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Utilizing crude waste glycerol in the bio refinery: glycerol gels for *in situ* substrate delivery to whole cell biocatalysts.

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Supporting Information: Materials and methods, bacteria cultivation experiments.

Synopsis. Glycerol gels provide a method of delivering glycerol to biocatalysts without the need for glycerol pre-purification.

Keywords: crude glycerol, *in-situ* substrate delivery, whole cell biocatalysts, sol-gel immobilization, 1,3-propanediol.

Abstract

Sol-gel methods were used to facilitate the *in-situ* controlled delivery of glycerol as a substrate for whole cell biocatalysis. The entrapment of the polyol within a silica gel matrix enabled the utilization of crude and moderately toxic bio-derived substrates that could not be used in fed-batch mode. Different grades of glycerol were incorporated in gels and fed to *C. butyricum* for 1,3-propanediol production and *E.coli* for recombinant protein production. Of particular note was the successful use of crude glycerol gels with *E.coli*. By virtue of gel immobilisation, previously toxic crude glycerol samples were rendered active; and the use of gel technologies was therefore shown to expand the range of biomass that can be utilised in whole cell biocatalytic processes. Specific matching of the acid used in the gel synthesis with the fermentation medium further supported cell growth.

Introduction

Biocatalysts can be very susceptible to poisoning, or the suppression of activity, by toxic impurities in the substrate. Impurities are common in bio-derived feedstocks, and can vary significantly from batch to batch. This can require the use of low substrate concentrations, as the substrate feed must be diluted down, or in extreme cases prevent the use of the biotransformation altogether. Dilution leads to a substantial increase in process costs, as the products must be separated from the aqueous waste. The production of high volumes of wastewater is not environmentally acceptable and therefore the aqueous waste must go into recycling, significantly increasing processing steps. For these reasons the development of strategies to allow the use of impure substrates and/or more concentrated fermentation broths are key factors in the expansion of biocatalysis technologies.

There are several approaches employed to mitigate these problems; for instance, protection of biocatalysts by immobilization.^{1,2,3} biphasic systems,^{4,5,6} or the application of biocatalysts in non-conventional media. However, most of these methods require modification of the biocatalyst itself, or require the use of non-environmentally friendly solvents, both of which can reduce activity and / or selectivity.

In an effort to increase the substrate scope of our biocatalysts, we investigated the application of biocompatible gels for substrate delivery. These gels contain the substrate within a solid matrix, which gives them the potential to retain impurities locked inside, whilst delivering the substrate *in situ*. If the matrix has a higher affinity for undesirable impurities than the desired substrate, the matrix will act as the toxic substrate binder. The gels were made using three components; silica, glycerol and a biocompatible acid such as citric or tartaric acid.

Crude glycerol is a waste product from the production of biodiesel. Biodiesel is produced by the transesterification of triglycerides into fatty acid alkyl (usually methyl) esters in the presence of an alcohol, with an acid, base or enzymatic catalyst.⁷ The most common method of biodiesel synthesis is catalysed by a homogeneous base (Scheme 1); after the process the base is retained in a crude glycerol-containing phase which spontaneously separates from the biodiesel. Biodiesel is of particular interest as it can be produced from waste cooking fats and oils, and the huge quantities of waste fats and oils produced from industrial sources are challenging to dispose of.⁸ Crude glycerol is generated at about 10% (w/w) of biodiesel⁹

and is produced with varying purity. The production of biodiesel has increased significantly in the past few decades.^{10,11,12}

The disposal or purification of crude glycerol is comparatively expensive,¹³ making these raw material management routes economically as well as environmentally unfavourable. A convenient solution as explored here would be to utilise non-purified (crude) glycerol for the production of useful chemicals via fermentation processes. The development of new technologies that allow for the efficient use of crude glycerol will therefore support the commercialization and increase the sustainability of biodiesel production.

Glycerol is a useful raw material for the production of value added chemicals, in particular through bioconversion by microbial fermentation. A number of such promising applications include conversion to 1,3-propanediol,^{9,10,14-16} succinate,¹⁷ ethanol,^{18,19} butanol²⁰ and hydrogen.²¹ The product of interest in this study is 1,3-propanediol, as we have shown that 1,3-PD can be converted into a range of value-added chemicals in downstream chemocatalytic hydrogen borrowing reactions.^{14,15,22,23}

In previous studies *Clostridium butyricum* represented the most promising 1,3-propanediol producer among the *Clostridia* genus.²⁴ The main advantages of the species are higher 1,3-PD yields (up to 0.64 mol/mol of glycerol)⁹ and shorter fermentation times. In addition, compared to other 1,3-PD producers (e.g. *C. frendii*, *K. penumonie*), glycerol dehydratase of *C. butyricum* (enzyme from glycerol - 1,3-PD pathway) doesn't require coenzyme B₁₂, which represents a significant economic benefit.^{25,26}

Scheme 1. Alkaline catalysed transesterification of fats and oils to produce biodiesel and glycerol.

