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CULTIVATION OF *Lactococcus lactis* IN A POLYELECTROLYTE-NEUTRAL POLYMER AQUEOUS TWO-PHASE SYSTEM.

Ulla Dissing and Bo Mattiasson*
Department of Biotechnology, Chemical Center, Lund University, P.O.Box 124,
S-221 00 Lund, Sweden.

Key Words: aqueous two-phase system, (hydroxyethyl)cellulose, poly(ethylene imine), *Lactococcus lactis,* extractive bioconversion, in situ product recovery.

Abbreviations: PEG; poly(ethylene glycol), PEI; poly(ethylene imine), HEC; (hydroxyethyl)cellulose.

Abstract

The possibility to cultivate *Lactococcus lactis* in aqueous polymer two-phase system has been investigated. The phase system was made up of poly(ethylene imine) and (hydroxyethyl) cellulose. Long lag phases were needed for the microorganism to adapt to the polymer rich media. Cells favoured the (hydroxyethyl)cellulose rich top phase or they accumulated at the interface, while lactic acid showed affinity for the poly(ethylene imine) rich phase.

Introduction

Lactococcus lactis excretes lactic acid to the growth medium to concentrations that are inhibitory to its growth. Integration of product removal with the fermentation is a means to reduce the end product inhibition and thus increase the productivity (Srivastava *et al.*,1992; Schöller *et al.*, 1993; Hjörleifsdottir *et al.*, 1990; Mattiasson and Holst, 1991). One such technique is extractive fermentation which can be operated both on small and large scale. Ion pair extraction with organic solvents has been tried, however, many extractants were found to be toxic to the microorganisms (Seevaratnam *et al.*, 1991; Hano *et al.*, 1993; Yabannavar and Wang, 1991).

Aqueous two-phase systems provide a biocompatible environment for the cells and have a potential to be used for extractive fermentations (Albertsson, 1986; Andersson *et al.*, 1988). The aqueous polymer systems reported so far have mostly been made of uncharged polymers such as poly(ethylene glycol) (PEG) and dextran, or polymer-salt systems such as PEG-potassium phosphate or PEG-magnesium sulfate. The distribution of cells between the phases depends on the surface properties of the cells, such as charge and hydrophobicity. In order to establish extraction of inhibiting substances the cells must partition to only one phase. This is fairly easy to accomplish since particulate matter often show one-sided partitioning. Small molecules, however, often partition evenly between the phases. In such cases, an uneven phase volume ratio can be used in order to achieve maximal extraction of the substance from the phase with microorganisms.

Materials and methods

Materials

(Hydroxyethyl)cellulose 20 (viscosity 0.02 Pa s 2% in water 20 °C) was obtained from Serva Feinbiochemica, Heidelberg, Germany and poly(ethylene imine) (Mw= 50 000-60 000) from Janssen Chimica, Beerse, Belgium. All other chemicals were of analytical grade. The microorganism used in this investigation was *Lactococcus lactis* 65.1 obtained from Swedish Diary Association, Lund, Sweden.

Culture media

The culture medium for cultivating *L. lactis* contained in g/l: glucose 10; yeast extract 5.0; tryptone 5.0; casamino acids 1.0; K_2HPO_4 2.5; KH_2PO_4 2.5; $MgSO_4 \cdot 7H_2O$ 0.5. The pH was 6.5. Glucose and magnesium solutions were autoclaved separately. The inoculum of the organism was prepared in the same medium.

Preparation of two-phase systems

Stock solutions in water of PEI (6% w/w) and HEC (3% w/w) were prepared and then stored at 4 °C. HEC demanded heating in order to dissolve. The PEI solution was adjusted to desired pH using H_2SO_4. PEI and HEC solutions were mixed and diluted with distilled water to the desired concentration of polymers for making the two-phase system.

