



# Repeated production of L-xylulose by an immobilized whole-cell biocatalyst harboring L-arabinitol dehydrogenase coupled with an NAD<sup>+</sup> 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.

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## 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  $\alpha$ -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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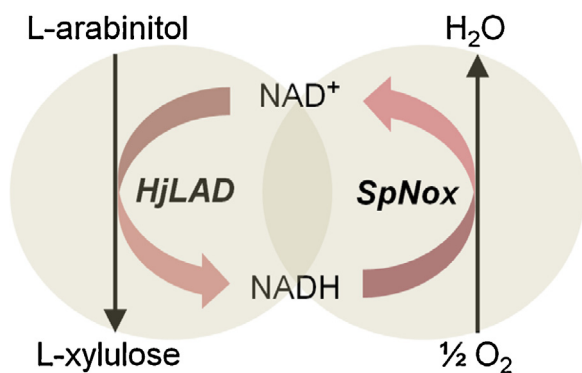


Fig. 1. A schematic illustration of biocatalytic L-xylulose production using *E. coli* whole cells harboring HjLAD coupled with SpNox a cofactor regeneration enzyme.

expensive nicotinamide cofactor, nicotinamide adenine dinucleotide (NAD<sup>+</sup>, 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<sup>+</sup>, 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*<sub>SpNox</sub> and *E. coli*<sub>HjLAD</sub>, respectively) were cultured at 37 °C in LB medium supplemented with kanamycin (50 μg mL<sup>-1</sup>). 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*<sub>HjLAD</sub>, 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<sub>DCW</sub>)L<sup>-1</sup>. The effect of L-arabinitol concentration on the rate of L-xylulose production from *E. coli*<sub>HjLAD</sub> 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<sup>+</sup>. 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*<sub>HjLAD</sub> and *E. coli*<sub>SpNox</sub>. The reaction conditions for the coupled system were the same as those for production of L-xylulose by whole-cell *E. coli*<sub>HjLAD</sub> alone, with the exception that 3.0 g<sub>DCW</sub> L<sup>-1</sup> of whole-cell *E. coli*<sub>SpNox</sub> was also present in the cell suspension. The two *E. coli* strains were coupled at 1:1 (i.e., 3.0 g<sub>DCW</sub> L<sup>-1</sup> 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<sub>2</sub> 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<sub>2</sub> (0.1–0.4 M), and initial biomass (1.25–5 g<sub>DCW</sub> L<sup>-1</sup>). For coupling, we utilized a 1:1 ratio of *E. coli*<sub>HjLAD</sub> and *E. coli*<sub>SpNox</sub>. 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<sub>DCW</sub> mL<sup>-1</sup> 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<sub>2</sub> 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<sub>2</sub> 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.

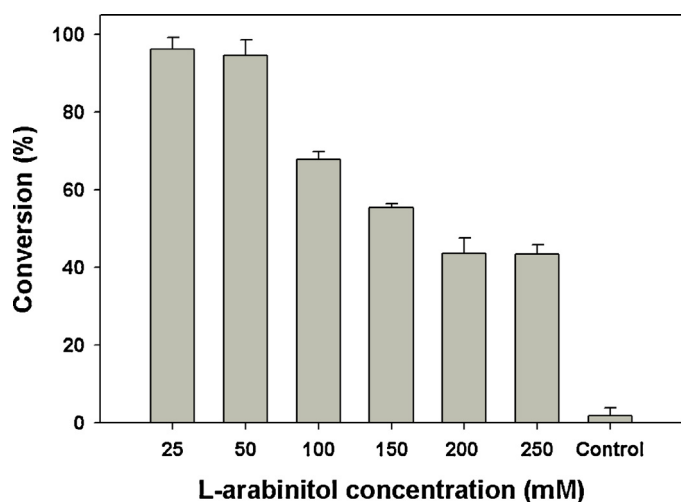


Fig. 2. Effects of substrate concentration on the production of L-xylulose using whole-cell *E.coli*<sub>HjLAD</sub>. Control is the conversion at 50 mM L-arabinitol obtained by whole cell *E. coli*-pET28a.

