Catalytic Biofilms on Structured Packing for the Production of Glycolic Acid

Li, Xuan Zhong¹, Bernhard Hauer², and Bettina Rosche¹*

¹School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney NSW 2052, Australia
²Institute of Technical Biochemistry, University of Stuttgart, Allmandring 3, 70569 Stuttgart, Germany

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While structured packing modules are known to be efficient for surface wetting and gas–liquid exchange in abiotic surface catalysis, this model study explores structured packing as a growth surface for catalytic biofilms. Microbial biofilms have been proposed as self-immobilized and self-regenerating catalysts for the production of chemicals. A concern is that the complex and dynamic nature of biofilms may cause fluctuations in their catalytic performance over time or may affect process reproducibility. An aerated continuous trickle-bed biofilm reactor system was designed with a 3 L structured packing, liquid recycling and pH control. *Pseudomonas diminuta* established a biofilm on the stainless steel structured packing with a specific surface area of 500 m² m⁻³ and catalyzed the oxidation of ethylene glycol to glycolic acid for over two months of continuous operation. A steady-state productivity of up to 1.6 g l⁻¹ h⁻¹ was achieved at a dilution rate of 0.33 h⁻¹. Process reproducibility between three independent runs was excellent, despite process interruptions and activity variations in cultures grown from biofilm effluent cells. The results demonstrate the robustness of a catalytic biofilm on structured packing, despite its dynamic nature. Implementation is recommended for whole-cell processes that require efficient gas–liquid exchange, catalyst retention for continuous operation, or improved catalyst stability.

Key words: Biofilm, structured packing, trickle-bed reactor, biocatalysis, *Pseudomonas*, ethylene glycol

Many microbial strains are able to grow as single-species biofilms on various surfaces [8, 23]. Biofilms can be regarded as living catalysts that regenerate by cell division and potentially have high resistance towards reactants [11, 25]. For these reasons, biofilms have been proposed as robust, self-immobilized, and self-regenerating catalysts [33], and numerous recent studies have exemplified the potential of biofilms for the production of chemicals [8–10, 13, 25, 32].

Various biofilm reactor configurations are available and their characteristics have been reviewed [33]. Trickle-bed reactors entail a downward flow of liquid through a static bed of catalyst-containing packing materials, and offer catalyst retention, high throughput of liquid and gas, low shear force, and therefore little catalyst wear [30]. They have been used in biofilm processes for off-gas and wastewater treatment as well as in productive catalysis [12, 14, 16, 21, 32]. A commercial example for the latter is the traditional production of vinegar by acetic acid bacteria on a packing of wood chips [5]. Trickle-bed reactors have been operated with random packing, which is made up of small individual elements packed in a random fashion. Flow channelling, incomplete catalyst wetting, poor mass transfer, reactor flooding, and blockage are areas of concern [7, 16, 30, 31]. Owing to these limitations, the trickle-bed biofilm reactor is not regarded as a suitable reactor configuration for high-rate production processes [29].

Structured packing refers to compact modules typically made of corrugated metal sheets or gauze that are designed to optimize surface wetting and contact between liquid and gas phases for processes of separation and surface catalysis. Structured packing has been applied in absorption and distillation processes since its development in the 1960s [37], and more recently in chemical catalytic processes [19, 31]. Whereas submerged structured packing made from plastic has already been tested in biofilm reactors in the field of wastewater treatment [34], we have suggested the use of structured packing in biofilm reactors for the synthesis of chemicals [33].

In comparison with conventional random packing, structured packing improves flow distribution and thereby minimizes pockets of stagnant fluid and flow channelling, resulting in higher catalyst efficiency and reactor performance [7]. With a high void fraction, structured packing allows for
high liquid and gas loading and is particularly suitable for processes that require efficient transfer between gas and liquid phases [1, 7]. A high void fraction in structured packing may also reduce the chance of reactor blockage by biomass. Whereas trickle-bed reactors with random packing are often limited to a liquid/gas cocurrent flow to reduce the risk of reactor flooding [35], structured packing can be operated in a counter-current flow because of the larger void volume [2]. Counter-current flow may be beneficial for reactions that are limited by product inhibition or by the thermodynamic equilibrium [2].

