Alcohol Production from Cheese Whey Permeate Using Genetically Modified Flocculent Yeast Cells

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Abstract: Alcoholic fermentation of cheese whey permeate was investigated using a recombinant flocculating *Saccharomyces cerevisiae*, expressing the *LAC4* (coding for β-galactosidase) and *LAC12* (coding for lactose permease) genes of *Kluyveromyces marxianus* enabling for lactose metabolization. Data on yeast fermentation and growth on cheese whey permeate from a Portuguese dairy industry is presented. For cheese whey permeate having a lactose concentration of 50 gL^{-1}, total lactose consumption was observed with a conversion yield of ethanol close to the expected theoretical value. Using a continuously operating 5.5-L bioreactor, ethanol productivity near 10 g L^{-1} h^{-1} (corresponding to 0.45 h^{-1} dilution rate) was obtained, which raises new perspectives for the economic feasibility of whey alcoholic fermentation. The use of 2-times concentrated cheese whey permeate, corresponding to 100 gL^{-1} of lactose concentration, was also considered allowing for obtaining a fermentation product with 5% (w/v) alcohol. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **72**: 507–514, 2001
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INTRODUCTION

Cheese whey is a by-product of dairy industries, which presents rather high pollutant characteristics and is produced in high amounts. Whey disposal has been under consideration for several years. The first option considered was drying it and using it as an additive in food or as feed. In this way, the discharge problem was solved but no value was added. As cheese whey has a high content of protein (1% w/v) and lactose (5% w/v), it is appropriate to consider this material as a source of added value compounds and not just as an effluent. The use of whey as a raw material for production of a variety of products (ethanol, biomass, acid lactic, etc.) has been considered by several authors (Compagno et al., 1993; Feijoo et al., 1999; Mann, 1980; Maullu et al., 1999; Mehaia and Cheryan, 1986; Michel et al., 1987;

Prenosil et al., 1987; Rech et al., 1999; Sonawat et al., 1981; Tahoun et al., 1987). Nowadays, after whey powder and demineralized whey powder, the third major products obtained from cheese whey are whey protein concentrates (WPC) (Horton, 1996). The nutritional and medical characteristics of WPC are generally accepted and the market for WPC is increasing not only for the concentrate as a whole, but also for individual proteins (as β-lactoglobulin or α-lactalbumin) or even peptides resulting from protein hydrolysis. When producing WPC, typically by ultrafiltration, a lactose-rich fraction, the cheese whey permeate, is obtained. This can be used directly as feed, to produce lactose, or as substrate for fermentation such as alcoholic fermentation (Moulin et al., 1980; Moulin and Galzy, 1984). The lactose, the largest component in whey, is the most problematic to dispose of economically (Horton, 1996)

The metabolization of lactose by recombinant *S. cerevisiae* has been addressed by several authors (Domingues et al., 1999a; Farahnak et al., 1986; Kumar et al., 1992; Porro et al., 1992; Rubio-Teixeira et al., 1998; Sreekrishna and Dickson, 1985; Tretiak et al., 1998) but only some have addressed lactose fermentation (Domingues et al., 1999b; Compagno et al., 1995; Ramakrishnan and Hartley, 1993). Microorganisms other than *S. cerevisiae* have been considered for lactose fermentation (Guimaraes et al., 1992; Teixeira et al., 1990) and ethanol is currently produced from whey in countries such as New Zealand (Mawson, 1987) or from deproteinized cheese whey in Ireland (Barry, 1982; Lyons and Cunningham, 1980;) and in the United States (Walker, 1982). Efforts have been made to improve the productivity for cheese whey permeate alcoholic fermentation. For an optimized batch process with a *Kluyveromyces fragilis* strain adapted to high lactose concentrations, using as substrate demineralized concentrated cheese whey permeate (CCWP) with 24% lactose content, a productivity of 3.2 g L^{-1} h^{-1} was obtained corresponding to a 108.8 g L^{-1} ethanol concentration (Gawel and Kosikowski, 1978). For continuous processes, Jassens et al. (1984) reported an ethanol productivity of 7.1 g L^{-1} h^{-1} for *K. fragilis* operating with cell recycling at $D = 0.15$ h^{-1} and for CCWP with 100 g L^{-1} lactose. For immobilized continuous processes ethanol productivity values such as 1.1 g L^{-1} h^{-1} for im-