There are many microorganisms that can utilise glycerol as an energy source due to the natural occurrence of glycerol in nature.^{27,28} Traditionally carbohydrates such as sugars and starches are used in industrial fermentation processes, thus competing with the food chain.

The use of a waste material such as crude glycerol could be considerably cheaper, more ethical and more environmentally friendly.²¹ However, without purification, the use of crude glycerol in biocatalytic fermentation can be a challenging process, due to the myriad of impurities present.²¹ The composition of crude glycerol can vary depending on the feedstock and the method of transesterification,²⁹ but the main impurities are free fatty acids, inorganic salts and methanol.¹⁰ Many studies have shown that the existence of these impurities has an inhibitory effect on microbial growth.^{10,30-33}

The presence of impurities in substrates derived from biomass can suppress whole cell biocatalysis, or even kill the microbes. High substrate concentrations can also be detrimental to the reaction at concentrations that are considered normal in chemocatalysis. Typically, fermentation reactions occur at very dilute concentrations to avoid poisoning the biocatalyst.

Most production processes for fermentation fall into three categories: batch, fed-batch and continuous.³⁴ Batch fermentation suffers from continually decreasing concentrations of substrate, with increasing biomass and product concentrations. Continuous fermentation processes typically have a constant biomass and product concentration, where the substrate is a constant although at growth-limiting concentrations. This gives a controlled growth rate; however, it does not result in optimum conditions. Under these conditions any impurities in the substrate will build up over time. In fed-batch fermentation, certain substrates are added as necessary during the process, often maintaining optimal substrate concentration for the maximum production of product, under these conditions impurities, products and side products accumulate. The most common way to attempt to regulate substrate concentration is to supply a steady feed. Our approach, the entrapping of the substrate in a matrix offers the additional benefits of controlled delivery of the substrate and retention of unwanted impurities within the material.

In this study glycerol gels were synthesised via the sol-gel process to explore the potential of immobilised glycerol silica gels as a substrate delivery system for glycerol. In systems using bacteria which feed on glycerol to produce value added chemicals, the limiting factor for the reaction can often be the necessity for a very dilute system with a low concentration of glycerol. Too high a concentration of glycerol can poison the bacterial cells and inhibit the reaction. Therefore, the gel was designed to deliver glycerol into the solution gradually over

a period of time. The glycerol chosen for immobilisation was from three different sources and with different levels of purity. A secondary aim was to investigate the effects of immobilisation of the glycerol on the impurities present in the glycerol samples. The gels were made using glycerol as the template in a one-pot low temperature silica gel synthesis by the sol-gel method.

Results and discussion

Several different grades of glycerol were trialled for entrapment within SiO₂ gel matrices, to create materials for glycerol delivery into bacterial cultures (S.I. S1 materials and methods). Glycerol samples that were successfully incorporated into a silica gel included pure glycerol **P** (99.5 % purity), industrial glycerol **I** (97 % purity) and crude glycerol **C** (76.9 % purity). The gels were formed by mixing glycerol with an acid catalyst in ethanol, followed by the addition of Tetraethoxy orthosilicate (TEOS). The gels were aged for over 4 weeks before use. Natural acids (citric, tartaric) were employed as the acid catalysts of gelation, these were chosen to maximise the compatibility of the resultant materials with bacterial cultures, as biological organisms are typically sensitive to acids and pH changes. The amount of acid added was limited by the solubilities of the acids in the ethanol/glycerol/TEOS mixture.

Synthesis of glycerol gels

Synthesis of pure glycerol (P) gels: Stable gels were found to form with glycerol (e.g. Figure 1), ethanol and TEOS (glycerol:TEOS 1:1) containing various quantities of each acid. Citric acid (CA), and L-(+)-tartaric acid (TA) catalysed glycerol gels typically gelled in 8-16 hours. Further experimental details are available in the S.I. (S1.2).