### 2.7. Analytical methods

Samples taken from the reaction mixture were analyzed by HPLC using an Ultimate 3000 (Dionex, CA, USA) system equipped with a Shodex SUGAR SP0810 column (Showa Denko, K. K., Kawasaki, Japan) and an evaporation light scattering detector (ESA6700, MA). Water served as the elution buffer; elution was performed at a rate of 1 mL min<sup>-1</sup> at a column temperature of 80 °C. The evaporation and nebulizer temperatures were 50 °C and 65 °C, respectively. Under these operating conditions, the L-arabinitol and L-xylulose retention times were 9.8 and 16.4 min, respectively. Quantification of L-xylulose samples derived from the whole-cell-mediated reactions was performed using external calibration standards and five different L-xylulose concentrations. Each reaction was performed and analyzed three times, and the reported values are the average of the three measurements, along with the associated standard deviation.

## 3. Results and discussion

### 3.1. Optimization of L-xylulose production using a free whole-cell system

To optimize the experimental conditions to achieve high L-xylulose yields, we examined the effects of substrate concentration, pH, and initial cell density on the conversion of L-arabinitol to L-xylulose by *E. coli*<sub>HjLAD</sub>. We then examined L-xylulose production in a coupled system containing both *E. coli*<sub>HjLAD</sub> and *E. coli*<sub>SpNox</sub>.

We first investigated the effect of substrate concentration on the conversion by varying L-arabinitol concentration. Substrate concentration is an important parameter in determining the rate of biocatalysis; high substrate concentrations may inhibit biocatalytic conversion [11,13]. In the present study, the conversion efficiency decreased as the substrate concentration increased (Fig. 2). This result is consistent with a previous study of L-xylulose production using resting cells expressing xylitol dehydrogenase, where Takata et al. achieved approximately 53% conversion with a substrate concentration of 2% (133 mM), but only achieved a 36% conversion rate with a substrate concentration of 5% [12]. To further improve the conversion of L-arabinitol to L-xylulose under conditions of high substrate concentration, we investigated the effect of pH on L-xylulose yield. pH-optimization reactions were performed using 150 mM L-arabinitol as the substrate and 2.5 g<sub>DCW</sub> L<sup>-1</sup> of *E. coli*<sub>HjLAD</sub>

