Journal of Industrial Microbiology & Biotechnology (2000) 24, 285–290

www.nature.com/jim

Industrial media and fermentation processes for improved growth and protease production by *Tetrahymena thermophila* BIII

J de Coninck[1], S Bouquelet[2], V Dumortier[1], F Duyme[3] and I Verdier-Denantes[1]

[1]*Institut Supérieur d'Agriculture, Laboratoire de Microbiologie des Fermentations, 41 rue du Port, 59046 Lille Cedex, France;* [2]*Université des Sciences et Technologies de Lille, Laboratoire de Chimie Biologique, UMR CNRS 8576, 59655 Villeneuve d'Ascq Cedex, France;* [3]*Institut Supérieur d'Agriculture, Laboratoire d'Informatique et de Statistique, 41 rue du Port, 59046 Lille Cedex, France*

Tetrahymena thermophila **was cultivated on industrial by-product media. The composition of the best medium (with milk proteins) was optimised by a central composite design for growth and protease secretion. The optimal combination [1.07% (w/v) of yeast extract and 0.99% (w/v) of skimmed milk] improved biomass production by 46%. In a fermentation strategy, the pH must be regulated to produce no cell damage, lengthening the stationary phase and resulting in a more abundant protease production. To increase cell concentration and protease secretion, a continuous culture with cell recycling by microfiltration was successfully tested on ciliated protozoa.** *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 285–290.

Keywords: *Tetrahymena thermophila*; industrial media; fermentation process; protease secretion

Introduction

The potential of protozoa as sources of commercially produced enzymes is clearly established [20]. Among ciliated protozoa, *Tetrahymena* has a biotechnological potential, particularly in the secretion of digestive hydrolases [13]. North [15] reported that *Tetrahymena* produced specific proteases with high activity rates in eucaryotic organisms. To date, they have not been exploited as sources of enzymes for industrial production and use. An explanation for this under utilisation is that protozoa are more difficult to culture on a large scale than bacteria or fungi.

Studies have been conducted to obtain mass cultures of *Tetrahymena* and to develop industrial axenic media [2,8,18]. But only a few fermentation strategies yielding biomass comparable to bacteria and yeast cultures have been developed for this organism.

In this study, we were interested in optimising growth of *Tetrahymena thermophila* on industrial media, improving the secretion of enzymes, especially proteases, and developing a fermentation strategy for this production.

Materials and methods

Strain and cultivation

Tetrahymena thermophila BIII was obtained from the Carlsberg Institute, Copenhagen. Batch fermentations were carried out in 2, 20 or 100-L fermentors (Biolafitte, Saint Germain en Laye, France) with turbine impellers and equipped with digital control units. The stirrer speed was limited to 300 rpm to avoid cell damage. The temperature was kept constant at 28°C. Aeration rate was maintained at 1 vvm. Working volumes were 1 L for 2-L fermentors, 12 L for 20-L fermentors and 60 L for 100-L fermentors. All experiments, except scaling-up, were carried out in 2-L fermentors. Before experimentation, cells were adapted to the different media by successive cultivations in 125-ml flasks with 10 ml medium over 1 week. For culture in 2-L fermentors, cells were cultivated in 1-L Erlenmeyer flasks with 100 ml medium before being inoculated into the 2-L fermentor. Inocula were always exponentially growing cultures to obtain 10 000 cells ml^{-1} in the fresh medium.

Media

Mye medium [18] was composed of 1% w/v yeast extract and 1% w/v skimmed milk, Ppys medium contained 0.75% w/v yeast extract, 0.75% w/v proteose peptone and salts [16]. Yeg medium contained 1% w/v yeast extract and 0.5% glucose [5]. Yex medium contained 1% w/v yeast extract and 4.5% w/v maltodextrins (glucidex 12^R, Roquette Freres, Lestrem, France). Yep medium contained 1% w/v yeast extract and 0.3% w/v potatoe solubles (Roquette Freres). Yem medium contained 1% w/v yeast extract and 0.3% w/v corn soluble (Roquette Freres). Fermentors containing 1 L of medium with 0.001% v/v antifoam (Sigma, St Quentin Fallavier, France, ref. A6426) were autoclaved for 25 min at 120°C.

