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Evaluation of parallel operated small-scale bubble columns for microbial process development using *Staphylococcus carnosus*

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Abstract

Shake flasks and pH-controlled small-scale bubble columns were compared with respect to their usefulness as a basic tool for process development for human calcitonin precursor fusion-protein production with *Staphylococcus carnosus*. Parallel control of the pH (and making use of the base addition data) is necessary to study the effects of medium composition, to identify pH-optima and to develop a medium, which minimizes the acid excretion of *S. carnosus*. This medium with glycerol as energy source and yeast extract as carbon and nitrogen source resulted in cell dry weight concentration in shake flasks of 5 g l^{-1}, which were thus improved by a factor of 10. Cell dry weight concentrations of up to 12.5 g l^{-1} were measured in the batch process with pH-controlled small-scale bubble columns due to their higher oxygen transfer capability. In contrast to shake flasks it was demonstrated, that the batch process performance of recombinant *S. carnosus* secreting the human calcitonin precursor fusion-protein was identical within the estimation error in pH-controlled small-scale bubble columns compared to the stirred-tank reactor.

Keywords: Small-scale bubble columns; Shake flasks; Parallel pH-control; *Staphylococcus carnosus*; Human calcitonin

1. Introduction

Shake flasks are widely used as simple bioreactors for the screening of micro-organisms as well as in basic studies for microbial process development. On a technical scale, microbial reactions are primarily carried out in stirred-tank reactors. The most important reaction-engineering differences between the simple shake flask and a stirred-tank

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are a lack of control of important process variables (such as pH and pO_2) and lower volumetric oxygen transfer coefficients k_La due to surface aeration in the shake flask (Henzler and Schedel, 1991). Low concentrations of aerobic cells with reduced cellular activities are inevitable in shake flask experiments compared to stirred-tank reactors as a result of batch operation, uncontrolled changes in pH, reduced oxygen transfer rates and low gas exchange capacity. Due to these characteristics, basic studies for microbial process development on a shake flask scale may be ineffective, because important relationships between reaction conditions and microbial metabolism may not be identified. As a consequence, in many cases time-consuming sequential investigations are required using lab-scale stirred-tank reactors.

Recently, small-scale bubble columns with high oxygen transfer capabilities (k_La up to 0.2 s^{-1}) were developed as a new parallel reactor device (Weuster-Botz and Altenbach-Rehm, 1997; Weuster-Botz et al., 1998; Altenbach-Rehm et al., 1999). Bubble columns are mass transfer and reaction devices in which a gas is brought into contact with a liquid phase. A characteristic feature of bubble columns is that the gas flow is dispersed in the form of bubbles in the liquid phase. Bubble column reactors are characterized by their simple design and the complete absence of mechanically moving internals. Small-scale bubble columns (liquid volume of 200–400 ml) can therefore be used in a simple manner as 'shake flasks without shakers'. In comparison to shake flasks, the small-scale bubble columns do not need to be moved, the reaction volume does not need to be reduced to achieve high oxygen transfer rates, simple pH measurement or pO_2 measurement are possible and an interruption of the gas supply by off-line sampling can be avoided.

To overcome the lack of pH-control in parallel operated bioreactors, a parallel feeding and pH-control assembly was recently developed with a minimum of 'hard-ware' (Altenbach-Rehm et al., 1999; Drescher et al., 1999). The pH is measured individually in the bioreactors using standard autoclavable pH-probes. A precise syringe pump with a base or acid reservoir is connected via a substrate distribution system to individual 2/2-way miniature valves, one for each small-scale bioreactor. One individual miniature valve is opened with all other valves closed if a small amount of base or acid has to be added to the individual reactor. Parallel control of individual predefined pH set-points is achieved by a software controller.

Within the last decade, the gram-positive bacterium *Staphylococcus carnosus* was investigated as expression and secretion system for foreign protein production, because *S. carnosus* is almost devoid of soluble extracellular proteases (Götz, 1990; Meens et al. 1997). Product examples are the secretory production of a lipase, human proteins, an antibody fragment (Pschorr et al., 1994) and fragments of the anti-lysozyme antibody D1.3 (Schnappinger et al., 1995). Low cell mass concentrations of up to 8 g l^{-1} cell dry mass as well as low recombinant product concentrations of about 20 mg l^{-1} were reported by application of batch and fed-batch fermentations of the recombinant *S. carnosus* in stirred-tank reactors with complex media (Lechner et al., 1988; Voit et al., 1989, 1991; Falk et al., 1991).