mobilized *K. fragilis* and 4.5 g L^{-1} h^{-1} for *S. cerevisiae* coimmobilized with β-galactosidase have also been reported (Hahn-Hägerdal, 1985). Terrel et al. (1984) reported an ethanol productivity of 13.6 g L^{-1} h^{-1} for CCWP with 150 gL^{-1} lactose concentration operating at continuous operation with cell recycling at $D = 0.2$ h^{-1}. However, this was attained with an *S. cerevisiae* strain (mutant strain showing no catabolite repression) and thus the CCWP had to be hydrolyzed to glucose and galactose prior to use. Nevertheless, it is our belief that by using high cell density systems with flocculent yeast cells the productivity of alcoholic fermentation of cheese whey permeate can be improved. The use of flocculent cells with an adequate bioreactor design allows for high biomass concentration inside the bioreactor, making possible continuous operation at higher dilution rates and thus higher ethanol productivity. In addition, the downstream processing is greatly facilitated due to cell sedimentation characteristics. Moreover, the main drawback associated with continuous operation, the contamination problem, is not relevant when working with this system at sufficiently high-dilution rates (Domingues et al., 2000).

In this work, a recombinant and flocculent *Saccharomyces cerevisiae* was used to continuously ferment cheese whey permeate in a high cell density system allowing for high ethanol productivity.

MATERIAL AND METHODS

Microorganism

A recombinant *Saccharomyces cerevisiae* NCYC869-A3/T1 flocculent strain expressing the *LAC4* (coding for β-galactosidase) and *LAC12* (coding for lactose permease) of *Kluyveromyces lactis* was used. The construction of the recombinant strain has previously been described (Domingues et al., 1999a).

Culture Media and Batch Cultures

The recombinant yeast was maintained at 4°C on slants or at −80°C in permanent culture of YNB selective medium having the following composition: 6.7 gL^{-1} yeast nitrogen base (w/o amino acids), 20 gL^{-1} lactose. SSLactose medium, of the following composition: KH$_2$PO$_4$ 5 gL^{-1}; (NH$_4$)$_2$SO$_4$ 2 gL^{-1}; MgSO$_4$.7H$_2$O 0.4 gL^{-1}; yeast extract 1.0 gL^{-1}; lactose 50 gL^{-1}or 100 gL^{-1} was used. Sweet cheese whey permeate (CWP) obtained by ultrafiltration from a Portuguese dairy with a lactose concentration of approximately 50 gL^{-1} was initially assayed. The CWP was further concentrated by nanofiltration as the retentate and named concentrated cheese whey permeate (CCWP). The CCWP with approximately 100 gL^{-1} lactose was also tested. The feed lactose concentration for the experiments done with CWP and CCWP was always measured. Yeast cultures were carried out at 30°C by rotary shaker with 150 rpm agitation, in 100 mL of culture medium in 250-mL Erlenmeyer flasks. Alternatively, a 2-L bioreactor (Braun, Biostat® M) fitted with agitation and aeration control, temperature measurement and control, pH measurement and control was used. The temperature was maintained at 30°C, the pH at 4.0 by automatic addition of an ammonia solution, agitation speed was set at 150 rpm and the bioreactor was aerated with filtered air at a flow rate of 0.1 vvm. The fermentor vessel and tubing were autoclaved for 20 min at 121°C.

Continuous-Culture Experiments

An airlift bioreactor of the concentric draft tube made of Perspex (Fig. 1) with a working volume of 5.5 L was used (Domingues et al., 1999b). The regulation system allows for: temperature control at 30 ± 1°C; foam-level control by addition of antifoam (Sigma A-5551); and pH control by automatic addition of ammonia, being the set-point fixed at pH 4.0 ± 0.1. The system was aerated with filtered air at a flow rate of 0.0800 to 1.0000 ± 0.0002 vvm. Flow rate was controlled using a Hastings mass-flow controller. It is worth noting that an increase in the air-flow rate was needed when increasing dilution rate to prevent the bioreactor from stalling. Dilution rate was varied during the experiments in the range 0.1 to 0.55 h^{-1}.

