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Repeated production of L-xylulose by an immobilized whole-cell biocatalyst harboring L-arabinitol dehydrogenase coupled with an NAD^+ regeneration system

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ABSTRACT

The biotransformation of L-arabinitol into L-xylulose was investigated using whole-cell biocatalysts. Efficient production of L-xylulose was accomplished using *Escherichia coli* that expressed L-arabinitol dehydrogenase (LAD). The production yield was enhanced by coupling LAD with a cofactor-regenerating NADH oxidase in an *E. coli* whole-cell biocatalyst system. Factors affecting the production of L-xylulose by this whole-cell biocatalyst system include reaction pH, maximal cell loading, and cofactor regeneration. Under optimized conditions, the conversion of L-arabinitol into L-xylulose was achieved with above 96% efficiency, a rate much higher than that reported in previous studies. Furthermore, the whole-cell system could be immobilized on calcium alginate; the immobilized cells showed good operational stability, retaining their relative productivity at 65% after 7 cycles of successive re-use.

1. Introduction

Rare sugars are unique monosaccharides or sugar derivatives, that occur only rarely in nature. These rare sugars play crucial roles as recognition elements in bioactive molecules [1,2]. They have a wide range of uses, from sweeteners to functional foods and potential therapeutics [3]. Specifically, L-xylulose is a rare sugar that can be used as a potential inhibitor of multiple α-glucosidases, and may also be used as an indicator of hepatitis or liver cirrhosis [2,4].

The application of microorganisms for reductive whole-cell biotransformation has become an important method in chemoenzymatic synthesis, and has several advantages over the use of isolated enzymes [5,6]. Whole-cell biotransformation uses enzymes without cost-intensive enzyme purification steps. A whole-cell system can also regenerate cofactors in vivo via the addition of inexpensive electron-donating co-substrates. Additionally, the catalysts in such reactions demonstrate extended lifetimes [7]. Microbial production of L-xylulose has been studied previously: using a resting cell reaction, the production of L-xylulose from xylitol has been described in *Pantoea ananatis* [8], *Alcaligenes* sp. 701B [9], and *Bacillus pallidus* Y25 [10]. Furthermore, the genes encoding the xylitol dehydrogenase from *P. ananatis* ATCC 43,072 [11] and *B. pallidus* [12] have been cloned, and recombinant strains containing these genes have been used to produce L-xylulose. High-efficiency conversions of xylitol to L-xylulose (~70% conversion) have previously been achieved by a recombinant *Escherichia coli* strain; however, this conversion was only achieved under low (<67 mM) xylitol conditions [13]. low concentrations were used because an initial concentration of xylitol over 100 mM would inhibit xylitol-4-dehydrogenase activity [11,13], and the thermodynamic equilibrium between xylulose and xylitol has been shown to be strongly on the side of xylitol [11,14]. To the best of our knowledge, there have been no studies on the production of L-xylulose production using a cofactor-regeneration system. Additionally, there have been no reports on the use of immobilized whole cells for the conversion of L-arabinitol to L-xylulose.

L-arabinitol 4-dehydrogenase (EC 1.1.1.12) from *Hypocrea jecorina* (HjLAD) is an enzyme in the L-arabinose catabolic pathway of fungi, catalyzing the conversion of L-arabinitol into L-xylulose [15,16]. However, HjLAD requires a stoichiometric amount of the

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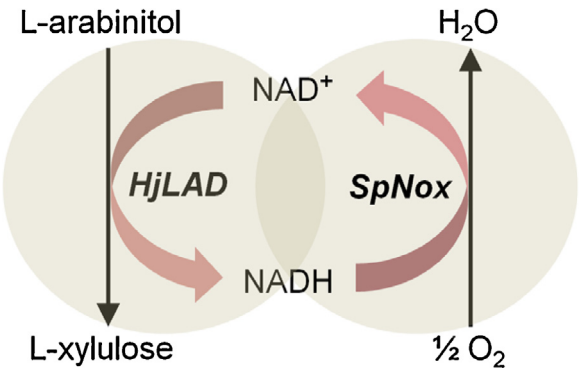

Fig. 1. A schematic illustration of biocatalytic L-xylulose production using *E. coli* whole cells harboring HjLAD coupled with SpNox a cofactor regeneration enzyme.

expensive nicotinamide cofactor, nicotinamide adenine dinucleotide (NAD^+, the oxidized form of NADH) in order to carry out the L-arabinitol-to-L-xylulose conversion. NADH oxidase from *Streptococcus pyogenes* (SpNox) displays high cofactor-regeneration activity [17,18]. In the present study, we used whole *E. coli* cells expressing HjLAD for the production of L-xylulose. Furthermore, we coupled whole *E. coli* cells expressing HjLAD with whole *E. coli* cells expressing SpNox (Fig. 1) for cofactor regeneration to achieve a higher conversion than that reported in previous studies. Additionally, the biocatalyst system containing *E. coli* cells harboring HjLAD and SpNox was immobilized using calcium alginate to improve the reusability of the system for multiple cycles of L-xylulose production.

