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Fermentation conditions increasing protease production by *Serratia marcescens* in fresh whey

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Abstract

Extracellular protease production in fresh whey fermented by *Serratia marcescens* was increased by studying the influence of the most important physico-chemical operating variables. Fermentations were carried out in Erlenmeyer flask and in an automatic controlled bioreactor (Bioflo III), obtaining in the latter case the highest protease levels (8,800 EU/mL ± 484 DS) when working under optimized conditions. Two kinds of proteases were determined in the supernatant, a major metalloprotease (84% ± 2 DS) and a minor serine protease (13%+/-2 DS). In all cases cell growth and protein degradation were modelled, and the effect of oxygen concentration on cell growth was determinate by Bioflo III and was also modelled.

Key words: Fresh whey, fermentation, proteases, Serratia marcescens.

Condiciones de fermentación para el incremento de la producción de proteasas por *Serratia marcescens* en lactosuero fresco

Resumen

El estudio de la influencia de las principales variables fisicoquímicas de operación relacionadas con la producción de proteasas extracelulares por *Serratia marcescens* permitió establecer las mejores condiciones experimentales para su producción tanto en matraces Erlenmeyer como en el biorreactor controlado (Bioflo III). Los máximos niveles de actividad proteolítica (8.800 UE/mL ± 484 DS) se alcanzaron en el Bioflo III bajo condiciones óptimas de producción. Dos tipos de proteasas se determinaron en el sobrenadante, una metaloproteasa mayoritaria (84% ± 2 DS) y una serinproteasa minoritaria (13% ± 2 DS). En todos los casos el crecimiento celular y la degradación de sustrato fueron modelizados. El efecto de la concentración del oxígeno disuelto sobre el crecimiento celular de *Serratia marcescens* determinado en el Bioflo III fue también modelizado.

Palabras clave: Lactosuero fresco, fermentación, proteasas, Serratia marcescens.

Introduction

Whey is a by-product of cheese manufacturing produced in enormous quantities, up to 110 millions of tons per year worldwide [1]. Its chemical composition has enabled its use as a functional additive in several foods [2-4]. However, whey is still a little economic value and several studies have been carried out in search of new applications. One possibility to be assessed is the use of whey to obtain products of high economic value by fermentation, being an example the production of microbial protease.

A huge amount of widespread microorganisms naturally existing in different environments

are able to produce proteases. These enzymes are used in several industries, like those of food, detergents and pharmacy, as well as more recently in the treatment of leather and clinical and analytical research [5]. All these applications have created an extensive market for these enzymes, leading to proteases representing 60% of the total sales of industrial enzymes worldwide. As a matter of fact, industrial enzyme sales are estimated at one billion dollars per year, so the economic importance of these enzymes production seems obvious [5]. Their variety, specificity of action, physiological and biotechnological applications and the high price achieved in the market are powerful appeals that lead to search for new protease producing sources and the optimisation of known processes for their production.

Protease production by *S. marcescens* has been described in previous research papers [6, 7, 9], studies have been developed based on regulation-excretion mechanisms [8, 10, 11], characterization [6], purification [12, 13] and genetic analysis of these enzymes [14]. *S. marcescens* produces a potent extracellular metalloprotease, which is widely used as an anti-inflammatory agent [5]. This behaviour has been observed in synthetic fermentation and also when using non-conventional media, such as molasses or whey powder [7, 8, 14]. The good results obtained when employing the protease production rehydrated whey powder inoculated with *S. marcescens* [7, 9], have proved the potential of this dairy by-product as a substrate to produce this enzyme. However, it appears necessary to identify and adjust the physico-chemical variables that influence this system, in order to improve the efficacy and profitability of the process. The effect of operating variables has been already studied for this bacterium in other systems [15], and also for the present one [9]. However, all the existing studies of *S. marcescens* grown in whey have been carried out starting out with whey powder [9]. During the dehydration process, whey undergoes treatment at a high temperature that produces important changes in its physico-chemical characteristics. Some whey nutrients that induce protease synthesis might eventually be lost during this process, which is avoided when fresh whey is employed. Therefore, the present study focuses on achieving a better knowledge of how the most important physical and chemical operating variables influence protease production by fresh whey fermentation using *S. marcescens*, in order to make an industrial application feasible and attractive.

