Separation by Microfiltration of *Pseudomonas Aeruginosa* Cells from the Culture Broth

CAMELIA UNGUREANU[1], ROMULUS DIMA[1], ADRIAN ONU[2], ALINA MIHALCEA[2]

[1] University Politehnica of Bucharest, 313 Splaiul Independenţei, 060042, Bucharest, Romania

[2] National Institute of Research and Development for Microbiology and Immunology "Cantacuzino", 103 Splaiul Independenţei, 050096, Bucharest, Romania

The paper presents the experimental research to redesign the separation stage of a bacterial immune modulator of pharmaceutical use, to be integrated into the GMP (Good Manufacturing Practice) small scale bio product manufacture aseptic, closed flow. It will be studed the separation characteristics for each technological operation, the membrane regeneration capacity and evaluated the period of working time.

Keywords: Pseudomonas aeruginosa, GMP, microfiltration, membrane fouling, membrane cleaning

The European Union imposes a set of requirements, which represent directives, for the manufacture of therapeutic bioproducts. These directives are in a continuous revision, completion and evolution according to the market demands and the technological development and are also oriented towards assuring medicine products' quality by applying a complex set of conditions [1]. These requirements are found in the Directive CE 2003/94/EC from October 2003 (GMP for medicinal products of human use) and are presented in PHARMACEUTICALS (EUDRALEX) VOL. 4 (Medicinal Products for Human and Veterinary Use: GMP). A great value for the manufacture of the biological medicinal products of human use has the Annexe 2 (Manufacture of Biological Medicinal Products for Human Use) [2].

The Good Manufacturing Practice (GMP) [3] rules are applied for the entire preparation technological flow as well as the rules for the environment protection; these requirements finally introduce constraints in the production of medicinal bioproducts of human use. Among others, the GMP regulations application objective is the reduction to a minimum level of the contamination risks due to the adequate production equipment use.

This paper describes the separation phase for the preparation of an immune modulator, already on the market [4], obtained as an alcoholic extract from the *Pseudomonas aeruginosa* bacterium cells. The separation is done after the bioprocessing and must be operated in accordance with the GMP norms, which means to introduce technological changes in the former manufacture flow (fig. 1).

The separation of the *Pseudomonas aeruginosa* bacterium cells from the cultivation medium was done until now by the broth centrifugation. In order to assure an aseptic processing in a closed flow, a technological modification was proposed, which means the replacement of this operation with an adequate membrane technique, method that is recommended by the good manufacturing practice. It is already known that the membrane separation techniques have increasing important applications in the modern biotechnologies [5].

The membrane separation are being frequently used as methods for the sterile separation of many biological products for pharmaceutical use; but the main factor which limits the applicability and efficiency of these membranes is the alteration in time of its characteristics, phenomenon known as membrane fouling. From this point of view the culture media are typical examples of complex mixtures (including proteins, polyphenols, hydrocarbons, colloids, salts) with high tendency of fouling. The phenomenon mainly manifests itself through the reduction in time of the permeate flow as an effect of the membrane resistance increase. This is determined, from case to case, by different causes: the pore blocking, the polarization of the concentration and the formation of a precipitation layer [6]. Therefore, the permeate flux can be described by the following relationship:

$$J = \frac{\Delta p}{\mu\left(R_m + R_p + R_c\right)} \quad (1)$$

As a rule, for the separations through microfiltration, the influence of the concentration polarization on the reduction of the permeate flux is considered negligible due to the greater dimension of the retained particles.

For the operation at a constant pressure difference, the initial value of the permeate flux mainly depends on the membrane resistance. Together with the increase of the separation duration the additional resistances grow due to