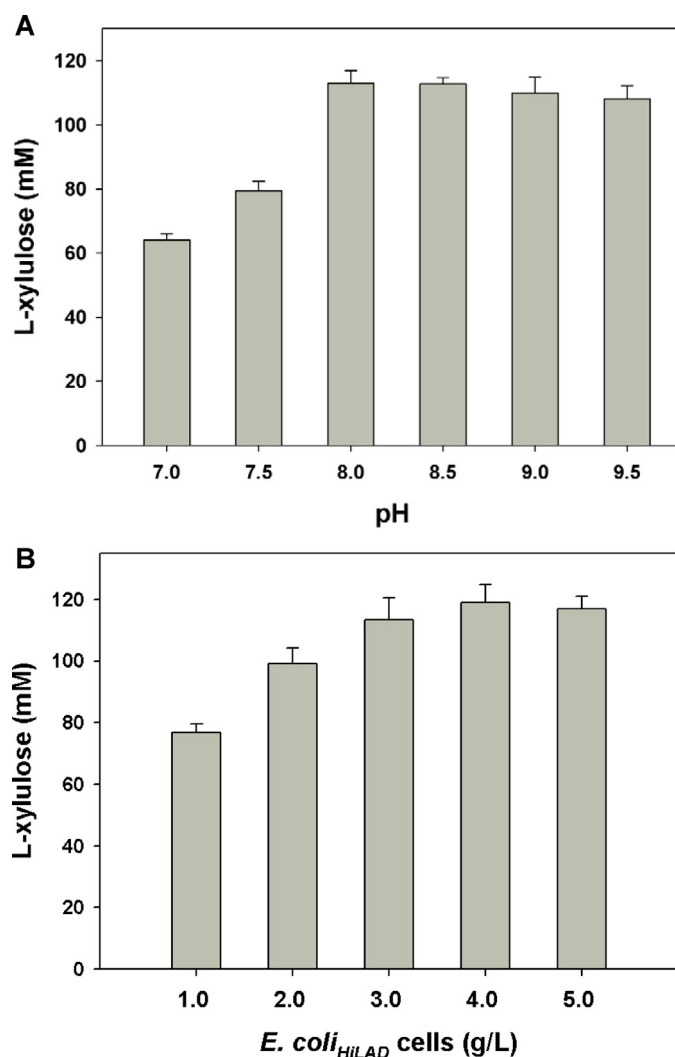


Fig. 3. Effects of biocatalysis conditions on the production of L-xylulose. (A) pH; (B) amount of whole *E. coli*<sub>HjLAD</sub> cells used for production.

as the biocatalyst, and were carried out under different pH values (Fig. 3A). After 6 h of reaction with *E. coli*<sub>HjLAD</sub>, it was demonstrated that L-xylulose production yield increased with the rise in buffer pH from 7.0 to 8.0. However, increasing the pH beyond 8.0 decreased production of L-xylulose. Considering that the SpNox enzyme has an optimum pH of 7.0, we chose pH 8.0 as the optimal buffer pH for L-xylulose production. Variation in buffer pH may not only influence the selectivity and activity of the enzymes, but may also influence the regeneration of the cofactor present in the microbial cells, which in turn affects the rate of biotransformation [21]. Further optimization was performed by investigating the effect of *E. coli*<sub>HjLAD</sub> cell density on L-xylulose production yield (Fig. 3B). The conversion of L-arabinitol to L-xylulose increased in a linear manner as cell density increased within the range of 1–3 g<sub>DCW</sub> L<sup>-1</sup>. When the DCW used in the reaction was increased above 3.0 g L<sup>-1</sup>, the production of L-xylulose remained at approximately the same level, indicating that the optimal cell loading was 3.0 g L<sup>-1</sup>.

To enhance the bioconversion of L-arabinitol into L-xylulose, a simple and efficient cofactor-recycling method has been developed by coupling two different recombinant *E. coli* cell lines, *E. coli*<sub>HjLAD</sub> and *E. coli*<sub>SpNox</sub>, in a 1:1 ratio. As shown in Fig. 4, the yield obtained from 150 mM L-arabinitol by whole-cell *E. coli*<sub>HjLAD</sub> was 79% (118 mM L-xylulose), which was markedly enhanced to 96% by coupling these cells with *E. coli*<sub>SpNox</sub> (144 mM L-xylulose).

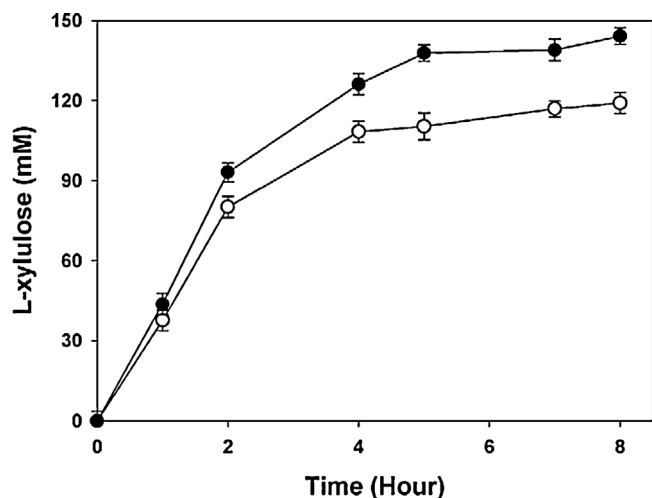


Fig. 4. The time course of L-xylulose production using whole *E. coli<sub>HjLAD</sub>* cells (open circles) and whole *E. coli<sub>HjLAD</sub>* cells coupled with whole *E. coli<sub>SpNox</sub>* cells (filled circles).

