Removal of biofilms formed on ion-exchange membranes in ultrafiltration permeates of secondary dairy raw materials

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Abstract. The modelling of biofilm development on MK-40 and MA-41П ion-exchange membrane coupons was conducted in skim milk, sweet whey and acid whey ultrafiltration permeates. The membranes were suspended in permeates for up to five days at the room temperature. The incubation was followed with cleaning procedure including water rinsing, acidic, enzymatic and alkaline steps. The efficiency of each step was estimated by the optical densities of waste cleaning solutions relative to original values prior to cleaning. Cleaning efficiency was influenced by the growth medium, type of membrane and incubation time. The biofilms formed in acid whey permeate showed the highest resistance to the introduced cleaning procedure. Moreover, no increase in the optical density of cleaning solutions was observed for 24-h-old biofilms formed in acid whey permeate. Generally, biofilms demonstrated better attachment to MA-41П membranes. The acidic cleaning step appeared to remove the highest amount of biofilm material from the membrane. However, in some cases, the full cleaning procedure was insufficient to remove a biofilm.

1. Introduction
Formation of biofilms on the surfaces of industrial equipment significantly influences quality and safety of dairy products [1, 2]. In membrane processing of secondary dairy raw materials (SDRM), the issue of biofilms development is of a special importance. Application of membrane separation techniques to dairy components allows economically efficient processing of skim milk and whey. The resulting products, particularly, whey protein concentrates and demineralised whey powder, are widely utilised in food industry, while lactose and its derivatives are used in pharmaceutical industry and for production of functional food products [3]. However, membrane processing of SDRM leads to the deposition of membrane fouling layer including proteins and mineral salts. This fouling promotes attachment of bacteria present in SDRM. Formation and development of biofilms is affected by numerous factors such as composition and properties of microflora in the feed (taxa, morphology, and metabolism), properties of membrane surface (roughness and hydrophobicity), and properties of medium (pH, concentration of nutrients, temperature etc.) [4].

Generally, biofilms irreversibly attach to the membranes within 8–10 h, and from this point their removal using routine cleaning protocols becomes challenging. Membrane processing of milk and whey is usually performed in 24-h cycle with CIP cleaning applied to restore membrane transport characteristics and hence the capacity of equipment. Emergence of biofilms along with concentrating of organic and inorganic components in retentate may cause drastic drop in transmembrane flux and 10–
15% increase in pressure. As the biofilm matures, its outer layers consolidate and form even less permeable hydrophobic barrier; concentration polarization progresses and calls forth membrane biodegradation. Subsequently, the lifetime of membranes shortens, plant capacity lowers, consumption of energy and cleaning agents grows, quality and safety of a product are compromised, early failure of equipment becomes probable [4].

In this regard, a special attention is paid to the enhancement of cleaning protocols by varying regimes, introduction of enzymes for an efficient breakdown of the multicomponent biofilm matrix, and selection of disinfectants capable of killing microorganisms in their attached form. Recently, a biological approach emerged in this field, revealing the quorum signals in biofilm development and proposing inhibitors of quorum sensing [4]. Formation of biofilms in SDRM is well-studied for ultrafiltration (UF) and reverse osmosis (RO) [4, 5–10].

The influence of temperature (20 and 50°C) and pretreatment (pasteurization, cross-flow microfiltration) on UF of sweet whey using ceramic membranes (MWCO 20 kDa) was investigated in the work [5]. The highest flux and the lowest membrane resistance were registered at 20°C, a reversible biofouling was primarily affected by protein deposition, while at 50°C mineral salts could play an important role in fouling formation. Whey microfiltration through 0.5 μm ceramic membrane led to a significant increase in flux and lower fouling. Authors note that the UF of raw and microfiltered whey at 20°C was more efficient in comparison with the UF of pasteurized whey at 50°C.

Formation of Bacillus sp. biofilms on RO membranes in skim milk UF permeate (MP), whey, and both substrates were compared in [6]. The authors report difference in structure and resistance to standard cleaning procedure among three kinds of biofilms. Number of Bacillus cells in 48-hour biofilms grown in MP (5.39 log CFU/cm²) was much higher than that in biofilms grown in whey (3.44 log) or biofilms grown in both substrates (4.54 log). A six-stage cleaning procedure was followed by the reduction of CFU/cm² to 2.54 log in MP biofilms, to 1.82 in whey biofilms, and to 2.14 in biofilms grown in both substrates. The authors conclude that the biofilms formed in MP are more resistant to cleaning than those formed in whey and whey/MP.