The aim of this study was to design and operate an aerated trickle-bed biofilm reactor with structured packing for continuous biotransformation. Reactor performance and reproducibility were investigated using glycolic acid production by *Pseudomonas diminuta* as a model process. Glycolic acid is a fine chemical used in the adhesive, metal cleaning, textile, leather processing, and cosmetics industries, and microbial conversion of ethylene glycol has been considered an attractive method for glycolic acid production [17, 41].

**Materials and Methods**

**Bacterial Strain and Media**

*Pseudomonas diminuta* ATCC 19146, also known as *Brevundimonas diminuta* [36], is available from the American Type Culture Collection. The inoculation medium contained per liter 1 g “Lab-Lemco” powder, 2 g yeast extract (both from Oxoid, Hampshire, England), 5 g peptone (Merck, Darmstadt, Germany), 5 g NaCl, and 1 g polypropylene glycol (PPG1000) as antifoam. The pH was 7.4 without adjustment. The biotransformation medium consisted of M12 medium [24] supplemented with 100 mM ethylene glycol as the biotransformation substrate, 1 g/l casamino acid, 4 mg/l cystine, and 50 mM potassium phosphate buffer at pH 7. Each medium component was dissolved in reverse osmosis water, sterilized separately, and combined in 20 L bottles to achieve the listed concentration. The final pH was 7 ± 0.1 without further adjustment. The stock culture of *P. diminuta* was grown to stationary phase in inoculation medium without PPG and was stored in 20% (w/v) glycerol at −80°C.

**Biotransformation of Ethylene Glycol in Planktonic Batch Cultures**

Cells were grown to stationary phase in biotransformation medium at 30°C and 150 rpm. Cells were resuspended in biotransformation medium (50 ml in a 250 ml Erlenmeyer flask or 5 ml in a 50 ml Falcon tube) buffered with 250 mM MES at an initial pH of 7.0 and OD₆₀₀ adjusted to 5.0. Cultures were incubated at 30°C and 150 rpm for various times and were analyzed by HPLC.

**HPLC Analysis**

Biotransformation samples and external standard solutions of ethylene glycol and glycolic acid were prepared for HPLC analysis by mixing with equal volumes of 90% (v/v) acetonitrile containing 10 mM xylitol as an internal standard, followed by centrifugation (13,000 rpm, 5 min) for removal of biomass including precipitated protein. HPLC analysis was performed on a Bio-Rad Aminex HPX-87H ion exclusion column (300 × 7.8 mm) at 30°C with refractive index detection, using 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 ml/min.

**Reactor System Assemblage**

The biofilm reactor system is illustrated in Fig. 1A and consisted primarily of a trickle-bed reactor and a recycling vessel. The 6 L trickle-bed reactor was a cylindrical glass unit with a height of 30 cm and an inner diameter of 16 cm, fitted with a 17 cm (height) × 15 cm (diameter) cylindrical stainless steel structured packing (Montz-pak A3-500; ACS, USA) as biofilm substratum (Fig. 1B). The total volume of the structured packing was 3 L, with a cross section area of 0.0177 m² and a specific surface area of 500 m²/m³. Medium was supplied onto the packing at a flow rate of 20 l/h via a peristaltic pump (Watson Marlow 603S, USA) through a two-piece mist spray nozzle (Gardena, USA) and this corresponded to a liquid loading rate of approximately 1,100 l m⁻² h⁻¹. Air was sterilized...
through a 0.22 µm air filter unit (Millipore, USA) and was supplied in counterflow at 2.4 l/min (0.4 vvm).

