High cell density reactor for the production of *Lactobacillus plantarum*

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Key words: Flocculation; Fluidized bed reactor; *Lactobacillus plantarum*; Lactic starter culture

SUMMARY

The production of a flocculent strain of *Lactobacillus plantarum* was performed in a high cell density reactor: a fluidized bed reactor (FBR) with a settler and an external cell recirculation. Two variables were assessed, the recirculation rate (R) and the dilution rate (D). The effect of the latter is much more important than the effect of the former in ensuring a quick start up in the flocculation process. The cell volumetric productivities obtained with this system increase directly with dilution rate and recirculation rate. The values of cell volumetric productivities obtained are considerably higher than those obtained in continuous stirred tank reactors (CSTR) and much higher than in batch reactors.

INTRODUCTION

Lactobacillus is an important group of microorganisms used for industrial production of a variety of milk-derived products, crakers, fermented vegetables, meats and crop silages. They are known to possess therapeutical benefits and as such, are also used as intestinal flora controllers. Nutritional advantages and production of flavours and bacteriocins are other attributes of these bacteria [6,9,13,20].

Lactobacillus plantarum, the microorganism used throughout this work, is a facultative heterofermentative *Lactobacillus*, which means that it produces mainly lactic acid from hexoses but can produce lactic and acetic acids depending on the level of substrate and oxygen in the medium [8]. Care must be taken to prevent a change from homo to heterofermentative behaviour during its production. This strain is widely used as a silage starter for different vegetables and crops.

The production of these lactic starter cultures has been traditionally done using batch fermentation followed by centrifugal separation, ultrafiltration and spray-drying. Continuous and/or dialysis cultures fermentation with lactic *Streptococci* have also been attempted [11,15].

Higher cell concentrations can be obtained by using bioreactor systems with retained biocatalysts like microorganisms, plant or animal cells [19]. Cell flocculation is one of the retention techniques that has been used, not only with yeasts–which have the most well studied flocculation phenomena–but also with bacteria, filamentous fungi and animal cells [4]. Cells that have a natural tendency to form flocs can be pushed to a higher aggregation state by changing the fermentation conditions and the hydrodynamics of the reactors where they are produced [5,14].

Tower reactors or column fluidized bed reactors are systems where flocculent microorganisms can accumulate and so be produced with high cell volumetric productivities [18].

Fluidization has many advantages for cell production. As there are low pressure drops, fluidized bed reactors require less energy for pumping the fluid phase; furthermore, at low Reynolds numbers (higher than, approximately, 1) this reactor type has the highest energetic efficiency (transport efficiency/energy dissipated) in comparison with continuous stirred tank reactors (CSTR) or packed bed reactors [7]. Fluidization keeps the particles separated allowing good mass and heat transfer characteristics and diminishes the problems of plugging. Finally it allows work to be carried out at dilution rates higher than that responsible for wash-out and thus increase productivities.

This paper reports experiments performed in order to obtain a lactic starter culture more efficiently by exploring the use of a fluidized bed reactor (FBR) with an external cell recycle using a flocculent strain of *L. plantarum*.

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MATERIALS AND METHODS

Microorganism. The culture used was *Lactobacillus plantarum* 7, a flocculating strain supplied by Profs. V. Bottazzi and S. Cocconcelli (Istituto di Microbiologia, Univ. Cattolica del Sacro Cuore, Piacenza, Italy). Culture was kept at 4 °C in MRS medium [12] and transferred monthly.

Culture media. Experiments were performed with MRS medium which contained in 1 liter of tap water: tryptone, 10 g; beef extract (Difco), 1 g; yeast extract, 5 g; K_2HPO_4, 2 g; sodium acetate, 5 g; di-ammonium hydrogen citrate, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.05 g. Tween 80 was omitted, as it could interfere with the flocculation process. Glucose was used as carbon source.

Cell concentration determination. Dry weights of biomass were determined using 5 ml samples of broth culture filtered through a membrane (0.2 μm) and dried for 72 h at 105 °C. When needed, turbidities of broth cultures were expressed as optical densities (OD) at 540 nm. A calibration curve was constructed by plotting optical densities against dry weights.

Glucose, lactic and acetic acids determination. Glucose, ethanol, lactic and acetic acids were determined by HPLC with a Shodex Sugar SH-1011 column (Macherey-Nagel, F.R.G.), using 0.01 N H_2SO_4 as the eluent, and a flow-rate of 1.0 ml/min at 50 °C; a refraction index detector was used (ERC-7511, Erma Inc., Japan).

