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Viability of spores of Aspergillus awamori immobilized in cryogels

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ABSTRACT

The work consisted of immobilizing *Aspergillus awamori* spores in polyvinil alcohol (PVA) at different concentrations 10, 12 y 15% (w/v). The viability of the immobilized spores was compared to the free spores, by the conventional plate counting method. The imobilization technique was by entrapment, it consisted of a suspension of spores in the polymer and then it was frozen at -18º C for 24 hours. Viability was similar in immobilized and frees pores, this indicates that there are probably no toxic effects of the polymer and that exposure to low temperatures does not damage the structure of the spores. Spores leakage was different in each case of immobilization at 24 hours, the least leakage was with spores at 15% and the highest at 12%. At 96 hours, the lowest was with spores immobilized at 10%, followed by 15% and the highest spores immobilized at 12%, thus remaining until 120 hours.

INTRODUCTION

In recent decades, most research on immobilized cells has been done with porous supports formed around the cells. Synthetic polysaccharides and polymers can be gelled allowing cell entrapment, where the loss of cell viability is minimal and allows a high biome load; obtaining different shape and diameters of the pores in the cryogel where it varies from 1 to 1. 5 mm. (E. N. Efremenko et al., 2006).

The technique of entrapment in gels with natural polymers is a technique where damage to cells is minimized. The disadvantage is the low mechanical strength and limited permanence of the gel structure that is easily destroyed by cell development and the production of carbon dioxide(CO_2),as well as in immobilization in application in wastewater treatment processes (Willaert & Baron, 1996).

The application of synthetic polymers for the immobilization of living cells has benefits compared to natural polymers for porosity, ionic, hydrophobic or hydrophilic properties and their mechanical strength.

Polyvinyl alcohol is a vinyl raw material and is a low-cost material, non-toxic to microorganisms and can be used to trap living cells. The solution becomes gelatinous by freezing and the strength of the gel increases during freezing and thawing interactions.

Cryogels in polyvinylalcohol, which are prepared by freezing-thawing concentrated aqueous solutions of the polymer, possess definite advantages compared to other hydrogels commonly used for the same purposes. Similar benefits are as follows: (i) polyvinyl alcohol

cryogels have very high micro and macroporosities that provide favorable conditions for unimpeded mass transfer of substrates and metabolites; (ii) the rheological characteristics of the non-fragmentary matrix are excellent and allow the use of these carriers in most reactor types; (iii) the thermostability of polyvinyl alcohol cryogels exceeds that of other commonly used thermoreeversible gel carriers; (iv) cryogels are highly resistant to biological degradation, in addition to being of low sensitivity to the compositions of culture media; (v) Polyvinyl alcohol itself is a biologically compatible, non-toxic and readily available low-cost polymer (Lozinsky & Plieva, 1998).

Cell immobilization can be defined as "the physical confinement or localization of intact cells in a region without loss of desired biological activity" (El-Mansi et al., 2011).

It is estimated that the proposal for the use of cryogels in microbiology begun in 1980, when they were suggested as matrices for cell immobilization by the technique of mechanical entrapment. However, its application was made recently. Generally, the immobilization of microorganisms via mechanical entrapment consists of a suspension of spores or pellets mixed homogeneously with the polymer and then subjected to sub-zero temperatures (Lozinsky & Plieva, 1998).

The main objective of this work was to demonstrate that the spores of *Aspergillus awamori* could be immobilized in polyvinyl alcohol, their viability was evaluated by the conventional method of plate counting. The viability of spores immobilized in polyvinyl alcohol at different concentrations (10, 12 and 15 % w/v) was compared with spores in the free state. The immobilization procedure was simple, consisting of a suspension of spores in the polymer, homogenized and cryogelized at -18ºC for 24 hours.

The results show that the entrapment immobilization technique is promising and does not affect the viability of the microorganism's spores. However,it is necessary in future studies to evaluate kinetic and metabolic parameters.

METHODOLOGY

This research work was carried out in the Microbiology area of the Biotechnology Laboratory of Universidad Nacional Autonoma de Nicaragua, Managua (UNAN-Managua).

The isolation of *Aspergillus awamori*, was made of samples of soils contaminated by artisanal mining activity in Nicaragua, in the department of Chontales (Santo Domingo). The mushrooms were grown in plates with PDA + Chloramphenicol agar at 27 ± 1°C for five days.

Spore count

For the spore count, a Neubauer camera and a Trinocular BX43 microscope with a magnification of 40X were used. The suspension of spores was made by the drag technique, was homogenized in test tubes of 16 x 150 mm with 10 ml of distilled water and sterile and stirred for 15 seconds in Vortex at 150 rpm,with automatic micropipettes were measured 20 ul to perform the count. The density of the spores with which we worked was $1x10^6$ spores/ml, both for free and immobilized spores. The result was expressed in number of spores per ml. (E. Efremenko et al., 2006)

Immobilization of Spores of Aspergillus awamori

Polyvinyl alcohol (molecular weight is 85,000-124,000 99% + % dehydrolized)) homogenized with spores at three concentrations (w / v) of 10%, 12% and 15%, sterilized at 121°C for 15 minutes. The procedure for suspending the spores was the same one used in the spore count.