For start-up of continuous culture, cells were grown in a 2-L Erlenmeyer flask filled with 1 L of culture medium, for 24 h. The cell suspension was then aseptically transferred to the bioreactor, which was kept in batch operation for 24 h before switching on the feeding. Once pseudo-stationary
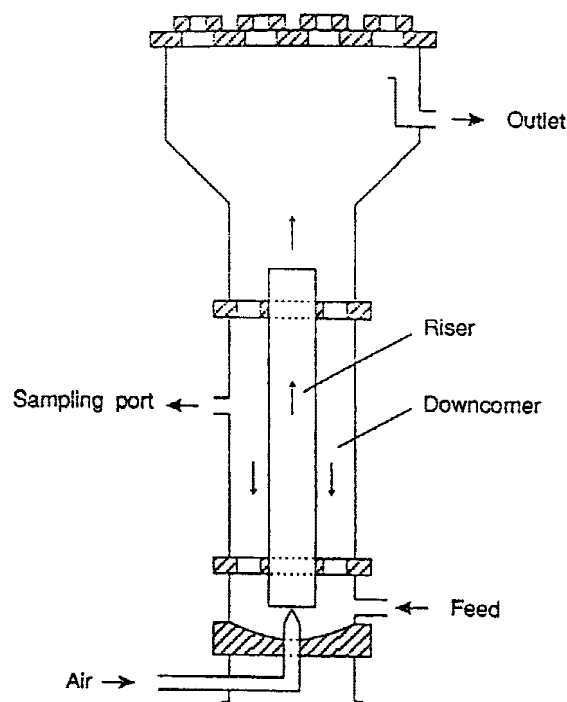

Figure 1. Schematic representation of the airlift bioreactor.

state with semisynthetic medium was attained, the bioreactor was fed with sweet cheese whey permeate. A pseudo-stationary state is reached whenever lactose consumption and ethanol production have reached constant values. For the concentrated whey permeate the start-up of the continuous operation was made after a 24-h batch culture in the bioreactor with concentrated cheese whey permeate.

Analysis

The cell concentration, i.e., biomass concentration, was estimated by the dry weight method. The dry weight cell concentration was determined by filtering the sample through 0.2-μm filter paper and then dried at 105°C for 24 h. The lactose concentration was determined by the dinitrosalicylic acid method (Miller, 1959). Ethanol concentration was measured by an HPLC method (Poyspher® CH CA column, Merck). The solvent used was 0.1 N H_2SO_4, at a flow rate of 0.7 mL min^{-1}, while detection was effected with a refractive-index detector. Temperature was maintained at 60°C. Cell viability was estimated by the vital methylene blue staining method and direct cell counting in a Neubauer chamber.

Screening for Contaminations Assay

As cheese whey permeate was not autoclaved before being fed into the bioreactor, screening for contaminants was performed. This was done by sample spreading on lysine plates (Oxoid CM191). Lysine medium is a synthetic medium for the isolation and enumeration of wild yeasts encountered in brewing. On this medium, *S. cerevisiae* growth is suppressed because no normal *S. cerevisiae* or *S. carlbergensis* strains utilize lysine while many other types of yeast, including wild yeasts, do so. When using concentrated whey permeate the contamination assay was not done. Instead the concentrated whey permeate was acidified during its preparation, pasteurized at 60°C for 30 min, and stored at 4°C until use.

Flocculation Assay

The flocculation assay was done as previously described (Domingues et al., 1999b). Cells were washed twice with 15 gL^{-1} NaCl, pH 3.0, solution. A 24-mL volume of cell suspension was placed on a 25-mL graduated cylinder. Then, 1 mL of a 100 m*M* CaCl$_2$, pH 3.0, solution was added and the solution immediately mixed by inversion 18 times. Samples of 200 μL were taken at the 20-mL level at different times. To ensure floc dispersion, samples were treated with NaCl solution before OD reading at 620 nm (Soares et al., 1992). The normalized cell concentration defined as the ratio between actual and initial concentration was plotted against sedimentation time. A sedimentation profile was then obtained.

Effluent Characterization

The chemical oxygen demand (COD), biochemical oxygen demand (BOD$_5$), total suspended solids (TSS), and total nitrogen were analyzed according to standard methods (Clesceri et al., 1989).

Cations Measurement

The cations (Na$^+$, K$^+$) concentration was measured by atomic absorption spectrometry (Clesceri et al., 1989).