This paper deals with the evaluation of parallel operated small-scale bubble columns as basic tool for the development of a process for production of human calcitonin precursor peptide using *S. carnosus* as expression and secretion system (Dilsen et al., 2000). First of all, the necessity of pH control was evaluated by studying process performance of shake flasks and small-scale bubble columns with parallel pH-control. Secondly, the effects of the oxygen transfer capabilities of shake flasks and small-scale bubble columns on process performance were investigated. Finally, process performance (growth of *S. carnosus* and secretory production of the recombinant fusion-protein) in small-scale bubble columns was compared to the process performance in a stirred-tank reactor.

2. Materials and methods

2.1. Micro-organism and medium

The wild type strain *S. carnosus* TM300 (DSM 20501) and recombinant *S. carnosus* pXPhCT2

were used in this study. The recombinant strain was a *S. carnosus* TM300 containing the plasmid pXPhCT2 which is a derivative of pXR2 (Meens et al., 1997). The plasmid pXPhCT2 carries the gene ligated into *Xba*/*Hin*dIII-digested pXR2 coding for a fusion-protein consisting of two synthetic human calcitonin precursor repeats that are connected by a linker sequence fused to the pre-pro part of *S. hyicus* lipase. The gene coding for the two synthetic human calcitonin precursor repeats was kindly provided by Novartis Pharma K.K., Takarazuka, Japan. Expression was controlled by a xylose-inducible, glucose-repressible promotor derived from *S. xylosus* (Sizemore et al., 1991; Wieland et al., 1995).

LB culture medium consisted of 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 5 g l^{-1} NaCl and 1 g l^{-1} NaH_2PO_4. Further complex culture medium consisted of up to 1.5% yeast extract (Merck, Darmstadt), 0.4066 g l^{-1} $MgCl_2 \cdot 6H_2O$, 0.1029 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.01 g l^{-1} $MnCl_2 \cdot 4H_2O$, 1.35 mg l^{-1} $FeCl_3 \cdot 6H_2O$, 0.14 mg l^{-1} $ZnCl_2$ and 20 mM K_2HPO_4/KH_2PO_4 buffer pH 7.0. All media contained either 1% (w/v) glucose for repression or 0.5% (w/v) xylose for induction and 10 mg l^{-1} chloramphenicol for the producing strain. A pH of 7.0 was adjusted with 45% (w/v) NaOH. Media components were sterilized separately for 20 min at 121°C. Chloramphenicol was stored at −20°C as stock solution (50 g l^{-1} in pure ethanol) and added after sterilization.

The defined medium consisted of 10 g l^{-1} carbon source, 1.321 g l^{-1} $(NH_4)_2SO_4$, 0.4066 g l^{-1} $MgCl_2 \cdot 6H_2O$, 0.1029 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.01 g l^{-1} $MnCl_2 \cdot 4H_2O$, 1.35 mg l^{-1} $FeCl_3 \cdot 6H_2O$, 0.14 mg l^{-1} $ZnCl_2$, 1 mg l^{-1} of each proteinogenic amino acid, 1 ml l^{-1} trace element solution (0.3 g l^{-1} H_3BO_3, 0.1 g l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.2 g l^{-1} $CoCl_2 \cdot 6H_2O$, 0.01 g l^{-1} $CuCl_2 \cdot 2H_2O$, 0.02 g l^{-1} $NiCl_2 \cdot 6H_2O$ and 0.03 g l^{-1} $Na_2MoO_4 \cdot 2H_2O$), 5 ml of a vitamins solution (10 mg l^{-1} riboflavine, 50 mg l^{-1} thiamine, 50 mg l^{-1} nicotin acid, 50 mg l^{-1} pyridoxin-HCl, 0.1 mg l^{-1} biotin, 0.2 mg l^{-1} folic acid and 1 mg l^{-1} vitamin B12). The pH was adjusted to pH 7.0 by adding 45 % (w/v) NaOH. Defined media were sterilized by micro-filtration before use. The buffered defined medium was supplemented with 20 mM K_2HPO_4/KH_2PO_4 buffer of pH 7.0.

