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Encapsulation of *Saccharomyces cerevisiae* in hydrogel particles based gellan ionically cross-linked with zinc acetate

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ABSTRACT

The purpose of this study is to obtain ionically cross-linked gellan particles containing immobilized yeast cells. The zinc acetate was chosen as a cross-linking agent due to its beneficial role in the growth and nutrition of yeast cells and also due to the advantages of its use in fermentation processes. The physicochemical and morphological properties as well as the fermentative activity of the obtained particles were studied. The zinc acetate concentration has an influence on particles stability in water, on the mechanical stability and also on the swelling degree. The morphology of the obtained particles was analyzed by SEM and proves that the yeast cells are immobilized in large numbers in the polymeric matrix. The cell viability is maintained at high values after several fermentation cycles. These obtained micro-bioreactors were tested in terms of fermentative activity and the fermentation process was optimized by studying the influence of several factors. The gellan gum particles can be easily recovered by filtration and they can be reused in repeated fermentation cycles. The results obtained in the presence of these cross-linked particles are comparable to those achieved in the free yeast fermentation.

1. Introduction

Immobilization of yeast cells in various macromolecular carriers, in order to obtain ethyl alcohol is a research field that has expanded due to its attractive technique and to its economic benefits. Continuous processes are preferred as they are characterized by a substantially improved efficiency, a higher productivity and lower operating costs [1,2].

Saccharomyces cerevisiae are unicellular microorganisms, eukaryotic, classified as fungus and they are also known as baker's yeast or beer yeast. Yeast cells reproduce asexually by a process called budding at every 90 min and their diameter is usually between 3 and 4 μm. When the yeast cells are stored under adverse conditions, such as for example lack of nutrients in the medium or high temperatures, they do not die but are undergoing a process called sporulation. Yeast spores can withstand long periods without nutrients, at the low and high temperatures, until the conditions are appropriate for reproduction and then they start to sprout all over again [3].

Moreover, these cells are chemoorganotrophs and they use, as an energy source, sugars such as hexoses and disaccharides. In the presence of oxygen, they are aerobic microorganisms but they can be anaerobic when are subjected to fermentation for the production of ethanol. Optimal conditions for the proliferation of yeast cells are represented by a temperature between 30 and 33 °C [4] and a pH range of 4 to 6 [5–7].

The techniques involved in the encapsulation of the microbial cells in the polymeric matrix are emulsification [8,9], extrusion [10], complex coacervation [11], spray drying [12], gel entrapment [13] and polymerization by radiation [14]. Each of these techniques has different advantages and disadvantages. The optimal technique depends on the type of polymer used for encapsulation, on the microorganism and on the desired properties of the immobilized product. For microbial cells, the extrusion is the most often used method due to the mild processing conditions that enable optimal encapsulation and does not affect the viability of microorganisms. This method is usually used in the case of alginates and carrageenan, and involves the extrusion in small droplets of the biopolymer solution containing the yeast cell suspension in the cross-linking agent solution [15].

The obtained particles with immobilized yeast cells are larger than the free cells and can be recovered easily from the reaction medium

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by filtration. These particles must be stable under various conditions such as pH, osmotic pressure and turbulence. Besides protecting the encapsulated cells from unfavorable environment, the polymer matrix must ensure the efficient exchange of nutrients and metabolites with the environment. Micro-bio-reactors efficiency depends on their specific surface area, porosity, swelling capacity and stability [16,17]. The recovered particles can be reused in the subsequent fermentation process as shown by Tan et al. [9] for gellan particles.

Polysaccharides have been shown to be effective in immobilizing microbial cells because they are biodegradable, non-toxic and preserve the cell viability over extended periods of time [18]. Gellan is a water soluble microbial exopolysaccharide, produced by the bacterium *Sphingomonas paucimobilis*, with a molecular weight varying between $1–2 \times 10^6$ g·mol^{-1} for the high-acyl gellan and $2–3 \times 10^5$ g·mol^{-1} for low-acyl gellan [19,20]. Is a linear anionic biopolymer having tetrasaccharide repeating sequences and consisting of two residues of β-D-glucose, one of β-D-glucuronic acid and another of α-L-rhamnose in a ratio of 2:1:1 [21,22].

Gellan is a biocompatible, biodegradable and non-toxic polysaccharide with high stability at pH values ranging from 2 to 10 [23]; it is resistant to enzymes such as pectinase, amylase, cellulase, lipase and papain [20,24], but it is significantly degraded in the presence of galactomannanase [25]. Gellan have been used to immobilize yeast cells and to obtain a biocatalyst with real perspectives of being used for the sparkling wine production technology. Spherical particles, with immobilized yeast cells, were obtained by extruding the gel containing a suspension of yeast cells through a capillary in a bath of $CaCl_2$ solution (the ionic cross-linking agent) [26]. Tan et al. [9] have immobilized yeast cells, using gellan gum as a support, with an emulsification method and have demonstrated the possibility of reusing the obtained micro-bioreactors. The production of ethanol during the first three cycles was comparable to that of free yeast. Micro-bioreactors are stable and readily recovered from the fermentation medium by filtration and can be reused for at least 10 cycles of fermentation with relatively high yield of ethanol.

