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Amine-based aqueous polymers for the simultaneous titration and extraction of lactic acid in aqueous two-phase systems

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Abstract

The partitioning of 10% (w/w) lactic acid in ethylene oxide propylene oxide (EOPO) random copolymers and dextran T500 aqueous two-phase systems was studied. An analysis of variance design was applied to investigate the effect of pH, polymer concentration, and addition of polyethyleneimine to the aqueous two-phase systems. The lowest lactate partition coefficient of 0.09 was obtained at pH 6 in the systems containing 7.2% (w/w) polyethyleneimine. The use of polyethyleneimine as titrating base during the fermentative production of lactic acid was evaluated in batch fermentations with 100 g/l glucose. Yield and productivity of polyethyleneimine titrated fermentations compared with those obtained in fermentations titrated with NaOH and KOH.

Keywords: Aqueous two-phase systems; Lactic acid

1. Introduction

Recovery of lactic acid is impeded by the complex nature of fermentation broths, by dilute product streams, and by the physico-chemical properties of lactic acid itself which cannot be distilled and which is difficult to crystallize. At present, the most widely used process for the recovery of lactic acid involves precipitation and filtration of the calcium salt of the acid [1]. Treatment of the precipitate with sulfuric acid leads to preferential precipitation of $CaSO_4$ which is filtered off. Concentration by water evaporation and purification by crystallization are used to achieve the final product specifications. This method has the disadvantage to irreversibly consume the base used for the titration of lactic acid during the fermentation and the sulfuric acid, to leave, as waste, large quantities of sulfate giving rise to disposal problems. Moreover, lactic acid crystallizes with great difficulty and low yield. The yield and purity of the final product have been improved [2], but the process remains time consuming, and operationally complex.

Alternative techniques, such as extraction and sorption, have been developed. Extraction of lactic acid can be performed by three different extractant categories: (i) carbon-bonded oxygen-bearing extractants, (ii) phosphorous-bonded oxygen-bearing extractants, and (iii) high-molecular-mass aliphatic imines [3]. The first two categories are based on the solvation of the acid by the donor bonds resulting in weak and non-specific interactions between the acid and the solvent, thus they are not recommendable for lactic acid extraction. In the third category, a specific reaction of proton transfer between the lactic acid and the imine occurs, and allows a further ion-pair interaction between the acid and the imine, bringing the lactic acid to the organic phase. This latter

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process has been intensely studied [4–8], technically improved [9–12], and patented [13,14]. In extractive fermentation, these systems have a main drawback because of their toxic effect on organisms [15]. Therefore immobilization of the amine-based compounds in solid sorbents has been used in lactic acid production [16,17]. The low capacity of the resins, typically between 0.1 and 0.2 g lactic acid/g resin, and the fact that they are solid and must be continuously added to the fermentor as lactic acid is being synthesized, makes these processes difficult to operate.

Aqueous two-phase systems (ATPS) are used for liquid–liquid extraction, and for bioconversion of different substances [18,19]. The uneven partitioning of cells in an aqueous two-phase system (ATPS), allows the removal of the product from the cell-free phase. ATPS have been used for the production of bulk chemicals [19–21], and specifically for the production of lactic acid [22–24]. However, an even distribution of lactic acid between the two phases makes the process economically non-viable. Polyethyleneimine (PEI), has been used as a phase-forming polymer in ATPS [25], and lactic acid bacteria (LAB) have been cultivated in PEI–hydroxyethyl cellulose (HEC) [26,27]. However, no major improvement with respect to reference fermentations was observed since PEI had to be titrated with sulfuric acid prior to be used as phase-forming polymer. This resulted in high sulfate concentrations in the culture broth and poor lactic acid partitioning.

In the present study, the partitioning of lactic acid in ATPS based on ethylene oxide propylene oxide (EOPO) random copolymers was investigated. EOPO copolymers are easily recyclable by temperature-induced phase separation [28] and, thus, of great technical interest. The influence of pH, polymer concentration, and the presence of PEI on the partitioning of lactic acid was studied in systems containing high concentrations of lactic acid (10% w/w). The influence of phosphate buffer on the interaction between PEI and lactic acid in ATPS was also investigated. Moreover, PEI was used as titrating base to control the pH during the fermentative production of lactic acid, and its performance was compared to that of the more traditional bases, NaOH and KOH.

2. Experimental

2.1. Chemicals

Polymer stock solutions were prepared in ultrapure water (Milli-RO4 water purification system, Millipore, Bedford, MA, USA). PEI low-molecular-mass (PEI_{lmw}) (M_r~2000) 50% (w/w) solution in water, and water-free PEI high-molecular-mass (PEI_{hmw}) (M_r~25 000) were purchased from Aldrich (Milwaukee, WI, USA). $EO_{50}PO_{50}$ (50% ethylene oxide; 50% propylene oxide random copolymer, M_r~3900) was from International Speciality Chemicals (Southampton, UK), $EO_{30}PO_{70}$ (30% ethylene oxide; 50% propylene oxide random copolymer, M_r~5000) was from Shearwater Polymers (Huntsville, AL, USA), and dextran T 500 (DEX) was from Pharmacia Biotech Norden (Sollentuna, Sweden). DL-Lactic acid 85% in water was obtained from Acros Organics (Pittsburgh, PA, USA). Lithium-lactate from Merck (Darmstadt, Germany) was used to adjust the pH of the lactate stock solutions. Melaphosphoric acid (pro analysis grade), sodium dihydrogen phosphate (extra pure grade), and disodium hydrogen phosphate (extra pure grade), from Merck were used to prepare phosphate stock solutions at different pH.