Feed stocks prepared by overnight culture with 1% (w/v) glucose in LB-medium containing 10 mg l^{-1} chloramphenicol (1% (v/v) inoculum of 50 ml medium in 1 l shake flasks, 37°C, 150 rpm) were diluted 1:2 with sterile glycerol after harvest and stored at −80°C.

2.2. Analytical methods

The human calcitonin precursor peptide (PhCT2) concentration was measured by ELISA using polyclonal IgG antibodies for hCT (ICN, Germany) in 96 well plates (Maxisorb, Nunc). Each well was filled with 100 μl catching buffer (10 mM Tris/HCl pH 8, 250 mM NaCl). 100 μl supernatant was filled in the first line of the plate, mixed, and diluted 1:2 in each column. After 1 h shaking at room temperature each well was washed for three times with 200 μl washing buffer (10 mM Tris/HCl pH 8, 250 mM NaCl, 0.1% (w/v) BSA, 0.05% (v/v) Tween 20). Unspecific binding sites were blocked with 200 μl blocking reagent (10 mM Tris/HCl pH 8, 250 mM NaCl, 1% (w/v) BSA) for 1 h. After three times washing 100 μl of hCT antibody solution (20 μl hCT antibody per 10 ml washing buffer) was added and the plate incubated overnight at 4°C. 100 μl anti-IgG conjugated with alkaline phosphatase solution (4 μl anti-IgG conjugated with alkaline phosphatase solution per 10 ml washing buffer) was added after three times washing and incubated once more for 1 h. After washing substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2 \cdot 6H_20$, 225 mM NaCl and 10 mM *p*-nitrophenylphosphate, pH 10 adjusted with 32% HCl) was added and extinction at 405 nm measured with a thermomax plate reader (BMK-ZZZ, Beckman, Germany). If the extinction of the standard (2 μg PhCT2 ml^{-1}) reached 0.5, the PhCT2 concentration was calculated with at least two values per sample in the linear range 0.01–0.25 μg ml^{-1}. The PhCT2 standard provided from the Institute of Enzyme Technology, University of Düsseldorf, Germany and samples were measured twice.

Organic acids were analyzed by cation exchange HPLC using an Aminex HPX-87H column

(300 × 7.8 mm^2, Biorad, Munich, Germany) isocratically eluted with a flow rate of 0.5 ml min^{-1} at 40°C with 0.1 M H_2SO_4. Detection of UV adsorption was performed at 215 nm.

Cell dry weight was determined gravimetrically and gave a linear relationship to the optical density at 600 nm in a 1-cm cuvette (0.267 g OD_{600}^{-1}).

All chemicals were of analytical grade and used as supplied from Boehringer-Mannheim, Sigma, Riedel-deHaen, and Merck.

2.3. Reactors and reaction conditions

Investigations on a shake flask scale with a total volume of 0.1 l or 1 l scale were carried out as follows: Each flask was filled with sterilized medium (10% of the total volume), inocculated and incubated at 37°C in a rotary shaker (100–150 rpm).

S. carnosus was cultivated in up to 16 parallel operated small-scale bubble columns with a working volume of 200–400 ml in an incubator chamber (PROFORS, Infors HT, Bottmingen, Switzerland). For gas distribution, a porous plate with a diameter of 60 mm made of sintered glass with a pore size of 10–16 μm or stainless steel with laser-drilled pores of 60 μm were applied. The bubble columns were equipped with a port for a pH-probe, a sampling port with a septum and a culture tube closure at the top with rubber stoppers for the connection of feed tubing. Humidified air was used for aeration to reduce the evaporation rate during cultivation. The gas flow rate of each bubble column with integrated sterile filter was controlled manually in the range 0–5 vvm using a flow-meter with a needle valve. The pO_2 was measured in one of the parallel-operated small-scale bubble columns as reference.