RESULTS AND DISCUSSION

Figure 2 shows the time course of cell, lactose, and ethanol concentrations, using SSmedium or cheese whey permeate (CWP) as substrate, in shake-flask cultures of the recombinant strain. As can be observed from Figure 2, the fermentation time is similar for both culture media, although a
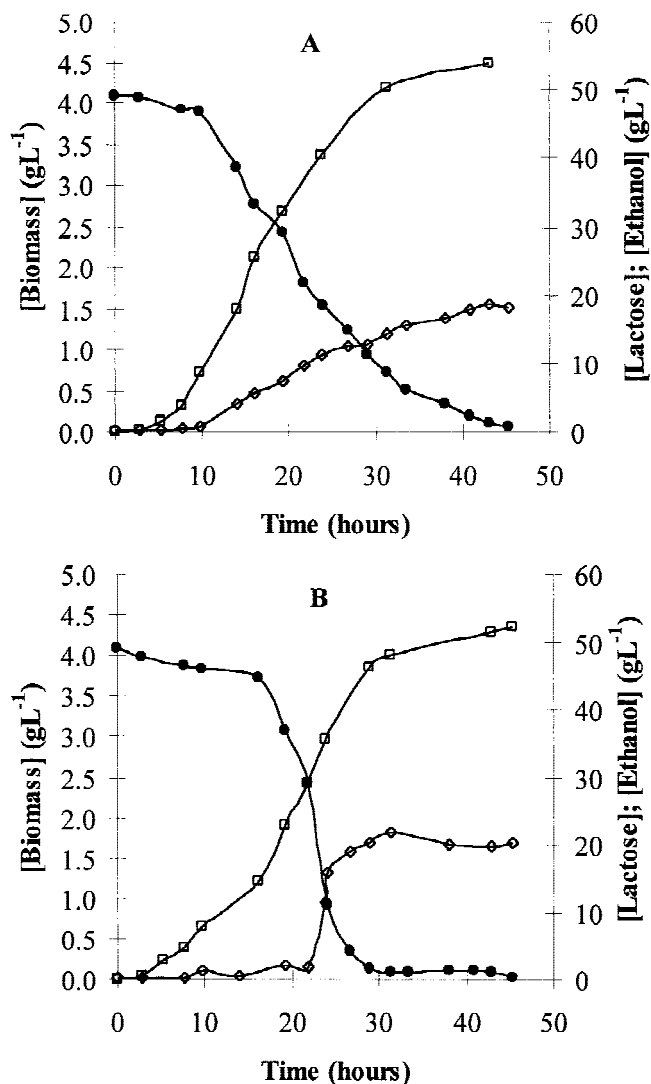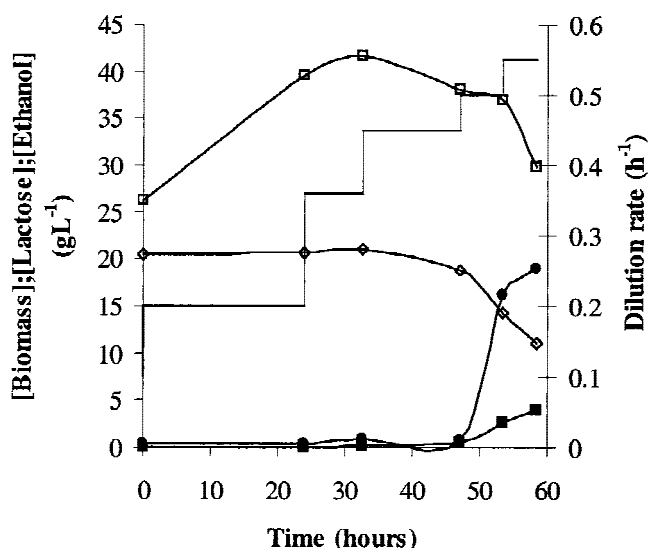

Figure 2. Cell ($\square$), lactose ($\bullet$) and ethanol ($\diamond$) concentration profiles for SSmedium (A) and cheese whey permeate (B) in shake-flask culture.

more pronounced lag phase is observed for CWP. After the lag phase, the consumption and ethanol production rates are higher for CCWP medium. This is probably due to the medium composition difference that might be richer in the case of CWP (SSlactose medium has just 1 gL^{-1} yeast extract). In less than 30 hours total lactose consumption in CWP was achieved. Moreover, the percentage of theoretical conversion obtained with CWP was comparable with the one obtained with semisynthetic medium (80% of the theoretical value). These data support the feasibility of using CWP as substrate for alcoholic fermentation without addition of any nutrient. This is important because additional nutrients can hinder economic feasibility in industrial applications.

