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NEW CONCEPT FOR THE OBTENTION OF BIOPOLYMERS-BASED FOOD BIOFILMS

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Abstract

A new modern concept for a biopolymer-based biofilm formulation is presented. The biofilm contains (1) NaCl, (2) polyamphionic extracellular polysaccharide with thickening properties and antiviral action and (3) extracellular protein with antimicrobial properties. The two biopolymers are obtained at the fed-batch cultivation of the haloarchaeon Haloferax mediterranei*, the optimal conditions for maximal polysaccharide formation being on substrate with glucose as limiting agent in the fed-batch phase. The biopolymers distribution, analysed using microscopy, is more uniform in the biofilms obtained by drying in air at room temperature (20*°C*) as at 60°C.*
***Keywords**: biofilm, EPS, halocin H4, NaCl, Haloferax mediterranei*

Introduction

Biopolymer-based packaging contains raw materials originating from agricultural and marine sources. Some microbial polysaccharides are already used as biopolymer-based films and coatings, as alginates produced by bacteria (Cha and Chinnan, 2004).

In the last years, an increased interest on extremophylic microorganisms as polysaccharides producers was shown (Oren, 2002). The extreme halophylic archaeon (haloarchaeon) *Haloferax mediterranei* is such a microorganism, growing at extremely high concentrations of NaCl (between 18% and 25%), at neutral pH (7-7.4) and temperatures between 35 and 45°C (Anton et al., 1988). The haloarcheaon produces two valuable extracellular biopolymers: polysaccharides (EPS) and proteins.

The unique EPS of *H. mediterranei* are of special interest due to their composition and properties. They have uronic groups and half sulphate esters groups responsible for antiviral action (Sutherland, 1994). The rheological measurements on EPS revealed that the polysaccharide has pseudoplastic behaviour, strong ampholytic character and the biopolymer maintains his structure at high

temperature, being resistant at 100°C longer as xanthan (Mironescu, 2006). Viscosity is stable in a wide pH-range with a maximal value at neutral and slightly basic pH. Due to the adaptation to high salt concentrations, EPS are resistant to salinities up to 40% NaCl (Boan et al., 1998). The polysaccharide has good binding capacity of mono- and divalent ions (Mironescu and Mironescu, 2004).

The extracellular protein produced by the archaeon, named halocin H4 (Shand et al., 1999) shows bactericide action, especially against other archaea (Mesenguer et al., 1991) (Kis-Papo and Oren, 2000).

Due to his extreme salt tolerance, the microorganism can be grown without sterile precautions, which can obviously reduce production costs (Oren, 2002). This characteristic combined with the production of the two valuable biopolymers make the cultivation of this archaeon attractive for industry. No studies on the use of extracellular biopolymers synthesised by *H. mediterranei* as biofilm in the food packaging are presented in the literature.

In this work, the concept for obtaining of a new food biofilm containing two biopolymers (EPS and halocin H4) is developed. First, the bioprocess for biomass and metabolites production is analysed. Second, the biofilm formation process and the biopolymers distribution in biofilm in different drying conditions are studied.

Experimental

Cultivation: EPS was produced by the cultivation of *Haloferax mediterranei* strain ATCC 33500 in a cultivation plant (Mironescu et al., 2004). A standardised cultivation was provided by a process control system based on LabVIEW, which allowed the control of aeration, pH and temperature.

The cultivation was conducted in fed-batch mode. A complex medium with (in g/l): NaCl 125; $MgCl_2$ 50; K_2SO_4 5; $CaCl_2$ 0.133; yeast extract 5; peptone 5; was used. In the batch phase, 4.5 g/l glucose was added in medium and during the fed-batch phase the glucose concentration was maintained at very small levels (0.1-0.3 g/l).

Glucose, biomass and biopolymers quantification: Glucose concentration was measured using the enzymatic test Glucose Liquicolor (Human, Wiesbaden, Germany).

For the biomass determination (measured as cell dry weight), sample broth was centrifuged at 11000 rpm, 4°C for 30 minutes. In order to remove

the salts without cells lysis, the residue was washed twice with NaCl 10% and 5% solutions (Mironescu et al., 2003). After each washing, the samples were centrifuged (11000 rpm, 4°C for 20 minutes). The washed cells were then dried at 80°C for 24 h, the remaining salt crystals at the surface were removed with distilled water and dried again to constant weight.

