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PVA-gel (Lentikats®) as an effective matrix for yeast strain immobilization aimed at heterologous protein production

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Abstract

Three different yeast strains, namely *Saccharomyces cerevisiae mnn1mnn9*, *Kluyveromyces lactis* JA6/GAA and *Zygosaccharomyces bailii* [pZ$_3$klIL-1β], were entrapped in polyvinyl alcohol (PVA) gel particles, obtained following the commercially available immobilization kit named Lentikat®. After immobilization in the PVA-gel particles, yeast cells remained viable: colonization of the gel matrix reached up 100 mg d.w. of cells cm^{-3} gel for all the strains examined.

Lentikats® of *K. lactis* JA6/GAA and *Z. bailii* [pZ$_3$klIL-1β] showed to be suitable for the continuous production of glucoamylase and interleukin 1β, respectively, when employed under non-selective conditions. They were of easy handiness and showed excellent mechanical properties during prolonged operation in stirred tank reactors.

Keywords: Immobilization; Polyvinyl alcohol; Yeast; Heterologous proteins

1. Introduction

Entrapment of cells in a proper support matrix is one of the most widely used methods of cell immobilization. Gels of agar, agarose, κ-carrageenan, alginate and chitosan are the most popular matrices when immobilized cells are to be employed in the production of metabolites, since entrapment in these gels is known to preserve cell integrity. However, natural polymeric gels are very sensitive to abrasion and often chemically unstable [1].

Polyvinylalcohol (PVA) is nontoxic to organisms and can be cheaply produced at industrial scale. Furthermore, it is not a carbon source for most microrganisms and is not infected by them. The use of PVA for cell immobilization has attracted much attention. Most of the cases cells are embedded in PVA cryogels obtained by means of a freeze-thawing technique, and sometimes cell viability is not fully preserved [2]. Also other immobilization methods using PVA may have undesiderable effects on the entrapped cells, since gelation occurs under UV irradiation [3], or in highly acidic solutions (PVA-boric acid method) [4]. The problems occurring in the PVA-boric acid method were prevented through esterification of PVA with phosphate solution; this technique was employed to immobilize denitrifying sludges [5] and, more recently, a microbial consortium for decolorization of azodye [6].

In the present report, aiming at developing immobilized yeast cell systems to be employed in the continuous production of heterologous proteins, we have taken into consideration an immobilization procedure which allows PVA gelation to occur under mild conditions.

The immobilization kit Lentikat®, commercialised by geniaLab (Braunschweig, Germany) [7], satisfies this requirement. In fact, the patented Lentikat®Liquid (a solution of 10%, w/v PVA) offers the possibility to entrap cells in stable hydrogels, obtained by dehydration in the absence of chemical reaction starters. The lenticular form of the gel par-

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ticle (named Lentikat®) obtained following gelation of the PVA solution has an optimised geometry which is claimed to reduce mass transfer resistance in the matrix. Moreover, using a Lentikat®Printer a reproducible large-scale production of gel particles of the same size can be obtained. This immobilization technique was reported to preserve cell viability in the case of bacterial cells [8].

In this work, we employ the Lentikat® technique to entrap three different yeasts: the mutant *mnn1mnn9* of *Saccharomyces cerevisiae* [9], and two recombinant strains, namely *Kluyveromyces lactis* JA6 and *Zygosaccharomyces bailii* ATCC36947 transformed with the glucoamylase gene (GAA) of *Arxula adeninivorans* [10], and with the human gene of interleukin 1β (IL-1β) [11], respectively.

The *N*-glycosylation defective mutant *mnn1mnn9* of *S. cerevisiae* [9], characterized by a high permeability of the cell-wall, was chosen to test the above mentioned immobilization technique in the case of a yeast strain able to secrete external invertase [12] in the medium.

K. lactis JA6/GAA and *Z. bailii* [pZ$_3$klIL-1β] entrapped in Lentikats® were incubated in an appropriate culture medium under selective conditions to promote cell colonization of the matrix and enhance cell-loading up to the maximum. Then, both the PVA-gel particles of *K. lactis* JA6/GAA and *Z. bailii* [pZ$_3$klIL-1β], were employed, at laboratory scale, in a continuous stirred tank reactor (CSTR), for the production of glucoamylase and interleukin 1β, respectively. Experiments were carried out at different dilution rates under non-selective conditions, since cell immobilization in a gel matrix is known to prevent plasmid loss [13].

