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Experimental Analysis of a Product Inhibited Fermentation in an Aqueous Two-Phased System

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ABSTRACT

Two aqueous two-phased sytems were investigated to determine their ability to reduce product inhibition in the acetone-butanol-ethanol (A-B-E) fermentation. In an attempt to avoid the high cost of fractionated dextran, an industrial-grade dextran (DEX) and a hydroxylpropyl starch polymer, whose commercial name is Aquaphase PPT (APPT), were tested as the copolymer with polyethylene glycol (PEG) to form the two-phased fermentation broth. The two-phased fermentation performances in the DEX-PEG and APPT-PEG two-phase systems were compared to a single-phased conventional fermentation through a series of batch runs. Also, the effects of the phase forming polymers on *Clostridium acetobutylicum* were investigated. With the butanol partition coefficient of 1.3, which is defined as the concentration ratio of butanol in the continuous and dispersed phases, the butanol yield with the two-phased system was increased by 27% in comparison to the conventional fermentation.

Index Entries: Two-phased fermentation; polyethylene glycol; dextran; aquaphase PPT; partition coefficient.

INTRODUCTION

A problem with the majority of fermentation processes is low product concentration in the final fermentation broth. In particular, this problem is pronounced with solvent fermentations because of the significant prod-

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uct inhibitory effects on microorganisms. This is the major reason that low-valued chemical production via fermentation processes is economically unattractive. However, a significant increase in the price of petroleum, the major feedstock for low-valued chemicals, along with process improvements that can increase the final product concentration, can make fermentation processes more competitive in the future.

The detrimental effects of product inhibition on the microorganisms, which, in turn, cause low product concentrations, result in high downstream processing cost. In the ethanol fermentation, a concentration of 5–8 wt% causes inhibition (*1*), whereas in the A-B-E fermentation with *Clostridium acetobutylicum*, a total solvent concentration of 2 wt% (*2*), or a butanol concentration of 10–15 g/L, causes significant product inhibition (*3–6*).

End-product toxicity effects can be reduced either by an *in situ* removal of the toxic products, or by mutation, adaptation, and selection of solvent-tolerant strains (*1,7*). A vacuum distillation process has been used for the *in situ* removal of ethanol (*8–11*). Also, liquid–liquid extraction has been used for the *in situ* removal of toxic products from the fermentation broth. Liquid–liquid two-phased systems can be generated in several different ways: mixing water with organic solvents, e.g., carbon tetrachloride, *n*-hexadodecanol, *n*-dodecanol, and so on (*12–17*), mixing water with phase forming polymers, e.g., dextran, polyethylene glycol, ficoll, methylcellulose, Aquaphase PPT, and so forth (*18–27*), and mixing a phase forming polymer with salts, e.g., polyethylene glycol and phosphate (*28*). The aqueous two-phased system obtained by the addition of dextran and polyethylene glycol has been widely used for the purification (isolation) of proteins (*29–31*), e.g., γ-Interferon, enzymes, and so on. Recently, the aqueous two-phased systems have been used for an extractive bioconversion, i.e., a liquid-phase immobilization was formed by confining the enzymes and substrate to the dispersed phase while the products partition into the continuous phase. These products can be recovered by repetitive partitioning steps or by continuous extraction procedure. Alcohol production by yeast (*20*) and the bioconversion of cellulose to glucose by the combined action of cellulase and α-glucosidase (*32*) in the PEG-DEX aqueous two-phased system are good examples of the repetitive partitioning and the continuous extraction procedures for the product recovery, respectively. Two reasons for the wide application of PEG and DEX as the phase forming polymers are their well-known physical and chemical properties, and their highly biocompatible environment. This is caused by the high water content in each phase, i.e., 65–95%, when compared to the water-organic solvents.

One important reason for the limited application of the aqueous two-phased system to fermentation processes, especially for low-valued products, is the high cost of fractionated dextran. In this work, industrial-grade dextran and Aquaphase PPT, i.e., a new hydroxylpropyl starch polymer