2.2. Partitioning of lactic acid

The partitioning of lactic acid was determined in different ATPS. To detect the effects of different variables and their interactions on the partitioning of lactic acid, an analysis of variance (ANOVA) design was used. The influence of pH, polymer concentration, and type of top-phase-forming polymer on the partitioning of lactic acid was investigated. The phase systems were made up by mixing appropriate amounts of polymer stock solutions with lactic acid stock solutions, which had been previously pH adjusted (PHM82 standard pH meter, Radiometer, Copenhagen, Denmark), by mixing appropriate amounts of lactic acid and lithium lactate, and adding distilled water to 10.0 g. The tubes were capped and gently shaken for 10 min. The phase separation was allowed to occur for 5 h at 24°C. When the influence of PEI_{lmw} on the partitioning was

investigated, lactic acid and PEI were mixed in the same stock solutions, but in different proportions to give the right pH. In systems containing PEI_{lmw} the effect of the concentration of phosphate buffer was also investigated. Phosphate stock solutions were made by mixing melaphosphoric acid, sodium dihydrogen phosphate, and disodium hydrogen phosphate in different proportions to yield the final pH. In all the cases the lactic acid concentration in the ATPS was about 10% (w/w).

2.3. Bacteria and culture conditions

Lactococcus lactis subsp. *lactis* 19435 (*L. lactis*) was obtained from the American Type Culture Collection (Rockville, MD, USA). Bacteria were precultured overnight in a growth medium containing per litre: peptone from soymeal, 5.0 g; peptone from meat, 2.5 g; peptone from casein, 2.5 g; yeast extract, 2.5 g; meat extract, 5.0 g; ascorbic acid, 0.5 g; Na-β-glycerophosphate, 19.0 g; magnesium sulfate, 0.25 g; glucose, 10.0 g. The growth medium and the glucose were autoclaved separately. When PEI was used as titrating base, the preculture medium contained 5% (w/w) PEI. Fermentations were performed in a New Brunswick Bioflo III fermentor (New Brunswick Scientific Co., Edison, NJ, USA) with pH control at 6.0. Temperature was maintained at 30°C and the stirring speed was 200 rpm. Fermentations were performed in 1.5 l working volume containing growth medium supplemented with 100 g/l glucose. Four different fermentations were performed and titrated with 25% (w/w) of NaOH, KOH, PEI_{lmw}, and PEI_{hmw}, respectively.

2.4. Analysis

All analyses were performed by HPLC. Eluent flow was provided by a LC pump (LC 6A Model, Shimadzu Corporation, Kyoto, Japan). Samples were injected in the flow line by an autosampler (Marathon Model, Spark Holland, Emmen, The Netherlands). The separation of glucose and fermentation products in fermentation samples, and the determination of lactic acid from lactic acid partitioning in EOPO–DEX systems, was performed at 60°C on a prepacked cation-exchange column (Aminex HPX 87-H, 300×7.8 mm I.D.; Bio-Rad laboratories, Richmond, CA, USA). The mobile phase was 0.005 *M* H_2SO_4. The analytes were detected with a differential refractometer (RID 6A Model, Shimadzu).

For the analysis of EOPO, PEI_{lmw} and lactate in samples from the partitioning studies in EOPO–DEX systems containing PEI_{lmw}, a prepacked gel exclusion chromatography column (Ultrahydrogel 500, 300×7.8 mm I.D.; Waters, Millipore Corporation, Milford, MA, USA) was used at 22°C [24]. The flow-rate was 0.6 ml/min in all cases. Standards were injected separately before the samples. Quantification was through computer integration of the area under each chromatographic peak using the EZChrom chromatographic data system software package (Scientific Software, San Ramon, CA, USA).

2.5. Analysis of the data

A statistical approach was used for the analysis of the data of lactate partitioning. The simplest version of ANOVA was applied. A linear model is used to discriminate if difference between experimental values is due to experimental error or to the effect of the different levels of each factor, that is, the different conditions in which the variable of interest is studied. There are three conditions that must be fulfilled in order to be able to use this model. (i) The distribution of the experimental error is a normal distribution centred at zero. (ii) The experimental error is independent. (iii) The variances are homogeneous. To check the validity of the model simple tests were performed: (i) a frequency diagram of the residuals was plotted, giving in all the cases a normal-like diagram centred at zero; (ii) residuals were then plotted against dependent variables and no specific trends could be observed in any case; (iii) finally a plot of residuals against the expected value of response did not show any trend in any of the cases.