For the biopolymers quantification, the cells were removed from the medium through centrifugation at 8000 rpm, 4°C 10 minutes. For EPS measurement, a colorimetric method was used, after salts removal through dialysis (Mironescu et al., 2003). Proteins were determined using the Biuret method.

Biofilm obtaining and analysis: The biofilm was obtained using a easy technique. After cell removal trough centrifugation at 10000 rpm, 4°C 30 minutes and dilution with distilled water to 5 g/l EPS, two solutions for the water removal from the supernatant were tested:

- treatment at 20°C on air on plates with initial liquid height of 1 mm;
- treatment at 60°C with air on plates with initial liquid height of 1 mm.

The aspect of biofilm in various conditions was analysed through optical microscopy using basic fuxine for staining. The digital images obtained were processed with the image manipulation program GIMP 2.

Results and Discussions

The results obtained at the cultivation in media with 4.5 g/l in the batch phase and 0.1-0.3 g/l in the fed-batch phase are presented in figure 1. After a lag phase (around 12 h – not shown in figure 1), the cell population begin to grow and, after a small quantity of biomass is produced, EPS synthesis begins, too. The biomass and polysaccharide synthesis occur in the exponential growth phase of the batch stage, the production of halocin H4 being not significant.

(Mironescu, 2006) showed that EPS is produced with maximal yield in fed-batch cultivations with very small glucose concentration in bioreactor during the fed-batch phase. So, the fed-batch stage is started when the substrate is exhausted in glucose (0.1-0.3 g/l) and this concentration is maintained constant during the fed-batch phase. After the initiation of the fed-batch phase biomass and metabolites have different evolutions. The biomass concentration increases at the beginning and then remains constant at values around 5 g/l, whereas

the EPS concentration increases very much from 5.69 g/l at the beginning of the fed-batch phase to 14.37 after 23 h of feeding with substrate. It can be concluded that when glucose is limiting in the nutritive substrate, the microbial metabolism is oriented on polysaccharide formation, as predominant metabolite, followed by the synthesis of extracellular proteins. When the maximal polysaccharide concentration is attained (14.5 g/l in 48 h after the fed-batch phase was started), the metabolism commutes to biomass formation.

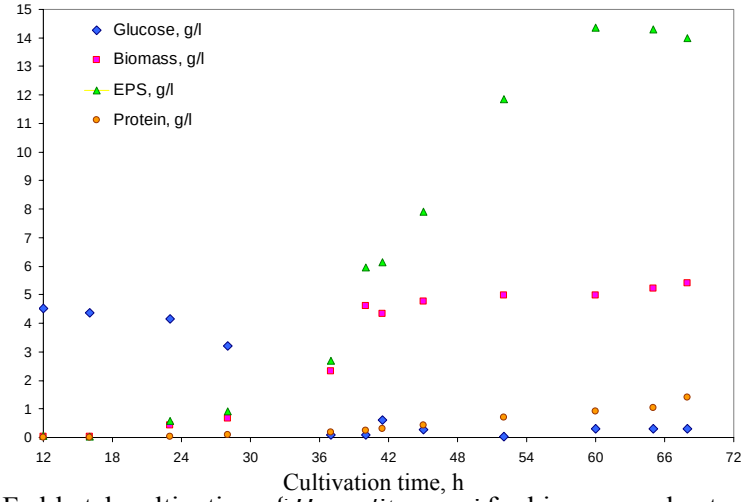

Fig. 1. Fed-batch cultivation of *H. mediterranei* for biomass and extracellular biopolymers production. Cultivation conditions were: glucose concentration in the feeding medium 100 g/l; aeration rate 1 l/min.; partial pressure of oxygen in the fed-batch phase 25%

Halocin H4 is produced in very small quantities in the batch phase. During the fed-batch stage having glucose as limiting agent, the protein synthesis is stimulated, around 1.5 g/l being obtained after 48 h of feeding. Salts, EPS and halocin H4 are present in the liquid obtained at the end of fed-batch cultivation after biomass separation through centrifugation. This mix could be used as biofilm source. For the biofilm production, a new concept, presented in figure 2, was developed.