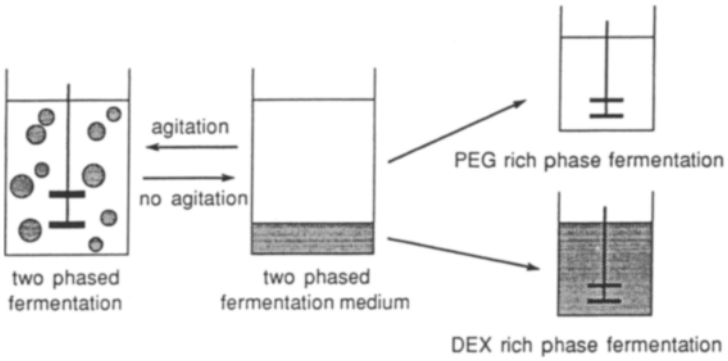

Fig. 1. Schematic description of fermentation systems.

developed by Tjerneld et al. (25–27), are investigated with PEG as substitute polymers for the fractionated dextran. This could significantly reduce the cost for this process. The fermentation characteristics of the PEG-DEX and the PEG-APPT two-phased systems were compared to a single-phased conventional fermentation process for A-B-E fermentation.

Also, the effects of phase forming polymers on *Clostridium acetobutylicum* were examined. This was done by comparing the fermentation performances of single-phased polymer systems with both the two-phased and conventional single-phased fermentation processes. These single-phased polymer systems were formed by separation of top and bottom phases of a settled two-phased system. This is illustrated in Fig. 1.

MATERIALS AND METHODS

Microorganism and Media

The microorganism used was *Clostridium acetobutylicum* ATCC 824. The freezed-dried cell pellet was activated with RCM ("Reinforced Clostridium Medium"). The spore formation characteristics of *Clostridium acetobutylicum* ATCC 824 enable long-term storage as stock cultures without genetic variation. The growth medium contains the following ingredients in 1 L of deionized water: glucose, 10 g; yeast extract, 5.0 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $MnSO_4 \cdot H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; asparagine, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; cysteine, 0.5 g. The concentrations of glucose and $(NH_4)_2SO_4$, were changed to 55 or 75 g/L and 10 g/L, respectively, for the fermentation experiments. All inoculum development processes were performed by using a syringe to prevent oxygen contamination.

FERMENTATION EXPERIMENTS

Phase Forming Polymers

Carbowax PEG 8000 (average mol. wt. approx 8000) was purchased from Fisher Scientific, and industrial-grade dextran (average mol. wt. approx 15,000–20,000) was purchased from the Sigma Chemical Company. Aquaphase PPT (average mol. wt. approx 30,000–35,000) was purchased from Perstorp Biolytica, Perstorp, Sweden.

Description of Two-Phase System

Concentrations of 5 (w/v)% industrial-grade dextran, 5 (w/v)% Aquaphase PPT, and 10 (w/v)% Carbowax PEG 8000 were used to form the aqueous two-phase systems, and volume ratios of the top and bottom phase, i.e., V_t/V_b of 5.7 and 8.0, were obtained with the PEG-DEX and the PEG-APPT two-phase systems, respectively. The compositions of the two phases were 19.5% DEX and 2.5% PEG for the dispersed phase, and 2.8% DEX and 10.0% PEG for the continuous phase. The partition coefficients in the PEG-DEX two-phase system were 1.22, 1.14, and 1.30 for acetone, ethanol, and butanol, respectively.

Fermentation Medium

Five different types of fermentations were investigated. In addition to conventional single-phased fermentations and two-phased fermentations with the PEG-DEX and PEG-APPT two-phase systems, single-phased fermentations in the PEG-rich top phase, and the DEX-rich bottom phase were also performed. The uneven distribution of sulfate and phosphate in the aqueous two-phase system, which is caused by the greater tendency of PEG over DEX to exclude these ions (*33,34*), was maintained in the top- and the bottom-phase fermentation experiments by allowing the two-phased fermentation medium to stand for 24 h before separating, and collecting the top and the bottom phases (*see* Figure 1).

Fermentation

A Bioflow I fermentor (New Brunswick Scientific Co.) with a working vol of 1 L was used. The agitation and temperature were controlled at 300 RPM and 37°C, respectively. The pH was controlled to stay above 4.5 by the automatic addition of 6*N* NaOH. Antifoam AF Emulsion (Dow Corning Corp.), a water-dilutable, 30% silicone defoamer, was added from the top of the fermentor to avoid a foaming problem. Nitrogen gas was sparged throughout the fermentation to ensure anaerobic conditions. A Friedrichs type condenser was used to prevent stripping of solvent products from the nitrogen gas stream.

