Effect of Oxygen Supply on Metabolism of Immobilized and Suspended *Escherichia coli*

Emel Inanç,[1] Judith E. Miller,[2] and David DiBiasio[3]*

[1]Department of Molecular Biology and Genetics, Bilkent University, 06533 Bilkent, Ankara, Turkey, and Departments of [2]Biology and Biotechnology and [3]Chemical Engineering, Polytechnic Institute, Worcester, Massachusetts 01609

The effect of reduced oxygen supply on the production of a recombinant protein (plasmid-encoded β-galactosidase) was investigated in *Escherichia coli*. A novel modified bubble tank reactor was used to provide a direct comparison between immobilized and suspended cells in identical environments except for the immobilization matrix. Decreased oxygen supply led to increased β-galactosidase synthesis by both immobilized and suspended cells. Immobilized cells produced similar amounts of β-galactosidase as the suspended cells. Lactose consumption and acetate production, on a per cell basis, were significantly higher in immobilized cells, suggesting that immobilized cells utilized fermentative metabolism. However, a transport analysis of the immobilized cell system showed that immobilized cells were not subject to either external or internal mass transfer gradients. © 1996 John Wiley & Sons, Inc.
Key words: *E. coli* • alginate immobilization • oxygen transport • mass transfer • acetate

INTRODUCTION

Whole cell immobilization has been shown to increase product synthesis and improve product recovery in fermentation systems. The specific rate of ethanol production by immobilized *Saccharomyces cerevisiae* grown in a packed bed reactor was 45% greater and glucose uptake was twice that of suspended cells, while the immobilized cells exhibited a lower specific growth rate (Doran and Bailey, 1986; Galazzo and Bailey, 1990b). *Escherichia coli* grown in calcium alginate coated glass beads in a packed bed reactor synthesized 1.6 (aerobic growth) and 4.9 (microaerophilic growth) times as much β-galactosidase per cell in response to induction as did suspended cells. The cell yield from glycerol for immobilized cells was half that for suspended cells while glycerol was consumed at twice the rate. Elevated quantities of acetate, pyruvate, and lactate were produced (Zhang et al., 1989).

Effective oxygenation of entrapped cells is a major problem because of potential mass transfer limitations. Ogbonna et al. (1989) showed that small bead diameter

and low cell densities were required if the center of the beads as to receive enough oxygen to support aerobic processes. Limited oxygen supply causes *E. coli* to direct carbon flow to fermentative metabolism (Han et al., 1992; Ko et al., 1993). As a result, cells produce acetate via fermentation, instead of CO_2 via the tricarboxylic acid (TCA) cycle. In a similar manner, yeast produces ethanol when respiration capacity is limited (Doran and Bailey, 1986).

In many studies it is not clear whether the metabolic changes observed for immobilized cells are the result of mass transfer limitations or changes in intrinsic metabolism caused by other effects of immobilization such as stress response or reduced water activity. The objective of our study was to investigate the effects of limited oxygen supply on metabolism, especially β-galactosidase synthesis, by immobilized and suspended *E. coli*. In most prior studies the immobilized cells were placed in packed beds (Kavel et al., 1985; Osuga et al., 1984) or fluidized beds while suspended cells were grown in agitated stirred tanks. In this research, the same reactor system was used for both immobilized and suspended cells. This comparison study tests the hypothesis that mass transfer limitation caused by immobilization is responsible for the metabolic changes observed in immobilized cells.

MATERIALS AND METHODS

Bacterial Strain and Culture

The bacterial strain used in this work, *E. coli* RDP146 ($\Delta lac\ pro\ thi$) containing plasmid pBRP1 (*lac* i[+] *lac* z[+] *lac* y[+] *Tet*[R]) that codes for the enzyme β-galactosidase, was kindly provided by Dr. William D. Taylor (Department of Molecular and Cell Biology, Pennsylvania State University). The strain was grown in Luria Broth (Miller, 1972) at 37°C overnight to the stationary phase (5 mL), pelleted and resuspended in 1 mL 1% peptone/40% glycerol (w/v), transferred into small sterile screw cap vials, and stored at −25°C. Blue colonies on Xgal (Miller, 1972)

agar plates incubated at 37°C and stored at 4°C were periodically selected for adaptation growth in lactose minimal medium (LMM). Ten milliliters of LMM was inoculated with one loop of cells from a plate and incubated for 24 h at 37°C, with shaking. After two more cycles of growth for 10 h each, 50 mL of cell suspension in the mid-exponential phase (600-nm optical density, OD_{600} = 0.60) was used to inoculate 500 mL of the fresh LMM medium in the fermentor (Multigen, New Brunswick Scientific Co.) for suspended cell experiments, or to obtain a cell pellet for immobilization experiments.

