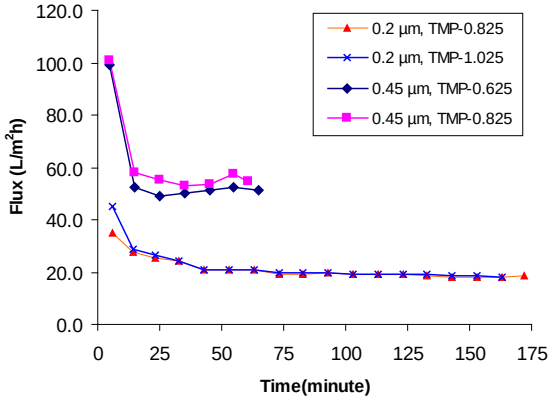

Fig 1. Permeate flux at different TMP and MWCO during micro-filtration

It was found from the study that membrane fouling (%) of 0.2 µm membrane was more than four times higher than that of 0.45 µm membrane on both TMPs (0.825 and 1.025) applied and the average enzyme activity of the permeate of 0.2 µm membrane (17.3 and 18.1 CMCase, U/ml) showed about 16% higher than that of 0.45 µm membrane (15 and 14.7 CMCase, U/ml), where the enzyme activity of the broth before microfiltration was 14.5 CMCase, U/ml. Benitez et al. [28] also reported same pattern of fouling trend for the lower MWCO membranes. The cake layer formation on the membrane surface was caused by the rejection of different suspended matters of the bio-converted enzyme sample [29]. It can be concluded from other findings that fouling is very interrelated with the rejection of suspended matters and large biomolecules [29]. Many researchers described that favorable fouling, in turn, would lead to better rejection of the suspended matters present in the feed sample [30]. Therefore, based on the results in Fig. 2, 0.2 µm membrane under 1.025 bar of TMP was found to be the best pretreatment strategy for the ultrafiltration process.

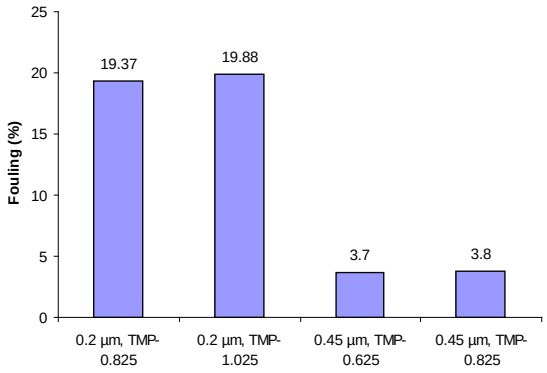

Fig 2. Fouling (%) of the membranes at different TMP and MWCO during micro-filtration

B. Effect of TMP and MWCO on the Permeate Flux Profile during UF

Four different MWCO such as 1, 5, 10, and 30 KD of polyethersulphone membrane were investigated to evaluate the effect of TMPs applied. A 1 KD membrane showed very low permeate flux under long time of operation (more than 10 hrs) per operation. On the other hand, operation with 30 KD membrane was finished very quickly under even lower TMP with very little resistance and very low retention and concentration of enzyme. Since concentration is the target of this study, therefore, a favorable fouling is expected for optimum retention. Therefore, 1KD and 30 KD were dropped for further experiment. The influence of different applied pressures on the two membranes (5 KD and 10 KD) with different MWCO was investigated. The variation in permeate flux was observed over time at different applied pressure (Fig 3). The results indicated the influence of TMP on MWCO of membrane differently for the two different membranes.

For 5 KD MWCO membrane, it was found negative effect on the permeate flux all along. The applied pressure did not even help to increase the initial flux. In addition, the filtration rate became almost half when higher pressure was applied. This lowering trend is attributed especially for the lower MWCO membranes in ultrafiltration [31]. The larger particles

present in the solution exerted a greater lateral lift away from the membrane surface which resulted in a stratification of smaller particles on the membrane surface leads to the lowering even of initial flux when higher pressure was applied [32].