Cultivation of *L. lactis* in HEC/PEI- H_2SO_4 two-phase systems

Autoclaved two-phase system of pH 6.5 was mixed with autoclaved nutrition medium to give the final concentrations of polymers: PEI, 3% and HEC, 0.75% (w/w). The system was inoculated with *L. lactis* cells and placed in a water bath with shaking at 30 °C (cultivation volumes were 40 ml). Different concentration of nutrients were used: 5g/l of glucose and half the concentration of other components as described earlier; 10 and 20g/l of glucose and the concentration of other components as described earlier. For each of these cultivations identical references without the two-phase system (distilled water was used in place of polymer solutions) were studied.

Preparation of cells for partition experiments

L. lactis were cultivated in a water bath with shaking at 30 °C. The cells were harvested by centrifugation at 10 000*g for 10 minutes, washed once by suspension in distilled water and centrifuged again.

Partition experiments

Cells (0.3g/l, dry weight) were suspended in a two-phase system from which aliquots of 2g were with-drawn and put in graded (0.1 ml) test tubes. Solution of Na_2SO_4 or sodium phosphate buffer of the same pH as the two-phase system was added and the mixtures were diluted with distilled water to give the final polymer concentrations: PEI, 3% and HEC, 0.75% (w/w). The tubes with the phase systems were shaken and subsequently left for 60 minutes for the phases to separate. The phase volumes were read visually, top and bottom phases were withdrawn and analysed for cell content.

Analytical methods

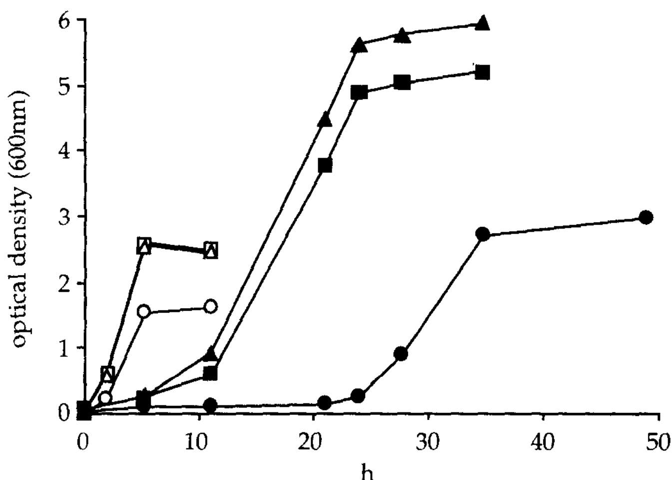
Cell densities were measured both by way of optical density at 600 nm and as dry weights. Optical density was measured on samples prepared by adding 0.5 ml of 0.5 M Tris-HCl, pH 8.5 to 0.2-0.5 ml phase system with cells and adjusting with distilled water to one ml. Dry weights of biomass were measured by filtration through a membrane filter (0.45 µm), washing with 0.9% NaCl and then drying for 90 minutes at 105 °C before being weighed. Lactic acid was measured using an enzymatic procedure (Sigma Chemical Co., St Louis, Missouri, USA, No. 726UV/826UV).

Results and discussion

This paper reports on the possibility to use aqueous two-phase system for cultivation of *Lactococcus lactis*. The two-phase system used in this study was composed of PEI (Lukovkin *et al.*, 1973) and HEC (Reuben, 1984) and separates in a HEC rich top phase and a PEI rich bottom phase (Dissing and Mattiasson, 1993). PEI is positively charged with a charge density that depends on pH. If the lactate produced by the microorganism forms ion pairs with the positive charges on PEI, the two-phase system would be suitable for extractive fermentation.

The cultivation of *L. lactis* in PEI/HEC (3% (w/w) and 0.75% (w/w) respectively) polymer two-phase system was successful (Figure 1), although long lag phases were obtained. The cultivation of *L. lactis* in presence of only HEC, did not differ from the reference cultivations i.e. without the polymers. Thus, the obtained lag phase must depend on the presence of PEI. Prolongation of the lag phase has been observed earlier for cells in aqueous two-phase systems (Larsson *et al.*, 1988). *L. lactis* has very complex nutritional requirements and lower concentrations of nutrients (cultivations performed with 5g/l glucose) together with the presence of the two-phase system gave remarkably long lag phases.