Materials and Methods

Microorganism

S. marcescens (ATCC: 25419) was the microorganism employed in this work.

Inoculum development

The cells used for inoculation were previously grown for 12 hours (logarithmic phase) in 250 mL Erlenmeyer flasks containing 100 mL of Nutrient Broth (NB) (Biokar Diagnostic) (20 g/L) enriched with glucose (10 g/L) at 30°C and 200 rpm. Cells were separated from the broth by centrifugation at 13,000 g for 15 minutes, washed with distilled water and re-suspended in NaCl (9 g/L).

Culture medium

Fresh sweet whey, supplied by an industrial dairy (Reny-Picot, Asturias–Spain), was employed as the culture medium. The initial protein concentration was varied between 6.0 g/L and 12.0g/L and pH 6.1. In all cases, whey was sterilised by tangential microfiltration (0.33 μm at 1.5 bar), showing a decrease in the initial protein contents resulting in protein concentrations between 3.5 g/L and 7.4 g/L.

Fermentation conditions

Two kinds of fermenters were employed: 250 ml capacity Erlenmeyer flasks containing 100 mL of fresh whey and a 5.0 L capacity mechanically stirred bioreactor (Bioflo III, New Brunswick Scientific Co. Inc.), containing 2.6 L of fresh whey. Erlenmeyer flaks were employed to study the effect of pH, temperature and initial protein concentration and the bioreactor to study the effect of dissolved oxygen concentration.

Erlenmeyer flasks

– *Effect of temperature*: Microfiltered whey containing 5.8 g/L of protein was employed for this study. Initial pH was adjusted to 7.0 by adding NaOH (6.25 M). The cultures

were incubated at 200 rpm and 26°C, 30°C, 34°C, and 37°C.

- *Effect of pH:* Microfiltered whey containing 5.8 g/L of protein was employed for this study. pH values were adjusted at the beginning with NaOH (6.25M) and were controlled during fermentations by adding the amount of HCl needed (16.25 M) periodically. pH values tested were (7.0, 7.3, 7.6, and 8.0). The cultures were incubated at 200 rpm and 30°C.
- *Effect of initial protein concentration*: Microfiltered whey containing 7.4 g/L of protein was employed for this study. The initial protein concentrations tested were 4.0g/L, 6.0g/L, and 7.4 g/L. Initial pH was adjusted to 7.6 by adding NaOH (6.25 M). The cultures were incubated at 200 rpm and 30°C.

Bioflo reactor

Effect of dissolved oxygen concentration: In this case the initial protein concentration of microfiltered whey was 3.5 g/L. The Bioflo reactor was employed to study the effect of dissolved oxygen maintaining the medium during the fermentations at 5%, 10%, 30%, and 50% of saturation (8 mg of oxygen per litre at 30°C). These fermentations were carried out at 30°C and pH value was initially adjusted to 7.6 with NaOH (6.25 M). Dissolved oxygen and pH were automatically controlled by the equipment. The oxygen concentration was controlled by means of an air flow rate between 0.1-0.4 volumes of air per volume of medium (vvm) variable agitation between 200 rpm and 500 rpm, and pH was automatically maintained at 7.6 by adding HCl (16.25 M).

In all experiments whey was inoculated with *S. marcescens* to a final biomass concentration ranged between 0.10g/L and 0.13 g/L (dry weight).

Analytical methods

Cell growth determination

Culture samples were centrifuged at 16,000 g for 15 minutes and the pellet was washed twice with distilled water. Cells were finally suspended in distilled water. Bacterium growth was determined by measuring the absorbance of the cells suspension at 587 nm. Experiments were carried out in order to correlate this absorbance to the cell dry weight by using a calibration curve obtained at 587 nm [6, 9]. An absorbance value of 0.8 was shown to be 333 mg of cell dry weight per litre. Standard deviation of the data ranged between 0.001g/L and 0.009 g/L.