In the ANOVA table the degree of freedom (DF), the mean squares (MS), the experimental *F*, and the significance level for the experimental *F* (*P*), are shown. The experimental *F* value is the ratio be-

tween the MS associated to each factor or interaction over the MS associated to the residuals. *P* is a probability value, and is the area under the curve left to the right of the experimental *F* value in the *F* distribution curve [29]. A decision about the presence of significant differences between the levels of a factor is made at a certain probability level, which in this study was 0.05. Above this probability value, the observed differences between the levels of each factor or interaction cannot be considered significant, rather than due to experimental error. Below the probability threshold the differences between at least two of the levels of the studied factor can be considered significant, and due to the different experimental conditions. In the case where a factor contains more than two levels, the ANOVA results do not discriminate levels being significantly different from each other. In this case pairwise comparisons must be made. Tukey's and Scheffè's tests are most commonly used to check pairwise differences [29]. The partitioning data were analyzed with ANOVA and Tukey's and Scheffè's tests using Statistix software package (InVision Interactive, Palo Alto, CA, USA).

3. Results

3.1. Lactate partitioning in EOPO-DEX 500 ATPS

A three-factorial ANOVA design was applied to investigate the effect of pH, top polymer composition, and polymer concentration on the partitioning of lactic acid in EOPO–DEX ATPS. Four different pH levels were studied: 2.0, 2.8, 3.4, and 5.0. Two different top-phase polymers were used, $EO_{50}PO_{50}$ and $EO_{30}PO_{70}$, and three different polymer concentration levels, low (4.0%$EO_{50}PO_{50}$–6.0%DEX; 6.5%$EO_{30}PO_{70}$–4.0%DEX), medium (6.0%$EO_{50}PO_{50}$–8.0%DEX; 6.5%$EO_{30}PO_{70}$–6.5%DEX), and high (8.0% $EO_{50}PO_{50}$–10.0% DEX; 8.0%$EO_{30}PO_{70}$–8.0%DEX). The different polymer concentrations were chosen so that the phase systems were increasingly moved away from the critical point. The experimental results are shown in Figs. 1 and 2, where each point represents the average value of three replicates. The results of the statistical analysis of these data are shown in Table 1. According to the ANOVA results, the lactic acid partitioning in EOPO-based systems was influenced by pH and type of top-phase-forming polymer as seen by $p<$

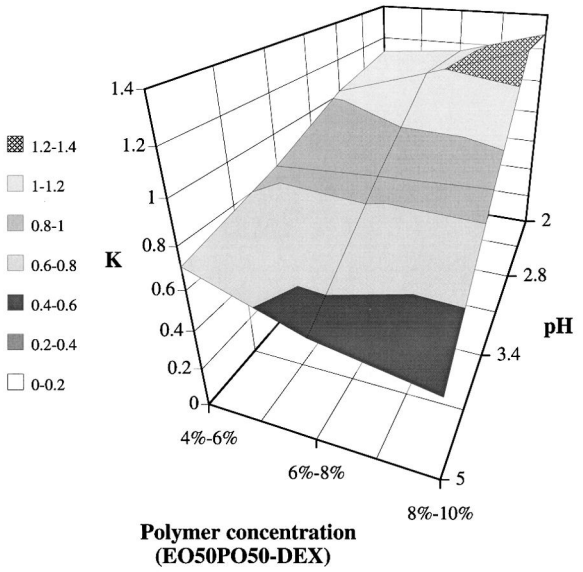

Fig. 1. Partitioning of lactic acid at 10% (w/w) in $EO_{50}PO_{50}$–DEX systems at different pH values and polymer concentrations. The different pH values were adjusted by mixing the appropriate amounts of lactic acid and lithium lactate.

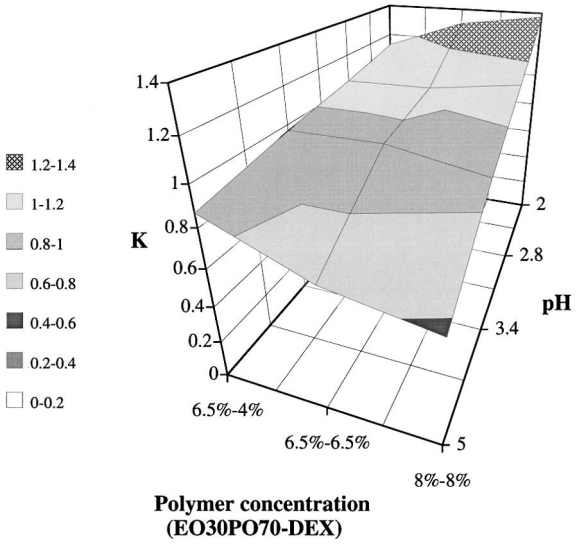

Fig. 2. Partitioning of lactic acid at 10% (w/w) in $EO_{30}PO_{70}$–DEX systems at different pH values and polymer concentrations. The different pH values were adjusted by mixing the appropriate amounts of lactic acid and lithium lactate.