Growth Medium

The composition of the LMM was modified from Zhang et al. (1989) and consisted of, per liter: 10.0 g β-lactose, 0.1151 g L-proline, 0.1 g vitamin B_1, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.312 g K_2HPO_4, 0.024 g NaH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 3.98 g tes N tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, 1.21 g tris(hydroxymethyl)aminomethane (all from Sigma).

Immobilization

The cells from the 50 mL preculture were centrifuged at 7000 rpm for 15 min in an IEC clinical centrifuge (model CL). The cell pellet was suspended in 50 mL of a 2% w/v sodium alginate solution (KELCO, Keltone-HV). The cell/alginate solution was dropped into a $0.2M$ $SrCl_2$ solution (Ogbonna, 1989b) by means of a 10-mL syringe fitted with a 21-gauge needle. Strontium alginate beads were allowed to cure in the $SrCl_2$ solution for 2 h, washed three times with sterile water, then immediately placed into the reactor. The average diameter of the strontium alginate beads was 2.84 mm, and approximately 3125 beads were obtained from 50 mL of cell/alginate suspension. The strontium alginate beads were stable for 16 h (Inanç, 1994). The initial cell concentration in the beads was 7.42 × 10^8, 13.75 × 10^8, 14.60 × 10^8, and 15.40 × 10^8 cells/mL of gel volume; final cell concentration was 10.25 × 10^9, 18.30 × 10^9, 28.30 × 10^9, and 27.10 × 10^9 cells/mL of gel volume at the initial dissolved oxygen concentrations of 0.235, 0.533, 0.800, and 1.070 mmol/L, respectively. Of the total cells immobilized, 0.01% escaped from the gel matrix at the beginning of the experiment, and total free cells at the end of the experiment did not exceed 0.05%.

Reactor System and Experimental Procedure

We used a modified bubble tank reactor (MBTR), consisting of a New Brunswick Multigen fermentor jar without an impeller. The MBTR allowed direct comparison of suspended and immobilized cultures under identical mixing conditions. The mixing was supplied by gas flow at 1.5 L/min. Mechanical agitation was not used because it caused gel breakage. The gas mixture was supplied

to the bioreactor from a perforated, steel, looped pipe placed in the fermentor vessel. The gas flow was adjusted to the desired initial dissolved oxygen concentration (0.235, 0.533, 0.800, and 1.070 mmol/L) by mixing oxygen and nitrogen gases. Dissolved oxygen was monitored with a New Brunswick Dissolved Oxygen Controller (model DO-81). The dissolved oxygen concentrations were calculated using the Henry's law constant (Bailey and Ollis, 1986) of 1.066 mmol/L at 37°C. The pH was controlled at 7.0 (Horizon Ecology Co., model 5997-20) with the addition of $1M$ NH_4OH and $0.5M$ HCl. The bioreactor, containing 500 mL of medium, was inoculated either with 50 mL preculture for suspended cell experiments or with 3125 strontium alginate beads for immobilized experiments.

Samples were taken at 2-h intervals from both suspended and immobilized experiments. For suspended cells, 8 mL of sample was taken and analyzed for viability, OD, and β-galactosidase. Three milliliters of the sample were centrifuged, and supernatant was stored in the freezer for later lactose and acetate analyses. For immobilized cells, duplicate samples of 40 beads were transferred from the MBTR into each of two tubes. The medium was removed from the beads with a pipet and saved for acetate and lactose assays. Ten milliliters of 4% w/v sodium citrate (citric acid sodium salt, Sigma) were added. The beads were incubated at 37°C and vortexed periodically for approximately 40 min until all the beads were completely dissolved.