Cell count and statistical analysis

Cell were counted electronically (Coulter counter Z1, Beckman Coulter France, Roissy CDG, France). Experiments were planned to use a variance analysis followed if necessary by the Newman–Keuls test [6,14]. When the variance analysis concluded that the results media were statistically different, the Newman–Keuls test enabled us to

Correspondence: J de Coninck, Institut Superieur d'Agriculture, Laboratoire de Microbiologie des Fermentations, 41 rue du Port, 59046 Lille Cedex, France. E-mail: j.deconinck@isa.fupl.asso.fr
Received 28 July 1999; accepted 20 January 2000

classify and to determine groups of averages. In each group, the averages are not considered different.

A central composite design was adopted to assess the combined effect of yeast extract and skimmed milk on generation time and maximal population (Table 1).

Data obtained were analysed by multiple regression using Minitab [11] software and the following equation—called a response model—was established to fit the data and calculate the optimal combination of yeast extract and skimmed milk:

$$y = b_0 + b_1(\mathrm{YE}) + b_2(\mathrm{MI}) + b_3(\mathrm{YE})^2 + b_4(\mathrm{MI})^2 + b_5(\mathrm{YE})(\mathrm{MI}) + \epsilon$$

y: generation time (min) or maximal population (10^6 cells ml^{-1}); b_0 to b_5: regression coefficients of the model; (YE): yeast extract in % w/v; (MI): skimmed milk in % w/v; (YE)(MI): interaction term; ϵ: error term (residual) supposed to be of null expected value and constant variance for the requirement for hypothesis testing. First partial derivatives were calculated to obtain optimal values or confidence regions of optimal values when simulation was necessary.

Enzyme assay

Protease secretion: The cells were removed from the medium by centrifugation at 16°C for 30 min at 300 × *g* (to avoid damaging cells). The proteolytic activity in the cell-free supernatant was measured spectrophotometrically using hemoglobin as the substrate with modifications to the procedure of Beynon and Bond [1]. The buffer was 0.2 M acetic acid–sodium acetate, pH 4.5. Substrate (1 ml) was added to 0.2 ml cell-free supernatant. After incubation at 55°C for 20 min, the reaction was stopped by adding 2 ml trichloroacetic acid (5% v/v). After 30 min, precipitates were removed by centrifugation at 4500 × *g* for 20 min and tyrosine was assayed as described previously [17]. The proteolytic unit corresponds to the amount of enzyme required for the release of 1 μmole of tyrosine per minute. Results were expressed as milliproteolytic units per ml (mU ml^{-1}).

Hexokinase assay (HK): HK was selected as a marker enzyme for the estimation of cell damage due to its high activity in the cytosol and good stability. HK activity was measured spectrophotometrically using glucose as the substrate according to the method of Kunst *et al* [10] in the presence of ATP and NADP at pH 7.6. Global HK activity in cultures was measured after lysis of cells. HK activity in the cell-free supernatant was measured after removal of the cells from the medium by centrifugation at 16°C for 30 min at 300 × *g* (to avoid damaging cells).

Substrate (0.5 ml) was added to 0.5 ml of cell-free supernatant. After incubation at 25°C for 20 min, the reaction was stopped by heating the mixture to 100°C for 2 min. Then the reaction product, glucose-6-phosphate, was oxidized by 1.0 ml glucose-6-phosphate dehydrogenase. After a 20-min incubation at 25°C, NADPH was determined spectrophotometrically at 340 nm. One HK unit corresponds to the amount of enzyme required for the release of 1 mmole of NADPH or glucose per minute. Results were expressed as millihexokinase units per ml (mU ml^{-1}).