A parallel feeding and pH-control assembly was used for individual pH-control (fed-batch-pro, DASGIPmbH, Jülich, Germany). A maximum base flow of 3 ml min^{-1} and a stop time of 20 s with opened miniature valve after each individual base addition were chosen to achieve an optimal tracking of the preset pH set-points.

The medium with 10% inocculum (v/v) was filled aseptically into the bubble columns. After transfer of the bubble columns into the incubator, aeration was started immediately via plug-in valves on the base tray.

Additionally, batch cultivations of *S. carnosus* were performed in a stirred-tank reactor with two flat-bladed disk turbines with six disks each on a laboratory scale (LABFORS, Infors HT, Bottmingen, Switzerland). The fermentation unit was equipped with standard measuring and control units (T, P, pH, pO_2, stirrer speed, air supply). pH was controlled by the addition of 4 M NaOH or 1 M HCl.

All reactors were inocculated with an overnight culture (10% of start volume). This pre-culture was prepared with 100 ml LB-medium and 10 mg l^{-1} chloramphenicol under repressed conditions (1% (w/v) glucose) in 1 l shake flasks at 37°C and 150 rpm.

3. Results and discussion

Batch-growth of the wildtype *S. carnosus* TM300 was investigated using a defined unbuffered growth medium with 10 g l^{-1} glucose as carbon source in shake flasks. Within 24 h a cell dry weight concentration of 0.5 g l^{-1} was achieved. The pH in the medium declined down to pH 4.5. Adding 20 mM of phosphate buffer to this growth medium resulted in higher cell dry weight concentrations (1.0 g l^{-1}) within 24 h. As the pH at harvest was the same as before (pH 4.5), it was concluded, that growth of *S. carnosus* may be inhibited at pH 4.5. This was confirmed by growth studies of *S. carnosus* TM300 using pH-controlled small-scale bubble columns and a stirred-tank reactor. At a set-point of pH 7.0 cell dry weight concentrations of about 3 g l^{-1} were measured after a process time of 15 h (Fig. 1). Within the estimation error no differences in process performance were observed in the stirred-tank reactor compared to the small-scale bubble columns.

Due to this finding, the effect of pH on growth rate of *S. carnosus* was studied with one set of parallel operated small-scale bubble columns. The pH-set-point was varied in the individual small-scale bubble columns between pH 4.0 and pH 9.0 (Fig. 2). The parallel pH-control assembly re-

sulted in a precise tracking of the individual pH set-points during acid excretion by *S. carnosus*. An increase of the pH could be observed after the initially added glucose was consumed. The reproducibility of the process performance of *S. carnosus* in pH-controlled small-scale bubble columns is demonstrated by simultaneous plotting of the off-line measured cell dry weight concentrations, the on-line measured pH and base addition as function of process time. Each experiment was performed twice at the same time.

Estimation of growth rates was based on the off-line measured optical densities. No growth of *S. carnosus* was observed at pH 9.0 (Fig. 3). At pH 4.0 the growth rate was low ($\sim 0.2\ h^{-1}$). Growth rates of up to 0.75 h^{-1} were observed between pH 5.0 and pH 8.0, indicating a broad pH-optimum for growth of *S. carnosus*.

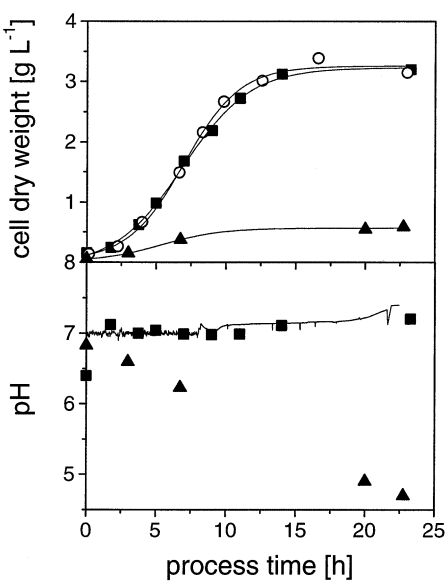

Fig. 1. Growth of *S. carnosus* TM300 in shake flasks (▲), pH-controlled bubble columns (■) and stirred-tank reactor (○) with glucose as carbon source (10 g l^{-1}) and a defined growth medium at 37°C (the online measured pH-signal of one of the small-scale bubble columns, as well the offline measured pH of a bubble column (■) and a shake flask (▲) are indicated).