Protease assays

Culture samples were centrifuged at 16,000 g for 15 minutes and the supernatant was retained for analysis. Total and specific protease activities on cell-free supernatant were spectrophometrically analysed by the Azocasein method [7, 9, 16, 17, 27]. The reaction was carried out with 480 μL of Azocasein solution (10 g/L in tris-glycin buffer, 0.2 M, pH 9.0 and 2mM $CaCl_2$) and 120 μL of sample, at 45°C for 1 h. The reaction was stopped by adding 600 μL of trichloroacetic acid (100 g/L), the reaction mixture was centrifuged at 16,000 g for 15 min. A volume of 200 μL of NaOH (1.8 M) was then added over 800 μL of the supernatant and absorbance was measured at 420 nm. Class-specific inhibitors of serineprotease (phenylmethylsulfonyl fluoride (PMSF) 4mM) and metalloprotease (1,10-phenanthroline 5mM) [13, 17, 27] were employed to determine the specific kinds of proteases produced. The determination of the specific activities was performed by the addition of specific inhibitor to the sample and they were maintained at room temperature for 15 minutes before being assayed. All the samples with absorbance higher than 0.5 were diluted with tris-HCl buffer, 50 mM, pH 7.6 and analyzed once again. One unit of protease activity was defined as the amount of enzyme that causes an increase in absorbance at 420 nm of 0.1 units in one hour. Standard deviation of the data ranged between 0.2 EU/mL and 484 EU/mL.

Protein determination

Total protein concentration of the cell-free culture supernatant was analysed by the Lowry Method [18]. Bovine serum albumin was used as the standard. Standard deviation of the data ranged between 0.002 g/L. and 0.05 g/L.

All experiments and analytical determinations were performed by triplicate.

Results and Discussion

Temperature effect

In previous studies, clear discrepancies had risen concerning the optimum temperature for protease production by *S. marcescens*. Depending on the author, the values of 30°C or 37°C were employed for incubation [7-9, 12, 13, 17]. Romero *et al.* [13] reported that, although cell growth was higher at 36°C, protease production improved at 30°C when whey powder was employed as substrate. In the present work, a study was developed to set up the most adequate temperature when fresh sweet whey was employed by testing four temperatures: 26°C, 30°C, 34°C and 37°C. The results of these fermentations show significant differences in cell growth and protease production (Figure 1).

Although the time necessary to reach the stationary growth phase was similar in all cases, a slight but progressive increase in the maximum biomass concentration was observed with the increase of temperature. The highest biomass values were achieved when fermentation was carried out at 37°C (Figure 1.d). However, the protease activity level achieved at 30°C (2,884 EU/mL ± 129SD) was 1.68 fold higher than the one achieved at 37°C (1,715 EU/mL ± 86 SD). The lowest protease activity level (715 EU/mL ± 29SD) were achieved when fermentation was carried out at 26°C. It seems to indicate that, although an increase in temperature gives rise to higher cell growth, protease production is preferred at 30°C; hence the latter temperature was selected as optimal.

The highest values of protease activity were achieved at the end of the exponential growth phase or during the stationary phase, which indicates that protease is a metabolite that is not completely associated with cell growth.

In Figure 1.c. a decrease in protease activity levels was observed after having reached the maximum value. The same behaviour was found in prior studies on protease production with *S. marcescens* [8, 19]. Experiments carried out with

