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Effects of immobilization on growth, substrate consumption, β-galactosidase induction, and byproduct formation in *Escherichia coli*

Xiaojun Zhang[1,*], Scott Bury[1,**], David DiBiasio[2], and Judith E. Miller[1]

Departments of [1]Biology and Biotechnology and [2]Chemical Engineering, Worcester Polytechnic Institute, Worcester, MA, U.S.A.

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SUMMARY

Some metabolic properties of both suspended and immobilized aerobically and anaerobically growing *Escherichia coli* cells were investigated. Metabolic activity was found to be substantially different when *E. coli* cells were immobilized in alginate. Cells grown immobilized in alginate, and then released from the gel, synthesized 1.6 (aerobic growth) and 4.9 (anaerobic growth) times as much β-galactosidase per cell in response to induction as did suspended cells. Under both aerobic and anaerobic conditions, the cell yield from glycerol for immobilized cells was half that for suspended cells. At specific growth rates that were not significantly different from those of suspended cells, immobilized cells consumed glycerol at twice the rate of suspended cells. Immobilized cells produced elevated quantities of acetate, pyruvate, and lactate. Interpretation of these findings is discussed in terms of the kinetics of energy metabolism and the regulation of inducible protein synthesis in *E. coli*.

INTRODUCTION

The effects of cell immobilization on the metabolism of the immobilized organisms have recently been debated in the literature. Although several investigators have shown increased intrinsic rates of metabolic activity, other claims of such increases have been clouded by improper monitoring of microbial growth (as pointed out by Doran and Bailey [3]).

Higher growth rates of immobilized cells have been reported for *Escherichia coli* [5]. Lower growth rates, on the other hand, have been reported for *Saccharomyces cerevisiae* [3,16], and *Bacillus amyloliquefaciens* [14]. In Doran and Bailey's [3] study,

* Present address: Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06268, U.S.A.
** Present address: Department of Chemical Engineering, Rice University, Houston, TX 77251, U.S.A.
Correspondence: Judith E. Miller, Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA 01609-2280, U.S.A.

the specific growth rate and yield of cell biomass for immobilized *S. cerevisiae* cells were reduced by 45% and 33% compared to those of free cells.

Increased oxygen uptake was reported for immobilized *E. coli* [17], and *S. cerevisiae* [9]. In contrast, decreased oxygen uptake has been reported for the same organisms [1,7,14].

Enhanced substrate consumption as a result of immobilization by adsorption has been reported for *Saccharomyces* yeasts [3,13]. The latter workers showed that immobilized *S. cerevisiae* consumed glucose twice as fast as suspended cells.

Altered macromolecular composition and higher DNA content for immobilized cells, compared to suspension grown cells, were observed for *S. cerevisiae* [3,15]. Immobilized *E. coli* exhibited unchanged DNA content but higher RNA content [6].

Higher specific production of ethanol from glucose has been reported for *Saccharomyces* sp. [3,13,16]. *E. coli* adsorbed on ion exchange resin exhibited a shorter lag phase for the induction of enzymes responsible for the utilization of lactose or xylose [6].

These observations are significant because immobilized cells have the potential for increasing utility in the production of high-value recombinant products. When cells are immobilized, low yields and product titers normally achieved in continuous culture can be elevated by utilizing high cell density, and stability of recombinant plasmids, which is often insufficient during multiple generations of continuous culture growth, can be improved. If it is generally true that microorganisms immobilized under gentle conditions also exhibit elevated levels of metabolic activity generally and product formation specifically, then the possibility exists for improving the productivity, yield, and product titers of recombinant product formation. *E. coli* has been commonly used as the host for the production of many commercially important recombinant products. The knowledge as to whether gentle immobilization generally affects protein synthesis by *E. coli* is important but lacking.

This study was undertaken in order to compare the inducible synthesis of β-galactosidase and the utilization of glycerol by suspended and immobilized cells under both aerobic and anaerobic growth conditions.

MATERIALS AND METHODS

Organism

E. coli K-12 wildtype (F^+) (CGSC 4401) was provided by the *E. coli* Genetic Stock Center, Department of Biology, Yale University.