The concept used for the biofilm formulation has three levels and three directions. The first direction is the use of NaCl already existent in the cultivation media and the building of biofilm directly from the supernatant obtained after cells removal. This solution decreases the

waste products quantity and the production costs, making the bioprocess cheaper and easier. In the same time, the salts present in biofilm have conserving action on food.

The second direction of the biofilm formulation is the use of EPS which offer many advantages:

- EPS synthesised by the extreme halophylic microorganism is adapted to very high salinity levels (between 125 and 250 g/l). Taking into account this particular adaptation, very high NaCl concentrations in the biofilm concentrations are not affecting the biopolymer structure.
- EPS contains already sulfonated groups, which show antiviral action.
- The polysaccharide is a good thickening agent, due to its high viscosity in saline environments.

The third compound used for the biofilm formulation is halocin H4, which has antimicrobial activity.

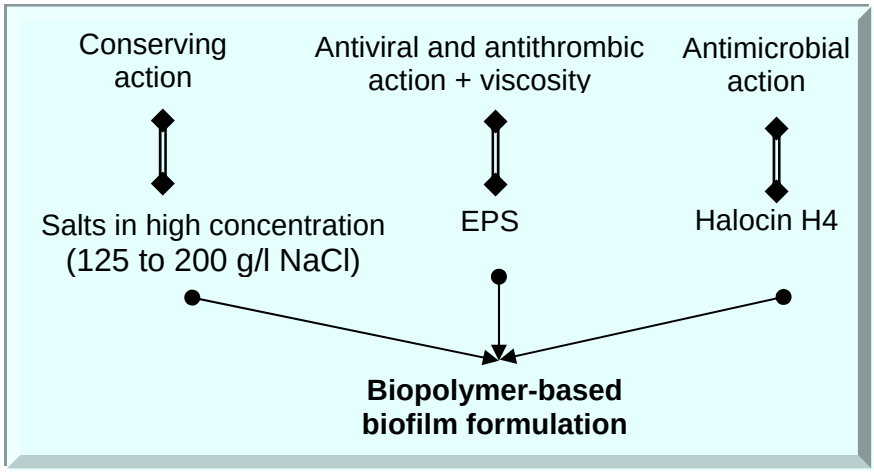

Fig. 2. Concept used for the formulation of a new biopolymer-based biofilm produced from the extracellular metabolites synthesised by the haloarchaeon *H. mediterranei*

Using this new concept, the biofilm has important characteristics:

- is natural and biodegradable;
- can be obtained in unlimited quantities, independent on season and in short time;

- shows conserving, antiviral and antimicrobial activity. Due to this action, the biofilm can be used as protective coating against viruses and microorganisms.

The choice of materials for a film or coating is largely dependent on its desired function. The properties of the biofilm proposed here are based on the presence of high NaCl concentration as conserving agent and on formation of a biopolymeric coating surface due to the presence in high amount of polysaccharide with good thickening properties (Mironescu, 2006). So, in the food industry the new developed biofilm could be adequate for foods preserved with salt as fish, meat and cheese. Further investigations on the antiviral and antimicrobial effectiveness of biofilm, on the interaction with food products and on its physical properties are necessary.

The biofilm obtaining is studied using images analyses of the biofilms formed in two conditions tested. An image of cells embedded in a biopolymeric film at the end of the batch phase is presented in figure 3. EPS and halocin H4 are not distinctive coloured.

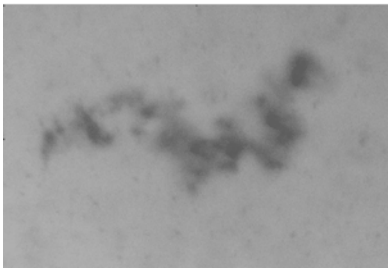

Fig. 3. *H. mediterranei* cells entrapped in the extracellular biopolymer film. The cells are intense red coloured with fuxine (dark-grey in the unprocessed image), whereas the biofilm has pink colour (hell-grey in the unprocessed image).