The suspension of the spores ($1x10^6$) to the solution of polyvinyl alcohol was homogenized, then transferred in falcon tubes of 25 ml, containing plastic molds of 2.5 x 2.5 cm. Cryogelling was carried out at 18°C for 24 hours, in a Fisher Scientific Isotemp 6200 R28 thermostat previously calibrated to -18°C and conditioned with 96% alcohol.

Activation

For the activation of the spores, Erlenmeyer of 1000 ml was used, containing 500 ml of culture medium composed of (g/liter): Glucose 120; $(NH_4)_2SO_4$,3.0; $MgSO_4 \times 7H_2O$, 0.3; $ZnSO_4 \times 7H_2O$, 0.05; KH_2PO_4,0.2. It was adjusted to pH 6.0 and sterilized at 121°C for 15 minutes. To suspend the molds with the spores immobilized at different concentrations of polyvinyl alcohol (10%, 12%, 15%) previously cut and washed with sterile distilled water. The goal of activating spores is for them to adapt to their new germination temperature and reproduce (E. Efremenko et al., 2006).

The aeration of the culture of immobilized and free spores was performed in orbital agitator (AO-400 Bunsen, Spain) at 100 rpm. To ensure the temperature, the agitator was introduced into fisher scientific Isotemp 3720A incubator at 30 °C for four days. This procedure was done in triplicate.

Viability of spores

To determine the viability of the spores, Erlenmeyer of 250 ml (containing 100 ml of culture medium) was used to suspend the molds with the spores immobilized and activated at different concentrations of PVA, as well as the viability of free spores, the aeration of the immobilized and free spores, was performed in orbital agitator (AO-400 Bunsen, Spain) at 100

rpm. To ensure the temperature, the agitator was introduced into Fisher Scientific Isotemp 3720A incubator at 30°C for five days. This procedure was done in triplicate. The culture medium used was the same as the activation (g/l): Glucose 120; $(NH_4)_2 SO_4$,3.0; $MgSO_4 7H_2O$, 0.3; $ZnSO_4 7H_2O$, 0.05; KH_2PO_4,0.2. It was adjusted to pH 6. 0. This procedure was done in triplicate.

Viability of immobilized and free spores

Viable spores were determined by plate count, without successive dilutions (inoculation was direct from Erlenmayer to plaque) in duplicate,0.5 ml of culture medium was inoculated in plates containing PDA agar, samples were collected every 24 hours or for five days.

Results

Figure 1 shows the viability of free and immobilized spores of *Aspergillus awamori*. The exponential phase of the free spores (control) was after 72 hours for polyvinyl alcohol concentrations of 12 and 15 (% w/v). The exponential phase of the spores immobilized at 12 (%w/v) was similar to the free ones. The spores immobilized at 15 (% w / v) began their exponential phase at 24 hours, but their declination phase was approximately at 96 hours, this behavior is probably due to the size of the pores of the cryogel there was better transfer of substrates and other elements. The free spores and those immobilized at 10 (% w / v),behaved similarly in their exponential fase, however, the delay in their exponential phase is a disadvantage compared to spores immobilized at higher concentrations of polyvinylalcohol, if the spores of *Aspergillus awamori* are used in industrial microbiology, it would be necessary to assess if the enzyme to be obtained will be extracellular or endocellular. If they are to be applied in the middle of the micro-organism, it would have to be assessed whether the duration of the adaptation stage of the micro-organism should be greater or shorter. In a study conducted with *Rhizopus orizae* where its spores were immobilized in polyvinyl alcohol, its kinetic and metabolic parameters were evaluated. The results show that immobilized spores develop slightly slower than free spores (E. Efremenko et al., 2006).

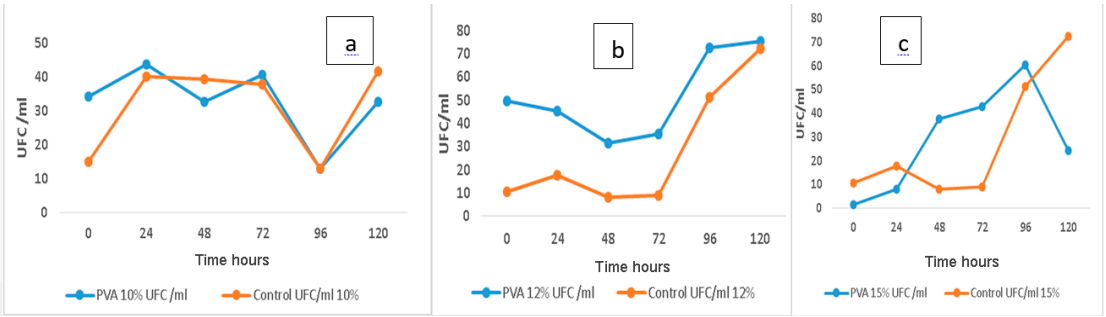

Figure 1.