Assays

Samples were assayed in duplicate for cell viability and in triplicate for β-galactosidase. Both assays had an average deviation of less than 10%. Cell viability was determined by diluting 100 μL of culture in PBS (phosphate buffered saline) solution (Miller, 1972) plating on MacConkey Agar (Difco), and incubating at 37°C for 20 h. Viable cell density plotted against OD_{600} showed a linear relationship at all oxygen levels. OD was measured at 600 nm using a Unicam SP 1800 ultraviolet spectrophotometer. Lactose was measured with a YSI model 27 glucose analyzer (Inanç, 1994). Acetate was analyzed with a Perkin–Elmer Sigma 3 gas chromatograph equipped with a flame ionization detector. The column used for this purpose was a 100/200 Chromasorb 101, 0.5% free fatty acid phthalate (FFAP). The injector and detector temperatures were both 185°C and the column (oven) temperature was 170°C. The carrier gas was 50 mL/min nitrogen (Inanç, 1994). β-Galactosidase was assayed according to the method of Miller (1972), except that the calculation used was 1 unit β-galactosidase liberates 1 μmol orthonitrophenol/min at 28°C giving OD_{420} = 0.0045.

RESULTS

The specific growth and respiration rates of both suspended and immobilized cells increased with increasing

initial dissolved oxygen concentration. The specific respiration rate (Q_{res}) was calculated from the slope of dissolved oxygen/cell versus time transient. A linear relationship between the specific growth rate and initial dissolved oxygen concentration was observed (not shown).

The β-galactosidase production reached a maximum after about 4–6 h in both suspended (Fig. 1A) and immobilized (Fig. 1C) cells while the cells were in the early exponential phase (Fig. 1B,D). The highest β-galactosidase level was achieved with the lowest initial dissolved oxygen concentration (0.235 mmol/L) (Fig. 1A,C). A fourfold increase in initial dissolved oxygen concentration decreased the maximum level of β-galactosidase by one-third in both suspended and immobilized cells (Table I). The β-galactosidase production by immobilized and suspended cells was similar at each dissolved oxygen concentration.

Limited oxygen supply also affected lactose consumption and acetate production. The highest lactose consumption was observed with the lowest oxygen supply (Fig. 2). When oxygen supply decreased from the highest to the lowest value, lactose consumption increased up to 3.6 times in suspended and up to 2.8 times in immobilized cells (Table I). Immobilized cells con-

sumed more than twice as much lactose as suspended cells at each oxygen level.

The cells produced significant amounts of acetate only after the first 4–6 h of any experiment. As with lactose consumption, acetate production increased with decreasing dissolved oxygen concentration (Fig. 2B,D). Immobilized cells produced more acetate per cell than suspended cells, up to 2.8-fold at the highest oxygen concentration. The difference between the acetate production by immobilized and suspended cells was very small at the lowest oxygen level. The yield of acetate on lactose was the same for both immobilized and suspended cells, but the yield increased with decreasing oxygen supply (Table I).