Feedstock was introduced into the reactor system at a flow rate of 0.04–0.1 l/h via the recycling vessel. The recycling vessel was a modified 1 L Quickfit glassware with a flat flange lid and Quickfit ports. Liquid from the recycling vessel was delivered into the trickle-bed reactor via the spray nozzle, and was returned to the recycling vessel by gravity. Another peristaltic pump drew effluent out of the recycling vessel to maintain a constant liquid volume of 100 ml within the recycling vessel. Since the liquid holdup volume within the packing and tubing was approximately 200 ml, the liquid volume in the entire reactor system was 300 ml. A pH controlling unit (Leeds & Northrup, USA) was connected to the recycling vessel, and the reactor pH was kept at 6.9 by the addition of 1 M NaOH. Rapid liquid exchange within the recycling vessel facilitated liquid mixing.

Preliminary reactor runs revealed that silicon tubing was prone to fouling and blockage. Tygon tubing (Norton, USA) was hence used in the reactor system except for the sections within peristaltic pump heads where neoprene tubing (Watson Marlow, UK) was needed for added durability. The external and internal tubing diameters were 1/4” and 1/8”, respectively. The sampling port was a 3-way valve with attached vial, which allowed sterile sampling. Reactor components and tubing were sterilized in an autoclave at 121°C for 90 min and then aseptically assembled, and tubing connections were secured with cable ties. For cleaning after a reactor run, the packing was removed from the trickle-bed reactor and soaked in a 1–2% alkaline detergent solution (Pyroneg, Crown Scientific, Australia) at 100°C for approximately 5 h, followed by rinsing with RO water.

Reactor Inoculation and Continuous Biotransformation

The reactor system was operated in a 30°C constant temperature room. It was inoculated by adding 300 ml of inoculation medium containing 0.1% (v/v) P. diminuta stock culture, followed by liquid circulation between the trickle-bed reactor and the recycling vessel at a flow rate of 20 l/h without feedstock addition. After 24–27 h, biotransformation medium was delivered into the reactor system at flow rates between 0.04 and 0.1 l/h which corresponded to dilution rates of 0.14–0.33 h⁻¹. Samples were taken regularly via the sampling port for optical density measurement at 600 nm and for quantification of ethylene glycol and glycolic acid by high-performance liquid chromatography (HPLC).

Isolation and Characterization of Cells from Biofilm Reactor Effluent

A sample was aseptically collected from the sampling port and plated in different dilutions onto agar plates made with the biotransformation medium. Single colonies were observed after incubation at 30°C for 1–2 days. Twenty randomly picked single colonies were transferred to fresh agar plates for purification. To test for possible contamination of the reactor, the 16s rRNA gene of the isolates was amplified by colony PCR using the primer pair 16S-27F (AGAGTTTGATCCTGGCTCAG)/16S-1494R (TACGGCTACCTTGTTACGAC) [20]. Sequenced PCR products were compared with known sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST, NCBI, http://www.ncbi.nlm.nih.gov/blast). Furthermore, it was tested in 5 ml batch culture biotransformations of ethylene glycol whether biofilm reactor-derived cells yielded varying phenotypes of productivity when compared with cells derived from a planktonic culture. The batch cultures were inoculated with colonies of the biofilm effluent isolates, or with colonies grown from a planktonic batch culture, or with the stock culture.

RESULTS

Glycolic Acid Production in Planktonic Culture

Planktonic cells of P. diminuta, a Gram-negative aerobic bacterium [36], converted approximately 80% of 100 mM ethylene glycol in 48 h, which corresponds to an average glycolic acid productivity of approximately 0.12 g l⁻¹ h⁻¹. The rate of ethylene glycol biotransformation decreased after 48 h, and a total of 90 mM ethylene glycol was converted to glycolic acid after 4 days with nearly 100% molar yield of product per consumed substrate (Yp/s). Neither oxidative intermediates nor other by-products were detected by HPLC. Since P. diminuta is also a strong biofilm former [23] and developed biofilms on stainless steel materials, this organism was chosen as the catalyst to investigate the potential of structured packing in a trickle-bed biofilm reactor.