Table 1 Levels of combined factors chosen for the central composite design

	Yeast extract (% w/v)	Skimmed milk (% w/v)
Minimum	0.5	1.0
Maximum	1.5	2.0

Results

Mass culture improvement

In the first stage, simplification of the media led to use of a medium containing only yeast extract as nitrogen and nucleotide source. In the second stage, to improve biomass production, addition of the following components was tested: glucose, skimmed milk, maltodextrins and potato or corn solubles.

Maximal populations in skimmed milk-based medium (Figure 1) were improved by 30% and generation times were shortened. The Newman–Keuls test indicated that Mye was the best medium for maximal cell production and in the same group of averages, generation times were shortened in Mye, Yem and Ppys.

Mye composition was optimised with a central composite design. Models of response surfaces (Figure 2) were determined using a multiple regression analysis (Table 2). Regression models could only be used between the levels

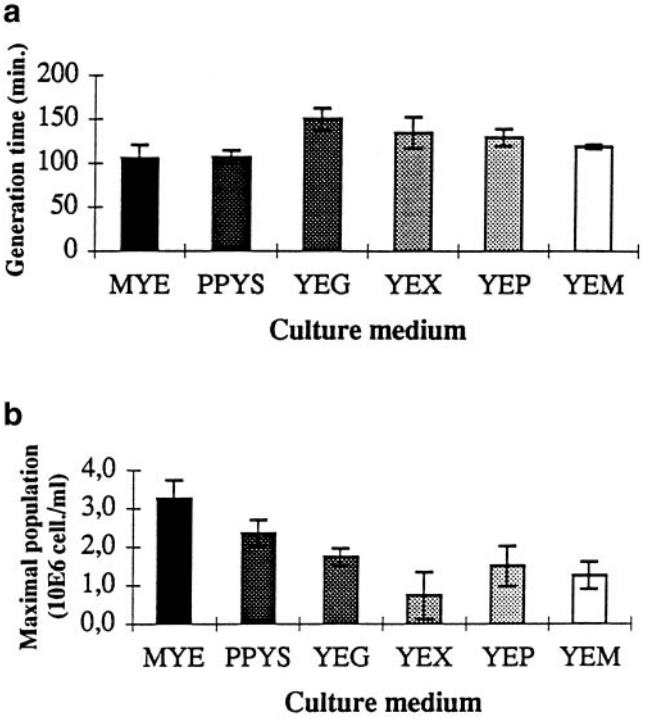

Figure 1 *Tetrahymena thermophila* growth: maximal populations (a) and generation times (b) on complex media.

