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Optimization of the microbial synthesis of dihydroxyacetone in a semi-continuous repeated-fed-batch process by in situ immobilization of *Gluconobacter oxydans*

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Abstract

The effect of in situ immobilization of *Gluconobacter oxydans* on a novel carrier material in a repeated-fed-batch operated packed-bed bubble-column bioreactor for the production of the fine chemical dihydroxyacetone was investigated experimentally. The carrier material were biocompatible, durable, coated Ralu-rings. The coating was a porous silicone matrix with satisfactory wetting characteristics. Settling of cells was relatively rapid. The cells were protected from abrasion caused by shear forces. A sufficiently high oxygen supply rate to the immobilized cells was provided due to the high oxygen permeability of the silicone matrix. The immobilized biomass was estimated to be about 65% of the total biomass contained in the bioreactor after 18 days of operation. The observed space-time yield was approx. 76% higher compared to a similar process which was performed without an optimized fermentation medium. Compared to previous experiments with a trickle-bed bioreactor, the space-time yield was approx. 3.7 times higher. A typical time course of the immobilization process was observed: after an induction phase, a transition phase followed which later on gave way to a nearly linear accumulation phase. A stationary phase with regard to the amount of immobilized active cells, however, was not reached. Hence, a higher bioreactor performance than observed could be expected at longer operation times.

Keywords: Dihydroxyacetone; *Gluconobacter oxydans*; In situ immobilization; Optimization; Repeated-fed-batch process

1. Introduction

The conventional process of the industrial microbial production of the fine chemical dihydroxyacetone (DHA) via *Gluconobacter oxydans* is the fed-batch operation in a stirred tank bioreactor. However, the disadvantages of this operation mode are the necessity of cleaning, sterilization, and inoculation procedures after each fed-batch cycle. A favoured alternative to the fed-batch fermentation is represented by the semi-continuous repeated-fed-batch operation [1]. The procedure for the repeated-fed-batch operation is as follows: The fermentation with controlled substrate feeding is performed until a pre-determined DHA concentration threshold value is reached. This DHA threshold value corresponds to a product concentration at which the culture is not irreversibly growth inhibited. Then, most of the fermentation broth is removed and the bioreactor is replenished with fresh medium. The residual broth volume serves as inoculate for the next cycle. A way to intensify and optimize this process is to increase the amount of active biomass in the reactor via recycling of whole cells. This procedure enables a decoupling of the residence times of cells and liquid in the bioreactor. The main advantage of cell recycling is the achievement of a higher space-time yield via the increased active biomass density in the bioreactor. Such approaches using whole cell recycling have already been pursued for decades for numerous applications in the area of biodegradation. However, in comparison, the number of such applications in the area of microbial production processes is much smaller. Basically, two concepts exist: (i) the immobilization of cells on a suitable carrier material which is contained in the bioreactor or (ii) the recycling of cells in an external loop equipped with a cell separation unit (e.g. a membrane unit or a centrifuge) [2,3]. However, in some of the latter cases, repeatedly observed phenomena like membrane fouling, shear forces, and insufficient oxygen supply may adversely affect the process. On the other hand, Merck KGaA, Darmstadt, Germany [4] demonstrated in a patent a promising system with cell recycling via an external loop. The first mentioned approach

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was pursued using carrier materials such as porous particles [5,6] or gel beads made of biopolymers [7,8]. However, it turned out that the settling of cells on the interior surface of the porous particles was not satisfactory in many cases due to insufficient oxygen supply into the deeper biofilm layers. Furthermore, many biopolymer gel beads did not exhibit the required operational stability. Therefore, cross-linking was examined in order to improve the stability of the gel beads [9]. A review on the feasibility and operational stability of immobilized microbial cell systems was published by Freeman and Lilly [10]. The utilization of simple surface carrier materials was examined as early as 1979. Here, an improved microbial production process of citric acid using wood chips as the carrier material of whole immobilized cells was successfully demonstrated [11]. Comparable in situ immobilized whole cell systems have been examined in long-term experiments and proved to be stable for several months [12,13,14]. Hence, the feasibility of the concept was proven. In addition to reactor systems with immobilized whole cells, successful applications using immobilized enzymes were reported in the literature [15,16]. It was shown in a previous work that high productivities coupled with high enantiomeric excess values were reached [17].