Process Development

Being an aerobic organism, P. diminuta requires oxygen for respiration. Preliminary results indicated that vigorous aeration, efficient liquid distribution to biofilms, as well as pH control were necessary for the oxidation of ethylene glycol to glycolic acid by P. diminuta biofilms (data not shown). It has been suggested that the reaction rate in a trickle-bed reactor is proportional to the fraction of the reactor packing surface that is effectively wetted by the flowing liquid [28]. Therefore, the structured packing Montz-pak A3-500 (Fig. 1B) was tested as a biofilm substratum, because this packing is made from multiple layers of corrugated and pierced stainless steel wire gauze (Fig. 1C) that are arranged to create efficient contacting surface between the solid, liquid and gas phases. According to the manufacturer’s description, the special wire mesh has a capillary effect, which provides excellent wettability of the packing surface (http://www.acsseparations.com; ACS, USA). It was originally designed for chemical fractionation and has a specific surface area of 500 m²/m³. By displacement of water, the void fraction within the packing material was measured to be 96%. Such a large void fraction will allow substantial biomass accumulation without reactor blockage.

To ensure good nutrient and substrate delivery to attached biofilms on the structured packing, it was necessary to select a liquid distributor that could achieve sufficient wetting of the packing module. During preliminary testing, a two-piece mist spray nozzle produced a full-cone liquid distribution that covered the structured packing cross section area, as judged by visual inspection, and was hence chosen for this study. A flow rate of 16–24 l/h was
required to sustain such liquid distribution by the spray nozzle.

Although trickle-bed reactors can be operated with or without medium recycling, the recycling vessel was introduced as a key element in the reactor configuration for several reasons. Aside from its functions in feedstock delivery, effluent removal, and pH control, the recycling vessel was essential in maintaining the relatively high flow rate (16–24 l/h) required by the mist spray nozzle for optimal liquid delivery. High liquid loading provides better flow distribution and mass transfer efficiency within a trickle-bed reactor [1, 7] and may support higher productivity. The incorporation of a recycling vessel within the reactor system also allowed the use of concentrated substrate in the feedstock with potential for higher product concentration compared with the reactor without a recycling vessel.

**Glycolic Acid Production in the Structured-Pack Biofilm Reactor**

Three continuous biotransformations of ethylene glycol were performed independently in the biofilm reactor to evaluate process reproducibility and robustness. Fig. 2 shows a biotransformation profile at a dilution rate of 0.33 h⁻¹. After an inoculation period of 26 h, 100 mM ethylene glycol was fed and the concentration of glycolic acid increased to 55 mM within the next 165 h, reaching a steady-state productivity of 1.4 gl⁻¹ h⁻¹. The sum of ethylene glycol and glycolic acid concentration remained slightly below 100 mM, which indicates a minor gap in molar balance of approximately 5%. However, glycolic acid was the only product detected. During steady state, the effluent optical density was below 0.05; thus little biomass was present in the liquid phase and glycolic acid productivity was mostly attributed to biofilm. No pronounced biofilm dispersal event was observed during the entire time course. At 191 h, microbial growth was discovered in the feedstock reservoir and it was replaced with a new bottle of feedstock. It was not clear at this stage whether the contamination had prevented further increase of productivity, but the repeat runs showed similar productivities in the absence of contamination (Fig. 4). Despite the event of feedstock contamination, the productivity in this run remained steady for over 700 h. This demonstrated the robustness of established *P. diminuta* biofilm in a non-sterile operating environment.