Table 1
ANOVA table of lactic acid partitioning in EOPO–DEX systems at different pH values, top-phase polymers and polymer concentration

Source	DF	MS	*F*	*P*
pH (A)	3	1.254	179.89	0.000
Top-phase polymer (B)	1	0.035	5.03	0.029
Polymer concentration (C)	2	0.005	0.67	0.514
A*B	3	0.058	8.26	0.000
A*C	6	0.089	12.75	0.000
B*C	2	0.007	0.99	0.380
A*B*C	6	0.010	1.47	0.209
Residual	48	0.007		

K_{lac} was the response variable. Three replicates for each experimental condition allowed the analysis of statistical interactions, which were expressed as the product of the main factors. See text for details.

0.05. The polymer concentration had no significant net influence on K_{lac} ($p>0.05$). A statistical interaction between pH and type of top-phase-forming polymer significantly influenced K_{lac} ($p<0.05$). To detect pairwise significant differences between the means within the factors, and the statistical interactions, both Scheffè's and Tukey's test were applied and gave the same results. Thus, K_{lac} values were significantly different at each pH level. All the contrasts of the statistical interaction between pH and top polymer were performed and shown to be significant. The statistical interaction between pH and polymer concentration, was found to be significant in all cases except for the polymer concentration at pH 3.4.

In ATPS using $EO_{50}PO_{50}$ as top-phase-forming polymer, K_{lac} changed from 1.11 at pH 2.0 to 0.71 at pH 5.0 at low polymer concentration. At high polymer concentration K_{lac} changed from 1.29 at pH 2.0 to 0.42 at pH 5.0 (Fig. 1). When the top-phase polymer was substituted by $EO_{30}PO_{70}$, K_{lac} changed from 1.13 at pH 2.0 to 0.87 at pH 5.0 at low polymer concentration. At high polymer concentration the change was from 1.38 at pH 2.0 to 0.57 at pH 5.0 (Fig. 2). Independently of the top-phase-forming polymer, and polymer concentration, a qualitative change in the partitioning characteristics of lactic acid occurred in the region between pH 2.8 and pH 3.4. In this region, as the pH increased, lactic acid moved from the top phase to the bottom phase.

3.2. Lactate partitioning in EOPO-DEX 500 ATPS containing PEI

The effect of PEI_{lmw} on K_{lac} in EOPO–DEX systems was investigated using a two-factorial ANOVA design. The first factor, here called 'pH factor', is a combination of pH and PEI concentration, since PEI was used to adjust the pH of the different systems so that the PEI concentration increased with pH. This factor was studied at six different pH levels: 2.8, 3.4, 4.0, 4.5, 5.0 and 6.0. The polymer concentration factor was studied at three different levels, low (6.5%$EO_{30}PO_{70}$–4.0%DEX), medium (6.5%$EO_{30}PO_{70}$–6.5%DEX), and high (8.0%$EO_{30}PO_{70}$–8.0%DEX). The different polymer concentrations were chosen so that the phase systems were increasingly moved away from the critical point. The experimental results are shown in Fig. 3, where each point represents the average value of three replicates. The results of the statistical analysis of these data are shown in Table 2. According to the ANOVA results, the lactic acid partitioning in EOPO-based systems containing PEI was influenced by pH and the polymer concentration ($p<0.05$), and the statistical interaction between these two factors was significant ($p<0.05$). To detect pairwise significant differences within the factors, and the statistical interactions, both Scheffè's and Tukey's test were used and gave the same results. Thus there was no significant difference between low and medium polymer concentrations, but between them and high polymer concentration. Conversely, all levels of the pH factor were significantly different. The effect of the polymer concentration was significantly influenced by pH only at pH 2.8, 3.4 and 6.0. On the other hand, the 'pH effect' was influenced by the polymer concentration at low and high concentrations, but no significant statistical interaction was observed at medium polymer concentration.

Maximum K_{lac} values of 1.04 were obtained at medium and high polymer concentrations at pH 2.8, corresponding to a PEI concentration of 2.2% (w/w). Minimum values of 0.09 were obtained at low and medium polymer concentrations at pH 6, corresponding to a PEI concentration of 7.2% (w/w). At low and medium polymer concentrations, the K_{lac} curve had a inflexion point between pH 4.0 and 4.5,