Reactors. Batch and continuous stirred tank reactor (CSTR) studies were performed in 2-l Setric fermentor (SGI, France) allowing for temperature and pH control.

The fluidized bed reactor (FBR) was a cylindrical column, 80 cm in height and 4 cm in diameter with an external settler allowing recirculation (Fig. 1). Temperature was controlled by water jacket and the pH value was maintained at the desired value by a pH control system, using a pH electrode placed at the top of the column and an ammonia injection in the middle of the column.

RESULTS AND DISCUSSION

FBR

The FBR was operated with a system volume of 1760 ml, the temperature was maintained at 31 °C and pH was controlled at 5.5. Samples were taken at three sampling ports (1, 3 and 4 in Fig. 1). Effluent stream samples coincided with sampling port 4.

Preliminary experiments [3] were performed in the column using different dilution rates, without emptying the column between runs. The settler was emptied before the change in dilution rate so that the accumulation of biomass could be measured.

A different approach was used in this work, where recirculation rate was also varied. To test the start up of the flocculation process and steady state conditions, the column was washed, sterilized and inoculated before each run. The fermentation broth always had the same sugar concentration, 70 g/l.

The efficiency of flocculation in the system was assessed by a flocculation factor defined as:

$$\text{Flocculation factor} = \frac{\text{Cell concentration in the column samples}}{\text{Cell concentration in the effluent samples}} \quad (1)$$

In FBR tests, two operational parameters were studied to optimize the production of cells: the recirculation rate (R) and the dilution rate (D).

Recirculation rate

Different recirculation rates in FBR were maintained by the use of a peristaltic pump. The inlet feed rate, I (m/h) of the MRS medium was controlled with a peristaltic pump, and the same dilution rate, 0.76 h^{-1}, was used for all these experiments. Operational conditions for the experiments are summarized in Table 1.

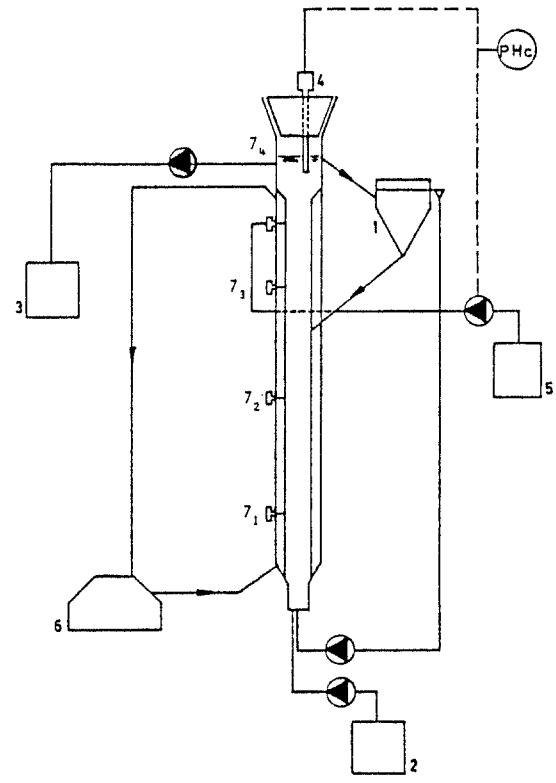

Fig. 1. Fluidized bed reactor. 1, Settler; 2, Feed vessel; 3, Overflow vessel; 4, pH electrode; 5, Base vessel; 6, Temperature control; 7, Sampling ports (1, 2, 3 and 4).

Fig. 2 and 3 represent the growth curves obtained with different recirculation rates at levels 1 and 3 in the column, corresponding to heights of 19.5 cm and 53.5 cm above the bottom of the column, respectively.

Both growth curves show the same pattern of cell production in FBR: an initial phase without flocs, where cell concentration at levels 1, 3 and 4 are very similar; a flocculation phase follows, characterized by a sudden change in cell concentration as the flocs appear along the column, starting from the bottom, and finally a stationary phase establishes itself with some instability.

As can be seen from Fig. 3, at recirculation rates near 21.29 m/h, the flocs do not attain level 3, as for hydrodynamic reasons, they enter the settler return tubing. Thus, under such conditions part of the column was not being used.