Fig.1
Cultivations of *L. lactis* in shake flasks without pH regulation: filled symbols with PEI/HEC two-phase system (3%/0.75%); and open symbols without. Glucose concentrations were (o) 5g/l; (□) 10g/l and (Δ) 20g/l. The cultivation with 5g/l glucose had half the concentration of the nutrients as described in materials and methods while 10g/l and 20g/l had the concentrations as described.



The growth of *L. lactis* was inhibited by its product lactic acid and the resulting low pH (cultivations were done on shaking water bath without pH regulation). Since PEI has buffering capacity, it was found that pH at the end of the fermentations were higher for all cultivations in two-phase system as compared to those without (Table 1). The cultivations made at higher glucose concentration (20g/l), without two-phase system, appear to be inhibited before all glucose has been fermented as no increase in cell density and lactic acid concentration was obtained as compared to cultivations in 10 g/l glucose. These data can be compared with those of the cultivations performed in the two-phase systems which showed an increase in biomass and lactate concentration when the glucose concentration was increased from 10 to 20 g/l. The concentration of lactic acid measured at the end of the cultivations were sometimes (for the long time cultivations with two-phase systems) higher than theoretically possible, which probably was a result of evaporation of water from broth. (This would also have affected the measured

cell density).

Table I Effect of PEI/HEC two-phase system on the cultivation of *Lactoccoccus lactis.*

glucose (g/l)	two-phase system	pH	lactic acid (g/l)	dry weight (g/l)	Yp/x
5	-	4.3	5.1	0.55	9.3
10	-	4.4	8.0	0.86	9.3
20	-	4.3	7.8	0.88	8.9
5	+	5.6	5.7	1.2	4.8
10	+	5.0	11	1.8	6.1
20	+	4.6	19	*	*

(*; value not measured)

All values measured at the end of the cultivations: (+) when two-phase systems were present; (-) absent. The cultivations with 5g/l glucose had half the concentration of other nutrients as described in materials and methods while 10 g/l and 20g/l had the concentrations as described. The yield coefficient Yp/x is calculated from p; lactic acid and x; dry weight.

The yield coefficient Yp/x (lactic acid/cell mass) for a fermentation can differ due to growth at different pH-values. *Lactobacillus delbrueckii* was shown to give a lower yield factor Yp/x when cultivated at higher pH (Luedeking and Piret, 1959). Also *L. lactis* had lower Yp/x when cultivated at higher pH i.e. in the two-phase systems.

If extractive bioconversions are to be performed with aqueous two-phase systems, one sided cell partition is desired. The PEI/HEC systems have been found to be sensitive to pH and the nature and concentration of ions (especially multivalent anions) (Dissing and Mattiasson, 1993). Therefore, the partition behaviour of *L. lactis* in PEI/HEC systems was studied at various concentrations of Na_2SO_4 and sodium hydrogen phosphate at pH 5.5 (salts that are present in culture media) and the results are presented in Figure 2.

As partition of cells is difficult to express in K values (concentration in top phase/ concentration in bottom phase) due to cell gathering at the interface, the percentage of cells added to the system present in either phase was used to describe the partition behaviour. The systems used were fairly close to the binodial, therefore the settling times of the phases, 60 minutes in some cases (systems with low concentration of Na_2SO_4 and sodium hydrogenphosphate), were long. In the cultivation experiments, however, additional media components influenced the system so that a more efficient phase separation took place.

L. lactis avoids the PEI-rich bottom phase and the addition of Na_2SO_4 and sodium hydrogen phosphate did not change the amount of cells partitioned in the bottom phase even though the phase volume ratios changed. The accumulation of cells at the interface was however affected by the addition of salts. As it could not be excluded that sedimented cells from the top phase had been gathered at the interface the partitioning of cells to the top phase