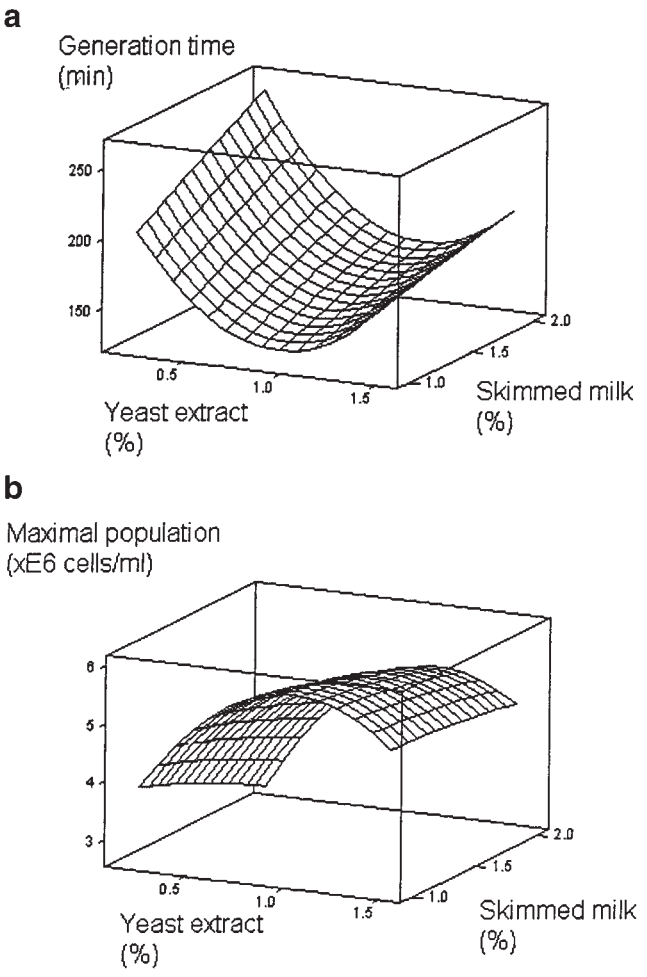

Figure 2 Response surfaces obtained for (a) generation time and (b) maximal population by means of central composite design for each combination of yeast extract and skimmed milk.

Table 2 Regression models linking generation time (min), maximal population (10^6 cells ml^{-1}) and skimmed milk and yeast extract (coefficient rounded)

Combined factors	Regression equation
Skimmed milk and yeast extract	GT = 219.1 − 229.8 (YE) + 73.8 (MI) + 135.5 $(YE)^2$ − 4.1 $(MI)^2$ − 61.3 (MI)(YE)
Skimmed milk and yeast extract	MP = 3.1 + 5.5 (YE) − 0.4 (MI) − 2.9 $(YE)^2$ − 0.2 $(MI)^2$ + 0.76 (MI) (YE)

GT (the response): generation time; MP (the response): maximal population.

of combined factors chosen for the central composite design (Table 1).

Optimal values of yeast extract and skimmed milk were determined and then optimal values of generation time and maximal population were calculated (Table 3). The optimal combination was 1.07% (w/v) of yeast extract and 0.99% (w/v) of skimmed milk and maximal cell production was improved.

Table 3 Best combinations of yeast extract and skimmed milk and corresponding optimal generation times and maximal populations

Optimal conditions	Generation time (min)	Maximal population (10^6 cells ml^{-1})
	132	5.88
Yeast extract (% w/v)	1.07	1.09
Skimmed milk (% w/v)	0.99	1.07

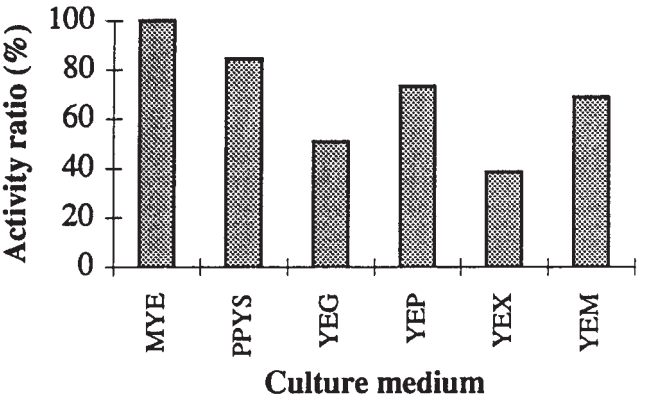

Figure 3 Protease secretion on different media.

Best medium for protease production

The different media were tested for their effect on protease secretion (Figure 3). The highest protease secretion was obtained on Mye (20% higher than on other media), indicating that milk proteins enhance protease secretion.

Improvements to culture techniques for growth and protease production

Scaling-up: We have compared *T. thermophila* BIII growth both in static cultures and in batch fermentors (Table 4). Cultivation in fermentors improved *Tetrahymena* culture: maximal populations were increased by 70% probably because aeration and agitation improved oxygen transfer in the medium.