Media

Glycerol minimal medium (GMM) consisted of glycerol 2.0 g/l, K_2HPO_4 10.5 g/l, KH_2PO_4 4.5 g/l, $(NH_4)_2SO_4$ 1.0 g/l, Na-citrate · $2H_2O$ 0.5 g/l, $MgSO_4$ · $2H_2O$ 0.24 g/l, vitamin B_1 2.0 mg/l, adjusted to pH 7.0.

Preliminary experiments showed that active cell growth is necessary for a high specific β-galactosidase synthesis level (data not shown). Therefore, experiments were carried out to design a growth medium compatible with our experimental needs.

Ca-alginate is unstable in media containing high concentrations of PO_4^{3-}, a standard medium buffer. The minimal concentration of phosphate in the medium necessary for normal cell growth, and which preserved the stability of Ca-alginate for 12 h, was 2 mM, compared with the 90 mM used in phosphate buffered media such as GMM. Tes-Tris (17 mM-10 mM) buffer preserved the stability of Ca-alginate gel and supported slightly better growth of *E. coli* than did phosphate buffer, even at concentrations far higher than those required as buffer (data not shown).

Glycerol was chosen as the carbon source since its metabolism does not interfere with the induction of β-galactosidase. In order to help reduce the effects of possible concentration gradients, glycerol concentration in the medium was maximized. This would prevent the limitation of cell metabolism due to low substrate concentration. We determined that glycerol levels in the range of 2 to 20 g/l were optimal for cell growth (data not shown).

As a result of these preliminary experiments, a growth minimal medium specifically designed for comparison of metabolic properties of free and im-

mobilized cells was established and designated as comparison growth minimal medium (CGMM): glycerol 20.0 g/l; Tes-N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (Nutritional Biochemical Corp.) 3.98 g/l; Tris (hydroxymethyl) aminoethane 1.21 g/l; $(NH_4)_2SO_4$ 1.0 g/l; K_2HPO_4 0.312 g/l; $MgSO_4 \cdot 7H_2O$ 0.2 g/l; NaH_2PO_4 24 mg/l; vitamin B_1 2 mg/l; adjusted to pH 7.0.

Storage and pregrowth

The culture was maintained in LB liquid medium [12] with glycerol (40% w/v) at –20°C. Colonies on LB agar plates at 4°C were periodically picked for adaptation growth in GMM. Cells adapted in GMM for three passages were stored at 4°C for inoculation of experiments.

Immobilization

A culture growing exponentially (OD_{600} = 0.6–0.7) in CGMM at 37°C, 200 rpm was used for immobilization or direct inoculation. For immobilization, the pregrown cells were collected by centrifuging the culture in an IEC clinical centrifuge (Model CL) set at 7 for 20 min. The cells were mixed into a solution of 2% (w/v) Na-alginate (Fisher) dissolved in distilled water at 37–38°C. Glass or Lexan (McMaster) beads 3 mm in diameter were dip-coated in the gel–cell mixture, dumped into a solution of 0.1 M $CaCl_2$, and left standing at room temperature for 5–6 h. The volume of gel coated onto each glass bead (determined by displacement) was about 6.9 μl which gave a gel layer about 200 μm thick. Uniform thickness of the gel layer was confirmed by microscopic examination. The reason for surface coating beads with gel was to eliminate intraparticle diffusion effects often found in solid gel beads.

For suspended cell experiments, cells pregrown as described above were spun down, resuspended in 0.1 M $CaCl_2$, and left standing at room temperature for 5–6 h.

Reactor and its operation

The original intention was to use a fluidized bed reactor for immobilized cells in order to minimize mass transfer limitations. In trial runs, however, the gel-coat layer on glass beads was not stable enough to withstand the minimum fluidization velocity. Consequently, a packed bed glass column reactor, with an effective packing length of 8 cm and diameter of 2.5 cm, was constructed for experiments with immobilized cells. The reactor held approximately 900–1000 coated beads. The column reactor was operated in a recirculation loop with a fermentor; the system contained 400 ml CGMM. Under these conditions, conversion per pass never exceeded 1.9%, and averaged 0.6%. Suspended cells were grown in the fermentor, with no medium recirculation through the packed column.