**Effect of Dilution Rate on Glycolic Acid Production**

Fig. 3 presents another biotransformation operated under the same conditions as the biotransformation shown in Fig. 2, except for the variation of dilution rate. At the initial dilution rate of 0.33 h⁻¹, the concentration of glycolic acid increased to 56 mM within 180 h after the inoculation phase, reaching a steady-state productivity of 1.4 gl⁻¹ h⁻¹ (steady-state 1). This result is very similar to the former run (Fig. 2) and therefore reveals that the feedstock contamination in the former run did not limit the steady-state productivity of the reactor system.

The dilution rate was varied, as indicated in Fig. 3, at values higher than the specific growth rate of a planktonic batch culture (0.11 h⁻¹) to promote wash-out of planktonic cells from the reactor. Phases of steady-state productivity
are marked for each dilution rate. Averages of substrate and product concentrations, productivity, Yp/s, and molar balance for each steady-state are presented in Table 1. A drop in dilution rate from 0.33 h\(^{-1}\) (steady-state 1) to 0.2 h\(^{-1}\) (steady-state 2) increased the average glycolic acid concentration from 56 mM to 71 mM; however, the productivity decreased from 1.4 g l\(^{-1}\) h\(^{-1}\) to 1.1 g l\(^{-1}\) h\(^{-1}\). Further decrease in dilution rate to 0.14 h\(^{-1}\) did not increase the glycolic acid concentration (steady-state 3). Thus, the highest substrate conversion achieved in the system was an average of 71% of fed substrate. Raising the dilution rate back to 0.33 h\(^{-1}\) restored the reactor productivity and decreased the glycolic acid concentration (steady-state 4). Overall, the results indicate that steady-state productivity was positively correlated to the dilution rate within the experimental range.

Ethylene glycol biotransformation at the 0.33 h\(^{-1}\) dilution rate (Table 1, steady-states 1 and 4) resulted in lower Yp/s values compared with the Yp/s values at lower dilution rates (Table 1, steady-states 2 and 3). This is reflected in a 6–11% gap of molar balance that only occurred at the higher dilution rate, and such molar balance gap was also observed in the previous run (Fig. 2). It is not known if, at the higher dilution rate and increased availability of nutrient, some of the ethylene glycol was built into the biomass. For example, EPS synthesis can be influenced by carbon source and nutrient availability [39].

Three biofilm dispersal events (Fig. 3, D1-D3) were observed over the 64 days of biotransformation, evident by increased optical density in the reactor effluent. Medium recycling interruptions for up to 24 h due to nozzle orifice blockage may have triggered the 1\(^{st}\) and 3\(^{rd}\) biofilm dispersal events at the time points of 775 h and 1,269 h, respectively. The biofilm quickly recovered from these two events, with only minor impact on reactor productivity. The 2\(^{nd}\) biofilm dispersal event (D2) occurred between 886 h and 989 h (Fig. 3). Prior to the dispersal event, the biofilm reactor had been operated at the dilution rate of 0.14 h\(^{-1}\) for nearly 400 h. Prolonged nutrient limitation as a result of low dilution rate may have initiated biofilm dispersal towards the end of steady-state 3. Subsequently, a sudden increase in available nutrient due to the large increase in dilution rate from 0.14 to 0.33 h\(^{-1}\) may have triggered further
biofilm dispersal. The initial productivity after this biofilm dispersal event was over 20% lower compared with the productivity under the same dilution rate in steady-state 1, which may indicate temporary loss of reactor productivity as a result of biofilm dispersal. It is promising that the reactor productivity recovered and increased to an average of 1.6 g·L⁻¹ h⁻¹ within 250 h (steady-state 4), as this again illustrates the robustness of the biofilm reactor system.

Continuous biotransformation of ethylene glycol to glycolic acid was maintained for 64 days before a decision was made to terminate the experiment. The stainless steel structured packing was covered with a thick biofilm visible to the naked eye. The biofilm mass on the structured packing was not quantified owing to the relatively much higher weight of the structured packing and the difficulty in removing representative samples from this 3 L compact module.