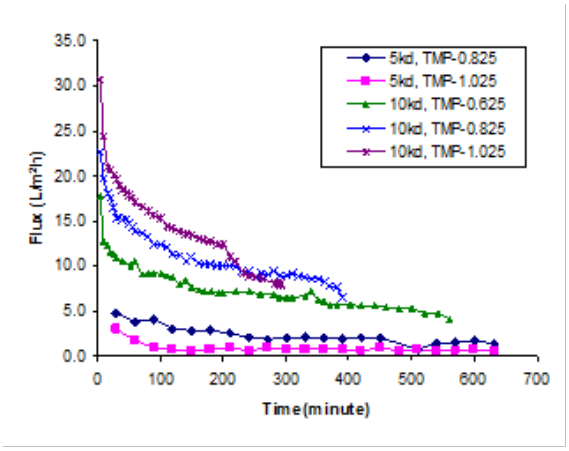

Fig 3. Effect of TMP and MWCO on the permeate flux during ultra-filtration

However, both initial and final permeate flux were increased with the increasing pressure when higher MWCO membrane (10 KD) was employed. In this study, the highest initial and final permeate flux was 30.72 L/m^2-h and 8.12 L/m^2-h, respectively when the TMP was 1.025, whereas, the lowest initial and final permeate flux was 17.76 L/m^2-h and 4.08 L/m^2-h, respectively when the TMP was 0.625 bar. This trend can be easily explained by the Darcy's law that the increasing pressure gradient increases permeate flux. Results of many researchers [33] also acknowledge the current findings.

Though, the influence of pressure on the initial and final permeate flux were found different for the membrane with different MWCO, reduction of permeate flux through out the progression of operation was observed for both of the membranes. However, the rate of reduction of permeate flux was very sharp initially for the higher MWCO (10 KD) membrane followed by gradual decline as shown in the Fig. 3 whereas the 5 KD membrane showed the reduction smoothly. These types of difference in the reduction of permeate flux for the membranes with different MWCO were also reported by Tang et al. [34]. The reduction of permeate flux for 5KD membrane was gradual because the reversible fouling layer was not formed easily on the surface of the membrane under this operational condition. The sharp reduction in permeate flux in the initial stage of ultrafiltration for the membranes with moderate porosity (10 KD-40 KD) were reported by many researchers, especially in this type of operational conditions [12]. Moreover, it is a common trend when the polysulphone membrane is employed in the ultrafiltration of the sample that contain substantial amount of protein [35]. In this current study, the crude enzyme sample before UF contained 3.0 mg/ml of protein. According to Nakatsuka and Michaels [35] the rapid flux decline is a consequence of permeability reduction due to adsorption of protein on the pure walls and formation of a low–hydraulic-permeability layer of retained protein on the membrane surface. The gradual decrease after the sharp decrease of flux was due to the particle deposition and protein adsorption on the surface and inside the pores of the membrane. Formation of cake layer due to the particle deposition, consolidation of fouling materials on the inner side of the membrane resulted the extended period of constant flux [36].

C. Effect of TMP and MWCO on the Content of COD, Protein and Enzyme of Permeate during UF

Cellulase activity, protein content and COD removal of the permeate after UF are presented in Fig. 4 and COD reduction, protein content and enzyme activity profile in the permeates during UF was shown in Fig. 5, Fig. 6, Fig. 7, respectively. The results in Fig. 4 show that the smaller MWCO membrane (5 KD) was found to be efficient in reduction of COD than the 10 KD membrane at any TMP. The 5KD membrane could reduce COD more than two times than the membrane of 10 KD. The protein and enzyme concentration also showed the similar trend as COD, as it was expected.