For the purpose of determining the recirculating velocity of medium through the packed column necessary to prevent substrate limitation, cell viability and glycerol consumption were monitored under experimental conditions and at different recirculating velocities. At medium recirculating velocities of 7, 14, and 28 cm/min, the growth rate of immobilized cells was identical. A recirculating velocity of 14 cm/min was chosen for the comparison experiments. Because we used both a glycerol concentration and a medium flow rate well above the minimum necessary to support optimum growth, we conclude that no external diffusion limitations of oxygen or glycerol existed. Since beads were surface coated with a thin layer, and since cell concentrations were relatively low, no internal diffusion limitations were anticipated. This was confirmed by a calculation which showed the effectiveness factor to be 1.

Comparison experiments

Comparison experiments were carried out at 37°C under both aerobic and anaerobic growth conditions, and with both free and immobilized cells. Aerobic conditions were obtained by pumping sterile air into the medium reservoir at a rate of 1000 ml/min during the experiment. In typical experiments of this type, dissolved oxygen was maintained at greater than 60% of saturation for the duration of the runs. Anaerobic conditions were obtained by pumping sterile nitrogen gas into the medium at a rate of 1000 ml/min for 30 min prior to and 500 ml/min during the experimental time period of 8 h.

Samples were taken at 2 h intervals in both cases. For suspended cells, culture was used directly for viability determination. 4 ml culture was concentrated 4:1 by centrifuging for 15 min and pipetting off 3 ml supernatant. The removed supernatant was stored at 4°C for the glycerol assay and the concentrated culture was used immediately for β-galactosidase induction. In the case of immobilized cells, medium samples were collected from the fermentor, centrifuged to remove cells (medium samples never contained more than 100 viable cells/ml), and stored at 4°C for the glycerol and byproduct assays. Samples consisting of about 100 gel-coated glass beads were taken from the top portion of the column. Immobilized cells were recovered by solubilizing the gel layer in 1% Na-citrate (J.T. Baker Chemical Co.) solution shaken at 150 rpm for 20 min at 37°C. After the bare glass beads were removed, the cell pellet was resuspended in 1 ml CGMM and the mixture of cells and 1% Na-citrate was centrifuged for 15 min. The cell pellet was resuspended in 1 ml CGMM; this suspension was used for determination of viability and β-galactosidase induction.

Inducibility of β-galactosidase synthesis by suspended and immobilized cells was monitored throughout the experimental time period. For induction, 0.01 ml isopropyl-β-D-thiogalactoside (IPTG) (Sigma) in distilled water was added to 0.5 ml cell suspension described above for a final IPTG concentration of 0.05 M. The mixture of cells and inducer was incubated at 37°C for 20 min.

Assays

Cell viability was determined by diluting in saline, plating on LB plates and incubating at 37°C for 20 h. Dry weight of free and immobilized cells was determined by standard methods. Glycerol was assayed with a chemical method derived from the one used by Beckett [2]. β-galactosidase was assayed according to the method of Miller [12] using *o*-nitrophenyl-β-D-galactoside (ONPG) (Sigma).

Acetate, ethanol, and succinate were assayed on a gas chromatograph (Perkin-Elmer Sigma 3) equipped with a flame ionization detector. Acetate and succinate were assayed on a 6′ × 1/8″ stainless steel column packed with 10% SP-1000/1% H_3PO_4 on 100/120 Chromasorb WAW (Supelco). For acetate, the oven temperature was 130°C, the injector and detector temperatures were 250°C, the carrier gas was N_2 at 30 cc/min, and the sample was 3.5 μl of acidified (17 μl 50% v/v H_2SO_4 per ml) culture supernatant. Succinate was assayed under the same conditions except the oven temperature was 140°C. Succinate samples were prepared according to Miller et al. [11]. Ethanol was assayed on a 6′ × 1/8″ Porapak 80 column under the same conditions as acetate. 2 μl of supernatant were injected directly. Pyruvate and lactate were assayed by lactate dehydrogenase-based spectrophotometric kits (Sigma).