Table 4 Influence of culture-type on *Tetrahymena* growth (Yeg medium, *n* = number of experiments)

Culture type	Generation time (min)	Maximal population (cells ml^{-1})
Flasks	188 (n = 5)	0.6×10^6 (n = 5)
Erlenmeyer flasks	172 (n = 5)	0.5×10^6 (n = 5)
Fermentors		
2 L	150 (n = 5)	1.7×10^6 (n = 5)
20 L	158 (n = 3)	1.9×10^6 (n = 3)
100 L	162 (n = 2)	1.7×10^6 (n = 2)

pH regulation: When pH was not regulated, the stationary phase was shortened, and the cells were lysed. Initially the Mye medium pH was 6.8 however, during fermentation, the pH reached 8.5 because ammonia was liberated. If the pH was regulated at 6.8, the stationary phase was lengthened about 100 h and maximal cells were improved by 55%. The generation time was not changed (Figure 4).

In pH-regulated cultures, protease secretion was more constant but lower than in unregulated cultures because in this culture, cells were damaged at the end of fermentation, and liberated intracellular enzymes and fermentation were stopped.

Continuous culture with cell recycle: Different processes were tested to produce high cell density as well as increased product yield. In a continuous culture system, high cell density was obtained by retaining or recycling the cells. We chose recycling technology where cells were separated from the liquid phase through microfiltration membranes and recycled back to the fermentor (Figure 5).

The fermentation system was a single continuous stirred tank reactor (pH regulated) employing polysulfone microfiltration membranes (SETRIC, Toulouse, France; average porosity: 0.55 μm; filtration surface: 0.4 m^2) for cell recycling. Pressure drop along the membranes varied from 0.5 to 1 bar. Cell density reached 1.24×10^7 cells per ml at stationary phase and was constant for 123 h (Figure 6). In addition, we measured the activity of the intracellular marker enzyme, HK, during fermentation. HK activity in the extracellular medium never exceeded 1% of the total activity indicating that cell lysis was very low. Protease secretion rates were lower than in batch fermentation but the enzyme was constantly secreted.

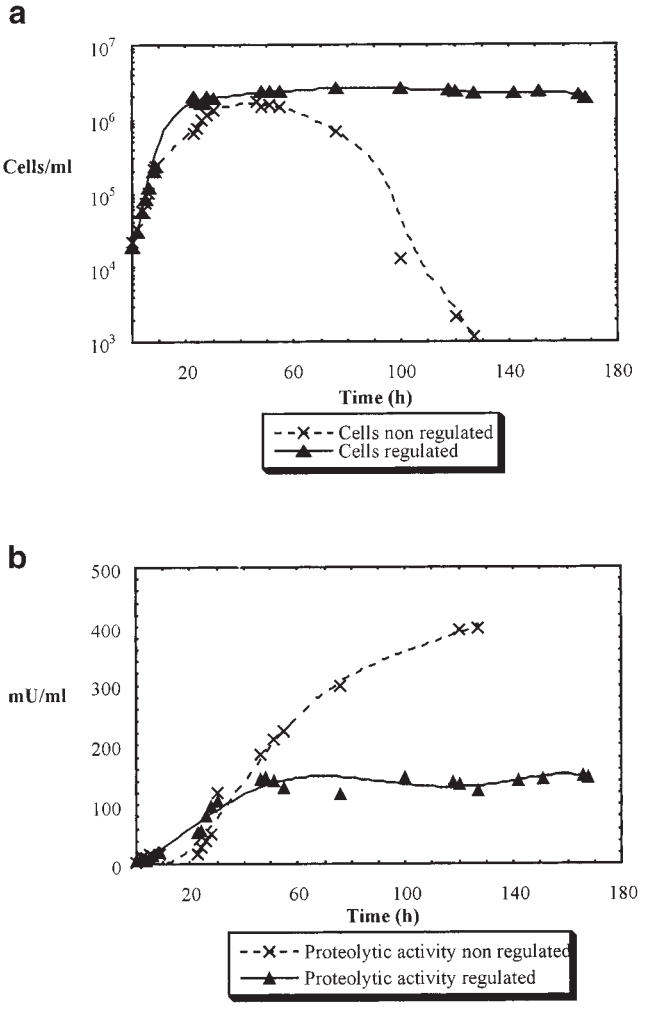

Figure 4 Influence of pH (regulated or not) on growth (a) and protease production (b) of *T. thermophila*.