DISCUSSION

We proposed that the mass transfer limited supply of oxygen in the immobilized systems causes a switch of carbon flow from oxidative metabolism to fermentative metabolism. As a result of the less efficient metabolism of lactose under oxygen-limited conditions, lactose uptake would increase, which could, in turn, elevate induction of the *lac* operon and stimulate β-galactosidase synthesis. If this hypothesis is right, when oxygen supply
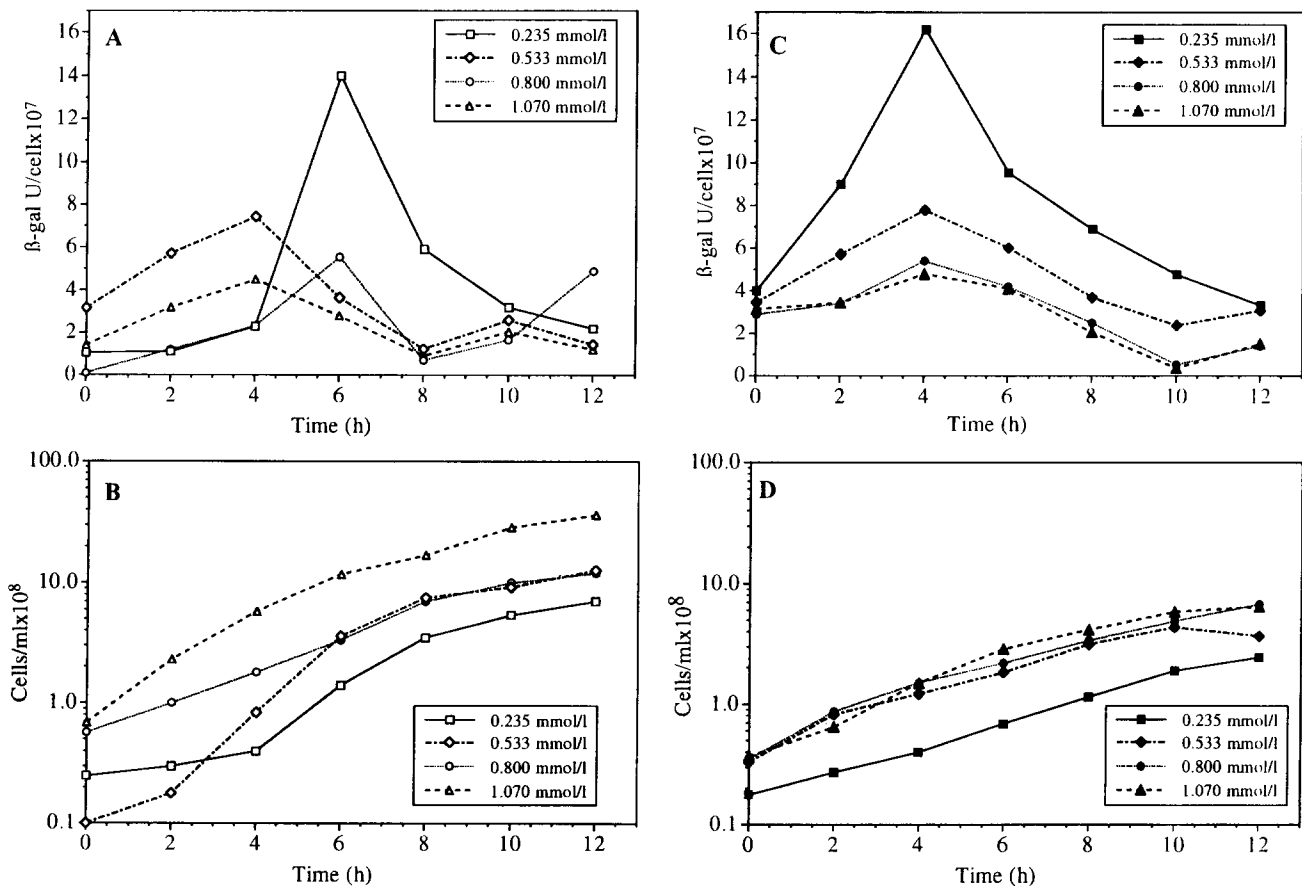

Figure 1. Effects of oxygen supply on (**A**) β-galactosidase production of suspended cells, (**B**) growth of suspended cells, (**C**) β-galactosidase production of immobilized cells, and (**D**) growth of immobilized cells. Initial oxygen concentrations ranged from 0.235 to 1.070 mmol O_2/L.

Table I. Comparison of suspended and immobilized cells.

DO_i (mmol/L)	μ (/h^1)		Q_{res} (mmol/cells h) × 10^{12}		Lactose consumption (g/ cell) × 10^{12}		Acetate production (g/cell) × 10^{12}		$Y_{acetate/lactose}$ (g acetate/ g lactose)	
	SUS	IMM	SUS	IMM	SUS	IMM	SUS	IMM	SUS	IMM
0.235	0.315	0.188	ND	2.95	6.25	12.90	1.65	1.69	0.41	0.44
0.533	0.365	0.244	ND	3.59	2.88	8.90	1.11	1.60	0.38	0.34
0.800	0.409	0.291	2.63	4.49	2.69	4.81	0.91	1.57	0.34	0.34
1.070	0.440	0.336	3.60	5.62	1.73	4.57	0.37	1.04	0.22	0.23

DO, dissolved oxygen; SUS, suspended cells; IMM, immobilized cells; ND, not determined, oxygen consumption was too rapid.

decreases, lactose consumption, β-galactosidase synthesis, and acetate production should increase while the specific growth rate decreases. Also, if immobilized cells experience lower oxygen concentrations than suspended cells (due to transport limitations), they would produce more β-galactosidase and acetate, consume more lactose, and grow slower than suspended cells.