It is known that the yeast cells require a wide range of metals to their proliferation and metabolism [27]. In the fermentative processes, especially in the beer industry, the key metal ions are magnesium and zinc as they act as cofactors for different metabolic and biosynthetic enzymes, including glycolytic enzymes and alcohol dehydrogenase [28,29]. The heavy metals, even at micromolar concentrations, can be toxic to the yeast cells [30]. An environment deficient in zinc ions can lead to slow or incomplete fermentation and this issue was observed in the beer industry. The zinc ions play a significant role in the metabolism of the yeast cells, not only because it is an enzymatic activator but also because it can boost the absorption of maltose and maltotriose in the yeast synthesis process thus achieving high fermentation yields [29–31]. These ions may have also a beneficial role on the stability and dynamics of the cell membrane. When yeast is facing changes in the environment, such as a nutrient or metal ions deficiency, accumulation of toxic metabolites or the temperature variation, the plasma membrane must adapt before internal structure. Maintaining membrane fluidity is an essential factor in maintaining its functions [32]. Yeast cells have the ability to absorb, very efficient, essential minerals and to exclude the non-essential ones. Zinc ions can be absorbed by the cells in the fermentation medium in order to perform essential physiological roles [27].

The aim of this paper is to obtain ionically cross-linked gellan particles with immobilized yeast cells, by the extrusion method. The originality of this work consists in the use of zinc acetate as a cross-linking agent in order to obtain gellan particles with immobilized yeast cells and the study of various factors that can influence the fermentation process. The zinc acetate was used as cross-linking agent as is not toxic, it has a positive effect on the yeast cells metabolism and plays an important role in the fermentation processes. The morphological and the physico-chemical characterization of the obtained particles was achieved and the fermentative activity was tested and compared with that of the free yeast. Micro-bioreactors cross-linked with zinc ions and with immobilized yeast cell can be an approach for solving the zinc deficiency, problem which faces brewers.

2. Materials and methods

2.1. Materials

In this study was used deacetylated Gellan Kelkogel characterized by an Mw = 2.351×10^5 g/mol, determined at 25 °C by using a method described by Reed et al. [33]. Zinc acetate and Glucose were procured from Sigma Aldrich and used as received.

2.1.1. Cell yeast type

For the immobilization, was used a commercial bakery yeast strain (Pakmaya, Romania) which is found in a compressed form and has a water content of 70%. *Saccharomyces cerivisiae* cell characteristics are: diameter: 2–8 μm; volume: $0.8–2 \times 10^{-3}$ cm^3; weight: $0.2–0{,}4 \times 10^{-10}$ g; number of cells/g: $8–14 \times 10^9$; specific surface: $8–14 \times 10^9$ (m^2/g).

This type of yeast was chosen as it represents a type of yeast cell biomass of the species (top fermenting yeast) composed of living cells capable of producing sugars fermentation. These yeast cells easily absorb the hexoses (glucose), and then the sucrose and the maltose [34–39]. To achieve immobilized microorganisms with high performance assets in the fermentation process is recommended that these be allowed to grow directly into the gel [40,41].

2.2. Methods

2.2.1. Preparation of gellan particles with and without immobilized yeast cells

In order to obtain ionically cross-linked gellan particles with zinc acetate, 0.25 g gellan was dispersed in 25 mL bi-distilled water and the temperature was fixed at 85 ± 1 °C until all gellan was dissolved. The formed polymer solution was extruded dropwise using a syringe and a capillary (injection needle), with the size of 0.6 × 30 mm (22 gauges), into a cross-linking bath, with a volume of 125 mL, containing zinc acetate solutions with different concentrations (from 0.05 to 0.3%). Because the gellan solution do not form stable particles at the time of extrusion into the cross-linking bath, 1.25 mL zinc acetate, of different concentrations (from 0.02 to 0.1%), was added to the aqueous gellan solution prior to extrusion. The obtained gellan particles were washed with bi-distilled water in order to remove the zinc acetate excess and these particles were kept at 4–6 °C in tightly closed containers until further characterization. For yeast cell immobilization in gellan particles was used the same method as described above. Prior to extrusion, a suspension of yeast cells, obtained from 1.25 g yeast and 5 mL of water, was added into the gellan solution at a temperature of 38–40 °C. The temperature at which the extrusion of the polymer solution is carried out is 30 °C. The extrusion process takes approximately 15 min and the particles obtained are spherical and have a diameter of about 2–2.5 mm. From the total quantity of the yeast only 30% represents yeast cells. The amount of yeast cells in 1.25 g yeast used as previously indicated is therefore equal to 0.375 g and the ratio of yeast cells/gellan is 1.5:1 (w/w). The obtained particles are left in the cross-linking bath for 2 h, and 12 h, respectively and then were washed with bi-distilled water in order to remove the zinc acetate excess and these particles were kept at 4–6 °C in tightly closed containers until further characterization.

2.2.2. Particles stability in aqueous medium

The stability of the gellan particles with and without immobilized yeast cells was determined not only in the cross-linking solution (supernatant) but also in bi-distilled water. It is considered that from the less stable particles, polymer fragments can be drawn and this causes the