RESULTS

Aerobic comparison experiments

A typical set of results from the aerobic growth of suspended and immobilized *E. coli* cells is summarized in Figs. 1–3; the average of two experiments is shown in Table 1. Immobilized *E. coli* cells consumed glycerol twice as fast as did suspended cells, but concomitant with the increased rate of glycerol uptake, there was a substantial decrease in their cell yield on glycerol. Specific growth rate did not change significantly upon immobilization. Thus the immobilized cells channelled a smaller fraction of glycerol into production of biomass although they

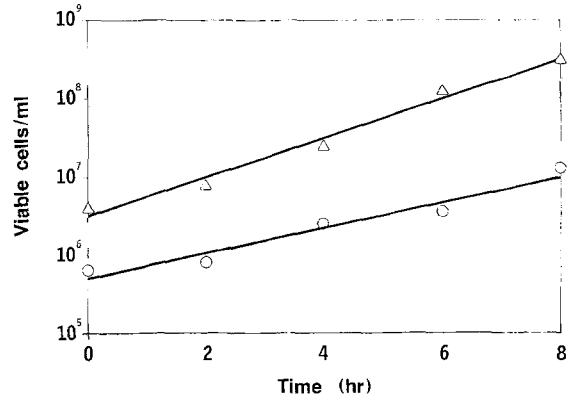

Fig. 1. Aerobic growth of suspended and immobilized cells. △, suspended cells; ○, immobilized cells.

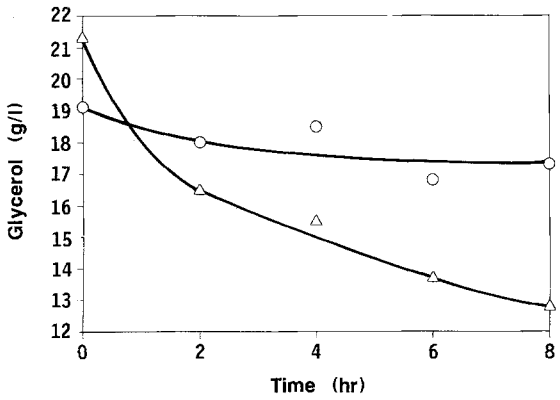

Fig. 2. Glycerol consumption during aerobic growth shown in Fig. 1. △, suspended cells; ○, immobilized cells.

consumed glycerol more rapidly. Immobilized *E. coli* cells exhibited a 1.6-fold higher inducible synthesis level of β-galactosidase than did suspended cells throughout the experimental time period.

In a control experiment, suspended *E. coli* incubated in 1% Na-citrate (such as during recovery of immobilized cells) did not show increased β-galactosidase inducibility. Thus the increase of β-galactosidase synthesis level in immobilized cells seems to be caused directly by immobilization in alginate.

Anaerobic comparison experiments

A typical set of results from anaerobic growth of suspended and immobilized *E. coli* cells are presented in Figs. 4–6; the average of two experiments is shown in Table 2. The results were qualitatively

Table 1

Metabolism during aerobic growth of suspended and immobilized cells[a]

	Suspended	Immobilized
μ (h^{-1})	0.54 ± 0.05	0.51 ± 0.02
Glycerol uptake rate (g/h cell × 10^{12})	4.2[b]	8.2 ± 2.1
Cell yield on glycerol (g/g)	0.059[b]	0.032 ± 0.008
Average[c] β-gal activity per cell normalized to suspended cell culture	1.00	1.61

[a] Values are mean values from two experiments, ± standard deviation of the mean.
[b] Data from one experiment only.
[c] Averages of the values for the 0, 2, 4, 6, and 8 h time points.

similar to the aerobic experiments described above: immobilized cells showed increased glycerol uptake, reduced cell yield on glycerol, and higher inducibility of β-galactosidase than did suspended cells. Specific growth rates of suspended and immobilized cells were the same.

As expected, growth rate and cell yield were lower, and glycerol consumption was higher, under anaerobic conditions than under aerobic conditions. β-galactosidase inducibility was somewhat lower, but the increase in inducibility upon immobilization was more dramatic, under anaerobic conditions.

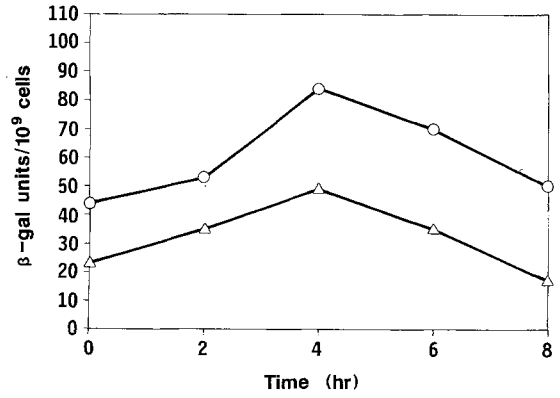

Fig. 3. β-Galactosidase inducibility during aerobic growth in Fig. 1. △, suspended cells; ○, immobilized cells.