In our experiments, as expected, lactose consumption, β-galactosidase production, and acetate production increased and growth rate decreased with reduced oxygen supply in both immobilized and suspended cells (Table I). Also as expected, immobilized cells appeared to me-

tabolize fermentatively to a greater extent than did suspended cells, i.e., at a given oxygen level they consumed more lactose and produced more acetate. These results are analogous to those of Galazzo and Bailey (1990a) who showed that glucose uptake and ethanol production by immobilized cells were elevated compared to suspended cells. *E. coli* used oxidative metabolism preferentially if there was enough oxygen as shown by the Q_{res} results (Table I). When the oxygen decreased, cells switched to fermentative metabolism and because fermentative metabolism is less efficient, lactose consumption increased. Because β-galactosidase is induced by
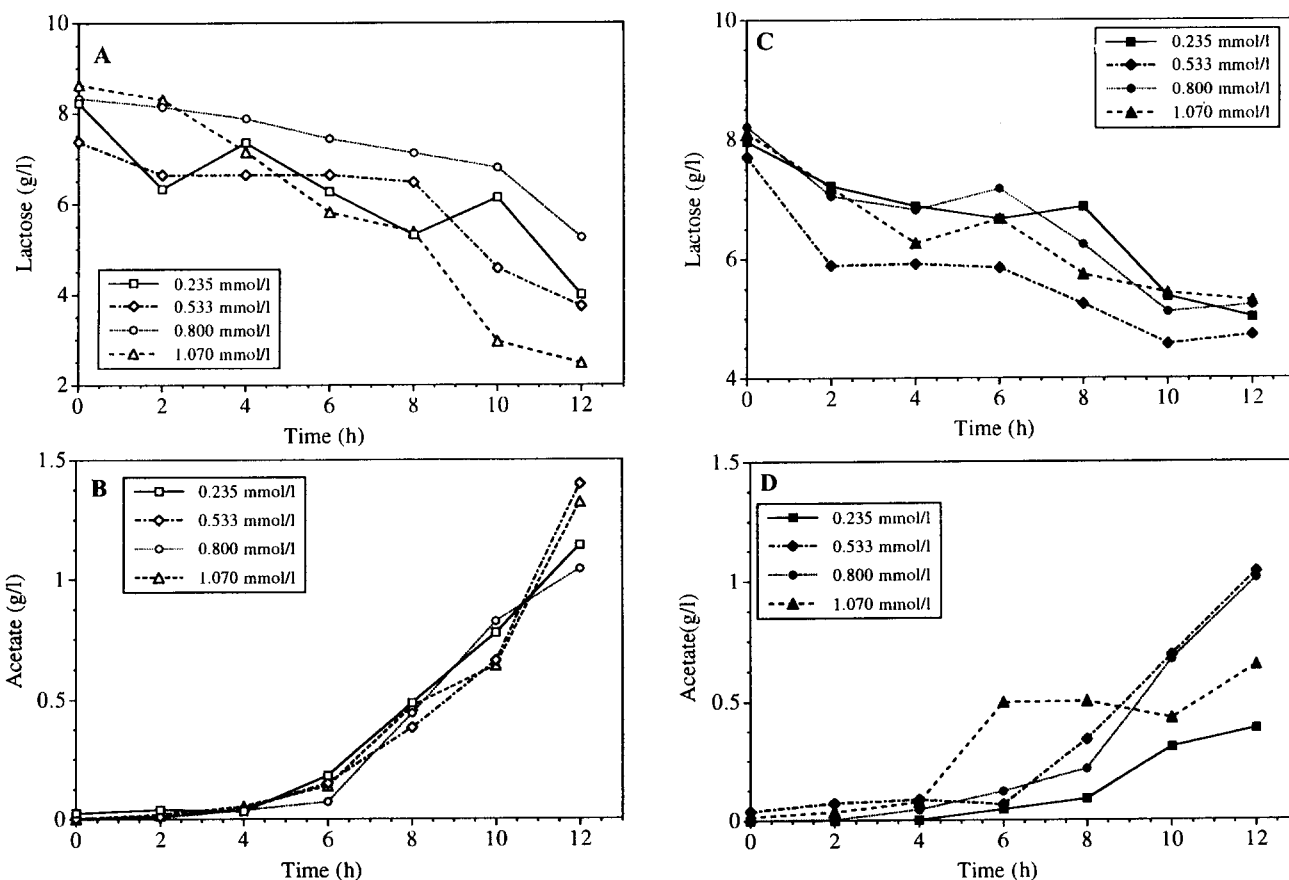

Figure 2. Effects of oxygen supply on (**A**) lactose consumption of suspended cells, (**B**) acetate production of suspended cells, (**C**) lactose consumption of immobilized cells, and (**D**) acetate production of immobilized cells. Initial oxygen concentrations ranged from 0.235 to 1.070 mmol O$_2$/L.

lactose, the highest β-galactosidase production was achieved with the lowest dissolved oxygen concentration for both immobilized and suspended cells (Fig. 1A,C).