The formation of biofilms in UF of SDRM was studied by means of metagenomic analysis targeting 16S RNA gene [7–10]. It was found that the feed type (milk, whey, water) determined 58.6% difference between microbial populations found on the membranes. Preliminary pasteurization, clarification or UF of whey also affected the diversity of bacteria attached to the membranes [7]. Metabarcoding in combination with real-time PCR showed that the feed microflora significantly influenced bacterial composition in early membrane biofilms formed during UF of milk and whey. Feed type as well as temperature of filtration and microbial environment of the processing plant contribute to the biofilm composition [8]. A laboratory study showed that the type of membranes affected biofilm formation in UF of milk less than the casein layer that rapidly grows on a membrane [9]. In the modelling of the industrial plant for UF of pasteurized milk, it was established that the inhibition of thermophiles growth at 50°C demands limiting of the processing time to 10 h, while the least growth was observed at 15°C [10].

Electrodialysis allows to control salinity and acidity in whey. However, it is typically a long batch-mode process, and different groups of microorganisms may proliferate and form biofilms during electrodialysis processing. In [11] authors studied the influence of ED temperature on the microbiological properties of various feed streams. The study involved single-strength and condensed SRDM: sweet whey, acid whey and skim milk UF permeate. It was found that ED at 15°C did not affect microbial growth significantly. ED at 22°C slightly promoted growth of some groups of microorganisms, while ED at 30°C led to significant increase in mesophilic cells count for single-strength sweet whey (ΔlgN = 0.49), yeasts cell count for single-strength (ΔlgN = 1.22) and concentrated (ΔlgN = 0.65) acid whey, and yeasts for single-strength permeate (ΔlgN = 0.50). The microbial growth, including yeasts and spore forming bacteria, and volatile microbiological properties of different SDRM in ED processing could be connected to the formation of biofilms on ion-exchange membranes.

The aim of this work was to model removal of biofilms formed on ion-exchange membranes in UF permeates of milk, sweet and acid whey.
2. Materials and methods

Samples of SDRM were obtained from an industrial-scale UF unit (GEA Filtration, Denmark) equipped with 10 kDa polymer spiral-wound membranes 6338 HFK-131 (Koch Membrane Systems, USA). Skim milk permeate (MP) was obtained from cheesemilk production (10 °C, volume concentration factor 1.4). Sweet whey permeate (SWP) was obtained from UF of rennet cheese whey (10 °C, volume concentration factor 3.0) after removal of casein fines in a vibrating screen. Acid whey from tvorog production was clarified by separation and subjected to UF (10 °C, volume concentration factor 3.0) to produce acid whey permeate (AWP) used in the study. The SDRM were kindly provided by a local dairy manufacturer (Stavropol’sky Dairy Plant JSC). The properties of permeates used in the study are given in the Table 1.

|                         | Skim milk UF permeate | Sweet whey UF permeate | Acid whey UF permeate |
|-------------------------|-----------------------|------------------------|-----------------------|
| Total solids, %         | 4.9 ± 0.1             | 4.9 ± 0.1              | 4.8 ± 0.1             |
| pH                      | 6.39 ± 0.15           | 6.42 ± 0.15            | 4.65 ± 0.05           |
| QMAFA/nM, CFU/cm³       | (5.6 ± 0.9) × 10⁴     | (1.4 ± 0.5) × 10³      | (2.9 ± 0.5) × 10³     |
| Coliformes, CFU/cm³     | (2.7 ± 0.3) × 10²     | (2.9 ± 0.4) × 10³      | not detected in 1 cm³ |
| Yeasts, CFU/cm³         | (4.1 ± 0.5) × 10²     | (2.8 ± 0.1) × 10²      | (2.2 ± 0.6) × 10²     |
| Molds, CFU/cm³          | 0±21                  | 17±60                  | 110±620               |

The ion-exchange membranes used in the study were heterogeneous MK-40 (cation-exchange) and MA-41II (anion-exchange) membranes (Shchekinoazot, Russia).

The 26×76 mm coupons of ion-exchange membranes were placed in Petri dishes and submerged in 25 ml of permeate for a period of up to 5 days at the room temperature in order to model biofilm formation. Further, the following cleaning procedure was applied to the membrane coupons. A membrane was placed into 100 ml of distilled water or cleaning solution and held there with stirring for 5 min.