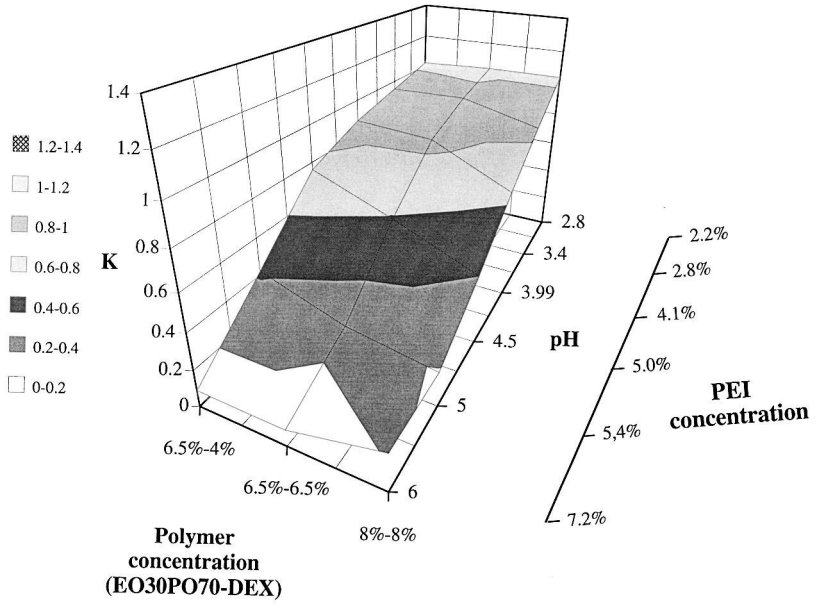

Fig. 3. Partitioning of lactic acid at 10% (w/w) in $EO_{30}PO_{70}$–DEX systems at different pH values and polymer concentrations. The different pH values were adjusted by mixing the appropriate amounts of PEI to the different lactic acid stock solutions, thus PEI concentration is also changing with the pH.

from which the slope of the curve became steeper as pH increased (Fig. 3).

Since the polymer content of the phases was determined, the natural logarithm of the partition coefficients obtained at the different pH values (Fig. 3) were plotted against the ratio of the $EO_{30}PO_{70}$ concentration in the top phase and the overall system ($EOPO_{top/tot}$) for the respective systems at the different pH values obtained by PEI titration (Fig. 4). In these systems there was a linear relation between the natural logarithm of K_{lac} and the $EOPO_{top/tot}$,

$$\ln K_{lac} = 0.48(\pm 0.08) - 0.33(\pm 0.02) \cdot EOPO_{top/tot} \quad (r^2 = 0.954) \quad (1)$$

and the natural logarithm of K_{PEI} and $EOPO_{top/tot}$,

$$\ln K_{PEI} = 0.62(\pm 0.07) - 0.43(\pm 0.02) \cdot EOPO_{top/tot} \quad (r^2 = 0.968) \quad (2)$$

The relation between K_{PEI} and K_{lac} was also linear in these systems (Fig. 5),

$$K_{lac} = 0.18(\pm 0.05) + 1.00(\pm 0.09) \cdot K_{PEI} \quad (r^2 = 0.964) \quad (3)$$

Table 2
ANOVA table of the lactic acid partitioning in $EO_{30}PO_{70}$–DEX systems containing the necessary amount of PEI to adjust the systems to different pH values

Source	DF	MS	*F*	*P*
pH (A)	5	1.151	238.14	0.000
Polymer concentration (B)	2	0.066	13.70	0.000
A*B	10	0.033	6.91	0.000
Residual	36	0.005		

The effect of pH and polymer concentration were simultaneously studied. K_{lac} was the response variable. Three replicates for each experimental condition allowed the analysis of statistical interactions, which were expressed as the product of the main factors. See text for details.

3.3. Effect of phosphate on K_{lac} in PEI-containing ATPS

Since phosphate buffers are used to provide the right osmotic conditions in fermentations, the effect of Na-phosphate on K_{lac} in PEI_{lmw}-containing sys-

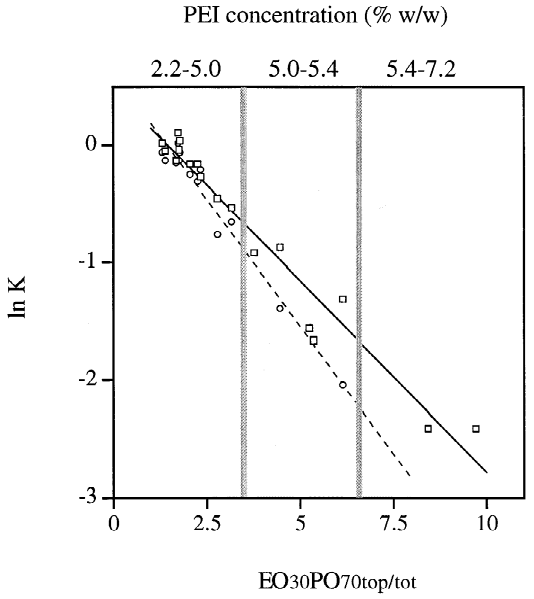

Fig. 4. Relation between $\ln K_{lac}$ (□) and $\ln K_{PEI}$ (○) with $EO_{30}PO_{70top/tot}$ in $EO_{30}PO_{70}$–DEX systems containing 10% (w/w) lactic acid and different concentrations of PEI_{lmw} used to adjust the ATPS to different pH. The continuous line corresponds to $\ln K_{lac}$-fitted data (Eq. (1)), and the dotted line to $\ln K_{PEI}$-fitted data (Eq. (2)). The experimental partitioning points correspond to those obtained in the study of the influence of pH and polymer concentration (Fig. 4).