Figure 1. Glycerol gels made using (left) citric acid, (right) L-(+)-tartaric acid

The acids had different solubilities in the glycerol/TEOS/ethanol mixture and the amount of each acid added was tested to saturation. Citric acid and L-(+)-tartaric acid could be dissolved in sufficient quantity to catalyse the gelation and form a stable silica gel.

Synthesis of industrial glycerol (I) gels: These gels were prepared in a similar manner to the pure glycerol gels (S.I. S1.3). The gels had a very similar appearance to the pure glycerol gels, although a light yellow colour was observed, the colour originated from the impure glycerol.

Synthesis of crude glycerol (C) gels: Crude glycerol was entrapped by both citric and L-(+)-tartaric acid catalysed sol-gel techniques, using the same methods used for pure (P) and industrial (I) glycerol (S.I. S1.4). The synthesis of these gels was initially hindered by the low solubility of the crude glycerol in the TEOS/ethanol mixture, glycerol P and I were both transparent and viscous liquids, glycerol C was a semi-solid, opaque and dark brown in colour (Figure 2).

Figure 2. Left to right: comparison of **I** (LHS) and **C** (RHS) glycerol gels.

A standard 1:1:1 ratio of TEOS, glycerol and ethanol, as used in the synthesis of glycerol I gels, appeared to leave some unincorporated crude glycerol when the gel formed, creating a non-homogenous gel with sections of non-fully entrapped glycerol (Figure 2 RHS). The relative proportions of glycerol and ethanol were varied in order to maximise incorporation of the crude glycerol within the silica matrix. The most stable glycerol gels were selected for further study (S.I. S2.1 Tables S3-5).

Glycerol release to biocatalytic bacteria

Glycerol gels containing different purities of glycerol (**P**, **I** and **C**) and catalysed by tartaric or citric acid, (S.I. Tables S3-5), were used to feed glycerol to bacteria within fermentation broths, (Figure 3). The fermentation media provide the bacteria with a nitrogen source, water, various salts, and micronutrients optimised to support cell growth. The fermentation media for *C. butyricum* and *E.coli* are referred to as Y5 medium and semi-defined medium respectively, (S.I. S2.2, S2.3).

Figure 3. Schematic of glycerol delivery

The dissolution of glycerol from the glycerol gels took place in the liquid phase fermentation media for *C. butyricum* and *E. coli* and was measured by HPLC. Control experiments used a fermentation medium that contained non-immobilised pure glycerol at concentrations approximately equal to the amount contained within the glycerol gels. The micro-cultivation experiments were carried out in 96 well microtest plates, with a working volume of 200 μL . The gels were weighed into each well before being exposed to UV light for 12 hours in a laminar chamber for UV sterilisation to prevent contamination before cultivation. The specific media (*E. coli* or *C. butyricum*) was pipetted into the wells, followed by inoculation with the bacterial cells, which were then cultivated for 16 hours. The optical density at 600nm, an indication of cell growth, was measured every 5 minutes, and after cultivation pH was measured in each well samples, which were then submitted for HPLC analysis.

The use of glycerol gels to feed *C. butyricum*

C. butyricum can be used to convert a biomass feed to a renewable platform chemical. Testing focussed on the comparison of gels containing glycerol **I** and **C** as the purification of glycerol to a high purity (**P**) would not be commercially viable, given the relatively low value of chemical products obtained.

Release of glycerol **I** into the *C. butyricum* medium: Dissolution experiments were carried out to observe glycerol release into the Y5 medium from immobilized glycerol **I** gels, (Figure 4). The dissolution of glycerol from the gels into the Y5 medium showed similar patterns to the dissolution into water, so it can be assumed that the other non-water components of the Y5 medium do not significantly interfere with the glycerol delivery. The L-(+)-tartaric acid catalysed gels gave the highest release of glycerol. The slight differences seen between the parallel experiments for both the citric acid and L-(+)-tartaric acid gels (CA-A, CA-B and TA-A, TA-B) were due to the slightly different amounts of glycerol per gel (S.I. Table S4). The difference observed between the citric and the L-(+)-tartaric acid methods of gelation can be explained by the differences in the total weight of each gel used, as the citric acid catalysed gels had a lower wt % of glycerol.

Figure 4. Glycerol concentration released from glycerol **I** gels to the *C. butyricum* media per time. Two duplicates of each type were tested. Conditions: 35 °C, 250 RPM, reaction volume 1 ml, gel concentration 40 g/L.