Analytical Procedures

An HP 5890 gas chromatograph equipped with a teflon™-lined stainless-steel column, 1.85 m×0.32 cm outside diameter, packed with Chromosorb W-AW coated with 10% AT 1000 (Alltech Associates), and an HP 3396A integrator were used for the analysis of solvents and acids. The gas chromatograph operating parameters were: injection port temperature: 250°C; detector temperature: 250°C; column temperature: programmed between 100–180°C with 16°C/min increment; detector type: flame ionization; carrier gas: He at the flow rate of 30 mL/min; H_2 and air flowrates to FID: 40 and 200 mL/min, respectively.

The samples for the gas chromatograph were prepared by adding 10 μL of 6*N* HCl to acidify, and 100 μL of iso-butanol as an internal standard to 900 μL of cell-free fermentation broth. Concentrations of five components, i.e., acetone, ethanol, butanol, acetic acid, and butylic acid, were calculated by the internal standard calibration method based on peak height. Biomass concentration was estimated by the calibration curve between dry cell weight and optical density. The optical density was measured by Spectronic 21 (Milton Roy Company) at 610 nm. The glucose concentration was measured by YSI Model 27 (Yellow Springs Instrument Company, Inc.).

RESULTS AND DISCUSSION

Cell partitioning in the aqueous two-phase system was observed with a phase contrast microscope (10×40 magnification). It was found that all of the cells were confined to the dispersed phase, i.e., either the DEX-rich or the APPT-rich droplet phases. This complete cell partitioning to the dispersed phase, where the butanol concentration is approx 25% lower than in the continuous phase, was expected to produce increased butanol formation when compared to the conventional single-phased fermentation. However, as seen in Tables 1 and 2, the PEG-APPT two-phased system did not increase butanol concentration. This result was caused by the breakdown of the PEG-APPT two-phased system to a single-phased system at about 15 h into the batch. This event was repeated several times, so the PEG-APPT system was discontinued. Further, Tables 1 and 2 show an increased butanol level for the PEG-DEX two-phased system. However, since there was no residual glucose for any of the systems listed in Table 1, it appeared that glucose depletion, and not a toxic level of butanol, caused the fermentation to terminate. Therefore, the remaining fermentations were performed with higher glucose concentrations. The data given in Tables 3 and 4, and shown in Fig. 2, show a clear increase in butanol level for the PEG-DEX two-phased system when compared to the con-

Table 1
Comparison of Fermentation Performance (Low Glucose Concentration)*

System	Initial glucose	Residual glucose	Max. cell mass	Max. butanol	Total solvents	Final butyrate
Conventional	53.42	0.0	3.7042	9.273	14.721	0.944
PEG-DEX	54.23	0.0	4.4249	10.581	16.447	1.671
PEG-APPT	54.28	0.0	4.1327	9.088	16.628	0.365

*All concentrations are in g/L U.

Table 2
Comparison of Fermentation Efficiency (Low Glucose Concentration)

System	$Y_{bt/glu}$	$Y_{dw/glu}$	$Y_{bt/dw}$	$Y_{sol/glu}$
Conventional	0.1736	0.0693	2.5051	0.2756
PEG-DEX	0.1951	0.0816	2.3910	0.3033
PEG-APPT	0.1674	0.0761	2.200	0.3063

ventional single-phased system. However, there are at least two physical differences to be noted when these two systems are compared. One difference is the environment for the cells, i.e., in the two-phased system, the cells reside in a liquid phase enriched in dextran. The other difference with the two-phased system is the transfer of butanol from the dextran-rich droplet phase to the polyethylene glycol-rich continuous phase. In an attempt to determine the relative effect of these physical differences, a dextran-rich, single-phased fermentation was performed with media composition identical to the dextran-rich dispersed phase from the two-phased system. A comparison of the DEX-rich, single-phased fermentation with the conventional single-phased fermentation will illustrate the effect of increased dextran concentration on cell growth and butanol formation. The data in Tables 3 and 4, and Figs. 3, 4, and 5 indicate very comparable cell growth, butanol formation, and substrate consumption profiles for the DEX-rich, single-phased and conventional single-phased fermentations. Therefore, except for a small delay in the cell growth and butanol formation profiles possibly owing to the increased viscosity, increased dextran concentration appears to have no effect on the fermentation.

The effect of the mass transfer to the continuous phase is illustrated by comparing the DEX-rich, single-phased fermentation with the PEG-DEX two-phased fermentation. In this comparison, the polymer composition of the cells' environment is identical for both systems, and only the transfer of butanol to the continuous phase distinguishes these fermentations. The data in Tables 3 and 4, and Figs. 3-5 indicate increases in both butanol level and yield (37%) caused by mass transfer to the second phase.