Due to the large oxygen requirements of the strain *G. oxydans* used in the present study, it must be ensured that a large enough mass transfer to the immobilized cells is maintained at all times. In a previous study, Hekmat et al. [1] showed that the in situ immobilization of *G. oxydans* in a 85 l pilot-scale trickle-bed reactor was feasible. The utilized carrier material in this work were hydrophilized uncoated polypropylene Ralu rings. These carriers yielded a high specific surface area, a homogeneous radial distribution of fluids, and a low pressure drop. It was expected that the hydrodynamics of the trickle-bed reactor ensured mild conditions, e.g. small shear forces, in order to facilitate the settling of cells and the formation of a biofilm. However, it was observed that the biofilm formation rate was relatively small and only parts of the carrier material were covered by the biofilm. Hence, the immobilization was unsatisfactory. However, the immobilization procedure still led to an increase of the product formation rate of about 75% during a 17 days operation [1]. The aim of the present study was to develop and test a suitable coating for an enhanced carrier material which yields a fast and evenly distributed immobilization of cells. The immobilization matrix should be robust enough to enable an operation in a packed bubble-column instead of a trickle-bed reactor. The packed bubble-column bioreactor is favourable since the portion of fermentation broth is significantly larger compared to a trickle-bed reactor, thus, resulting in higher space-time yields. The requirements for a suitable coating material were as follows: (1) chemically inert, biocompatible, and durable; (2) thermally stable in order to withstand 121 °C; (3) mechanically stable; (4) highly porous to facilitate cell immobilization as well as mass transfer into the deeper layers of the matrix. A coating material with such properties has been developed by Muscat [18]. This material was a silicone mixture with the addition of salt with a given narrow particle size distribution prior to polymerisation. The salt particles were dissolved after polymerisation to generate a silicone matrix with a given porosity. Furthermore, titanium oxide was added during the fabrication process in order to improve the wetting characteristics by reducing the contact angle between liquid and solid phase [19]. Titanium oxide is biocompatible and chemically inert and therefore well suitable. A further advantage of the chosen carrier matrix was the fact that silicone exhibits a relatively high oxygen permeability so that oxygen supply is carried out not only via the liquid phase but also via the solid silicone matrix.

According to the work of Bettin [19], the permeation coefficient of oxygen in silicone was 3.5×10^{-10} m^2 s^{-1}. This value is about five-fold higher than the permeation coefficient of oxygen in water. Hence, it could be expected from the chosen set-up that the oxygen limitation effect on the immobilized obligate aerobic cells of *G. oxydans* was reduced.

2. Materials and methods

2.1. Microorganisms and media

G. oxydans is an obligate aerobic Gram-negative bacterium and belongs to the family of acetic acid bacteria. These microorganisms are able to synthesize a variety of different polyols, such as L-sorbose from D-sorbitol or D-gluconic acid from D-glucose [20]. This work focuses on the production of DHA from glycerol. The reaction is catalysed by a membrane-bound dehydrogenase and is carried out for the purpose of energy generation. The *G. oxydans* industrial strain M1136 was used which was kindly provided by Merck KGaA, Darmstadt, Germany. In order to use genetically consistent material, the strain was conserved at −20 °C in a sorbitol medium containing the following components: 2.0 kg m^{-3} $(NH_4)_2SO_4$, 0.1 kg m^{-3} K_2HPO_4, 0.9 kg m^{-3} KH_2PO_4, 1.0 kg m^{-3} $MgSO_4{\cdot}7H_2O$, 2.5 kg m^{-3} yeast extract, and 10 kg m^{-3} sorbitol. The culture was transferred from the cryoculture to flask cultures every month. Ohrem [21] reported that $CaCl_2$ had a positive effect on growth of *G. oxydans*. In addition, Bauer [22] discovered that the addition of sorbitol as a secondary substrate had a positive effect both on growth on glycerol and on the DHA production rate. Therefore, for the experiments in the laboratory-scale fermenters, a medium as described above was used which contained a controlled amount of 5–20 kg m^{-3} glycerol and, in addition, 1.5 kg m^{-3} $CaCl_2$.

2.2. Analytical methods

Optical density (OD) was measured at 578 nm with a Lambda 2 spectrophotometer (Perkin Elmer, Wiesbaden, Germany). The samples were centrifuged at 5000 rpm for 5 min. The following relation resulted after calibration: biomass dry weight (kg m^{-3}) = 0.247 × $OD_{578\ nm}$. Glycerol and DHA concentrations were determined using an on-line high performance liquid chromatograph (HPLC) Beckman System Gold (Beckman Coulter GmbH, Krefeld, Germany). A Rezex RCM Monosaccharide calcium-column (Phenomenex, Aschaffenburg, Germany) was employed for the measurements. The eluent was bi-distilled water. The elution flow rate was 0.6 ml min^{-1} [22].

2.3. Description of bioreactor system

The bioreactor system was a laboratory-scale bubble-column with a height of 300 mm and an inner diameter of 100 mm modified from equipment which originated from Bioengineering AG, Wald, Switzerland. The total volume of the bioreactor was 2 l. The experiments were performed in a fully automated way by using a microcomputer. Bettin [19] investigated the limiting and inhibiting effects on growth by glycerol. It was found that the optimum glycerol concentration range for growth and product formation was 5–20 kg m^{-3}. Therefore, the substrate glycerol was fed to the repeated-fed-batch process by computer control in order to keep the glycerol concentration within this range at all times. The liquid volume was kept nearly constant during the repeated-fed-batch