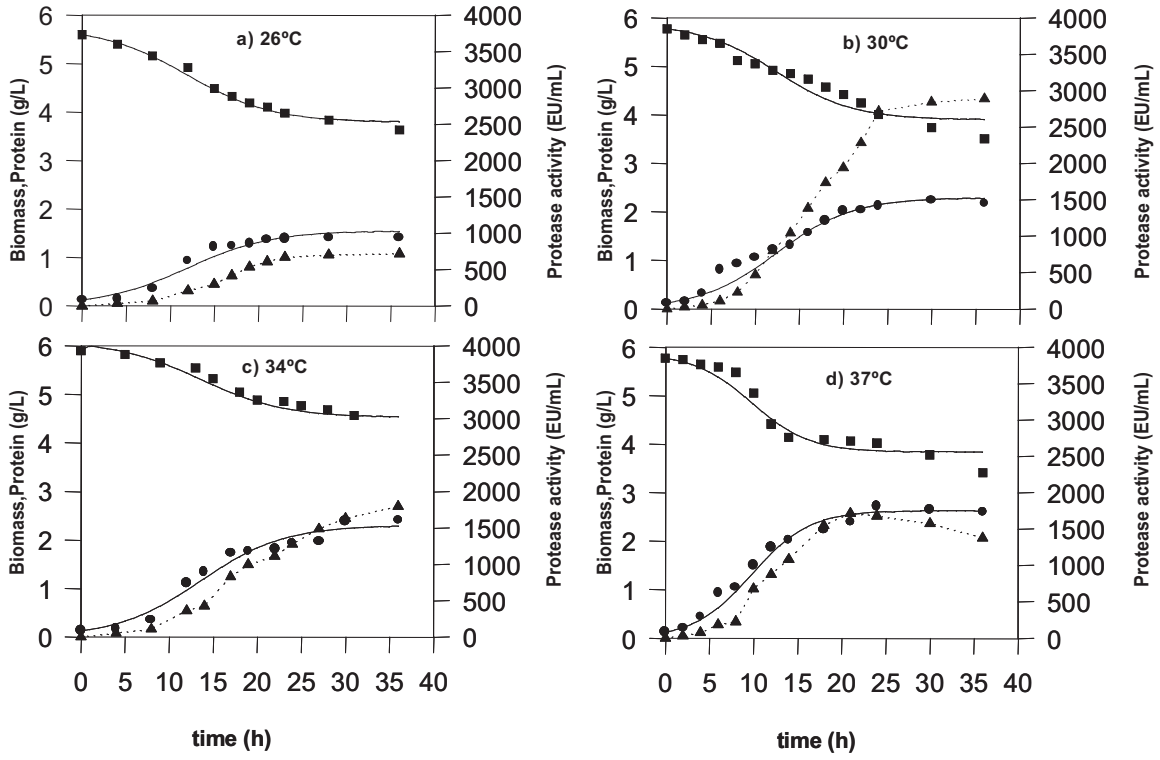

Figure 1. Temperature effect on cell growth (●), protease production (▲) and protein consumption (■) during flask fermentations: a) 37°C, b) 30°C, c) 34°C, d) 37°C. Solid lines show the theoretical data obtained by fitting experimental data to Eq. 1 and 2.

specific inhibitors showed that both kinds of protease, metallo and serineprotease, suffered a decline when they were maintained at working conditions for several hours (data not shown), which confirms the studies carried by Braun and Schmitz, that is [8,19], that protease may suffer selfdegradation. This decrease in protease activity was also observed in subsequent experiments.

pH Effect

The experimental results showed that when fresh whey is inoculated with *S. marcescens*, pH of the medium increases during fermentation from 6.2, which is the pH of fresh whey, to approximately pH 8.5 at the end of the fermentation. This increase is due to the ammonia produced as a consequence of the aminoacids catabolism released by protein hydrolysis [18]. Under these conditions, the lag phase of cell growth is very long (around 6 hours), considering the fact that the best pH for *S. marcescens* growth is higher than pH 6.2 employed in this case [6,7,9]. The results could also demonstrate that, like previous studies [6,7,9] optimal pH for the production of proteases by *S. marcescens* is higher than 6.2; consequently, the time necessary to achieve the maximum protease activity is also very long (data not shown).

In order to minimize these periods of time and maximize protease production, the pH effect has been studied carrying out fermentations with controlled pH. The pH values were 7.0, 7.3, 7.6 and 8.0, all selected within the range described as suitable for S. marcescens growth [20]. The results for the different pH values can be observed in Figure 2. In all cases, lag phase of cell growth was very short (around 2 hours) and similar cell concentrations were achieved after 36 h, between 2.3g/L and 2.7 g/L (dry weight). At the same time, a decrease in protein concentration ranged between 30% and 39% for the different pH values were observed. In all cases, the highest degradation was observed during the exponential growth phase. However, the highest protease activity levels were achieved at the end of the exponential phase or during the stationary growth phase.