(a), (b) and (c) Viability of Aspergillus awamori spores immobilized in polyvinyl alcohol at different concentrations compared to the viability of free spores.

Figure 2 shows the viability of the spores with three concentrations of polyvinyl alcohol (PVA). At 24 hours the smallest leakage of spores from the pores of the polymer was 15%, after 72 hours the behavior varied or completely, at 96 hours the greatest leakage of spores was with PVA 12% and the lowest with PVA 10%. This behavior is closely related to the Growth Curve *of Aspergillus awamori,* nutrient transfer, and pore size after cryogelization.

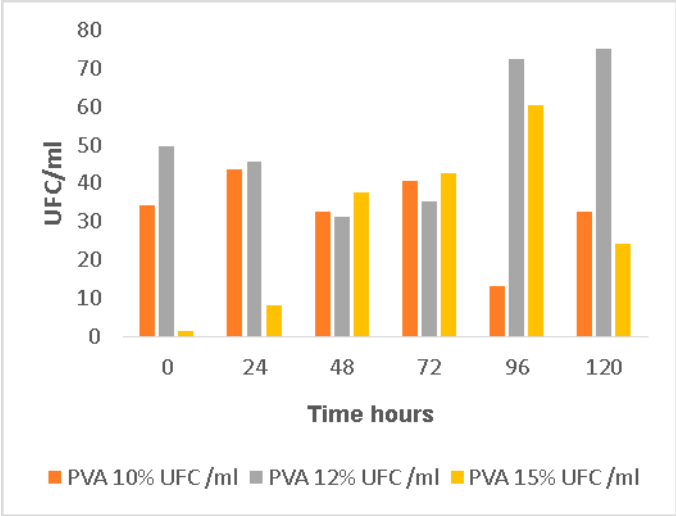

Figure 2.

Immobilized spores of Aspergillus awamori at different concentrations of polyvinyl alcohol.

Figure 3 shows that the pH decreased after 24 hours in all cases, at 48 hours the recorded pH was 2 in all trials. The change in pH is associated with metabolic and kinetic parameters which indicates that the substrate was consumed.

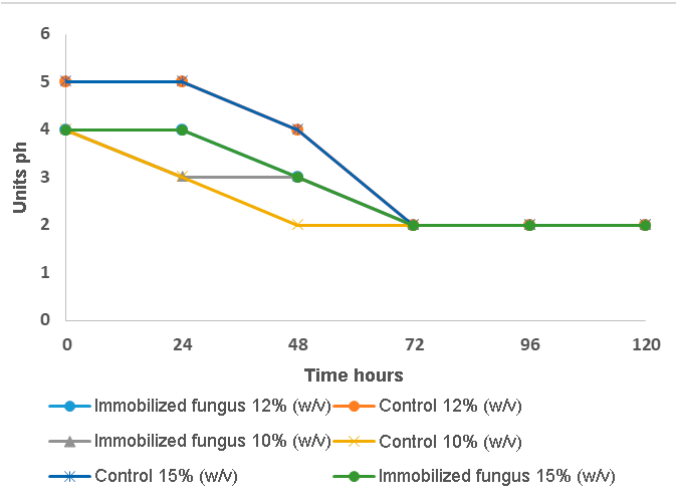

Figure 3.

Decrease in pH in the culture medium of immobilized and free spores.

DISCUSSION

The difference between the kinetic and metabolic parameters of free and immobilized spores trapped in PVA was not evaluated in this work. Efremenko comparatively analyzes these parameters in *Rizophus orizae* immobilized in PVA, the productivity of immobilized and free cells associates them with the limitations of mass transfers between immobilized and free cells. Probably, the macro pore created during the cryogelization of the polymer determines the formation of an internal structure with lower cell density, but with greater accessibility to substrate and oxygenation. (E. Efremenko et al., 2006)

The different concentrations of the polymer directly influence its porosity. The combination of properties, the controlled structure of macro pores, biocompatibility, chemical and mechanical properties open up new applications in Microbiology. (Plieva et al., 2008).

The variation in the viability of the immobilized spores is probably due to the size of the pores which is directly determined by the concentration of the polymer and the temperature at which the polymer was cryogelized with the cell suspension. Comparing the viability of immobilized and free spores in each case it is shown that the concentration of the polymer and the temperature do not affect its viability, however, it is necessary in future studies to measure the kinetic and metabolic parameters of both cases.

CONCLUSIONS

The technique of entrapment and cryogelization is promising for applications in industrial and environmental microbiology, it could also be applied as a method of preservation of microorganisms.

It is a precedent of non-invasive, non-toxic, economical and easy to manufacture immobilization techniques.

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