Finally, the effect of increased concentration of PEG is explained by performing PEG-rich, single-phased fermentation. As shown in Fig. 1, this phase was obtained by collecting the PEG-rich top phase of a settled

Table 3
Comparison of Fermentation Performance (High Glucose Concentration)*

System	Initial glucose	Residual glucose	Max. cell mass	Max. butanol	Total solvents	Final butyrate
Conventional	78.43	5.58	5.0	9.94	15.41	0.64
PEG rich	73.20	0.0	3.04	12.23	17.92	0.92
DEX rich	74.08	0.0	5.18	9.32	14.06	0.66
Two phased	75.32	0.0	5.68	12.56	20.28	0.90

*All concentrations are in g/L U.

Table 4
Comparison of Fermentation Efficiency (High Glucose Concentration)

System	$Y_{bt/glu}$	$Y_{dw/glu}$	$Y_{bt/dw}$	$Y_{sol/glu}$
Conventional	0.1364	0.0686	1.9886	0.2115
PEG rich	0.1671	0.0404	4.1333	0.2448
DEX rich	0.1258	0.0687	1.8320	0.1898
Two phased	0.1728	0.0741	2.3324	0.2693

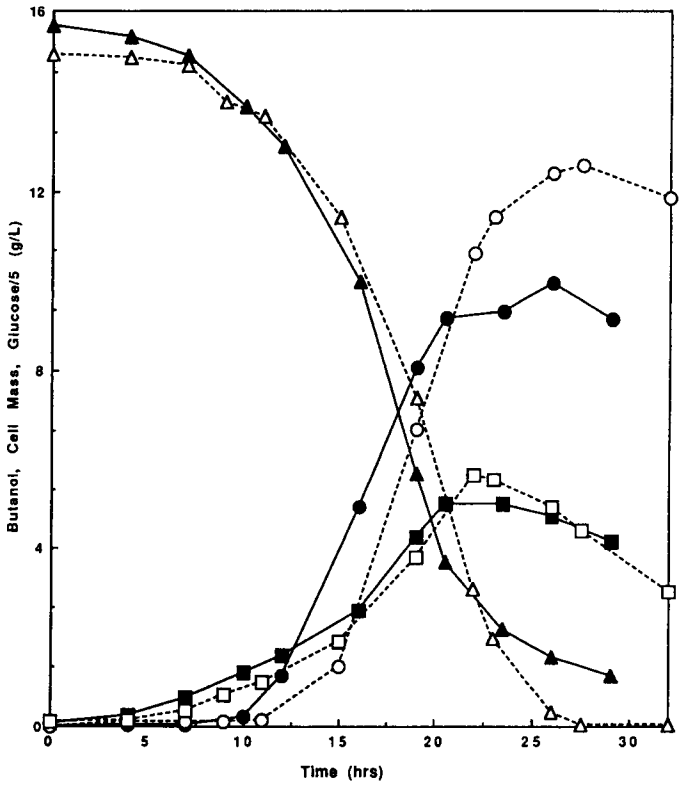

Fig. 2. Comparison between conventional and two-phased fermentation (PEG-DEX). —●— conventional, butanol; —■— conventional, cell mass; —▲— conventional, glu./5; --○-- two phased, butanol; --□-- two phased, cell mass; --△--two phased, glu./5.