Discussion

Skimmed milk was the best component tested for growth of *T. thermophila*, in agreement with previous works. [8,18]

With yeast extract, Plesner *et al* [16] used proteose peptone and salt solutions (Ppys medium). Dive *et al* [3] obtained highly reproducible results with *T. pyriformis*, *T. rostrata* and *T. thermophila* in a medium based on a hemoglobin hydrolysate (hb) and recommended this nitrogen source because it was a stable and defined source of amino acids. Ethuin [5] demonstrated that *T. pyriformis* and *T. thermophila* grew in media simply composed of yeast extract. Saliba *et al* [18] proved that skimmed milk (Mye medium) was a suitable substrate for mass cultivation of *T. pyriformis* and *T. rostrata*. Kiy and Tiedtke [8] improved Mye medium by glucose addition. Caillieret-Ethuin *et al* [2] showed that in industrial by-product media, *T. rostrata* gave best growth in media with dairy products.

In addition, the price of this medium was 75% lower than that of Ppys. Mye medium has never been optimised. In the present work the optimal combination for *Tetrahymena* growth was 1.07% yeast extract, 0.99% skimmed milk which increased biomass production 46%.

Protease production was also enhanced by skimmed milk, in agreement with Kiy and Tiedtke [8]. We suggest that casein particles stimulated protease secretion and hydrolysed proteins improved biomass. Zdanowski and Rasmussen [21] obtained evidence for a cell surface-bound peptidase, which could be involved in protein hydrolysis of the Mye medium.

Culture in batch fermentors improved biomass yield and scale-up can be successfully applied to *T. thermophila* for industrial purposes. We also propose that pH regulation produced undamaged cells during an extended stationary phase and resulted in abundant protease production.

To increase the cell yield in fermentors, immobilised techniques have been used with bacteria [4,19] or with *Tetrahymena* [7], but they present some disadvantages. Kiy and Tiedtke [9] developed a perfused bioreactor, as already described for cultivation of animal cell lines. *Tetrahymena* cells were retained in a perfusion module inside a bioreactor but this process was limited due to a decreased perfusion rate caused by blocking of the membranes. Cell recycling by microfiltration was used in yeast alcoholic fermentation in continuous operation and productivity was considerably improved [12]. The present work was the first time that continuous culture with cell recycling was successfully

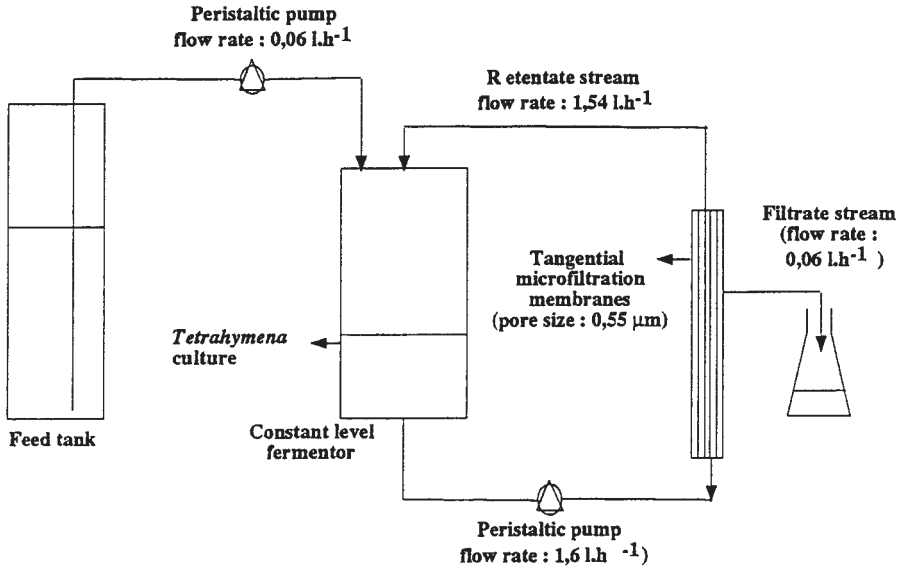

Figure 5 Schematic diagram of continuous fermentation coupled with tangential microfiltration.