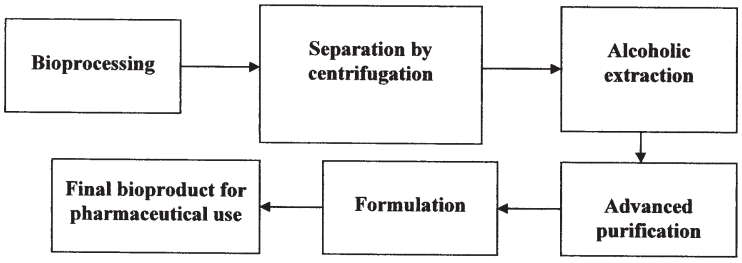
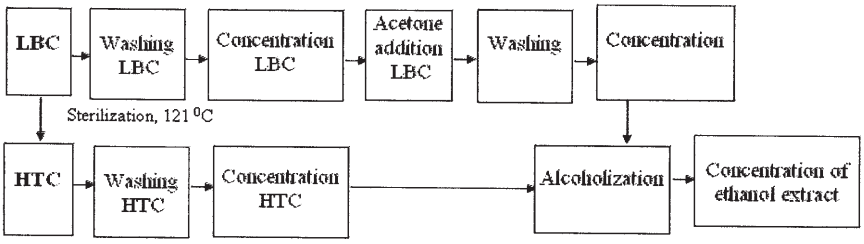


Fig. 1 Former technological flow of the immune modulator preparation

* *Tel.: 0723239120, ungureanucamelia@gmail.com*



LBC - Living Bacterial Cells
HTC -Heat Treated Cells

Fig. 2 Variants for obtaining the concentrated alcoholic extract

the pore blocking resistance or/and to the formation of a cake resistance at the membrane surface, which determines the modification of the relative importance of the three determined resistances. The time dependence of the permeate flux can be described through general relationships, specific to each type of the determined resistance [7]:

- membrane resistance-limited:

$$J = \frac{J_o}{1 + J_o K_m t} \quad (2)$$

- pore blocking resistance-limited:

$$J^2 = J_o \exp(-K_p t) \quad (3)$$

- cake resistance-limited:

$$J^2 = \frac{J^2}{1 + J^2 K_c t} \quad (4)$$

In the linear form the relationships (2-4) become:

- membrane resistance-limited:

$$\frac{1}{J} = \frac{1}{J_o} + K_m t \quad (5)$$

- pore blocking resistance-limited:

$$\ln J = K_p t + \ln J_o \quad (6)$$

- cake resistance-limited:

$$\frac{1}{J^2} = \frac{1}{J_o^2} + K_c t \quad (7)$$

The comparison of the experimental data with the three proposed models allows distinguishing the dominant mechanism which determines the membrane fouling for a given system.

Each of the fouling mechanisms depends on several factors, such as: the dimension of the membrane pores, the content of the solute and the distribution of its dimensions, the membrane material, the operating conditions, etc. For assuring the viability of a membrane separation process, the membrane fouling must be reduced as much as possible and this can be done through the use of new membrane materials, new modules configurations, the assurance of adequate flowing conditions, application of the regeneration procedures *in situ* or *ex situ* of the membrane, methods to be used from case to case, individually or coupled.

In order to replace the centrifugation with the separation through microfiltration, the decisive factors to take into account when building the proposed solution had been: the separation efficiency and the total duration of the separation, including the regeneration of the membrane, from the viewpoint of the duration and of the efficiency.

The data obtained from the experimental study had been used for the evaluation of the microfiltration module area needed to separate the whole volume of the bioprocess broth. A microfiltration module of this area will be added in the actual manufacture flow (scale up).

Experimental part

Methodology

The choice of the membrane separation method must be taken into account, function of the characteristic dimensions of the components to be retained. As mentioned before the immune modulator is extracted from the *Pseudomonas aeruginosa* cells [8], obtained through aerobic bioprocessing. This imposes the *separation* of these cells from the rest of the cultivation medium, *the concentration* and the *alcoholic extraction* of the active compound from the concentrated bacterial mass.

Pseudomonas aeruginosa is gram negative species, rod shaped, 1.2 – 2 μm long and 0.3 – 0.6μm wide from almost all natural waters. *Pseudomonas aeruginosa* is a biofilm former widely studied due to its ability to foul surfaces rapidly, its rapid reproduction rate, and its significance as a pathogen [9]. Taking into account these characteristics of the *Pseudomonas aeruginosa* bacterium, the chosen separation method was the microfiltration of the bacterial cells, with a membrane with the pores dimension of 0.2 μm.