In Figure 3 the different fermentation parameters measured during pseudo-stationary-state operation of the continuous bioreactor using cheese whey permeate (CWP) at increasing dilution rates are represented. The operation with CWP was initialized at 0.1 h^{-1} dilution rate after reaching pseudo-stationary state with semisynthetic medium. Each time a residual lactose concentration (< 1gL^{-1}) was obtained, the dilution rate was increased stepwise (up to 0.55 h^{-1}). Pseudo-stationary state values are the values obtained for each dilution rate, after feeding the bioreactor for five residence times at the same dilution rate or when residual lactose concentration is attained.

The cell concentration inside the bioreactor increased up to 0.36 h^{-1} dilution rate (D), reaching the maximum value of 42 gL^{-1} (dry weight), which is 10 times higher than the one obtained for shake-flask culture. For D above 0.45 h^{-1} the cell concentration inside the bioreactor slightly decreased and increased in the effluent. When increasing the dilution rate from 0.45 h^{-1} to 0.5 h^{-1}, the residual (< 1gL^{-1}) lactose

concentration inside the bioreactor raised to 16 gL^{-1}, indicating that 0.45 h^{-1} was the limiting dilution rate for total lactose consumption. Up to 0.45 h^{-1} dilution rate the ethanol concentration was constant, decreasing with the increase of residual lactose concentration. During all the period of operation with CWP the cell viability was above 90%.

Considering that at a given dilution rate, when residual concentration of lactose is achieved, a pseudo-stationary state has been reached, the specific lactose consumption rate and the specific ethanol production rate can be calculated and compared with the ones previously obtained (Domingues et al., 1999b) with semisynthetic medium. The results obtained with CWP overlapped the ones previously obtained with SSlactose medium (Domingues et al., 1999b) confirming not only that the metabolic state of viable cells in the flocs was kept constant at the dilution rates and operational conditions studied, but also that unsupplemented cheese whey ultrafiltrate is an adequate substrate for ethanol fermentation.

This is also confirmed by analysing two other parameters of major importance, that are the % theoretical conversion (η), expressed as
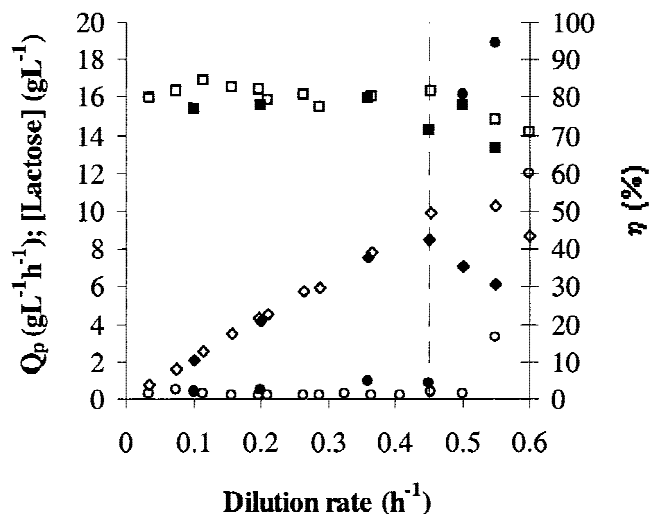
$$\eta(\%) = \frac{P}{(S_f - S)0.538} 100 \qquad (1)$$

and the ethanol productivity, defined as

$$Q_p = DP$$

In Figure 4, the ethanol productivity, residual lactose concentration, and % theoretical conversion for different dilution rates are presented and compared to the values previously obtained with SSlactose medium (Domingues et al., 1999b).

As can be observed, the two systems exhibit similar

Figure 3. Continuous fermentation time course of: lactose concentration (●); ethanol concentration (◇); cell concentration in the bioreactor (□) and in the effluent (■); at pseudo-stationary state with increasing dilution rates (−) for the recombinant *S. cerevisiae* NCYC869-A3/T1, using unsupplemented cheese whey permeate (CWP) as substrate.