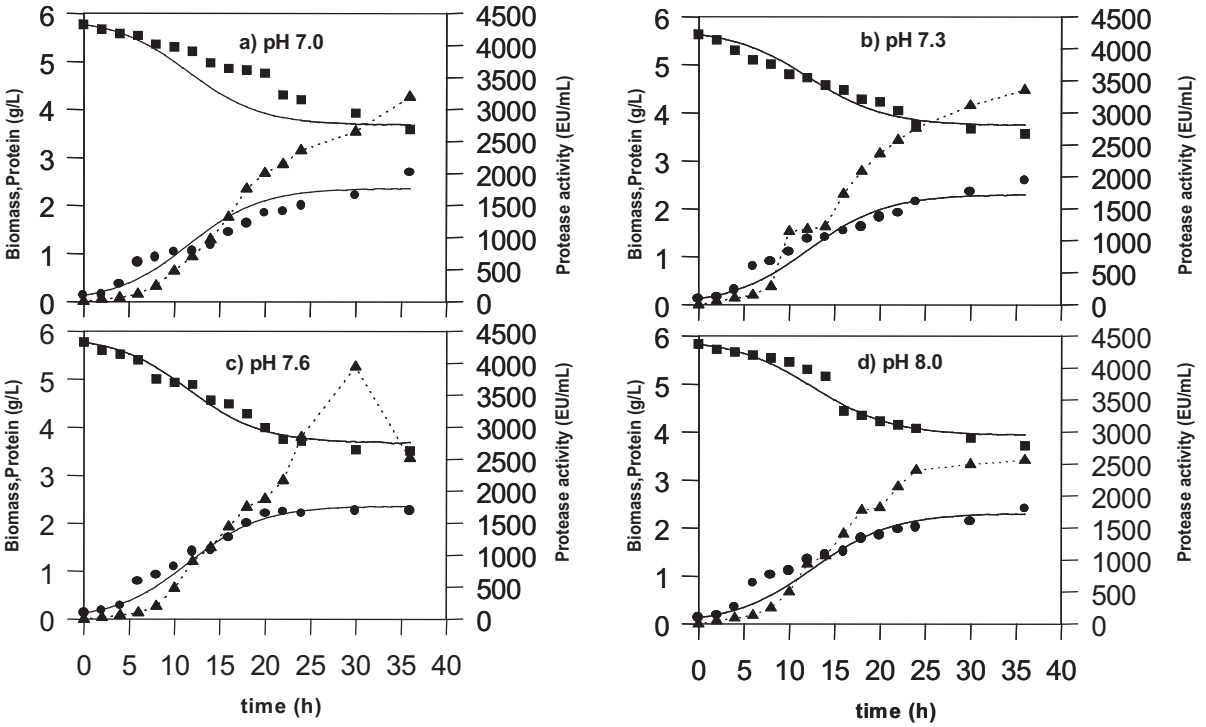

Figure 2. pH effect on cell growth (●), protease production (▲) and protein consumption (■) during flask fermentations: a) pH 7, b) pH 7.3, c) pH 7.6, d) pH 8. Solid lines show the theoretical data obtained by fitting experimental data to Eq. 1 and 2.

On the other hand, there are clear differences with regards to protease production. The highest value achieved was 3,940 ± 236 EU/mL, corresponding to a pH value of 7.6. High levels of production were also obtained for pH values 7.0 and 7.3 and at 36 hours protease production still showed a clear tendency to increase. In any case, 7.6 was selected as the optimal pH value because the highest levels of protease activity is obtained before, being this value slightly higher than those described and employed in previous studies [6, 9, 21]. When fermentation was carried out at pH 8.0, the achieved protease activity was only 2,500 EU/mL (Figure 2.d). These results prove the influence pH has on protease production by S. marcescens.

Initial protein concentration effect

Proteins are employed by *S. marcescens* when they are grown in whey as carbon and nitrogen sources. The purpose of this study was to determine how the initial protein concentration influences cell growth and protease production. Fermentations were carried out employing fresh whey with different initial protein concentrations. The results of these fermentations (Figure 3) show that when the initial protein concentration increases, the maximum cell concentration achieved also increases as well. In all cases, 40% of the initial protein content was consumed irrespective of the initial protein concentration. Surprisingly, a maximum cell growth was not accompanied by maximum protease production. In fact, maximum protease activity of 4,500 EU/mL ± 167 SD, was achieved in the fermentation that started out from a protein concentration of 6.0 g/L. However, significant differences in protease production were not seen among the three fermentations, since the lowest protease activity obtained was only approximately 13% lower than the highest one. It seems that a single optimum initial protein concentration does not exist, at least within the range of 3.5 g/L and 7.4 g/L (protein content in whey after microfiltration), and this is not a fundamental factor for protease production.