The pH-drop during cultivation of *S. carnosus* with glucose as sole carbon and energy source is caused by the production of organic acids (especially acetic acid and lactic acid). Varying the carbon sources (fructose, glycerol, and lactose, respectively) in shake flasks gave the same result as with glucose. The pH in each shake flask dropped down to pH 4.5 and identical cell dry weight concentrations of 1 g l^{-1} were measured after 24 h of incubation. The application of a parallel cultivation system with pH-control allowed discrimination between the carbon-sources. Within one set of parallel operated small-scale bubble columns it was found that the cell yield of the wild-type strain *S. carnosus* TM300 was just 50% with glycerol as carbon source (1.5 g l^{-1}) compared to the other carbon sources (3 g l^{-1}). If the recombinant strain was cultivated with lactose under induced conditions the cell yield was reduced to 50% (Fig. 4). Lactose is metabolized via the inducible lactose-pathway in *Staphylococci*. The reduced cell yield in the recombinant strain could be either caused by the introduction of the regulating elements of the xylose-operon or by a catabolite repressing effect of the inducer xylose on the lactose-pathway or by the metabolic burden of overexpressing PhCT2.

An estimation of the amount and dynamics of the production of organic acids by *S. carnosus* may be possible, if a correlation can be identified between the base addition by the pH-controller and the accumulation of organic acids. In fact, summing up the added NaOH during a batch cultivation of *S. carnosus* with pH-controlled small-scale bubble columns and calculating of the molar amount of OH^- ions gave a good correlation with the molar sum of organic acids (Fig. 5). It was demonstrated, that using glycerol or lactose as carbon sources the cellmass specific acid formation or OH^- ion addition for pH-control was much lower compared to glucose or fructose (factor 4 and 3.5, respectively). This finding offered the chance to design a medium for cultivation of *S. carnosus* which will cause a minimum of acid formation and will thus be suitable for shake flask cultivations as well. Using glycerol as energy