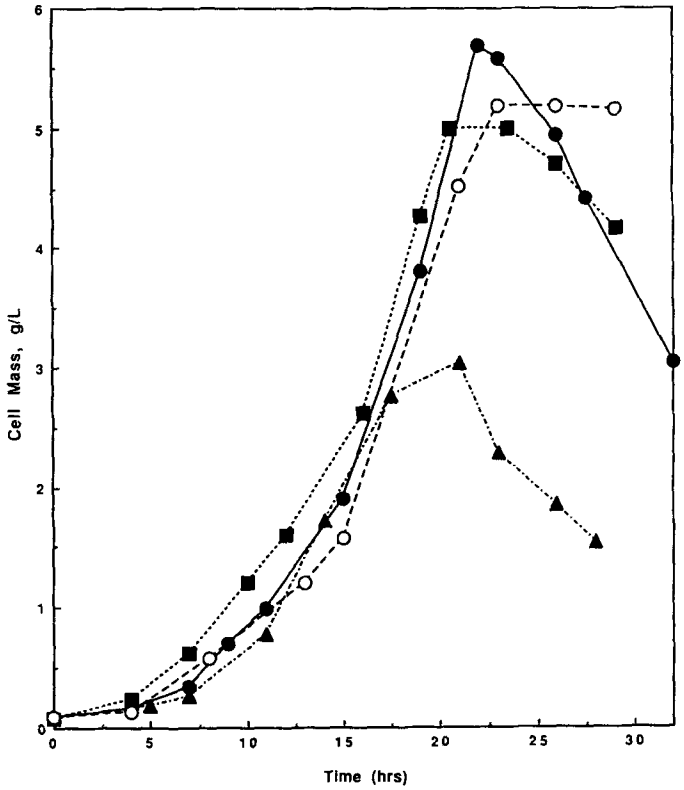

Fig. 3. Comparison of cell growth behavior. —●— two phased; —■— conventional; —▲— PEG rich; --○-- DEX rich.

two-phased system. This PEG-rich, single-phased fermentation is compared to both the conventional single-phased fermentation, which is polymer free, and the DEX-rich, single-phased fermentation. The data in Tables 3 and 4, and Figs. 3-6 indicate that the PEG-rich, single-phased fermentation has both lowered cell production and increased butanol formation compared to the other single-phased systems. These effects combined to give a dramatic increase in the butanol yield, $Y_{bt/bw}$, as shown in Table 4. In fact, the butanol level was comparable to that produced by the PEG-DEX two-phased system. These results may be explained by a variety of biological effects of PEG on cells, e.g., an ability to fuse cells (*35*), to decrease membrane fluidity (*36*), and to change liposome permeability (*37*). Of course, these higher PEG concentrations were not experienced by the cells in the PEG-DEX two-phased system, since the cells were always found to partition to the DEX-rich phase.

CONCLUSIONS

Both the PEG-DEX and the PEG-APPT aqueous two-phased systems were applied to the A-B-E fermentation in an attempt to reduce the toxic

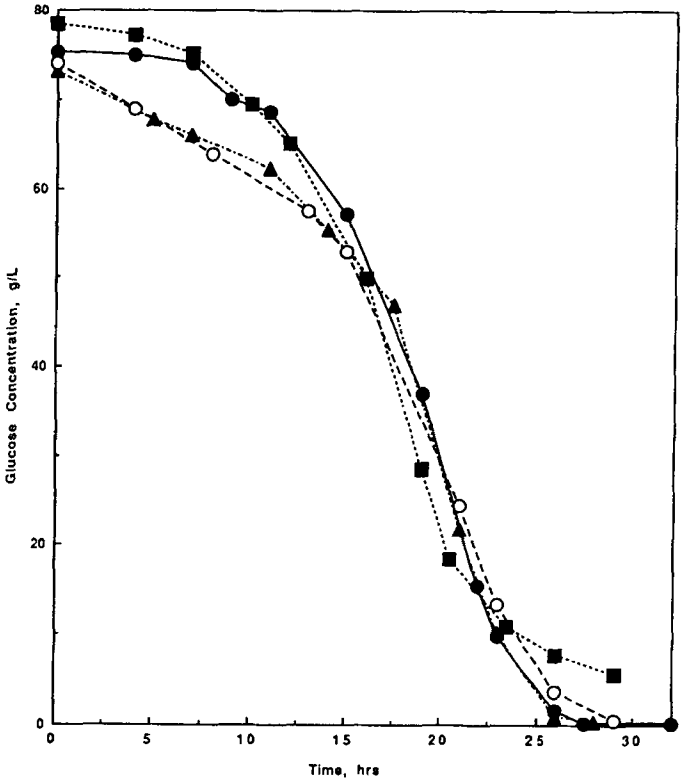

Fig. 4. Comparison of glucose consumption behavior. —●— two phased; —■— conventional; —▲— PEG rich; --○-- DEX rich.

effect of butanol. The PEG-APPT two-phased system broke down to a single-phased system during the course of the fermentation, with no improvement over the conventional single-phased fermentation. However, the PEG-DEX two-phased system increased butanol yield by 27% over the conventional fermentation (*see* Tables 3 and 4, Fig. 2). The effects of two physical differences between the PEG-DEX two-phased fermentation and the conventional single-phased fermentation, i.e., a cell environment with increased dextran concentration and the transfer of butanol from the dispersed phase, which contains the cells to the continuous phase, were explained by comparing a DEX-rich, single-phased system with both a conventional single-phased system (dextran effect) and the PEG-DEX two-phased system (mass transfer effect). The experimental data indicate that the improvement of the PEG-DEX two-phased system over the conventional single-phased system is primarily the result of mass transfer to the continuous phase. Also, the effect of increased PEG concentration in the cell environment produced by the PEG-rich, single-phased fermentation caused increased butanol concentration comparable to that produced by the PEG-DEX two-phased system.