In the former manufacture process the separated bacterial cells were inactivated through a treatment with acetone. The use of acetone presents important disadvantages the solvent consumption and the necessity of recovering it, the exposure of personnel to a toxic compound and the contamination of the air. The development of another technological solution, which avoids the use of this solvent without affecting the final product proprieties, would permit the removal of these disadvantages and would reduce the costs involving the solvent utilization and recovering.

For this purpose the cells suspension thermal treatment inactivation was studied to replace the acetone treatment. The thermal treatment was done through broth wet sterilization at 121°C. The final immune modulator product was tested for biological activity (the test of unspecific protection to mice) comparative with the product obtained through the standard method [10].

A first result of the preliminary experiment is also the good efficiency of the final product recovery (about 1000 mg product from 100 g of bacterium against about 100-150 mg product obtained from 100 g bacterium acetone treated). The biological activity of the product obtained through the cells thermal inactivation is at the same level compared with that of the product obtained through the standard inactivation procedure.

As a consequence for the lab tests two variants were considered. The two variants are different in terms of the separated raw material:

- Living Bacterial Cells (LBC) obtained from the bioreactor
- Heat Treated Cells (HTC) obtained from the LBC through sterilization at 121°C.

The operation steps for the suspension processing conforming to both variants are presented in figure 2, with the final scope the concentrated alcoholic extract obtaining.

Experimental set-up

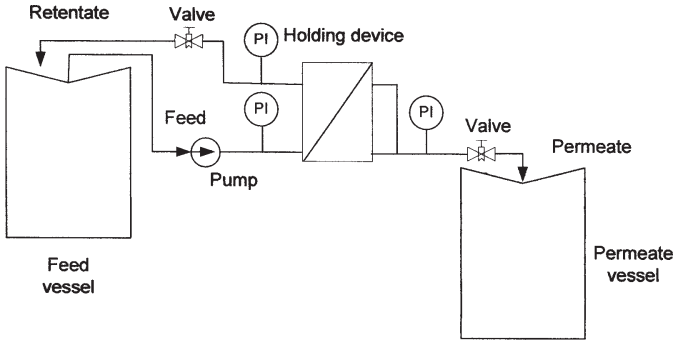


Fig.3. Experimental set-up

In figure 3 is presented the used experimental set-up.

The filtration cartridge was Sartorius with the following characteristics: total filtration area of 0.1 m^2; polymer membrane from polysulphone; Hydrosart MF/UF - Sartocon Slice Cassette; the pores diameter of 0.22μm.

Results and discussion

Processing of living bacterial cells

Washing the living bacterial cells

The membrane washing with water through the process of continuous diafiltration, has the objective to remove the compounds from the bioprocess medium; at the same time the needed water volume is to be considered when quantifying the total filtration area in the scale up operation. From the preliminary tests the efficiency of the washing was appreciated based on the concentration of the aminic nitrogen found in the collected permeate (fig.4), a value of less than 20 mg/100 g solution being satisfactory.

From figure 4 results that the aminic nitrogen decreases bellow the accepted limit after the use of 8L of water, which means a specific consumption of 1.6 L of washing water/ L of LBC.

The concentration of living bacterial cells

After the washing the suspension was concentrated to a volumetric report of concentration of VRC = 10, acquiring so a total volume of 0.5 L concentrated LBC.

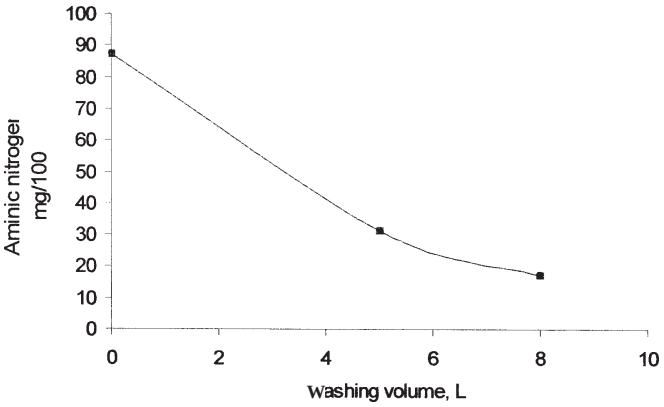


Fig. 4 The concentration of the aminic nitrogen in permeate by the continuous diafiltration

Treating the living bacterial cells with acetone

The treatment with acetone of the living bacterial cells, besides the inactivation of the bacterial cells (with direct consequences upon the further processing) can remove some extra cellular glicolipids produced by *Pseudomonas aeruginosa* with haemolytic effects. One uses an acetone volume 10 times higher than the volume of the concentrated bacterium mass. The total contact time was 30 min.