The change in pH was also monitored over time as the glycerol was released from each gel, as the gels contain a significant amount of acid (citric or tartaric) which could be released alongside the glycerol and disrupt the optimum pH conditions for the bacterial cultures. The glycerol gels were tested in the Y5 medium and the pH was set to 9.0 and 8.5 for CA and TA gels respectively (before sterilization by NH_3). Two parallels from each type of gel and two blanks were tested. The Y5 media with different pHs were also measured as blanks to observe the effect of sterilization on the pH values (without gel addition, Figure 5). Within the first half hour the CA and TA gels gave a sharp decrease in pH, this settled after two hours. This pattern was also seen in glycerol dissolution into water experiments, as the maximum release of glycerol is seen within the first two hours. The decrease in pH was significantly larger for the CA gels, which is expected as these gels had 3.2 mmol of acid per gel whilst the L-(+)-tartaric acid catalysed gels only contained 2.2 mmol of acid.

Figure 5. pH change in Y5 medium against time for Glycerol I gels in the *C. butyricum* medium at 35 °C, 250 RPM, reaction volume 1 ml, gel concentration 40 g/L.

Cultivation of *C. butyricum* using glycerol I gels: To counteract the pH decrease in the *C. butyricum* cultivation media, caused by the addition of the gels, different K_2HPO_4 and KH_2PO_4 concentrations were tested as pH buffers. The optical density (final $\text{OD}_{600\text{nm}}$) was

measured by microplate spectrophotometer to determine the increase in biomass by the end of the experiment (Table 1). The specific growth rate is then inferred from the optical density. For all the conditions tested *C. butyricum* bacteria were able to utilize the glycerol released from the gels and produce 1,3-propanediol, although productivity varied in comparison to the control reaction, which contained a fixed concentration of glycerol added at the start.

The gel concentration was set at 40 g/L to achieve approximately 15-20 g/L dissolution of glycerol, and the control reaction was the Y5 medium with 20 g/L of pure glycerol added at the beginning. CA-A; CA-B and TA-A gels were all biocompatible with *C. butyricum* to some degree. At the 5:5 and 10:0 concentration of $K_2HPO_4:KH_2PO_4$ buffer (g/L:g/L) the control reaction utilised a higher proportion of glycerol to produce a higher quantity of 1,3-PDO in comparison to the citric acid and L-(+)-tartaric acid gels tested (S.I. Table S6). At the 15:0 concentration of $K_2HPO_4:KH_2PO_4$, with the TA-A gel, the performance of the homogeneous and gel-fed reactions was very similar. The bacteria utilised 16.01 g/L of glycerol to produce 4.10 g/L of 1,3-PDO, compared to the control reaction where 16.59 g/L glycerol was utilised to produce 4.36 g/L 1,3-PDO. At 20:0 $K_2HPO_4:KH_2PO_4$ the two glycerol gels performed noticeably differently; the CA-B gel utilised 11.12 g/L glycerol to produce 4.10 g/L of 1,3-propanediol, whilst the TA-A gel out-performed the control experiment utilising 16.34 g/L glycerol and producing 5.59 g/L of 1,3-propanediol. In general specific growth rates and final ODs were lower for gel-fed reactions compared to the control reaction, indicating slower cell metabolism. This could be caused by inhibition arising from the gels themselves, or a result of the high concentrations of phosphates from the buffers.

The TA-A gel was the best performing glycerol I gel, able to produce more 1,3-PDO than the citric acid catalysed gels CA-A and CA-B. The higher molar quantities of acid used to create the citric acid gels, and the higher weight percent of glycerol in the TA-A gel (20 g/L compared to 15 g/L) (Figure 4) may contribute to this. Another factor was highlighted by Krysteva and co-workers,³⁵ who used wine vinasse containing sugars and residual acids from the fermentation of wine, as a substrate for *C. butyricum* for the production of H_2 , the assimilation of citric acid and tartaric acid by *C. butyricum* was found to be weak, however the microbes absorbed a higher proportion of citric acid at lower concentrations. In this study *C. butyricum* was found to perform better with tartaric acid industrial glycerol gels

than citric acid industrial glycerol gels. *C. butyricum* lacks all the necessary enzymes to completely metabolise citric acid, as it contains only NADP-specific isocitrate dehydrogenase (ICDH) and not L-malate dehydrogenase (MDH).³⁶ Another recent study found that *C. butyricum* is also missing succinate-CoA ligase and fumarate reductase enzymes, which are necessary for the citric acid cycle.³⁷ As well as the higher quantity of citric acid present compared to tartaric acid in the glycerol gels, the inability of *C. butyricum* to metabolise citric acid via the same pathway used by *E.coli* may help to explain the negative effect of citric acid. As different bacteria exhibit differing abilities to metabolise natural acids, choice of the right acid as the catalyst for sol-gel immobilisation of the substrate, may result in optimised catalytic performance.