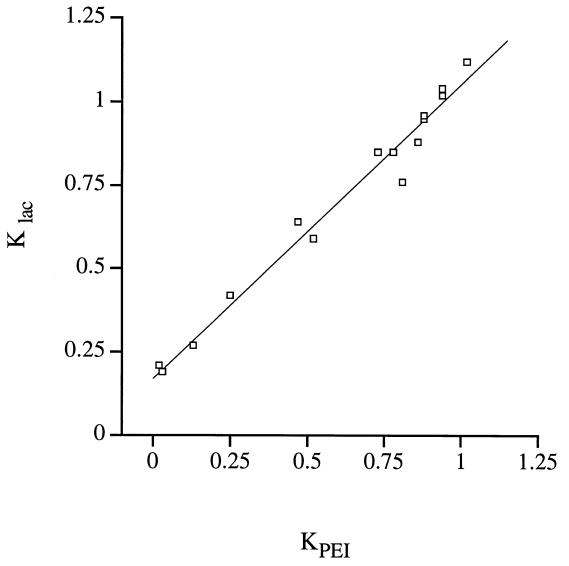

Fig. 5. Partitioning of lactic acid versus partitioning of PEI in $EO_{30}PO_{70}$–DEX systems. (□) Experimental points obtained at different polymer concentration and different pH; the straight line corresponds to the fitted data to Eq. (3).

tems was determined (Table 3) using a two-factorial ANOVA design. The 'pH factor', a combination of pH and PEI concentration, was studied at four different pH levels: 2.8, 3.4, 5.0 and 6.0. The phosphate concentration factor was studied at three different levels (g/l): 0, 5, and 20. According to the ANOVA results, the lactic acid partitioning was influenced by pH and the concentration of phosphate and the statistical interaction between these two factors was significant (Table 4). To detect pairwise significant differences within the factors and the statistical interactions, both Scheffè's and Tukey's tests were applied and gave the same results. Differences between K_{lac} were significant at all levels of the 'pH factor'. There were no significant differences for K_{lac} between 0 and 5 g/l phosphate, but between them and 20 g/l phosphate. The phosphate concentration effect was significantly influenced by pH at all pH values except 3.4.

Since the polymer content of the phases was determined, the natural logarithm of the partition coefficients obtained at the different pH (Table 3) were plotted against the ratio of the $EO_{30}PO_{70}$ concentration in the top phase and the overall system ($EOPO_{top/tot}$) for the respective systems at the different pH values obtained by PEI titration. In systems containing phosphate there was a linear relation between the natural logarithm of K_{lac} and $EOPO_{top/tot}$,

Table 3
Partition coefficient of lactic acid at 10% (w/w) and partition coefficient of PEI in 6.5% (w/w) $EO_{30}PO_{70}$–6.5% (w/w) DEX systems at different pH values and phosphate concentrations

pH	Phosphate concentration (g/l)					
	0		5		20	
	K_{lac}	K_{PEI}	K_{lac}	K_{PEI}	K_{lac}	K_{PEI}
2.8	1.04	0.94	1.02	0.79	0.98	—
3.4	0.96	0.88	0.94	0.78	0.78	0.53
5.0	0.27	0.10	0.59	0.47	0.29	—
6.0	0.09	—	0.43	0.31	0.26	0.10

The different pH values were obtained by adding the appropriate amount of PEI to the different lactic acid stock solutions, thus the PEI concentration changes with pH.

Table 4
ANOVA table of the lactic acid partitioning in $EO_{30}PO_{70}$–DEX systems containing phosphate buffer, and the necessary amount of PEI to adjust the systems to different pH values

Source	DF	MS	*F*	*P*
pH (A)	3	1.177	306.18	0.000
Phosphate (B)	2	0.087	22.55	0.000
A*B	6	0.036	9.35	0.000
Residual	24	0.004		

The effect of pH and phosphate buffer were simultaneously studied. Three replicates for each experimental condition allowed the analysis of statistical interactions, which are expressed as the product of the main factors. See text for details.

$$\ln K_{lac} = 0.62(\pm 0.09) - 0.31(\pm 0.02) \cdot \mathrm{EOPO}_{top/tot} \; (r^2 = 0.972) \quad (4)$$

The relation between K_{PEI} and K_{lac} was also linear in these systems,

$$K_{lac} = 0.25(\pm 0.09) + 1.0(\pm 0.2) \cdot K_{PEI} \; (r^2 = 0.901) \quad (5)$$

3.4. Polyethyleneimine as fermentation titrating base

The performance of PEI_{lmw} and PEI_{hmw} as titrating bases in the fermentative production of lactic acid was compared with two of the most commonly used titrating bases, NaOH and KOH. The growth curves of the fermentations in which NaOH and KOH were used were superimposable reaching maximum absorbance of about 11.5 a.u. (Fig. 6). When PEI_{lmw} was used, the growth curve followed the ones where NaOH and KOH were used, but reached a maximal absorbance of about 14 a.u. In the fermentation that was titrated with PEI_{hmw}, the growth curve showed an initial lag phase, followed by a logarithmic phase with a maximum absorbance of 12.5 a.u.