Washing the bacterial cells treated with acetone

The bacterial cells treated with acetone was later on concentrated to a VRC = 10. Then the acetone was washed with water through continuous diafiltration, until an acceptable content of acetone is measured in the permeate. The removal of the acetone is realised with a volume of water two times greater than the initial volume of the bacterial mass.

The alcoholic extraction of the active bacterial fraction

The extraction process with ethylic alcohol, which permits the separation of active compounds and the removal of the endotoxine contaminants (precipitated with ethylic alcohol), was done at a temperature of 60 °C for 30 min.

The concentration of the alcoholic extract

The alcoholic extract was concentrated through the elimination of the alcohol until a VRC = 10.

In figure 5 it is represented the duration time for each stage of the separation flow of the LBC. It results a total duration of 405 min, to which it should be added the durations of the treatment with acetone (30 min) and of the treatment with alcohol (30 min); resulting a final total duration of 465 min. A decrease of the permeate flux is remarked when using the membrane from approximated 1.25 L/min·m^2 to 0.63 L/min·m^2, which recommends a

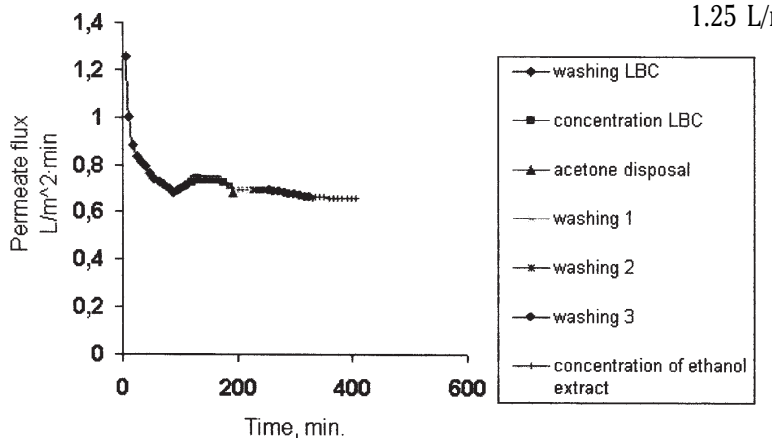


Fig. 5 Permeate flux for each phase of the LBC separation

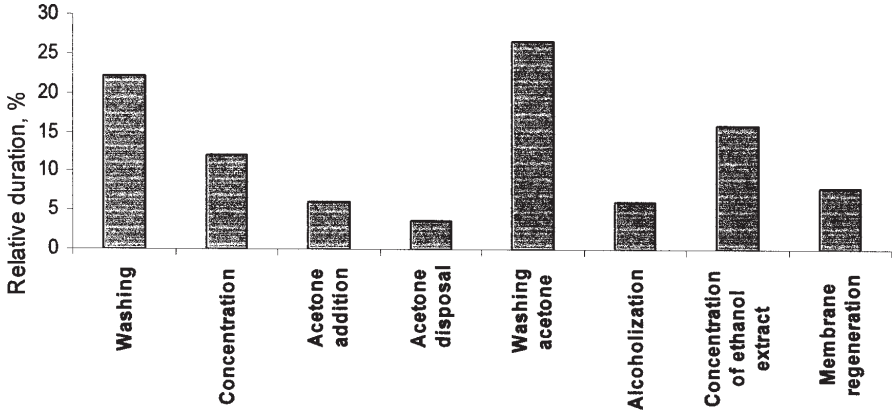


Fig. 6 Relative duration of main separation operations done for the LBC

membrane regeneration after the separation of the cells from the culture medium. The application of the protocol for the membrane regeneration took another 40 min, which rises the total time of the separation, concentration and purification procedures to 505 min.