Table 1 Final pH, utilized glycerol I and 1,3-propanediol concentrations, specific growth rate ($\mu(\text{h}^{-1})$) and final optical density after *C. butyricum* cultivation with immobilized glycerol. Four different K_2HPO_4 and KH_2PO_4 combinations in Y5 media were tested.

K_2HPO_4 : KH_2PO_4 (g/L:g/L)	Sample	Final pH	Utilized Glycerol I (g/L)	Final 1,3- PDO (g/L)	$\mu(\text{h}^{-1})$	Final $\text{OD}_{600\text{nm}}$
0.5:0.5	Control	5.43±0.07	16.59±0.52	4.36±0.13	0.25±0.01	0.71±0.02
5:5	CA-A	5.48±0.04	6.87±0.58	1.54±0.05	0.10±0.02	0.62±0.05
	CA-B	5.34±0.09	3.94±0.56	0.93±0.38	0.09±0.01	0.54±0.05
	TA-A	5.40±0.02	12.07±0.57	2.84±0.16	0.14±0.04	0.72±0.04
	TA-B	6.34±0.08	0.6±1.01	0.18±0.01	0.02±0.01	0.00±0.02
10:10	CA-A	5.39±0.04	4.14±0.59	1.10±0.06	0.09±0.01	0.51±0.16
	CA-B	5.34±0.05	8.66±0.93	1.21±0.03	0.09±0.03	0.60±0.07
	TA-A	5.39±0.03	12.55±0.46	2.67±0.09	0.14±0.02	0.61±0.02
	TA-B	6.27±0.07	0.31±0.98	0.18±0.01	0.02±0.00	0.00±0.02
15:0	CA-A	5.53±0.09	10.07±0.82	2.84±0.10	0.12±0.03	0.47±0.04
	CA-B	5.46±0.08	12.78±0.49	2.89±0.10	0.12±0.02	0.52±0.06
	TA-A	5.52±0.05	16.01±0.16	4.10±0.11	0.12±0.01	0.67±0.06
	TA-B	6.61±0.05	0.89±0.91	0.18±0.01	0.01±0.01	0.00±0.03
	CA-A	5.62±0.06	9.20±0.60	3.94±0.09	0.13±0.02	0.44±0.06
	CA-B	5.61±0.04	11.12±0.28	4.10±0.07	0.12±0.03	0.57±0.03

20:0	TA-A	5.86±0.02	16.34±0.78	5.59±0.05	0.11±0.01	0.37±0.20
	TA-B	6.85±0.03	2.51±0.97	0.19±0.01	0.02±0.00	0.00±0.01

Conditions: 35°C, 120 rpm, 5% (v/v) inoculation, gel concentration 40 g/L, working volume 200 µl. Control experiment contained standard Y5 medium with 20 g/L pure glycerol.

Release of glycerol C into the *C. butyricum* medium: Based on the previous results, tartaric acid was used as the catalyst to gel crude glycerol. Three different samples with different ratios of TEOS: glycerol (SI Table S5 entries 8-11), were tested for glycerol release and pH profile. To preserve the concentration of glycerol released at a concentration of approximately 20 g/L with 40 g/L of gel, the quantity of gel was increased for the ratios 1:0.5 and 1:0.25 to 80 g/L and 160 g/L respectively (SI, Figure S3). Adding double the quantity of 1:0.5 gel or quadruple the quantity of 1:0.25 gel gave a slightly higher glycerol dissolution compared to the 1:1 gel which gave approximately 20 g/L. The 1:0.1 ratio gel was determined to have too low a pH when scaled up to achieve the same glycerol concentration and so was not tested further.

The glycerol release profiles were similar for all purities of glycerol screened (**P**, **I** and **C**; S.I. Figures S1 & S3, glycerol release). The major difference observed in the use of the three different glycerol samples was the level of acid contamination. In the pH experiments on crude glycerol it was necessary to increase the initial pH in the Y5 media higher than was required for the glycerol **I** gels (S.I. Figure S4 pH change over time). The pH was adjusted to pH 9, 9.5 and 10 for gels with TEOS: glycerol **C** ratios of 1:1, 1:0.5 and 1:0.25 respectively. This was required to reach conditions for bioactivity as adding a greater quantity of gel represented the addition of more acid to the bioprocess. The best pH profile for *C. butyricum* cultivation was obtained with the 1:1 gel, which was selected for further testing.