Several previous studies on L-xylulose production have been reported. The highest L-xylulose yields from xylitol have been shown in *Alcaligenes* sp. strain 701B [9] and *P. ananatis* ATCC 43,072 [11]; L-xylulose yields from these strains were 80% and 94%, respectively. These results were obtained at a xylitol concentration of  $5 \text{ g L}^{-1}$  ( $\sim 32.8 \text{ mM}$ ). However, *Alcaligenes* sp. strain 701B [9] and *P. ananatis* [11] showed only 50% and 70% yields, respectively, at  $10 \text{ g L}^{-1}$  ( $\sim 65.7 \text{ mM}$ ) of xylitol. Furthermore, Poonperm et al. reported an 85% L-xylulose yield from *B. pallidus* Y25 when using 2% ( $\sim 131 \text{ mM}$ ) xylitol and incubating at  $50^\circ\text{C}$ ; it was reported that the higher bioconversion temperature could allow higher substrate solubility by decreasing the viscosity of the reaction mixture [10]. However, the requirement for increased temperature would increase the cost of industrial-scale production due to higher energy consumption. Additionally, L-xylulose produced by *B. pallidus* Y25 strain can be partially converted to L-xylose and L-arabinitol because of the intrinsic isomerase activity in *B. pallidus* [10]. Resting *E. coli* cells harboring the recombinant xylitol dehydrogenase gene of *B. pallidus* have been reported to produce L-xylulose from 2% xylitol, with a yield of approximately 53% [12].

In the present study, we obtained higher yields of L-xylulose than those reported previously. We have demonstrated that whole *E. coli* cells can effectively supply cofactors via their own cellular metabolism. However, the activity of this endogenous cofactor regeneration system is controlled by the robustness of the cellular physiology [22]. In the experiments performed in the present study, L-xylulose production was further enhanced by the presence of SpNox for cofactor regeneration in the whole-cell coupling system. In this cofactor-regeneration system, SpNox converts NADH to the required  $\text{NAD}^+$  for L-arabinitol oxidation by whole-cell *E. coli<sub>HjLAD</sub>*. This system enables the use of lower concentrations of the expensive cofactor  $\text{NAD}^+$ , and helps shift the reaction equilibrium towards the production of L-xylulose. Based on the results, a whole-cell biocatalyst with a cofactor regeneration system shows great potential for efficient L-xylulose production. In addition, we treated cells with toluene to study the effect of cell permeability on L-xylulose production [23]. However, the production yield was not enhanced by a more-permeable cell wall (data not shown).

### 3.2. Immobilization of *E. coli* cells expressing *HjLAD* and *SpNox*

Alginate is one of the best candidate matrices for cell entrapment, due to its good biocompatibility and processing capacity [24,25]. During entrapment of microbial cells, optimization of the

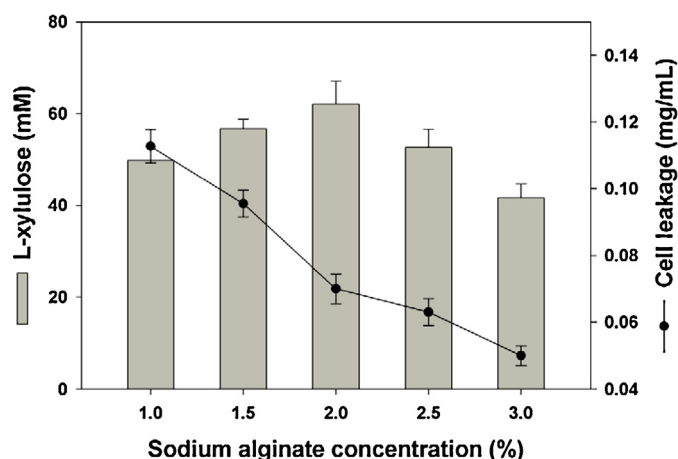


Fig. 5. Effect of sodium alginate concentration on cell leakage and L-xylulose production by immobilized whole cells.