An unexpected result was that β-galactosidase synthesis by immobilized cells was similar to that of suspended cells at each initial dissolved oxygen concentration. Because the immobilized cells consumed more lactose, more β-galactosidase was expected to be induced. Also, in a previous study by Zhang et al. (1989), immobilized E. coli produced more β-galactosidase than suspended cells. However, their comparison was made between two different reactor systems, packed bed and stirred tank reactors, so the cells grew in totally different environments. In our experimental system, the cells were in similar environments except that the immobilized cells were in a gel matrix. Also, the two sets of experiments employed different carbon sources (glycerol in Zhang et al.'s work and lactose in this work).

The mass transfer resistance encountered as a result of the immobilization matrix is an important issue in understanding the metabolism of immobilized cells (Karel et al., 1985). Because of the metabolic changes exhibited by immobilized cells, we suspected that immobilized cells were mass transfer limited with respect to oxygen. Our analysis included checks of the effects of bead presence on gas–liquid oxygen transport, external oxygen diffusion limitations, and internal oxygen diffusion effects.

The gas–liquid oxygen transfer was tested by measuring the gas–liquid oxygen transfer coefficient $k_L a$ using the static gassing out method (Perry et al., 1990). Each run was repeated three times. The average $k_L a$ values for immobilized and suspended cells were 54.13 and 54.66/h, respectively. Thus, aeration and mixing were essentially unchanged when gel beads were present.

The mass transfer coefficient governing oxygen transfer through the external layer surrounding a bead was calculated by assuming there was little shear between particles and fluid. Because beads and fluid were moving through similar circulation patterns in the MBTR, the local velocity difference between the beads and fluid was relatively small. The correlation was adapted from the slurry reactor literature (Fogler, 1992), where the Sherwood number is equal to 2.0. The external mass transfer coefficient (k_c) was calculated to be 1.76 × 10^{-6} m/s (Inanç, 1994).

The reaction rates were calculated from the slope of dissolved oxygen concentration versus time plots, and surface concentrations (C_c) of oxygen were calculated from Equation (1).

$$R_s = k_c a_c m (C_b - C_c) \tag{1}$$

where

$$a_c = 6/(d_p \rho_p). \tag{2}$$

The biocatalyst loading and density were 0.0784 and 1.05 g/mL. The difference between the surface and bulk (C_b) concentrations ranged from 0.012 to 0.030 mmol O_2/L for the highest (1.07 mmol/L) to the lowest (0.235 mmol/L) oxygen concentrations, respectively. Thus, external gradients were not significant.

The effectiveness factor was calculated to check internal diffusion limitations within the alginate beads. Standard methods for quantifying diffusion-reaction effects in spherical geometry were used (Fogler, 1992; Inanç, 1994). Calculated Thiele moduli were 0.125, 0.096, 0.034, and 0.030 for dissolved oxygen concentrations of 0.235, 0.533, 0.800, and 1.070 mmol/L, respectively. The effectiveness factors resulting from these moduli ranged between 0.9995 and 0.9997. Internal oxygen gradients were therefore minimal. This was reasonable because cell loading was low, and the diffusion coefficients of oxygen in water (2.5 × 10^{-9} m²/s; Fogler, 1992) and in alginate (1.8 × 10^{-9} m²/s; Ogbonna et al., 1991) are similar. In summary, we conclude that immobilized cells in this system were not oxygen transport limited, in contradiction to previous suppositions.

CONCLUSIONS

Unlike comparing a packed bed and stirred tank reactor, the modified bubble tank bioreactor enabled us to make an unambiguous comparison, with respect to reactor conditions, between immobilized and suspended cells. The MBTR provided a similar environment for both suspended and immobilized cells.

Reduced oxygen supply caused increased lactose consumption and increased acetate and β-galactosidase production in both immobilized and suspended cells. Growth rate decreased with reduced oxygen supply in both cases. Immobilization did not increase β-galactosidase production. Immobilization enhanced the fermentative metabolism of the cells at all dissolved oxygen concentrations.