Attempts to Cultivate *C. butyricum* using glycerol C gels: The use of crude glycerol **C** was observed to completely inhibit the biocatalytic activity of *C. butyricum*, (Figure 6, S.I. Tables S5 & S7). No cell growth was observed in any of the experiments. The entrapment of the crude glycerol in a silica matrix did not protect *C. butyricum* from the toxicity of glycerol **C**.

Figure 6. Cell death, Optical Density measurements when crude glycerol **C** was fed to *C. butyricum*.

The use of glycerol gels to feed *E. coli*

E. coli was chosen as the second testing model due to the potential of genetically engineered *E. coli* to produce recombinant enzymes from crude glycerol. These are high value products and could warrant the use of pure (**P**), purified industrial (**I**) or crude (**C**) glycerol. As the optimised medium for *E.coli* cultivation is different from that of *C. butyricum*, the glycerol release and pH change experiments were performed again into the optimised semi-defined medium. Pure glycerol release and pH change into semi-defined medium was prepared with an increased initial pH of 8.7 and 9.2 for TA and CA gels respectively (S.I. Table S3). Gel quantities of 40 g/L glycerol achieved approximately 15-20 g/L glycerol dissolution into the fermentation medium. The citric acid catalysed glycerol gels yielded a lower concentration of glycerol as there was a higher proportion of acid in these gels (S.I. Figure S5). The pH buffering capacity was much higher for the optimised *E. coli* BL21 medium compared to the Y5 medium used for *C. butyricum*, and the pH decrease from the gels in the *E. coli* medium wasn't as significant (S.I. Figure S4 c.f. Figure S6). Glycerol release was comparable in terms of final concentrations obtained, although in this case, the speed of release was marginally faster.

Cultivation of *E. coli* using glycerol P gels: The micro-cultivation of *E.coli* with 40 g/L immobilized glycerol P (S.I. Table S3), was tested with different amounts of NaH₂PO₄ and Na₂HPO₄ (Table 2). The glycerol utilized and the final optical density was highest for both CA and TA gels with the standard NaH₂PO₄ concentration as used in the control experiment (2.8 g/L). *E.coli* in the control experiment utilized 4.03 g/L glycerol whilst *E.coli* with TA gels utilized 7.19 g/L of glycerol, although with a slightly lower final optical density of 0.79 compared to 0.92; indicating less cell growth with TA gels compared to the non-immobilised pure glycerol control. The CA gels also utilised more glycerol compared to the control reaction, using 4.11 g/L glycerol and achieving an optical density of 0.69. This is significant as the control experiment contained 30 g/L of pure glycerol, whilst the gels at 40 g/L released 17-20 g/L for the CA and TA gels respectively (S.I. Figure S5). Although the release of glycerol from the CA and TA gels allowed *E.coli* to utilise more glycerol, the lower concentration and speed of the release of glycerol is possibly why the specific growth rates in these reactions were slower. When 5 g/L NaH₂PO₄ and 2.8 g/L Na₂HPO₄ were used with the CA and TA gels substantially less glycerol was utilised by *E.coli*, as well as giving a lower final optical density value indicating lower cell growth. In contrast to the experiments with 2.8 g/L NaH₂PO₄, the CA gels performed better than the TA gels, utilising higher concentrations of glycerol with higher cell growth.

Table 2. Final pH, utilized glycerol concentration, specific growth rate and final optical density after *E. coli* cultivation with immobilized glycerol P (40 g/L gel).

(g/L)	sample	Final pH	Utilized C _{glycerol} (g/L)	μ (h ⁻¹)	Final OD _{600nm}
2.8 NaH ₂ PO ₄	Control	6.55±0.09	4.03±0.57	1.62±0.02	0.92±0.03
	CA	6.74±0.03	4.11±0.97	0.92±0.15	0.69±0.11
	TA	6.82±0.03	7.19±2.20	0.85±0.07	0.79±0.14
5 NaH ₂ PO ₄	CA	6.68±0.04	2.67±0.84	0.76±0.07	0.71±0.11
	TA	6.79±0.02	0.85±2.16	0.49±0.21	0.61±0.17
2.8 Na ₂ HPO ₄	CA	6.40±0.06	1.29±0.63	0.79±0.18	0.79±0.09
	TA	6.68±0.03	0.17±3.41	0.73±0.14	0.41±0.07

Conditions: 37 °C, 240 rpm, 2.5% (v/v) inoculation, working volume 200 µl, gel concentration 40 g/L. Control experiment contained semi-defined medium with 30 g/L of pure glycerol and 2.8 g/L NaH₂PO₄.

