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Effect of substrate concentration, pH, and temperature on the activity of the complex glucose–fructose oxidoreductase/glucono-δ-lactonase present in calcium alginate-immobilized *Zymomonas mobilis* cells

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ABSTRACT

The action of the enzymes glucose–fructose oxidoreductase (GFOR) and glucono-δ-lactonase (GL), present in calcium alginate-immobilized *Zymomonas mobilis* cells, was characterized in relation to substrate concentration (0.05–2.0 mol L^{-1}), pH (5.2–9.7), and temperature (34–59 °C). Higher enzymatic activities were obtained at pH 7.8 and 8.2 and at 47 and 50 °C, which were 80% higher than the conditions presented in previously defined conditions for free cells, namely at pH 6.4 and 39 °C. Further analysis indicated that these findings are related to the diffusional barrier resulting from the calcium alginate beads, which hinder the transport of gluconic acid from the inner space of the beads to the external medium. This behavior was reproduced during the initial moments of bioconversion performed at pH 7.8 and 47 °C. Nevertheless, during the last hours of the process, the reaction stopped because of inadequate pH levels inside the beads. The results suggest that a variable pH – from 7.8 to 6.4 – and a constant temperature of about 47 °C are the best conditions for achieving good conversion yields and productivities.

1. Introduction

Glucose–fructose oxidoreductase (GFOR) and glucono-δ-lactonase (GL), periplasmic enzymes of the Gram-negative anaerobic bacterium *Zymomonas mobilis*, catalyze the bioconversion of fructose and glucose to equimolar amounts of sorbitol and gluconic acid, respectively [1].

Sorbitol is a polyalcohol with non-cariogenic properties that is widely used in the food and pharmaceutical industries [2–4]. The main applications of gluconic acid and its salts are in the food industry and in electroplating [5,6].

High yields in the formation of sorbitol and gluconic acid were obtained using the technique of cell immobilization, providing higher stability for the enzyme complex and facilitating a process that could be operated in a continuous mode [7,8]. Usually, calcium alginate [7,9], k-carrageenan [8,10], foam cubes [11,12] and other polymers [13] are used for immobilization support.

The effect of temperature and pH on the activity of GFOR was assessed for the purified enzyme [1], intact *Z. mobilis* [14], and permeabilized and immobilized cells [8]. In these works, pH values in the ranges of 5.7–6.5, 6.2–7.0, and 6.5–7.0, respectively, were referred to as optimal for the action of the enzyme. For temperature, the best results for both free and immobilized *Z. mobilis* cells laid between 39 and 42 °C [8,14]. From our own results in a previous work, the best results for GFOR/GL present in cetyl trimethylammonium bromide (CTAB)-permeabilized *Z. mobilis* cells were found at 43–45 °C and pH 6.4–6.8 [15].

As pH and temperature are critical parameters for any enzyme (taking into account the different conclusions reported for the GFOR/GL complex of *Z. mobilis*), the effects of these parameters on the activity of GFOR/GL present in calcium alginate-immobilized *Z. mobilis* cells were evaluated in this work. The best results obtained in these tests were assayed also under bioconversion conditions, envisaging the production of sorbitol and gluconic acid from fructose and glucose, respectively. Furthermore, the response of GFOR/GL activity to the concentration of substrates was assessed.

2. Materials and methods

2.1. Strain and cultivation conditions

Z. mobilis ATCC 29191 was maintained in liquid medium [16] and stored at 4 °C. Replicate cultures were made monthly. The liquid medium [16] used for maintenance, inocula growth, cell biomass,

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and enzyme production had the following composition ($g\,L^{-1}$): glucose, 20 (maintenance), 100 (inoculum), 150 (biomass and enzyme production); $(NH_4)_2SO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 1.0; Prodex Lac® yeast extract (Prodesa S.A., Brazil), 7.5. To prepare the inocula, $5\,g\,L^{-1}$ of $CaCO_3$ was added to the medium to avoid an excessive pH drop during the experiments. In this case, the initial pH was close to 6.2. Concentrated glucose solutions and $CaCO_3$ were separately sterilized and added to the medium before inoculation. Sterilization of nutrients and glucose solutions was accomplished by autoclaving at 1 atm for 15 min.

Inocula were prepared in 500-mL anaerobic bottles (with CO_2 release filters), filled with 450 mL of medium and kept under orbital agitation at $200\,min^{-1}$ (Certomat U, Sartorius Stedim Biotech, Germany) and 30 °C for approximately 10 h. Batch cultivation of *Z. mobilis* cells containing GFOR/GL was carried out in a 5.5 L stainless-steel bioreactor that was designed and built in our laboratory, equipped with two flat-blade impellers. The temperature was kept at 30 °C, the impeller speed was $450\,min^{-1}$, and the pH was maintained at 5.5 with $5\,mol\,L^{-1}$ NaOH using a Consort model R735 pH controller (Belgium). Cell mass was harvested from the medium by centrifugation at $6000\,min^{-1}$, at 4 °C, for 20 min, using a Sigma 4K-15 centrifuge (Sartorius Stedim Biotech, Germany). The cells were re-suspended in distilled water to a concentration of $50\,g\,L^{-1}$.

2.2. Cell permeabilization

Z. mobilis cells were permeabilized by mixing equal volumes of concentrated biomass and 0.2% (w/v) cetyl trimethylammonium bromide (CTAB) solution. This suspension was gently stirred for 10 min, as described by Rehr et al. [8]. The permeabilized cells were centrifuged and re-suspended to $50\,g\,L^{-1}$ with distilled water.