External and internal mass transfer limitations were found to be significant under the conditions of these experiments (low cell loading and small biocatalyst size). Thus, the metabolic differences between immobilized and suspended cells in this system cannot be attributed to mass transfer limitation and must be attributed to other effects such as stress response or water activity.

NOMENCLATURE

a_c	external surface area per mass of bead (m²/g)
C_b	bulk dissolved oxygen concentration (mmol/L)
C_c	surface concentration of oxygen (mmol/L)

d_p	bead diameter (m)
k_c	external mass transfer coefficient (m/s)
$k_L a$	gas–liquid mass transfer coefficient (per h)
m	biocatalyst loading (total weight of beads/volume of liquid in the reactor, g/mL)
MBTR	modified bubble tank reactor
R_s	oxygen consumption rate in Equation (1) (mmol/L h)
Q_{res}	specific respiration rate (mmol O_2/cell h)

Greek Letters

μ	specific growth rate (per h)
ρ_p	density of biocatalyst (g/mL)

References

Bailey, J. E., Ollis, D. F. 1986. Biochemical engineering fundamentals, 2nd ed. McGraw–Hill, New York, p. 319.

Doran, P. M., Bailey, J. E. 1986. Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. Biotechnol. Bioeng. **28**: 73–87.

Fogler, H. S. 1992. Elements of chemical reaction engineering, 2nd edition. Prentice–Hall, Englewood Cliffs, NJ, pp. 570, 668.

Galazzo J. L., Bailey, J. E. 1990a. Fermentation pathway kinetics and metabolic flux control in suspended and immobilized *Saccharomyces cerevisiae*. Enzyme Microb. Technol. **12**: 162–172.

Galazzo J. L., Bailey, J. E. 1990b. Growing *Saccharomyces cerevisiae* in calcium-alginate beads induces cell alterations which accelerate glucose conversion to ethanol. Biotechnol. Bioeng. **36**: 417–426.

Han, K., Lim, H. C., Hong, J. 1992. Acetic acid formation in *Escherichia coli* fermentation. Biotechnol. Bioeng. **39**: 663–671.

Inanç, E. 1994. An investigation of oxygen transfer limitations on the metabolism and growth of immobilized *Escherichia coli*. Ph.D. thesis, Worcester Polytechnic Institute, Worcester, MA.

Karel, S. F., Libicki, S. B., Robertson, C. R. 1985. The immobilization of whole cells: Engineering principles. Chem. Eng. Sci. **40**: 1321–1354.

Ko, Y.-F., Bentley, W. E., Weigand, W. A. 1993. An integrated metabolic modelling approach to describe the energy efficiency of *Escherichia coli* fermentations under oxygen-limited conditions: Cellular energetics, carbon flux, and acetate production. Biotechnol. Bioeng. **42**: 843–853.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Ogbonna, J. C., Matsumura, M., Kataoka, H. 1991. Production of glutamine by micro-gel bead immobilized *Corynebacterium glutamicum* 9703-T cells in a stirred tank reactor. Bioprocess Eng. **7**: 11–18.

Ogbonna, J. C., Matsumura, M., Yamagata, T., Sakuma, H., Kataoka, H. 1989a. Production of micro-gel beads by a rotating disk atomizer. J. Ferment. Bioeng. **68**: 40–48.

Ogbonna, J. C., Pham, C. B., Matsumura, M., Kataoka, H. 1989b. Evaluation of some gelling agents for immobilization of aerobic microbial cells in alginate and carrageenan gel beads. Biotech. Tech. **3**: 421–424.

Osuga, J., Mori, A., Kato, J. 1984. Acetic acid production by immobilized *Acetobacter aceti* cells entrapped in a κ-carrageenan gel. J. Ferment. Technol. **62**: 139–149.

Perry, R. H., Green, D. W., Maloney, J. O. 1990. Perry's chemical engineer's handbook, 6th edition. McGraw–Hill, New York, Sect. 27.

Zhang, X., Bury, S., DiBiasio, D., Miller, J. E. 1989. Effects of immobilization on growth, substrate consumption, β-galactosidase induction, and byproduct formation in *Escherichia coli*. J. Ind. Microbiol. **4**: 239–246.