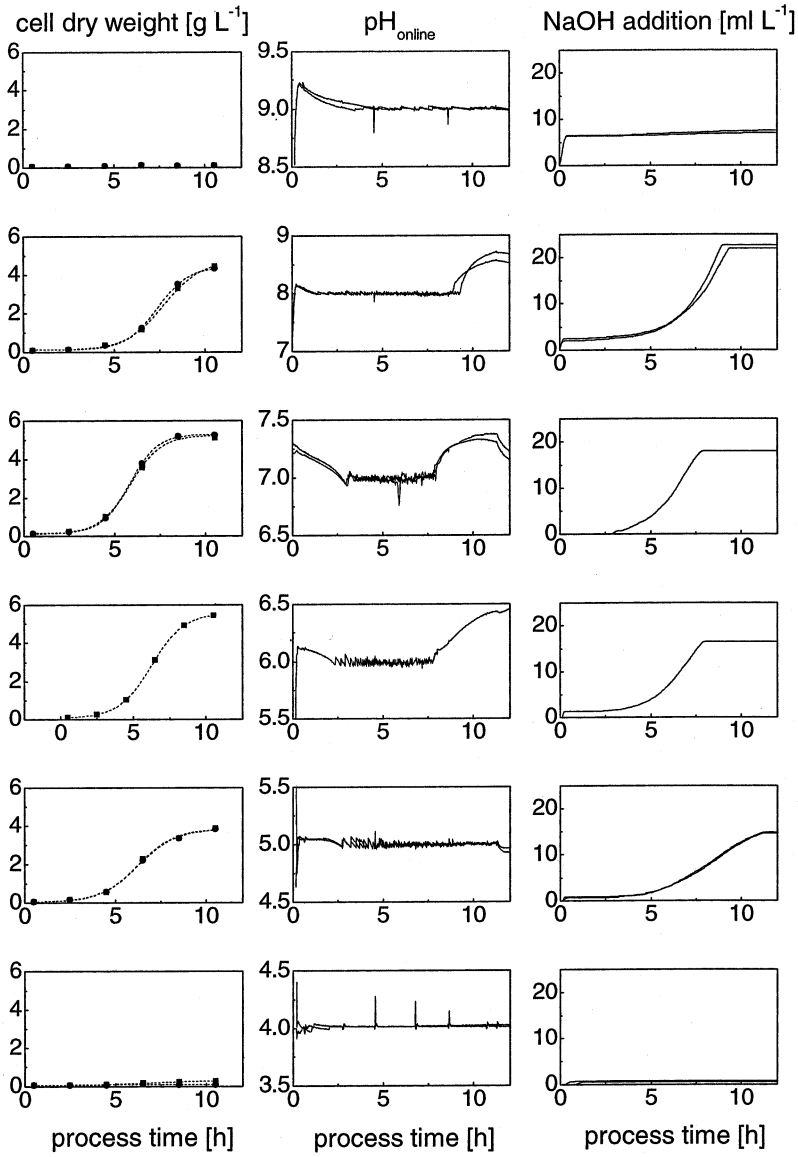

Fig. 2. Growth of *S. carnosus* TM300 as function of pH with varying pH set-points (9, 8, 7, 6, 5, and 4, respectively): offline measured cell dry weight, online measured pH and online measured base addition. Each experiment was performed twice at the same time (LB-medium, 10 g l^{-1} glucose, parallel operated and pH-controlled small-scale bubble columns, $V = 0.2$ l, $T = 37$°C).

source (50 g l^{-1}) and yeast extract as carbon and nitrogen source (15 g l^{-1}) minimal amounts of organic acids were formed on the shake flask scale. The pH was not changed significantly in batch runs (pH 7.0–6.8) and cell dry mass concentrations of up to 5 g l^{-1} were measured (improvement by a factor of 10).

As oxygen transfer rate is limited on a shake flask scale *S. carnosus* cells were cultivated in small-scale bubble columns with the same glycerol–yeast extract medium used before. The aeration rate was controlled manually in a way that oxygen tension did not fall below 30% air saturation. Due to the improvement in oxygen supply

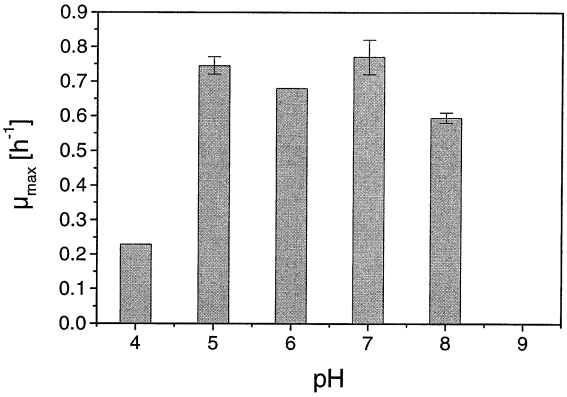

Fig. 3. Maximum growth rate of *S. carnosus* TM300 as function of pH (LB-medium, 10 g l^{-1} glucose, parallel operated and pH-controlled small-scale bubble columns, $V = 0.2$ l, $T = 37$°C, $n = 2$).

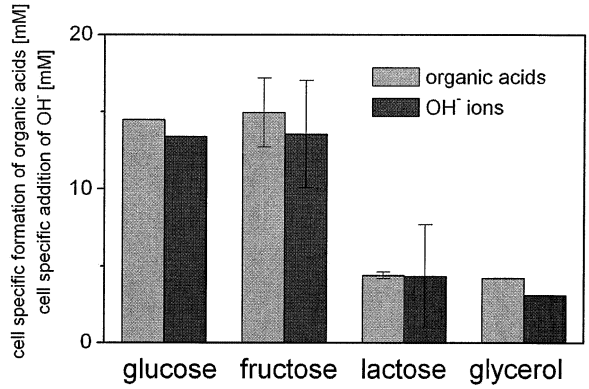

Fig. 5. Cell mass-specific formation of organic acids and addition of OH^- ions as function of the carbon source (10 g l^{-1}) in parallel operated and pH-controlled small-scale bubble columns ($t = 24$ h, $V = 0.2$ l, $T = 37$°C, pH 7.0, $n = 2$).