In all four fermentations the lactate concentration was above 75 g/l within 45 h (Fig. 7). The cumulative volumetric productivity showed two different patterns (Fig. 8). When NaOH and KOH were used, the cumulative productivity increased to a maximum of about 4 g/l h^{-1} within 6 h, and then decreased to 2.9 and 2.1 g/l h^{-1} for NaOH and KOH, respectively, when the yield was 0.8 g lactic acid/g glucose.

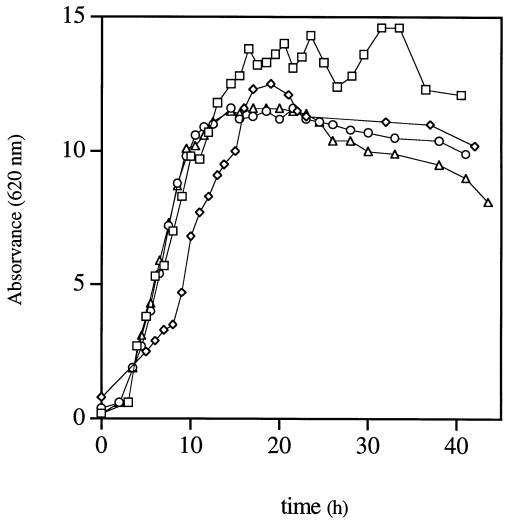

Fig. 6. Growth curves of *Lactococcus lactis* growing in a complete medium in which pH was controlled by the addition of (△) NaOH, (○) KOH, (□) PEI_{lmw}, and (◇) PEI_{hmw}.

When PEI was used, it took 11 h to reach the maximum productivities of 2.9 and 2.4 g/l h^{-1}, that decreased to 2.6 and 1.9 g/l h^{-1} for PEI_{lmw} and PEI_{hmw}, respectively, when the yield was 0.8 g lactic acid/g glucose.

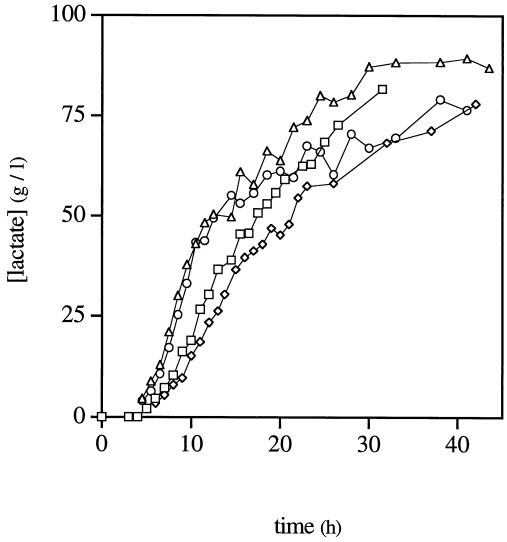

Fig. 7. Lactic acid concentration during the growth of *Lactococcus lactis* in a complete medium in which pH was controlled by the addition of (△) NaOH, (○) KOH, (□) PEI_{lmw}, and (◇) PEI_{hmw}.

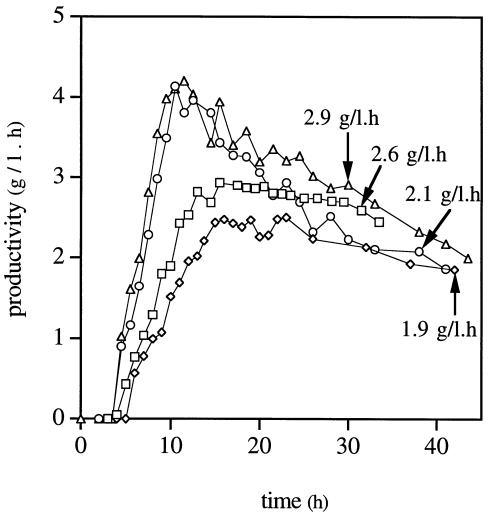

Fig. 8. Cumulative lactic acid productivity during the growth of *Lactococcus lactis* in a complete medium in which pH was controlled by the addition of (△) NaOH, (○) KOH, (□) PEI_{lmw}, and (◇) PEI_{hmw}. The productivity values correspond to the points were the yield achieved 0.8 g lactate/g glucose.