In 2006, Mironescu observed that in all bioprocesses realised in conditions similar with the cultivation presented in Figure 1, the polysaccharide concentration increases until a maximal value is attained and after that the concentration remains constant or even decreases. This behaviour could be correlated with the biofilm formation, which entraps very well the cells. So, when the EPS concentration increases to a value signalised by the cell to be maximal (probably the maximal concentration at which the oxygen still reach the cells) the production of polysaccharide stops.

After cells removal through centrifugation, the supernatant can be directly used for biofilm formulation, conform to the concept presented in figure 2. Images of biofilm obtained after drying at room

temperature (20°C) and at 60°C (considered as the maximal temperature which not affects the proteins structure) are presented in figures 4 and 5. As observed, biofilm forms in dried samples. The biopolymers entrap salts and other compounds from the nutritive broth used for cells cultivation. The biofilm is more uniform distributed when the drying is realised at 20°C (figure 4) as when higher temperatures (60°C – figure 5) are used. In the same time, the salt crystals formed are bigger in biofilms obtained during treatments at higher temperature. In conclusion, the biofilm formation is favoured by non-aggressive drying.

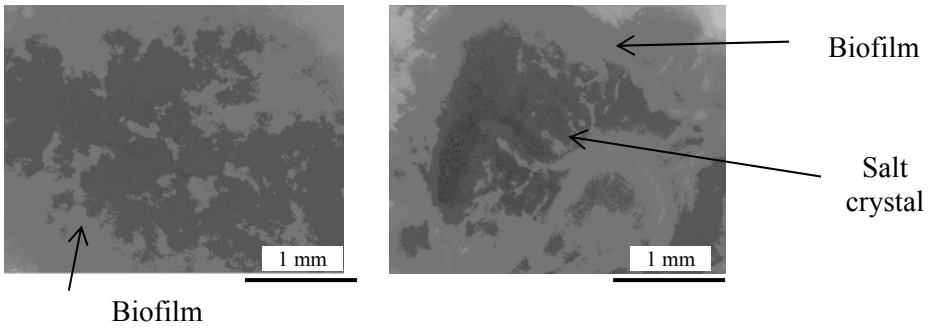

Fig. 4. Biopolymeric film obtained from the supernatant dried at 20°C. The biofilm has intense pink colour (hell-grey in the processed images), whereas salts and free-polymeric zones are not stained or stained in slight pink (dark-grey in the processed images).

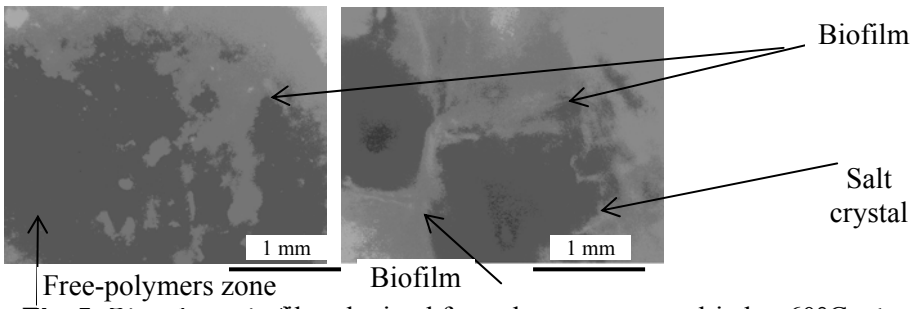

Fig. 5. Biopolymeric film obtained from the supernatant dried at 60°C. The biofilm has intense pink colour (hell-grey in the processed images), whereas salts and free-polymeric zones are not stained or stained in slight pink (dark-grey in the processed images).

Conclusions

At the fed-batch cultivation of the haloarchaeon Haloferax mediterranei having glucose as limiting agent, high amounts of extracellular polysaccharide are obtained (until 14.5 g/l), combined with 1.5 g/l extracellular protein. The two compounds, separated together with salts presented in the nutritive broth (with NaCl as major component), can form a biofilm at the drying with air. The microscopically techniques used for the analysis of biofilm formation reveal that, from the tested solutions, the treatment with air at 20°C give a good biofilm, which entraps small salts crystals.

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