The cleaning agents used were commercially available acidic solution Divos 2 (1%), proteolytic enzyme solution Divos 80-2 (0.1–0.2%) with buffer Divos 95 (0.5%), alkaline solution Divos 116 (0.5%). Water rinsing started the cleaning protocol and followed each reagent cleaning step. Cleaning efficiency was estimated by the optical densities of the waste cleaning solutions (relative to the original cleaning solutions). Optical density was determined using 1201 spectrophotometer (UNICO, USA) at 460 nm wavelength.

The microbiological analyses of SDRM were conducted using Petrifilms in accordance with national standards GOST 32901-2014 [12], GOST 33566-2015 [13], and MUK 4.2.2884-11 [14].

Quantity of mesophilic aerobes and facultative anaerobes (QMAFA/nM) was determined using Petrifilm Aerobic Count Plate (3M, USA). Petrifilms were incubated at 30 ± 1 °C for 72 ± 3 h; further, red colonies were counted on Petrifilms with a total number of colonies from 15 to 300.

Yeasts and molds were quantified using Petrifilm Yeast and Mold (3M, USA) after 72 ± 3 h of incubation at 24 ± 1 °C (for preliminary determination) and after 120 ± 3 h (for final determination). Round-shaped smooth-edged colonies were counted as yeasts; Petrifilms with a total number of colonies from 5 to 150 were counted. Colonies with diffuse edges and sharp centre were counted as molds. Petrifilms with a total number of colonies from 5 to 50 were counted. Yeast and mold colonies were distinguished by means of light microscopy.

Coliformes were quantified using Petrifilm Coliform Count Plate (3M, USA) after incubation at 37 ± 1 °C for 24 ± 2 h. Red colonies with gas bubbles were counted on Petrifilms with a total number of colonies from 15 to 150.

Experiments were conducted in 3–5-fold replicate. Statistic treatment of results and their graphical representation were performed using Excel 2010 software (Microsoft Corporation, USA).
3. Results and discussion

3.1. Skim milk permeate

Optical densities of solutions after cleaning membranes sustained in MP relative to optical density of original solutions is shown in figure 1. It can be seen that optical density of waste cleaning solutions increases for the first 72 h of biofilm growth, drops after four days and restores afterwards. Optical density of waste solutions after cleaning 24-h biofilm gradually decreased during multistep cleaning procedure. We can conclude that enzymatic cleaning was less effective for biofilm removal than other reagent cleaning steps. Also, acidic cleaning of 3-days-old biofilm on a cation-exchange MK-40 was the most effective.

Fixated biofilm layers (those left after first water rinsing, W1) built up on the MA-41II membrane for the whole 5-day experimental period. For the first two days, the attached layers were found to grow more than those rinsed with water. After 3–5 days of biofilm growth, the optical density of W1 showed a steep increase, which is probably due to the high turbidity of the residual medium.

The acidic cleaning became more significant in 3-days-old biofilm removal compared to that of earlier biofilms. However, 4-days-old biofilms were less susceptible to acidic step and more exposed to alkali.

![Figure 1](image-url)

**Figure 1.** Increase in the optical density of waste cleaning solutions after removal of biofilms formed on the membranes sustained in MP. W1, W2, W3, and W4 are consecutive water rinsing steps; Ac, En, Al are acidic, enzymatic, and alkaline cleaning steps, respectively; Σ is a summarized increase in the optical density of reagent cleaning solutions (Ac+En+Al). Error bars represent standard deviations.

The given results can be compared with the findings reported in [15], where biofilm formation and removal were modelled in similar conditions using glass plates. Optical density of waste cleaning solutions appears to be approximately two times higher after cleaning of membranes as compared to waste solutions from glass cleaning. This can be explained by the difference in surface properties of glass and ion-exchange membrane material. Indeed, membranes have by far more developed surface and provides better adhesion of cells. Alkaline solution was the least effective in removal of biofilms formed in MP on the glass surface, while biofilms on the membranes were resistant to enzymatic cleaning. However, for both surface types it was found that the optical density of W1 reaches maximum in 3 days of biofilm formation. This can be attributed to the development of friable upper biofilm layers and transition of cells to the plankton state.
3.2. Sweet whey permeate

Figure 2 represents optical density of waste cleaning solutions after the removal of biofilms formed in SWP.

![Figure 2](image)

**Figure 2.** Increase in the optical density of waste cleaning solutions after removal of biofilms formed on the membranes sustained in SWP. W1, W2, W3, and W4 are consecutive water rinsing steps; Ac, En, Al are acidic, enzymatic, and alkaline cleaning steps, respectively; Σ is a summarized increase in the optical density of reagent cleaning solutions (Ac+En+Al). Error bars represent standard deviations.