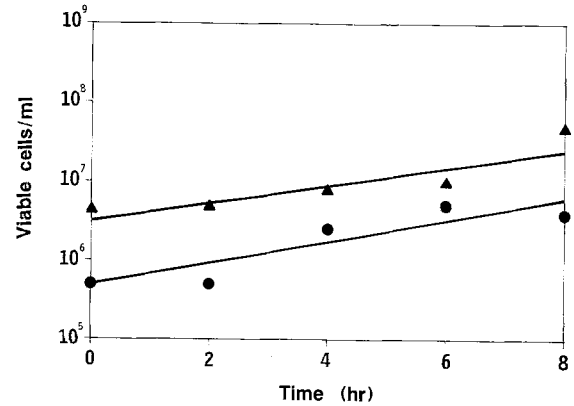

Fig. 4. Anaerobic growth of suspended and immobilized cells. ▲, suspended cells; ●, immobilized cells.

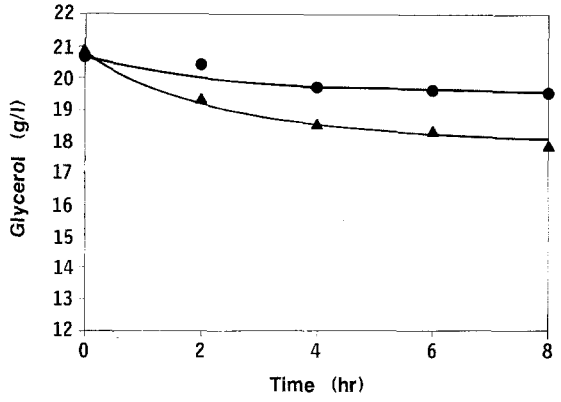

Fig. 5. Glycerol consumption during anaerobic growth shown in Fig. 4. ▲, suspended cells ●, immobilized cells.

Table 3 summarizes the quantitative comparison between immobilized and suspended cells in the aerobic and anaerobic experiments.

Carbon balance

In an attempt to account for the excess substrate which was consumed by immobilized cells, aerobically grown cultures of suspended and immobilized cells were analyzed for some typical byproducts of mixed acid fermentation (Fig. 7). On the average, immobilized cells produced three times as much acetate per mol of glycerol consumed as did suspended cells. Immobilized cells also showed elevated production of pyruvate and lactate, though at much lower levels than acetate. Neither ethanol nor succinate was detected in any of the supernatants. Of the carbon consumed by immobilized cells, 25 to 30% remains unaccounted for, whereas for suspended cells that figure is less than 10%.

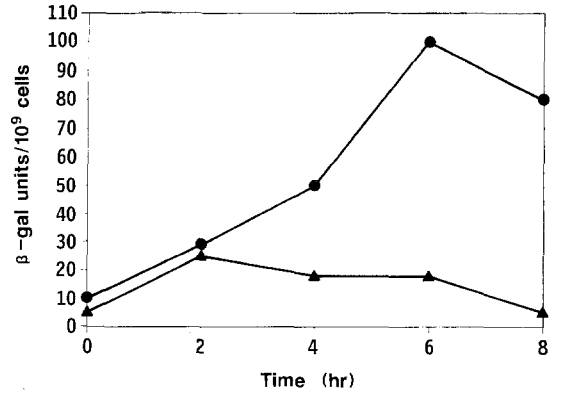

Fig. 6. β-Galactosidase inducibility during anaerobic growth in Fig. 4. ▲, suspended cells; ●, immobilized cells.

Table 2

Metabolism during anaerobic growth of suspended and immobilized cells[a]

	Suspended	Immobilized
μ (h^{-1})	0.27 ± 0	0.28 ± 0.02
Glycerol uptake rate (g/h cell × 10^{12})	20.9 ± 1.7	41.8 ± 3.7
Cell yield on glycerol (g/g)	0.012 ± 0.001	0.006 ± 0
Average[b] β-gal activity per cell normalized to suspended cell culture	1.00	4.90

[a] Values are mean values from two experiments, ± standard deviation of the mean.
[b] See note c, Table 1.