compared to the shake flask a cell dry mass concentration of 12.5 g l^{-1} was achieved after a process time of 18 h. This improvement by a factor of 2.5 compared to the results of the shake flasks demonstrates clearly, that growth of *S. carnosus* in shake flasks (under the operation conditions applied) is oxygen limited if the cell dry mass concentration exceeds ~ 5 g l^{-1}.

Process performance (growth of *S. carnosus* pXPhCT2 and secretory production of the recombinant fusion-protein) in small-scale bubble columns was compared to the process performance in a stirred-tank reactor. All reaction conditions were kept constant (medium, inocculum quality and quantity, T, pH, pO_2). It was demonstrated that dry cell mass concentration as well as the secretion of the recombinant fusion-protein

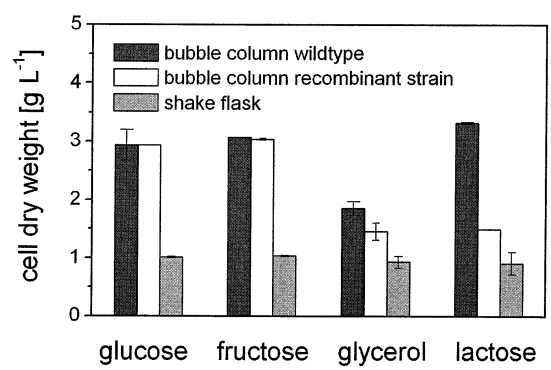

Fig. 4. Growth of *S. carnosus* TM300 and *S. carnosus* pXPhCT2 in shake flasks and pH-controlled bubble columns with glucose, fructose, glycerol or lactose as carbon source (10 g l^{-1}) and a defined growth medium ($T = 37$°C, pH 7.0, $n = 2$).

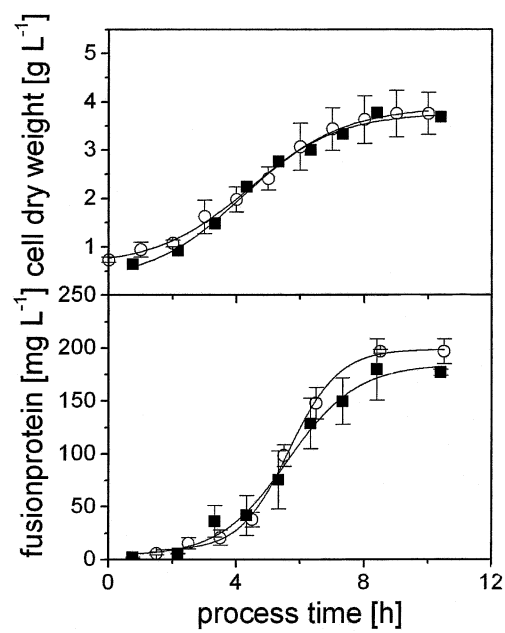

Fig. 6. pH-controlled batch cultivation of *S. carnosus* pXPhCT2 in small-scale bubble columns (■) with a liquid volume of 0.4 l and a stirred-tank reactor (○) with a liquid volume of 2 l using yeast extract as carbon and nitrogen source (initial conditions: 15 g l^{-1} yeast extract, 37°C, pH 7.0).

was identical within the estimation error (Fig. 6). The reproducibility of this batch process for the production of the fusion-protein with *S. carnosus* (initial addition of inducer, gas flow rate of 0.25 vvm, osmotic pressure 0.5 osmol kg^{-1}, pH 7.0, temperature of 37°C) was checked with parallel and sequential operated small-scale bubble columns. A standard deviation of 5% (3.7 ± 0.2 g l^{-1}) and of 4% (186 ± 7.5 mg l^{-1}) was measured with respect to the cell dry weight concentration and the fusion-protein PhCT2, respectively ($n = 6$).