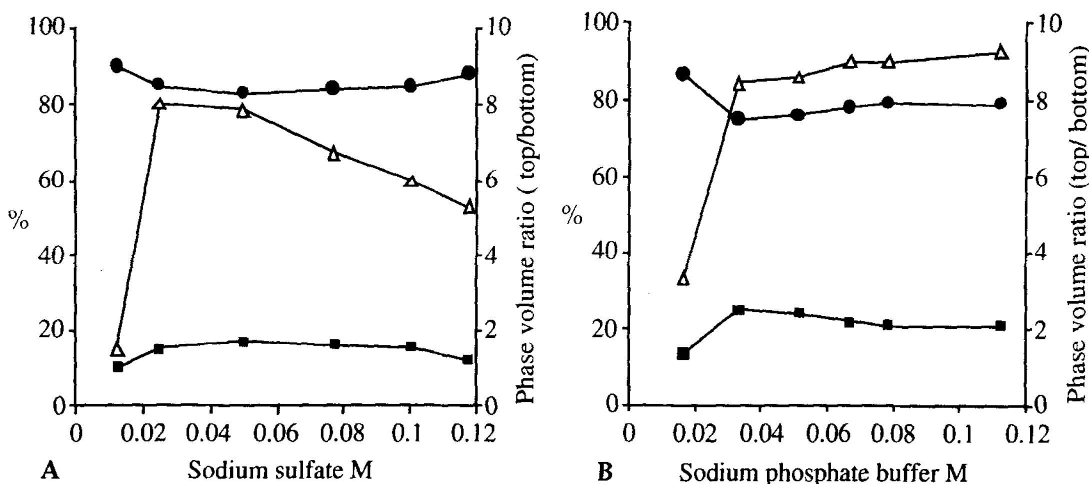


Fig. 2
Partitioning of *L. lactis* in PEI/HEC (3%/0.75%) two-phase system at pH 5.5 as a function of A: Na_2SO_4 concentration and B: sodium phosphate buffer (pH 5.5) concentration. % of the cells found in the top phase plus cells at the interface (●); % of the cells in the bottom phase (■) and the phase volume ratios of the resulting two-phase system (Δ).

and at the interface were combined. Even when cultivated in the two-phase system, *L. lactis* favoured the top phase and interface: 11% and 6% of the *L. lactis* cells were found in the PEI-rich bottom phase when cultivation was done at 5g/l glucose and 10 g/l glucose, respectively (the measurements were made at early stationary phase). The obtained phase volume ratios at the cultivations were 0.90-0.95. The culture medium, as described in materials and methods, contains 2mM sulfate and 40 mM phosphate. The contribution of these ions alone would, according to figure 2, give a large phase volume ratio. However, other components in the culture media and substances produced by *L. lactis* during growth, mainly lactic acid, decreased the phase volume ratio. (Observations have been done that monovalent anions counteract the formation of large phase volume ratios).

The partition coefficient K_L=lactic acid plus lactate in top phase/lactic acid plus lactate in bottom phase was measured to be 0.7- 0.8 for the cultivations. Since the cells partition preferentially to the top phase or accumulate at the interface a small K_L is prefered in order to get an efficient extraction by the bottom phase of PEI. Since the charge density of PEI increases at low pH, the extractive capacity of the bottom phase of PEI can increase at low pH. However, as pH is reduced lactate becomes protonated. Thus an optimal pH, with regard to the partition coefficient; K_L, probably exists. However, not only the pH will affect the K_L, also the presence of other anions in the culture media will affect the partition coefficient K_L.

The effect of cultivation in PEI/HEC system of higher polymer concentration on growth of *L. lactis* was studied. The polymer concentrations were increased to 4% PEI and 1% HEC respectively (lower nutrient concentration, 5g/l glucose), and no effect on either the lag phase or the cell density was observed. It is possible that even higher concentrations of polymers can be used, however, increased viscosity must then be considered.

The main objection to the use of the positively charged PEI in fermentations may be its inhibitory effect. Cationic polymers as PEI have been found to adsorb on cell surfaces

(Treweek and Morgan, 1977). Cultivations of *L. lactis* in PEI/HEC two-phase systems showed that *L. lactis* did not favour the PEI-rich phase which would indicate that such adsorption does not occur. This partition behaviour may explain why the phase system was not toxic. Microscopic observations showed that the morphology of the cells cultured in the two-phase systems was altered. They were seen to form big clusters while cells of *L. lactis* cultivated in abscence of two-phase systems predominantely formed small chains, see Figure 3.