Figure 4. Variation of ethanol productivity (◇, ◆), lactose concentration (○, ●) inside the bioreactor and % theoretical conversion (□, ■) with the dilution rate; open symbols for the bioreactor fed with SSlactose medium, closed symbols for the bioreactor fed with cheese whey permeate.

trends. Both systems have similar ethanol productivity at dilution rates up to 0.45 h^{-1}, after which the system fed with semisynthetic medium exhibits higher productivity. As already observed, this is mainly due to the higher wash-out dilution rate allowed for the bioreactor fed with semisynthetic medium. The wash out at a smaller dilution rate for CWP feed is caused by a slight deflocculation of the yeast strain. This is probably caused by the same factor that leads to deflocculation on the experiments with CCWP, further reported. In a previous study (Domingues et al., 1999b) it has been pointed out how unstable this system becomes when working at dilution rates above 0.45 h^{-1} and that it would be better to work at 0.45 h^{-1} even with loss of productivity than working at higher dilution rates. To prove that this difference was not caused by any kind of contamination, a screening for contaminations (twice a day) was done while operating with cheese whey permeate, as it was not sterilized. Bacterial contamination is easily noticed under microscopic observation, while yeast contamination is much more difficult to detect. Because flocculating yeast cells are used, the appearance of a strong turbidity in the culture medium would give a good indication of contamination. However, this is not straightforward because contaminant nonflocculent cells could also interact with the flocculent ones (Soares et al., 1992), and the flocculent cells could also for some reason deflocculate. As such, a screening for yeast contaminants was done with lysine differential medium during the operation and no contaminant was found, confirming the microbiological stability of these systems (Domingues et al., 2000).

As there are several profitable uses for proteins available from cheese whey, either by incorporation of the proteins into cheese products or by recovery and fractionation of these proteins, the permeate has theoretically little or no value (Moulin and Galzy, 1984). However, the volumes produced are so large that some treatment at least is necessary before their disposal as effluent. The proposed fermentation procedure, coupled with protein recovery, enables a reduction higher than 90% of whey chemical oxygen demand (see Table I). Moreover, the use of the fermentation procedure described herein allows for the attainment of these objectives at a lower cost than existing procedures (Maiorella and Castillo, 1984; Shama, 1988).

As lactose is present in the permeate at a relatively low concentration, its concentration before fermentation can be used to optimize the economics of ethanol recovery by distillation. If the cheese whey permeate (CWP) is concentrated 2 times a fermentation product with 5% (w/v) ethanol can be expected. This possibility was also studied, concentrating the permeate with a 150-300 Da cut-off nanofiltration membrane. In Figure 5, the results for batch fermentation of 2 times concentrated cheese whey permeate (CCWP) are shown. This fermentation was performed in a 2-L bioreactor with pH (set-point 4.0) and temperature (set-point 30°C) control, under 150-rpm agitation. In 30 h all the lactose (100 gL^{-1}) was metabolized, resulting in 53 gL^{-1} ethanol, clearly indicating that lactose is converted to ethanol with a conversion yield close to the expected theoretical value. It also indicates that the specific growth rate and ethanol specific production rate are not affected by the use of a high initial lactose concentration.

Taking this into account, continuous experiments were done with a 100 gL^{-1} lactose concentration in the feed medium. The first set of experiments was done with SSmedium (100 gL^{-1} lactose), with the dependence of ethanol productivity on dilution rate presented in Figure 6.

The maximum ethanol productivity is close to the one previously obtained with 50 gL^{-1} (Domingues et al., 1999b), but is now obtained at a lower dilution rate ($D = 0.23$ h^{-1}). However, when fed with concentrated cheese whey permeate (CCWP) flocculation was strongly impacted, and it became impossible to reach the above-mentioned dilution rate as deflocculated biomass was washed-out from the bioreactor, even when operating at such a low dilution rate as 0.077 h^{-1}. In Figure 7 the main results obtained with the continuous operation of the bioreactor fed with CCWP are summarized.

As can be observed from Figure 7, after 120 h of continuous operation with 2-times concentrated cheese whey permeate (CCWP), the system could not reach any pseudo-stationary state as biomass was continuously washed-out from the bioreactor. The increase of biomass concentration

Table I. Physicochemical characterization of cheese whey and of the final effluent (obtained after permeate alcoholic fermentation and distillation).[a]

| Parameter	Cheese whey	Final effluent	Reduction (%)
pH	5.5–6.5	3.8–4.3	—
COD (mg$_{O_2}$ L^{-1})	76 076	7 221	91
BOD$_5$ (mg$_{O_2}$ L^{-1})	67 067	2 133	97
TSS (mg L^{-1})	11 440	262	98
Total nitrogen (mg L^{-1})	1 329	705	47

[a]COD—Chemical oxygen demand; BOD$_5$—Biochemical oxygen demand; TSS—Total suspended solids.