The similarity of the obtained enzymatic activities in the different experiments indicates that the proteins are not the only inductors of the proteolytic metabolism of *S. marcescens* when it

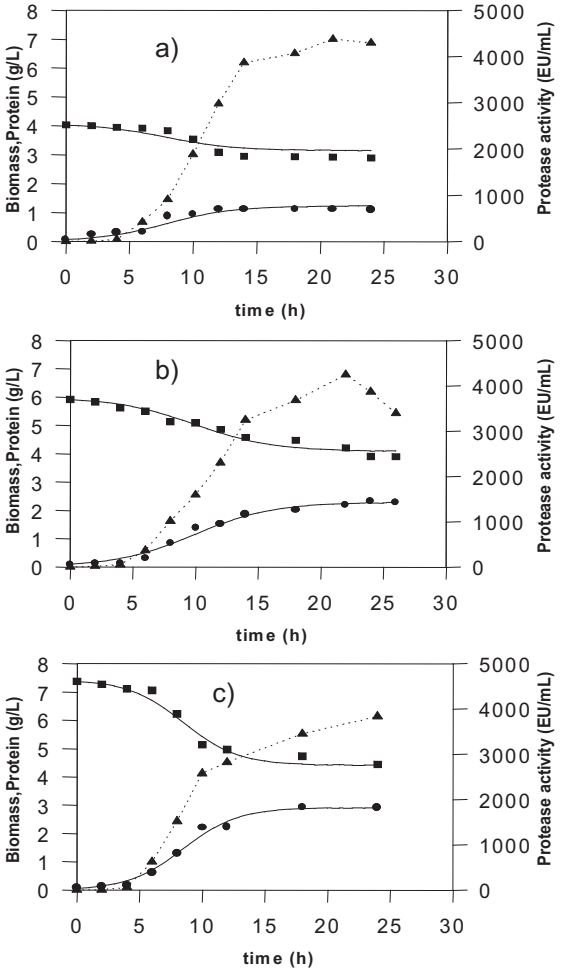

Figure 3. Initial protein concentration effect on cell growth (●), protease production (▲) and protein consumption (■) during flask fermentations. Initial protein concentrations (g/L): a) 4.0, b) 6.0, c) 7.4. Solid lines show the theoretical data obtained by fitting experimental data to Eq. 1 and 2.

is grown in fresh whey [14, 20]. These results corroborate experiments carried out with the bacterium *S. marcescens* and individual major whey proteins as sole substrate at the same concentration gave rise to different protease activity levels depending on the type of protein employed. The highest level of protease activity was achieved with bovine serum albumin, a slight increase in protease activity being obtained when D(+) calcium pantothenate or riboflavin were added to the medium [17]. The low levels of activity obtained with a mixture of individual whey proteins

eliminate the possibility of a positive synergic effect among these proteins on protease production. Compared to the high protease activity levels obtained when fresh whey is used as the culture medium, these results indicate that fresh whey must have a certain minor component that causes a higher level of production [17]. However, as previously mentioned, protein concentration influences cell growth directly, showing once more that protease production by *S. marcescens* is not fully associated with cell growth.

Dissolved oxygen effect

Considering that the dissolved oxygen present in the medium may drastically affect microorganism metabolism, a study was conducted on how its concentration influences cell growth and protease production. Previous studies [22-24] with other systems concur with the quantity of oxygen present in the culture medium during fermentation playing an important role in the production of these enzymes, as well as in the production of other metabolites. In fact, studies with *Bacillus licheniformis*, which is also an important protease producer, have shown that changes in the oxygen transfer rate influence the central pathways and consequently in the formation of the necessary intermediates precursors to produce serine alkaline protease [24]. This effect has also been studied for the production of chitinases by *S. marcescens* grown in a synthetic medium [23]. As the optimum oxygen concentration for protease production depends on the kind of microorganism and the conditions in which fermentation is carried out, this parameter has also been studied in the system considered in this research. Different oxygen concentrations were tested in a bioreactor containing 2.6 L of microfiltered whey, employing previously established optimal values of temperature and pH. The evolution of cell concentration, protein concentration and protease activity in the different cases assayed are shown in Figure 4. The optimal