Figs. 2 and 3 also show that as the recirculation ratio increases a faster flocculation process is obtained. This is apparent both a level 1 and 3, always taking place earlier at level 1. Higher cell concentrations in the stationary phase can be obtained at higher recirculation ratios.

The flocculation process seems, then, to be related to the mixing in the reactor. Better mass transfer conditions are established as the system changes from a situation nearing plug-flow (lower recirculation ratios) to a situation closer to CSTR (higher recirculation ratios). Increasing the number of passes in the column also illustrates this change in the column hydrodynamics.

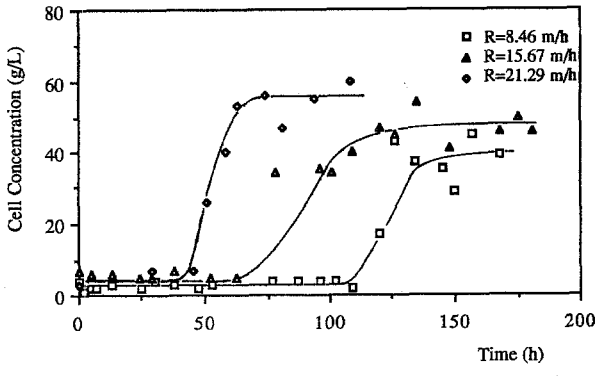

Fig. 2. Growth curves obtained in FBR at $D = 0.76\ h^{-1}$ and different recirculation rates (at level 1 in the column).

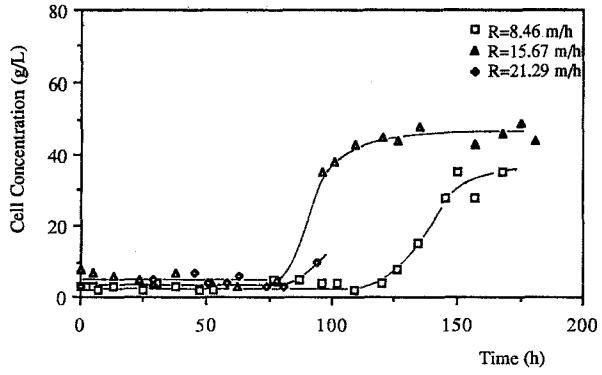

Fig. 3. Growth curves obtained in FBR at $D = 0.76\ h^{-1}$ and different recirculation rates (at level 3 in the column).

At higher recirculation rates more flocs are produced, their average size being smaller than at low recirculation ratios. Also more free cells and microscopic flocs are being washed out from the column. So, when the flocculation factor was calculated, at level 1, for the three experiments, an average of approximately 15 was reached in the flocculation phase.

A mass balance was used for calculating cell volumetric productivities obtained in the FBR:

$$\frac{dx}{dt} = Dx_{out} + \frac{dx_{reactor}}{dt} + \frac{\Delta x_{settler}}{\Delta t} \qquad (2)$$

where x = cell concentration, g l^{-1}; t = time, h; and D = dilution rate, h^{-1}

In Eqn. 2 the term Dx_{out} accounts for the free cells that are continuously being washed out. As in FBR cell mass is accumulated mainly by the flocs formed, the term $dx_{reactor}/dt$ is directly related to floc formation and growth during the experiment. The equation 2 also considers the accumulation of cells in the settler. This term, $\Delta x_{settler}/\Delta t$, was accounted for only at the end of each experiment,

TABLE 1

Operational conditions for FBR at $D = 0.76\ h^{-1}$

Recirculation rate (R) (m/h)	Recirculation ratio, r (R/I)[a]	Time of ascens. flow, Tp[b] (h)	Residence time, T_r ($1/D$) (h)	No. of passes T_r/T_p
8.46	16.15	0.089	1.32	14.78
15.67	29.91	0.049	1.32	26.85
21.29	40.64	0.037	1.32	35.56

[a] I = Inlet feed rate expressed as equivalent ascentional velocity in the column (m/h).

[b] T_p = length column/total ascensional velocity.

assuming constant rate of accumulation in the settler with time.

At the initial and final stages in the stationary phase cell volumetric productivity is mainly accounted for by cells being carried away from the system. Fig. 4 depicts the values of cell volumetric productivities at different recirculation rates, referring to samples taken at level 1. Higher cell volumetric productivities are obtained at higher recirculation rates.