4. Discussion

Partition coefficients well separated from 1 are required to use ATPS in extractive processes. In the present study, the partitioning of lactic acid was investigated in different ATPS to develop an ATPS for extractive lactic acid production. K_{lac} increased from values below 1 to values above 1 when the pH decreased from 5 to 2. An area around pH 3.4 in which K_{lac} was about 1, and where the polymer concentration had no effect, was observed. At pH values around the p*K* of the acid (the p*K* of lactic acid is 3.8) half of the lactic acid species are in the protonated form and half in the ionized form, which favours a distribution compensation yielding no net partitioning of the lactic acid. The polymer concentration effect depended on pH, thus at pH 3.4 there is no effect of polymer concentration, while above 3.4 increasing polymer concentrations increased K_{lac}, and below 3.4 increasing polymer concentrations decreased K_{lac}, which agrees with the observation that increasing polymer concentrations makes partitioning more uneven [30]. Two phenomena might be involved in the partitioning of high concentrations of lactic acid in EOPO–DEX systems. (i) Hydrophobic interactions, which make lactic acid partition to the relatively more hydrophobic top phase when the lactic acid is protonated (at pH below its p*K*), and to the relatively more hydrophilic bottom phase when it is charged (at pH above its p*K*). The partitioning of amino acids in EOPO–water systems has been shown to be related to its hydrophobicity when the pH of the systems was close the p*I* of the amino acid [31]. The lactic acid in the protonated form is more hydrophobic than the deprotonated lactate, which explains the partitioning to the EOPO-rich phase at pH below its p*K*. (ii) A salting-out effect which increases as pH increases and the main lactic acid form is lactate. It has been demonstrated that the two-phase area in EOPO–water systems containing different sodium carboxylates is increased as the hydrophobicity of the sodium carboxylate decreases, moreover, a strong repulsion between sodium acetate and EOPO was observed [32]. The salting out effects leads to a stronger partitioning of the lactate to the more hydrophilic dextran phase

To adjust the pH of the different phase systems, lactic acid was titrated with PEI (Fig. 3). Thus, as pH increased the PEI concentration increased and the lactic acid was progressively converted from its protonated form to its charged form. In the pH range used PEI was positively charged. The addition of PEI_{lmw} into $EO_{30}PO_{70}$–DEX systems gave a response surface which decreased from K_{lac} of about 1 at pH 2.8, down to K_{lac} of 0.09–0.2 at pH 6. Changes in the EOPO concentration in the top phase were observed by means of gel-exclusion chromatography, increasing concentrations of EOPO in the top phase were observed as the concentration of PEI increased from 2.2 to 7.2% (w/w) (Fig. 4). The partitioning of the charged PEI to the DEX-rich phase was increasing as PEI concentration increased in the system, leading to a strong partitioning of lactic acid to the DEX-rich phase, and a simultaneous transfer of water from the EOPO-rich phase to the DEX-rich phase. A linear relation between $\ln K_{lac}$ and $EOPO_{top/tot}$ was obtained (Fig. 4, Eq. (1)), showing that the EOPO concentration in the top phase increased and that simultaneously K_{lac} decreased with increasing PEI concentrations. The same linear relation was observed for $\ln K_{PEI}$, although here the slope was steeper (Fig. 4, Eq. (2)).

This shows how the increase in PEI concentration in the system leads both to an increase of its partitioning, and an increase in the concentration of the EOPO in the EOPO-rich phase having, as a result, a strong partitioning of lactic acid to the dextran phase together with PEI (Fig. 5).

Lactic acid in the protonated form will partition to the EOPO-rich phase in EOPO–DEX systems due to hydrophobic interactions. These interactions will be less important as pH increases due to PEI addition and the electrostatic interactions with the positively charged PEI will dominate. These are revealed by the linear relation between K_{lac} and K_{PEI} with a slope close to 1 suggesting a strong interaction between lactate and PEI (Fig. 5). The interaction between imines and lactate has been well characterized in aqueous–organic two-phase systems [5], and in chromatographic systems [33].

The observed changes in K_{lac} in the presence of phosphate can be explained by a more even partitioning of PEI between the phases (Table 3). The equation for the relation between K_{PEI} and K_{lac} had a slope of 0.88 in the absence of phosphate and a slope of 1.00 in the presence of phosphate, independent of the phosphate concentration.

PEI (M_r 50 000–60 000) (PEI_{50}) is a phase-forming polymer when titrated at neutral pH with di- or trivalent acids, but not when titrated with monovalent acids [25]. In the present work PEI_{lmw} was used as titrating base rather than as phase-forming polymer, and a lactic acid concentration of 82 g/l with a maximum productivity of 2.6 g/l was achieved. In PEI_{50}–hydroxyethyl cellulose (HEC) ATPS in which PEI_{50} was titrated with H_2SO_4 [26,27], K_{lac} was 0.48. However, when these ATPS were used for batchwise fermentative production of lactic acid, the maximum lactic acid concentration and the maximum productivity were only 70 g/l and 1.15 g/l h^{-1}, respectively. PEI being a strong base requires considerable amounts of H_2SO_4 to titrate to neutral pH and additional base must be added during the fermentation to titrate the produced lactic acid, with the effect that the extractive capacity of PEI decreases substantially with the competing effect of other salts, and that bacterial growth is impaired by increasing salt concentrations. Thus the cost of the polymers, and that of titrating chemicals add to the fermentation costs without any improvement in fermentation performance. Conversely, using PEI as titrating base improves the fermentation performance, reduces the operation costs and allows interaction between PEI and lactic acid free of interferences.

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