The comparative analysis of the duration of each phase (fig.6) indicates the fact that the weight of the acetone treatment phase is greater, which justifies, once again, the attempt to eliminate it through the processing of the heat treated cells.

The processing of the heat treated cells

The processing of the HTC supposes as working phases: the thermal inactivation of the bacterial cells, the washing and the concentration of the HTC, the alcoholic extraction and the concentration of the alcoholic extract.

The heat treatment of the bacterial cells was done according to the working methodology, the total duration of the treatment being 30 min. The initial suspension volume, as well as the washing procedures and its concentration were identical with the ones applied for the LBC.

The concentration of the aminic nitrogen in the permeate samples has a similar time evolution (fig. 7) for a specific water consumption equal to that used in the case of LBC.

According to the figure 8 it results a total duration of 206 min for the three phases; taking into account the thermal inactivation phase for the suspension (30 min), the alcoholization phase (30 min), as well as the final membrane regeneration (40 min), a total duration of 306 min for the processing of the heat treated cells was obtained, considerably lesser in comparison with the separation of LBC (fig.9).

The weight of each stage needed for the separation through the microfiltration of LBC until the alcoholic extract obtaining is presented in figure 10. It is important to remark that in contrast with the processing of the LBC, where the acetone treatment had the greatest weight, in this case around 70% from the total duration is owned by the washing and the initial concentration of the LBC.

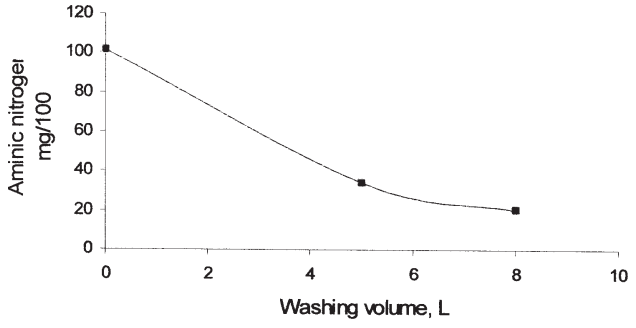


Fig. 7 The concentration of the aminic nitrogen in permeate at the continuous diafiltration

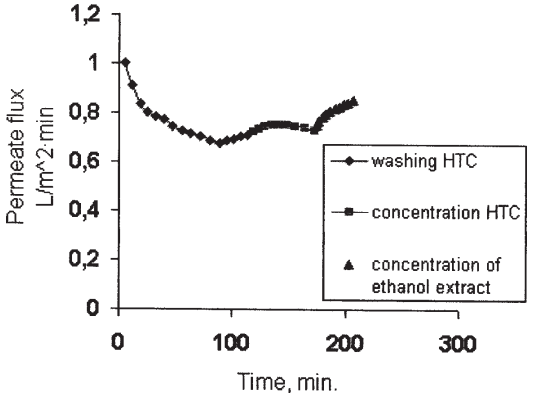


Fig. 8 The variation in time of the permeate flux at the separation of HTC

Fouling and regeneration of the membrane

The fouling of the membrane during successive stages of HTC separation is obvious, fact set off by the time variation of the permeate flux (fig. 8) with similar evolution to that determined for the LBC (an initial fast decrease followed by a slow one); this usually appears in the washing stage of the HTC. The reduction of the permeate flux is not linear, which can be explained due to some different involved mechanisms. Therefore for the first 89 min of the washing stage, realized at a constant pressure difference of $\Delta p = 1.15$ bar, the experimental data compared with the experimental models defined by the relationships (5)-(7) lead to the following conclusions:

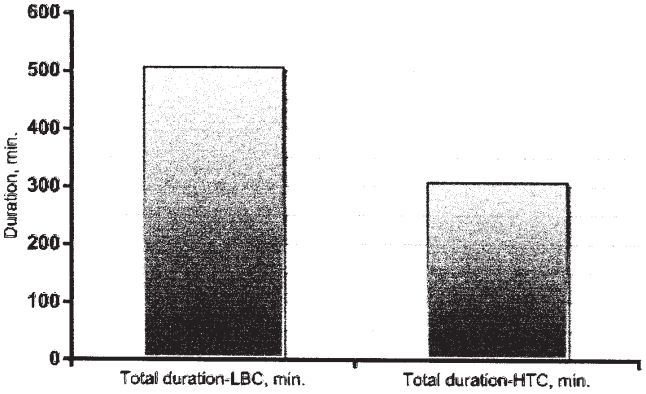


Fig. 9 Total duration – LBC versus HTC

- in the first 25 min from the beginning of the LBC washing operation the reduction of the permeate flux is due to the pores fouling (probably determined by constituents with dimensions smaller than the *Pseudomonas aeruginosa*, fact proven by the correlation of the experimental data with the model described in the relationship (6) (fig. 11,a);

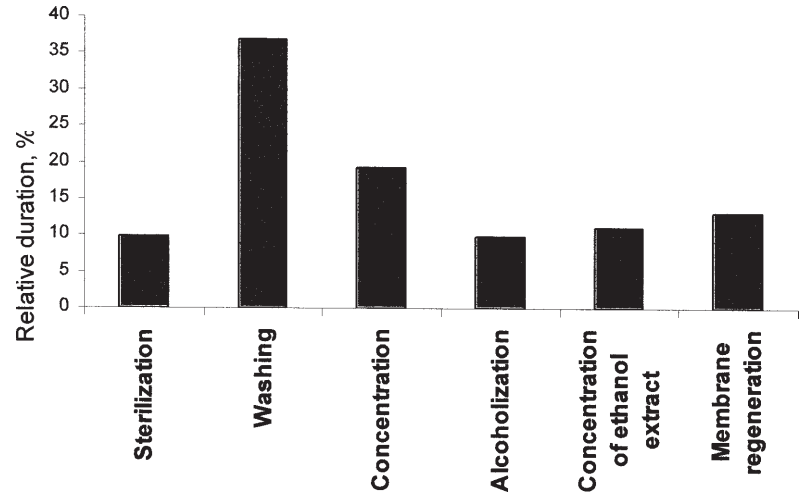


Fig. 10 Relative duration of main separation operations done for the HTC

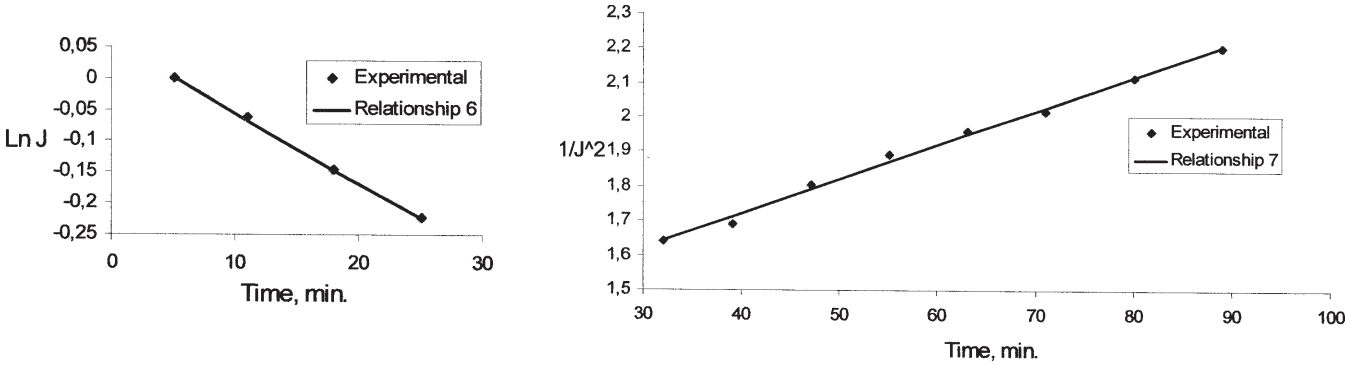


Fig. 11. The mechanisms for the membrane fouling in the washing phase of the LBC: a – pores fouling; b - the formation of the precipitate

- in the next 74 min the formation of a precipitate layer takes place on the surface of the membrane due to the progressive covering of its surface with cells from the bacterial suspension, which introduces the correlation of the experimental data with the model corresponding to the relationship (7) (fig.11,b);

-in the final stage of the LBC washing (last 45 min), the growth of the permeate flux is due to the increase of the through membrane difference pressure to 1.25 bar.