This clearly demonstrates, that parallel operated small-scale bubble columns with individual pH-control are a valuable tool for process development using *S. carnosus* as expression and secretion system for recombinant proteins (Dilsen et al., 2000).

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References

Altenbach-Rehm, J., Nell, C., Arnold, M., Weuster-Botz, D., 1999. Parallel bubble columns with fed-batch technique for microbial process development on a small scale. Chem. Eng. Technol. 22, 1051–1058.

Dilsen, S., Paul, W., Sandgathe, A., Tippe, W., Freudl, R., Thömmes, J., Kula, M.-R., Takors, R., Wandrey, C., Weuster-Botz, D., 2000. Fed-batch production of recombinant human calcitonin precursor fusion-protein using *Staphylococcus carnosus* as expression-secretion system. Appl. Microbiol. Biotechnol. 54, 361–369.

Drescher, T., Altenbach-Rehm, J., Weuster-Botz, D., 1999. Verfahren und Vorrichtung zur Serienkultivierung von Mikroorganismen. German Pat. DE 197 09 603 C2

Falk, M.P.F., Sanders, E.A., Deckwer, W.D., 1991. Studies on the production of lipase from recombinant *Staphylococcus carnosus*. Appl. Microbiol. Biotechnol. 35, 10–13.

Götz, F., 1990. Applied genetics in the gram-positive bacterium *Staphylococcus carnosus*. Food Biotechnol. 4, 505–513.

Henzler, H.J., Schedel, M., 1991. Suitability of the shaking flask for oxygen supply to microbiological cultures. Bioprocess Eng. 7, 123–131.

Lechner, M., Märkl, H., Götz, F., 1988. Lipase production of *Staphylococcus carnosus* in a dialysis fermentor. Appl. Microbiol. Biotechnol. 28, 345–349.

Meens, J., Herbort, M., Klein, M., Freudl, R., 1997. Use of the pre-pro part of *Staphylococcus hyicus* lipase as a carrier for secretion of *Escherichia coli* outer membrane protein A (OmpA) prevents proteolytic degradation of OmpA by cell-associated protease(s) in two different gram-positive bacteria. Appl. Environ. Microbiol. 63, 2814–2820.

Pschorr, J., Bieseler, B., Fritz, H.J., 1994. Production of the immunoglobulin variable domain REIv via a fusion-protein synthesized and secreted by *Staphylococcus carnosus*. Biol. Chem. Hoppe-Seyler 375, 271–280.

Schnappinger, D., Geissdörfer, W., Sizemore, C., Hillen, W., 1995. Extracellular expression of native human antilysozyme fragments in *Staphylococcus carnosus*. FEMS Microbiol. Lett. 129, 121–128.

Sizemore, C., Buchner, E., Rygus, T., Witke, C., Goetz, F., Hillen, W., 1991. Organization, promoter analysis and transcriptional regulation of the *Staphylococcus xylosus* xylose utilization operon. Mol. Gen. Genet. 227, 377–384.

Voit, H., Götz, F., Mersmann, A., 1989. Overproduction of lipase with *Staphylococcus carnosus* (pLipPS1) under modified gravity in a centrifugal field bioreactor. Chem. Eng. Technol. 12, 364–373.

Voit, H., Wenzig, E., Mersmann, A., 1991. Production of a microbial lipase by *Staphylococcus carnosus* (pLipMut2) in a bubble column reactor and a centrifugal field bioreactor. Bioprocess Eng. 6, 221–226.

Weuster-Botz, D., Altenbach-Rehm, J., 1997. Vorrichtung zur Serien-Kultivierung von Mikroorganismen bzw. Zellen in begasten Flüssigkeitssäulen. German Pat. DE 195 27 099A1

Weuster-Botz, D., Altenbach-Rehm, J., Hawrylenko, A., 1998. Verfahren und Vorrichtung zur Begasung von Medien sowie Begasungskörper. International Pat. Application PCT/DE98/03447.

Wieland, K.-P., Wieland, B., Götz, F., 1995. A promoter-screening plasmid and xylose-inducible, glucose-repressible expression vectors for *Staphylococcus carnosus*. Gene 158, 91–96.