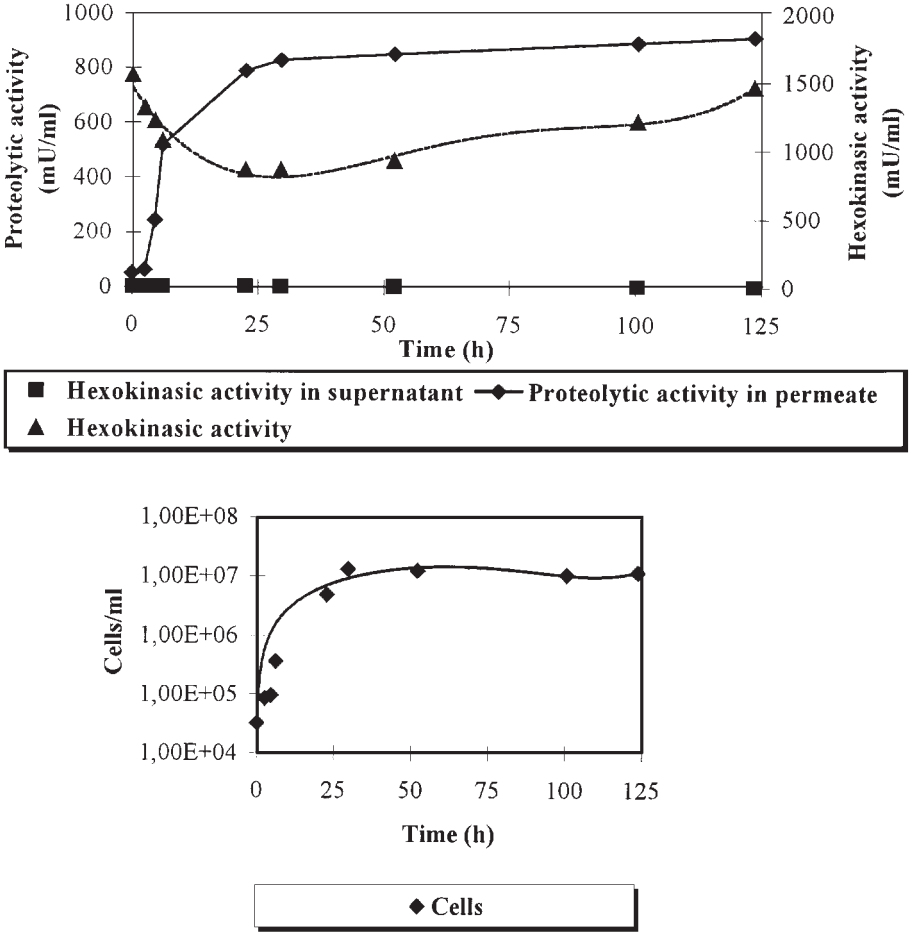

Figure 6 Growth kinetics of *T. thermophila* during continuous culture with cell recycle.

applied to protozoa. Additionally, our results indicate this process was an effective way to produce protease.

Acknowledgements

We gratefully thank Pr Thonart for use of the pilot-fermentors at the University of Gembloux (Belgium) and Mrs Ellis-Petit for helpful reading.