The influence of the flowing conditions upon the permeation speed was studied. It was found that the flux of permeate rises (relationship (1)), which initially determines an increase of the bacterial cells layer deposit on the membrane surface; the final result is the reach of a new stationary state (permeation flux virtually constant) as an effect of the realised equilibrium between the flux of solute towards the membrane surface and that moved by the rejected current (tangential flow). Due to operating procedure reasons, this evolution of the permeate flux also extends in the initial phase of the HTC concentrating stage (approximate minute 140), after which the flux presents a decrease tendency according to the relationship (7) (fig.12).

The initial increase of the permeate flux in the alcoholic extract concentration phase could be explained by the different viscosities of the continuous phase.

As a consequence to the facts mentioned above, the membrane regeneration was done through washing it both at the surface, and in the pores (taking into account the weight of this fouling mechanism).

The regeneration protocol is presented in figure 13, taking into account the initial value of the permeate flux.

A comparative analysis (LBC versus HTC) of the water fluxes after each regeneration stage shows that the efficiency of the regeneration is virtually the same, excepting the washing of the membrane surface, which seems to be greater for the HTC. Because in case of HTC irreversible fouling is greater, continuing the experimental research in this direction is imposed.

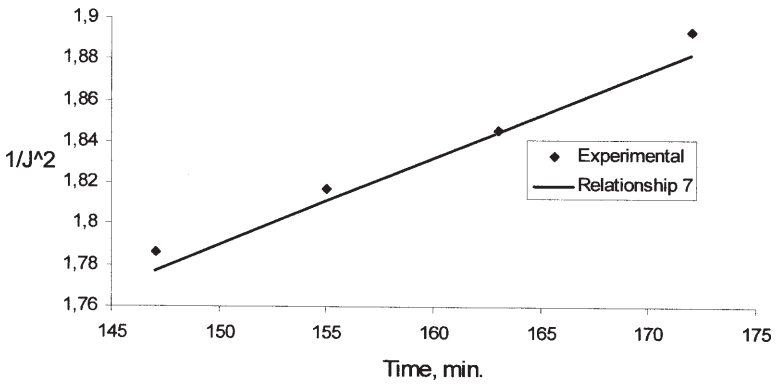


Fig. 12 The fouling mechanism in the concentration stage of the HTC

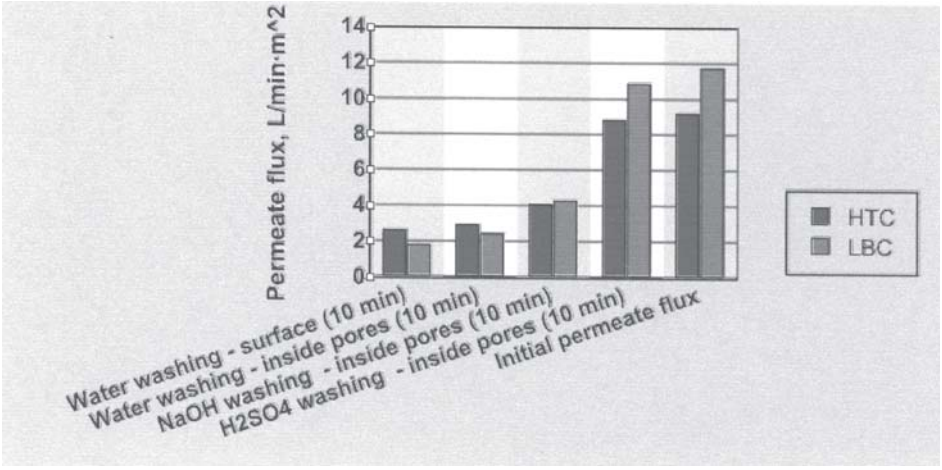


Fig. 13 The evolution of the permeate flux in the successive stages of the membrane regeneration