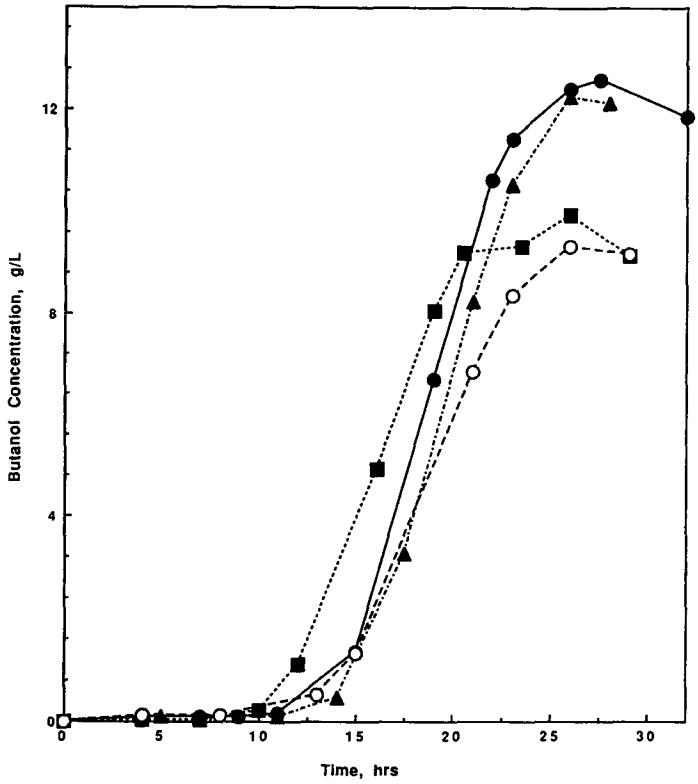

Fig. 5. Comparison of butanol formation behavior. —●— two phased; —■— conventional; —▲— PEG rich; --○-- DEX rich.

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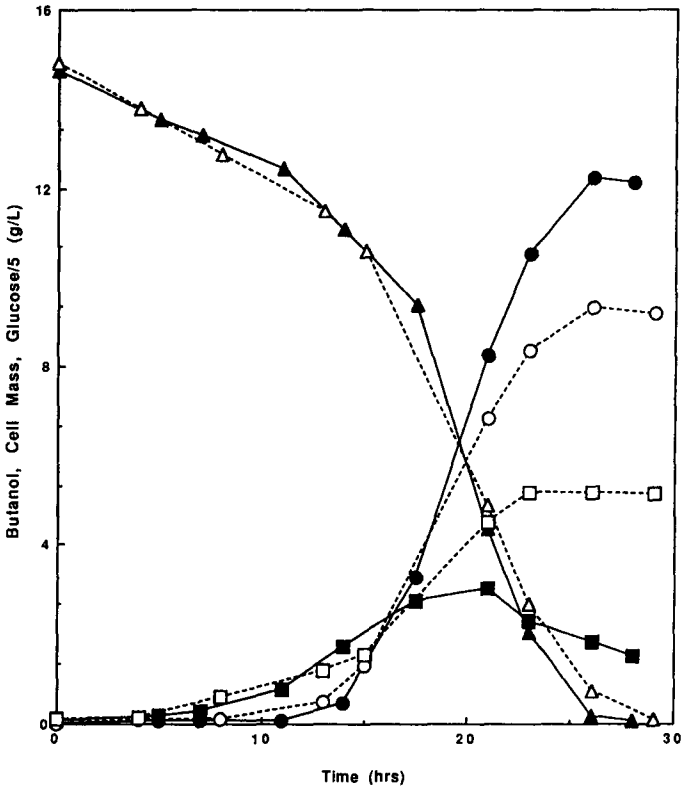

Fig. 6. Comparison between PEG-rich and DEX-rich phase fermentations. —●— PEG rich, butanol; —■— PEG rich, cell mass; —▲— PEG rich, glu./5; --○-- DEX rich, butanol; --□-- DEX rich, cell mass; --△--DEX rich, glu./5.

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