2.3. Immobilization method

In order to immobilize *Z. mobilis* cells in calcium alginate, equal volumes of concentrated cell suspension and 4% (w/v) sodium alginate (Algogel 5540, Degussa Flavours & Fruit Systems do Brasil Ltd.) solutions were added together. The mixture was kept under agitation for 2 h to achieve complete homogenization and then drop-wise poured through hypodermic needles onto a 0.3 M $CaCl_2$ solution under magnetic stirring in order to form calcium alginate beads in the presence of cells. The cell suspension and the beads thus formed were reticulated with 0.5% (w/v) glutaraldehyde for 15 min under magnetic stirring at room temperature. The average size of the beads was about 2 mm (diameter). The calcium alginate beads were kept at 4 °C in distilled water and were subsequently used in enzymatic activity and bioconversion comparative assays.

2.4. Enzymatic tests

The enzymatic activity of GFOR/GL, in *Z. mobilis* cells immobilized in calcium alginate, was determined on standardized conditions (substrate concentration, $0.7\,mol\,L^{-1}$; cell concentration, $10\,g\,L^{-1}$; temperature, 39 °C; pH, 6.4). The catalytic activity was evaluated under these standardized conditions on equimolar concentrations of glucose and fructose (from 0.05 to $2.0\,mol\,L^{-1}$), different pH values (5.2–9.7), and temperatures (34–59 °C). The thermal stability of the enzymatic complex present in calcium alginate beads was evaluated in tests that consisted of four successive 3 h bioconversion runs, at pH values of 6.4 or 7.8, and temperatures of 39, 43, and 47 °C. After each 3-h run, calcium alginate beads were treated with $0.3\,mol\,L^{-1}$ $CaCl_2$ to assure their hardness.

2.5. Bioconversion experiments

The bioconversion assays were performed in a 600 mL glass reactor containing 240 mL of $0.7\,mol\,L^{-1}$ equimolar glucose/fructose solution and $20\,g\,L^{-1}$ of previously permeabilized and immobilized cells. The temperature of the reactor was maintained at the desired value by placing it in a water bath. The pH was controlled by automatic addition of a $7.0\,mol\,L^{-1}$ NaOH solution through a pH controller (Consort R735, Belgium). A magnetic stirrer agitated the reaction medium.

The maximum specific rate of gluconic acid formation (ν_m, mmol gluconic acid g cell h^{-1}), which depends on the activity of GFOR/GL, was determined during the first hour of each bioconversion process.

All the values presented in graphs and tables are the mean values from two replications.

2.6. Analytical methods

Cell concentration was determined by measuring the optical density of cell suspensions at 560 nm. Turbidimetric measurements gave a linear relationship with dry cell mass for each case.

GFOR/GL activity, in calcium alginate-immobilized *Z. mobilis* cells, was determined by mixing an amount of calcium alginate beads (to give $10\,g\,L^{-1}$ of cell biomass) with a $0.7\,mol\,L^{-1}$ equimolar solution of glucose/fructose in a 300 mL reactor. The system was kept in a water bath, at the desired temperature, under magnetic agitation, with the pH being automatically controlled at 6.4 or 7.8 by the addition of $1\,mol\,L^{-1}$ NaOH. One unit of GFOR/GL was defined as the amount of enzymatic complex responsible for the production of 1 mmol of gluconic acid per hour under the assay conditions [8]. GFOR/GL activities in this work are presented in units per gram of dry cells ($U\,g^{-1}$).

The concentration of gluconic acid was stoichiometrically inferred from the volume of alkali used to control the pH value.

To estimate the average pH value within the alginate beads, samples of beads were collected from the bioconversion reactor, drained, and added to deionised boiling water to inactivate the GFOR/GL complex and, therefore, stop the reaction. The relationship between the volumes of alginate beads and boiling water was the same as that used in the bioreactor. After 24 h, the pH of the liquid phase was measured and these values were considered as an estimation of the internal pH of the alginate beads.

3. Results and discussion

3.1. Effect of substrate concentration on the enzyme activity of GFOR/GL in calcium alginate-immobilized Z. mobilis cells

Initially, the effect of substrate concentrations on the catalytic activity of the immobilized GFOR/GL from *Z. mobilis* was investigated. Increasing activities were observed with equimolar concentrations of glucose and fructose, up to $0.7\,mol\,L^{-1}$. Within the 0.7–$1.3\,mol\,L^{-1}$ range of substrate, similar activities were achieved (ca. of $5.4\,U\,g^{-1}$) whereas for substrate concentrations higher than $1.3\,mol\,L^{-1}$ the activity decreased (Fig. 1).

These values are quite lower than those obtained for free cells – $26\,U\,g^{-1}$, using $0.7\,mol\,L^{-1}$ of equimolar solution of glucose/fructose, and $33\,U\,g^{-1}$, with $1.3\,mol\,L^{-1}$ – at the same temperature and pH values [17]. Such a difference was probably due to the diffusional barrier of the immobilized system, with lower mass transfer rates than that of the free-cell system.

When hydrocolloidal materials are jellified they form a quasi-solid structure, depending on the type of gel material and gel concentration. These forms will retard the transport of a solute, reducing diffusion coefficients. The mass transfer behavior, besides