Figure 5. Cell (□), lactose (●) and ethanol (◇) concentration profiles for two times concentrated whey permeate (CCWP) in batch culture.
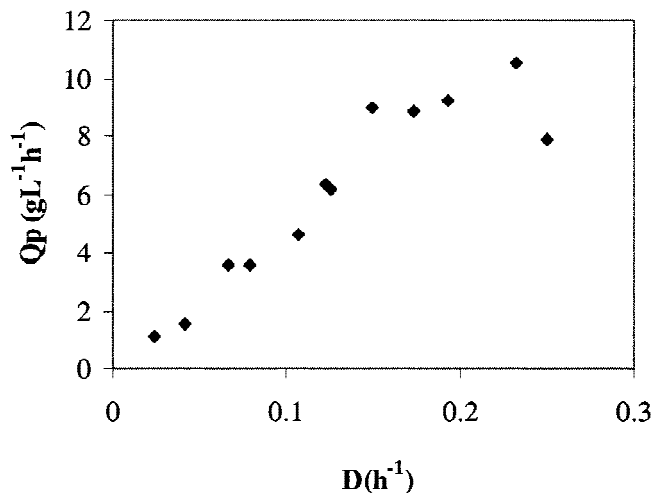
Figure 6. Variation of ethanol productivity with the dilution rate for the bioreactor fed with 100 gL⁻¹ lactose semisynthetic medium.

in the outlet was coincident with the decrease of flocculation ability as shown in Figure 8.

The decrease in the flocculation ability may be due to the concentration of salts present in the concentrated cheese whey permeate. An attempt to restore flocculation was made by adding calcium chloride, to a final concentration of 100 mM, to the CCWP at 190 h of continuous operation. Although a slight improvement was noticed both in the bioreactor performance as well as in the flocculation ability, it was far from being satisfactory.

The effect of cation concentration in flocculation is very well documented in the literature (Kihn et al., 1988; Kuriyama et al., 1991; Nishihara, 1982; Stratford, 1989; Stratford and Brundish, 1990; Taylor and Orton, 1973). It is generally accepted that at low concentration, ions promote flocculation while at high concentration they show an inhibitory effect. Sodium ions inhibit flocculation by compe-
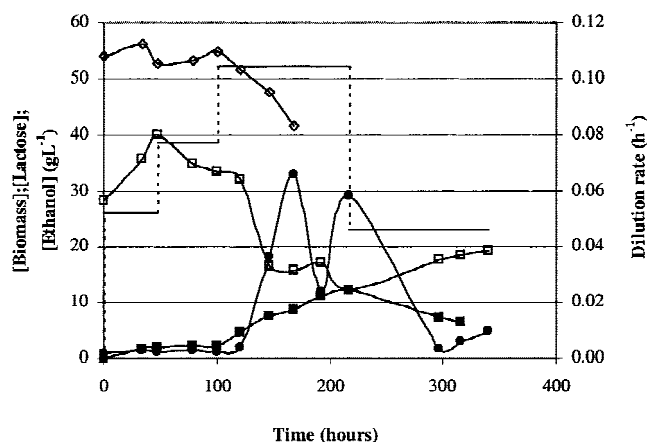
Figure 7. Continuous fermentation time course: lactose concentration (●); ethanol concentration (◇); cell concentration in the bioreactor (□) and in the effluent (■); with increasing dilution rates (–) for the recombinant *S. cerevisiae* NCYC869-A3/T1, using two times concentrated cheese whey permeate (CCWP) as substrate.
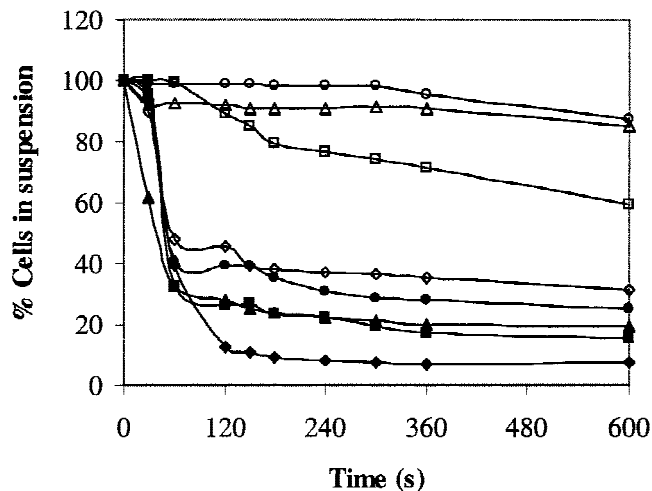
Figure 8. Sedimentation profiles of bioreactor cultured cells during operation with two times concentrated cheese whey permeate (CCWP): Continuous operation with semi-synthetic medium before feeding with CCWP (◆); Batch operation with CCWP (■); Beginning of continuous operation, $t = 0$ h (▲); $t = 35$ h (●); $t = 48$ h (◇); $t = 78$ h (□); $t = 147$ h (△); $t = 170$ h of continuous operation (○).