2. Materials and methods

2.1. Medium and chemicals

E. coli cultivation medium (Luria–Bertani, LB) was purchased from Duchefa–Postbus (Haarlem, The Netherlands). Sodium alginate, NAD^+, L-arabinitol and other commercially available chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless specified otherwise.

2.2. Cultivation of recombinant whole cell E. coli harboring HjLAD or SpNox

Recombinant *E. coli* strains harboring pET28a-SpNox [17] or pET28a-HjLAD [15] (hereafter referred to as *E. coli*$_{SpNox}$ and *E. coli*$_{HjLAD}$, respectively) were cultured at 37 °C in LB medium supplemented with kanamycin (50 μg mL^{-1}). To induce HjLAD or SpNox expression, isopropyl-β-d-thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.1 mM, and incubation was continued for 6 h with shaking at 25 °C. The induced cells were harvested by centrifugation at 4 °C for 15 min at 10,000 × *g*, followed by one rinse with phosphate-buffered saline, and the cell pellet was stored at −20 °C.

2.3. Production of L-xylulose from L-arabinitol by recombinant E. coli

To optimize the parameters of pH and cell density for production of L-xylulose from *E. coli*$_{HjLAD}$, the cell pellets described above were resuspended in a 50 mM potassium phosphate buffer or Tris–HCl buffer of varying pH (7.0–9.5), at a cell density of between 1.0 and 4.0 g dry cell weight (g_{DCW}) L^{-1}. The effect of L-arabinitol concentration on the rate of L-xylulose production from *E. coli*$_{HjLAD}$ was studied by varying the initial L-arabinitol concentration from 25 mM to 250 mM. The optimization of the pH and cell density parameters was conducted using 150 mM L-arabinitol and 3 mM NAD^+. Following determination of the optimum reaction conditions, the L-xylulose production reaction was carried in the coupled whole-cell biocatalyst system, containing both *E. coli*$_{HjLAD}$ and *E. coli*$_{SpNox}$. The reaction conditions for the coupled system were the same as those for production of L-xylulose by whole-cell *E. coli*$_{HjLAD}$ alone, with the exception that 3.0 g_{DCW} L^{-1} of whole-cell *E. coli*$_{SpNox}$ was also present in the cell suspension. The two *E. coli* strains were coupled at 1:1 (i.e., 3.0 g_{DCW} L^{-1} each). *E. coli* carrying the expression plasmid pET28a without the HjLAD or SpNox genes was used as the control strain. For all combinations of pH, cell density, and substrate concentration, biotransformation of L-arabinitol to L-xylulose was conducted at 30 °C with shaking at 200 rpm. Samples of the cell suspension (100 μl) were obtained periodically and were centrifuged at 16,000 × *g* for 20 min; the supernatants were used for high-performance liquid chromatography (HPLC) analysis of L-xylulose concentration.

2.4. Optimization of immobilization parameters

For the immobilization of *E. coli* in calcium alginate beads, we first determined the optimum parameters for bead formation. For the preparation of beads with the proper permeability and rigidity, parameters such as the sodium alginate concentration, the $CaCl_2$ concentration, and the initial cell mass required must be optimized. For this purpose, bead formation was tested under varying concentrations of sodium alginate (1–3% w/v), $CaCl_2$ (0.1–0.4 M), and initial biomass (1.25–5 g_{DCW} L^{-1}). For coupling, we utilized a 1:1 ratio of *E. coli*$_{HjLAD}$ and *E. coli*$_{SpNox}$. The initial biomass that could be loaded in the beads was investigated by incorporating different amounts of cells in the aqueous sodium alginate solution. The beads were cured for 2 h at 25 °C. The number of cells that leaked from the alginate beads was determined by measuring the optical density at 600 nm. The optical density value was then converted into mg_{DCW} mL^{-1} using a standard curve [19,20].

2.5. Immobilization of recombinant whole-cell E. coli

All immobilization steps were performed under sterile conditions. The cells were mixed thoroughly with 10 mL of sodium alginate solution (2% w/v final concentration). After proper mixing, the mixture was extruded dropwise through a syringe into 200 mL of 0.1 M $CaCl_2$ solution. The beads were allowed to harden in this solution for 2 h at 25 °C and were then washed with saline solution to remove any excess $CaCl_2$ and free cells. The average bead diameter was 2 mm. The calcium alginate beads with immobilized *E. coli* cells were then used for L-xylulose production.

2.6. Production of L-xylulose from L-arabinitol using immobilized recombinant E. coli

The conditions for production of L-xylulose by immobilized *E. coli* were similar to those for free *E. coli* cells, with the exception being the use of prepared beads. The reaction mixture was incubated with shaking, and samples were withdrawn at regular intervals for HPLC analysis. Repeated production experiments, in which the same batch of cells was used for multiple cycles of L-xylulose production, were performed under the same conditions. After each production cycle, liquid samples of the supernatant medium were collected by filtration, and the beads were washed three times with reaction buffer. Fresh substrate solution was then added to start a new cycle of the production reaction.