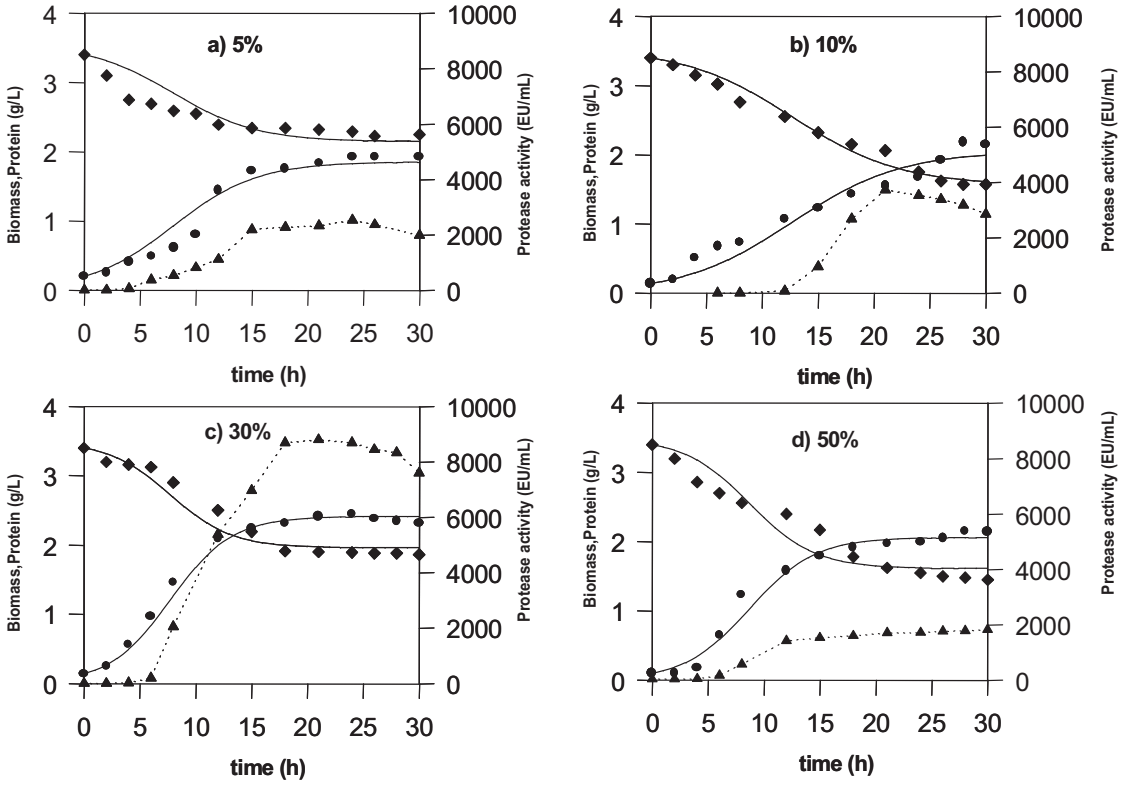

Figure 4. Dissolved oxygen concentration effect on cell growth (●), protease production (▲) and protein consumption (■) during bioreactor fermentations. Dissolved oxygen concentrations (% of saturation): a) 5, b) 10, c) 30 and d) 50. Solid lines show the theoretical data obtained by fitting experimental data to Eq. 1 and 2.

concentration for cell growth, as well as for protease production, appears to be 30% of dissolved oxygen. The difference in biomass concentration regarding other cases is hardly considerable. However, in the case of protease production, levels of 8,800 EU/mL ± 484 SD, were achieved when 30% of dissolved oxygen was employed, which are substantially higher values than those achieved for other oxygen concentrations and also over the protease activity levels obtained in the experiments carried out in flasks. The optimal dissolved oxygen concentration for protease production is within the range (20%-50% saturation) described as optimal for chitinase production by *S. marcescens* [22].

Types of proteases produced by *S. marcescens*

The results obtained for protease assays in the presence of inhibitors showed that 1,10 phenanthroline inhibited 84% ± 2SD of the total activity, indicating the presence of high levels of metalloproteases in the sample. Finally, phenylmethylsulfonyl fluoride, an irreversible inhibitor of serine protease, inhibited 13% ± 2SD of the total activity. These results suggest the production of only two kinds of proteases by *S. marcescens* in fresh whey (data not shown). This tendency was observed in all the systems studied. Similar results were obtained with whey powder and other proteinaceous substrates [13, 25].