tition with calcium ions for specific sites. Potassium and lithium ions are less potent inhibitors than sodium ions (Stratford, 1989). The concentration at which the inhibition occurs is highly strain dependent. Some authors note that at a concentration above 100 mM flocculation inhibition can be expected (Nishihara, 1982; Taylor and Orton, 1973) while others refer to a lower inhibition concentration (Kihn et al., 1988; Stratford, 1989) or a higher one (Kuriyama et al., 1991). Kihn and co-authors (1988) have tested the effect of cations on flocculation by the yeast strain *S. cerevisiae* NCYC869 (parental wild strain of the transformant T1 presented in this work). For this strain, sodium inhibits flocculation at concentrations from 10 to 100 mM while lithium and potassium did not inhibit flocculation below 200 mM. The sodium concentration present in the concentrated permeate is 20 mM, consistent with partial flocculation inhibition. The potassium concentration in concentrated permeate is 55 mM, which is below the inhibition flocculation concentration. However, Stratford and Brundish (1990) found that the inhibition caused by different salts are additive in their effects as noninhibitory concentrations of salts in combination caused inhibition of flocculation. Salts of potassium, which alone were noninhibitory, were found to be good inhibitors of flocculation in combination with other salts. Another important aspect pointed out by these authors was that inhibition by salts is time-dependent; prolonged incubation increased the degree of inhibition. Moreover, near-inhibitory salt concentrations were found to become inhibitory with prolonged incubation. This agrees with the results presented herewith, where the prolonged operation with concentrated whey permeate (CCWP) decreased the cell flocculation capacity. In summary, the concentration of salts present in CCWP affects the flocculation of yeast cells and does not allow for continuous operation at high cell

density with CCWP. If the salts are removed from the CCWP, it would be possible to attain at least the same productivity as with nonconcentrated whey permeate, but with a higher alcohol content in the end of the fermentation process.

CONCLUSIONS

Batch experiments showed that the recombinant *S. cerevisiae* strain used in this work was capable of utilizing cheese whey permeate as a carbon and energy source, fermenting 50 gL^{-1} or 100 gL^{-1} of lactose present in cheese whey permeate without any additional nutrient supply.

The continuous-airlift bioreactor, employing this flocculent yeast, is able to ferment lactose present in cheese whey permeate (CWP) to ethanol at a dilution rate of 0.45 h^{-1}. While producing ethanol, the recombinant *S. cerevisiae* strain cleared the cheese whey permeate of most organic substances, allowing for a significant reduction in the pollutant load of the cheese whey. For this system, ethanol productivity near 10 g L^{-1} h^{-1} with a bioconversion yield as high as 80% of the theoretical value and total lactose consumption was obtained. Ethanol productivity is largely superior to that reached with conventional methods (Lyons and Cunningham, 1980). For the continuous operation with 2 times concentrated cheese whey permeate (CCWP), inhibition of cell flocculation by salts was noticed, which hindered the operation at high cell density. If salts are removed, at least the same productivity as for the system fed with whey permeate can be expected, as confirmed by the data obtained with the continuous bioreactor fed with 100 gL^{-1} semisynthetic medium. Moreover, a 5% ethanol product is obtained, as shown for batch fermentation, minimizing the distillation costs.

NOMENCLATURE

P ethanol concentration
S lactose concentration

Subscripts

f feed concentration

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