DISCUSSION

Major shifts in metabolism occurred in *E. coli* cells following immobilization: (1) The specific growth rate for immobilized cells was not significantly different from that for suspended cells under aerobic or anaerobic conditions. (2) Immobilized *E. coli* cells took up glycerol at twice the suspended cell rate under both aerobic and anaerobic conditions. (3) The cell yield from glycerol for both aerobically

Table 3

Ratios of data from aerobic and anaerobic growth

	Immobilized/Suspended	
	aerobic	anaerobic
Specific growth rate	0.94	1.04
Glycerol uptake rate	1.95	2.00
Cell yield	0.54	0.50
Specific activity of β-gal	1.61	4.90

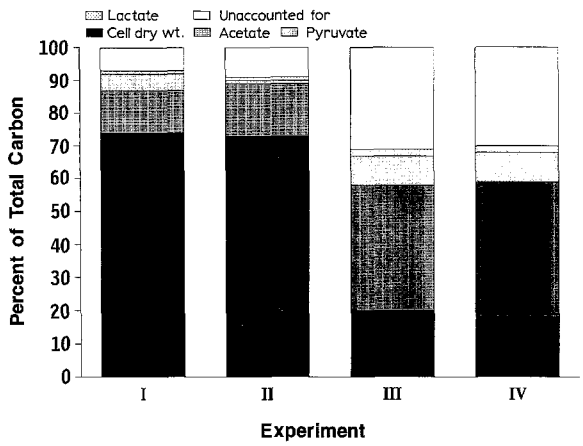

Fig. 7. Fraction of consumed glycerol accounted for at the end of the experiment. Each mol of glycerol consumed was assumed to be equivalent to 0.5 mol glucose. Cell mass accumulation was assumed to account for 1 g glucose for each 0.42 g dry weight produced. Acetate, pyruvate, and lactate were assumed to account for 1 mol glucose for each 2 mol byproduct produced. Experiments I and II were suspended cell runs; Experiments III and IV were immobilized cell runs.

and anaerobically growing immobilized cells was half that for suspended cells growing under the same conditions. (4) Aerobically growing immobilized cells exhibited an inducible synthesis level of β-galactosidase 1.6 times that of suspended cells; and anaerobically growing immobilized cells synthesized β-galactosidase at 4.9 times the level of suspended cells. (5) Production of the mixed acid fermentation byproducts acetate, pyruvate, and lactate was elevated in aerobically grown immobilized cells. A substantial portion of the carbon consumed by immobilized cells remains unaccounted for.

It has been suggested that immobilizaton results in enhanced cellular activity and substrate uptake because the cell benefits from increased nutrient availability at the solid–liquid surface or because the damage to the cell wall as a result of immobilization facilitates entry of substrates [4]. Doran and Bailey [3] proposed that increased glucose uptake rate resulted from greater transport activity in immobilized *S. cerevisiae* cells. These rationales are reasonable when the substrate supply in question is limited, but none of these apply to the present work since the glycerol concentration in the bulk medium was far more than enough for cell growth. Thus the substrate supply was not a limiting step for uptake and metabolic activity. Furthermore, the entrance of glycerol into the cell is an energy-independent process [8], and it is unlikely that immobilization affects the facilitator protein in such a way as to disrupt the internal/external equilibrium.

Mattiasson and Hahn-Hagerdal [10] suggested that the charge on support polymers reduces effective water activity in the vicinity of immobilized cells, thus increasing maintenance metabolism and reducing cell growth. From our results, and in accordance with this theory, it appears possible that the supply of carbon skeletons for non-growth related protein synthesis is increased as the growth anabolism is repressed in immobilized cells, and may lead to a higher β-galactosidase synthesis level.

This argument is supported by the finding that immobilized cells produced elevated amounts of acetate, pyruvate, and lactate, even under aerobic conditions. These byproducts are generally produced at elevated rates only when oxygen is limiting, or when central carbon catabolism is disrupted [11]. That oxygen is not limiting under our aerobic conditions is demonstrated by our dissolved oxygen measurements, and also by the fact that well under 10% of these byproducts is in the form of lactate, the only byproduct of the three that represents recovery of reducing equivalents during anaerobic growth. Taken together, the observations of greater β-galactosidase inducibility and elevated byproduct formation suggest that immobilization decouples the consumption of substrate from cell growth.

Experiments which extend these results to protein production by recombinant DNA-derived strains are in progress. In addition, the fate of the unaccounted-for carbon will be investigated by measurements of storage products and off-gas CO_2.

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