Kinetic fitting of cell growth and substrate consumption

Experimental results of cell growth were fitted to the Riccati [26] equation (1), whereas the substrate data were fitted to equation (2). Since it was observed that protein consumption was proportional to biomass growth, where X and S are the biomass and protein concentration respectively (g/L), t is time (h), k and τ ($1/X_{stationary}$) are kinetic parameters and $Y_{S/X}$ is the yield factor.

$$dX/dt = k\,X\,(1-\tau\,X), \quad (1)$$

$$dS/dt = -Y_{S/X}\,(dX/dt). \quad (2)$$

Fitting parameter values are shown in Table I, while the results of the simulation are

Table I
Kinetic parameter values obtained when flask and bioreactor fermentations data were fitted to Equations 1 and 2

Parameter	Conditions	K (h^{-1})	τ (L/g)	$Y_{s/x}$ (g/g)
Initial pH	7.0	0.20	0.39	0.79
	7.3	0.23	0.44	0.87
	7.6	0.25	0.42	0.94
	8.0	0.23	0.43	0.87
Temperature (°C)	26	0.21	0.64	1.26
	30	0.25	0.42	0.94
	34	0.21	0.43	0.68
	37	0.30	0.38	0.77
Initial protein concentration (g/L)	4.0	0.37	0.81	0.75
	5.9	0.32	0.44	0.83
	7.4	0.46	0.34	1.04
Dissolved oxygen concentration (%)	5	0.26	0.57	0.82
	10	0.29	0.49	0.89
	30	0.39	0.40	0.55
	50	0.37	0.43	1.07

shown as solid lines in Figures 1, 2, 3 and 4, as compared with the experimental data. The values of k range from 0.20 h^{-1} to 0.60 h^{-1} and the values of τ from 0.38 L/g to 0.89 L/g.

Although the fitting parameter values of the Riccati equation are not very distinct from those in the different experiments, some considerations may be highlighted in relation to the conditions of each experiment.

When the pH value is 7.0, the k value is slightly lower than in the experiments carried out at higher pH values, thus indicating slower cell growth. Regarding dissolved oxygen concentration in the medium, k took a maximum value of 30% dissolved oxygen and its values decreased for lower and higher oxygen concentrations. It seems that the presence of dissolved oxygen in the medium improves cell growth, but only until a certain concentration, above which the oxygen acts as an inhibitor. The k values obtained from the experimental results were fitted to an equation proposed by Andrews (as cited in 26) to treat substrate inhibition:

$$k = k_{\max} \frac{O}{K_s + O + O^2 / K_i}, \quad (3)$$

where O is the dissolved oxygen concentration (% of saturation) and k_{max}, K_s and K_i are constants. Figure 5, shows the results of the fitting values for k_{max}, (maxima saturation constant), K_s (saturation constant) and K_i (inhibitor constant) being 0.54 h^{-1}, 6.09 h^{-1} and 150 h^{-1}. However, note should be taken of the scarcity of the data to consider the significance of the parameters.

The fitting parameter $Y_{S/X}$ (proteins consumed per biomass generated) ranges from 0.55 to 1.30. This value does seem to present a direct dependence on the initial pH and protein concentration employed in the system during S. marcescens growth, whereas, the other variables do not seem to affect this parameter.

Conclusions

The high levels of protease production by *S. marcescens* in whey, under optimal conditions, have shown that fresh whey is a good substrate for protease production by fermentation. Initial protein concentrations do not seem to be a fundamental factor within the tested range and the results obtained were similar for initial protein concentrations between 3.5 g/L and 7.4 g/L. On the other hand pH, temperature and dissolved oxygen significantly influenced protease production, and the optimal values for maximum enzyme production were determined (pH 7.6, 30°C and 30% of dissolved oxygen saturation). Under these conditions, protease activity was increased until 8,800 EU/mL, making this system suitable for producing proteases at a higher scale. Finally, cell growth and protein degradation were modelised by the Riccati equation, and it was assumed that protein degradation was proportional to cell growth, although this could not be applied to protease production. Moreover, the influence of oxygen concentration was introduced in the model using Andrews' equation for substrate inhibition.

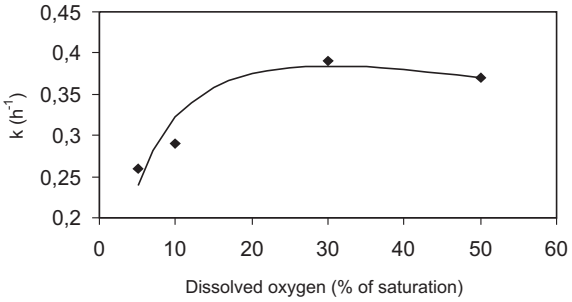

Figure 5. Dissolved oxygen concentration effect on cell growth. Solid lines show the theoretical data obtained by fitting k values presented in Table 1 to Equation 3.

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