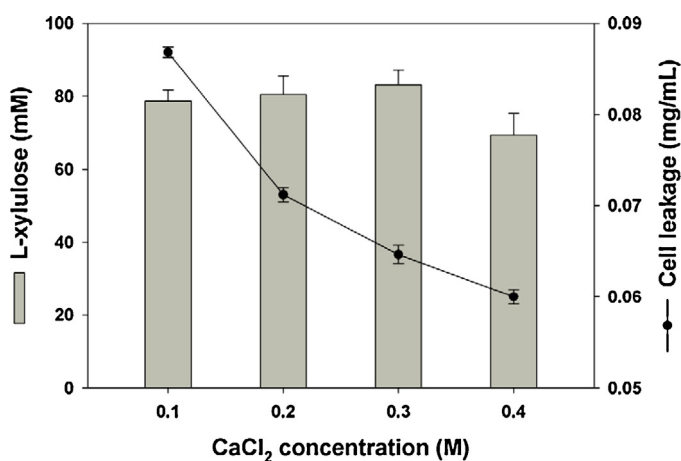


Fig. 6. Effect of  $\text{CaCl}_2$  concentration on cell leakage and L-xylulose production by immobilized whole cells.

immobilization conditions improves the rigidity and permeability of the beads [19]. We investigated the effects of specific parameters, such as alginate and  $\text{Ca}^{2+}$  concentrations and initial cell mass, on immobilization. Fig. 5 shows the effect of sodium alginate concentration on L-xylulose production and cell leakage from the formed alginate beads. The results indicate that beads made from 2% sodium alginate gave the maximum production of L-xylulose from *E. coli*. When the sodium alginate concentration was less than 1%, the resultant beads were fragile, released a significant amount of cells into the reaction medium, and were easily broken due to low mechanical strength. However, increasing the sodium alginate to concentrations higher than 2% hardens the beads, causing diffusion problems [26]. Furthermore, the mechanical strength of the alginate beads appeared to be dependent on  $\text{CaCl}_2$  concentration (Fig. 6). Lower concentrations of  $\text{CaCl}_2$  resulted in increased leakage of cells into the reaction medium, owing to the decreased rigidity of the beads. In contrast, increasing the  $\text{CaCl}_2$  concentration from 0.3 M to 0.4 M decreased cell leakage, but also reduced L-xylulose production. Considering the minimal cell leakage and maximum production of L-xylulose, 0.3 M was chosen as the optimum concentration of  $\text{CaCl}_2$  for the formation of alginate beads with suitable rigidity and permeability.

Whole *E. coli<sub>HjLAD</sub>* cells coupled with an equal amount of whole *E. coli<sub>SpNox</sub>* cells were used to study the effect of cell loading on the immobilization. In order to optimize cell loading or concentration in alginate beads, experiments were carried out using various

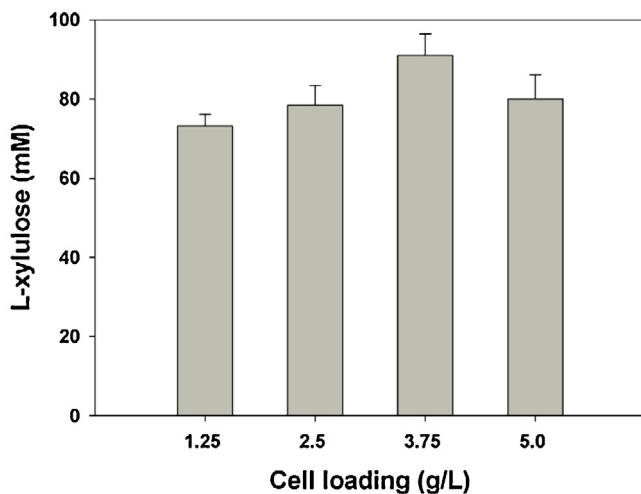


Fig. 7. Effect of initial cell concentration on cell leakage and L-xylulose production by immobilized whole cells.