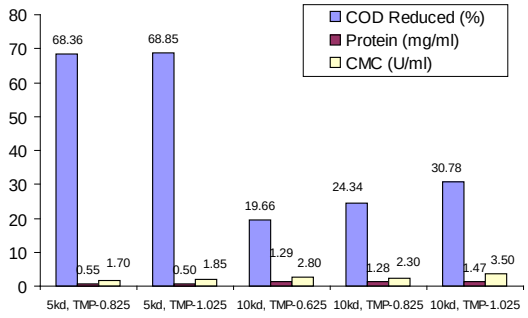

Fig 4. COD reduction, Protein and Enzyme concentration in the permeate after UF

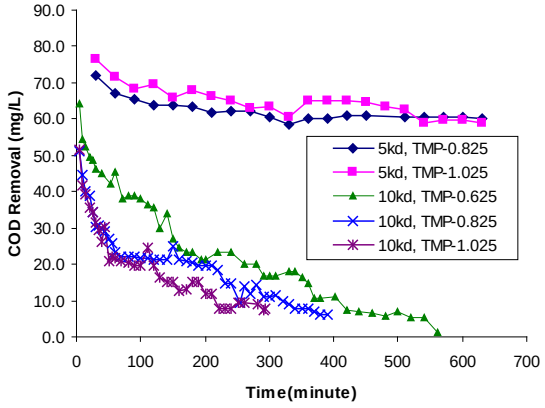

Fig 5. Effect of TMP and MWCO on the COD reduction (%) during ultra-filtration

These types of trend were reported by many researchers [15]. The highest COD reduction (68.85 %) that was achieved by 5KD membrane when TMP 1.025 bar was not even impressive, because ultra-filtration alone can not remove the dissolved organics completely [33] and the presence of higher concentration of protein in the permeate (Fig. 4) also suggested about the agreement with the findings of the

previous researchers. The current result also indicated that TMP could influence a little on the reduction of COD for specific membrane. The influence of pressure was not significant when TMP was raised from 0.825 to 1.025 bar with 5 KD membrane. But COD reduction was influenced by 25% and 20% when TMP was increased from 0.625 to 0.825 bar and 0.825 to 1.025 bar, respectively, for the membrane with 10 KD MWCO. Wu et al. [37] also reported around 20% increment in the reduction of COD due to TMP increment while Ahmad et al. [33] found no influence of TMP on COD reduction. Both of the researchers used POME as a feed sample for other purposes.

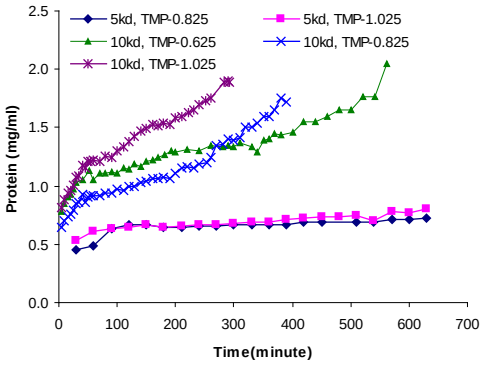

Fig 6. Effect of TMP and MWCO on the protein content of permeates during ultra-filtration

Profiles of COD reduction, protein content and enzyme activity in the permeates during UF was not reported in the previous researches. But the interesting observation worthy to be reported was that the reduction of the COD decreased (Fig 5) throughout the advancement of filtration for all the instances followed by the increment of protein (Fig 6) and enzyme concentration (Fig 7). This trend was sharp and less steady for the higher MWCO membrane, but it was gradual and steady for the membrane with lower MWCO (5 KD). This phenomenon is associated with the pattern of fouling layer formation on the membrane during ultrafiltration and the reduction of COD reduction was due to the increment of concentration polarization on the membrane surface [17].