Compatibility of crude glycerol gels with *E.coli*; cultivation of *E. coli* using glycerol **C** gels: The standard concentration of NaH₂PO₄ (2.8 g/L) was used in experiments with glycerol **C** gels. Both CA and TA catalysed crude glycerol **C** gels were screened. Gels with TEOS: glycerol ratio 1:1 were used and concentrations were tested at 20, 40, 60 and 80 g/L of gels (representing approximately 10, 20, 30 and 40 g/L of crude glycerol respectively).

Experiments with conventional (non-immobilised) crude glycerol **C** presented no cell growth above 10 g/L of crude glycerol, and very weak growth at lower concentrations (S.I. Figure S7).

E.coli bacteria were able to grow and utilise glycerol **C** when delivered via a gel (Table 3). In the control reaction, which used 30 g/L pure glycerol, the *E.coli* utilised 4.03 g/L glycerol with a final optical density of 0.94. The best performing gel was the CA glycerol **C** gel at 20 g/L, for which 3.54 glycerol was utilised to give a final optical density of 0.83. This shows that similar quantities of glycerol could be used by *E.coli* from the dissolution of crude glycerol from the gels, in comparison to the pure glycerol control reaction. The lower cell growth, and specific growth rate, is likely to be due to the lower concentrations of glycerol, 20g/L gel concentration typically yielded approximately 10g/L crude glycerol, in comparison to the optimised control, which contained 30 g/L pure glycerol. Increasing the gel to 40, 60 and 80 g/L gradually decreased cell growth. From these experiments, it is unclear whether this was caused by a higher concentration of released impurities (due to higher gel concentrations), by the decreased pH due to the higher quantities of acid from the gels, or by a combination of both factors. The TA gels were far less successful than the CA gel. This is the opposite of what was found to occur with the acids from the immobilised glycerol **I** with *C. butyricum*. This can be explained as the optimised fermentation medium for *E.coli* contains citric acid, which is necessary for the citric acid cycle in *E. coli*,^{38,39} and therefore the quantities present in the gel do not have as dramatic a negative effect on the *E.coli* as the addition of L-(+)-tartaric acid.

These results suggest that the silica gel immobilization of crude glycerol **C** at gel concentration 20 g/L supported cell growth and utilisation of glycerol as a carbon source. Gel-fed *E. coli* cells exhibited a higher tolerance to crude glycerol **C** than cells presented with glycerol **C** in the liquid phase. (S.I. Figure S7). This result shows that the use of silica sol-gel to immobilise crude glycerol is a viable one-step technique to turn a toxic, waste substrate in to a useable substrate without the need for purification.

Table 3. Final pH, utilized glycerol concentration, specific growth rate and final optical density after *E. coli* cultivation with immobilized glycerol **C** (CA 1:1 and TA 1:1) and 2.8 g/L NaH₂PO₄.

C _{Gel} (g/L)	Sample	Final pH	Utilized C _{glycerol} (g/L)	μ (h ⁻¹)	Final OD _{600nm}
-	Control	6.20±0.07	4.03±0.65	1.72±0.01	0.94±0.09
20	CA	6.70±0.09	3.54±1.16	0.37±0.20	0.83±0.02
	TA	6.47±0.03	1.41±0.54	0.55±0.25	0.76±0.03
40	CA	6.59±0.04	3.35±1.69	0.40±0.18	0.56±0.02
	TA	6.23±0.07	1.31±0.64	0.78±0.02	0.51±0.04
60	CA	5.34±0.11	1.74±1.25	0.37±0.04	0.22±0.02
	TA	5.32±0.09	0.33±2.17	0.46±0.33	0.36±0.09
80	CA	4.72±0.28	0.65±1.76	0.00±0.00	0.00±0.00
	TA	4.84±0.06	0.32±1.66	0.00±0.00	0.00±0.00

Conditions: 37 °C, 240 rpm, 2.5% (v/v) inoculation, working volume 200 μl, gel concentration 20, 40, 60, 80 g/L. Control experiment contained semi-defined medium with 30 g/L of pure glycerol and 2.8 g/L NaH₂PO₄.