Reproducibility of Biofilm Reactor Productivity
Reproducibility is a concern for biofilm processes, as variable biofilm growth and dispersal, physiological heterogeneity, and possible genetic instability of biofilm cells could lead to fluctuations in productivity [33]. Fig. 4 overlays the productivity profiles of three independent reactor runs at a dilution rate of 0.33 h⁻¹. It was apparent that the time profiles were very similar and a steady-state glycolic acid productivity of approximately 1.4 g·L⁻¹ h⁻¹ was achieved within the first 300 h of biotransformation. The reproducibility was high, even though there were interruptions in medium and air flow as well as periods of compromised protection from contamination. Thus, excellent reproducibility was accomplished by good process robustness.

Control Reactor Without Packing
During the first three reactor runs, biofilm growth was observed not only on the structured packing, but also in the connecting tubing and on the walls of the trickle-bed reactor and recycling vessel. To evaluate the role of structured packing as a biofilm substratum within the reactor system, the 4th reactor run was performed without any packing materials for comparison. The productivity profile is presented in Fig. 4. Similar to the previous reactor runs, the system was inoculated for 24 h, followed by biotransformation of 100 mM ethylene glycol at a dilution rate of 0.33 h⁻¹. Steady state was reached after approximately 180 h biotransformation at an average glycolic acid concentration of 11.7 mM. The steady-state productivity in the absence of packing was 0.29 g·L⁻¹ h⁻¹, which was approximately 20% of the productivities in the presence of the structured packing. It was maintained for over 150 h before active termination of the reactor. Since no packing material was present, the productivity was attributed to biofilm cells on the tubing and reactor walls as well as suspended cells. The available surface area within the reactor system was approximately 1.7 m², consisting of 1.5 m² from the structured packing and 0.2 m² from the connecting tubing and reactor walls. If equal biofilm thickness and biofilm activity are assumed across all the available surface area within the reactor system, biofilm cells on the tubing and reactor walls will contribute 12% of the system productivity. The remaining 8% of the productivity may be attributed to the increase in suspended cells in the reactor system, as indicated by a 3-5-fold increase in effluent OD compared with the reactor runs with packing. Hence, biofilm cells on structured packing could account for at least 80% of the productivity in the reactor system.

Table 1. Average steady-state data of the biotransformation shown in Fig. 3.

<table>
<thead>
<tr>
<th>Steady state</th>
<th>Dilution rate (h⁻¹)</th>
<th>Ethylene glycol conc. (mM)</th>
<th>Glycolic acid conc. (mM)</th>
<th>Yp/s (mol %)</th>
<th>Productivity (g·L⁻¹ h⁻¹)ᵇ</th>
<th>Molar balance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.33</td>
<td>38</td>
<td>56</td>
<td>91</td>
<td>1.4 ± 0.03</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>28</td>
<td>71</td>
<td>98</td>
<td>1.1 ± 0.03</td>
<td>99</td>
</tr>
<tr>
<td>3ᵃ</td>
<td>0.14</td>
<td>30</td>
<td>70</td>
<td>100</td>
<td>0.77 ± 0.03</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>0.33</td>
<td>27</td>
<td>63</td>
<td>85</td>
<td>1.6 ± 0.05</td>
<td>89</td>
</tr>
</tbody>
</table>

ᵇBiotransformation between 775 h and 799 h was affected by spray nozzle blockage, and data from this period were not included in steady-state 3 calculations. ᵃFor clearer presentation of the data, standard deviations are only shown for productivity.