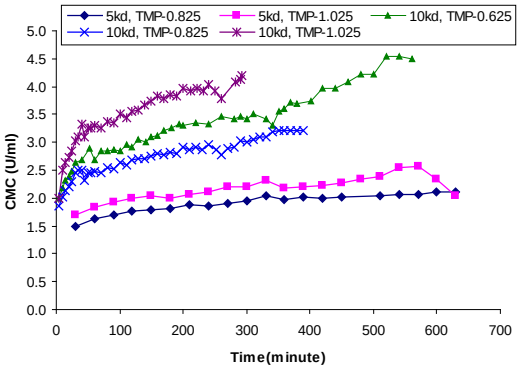

Fig 7. Effect of TMP and MWCO on the enzyme activity of permeates during ultra-filtration

D.Effect of TMP and MWCO on the Concentration of Cellulase Enzyme

The results in Fig. 8 show that the concentration of cellulase enzyme using 10KD MWCO was 8 times higher than the MWCO of 5 KD without much effect of the TMP. Very negligible effect was shown by the TMP on the concentration process, however, 10 KD membrane found to be the best when TMP was 1.025 bar and concentrated the enzyme from 15.41 to 140 CMC (U/ml) by 9 fold increase. This concentrated enzyme was retained in the 10.13% volume of the total feed enzyme solution with 89.95% rejection. No cellulase enzyme concentration process through UF has been reported recently. Roseiro et al. [24] reported their effort of concentration of cellulase enzyme by around 6 times using 10 KD hollow-fiber membrane.

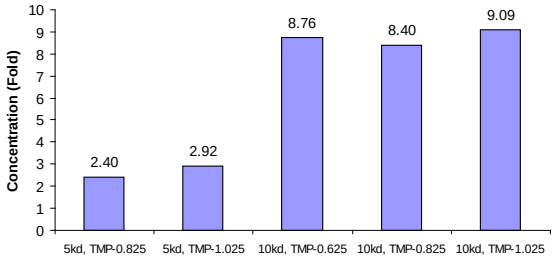

Fig 8. Effect of TMP and MWCO on the enzyme concentration during ultra-filtration

In this study, protein and enzyme concentration, were considered to evaluate the ultrafiltration process. During the investigation it was found that both rejection and fouling was higher and permeation was lower when 5 KD membrane was employed as compared to 10 KD MWCO was used (Fig. 9). The highest retention of protein, 42.82 %, was shown by the 5 KD membrane at 1.025 bar TMP. Both Tang et al. (2009) and Wu et al. (2007) reported about the recovery of protein from 61 % - 92 % at higher transmembrane pressure through UF. Considering the enzyme concentration 34.67 % protein retention found to be best, in this study, when cellulase enzyme was concentrated 9.09 times using 10 KD membrane at 1.025 bar TMP together with the 43.89 % and 21.44 % of permeation and fouling of protein, respectively.

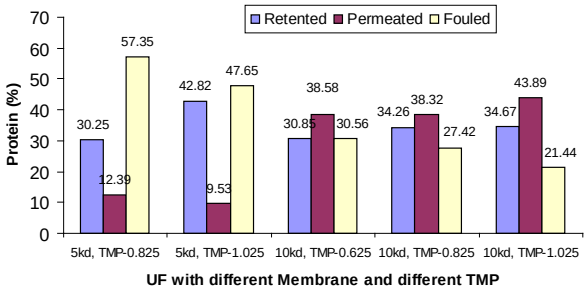

Fig 9. Retented, permeated and fouled protein (%) under influence of different TMP and MWCO during ultra-filtration

IV. CONCLUSIONS

In this study, microfiltration was used as a pretreatment process for the ultrafiltration of the cellulase enzyme broth to discard the suspended solids and unwanted macromolecules. A 0.45 µm MWCO of membrane was found to be the best

under the TMP of 1.025 (bar) as compared to the 0.2 µm based on the membrane fouling and protein concentration. The MF process was evaluated to find the MWCO of membrane with the TMP for maximum recovery of cellulase enzyme (concentration) with other parameters such as removal of COD and total protein content. The results showed that the MWCO of 10 KDa membrane was found to be the best (9-fold increase) under 1.025 (bar) of TMP with protein retention 34.67%, permeation 43.89% and fouling 21.44 %. This study offers a potential route of ultrafiltration system for the separation of cellulase obtained from the fermentation of waste based medium POME which could contribute to the further development of industrial scale of separation processes.

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