As was mentioned before, at high recirculation rates the mixing is stronger, resulting in smaller flocs and better mass transfer conditions. A situation of higher specific growth rate is then created, thus the system has higher cell volumetric productivities. Also, as conditions get closer to CSTR, the reactor becomes theoretically more performant for an autocatalytic reaction like microorganism production [1].

At the end of each stationary phase, the cell volumetric productivities are slightly larger than at the beginning; this is due to more cells being washed out from the system after the flocculation process getting well established, i.e., a larger X_{out} is obtained. A purge strategy will, of course, be required for a real steady state to be established; the observed phenomenon actually corresponds to a "natural" purge situation–but in this case no complete steady state should be expected.

As the aim of this work is the production of a starter culture, the lactic acid being formed by the cells is not strictly relevant. Nevertheless it enables us to assess the metabolic state of the culture. So, specific productivities (g acid/g cell per h) were calculated for every recirculation rate tested. The results are summarized in Fig. 5, based on samples obtained at level 1. Before the flocculation phase, specific productivities are similar to those obtained in CSTR. This behaviour should be expected at higher recirculation rates, corresponding to a reactor working closer to CSTR, but a small difference should be expected

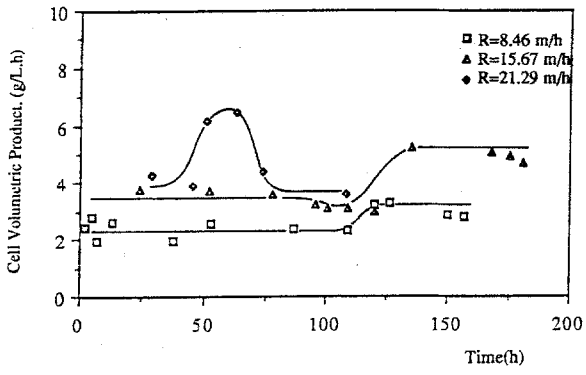

Fig. 4. Cell volumetric productivities obtained in FBR at $D = 0.76\ h^{-1}$ and different recirculation rates (at level 1 in the column).

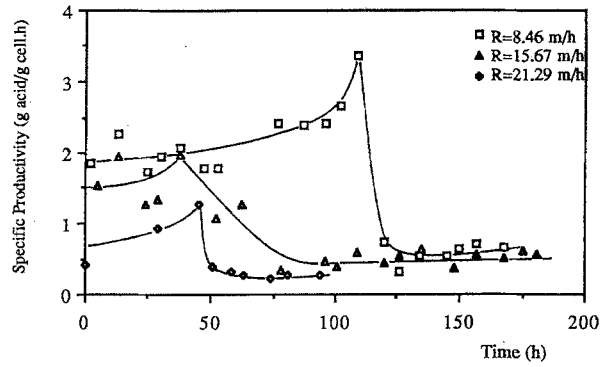

Fig. 5. Specific productivities obtained in FBR at $D = 0.76\ h^{-1}$ and different recirculation rates (at level 1 in the column).

at the lower recirculations corresponding to a plug flow regime. The most relevant fact is that after the flocculation phase, corresponding to a drop in specific productivities, their values are not very different for all the three recirculation rates. This is due to larger flocs being obtained at smaller recirculation rates; coupled with weaker mixing, this will result in a larger percentage of cells inside the flocs being unable to produce acid, as mass transfer problems arise. Thus, the viability of the cells in the flocs is dropping with an increase in floc size.

As higher cell concentrations, better cell volumetric productivities and better viabilities are obtained at higher recirculation rates, these should then be utilised in the column.

Dilution rate

Three different dilution rats were tested in the FBR for a recirculation rate of 8.46 m/h. The operational conditions for the tests are summarized in Table 2. Figs. 6 and 7 show the growth curves obtained with different dilution rates at levels 1 and 3 in the column. Again the same pattern of cell production for FBR as mentioned earlier is apparent: an initial phase without floc formation, a growth phase and a final stationary phase.

The effect of the dilution rate in the starting of the flocculation process and in the cell concentration obtained at the stationary phase is shown: increasing the dilution rate nearly five times from $0.24\ h^{-1}$ to $1.27\ h^{-1}$ the beginning of the flocculation process is obtained in a fifth of the time. Also the concentration at the stationary phase is higher when working at higher dilution rate. As before, the "jump" in cell concentration takes place later at level 3, specially at lower dilution rates, as the flocs begin to establish themselves at the bottom of column and the "wave" of floc production proceeds upward with time.