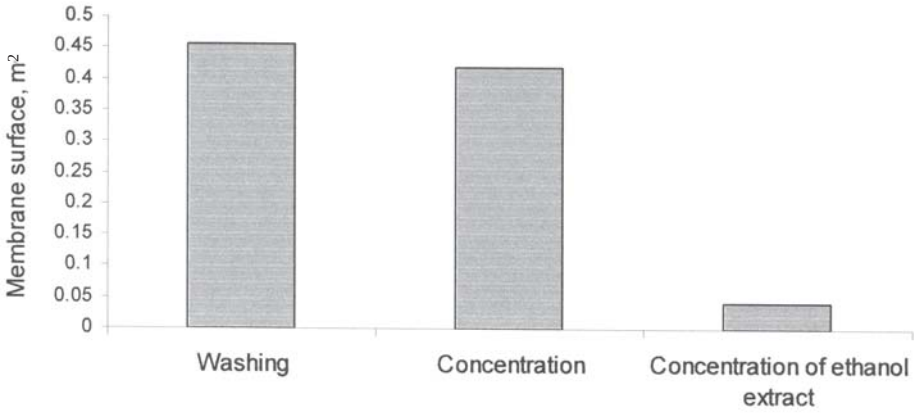


Fig. 14 The membrane area needed during each stage of HTC separation

Scaling the process

The scale-up of the process was done only for the processing of the sterilized bacterium mass as, on the basis of the experiments carried out, this proved to be a superior solution to the procedure currently applied at the scale of the industrial plant, in the immune modulator manufacture process.

The calculation hypothesis used to estimate the membrane surface of the microfiltration module needed to perform all the separation stages applied on HTC for maximum 8 h, are as follows:

- the stages of the total cycle of processing HTC at the industrial scale are the same as those defined for the lab study;
- the values of the permeate fluxes through the membrane, obtained on the basis of the experimental study, are reproducible at the industrial scale;
- the washing liquid volumes to be used at the manufacture scale are proportional to the processed suspension volume;
- the percent duration (reported to the total processing cycle) of each working stage at production scale is equal with the one determined by the experimental study.

Finally the processing of the heated bacterium mass in the separation module comprises three stages:

·washing the bacterium mass previously thermally inactivated;

·concentrating the bacterium mass;

·concentrating the alcoholic extract.

The results presented in figure 14 show that the stages of washing and concentration of HTC are decisive, both of them requiring the same membrane surface.

Conclusions

The paper presents the study of the separation procedure, according to the GMP requirements, needed to prepare a concentrated alcoholic extract of therapeutic bioproduct from the *Pseudomonas aeruginosa* bacterium cells, currently in manufacture. In order to reduce the infection hazard it was proposed to replace the centrifugation operation with the microfiltration, which eliminates the direct contact of bacterial cells with the environment. It was subsidiary tested the replacement of acetone treatment with thermal inactivation of the bacterial cells, with satisfactory results regarding the final product yield and the biological activity in comparison with the standard process. The new separation flow can be considered in conformity with the GMP requirements.

The comparative experimental study regarding the separation stages of the living bacterial cells versus the separation of the heat treated cells until the alcohol extract obtaining demonstrated the separation of the heat treated cells is to be applied due to a smaller total processing time, especially by reducing the number of phases.

Both processing procedures have as consequence a hard membrane fouling process. Experimental data prove that fouling is due to both the pore blockage and the building-up of a precipitate coat on the membrane surface. A membrane regeneration protocol was consequently proposed, which proved to be adequate.

The scaling up study determined a necessary membrane area for the microfiltration module from the manufacture flow of 0.5 m^2.

Notation

J – permeate flux ($m^3 m^{-2} s^{-1}$)
J_0 – initial permeate flux ($m^3 m^{-2} s^{-1}$)
Δp - pressure difference (Pa)
R_m – membrane resistance-limited (m^{-1})
R_p – pore blocking resistance-limited
R_c – cake resistance-limited
K_m, K_p, K_c – constants (s^{-1})
t – time (s)

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