Due to the evolution of W1, we concluded that easily removable upper biofilm layers were developing on the MK-40 membrane for the first 4 days of the experiment. However, optical density of W1 declined after 5 days of biofilm formation. Further reagent cleaning showed poor efficiency. In 3 days of growth, the contribution of upper biofilm layers and fixated layers to the optical density of waste solutions was similar. Further, material from 4–5-days-old biofilms was primarily transferred to W1 rather than reagent solutions.

It can be assumed that the first 3 days of biofilm growth entailed deposition of fixated layers. As the biofilm matured, upper layers grew more susceptible to water rinsing, the matrix was eventually released to the medium and thus provided new CFU capable to attach to membrane surface.

For MA-41П membranes, we observed increase in optical density of W1 waste solution which can be attributed to the growth of upper biofilm layers. On the other hand, optical density in waste reagent solutions dropped within the said period. This fact assumes the release of biofilm matrix to the medium and transition of its microbiota to the plankton state. Supposedly, in 4 days a partial reattachment of microorganisms and formation of new fixated layers occurred. By the end of experiment, the predominant increase in optical density was in waste W1 solution rather than reagent solutions.

The obtained data show that the acidic cleaning step was the most efficient in removal of the biofilms from membranes incubated in SWP. On the contrary, a lower amount of biofilm material was transferred to cleaning solution in enzymatic and alkaline cleaning steps.

3.3. Acid whey permeate

It was found that the optical density of cleaning solutions was not altered by the cleaning of membranes after 24-h incubation in AWP, i.e. a minimum of biofilm material was recovered in the resulting waste solutions. This can be due to low cleaning efficiency as well as special features of biofilm formation. In 2 days of incubation a gradually growing white film emerged on the surface of the medium. The membranes were disrupting the film on withdrawal, and its fragments were partially removed from membranes in W1 step.
The optical density of the waste solutions after cleaning of MK-40 samples exposed to AWP suggests that the majority of biofilm material was removed via initial water rinsing (figure 3). The optical density of W1 solution increased for the first 3 days of the experiment, then dropped at the day 4 and grew further. In turn, the washed material of the fixated biofilm layers provided much lower optical density in cleaning solutions with a maximum at the day 2. Moreover, cleaning efficiency decreased after each cleaning step. Visual examination of the cleaned membranes revealed that even the full cleaning procedure failed to provide complete biofilm removal. The latter observation corroborates low optical density of waste cleaning solutions.

The increase in optical density of waste W1 solution after cleaning of MA-41Π suggests the development of poorly attached biofilm layers with the maximum at the day 4 of incubation. For the reagent steps, optical density of the cleaning solutions peaked in 3 days of biofilm formation with a subsequent decline. Presumably, the biofilm matrix lost integrity and issued bound microorganisms into the medium. Acidic cleaning appeared the highest efficiency in case of MA-41Π membrane incubated in AWP, and the summarized optical density of waste reagent solutions was the highest observed in this study. This can be attributed to a more pronounced formation of fixated biofilm layers on the MA-41Π surface.

Figure 3. Increase in the optical density of waste cleaning solutions after removal of biofilms formed on the membranes sustained in AWP. W1, W2, W3, and W4 are consecutive water rinsing steps; Ac, En, Al are acidic, enzymatic, and alkaline cleaning steps, respectively; Σ is a summarized increase in the optical density of reagent cleaning solutions (Ac+En+Al). Error bars represent standard deviations.

4. Conclusions
The presented results confirm that biofilms development and removal are directly related to the type of feed and the properties of a substrate. E.g., optical density in case of MP was slightly lower than that in case of SWP or AWP. Also, optical density of cleaning solutions used for removal of biofilms from the glass was almost two times lower compared to the respective values this study. Cleaning of anion- and cation-exchange membranes had distinctive features. For all studied permeates, poorly attached layers are assumed to be prevalent in the structure of biofilms formed on MK-40, while more fixated biofilm material was intrinsic for MA-41Π.

The summarized optical density of waste reagent solutions suggests that the most fixated biofilm layers developed on MA-41Π in AWP. Generally, acidic cleaning led to the best observed biofilms removal. In some trials, specifically ones with SWP and AWP, it was visually observed that a biofilm was not completely removed. Therefore, mechanical treatment of the fouled membranes is assumed to provide thorough biofilm removal.
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