Considering the recirculation ratios, r, for these experiments we can see that their decrease quickens the flocculation process. This behaviour is the opposite to that

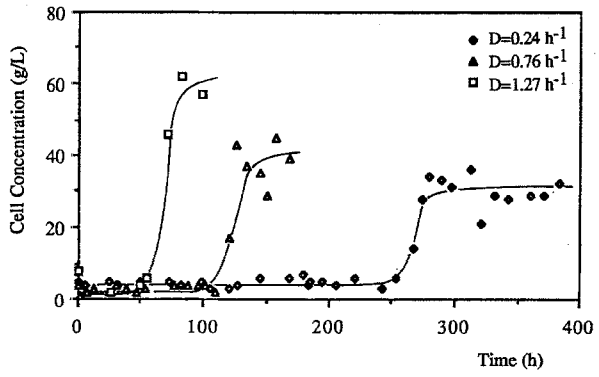

Fig. 6. Growth curves obtained in FBR at R = 8.46 m/h and different dilution rates (at level 1 in the column).

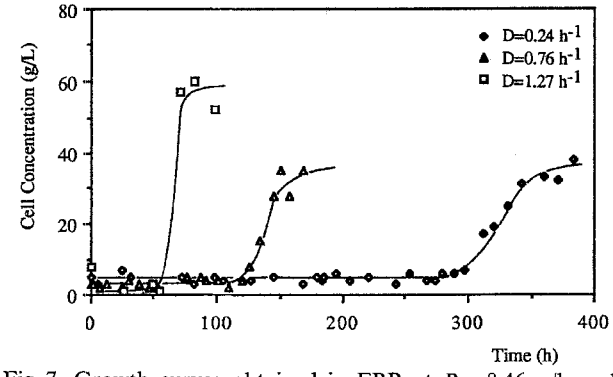

Fig. 7. Growth curves obtained in FBR at R = 8.46 m/h and different dilution rates (at level 3 in the column).

observed for the effect of the recirculation rate, where a direct relationship is apparent. (Table 1, Figs. 3 and 4). From this one might conclude that the main factor influencing flocculation is not the total ascensional velocity but the rate of substrate feed to the system and the rate of product removal from the column.

As the dilution rates increase cell volumetric productivity increases at all phases of the growth curve. This can be clearly seen in Fig. 8. Removal of inhibitory lactic acid and addition of substrate, as mentioned before, establish for better growth conditions for cells inside the column. Specific growth rates rise, thus higher cell volumetric productivities are obtained.

Specific productivities were also calculated for the different dilution rates. The results, shown in Fig. 9, refer to samples taken at level 1 in the column. A clear pattern can be observed: an initial phase where specific productivities are high followed by a drop that corresponds to the appearance of flocs and a new stable zone that corresponds to the final stationary phase in cell concentration.

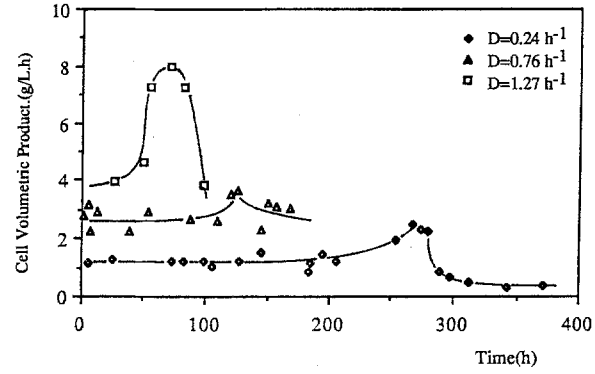

Fig. 8. Cell volumetric productivities obtained in FBR at R = 8.46 m/h and different dilution rates (at level 1 in the column).

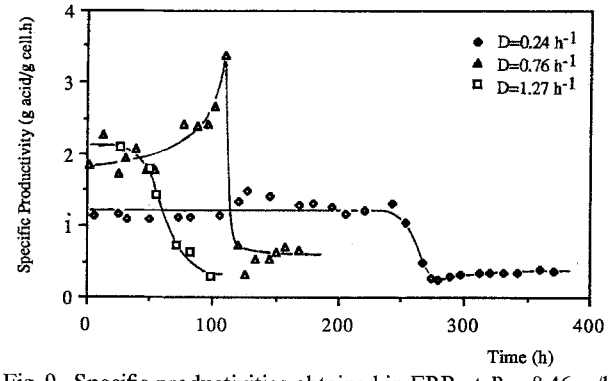

Fig. 9. Specific productivities obtained in FBR at R = 8.46 m/h and different dilution rates (at level 1 in the column).