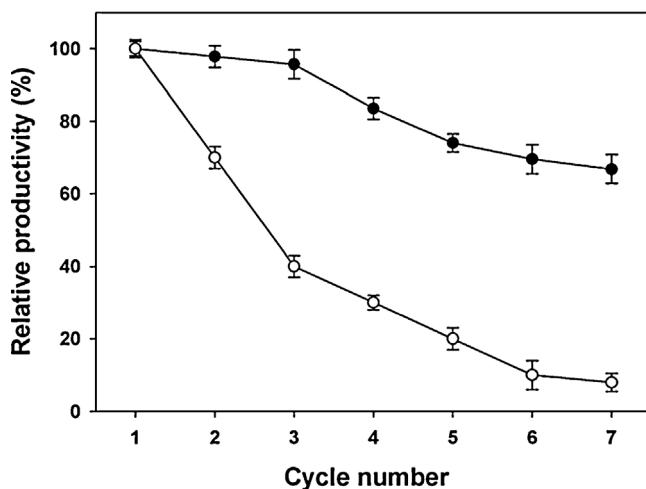


Fig. 8. Reusability of free (open circle) and immobilized whole cells (filled circle). The relative production yield of the cells in the first cycle was defined as 100%.

ratios of cells to beads (Fig. 7). The highest L-xylulose production was obtained using  $3.75 \text{ g}_{\text{DCW}} \text{ L}^{-1}$ , and the maximum conversion of L-arabinitol to L-xylulose reached 64%. When the same amount of immobilized whole *E. coli*<sub>HJLAD</sub> cells alone were used as the control, we obtained only a 32.5% conversion. This demonstrates that we observed a 50.7% improvement in the conversion using the immobilized, coupled whole-cell system. However, L-xylulose production was decreased in the immobilized, coupled whole-cell system compared to the free, coupled whole-cell system. This reduced conversion might be due to difficulty in diffusion of the substrate or transfer of the product through the calcium alginate matrix. Immobilization leads to modifications in the microenvironment, and various metabolic and morphologic alterations may be responsible for this modification [19].

### 3.3. Reusability of the immobilized and free cells for L-xylulose production

Cell immobilization offers the advantage of re-using the biocatalysts for repeated production reactions. The stability of the immobilized, coupled whole cells in a repeated batch reaction was investigated (Fig. 8). The immobilized beads were reused effectively for L-xylulose production. After 7 cycles of successive re-use, the

alginate beads retained good stability and maintained 65% of the yield obtained in the first cycle. In contrast, the free whole-cell system lost 60% of its ability to produce L-xylulose after only 3 cycles. In conclusion, their reusability gives the immobilized whole cells significant advantages over the free whole-cell system. Additionally, the use of immobilized cells as biocatalysts has the potential advantage of facilitating product separation, thus simplifying the process and lowering the cost of production. In conclusion, the efficient immobilized whole cell biocatalytic process shows enhanced operational stability compared to the free whole-cell system.

## 4. Conclusion

In the present study, the biocatalytic synthesis of L-xylulose was successfully performed using a coupled whole-cell system. Under optimal conditions and using an initial concentration of 150 mM L-arabinitol, the maximum L-xylulose yield reached 96%, a rate of conversion that was much more efficient than that obtained in previous studies. The use of immobilized cells as biocatalysts not only facilitates product separation, but also makes the whole-cell system recyclable and reusable, greatly simplifying the process and lowering the cost of production [21,27]. In this study, we investigated the ability of using the immobilized whole-cell catalyst for L-xylulose production and obtained 64% conversion yield, which is lower than obtained with the free whole-cell system. However, the immobilized cells demonstrated good operational stability, as supported by their high stability and reusability. The free cells almost completely lost the ability to convert L-arabinitol to L-xylulose after 7 cycles of successive re-use, while the immobilized cells maintained 65% of the conversion yield of the first cycle. These results may contribute to better industrial production techniques for L-xylulose.

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