Variation of Productivity Between Effluent Cell Subcultures
Previous studies have shown that detached biofilm cells could display different colony morphologies and specific activities [9, 18]. After 740 h of continuous operation as profiled in Fig. 2, cells from the biofilm reactor effluent were cultivated on agar plates. Over 3,000 colonies of biofilm effluent cells were examined and all displayed the typical smooth, round morphology. To investigate the activity of these cells, 20 colonies were randomly isolated for further characterization. All isolates were identified as P. diminuta, and thus no contaminating strains were detected. Surprisingly, only 6 out of the 20 biofilm effluent isolates displayed glycolic acid productivities that were within 20% of the P. diminuta stock culture, whereas the other isolates had up to 60% lower productivities (Fig. 5A). To exclude the possibility that the variation in glycolic acid productivity by different isolates is a phenomenon associated with P. diminuta rather than its biofilm effluent...
cells, an additional biotransformation experiment using isolates from a *P. diminuta* planktonic culture was carried out. Fig. 5B illustrates that all 10 isolates had similar glycolic acid productivity compared with the stock culture. Hence, although no biofilm-specific colony morphology was detected, phenotypic variation of metabolic activity was evident in *P. diminuta* biofilm effluent cells.

**DISCUSSION**

Previous trickle-bed biofilm reactor applications were exclusively based on the use of random packing, for example, wood chips, porous ceramics, and plastic Raschig rings. The present study explored the use of structured packing in a trickle-bed biofilm reactor for biotransformation. *P. diminuta* established an active and robust biofilm in the reactor system and catalyzed the continuous biotransformation of ethylene glycol to glycolic acid for over two months, and steady-state glycolic acid productivities of up to 1.6 gl⁻¹h⁻¹ were achieved.

Microbial production of glycolic acid from ethylene glycol has been previously reported. Optimized planktonic batch culture biotransformations by *Pichia naganishii* and *Rhodotorula* sp. over a 120 h production period yielded glycolic acid productivities of 0.88 and 0.92 gl⁻¹h⁻¹, respectively [17]. In comparison, the productivity was only 0.12 gl⁻¹h⁻¹ for a planktonic batch culture of the model strain used in this study, *P. diminuta*, and this indicates that the experimental conditions were not optimal or the strain is less active. In a recent study, planktonic resting cells of *Gluconobacter oxydans* produced a maximum of 1.86 gl⁻¹h⁻¹ glycolic acid in a 50 h fed-batch process with *in situ* product removal [41]. Whereas higher product concentrations (up to 110 g/l) were achieved in batch processes at a high substrate concentration [17], the productivity of the trickle-bed biofilm reactor in this study was comparable to optimized batch processes, and was sustainable over a longer production period. Furthermore, the productivity was within the range of values of recently reported biofilm processes for the production of other chemicals, as shown in Table 2.

It has been suggested that a particular concern for biofilm application is the possibility of fluctuation in biofilm productivity or product quality over the time course of a continuous process owing to the complex and dynamic nature of biofilms [33]. Process reproducibility is of great industrial importance. *P. diminuta* biofilms have displayed excellent long-term stability and reproducibility in the reactor system with structured packing. Several biofilm dispersal events were observed as a result of process interruptions. It is promising that process interruption for

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**Table 2.** Comparison of the current study with some recently reported studies on biofilm processes for the production of chemicals.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reactor configuration</th>
<th>Product</th>
<th>Duration (days)</th>
<th>Maximum productivity (gl⁻¹h⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zymomonas mobilis</em></td>
<td>Packed-bed reactor</td>
<td>Benzyl alcohol</td>
<td>2</td>
<td>0.9</td>
<td>[25]</td>
</tr>
<tr>
<td><em>Gluconobacter oxydans</em></td>
<td>Trickle-bed reactor</td>
<td>Dihydroxyacetone</td>
<td>17</td>
<td>1.0</td>
<td>[12]</td>
</tr>
<tr>
<td><em>Pseudomonas diminuta</em></td>
<td>Trickle-bed reactor</td>
<td>Glycolic acid</td>
<td>64</td>
<td>1.6</td>
<td>This study</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>Membrane reactor</td>
<td>Styrene oxide</td>
<td>50</td>
<td>2.9</td>
<td>[9]</td>
</tr>
<tr>
<td><em>Gluconobacter oxydans</em></td>
<td>Packed-bed bubble column reactor</td>
<td>Dihydroxyacetone</td>
<td>18</td>
<td>3.4</td>
<td>[13]</td>
</tr>
</tbody>
</table>
up to 24 h had only a temporary and minor impact on reactor productivity. Furthermore, the observed feedstock contamination had no apparent effect on reactor performance. It has been shown by Weuster-Botz et al. [43] that a biofilm reactor could be stably operated in a non-sterile environment and the authors attributed this to the wash-out of contaminating organisms. Since no contaminating strains were detected in the biofilm reactor effluent, it is possible that established \textit{P. diminuta} biofilms may have out-competed any potential contaminating organisms to achieve steady-state productivity for over 700 h. The displayed robustness of the catalytic biofilm is favorable in process application, as interruption and contamination may be inevitable during long-term production processes.