References

1 Beynon RJ and JS Bond. 1989. Proteolytic enzymes, a practical approach. IRL Press at Oxford University Press, Oxford, UK.
2 Caillieret-Ethuin P, F Duyme, F Tonon, J Jeanfils and J De Coninck. 1998. Optimisation of *Tetrahymena rostrata* growth using food by-products as nitrogen source. Biotechnol Techniques 12: 177–181.
3 Dive D, JM Piot, F Sannier, D Guillochon, P Charet and S Lutrat. 1989. Use of hemoglobin enzymic hydrolysates, prepared on a pilot-plant scale, as a nitrogen source for the cultivation of three species of *Tetrahymena*. Enzyme Microb Technol 11: 165–169.
4 Dhulster P, JN Barbotin and D Thomas. 1984. Culture and bioconversion use of plasmid-harboring strain of immobilised *E. coli*. Appl Microbiol Biotechnol 20: 87–93.
5 Ethuin P. 1993. Culture en masse de *Tetrahymena*: etude de milieux industriels et application à la culture en fermenteur du mutant thermodépendant *Tetrahymena thermophila* SJ180. Thesis, Université de Technologie de Compiègne, France.
6 Keuls M. 1952. The use of the studentized range in connection with an analysis of variance. Euphytica 1: 112–122.
7 Kiy T and A Tiedtke. 1991. Lysosomal enzymes produced by immobilized *Tetrahymena thermophila*. Appl Microbiol Biotechnol 35: 14–18.
8 Kiy T and A Tiedtke. 1992. Mass cultivation of *Tetrahymena thermophila* yielding high cell densities and short generation times. Appl Microbiol Biotechnol 37: 576–579.
9 Kiy T and A Tiedtke. 1992. Continuous high-cell-density fermentation of the ciliated protozoon *Tetrahymena* in a perfused bioreactor. Appl Microbiol Biotechnol 38: 141–146.
10 Kunst A, B Draeger and J Ziegenhorn. 1984. On hexokinase determination. In: Methods of Enzymatic Analysis, Vol 6 (Bergmeyer HU, ed), pp 163–172, Verlag Chemie, Weinheim: Academic Press, New York.
11 Minitab. 1998. On line Help. PA State College, Minitab Inc.
12 Mota M, C Lafforgue, P Strehaino and G Goma. 1987. Fermentation coupled with microfiltration: kinetics of ethanol fermentation with cell recycle. Bioproc Engin 2: 65–68.
13 Munro IG. 1985. Protozoa as source of commercialy produced enzymes. A review. Process Biochem 20: 139–144.
14 Newman D. 1939. The distribution of range in samples from a normal population expressed in terms of an independent estimate of standard deviation. Biometrika 31: 20–30.
15 North MJ. 1982. Comparative biochemistry of the proteinases of eucaryotik microorganisms. Microbiol Rev 46: 308–340.
16 Plesner P, L Rasmussen and E Zeuthen. 1964. Techniques used in the study of synchronous *Tetrahymena*. In: Synchrony in Cell Division and Growth (Zeuthen E, ed), pp 543–563, Intersci Publ, New York.
17 Rick W and WP Fritsch. 1974. On tyrosine determination in proteins. In: Methods of Enzymatic Analysis, Vol 2 (Bergmeyer HU, ed), pp 1046–1052, Verlag Chemie, Weinheim: Academic Press, New York.
18 Saliba R, D Dive and R Devis. 1983. An inexpensive and practical medium rearing mass cultures in *Tetrahymena*. Protistologica 19: 417–421.
19 Santos JAL, JMS Cabral and CL Cooney. 1992. Recovery of alkaline protease by membrane filtration. Effect of membrane type and addition of submicron sized charged particles. Bioproc Engin 7: 205–211.
20 Wheatley DN, L Rasmussen and A Tiedtke. 1994. *Tetrahymena*: a model for growth, cell cycle and nutritional studies, with biotechnological potential. BioEssays 16: 367–372.
21 Zdanowski MK and L Rasmussen. 1979. Peptidase activity in *Tetrahymena*. J Cell Physiol 100: 407–412.