The performance of the yeast cells entrapped in the PVA-gel particles is discussed.

2. Materials and methods

2.1. Strains and culture conditions

The strains employed were: *S. cerevisiae mnn1mnn9* (*MATa SUC*2 *mal mel gal*2 *cup*1 *mnn1mnn9*) [9], *K. lactis* JA6 (α *ade*1–600 *adeT*–600 *trp*1–11 *ura* 3–12) transformed with the multicopy plasmid vector pT32x carrying the glucoamylase gene from *A. adeninivorans* [11] and *Z. bailii* ATCC36947, transformed with the plasmid pZ$_3$klIL-1β, where the human IL-1β is functionally linked to the pre-leader sequence of the *K. lactis* kl killer toxin alpha-subunit. Said plasmid bears the kan^r cassette, conferring resistance to G418 [12].

The yeast biomass to be immobilized in PVA-gel was prepared by culturing cells aerobically at 30 °C in 500-cm^3 shake flasks (300 rpm) containing 200 ml of the appropriate medium. For *S. cerevisiae mnn1mnn9* and *Z. bailii*, YEP (1%, w/v yeast extract; 0.5%, w/v bactopeptone) medium was employed, containing 1 and 5% (w/v) glucose, respectively. In the case of *Z. bailii*, pH of the medium was adjusted to 5.0 and selective conditions were ensured by adding geneticin (G418) to a final concentration of 200 mg/l. *K. lactis* was cultured under selective conditions using the yeast-minimal salt medium (SD medium) with 1% (w/v) lactose as carbon source and the appropriate aminoacids, as described by Rose et al. [14].

2.2. Immobilization technique

Cells of each strain were collected by centrifugation after 24 h growth, washed abundantly and resuspended in 10 ml distilled water. According to the standardized procedure, 10 ml of cell suspension were added to 40 ml Lentikat® Liquid. Cell suspension contained 45 mg d.w. cells for *mnn1mnn9*, so that initial cell-loading was 0.9 mg cm^{-3} gel (initial low cell-loading). In the case of *K. lactis* JA6/GAA and *Z. bailii* [pZ$_3$klIL-1β], cell suspension contained 1400 mg d.w. cells so that initial cell-loading was 28 mg cm^{-3} gel (initial high cell-loading).

The mixture (cell suspension/Lentikats®Liquid) was then poured in a large (20 mm diameter) petri dish. The Lentikat®Printer with 400 wires was used to produce at the same time 400 droplets of uniform shape and size (diameter 2–3 mm). The wires were dipped into the mixture, then lifted and finally lowered on the surface of an empty dish to "print" controlled-sized droplets. Gelation of droplets was allowed to occur by drying them in a laminar airflow cabinet until their original mass was reduced of 75%, so that gel particles having a lenticular form were obtained. When gelation was completed, the Lentikat®Stabilizer solution was poured on the gel particles for re-swelling them to about the original size.

Lentikats® were kept in the stabilizer solution at 4 °C till the moment of using them.

2.3. Colonization of the PVA-gel particles and cell-loading determination

To allow cell colonization of the PVA-gel, Lentikats®, prepared as described above, were incubated in 500-cm^3 shake flasks (300 rpm) containing 200 ml of the appropriate medium (800 particles per flask). The medium was changed every 24 h. At fixed time intervals during incubation, the particles were collected and dissolved in tapered graduated tube by heating in a micro-wave oven. The volume of the resulting solution was measured once cooled at room temperature to evaluate the increase in size of Lentikats®.

Yeast cells, after their release from the PVA-gel, were harvested by centrifugation (3000 × *g*, 5 min) washed with distilled water and heated at 105 °C for dry weight determination. Cell-loading was expressed in terms of dry weight of biomass per gel volume (mg cell d.w. cm^{-3} gel).

2.4. Experiments in the continuous stirred tank reactor (CSTR)

PVA-gel particles containing entrapped cells either of *K. lactis* or *Z. bailii*, once reached the maximal cell-loading,