Being dynamic biological systems, biofilms consist of cells in different physiological states, which may harbour distinct metabolic pathways \cite{38, 42, 44}, and cells detached from biofilms could grow into colonies with different distinct metabolic pathways \cite{38, 42, 44}, and cells detached from a biofilm reactor, and up to 40\% difference in cellular-specific activity was evident for 8 isolates. Although no biofilm-specific colony morphology was detected in \textit{P. diminuta} biofilm effluent cells, a high occurrence of decreased glycolic acid productivity was observed in cultures grown from biofilm effluent cells (Fig. 5A). Such variations were not evident in \textit{P. diminuta} cultures that were not of a biofilm origin (Fig. 5B). It appeared that any variations in \textit{P. diminuta} biofilm cells did not decrease the reactor productivity over time or affect process reproducibility. Hence, even though the phenomenon of phenotypic variations is observed in many microbial biofilms \cite{4, 6, 9, 18, 27}, it may not necessarily have detrimental effects in an industrial application where process consistency and reproducibility would be important. A number of other studies have reported the continuous operation of biofilm processes for chemical production over several months without biocatalyst degeneration \cite{3, 22, 26, 32}. This confirms that despite the complex biofilm nature, long-term stability can be achieved for biofilm processes.

One limitation for the use of structured packing in a biofilm reactor is the difficulty in monitoring the biofilm. Since the structured packing consisted of one module, non-disruptive sampling of biofilm during reactor operation was not possible. As a result, process-relevant biofilm characteristics such as biofilm mass and structure, EPS content, and metabolic activity, as well as the interaction between biofilms and structured packing, were not monitored. Various questions may arise; for example, it is possible that the accumulation of biofilm biomass could lead to some flow channelling in structured packing with reduced hydrodynamics and mass transfer capacity. Future research in this field could benefit from the development of an innovative reactor design that allows the real-time monitoring of biofilms on structured packing.

Three areas of future application are recommended for the production of chemicals by microbial biofilms. Firstly, an increased tolerance of biofilms to toxic substrates and products may be of benefit for biotransformation reactions that involve solvents and other challenging reactants \cite{33}. Secondly, as a natural form of cell immobilization, biofilms facilitate continuous processes with reduced reactor down-time and allow catalyst retention for minimum biomass in the product stream. Replacing established continuous processes involving artificially immobilized whole-cell catalysts with biofilm processes may be of particular interest. Thirdly, structured packing is efficient for mass transfer between gas and liquid phases \cite{1, 7}, and use of structured packing as biofilm substratum may benefit biotransformations that require efficient gas–liquid exchange. For instance, structured packing may be ideal for various oxidation reactions involving molecular oxygen as a substrate as well as biotransformations by viable whole cells requiring oxygen for cellular respiration. Another example for gas-phase biotransformation is the microbial production of biofuels from synthesis gas, which has received growing commercial interest in recent years \cite{15, 40}. Synthesis gas contains a mixture of carbon monoxide and hydrogen, and a biofilm membrane reactor has been developed for the conversion of synthesis gas to ethanol, butanol, and other liquid products \cite{15}.

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