TABLE 2

Operational conditions for FBR at R = 8.46 m/h

D (h^{-1})	Feed rate (I) (m/h)	Recirculation ratio, r (R/I)[a]	Time of ascens. flow, Tp[b] (h)	Residence time, T_r ($1/D$) (h)	No. of passes T_r/T_p
0.24	0.162	52.13	0.093	4.17	44.80
0.76	0.524	16.15	0.089	1.32	14.78
1.27	0.896	9.43	0.085	0.79	9.26

[a] I = Inlet feed rate expressed as equivalent ascensional velocity in the column (m/h).
[b] T_p = length column/total ascensional velocity.

This last phase yields little differences in specific productivities for the different dilution rates. A similar pattern had been observed for different recirculation rates. As higher cell concentration and higher cell volumetric productivities are also obtained at high dilution rates, these seem to be the better operational conditions.

The results obtained in FBR can be compared with batch and continuous stirred tank reactor (CSTR).

Batch and CSTR

Batch and CSTR tests were performed at 31 °C and controlled pH at 5.5. Such results are summarized in Table 3.

No wall growth or floc formation was observed in batch experiments with or without pH control. It should be mentioned that the specific productivities considered for the batch tests refer to values of cell concentrations similar to those obtained in CSTR so that results can be compared. Times at which these specific productivities where obtained in batch reactor are also referred in Table 3.

The kinetics of flocculent *L. plantarum* 7 in continuous operation are characterized by the appearance of flocs and wall growth. This explains the apparently anomalous rise in cell concentration at high dilution rates [3]. Similar results have also been observed for other bacteria, namely aquatic [16,17].

Macromolecular components of bacterial surface like lipopolysaccharides, protein or exopolymers have already been shown to vary in quantity and composition with dilution rate in continuous cultures [10,14]. A change in surface composition of the cells of *L. plantarum* can explain this behaviour at high dilution rates.

Comparing the results between batch, CSTR and FBR in Table 3, one can see the striking difference in cell concentrations obtained at the different dilution rates for the two continuous reactors: the FBR always has largest cell concentration. Also higher cell volumetric productivities are obtained in FBR than in batch or CSTR. Specific productivities are lower in FBR after the flocculation process possibly due to the diffusional problems that exist inside the flocs, cutting the acid production and diminishing the viability of the cells.

TABLE 3

Comparative results between batch, CSTR and FBR

	Continuous reactors			Batch reactor
Dilution rate (h^{-1})				
Batch	–	–	–	
CSTR	0.21	0.80	1.04	
FBR (level 1)	0.24	0.76	1.27	
Cell concentration (g/l)				
Batch (t = 30 h)				11.9
CSTR	5.92	2.62	2.62	
FBR (level 1)	30.5	38.3	59.7	
Maxim. cell volum. product. (g/l per h)				
Batch (t = 12 h)				0.70
CSTR	1.2	2.1	2.7	
FBR (level 1)	2.3	4.3	8.0	
Specific prod. (g acid/g cell per h)				
Batch				
(2.5 g cell/l, t = 8 h)				0.605
(6.1 g cell/l, t = 11 h)				0.252
CSTR	0.74	1.59	1.92	
FBR				
(before floc)	1.23	2.30	2.30	
(after floc)	0.54	0.56	0.46	

Conclusions

The flocculation process depends on natural causes (genetic programming and life cycle) and on environmental causes (growth media, type and hydrodynamics of reactors). The mechanisms of floc formation by *L. plantarum* are not known. But production of exopolymers by lactic acid bacteria have already been reported [2]. These polymers could be responsible for the adhesion of cells to glass and for the net aggregation of cells. The eventual production of such exopolysaccharides by *L. plantarum* is being studied at the moment in our laboratory by NMR (nuclear magnetic resonance). Preliminary results seem to indicate the production of carbohydrate polymers by the strain *L. plantarum* 7.

No floc formation was observed in the batch studies with or without pH control. On the other hand floc formation was observed in both continuous systems used, CSTR and FBR. This shows that the type of operation used, batch or continuous, is very important in terms of flocculation phenomena.

The tests performed in FBR indicate that the hydrodynamics of the system is an important parameter for the control of the flocculation process: at high recirculation rates, high cell concentrations and cell volumetric productivities are obtained. Also, a quick start-up of the flocculation process is observed which might be important for a production process.