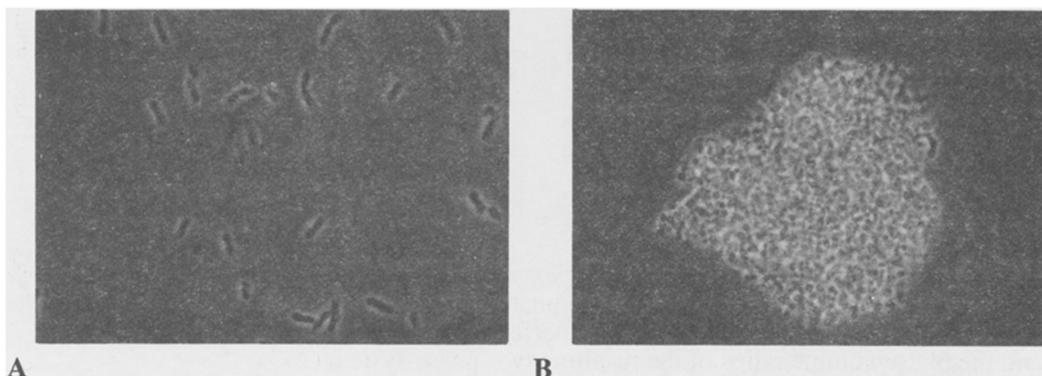


Fig.3
L. lactis cultivated without PEI/HEC two-phase system; A and with PEI/HEC two-phase system; B.

The present work shows the possibility to use PEI/HEC aqueous two-phase systems for the extractive fermentation of *L. lactis*. Both PEI and HEC are cheap bulk polymers. HEC is nontoxic, its biodegradability can be chosen (by its substitution degree of ethyleneoxide chains) only to be sensitive to cellulases. No waste problems of HEC can thus be foreseen. PEI on the other hand can probably easily be recovered by phase separation with phosphate ions at pH 6.5 (Dissing,U. and Mattiasson, B., manuscript in preparartion).

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References

Albertsson, P.Å. (1986). Partition of Cell particles and Macromolecules. Wiley Interscience, New York.
Andersson, E. and Hahn-Hägerdal, B. (1990). Appl. Microbiol. Biotechnol. (1988). 29, 329-33.
Dissing, U. and Mattiasson, B. (1993). Biotechnol. Appl. Biochem. 17, 15-21.
Hano, T., Matsumoto, M., Uenoyama, S., Ohtake, T., Kawano, Y and Miura, S. (1993). Bioseparation 3, 321-326.
Hjörleifsdottir, S., Holst, O. and Mattiasson, B. (1991). Bioprocess Engineering 6, 29-34.
Larsson, M., Arasaratnam,V and Mattiasson, B. (1988). J.Microb. Biotechnol. 3, 22-29
Luedeking, R. and Piret, E.L. (1959). J. Biochem. Microbiol. Technol. Eng. 1, 359-364.
Lukovkin, G.M., Pshezhetsky, V.S. and Murtazaeva, G.A. (1973). Eur. Polymer. J. 9, 559-565.
Mattiasson, B. and Holst, O. eds. (1991). Extractive Bioconversions. Marcel Dekker Inc. New York.
Reuben, J (1984). Macromolecules 17, 156-161.
Seevaratnam, S., Holst, O. and Mattiasson, B. (1991). Bioprocess Engineering 6, 35-41.
Schöller,C., Chaudhuri, J.B. and Pyle, D.L. (1993.) Biotechnol. Bioeng. 42, 50-58.
Srivastava, A., Roychoudhury, P.K. and Sahai, V (1992). Biotechnol. Bioeng. 39, 607-613.
Treweek, G. P. and Morgan, J.J. (1977). J. Colloid Interface Sci. 60, 258-273.
Yabannavar, V.M. and Wang, D.I.C. (1991). Biotechnol. Bioeng. 37, 1095-1100.