Experimental Section

Materials and methods

Three purities of glycerol were used, Glycerol **P** (pure) Glycerol **I** (industrial) and Glycerol **C** (crude) Glycerol **P** (≥ 99.5 % w, Sigma-Aldrich), Glycerol **I** (Industrial) (97 % purity supplied from Megara) and glycerol **C** (crude) (76.9 % purity, ENEA, crude glycerol).

CH₃OH (≥ 99.9 %, CHROMASOLV, for HPLC, Sigma-Aldrich), C₆D₆ (≥ 99.0 % w, Aldrich), ethanol (≥ 99.0 %, laboratory supplies and instruments Ltd.), citric acid monohydrate (≥99.7

%, Atom scientific), TEOS (≥ 97 %, TCI UK), L-(+)-tartaric acid (99 % Alfa Aesar), were used as received.

Silica glycerol gels were prepared as detailed in S1.2, S1.3, S1.4 and S2.1. The cultivation media were prepared as described in S2.2 (Y5 medium) and S2.3 (semi-defined medium). The trace element solution is detailed in S2.4. Glycerol release experiments were carried out in a laboratory thermomixer with standard working volume 1ml as detailed in S2.5. Cultivation experiments were carried out as described in S2.6, S2.7, S2.8 and S2.9. Experiments with E.Coli were performed as detailed in S2.10 and S2.11.

Conclusions

All the silica glycerol gels reported here were able to deliver glycerol into the fermentation media for the bacteria. Natural acids could be used as the catalyst for silica gel formation from TEOS. The acid leach from the glycerol gels could be balanced to some extent by utilising higher concentrations of phosphate buffer. At higher acid concentrations this is likely to have resulted in non-ideal conditions for *C. butyricum* activity. Using *C. butyricum* promising results were found from the use of tartaric acid catalysed immobilised industrial (97 % purity) glycerol in comparison to the non-immobilised pure glycerol control reaction. However, the immobilised crude glycerol (C 76.9 % purity) gels were found to be completely incompatible with *C. butyricum*, and the toxicity using the gels was found to be similar to the toxicity of equivalent concentrations of crude glycerol C fed as a solution (10 g/L was found to inhibit cell growth).

The use of immobilised crude glycerol gels was more successful with *E. coli*. *E. coli* bacteria utilised glycerol C from the 20 g/L citric acid catalysed gels with uptake of glycerol and cell growth only slightly lower than the optimised control using pure glycerol. *E.coli* was found to have a preference for the citric acid catalysed crude glycerol gels over the tartaric acid catalysed crude glycerol gels. This is consistent with the fact that the optimised fermentation medium for *E.coli* already contains citric acid.

We have demonstrated that glycerol containing silica gels effectively deliver substrate in a controlled manner to fermentation bioprocesses. The gel is formed in a simple one-step procedure which can transform a crude bio-renewable chemical from a toxic poison into a non-toxic feedstock. The most remarkable results were obtained using crude glycerol

entrapped in a citric acid catalyzed silica gel fed to *E.coli*. These microbes are suitable for recombinant protein production.

In our delivery system we have used TEOS as the starting material for silica, and replaced the acid (catalyst) component and solvent of the sol-gel process with food acids and crude waste glycerol, which acts as the solvent for the reaction, the template, and the substrate for biocatalysis. The materials are produced at room temperature without the need for additional solvents. The source of TEOS is an important consideration; TEOS can be produced by the direct reaction of ethanol with silicon over a catalyst.⁴⁰⁻⁴⁴ The production of silicon is still a relatively high-energy process however, this method removes the need for the use of chlorinated reagents and the ethanol could be derived from renewable resources (bioethanol). In the life cycle the production of waste silica is another important factor; if pure silica wastes are deposited in soils or in aquatic environments, silica dissolution occurs via erosion, in the presence of water (silicic acid formed).⁴⁵ The utilisation of silica is observed in biological systems such as fungi, bacteria⁴⁶ or diatoms.⁴⁷ It has been debated that the emission of SiO₂ by human endeavour contributes only a small percentage to changes in global silicon fluctuations, and has a small environmental impact, because of the high concentrations found in nature (silica in soils and aquatic environments).⁴⁸ The material we present here essentially comprises of SiO₂, glycerol and food acids. That being said once the gel has been in contact with the whole cell culture a pre-treatment to sterilise the cells could be required before waste production. Chemical recycling of silica is also possible; dissolution in alkaline solution would produce aqueous silicates, which can be reused.^{49,50}

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Figure for TOC entry:

Synopsis. Glycerol gels provide a method of delivering waste glycerol from biodiesel production to biocatalysts without the need for glycerol pre-purification.