Operating the FBR at different dilution rates demonstrated the strong influence of the dilution rate on the start up of the flocculation process. This can be explained by the difference in nutrient concentrations that the cells "see", which can influence the production of exopolymers, that may be responsible for the aggregation process. The results presented herein show that main factor influencing flocculation is not the total ascensional velocity but the rate of substrate feed to the system and the rate of product removal from the column.

Although a stronger influence of the dilution rate on the start-up has been shown, a compromise between the two variables has to be reached in the fluidized bed reactor to achieve a high production of a starter culture of *L. plantarum*. Purge strategy tests are being performed in our laboratory in order to establish real steady states in FBR, the purge being the product of the process.

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REFERENCES

1 Bailey, J. and D.F. Ollis. 1977. Biochemical Engineering Fundamentals, pp. 554–568, McGraw-Hill Book Company, New York.

2 Bottazzi, V. and P.G. Sarra. 1987. I batteri lattici nell'ecologia microbica dell tratto digerente. In: Aggiornamento di Microbiologia dei Batteri Lattici (Bottazzi, V., ed.), pp. 221–238, Centro Sperimentale del Latte, Milan, Italy.

3 Barreto, M.T.O., E.P. Melo and M.J.T. Carrondo. 1989. Starter culture production in fluidized bed reactor with a flocculent strain of *L. plantarum*. Biotechnol. Lett. 11: 337–342.

4 Calleja, G.B. 1984. Microbial Aggregation, CRC Press, Boca Raton, FL.

5 Calleja, G.B. et al. 1984. Aggregation–group report. In: Microbial Adhesion and Aggregation (Marshall, K.C., ed.), pp. 303–321, Dahlem Konferenzen 1984, Springer Verlag, Berlin.

6 Chassy, B. 1986. *Lactobacilli* in food fermentations. In: Biotechnology in Food Processing (Harlander, J.K. and T.P. Labuza, eds.), pp. 197–207, Noyes Publications, NJ.

7 Cheetham, P.S.J. 1983. The application of immobilized enzymes and cells and biochemical reactors in biotechnology. In: Principles of Biotechnology (Wiseman, A., ed.), pp. 194–195, Surrey University Press, London.

8 Condon, S. 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol. Rev. 46: 269–280.

9 Day, C.A. and S.G. Lisansky. 1987. Agricultural alternatives. In: Environmental Biotechnology (Forster, C.F. and D.A. Wase, eds.), pp. 234–294, Ellis Horwood, Chichester.

10 Ellwood, D.C. and D.W. Tempest. 1972. Effects of environment on bacterial wall content and composition. Adv. Microb. Physiol. 7: 43–47.

11 Lloyd, G.T. and E.G. Pont. 1973. An experimental continuous – culture unit for the production of frozen concentrated cheese starters. J. Dairy Res. 40: 149–155.

12 Man, J.C. de, M. Rogosa and M.E. Sharpe. 1960. A medium for the cultivation of *Lactobacilli*. J. Appl. Bact. 23: 130–135.

13 Marshall, V.M. 1987. Lactic acid bacteria: starter for flavour. FEMS Microbiol. Rev. 46: 327–336.

14 McEldowney, S. and M. Fletcher. 1986. Effect of growth conditions and surface characteristics of aquatic bacteria on their attachement to solid surfaces. J. Gen. Microbiol. 132: 513–523.

15 Porubcan, R.S. and R.L. Sellars. 1979. Lactic starter culture concentrates. In: Microbial Technology (Peppler, H.J. and D. Perlman, eds.), pp. 59–92, Academic Press, New York.

16 Rogers, P.L., L. Bramall and I.J. McDonald. 1978. Kinetics analyses of batch and continuous culture of *Streptococcus cremoris* HP. Can. J. Microbiol. 24: 372–380.

17 Schügerl, K. 1985. Bioreactor Engineering, vol. 1, pp. 185–189. John Wiley & Sons, Chichester.

18 Schügerl, K. 1989. Biofluidization: application of the fluidization technique in biotechnology. Can. J. Chem. Eng. 67: 178–184.

19 Scott, C.D., 1987. Immobilized cells: a review of recent literature. Enzyme Microb. Technol. 9: 66–73.

20 Steinkraus, K.H. 1983. Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. Antonie van Leeuwenhoek, 49: 337–348.